# ROLE OF PROLACTIN RECEPTOR TYROSINE PHOSPHORYLATION IN INTRACELLULAR SIGNALING

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## ABSTRACT

Prolactin (PRL) is a polypeptide hormone synthesized by the anterior pituitary as well as by a number of extrapituitary tissues. The hormone is an essential regulator of important biological processes. The best characterized role for PRL is its participation in the development of the lobuloalveolar system in the mammary gland. The biological actions of PRL are mediated in the cell by the membrane-bound prolactin receptor (PRLR), which is a member of the cytokine family of receptors. Binding of PRL to the PRLR induces receptor dimerization and activation of the constitutively associated Jak2 kinase, which leads to the tyrosine phosphorylation of the kinase as well as phosphorylation of tyrosine residues on the PRLR creating recruitment sites for SH2 containing signaling molecules. Among the signaling molecules recruited to the PRLR/Jak2 complex are the signal transducers and activators of transcription 5 (Stat5), the protein tyrosine phosphatase SHP-2 and the inhibitory protein SOCS-1. Work in this thesis was performed in order to understand the molecular basis behind the biological responses to PRL trough investigating the intracellular signaling mechanisms emanating from the PRLR.

Tyrosine phosphorylation of the PRLR, particularly of the most C-terminal tyrosine, was shown to play a critical role in the induction of PRL responsive genes. The mechanism of regulation of signaling activities by the C-terminal tyrosine of the PRLR was not known. The aim of the doctoral work was to investigate the signaling mechanisms controlled by the C-terminal tyrosine of the PRLR.

The signal transducer and activator of transcription 5 (Stat5) is an important mediator of PRLR signaling leading to the activation of PRL responsive genes. Work in this thesis established that Stat5 tyrosine phosphorylation is independent of PRLR tyrosine phosphorylation, interestingly however, Stat5 nuclear translocation was directly regulated by the C-terminal tyrosine of the PRLR. Additionally, the studies

demonstrated that the C-terminal tyrosine is a site for the recruitment of the protein tyrosine phosphatase SHP-2. A second site for the SHP-2 recruitment was determined to be the adaptor protein Gab2 that is tyrosine phosphorylated in response to PRL stimulation. Furthermore, the studies illustrate that the C-terminal tyrosine, through recruiting SHP-2 phosphatase, regulates the phosphorylation state of tyrosine 1007 of Jak2 kinase. Dephosphorylation of tyrosine 1007 in the activation loop of Jak2 by SHP-2 prevents the association between Jak2 and the inhibitory protein SOCS-1 leading to the maintenance of Jak2 kinase activity and the prevention of the ubiquitination and proteasomal degradation of Jak2 as well as the PRLR.

Collectively, work in this thesis resolves some of the molecular mechanisms implicated in PRLR signaling, specifically through the most C-terminal tyrosine of the PRLR. The findings are not only important for realizing the molecular basis of PRL physiological actions, such as mammary gland development, but also for understanding the signaling schemes utilized by members of the cytokine receptor superfamily.

## RÉSUMÉ

La prolactine est une hormone polypeptidique synthétisée principalement par l'hypophyse antérieure mais aussi par un certain nombre d'autres tissus non hypophysaires. Cette hormone est essentielle pour la régulation de nombreux effets biologiques. Le rôle le mieux caracterisé de la prolactine est sa participation dans le développement de la glande mammaire. Les effets biologiques de la prolactine dans ce tissu sont médiés par une interaction directe entre la prolactine et son récepteur membranaire, un membre de la famille des récepteurs aux cytokines. La liaison de la prolactine sur son récepteur induit la dimérisation de ce dernier ainsi que l'activation de la protéine kinase Jak2, qui lui est associée. Cette activation résulte en la phosphorylation du récepteur et de la kinase elle-meme, sur des résidus tyrosine qui constituent ainsi des sites de recrutement pour les molécules effectrices contenant des domaines SH2. Parmi ces molécules recrutées par le complexe récepteur/Jak2 se retrouvent le facteur de transcription Stat5, la protéine tyrosine phosphatase SHP-2 et la molécule inhibitrice SOCS1. La phosphorylation de la tyrosine C-terminale du récepteur joue un rôle critique dans la médiation des effets de la prolactine menant à la différentiation des cellules épitheliales de la glande mammaire ainsi qu' à la production des protéines du lait comme la β-caséine. Mon projet de thèse a consisté à caratériser les mécanismes moléculaires impliqués dans les voies de signalisation du récepteur de la prolactine, particulièrement au niveau du résidu tyrosine C-terminal du récepteur.

Nos résultats démontrent que ce residu C-terminal joue un rôle important dans l'activation du facteur de transcription Stat5. Nous avons en outre pu montrer que la phosphorylation de Stat5 est indépendente de la phosphorylation du récepteur. De façon intéreressante, nous avons aussi montré que la translocation de Stat5 dans le noyau est directement régulée par la tyrosine C-terminale du récepteur. Nos études démontrent

aussi que la phosphorylation de la phosphatase SHP-2 et son recruitement sur le récepteur de la prolactine dépendent aussi du résidu tyrosine en position C-terminale du récepteur. Un deuxième site de recruitement pour SHP-2 se trouve sur la protéine adaptatrice Gab2, qui est elle aussi phosphorylée en réponse à la prolactine.

Nous avons finalement aussi pu démontrer que la tyrosine C-terminale du récepteur de la prolactine est capable de réguler l'activité de la kinase Jak2, ceci en recruitant la phosphatase SHP-2, induisant ainsi la déphosphorylation de Jak2 sur sa tyrosine 1007. Cette déphosphorylation de Jak2 prévient son association avcc la protéine inhibitrice SOCS1, maintenant ainsi l'activité de la kinase et prévenant la dégradation de Jak2 et du récepteur par les mécanismes d'ubiquitination.

Le travail effectué durant ma thèse permet donc de mieux caractériser les mécanismes moléculaires impliqués dans les voies de signalisation du récepteur de la prolactine, particulièrement au travers de la tyrosine C-terminale du récepteur. Ces résultats sont importants pour notre compréhension du mode d'action de la prolactine mais aussi des autres membres de la famille des récepteurs aux cytokines.

## ACKNOWLEDGEM ENTS

I would like to thank my thesis supervisor, Dr. Suhad Ali, for her advice, criticism, guidance, and support throughout my years of study in her laboratory. She had a clear influence on the development of my scientific personality.

I wish to express my appreciation to all past and present members of my laboratory for their company, help, and friendship. Particularly, I wish to thank Ms. Naila Chughtai, Dr. Parham Minoo, Mr. Strachan Hartley, Ms. Zainab Nohi, Mr. Edourd Toutel, Mr. Imad Ghouli, Mr. Koji Ayoama, Mr. Sinisa Tomic and Mr. Lin Yang. Additionaly, I am grateful to the Formation de Chercheurs et l'Aide à la Recherche (FCAR) and the Royal Victoria Hospital Research Institute for the financial support during the course of my studies.

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## PREFACE AND CONTRIBUTION OF AUTHORS

The present thesis, consisting of five chapters, describes the role of prolactin receptor tyrosine phosphorylation, particularly of the most carboxy-terminal tyrosine, in the regulation of signal transduction events leading to the activation of gene promoters of prolactin responsive genes. Chapter I is a literature review which first presents the hormone prolactin and describes biological functions, then describes the molecular basis of signaling pathways of cytokine receptors signaling with emphasis on prolactin receptor signal transduction pathways.

Chapters II to IV, inclusively, are comprised of three scientific papers: two of which (Chapters II and III) are published papers, whereas Chapter IV is a manuscript submitted for publication. Reprint copies of Chapters II and III are included in the Appendix section.

Chapter II, Prolactin Receptor Regulates Stat5 Tyrosine Phosphorylation and Nuclear Translocation by Two Separate Pathways, is a first authored article for which I performed all the experiments presented. I wrote the publication under supervision of my supervisor.

Chapter III, Recruitment of the Protein Tyrosine Phosphatase SHP-2 to the Carboxy-Terminal Tyrosine of the Prolactin Receptor and to the Adaptor Protein Gab2, is a first authored article in which I performed all the experiments presented. I wrote the article in collaboration with my supervisor, Dr. Suhad Ali.

Chapter IV, SHP-2 Positively Modulates the Jak/Stat Pathway Downstream of the Prolactin Receptor by Preventing SOCS-1 Mediated Ubiquitination/Degradation Pathway,

is a first authored article for which I performed all the experiments presented. I wrote the article in collaboration with my supervisor.

Finally, Chapter V includes a detailed discussion of the relevance of the findings presented in this thesis and paves the way for future prospects in term of studies to understand the molecular basis of prolactin receptor signal transduction pathways. A general bibliography follows and includes the references for Chapters I and V. The references for Chapters II to IV, inclusively, are found at the end of each chapter.

## CLAIM TO ORIGINALITY

This thesis was written entirely by myself, Samir Ali, with the assistance of my thesis director, Dr. Suhad Ali. The papers presented in Chapters II and III were published by our laboratory and constituted, at the time of publication, original and previously unpublished data. The manuscript presented in Chapter IV was recently submitted. The results presented in this manuscript are original and unpublished.

Work presented in this thesis; (i) Stat5 tyrosine phosphorylation is independent of tyrosine phosphorylation of the PRLR, (ii) the C-terminal tyrosine of the PRLR regulates the process of Stat5 nuclear translocation, (iii) the C-terminal tyrosine of the PRLR is a site for recruitment of the protein tyrosine phosphatase SHP-2, (iv) PRL stimulation of the prolactin receptor induces the tyrosine phosphorylation of the adaptor protein Gab2, (v) SHP-2 associates with Gab2 adaptor protein (vi) the C-terminal tyrosine modulates the tyrosine phosphorylation state of Jak2 (vii) SHP-2 dephosphorylates tyrosine 1007 of Jak2 and prevents the association of the inhibitory protein SOCS-1 (viii) the prevention of Jak2 association to SOCS-1 prolongs the life time of Jak2 and the prolactin receptor, are all novel findings and constitute original contributions to the field of study.

# CHAPTER I. INTRODUCTION

## 1. Prolactin hormone

### 1.1 The family of PRL/GH/PL

The hormone prolactin (PRL) was discovered many decades ago as a pituitary factor that stimulates mammary gland development in rabbits and hence was called "pro-lactin". Various genetic (Niall et al., 1971; Miller and Eberhardt, 1983), functional (Nicoll et al., 1986; Goffin et al., 1996b), structural (Nicoll et al., 1986; Goffin et al., 1996b), and binding (Goffin et al., 1996b) studies revealed that PRL is closely related to two other factors, GH and placental lactogens, and therefore they all form a distinct family of proteins called the PRL/GH/PL family. More broadly, based on molecular and functional studies (refer to section on biological functions of PRL), PRL was placed within an extended group of proteins called the cytokine family (

Miller and Eberhardt, 1983).

### 1.2 PRL Gene and Regulation of Production

Production of PRL occurs at various sites. The majority of PRL, the blood circulating PRL, is secreted by the lactotrophic cells of the anterior pituitary under the control of the inhibitory factor dopamine, which originates from the hypothalamus and acts on target tissues in an endocrine manner (Meites, 1977). In addition to the pituitary PRL, production of extrapituitary PRL has been observed in various other tissues. For example, PRL is produced in the brain, deciduas, myometrium, lacrimal gland, thymus, bone marrow, and mammary epithelial cells (Posner et al., 1974). Therefore, extrapituitary PRL is present in several fluid compartments such as cerebrospinal fluid, amniotic fluid, tears, and milk (Posner et al., 1974). The biological significance of extrapituitary PRL remains under investigation. It is presumed to function through an autocrine/paracrine manner by exerting its effects on the secreting or adjacent tissues.

Human PRL gene is located on chromosome 6 (Owerbach et al., 1981) and is composed of five exons and four introns with a length of approximately 10 Kb (Truong et al., 1984). The PRL gene has two promoters, the pituitary promoter and the extra-pituitary promoter. The choice of promoter usage depends on the site of production of PRL. The pituitary promoter, covers ~5 Kb upstream of initiation site, or the extrapituitary promoter, covers ~5.8 Kb of the transcription initiation site (Berwaer et al., 1994).

### 1.3 Primary and Tertiary Structures of PRL

Mature human PRL is composed of 199 amino acids and has a molecular weight of 23 kDa (Cooke et al., 1981). The protein forms three intramolecular disulfide bonds (between cysteines 4-11, 58-174, and 191-199) (Truong et al., 1984). Studies on the secondary structure of PRL indicated that the hormone is an all-α-helix protein. Approximately 50% of the protein is in α-helices and the remainder folds into connecting loop structures (Waterman et al., 1978). Human PRL folds into four-helix bundle and to have up-up-down-down connectivity of the α-helices (Goffin et al., 1995; Goffin et al., 1996b; de Vos et al., 1992; Abdel-Meguid et al., 1987).

### 1.4 Forms of PRL

The major form of prolactin that is secreted by the pituitary gland is a 23 kDa hormone. However, there are a number of forms of prolactin that have been characterized in many mammals, including humans.

#### 1.4.1 Proteolytically Processed PRL

A number of cleaved forms of PRL have been characterized, including 14-, 16-, and 22-kDa prolactin variants. The 16-kDa fragment (amino acid 1-148) was first described in rat pituitary extracts (Mittra, 1980) and was later found in mouse (Sinha and Gilligan, 1984) and human (Sinha et al., 1985) pituitary glands. The 16-kDa prolactin is a product of

kallikrein enzymatic activity. Kallikrein is an estrogen-induced, trypsin-like serine protease that is found in the Golgi cisternae and secretory granules of lactotrophs (Powers, 1993). The 14-kDa $NH_2$-terminal fragment results from posttranslational cleavage of PRL that is processed in the hypothalamus and shares biological activity with the 16-kDa fragment (Clapp et al., 1993; Torner et al., 1995). Production and release of these proteolyticaly-processed forms of PRL from the pituitary gland is specific to female rats and sensitive to inhibition by dopamine (Anthony et al., 1993). Although these and other fragments have been found in pituitary gland and serum, more work is required to determine their physiological significance.

### 1.4.2 Dimerized and Oligomerized PRL

High-molecular-weight forms of PRL resulting from aggregation of PRL monomers with binding proteins, such as immunoglobulins through covalent and noncovalent bonds have been described. These high-molecular-weight forms were observed to have reduced biological activity (Sinha, 1995).

### 1.4.3 Phosphorylated PRL

Phosphorylation of prolactin on serine and threonine residues occurs within the secretory vesicles of lactotrophs just before exocytosis (Greenan et al., 1989). Phosphorylated prolactin isoforms have been isolated from bovine (Brooks et al., 1990) and murine (Oetting et al., 1986) pituitary glands and were shown to have much lower biological activity than nonphosphorylated prolactin (Wang and Walker, 1993). It was suggested that phosphorylated prolactin may serve as an autocrine regulator of prolactin secretion since it suppresses the release of nonphosphorylated prolactin from the pituitary (Ho et al., 1989). The process of phosphorylation of prolactin as well as the ratio of phosphorylated to nonphosphorylated isoforms is regulated throughout the estrous cycle (Ho et al., 1993). The physiological relevance of this ratio is not yet known. In a study, it

was shown that phosphorylated prolactin acts as an antagonist of PRLR and blocks signal transduction pathways (Brooks and Saiduddin, 1998; Coss et al., 1999), and proliferative activities initiated by unmodified prolactin on Nb2 lymphoma cells (Wang and Walker, 1993). Additionally, it was demonstrated that phosphorylation of serine 179 in vitro produced a PRL antagonist mimicking the naturally phosphorylated PRL (Chen et al., 1998a). Further investigation is needed to determine the biological role of phosphorylated prolactin.

### 1.4.4 Glycosylated PRL

Prolactin has been found to be glycosylated in the pituitary glands of mammals (Sinha, 1995). Glycosylation occurs through the linkage of a carbohydrate moiety to PRL either through nitrogen (*N*-glycosylation) or oxygen (*O*-glycosylation). Glycosylation of PRL lowers biological activity (Markoff et al., 1988; Sinha et al., 1991) as well as receptor binding and immunologic reactivity (Haro et al., 1990).

## 1.5 Biological Functions of PRL

### 1.5.1 Mammary Gland Development and Lactation

The process of mammary gland development remains to date the most investigated biological process influenced by PRL. Development of the mammary gland is an intricate process and occurs in defined stages involving the mammary epithelium and its surrounding stroma. The epithelium consists of a branched ductal system and a lobuloalveolar compartment. The ducts branch into decreasingly smaller ductules, which terminate in lobules. Lobules are composed of alveoli, which in turn consist of secretory epithelial cells that undergo functional differentiation with parturition. The ducts are surrounded by a continuous layer of contractile myoepithelial cells and the alveoli have a network of myoepithelium. These cells contract in response to oxytocin stimulation, which results in milk release.

The first stage in the development of the mammary gland which results in the formation of the ductal system is irreversible and occurs before birth under the influence of estrogen, GH and IGF-1. Then during puberty, and under the influence of PRL and progesterone, lobular buds branch off from the ductal system to form elementary lobules (Topper and Freeman, 1980; Kleinberg, 1997). The second stage of development is reversible and it occurs during pregnancy and lactation. At this stage, the lobuloalveolar epithelium undergoes extensive proliferation under the influence of PRL, placental lactogens, progesterone and produces the lobuloalveoli.

The unambiguous role of PRL in the process of mammary gland development was proven through using targeted disruption of genes of PRL, the prolactin receptor (PRLR), as well as the signal transducer and activator of transcription-5 (Stat5), an important mediator of PRLR signaling. The knockout of the PRL gene resulted in defective mammary glands where the gland had a normal ductal tree, however they failed to develop the lobuloalveolar growths (Horseman et al., 1997). Similarly, null mutations of the PRLR (PRLR -/- and PRLR -/+) gene resulted in mice that had severely compromised mammary gland development manifested by the lack of alveologenesis. The ducts branch less frequently than in wild-type animals and terminal end bud structures persist at the ductal ends without differentiating into the lobuloaveolar structures as observed in wild-type animals (Ormandy et al., 1997b). Finally, gene disruptions of Stat5 (Stat5a/Stat5b) resulted in equally affected mammary glands (refer to section 3.3.1.2.4, Stat Protein Activated in PRLR Signaling). Interestingly, null mutations of the progesterone receptor resulted in a similar outcome to the knockouts of the PRLR. The mammary glands of mice lacking the progesterone receptor arrested at the ductal stage and failed to produce the lobuloalveolar structures (Brisken et al., 1998). Therefore, PRL either acting individually or in concert with progesterone plays an

obligate role in the normal development of the mammary gland and the process of alveologenesis.

### 1.5.2 Reproduction

PRL regulates female reproduction by modulating the states of estrus and pregnancy. It mainly regulates the production and secretion of progesterone by the luteal cells of the ovaries (Matsuyama et al., 1990). Progesterone production is important for the implantation of the fertilized egg, for maintenance of pregnancy and for inhibition of ovulation (Armstrong et al., 1970; Ota et al., 1986; Berhman et al., 1970).

Studies on mice showed that PRL gene knockout produced completely infertile females (Gertler et al., 1996). Similarly, knockouts of the PRLR resulted in completely infertile females where egg development arrests immediately after fertilization (Ormandy et al., 1997b).

### 1.5.3 Other Functions

In addition to its roles in mammary gland development and reproduction, PRL has over 300 other separate functions in various species. In fact, the hormone has more biological functions than all other pituitary hormones combined (Bole-Feysot et al., 1998). Some of the other functions for PRL include growth and development, endocrinology and metabolism, brain and behaviour, water and electrolyte balance, and immuneregulation. Perhaps, the best indication of the wide range of biological functions of PRL in humans and other vertebrates is the nearly ubiquitous expression of the PRLR.

## 2 The Prolactin Receptor

### 2.1 PRLR is a Member of the Cytokine receptor superfamily

Prior to the cloning of the PRLR, it was initially expected to be a membrane-bound protein with a specificity and high affinity for PRL (Posner et al., 1974; Kelly et al.,

1974b; Kelly et al., 1974a). This was confirmed by cloning the rat PRLR cDNA (Boutin et al., 1988), which was followed by cloning PRLR cDNAs from other species. Sequence comparison between the PRLR and the receptors for various other cytokines and growth factors led to the identification of a family of receptors termed class 1 cytokine receptor superfamily. This superfamily includes receptors for several interleukins, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), Oncostatin M (OM), erythropoietin (EPO), thrombopoietin (TPO), gp130, and the obesity factor leptin (Taga and Kishimoto, 1992; Stahl and Yancopoulos, 1993; Finidori and Kelly, 1995; Wells and de Vos, 1996). Two main features distinguish the members of the cytokine receptor superfamily from other categories of membrane-bound receptors. First, they have a single transmembrane domain (see below, transmembrane domain). This is in contrast to the group of G-protein coupled receptors. The second feature is that they all lack an intrinsic kinase activity in contrast to receptor tyrosine kinases. In addition, all these receptors contain stretches of conserved domains, both in the extracellular and the intracellular regions.

**2.2 The PRLR Gene**

The first PRLR form to be cloned in the rat encoded a membrane bound receptor for PRL, however, when its sequence was compared to the GHR and the EPOR it was predicted that a longer form of the PRLR must exist. Indeed, further cloning efforts revealed that multiple isoforms of the membrane-bound PRLR exist in various species. Two forms of the PRLR have been identified in rat tissues, called the long and short forms. The long form consists of 591 amino acids long, whereas the short form consists of only 291 amino acids. The long form is encoded by a gene which is arranged into 11 exons spanning more than 70 Kb. The short form shares the same first nine exons with the long form however alternative splicing arises at the 10th and $11^{th}$ exons which leads

to a transcript that encodes a shorter variant of the PRLR, the short form (Kelly et al., 1991b). A third form of the PRLR was identified in a rat derived pre-T lymphoma Nb2 cell line and was subsequently called the Nb2 form. The Nb2 form is similar to the long form however it has a deletion removing a region of 198 amino acid from the cytoplasmic domain of the long form of the PRLR, hence the Nb2 form is composed of 393 amino acids. (Ali et al., 1992) (Figure 1.1).

Similar to the rat, a number of mouse PRLR forms exist. Two of the receptor forms have a similar size, being composed of 292 and 303 amino acids, and have identical extracellular and transmembrane domains, however, their cytoplasmic domains are different. A third PRLR from is truncated. These multiple PRLRs are expected to be due to alternative RNA splicing (Davis and Linzer, 1989).

Human tissues express multiple forms of the PRLR. In a study, long and short forms of the PRLR were isolated from the breast carcinoma cell line, T47D. The nucleotide sequences are identical except that the short form lacks 191 amino acids in the intracellular domain resulting from a frameshift mutation. Scatchard analysis revealed comparable affinity for PRL for both the long and the intermediate forms of the human PRLR (Kline et al., 1999). In a separate study, two short forms of the hPRLR (S1a and S1b) were identified and found expressed in several normal tissues and in breast cancer cell lines. These short forms were expressed as cell surface receptors with binding affinities similar to the long form. The two short forms are derived from alternative splicing of exons 10 and 11. S1a encodes 376 amino acids that contains part of exons 10 and 11. S1b encodes 288 amino acids that lacks all of exon 10 and contains part of exon 11. When expressed with the long form of the PRLR, both human short forms negatively affected long form activation of the β-casein gene promoter induced by prolactin (Hu et al., 2001).

## 2.3 Structure of the PRLR

### 2.3.1 The extracellular Domain (ECD)

Within a given species, whichever the isoform of the PRLR is, the extracellular domain is identical. In addition, the highest sequence similarities found among class I cytokine receptor superfamily members are within their extracellular domains. The extracellular domain of the PRLR, similarly to other cytokine receptors, is composed of a domain of approximately 200 amino acids (in rat and human PRLR it is 210 amino acids). This domain is called the cytokine receptor homology (CRH) region (Wells and de Vos, 1996). The CRH has two sub-domains of approximately 100 amino acids each called D1 and D2. These sub-domains resemble fibronectin type III modules (Kelly et al., 1991a, Bazan, 1990) and are the main determinants of ligand interactions with the receptors (Wells and de Vos, 1996; Kossiakoff et al., 1994). In addition to the fibronectin-like domains, two almost universally conserved features are found in the extracellular domains of all cytokine receptor superfamily members. The first feature is that they contain two pairs of disulfide-bridged cysteines in the D1 (N-terminal) subdomain (in human PRLR, Cys12-22 and Cys51-62). The second feature is found in the D2 subdomain (C-terminal) and it is a region called the "WS motif" (doublet Trytophan-Serine separated by one non-specific amino acid W-S-X-W-S) (Figure 1.1).

#### 2.3.1.1 Three-Dimensional Structure of the Extracellular Domain

Crystallographic analysis performed on the extracellular domain of the human PRLR, gave insights into the three dimensional structure of the domain (Somers et al., 1994). Each of the fibronectin-like subdomains, D1 and D2, has seven β-strands that fold into a sandwich formed by two antiparallel β-sheets. The first sheet is composed of three strands called strands A, B, and E, and the second sheet is composed of four strands called strands C, C9, F, and G (de Vos et al., 1992; Wells and de Vos, 1996; Somers et

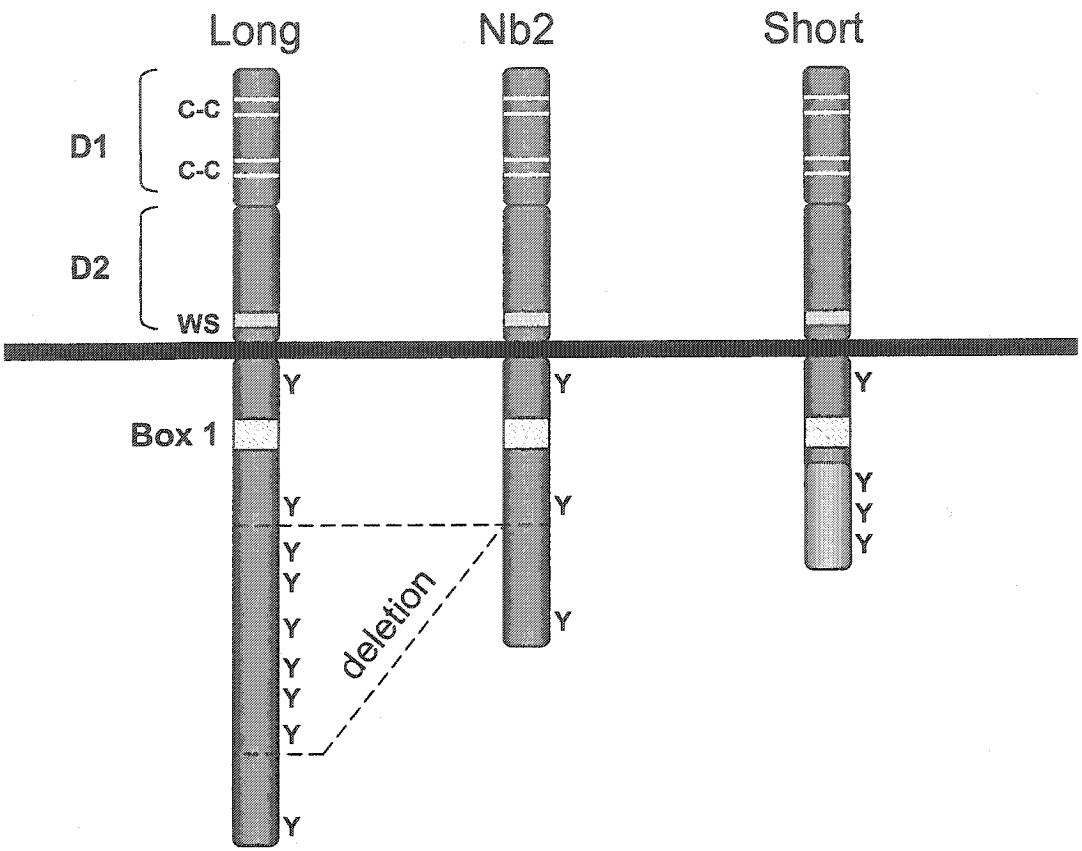


Figure 1.2 The long form of the PRLR has 9 intracellular tyrosines and is composed of 591 amino acids. The Nb2 form is encoded by a deletion mutant of the PRLR gene and is composed of 393 amino acids. The Nb2 form contains 3 intracellular tyrosines. The short form of the PRLR results from alternative splicing of the long form and it is composed of 291 amino acids. The intracellular region of the short form contains 4 tyrosines, the last three are different from the tyrosines present in the other two forms of the PRLR. The extracellular domains are identical in all forms and are formed by two fibronectin type III modules (D1 & D2), contain 4 cysteine residues that form disulphide bridges and a conserved WS motif. The box 1 region is present in all forms and it is the region of interaction with Jak2 kinase.

al., 1994) (Figure 1.1). The two subdomains are linked by a four-residue polypeptide. Based on sequence comparison for various cytokine receptor superfamily members the pattern of folding displayed for the PRLR is likely to be shared by several, or possibly all cytokine receptors (Bazan, 1990). A similar folding pattern was described for the extracellular domains of the human GHR (de Vos et al., 1992), the EPOR (Caravella et al., 1996; Livnah et al., 1996) and the α-chain of the interferon (IFN)-γ receptor (Walter et al., 1995).

**2.3.1.2 PRL Binding to the Extracellular Domain**

A limited number of studies have been performed to understand specific interactions between PRL and its receptor. The importance of the pair of disulfide-bonded cysteines and the WS of the extracellular domain of the PRLR in ligand binding was revealed by using mutational studies (Rozakis-Adcock and Kelly, 1991; Rozakis-Adcock and Kelly, 1992). A single mutation of any of the conserved cysteines led to a functionally inactive receptor (Rozakis-Adcock and Kelly, 1991). Similarly, mutations within the WS motif were detrimental to PRL binding affinity of the receptor (Rozakis-Adcock and Kelly, 1992; Baumgartner et al., 1994). Interestingly, structural data indicated that the WS motif of the PRLR is not involved in direct binding of PRL (Somers et al., 1994). Evidence gathered from functional studies performed on a number of cytokine receptors suggested that the WS motif is probably required for the appropriate folding of the extracellular domain rather than binding the ligand (Baumgartner et al., 1994, Quelle et al., 1992; Sandowski et al., 1995). In addition, based on sequence comparisons of the PRLR and a number of cytokine receptors, two tryptophan residues (in the rat PRLR, Trp72 and Trp139) may represent a feature required in ligand binding to these receptors (Goffin et al., 1995). The PRLR contains three asparagine-linked glycosylation sites in the extracellular domain but studies eliminated a role for these glycosylation sites in

ligand binding (Somers et al., 1994; Sandowski et al., 1995; Buteau et al., 1998; Ferrag et al., 1998; Pezet et al., 1997a)

### 2.3.2 The Transmembrane Domain

A distinctive feature that differentiates cytokine receptor superfamily members from other types of membrane-bound receptor forms (i.e. GPCRs) is the possession of a single transmembrane domain. The transmembrane region of the PRLR is a single-pass transmembrane domain of 24 amino acids in length composed of hydrophobic residues (in the rat PRLR, amino acids 211–234).

### 2.3.3 The Intracellular domain

Among the various cytokine receptors family members, the intracellular domains display more divergence than the extracellular domains. There is little homology except for two regions that show some level of conservation, named box 1 and box 2 (Kelly et al., 1991b; Murakami et al., 1991). The box 1 region is a membrane-proximal domain composed of 8 proline rich amino acids (conserve Proline-X-Proline motif) and hydrophobic residues (in the rat PRLR amino acids 243–250). The region is conserved in all PRLR isoforms. The second region, box 2, consists of a series of hydrophobic, negatively charged, and positively charged residues (in the rat PRLR amino acids 288–298). For the case of the rat PRLR isoforms, box1 is found in the long, Nb2 as well as short forms of the PRLR, whereas box 2 is not found in the short form (Kelly et al., 1991b). Another conserved feature among PRLR isoforms is the presence of a region containing a dileucine motif (in the rat PRLR, amino acids 259–260). This motif is suspected of being involved in receptor internalization (Vincent et al., 1997).

## 3 Signal Transduction by the PRLR

### 3.1 Activation of the PRLR

Various studies performed to understand the kinetics of activation of the PRLR indicated that the PRLR dimerizes upon ligand binding. Analyzing interactions between PRL and the extracellular domain of the PRLR by using surface plasmon resonance technology indicated the formation of 1:2 complexes (one ligand, two receptor molecules) (Gertler et al., 1996). Two regions of human PRL are involved in the binding to the PRLR. The first region, called site 1, is formed by a number of residues from helices 1 and 4 (Goffin et al., 1995; Kinet et al., 1996). The region, called site 2, is formed by residues from helices 1 and 3 (Goffin et al., 1995; Goffin et al., 1994). The formation of PRLR homodimers during the activation process occurs in a sequential manner. Initially, an interaction of binding site 1 of PRL takes place with one PRLR molecule leading to the formation of an inactive complex. Formation of this complex is followed by an interaction between binding site 2 with another PRLR molecule, forming an active complex composed of one hormone and two PRLR molecules in a homodimer (Goffin et al., 1996a).

### 3.2 Tyrosine phosphorylation

A number of studies performed using the three forms of the rat PRLR demonstrated that they all are membrane-anchored and bind prolactin with comparable affinity. However, only the long and the Nb2 forms are able to transduce PRL's signal (Lesueur et al., 1991; O'Neal and Yu-Lee, 1994). The signaling capacity of the PRLR was assessed by their ability to stimulate the transcription of milk protein gene promoters (β-casein and β-lactoglobulin) and the early immediate gene, interferon regulatory factor 1 (IRF-1). A specific function for the short form of the receptor has not been determined. One study suggested that this form may contribute to the mitogenic activity of prolactin. It was

demonstrated that both the short and long forms of the receptor induce the activation of the mitogen-activated protein (MAP) kinase pathway (Das and Vonderhaar, 1995). Therefore, it appears that, while both forms stimulate the cells to multiply, only the long form is able to transduce a differentiation signal.

Studies were also performed to understand the functional significance of the different regions of the receptor in mediating signaling to the responsive milk protein gene promoter. The proline-rich Box1 region is conserved in the three natural forms of the receptor and is necessary for the association/activation of Jak-2 (Lebrun et al., 1995b) (refer to section3.3.1.1, Jak Family of Cytoplasmic tyrosine Kinases). It was observed that after stimulation with prolactin, phosphorylation of both the receptor and Jak-2 take place and these phosphorylation events were required for activation of the responsive β-casein gene promoter (Lebrun et al., 1994). The exception is the short form of the PRLR, where the receptor itself was not phosphorylated despite the fact that Jak2 did become phosphorylated. Since the short form of the PRLR is not able to transduce PRL's signals, it appears that Jak2 activation alone without receptor phosphorylation is not sufficient for the transmission of the PRLR signal (Kelly et al., 1994). Therefore, tyrosine phosphorylation of the PRLR is critical for the mediation of PRLR signaling activity.

In a subsequent study examining the various PRLR tyrosines, the most C-terminal tyrosine residue of the PRLR was identified as the site of receptor phosphorylation that mediates activation of the β-casein promoter (Lebrun et al., 1995a). The C-terminal tyrosine in the long form of the PRLR, tyrosine 580, is equivalent in sequence to tyrosine 382 in the Nb2 form due to the genetic deletion which removes an internal motif present only in the long form of the PRLR resulting in the shorter Nb2 form (Ali et al., 1991). The C-terminal tyrosine in the Nb2 form appeared as the major, or possibly the only

phosphorylation site. In contrast, mutation of tyrosine 580 in the long form of the receptor did not affect the overall phosphorylation of the PRLR. In a study to determine the other potential tyrosine phosphorylation sites on the long form of the PRLR, it was determined that in addition to tyrosine 580, PRL is able to stimulate phosphorylation of tyrosines 473 and 479, and in cells overexpressing JAK2, also tyrosines 309, 402, and 515 can be phosphorylated (Pezet et al., 1997b).

Regardless of what other tyrosine residues are phosphorylated, the C-terminal tyrosine plays a critical role in PRLR signaling. Since the PRLR is known to form a dimer upon ligand stimulation, a study showed that the presence of the C-terminal tyrosine on both monomers of the dimerized PRLR complex is required to achieve a fully functional PRLR (Berlanga et al., 1997). Similarly, a separate study reported that mutation of tyrosine 382 within the intracellular domain of the Nb2 form of the PRLR in a dimer PRLR strongly decreases PRL induced proliferation of BA/F3 cells (Chang et al., 1998). In addition to the C-terminal tyrosine, other tyrosine residues of the PRLR may also participate in signaling. For example, a study suggested an important role of tyrosine 309 in the transcriptional activation of the IRF-1 promoter (Wang et al., 1997), possibly through Stat1 activation. Similarly, dimerization of a chimeric receptor composed of the GM-CSFR extracellular and the PRLR intracellular domain in which a single cytoplasmic domain lacks tyrosine 309 could only achieve partial activation of JAK2 and reduced proliferation potential of BA/F3 cells (Chang et al., 1998).

In addition to the role of receptor phosphorylation, a second determinant in signaling appears to be the dimerization between the different forms of the PRLR. Heterodimerization of any of the forms of the rat PRLR cytoplasmic domains, short/long, short/Nb2, long/Nb2, fails to induce proliferation of BA/F3 cells (Chang et al., 1998). This observation is of significance since tissues normally simultaneously express different forms of PRLR.

### 3.3 Signal Transduction by cytokine receptors

The PRLR, being a member of the cytokine receptor superfamily, not only shares structural homology with the other members of the family, but also shares many of the intracellular signaling components. All the biological and physiological functions of PRL result from binding of the hormone to its receptor in the respective target tissue, which leads to the activation of a cascade of intracellular signaling events leading to cellular responses such as activation of gene transcription.

#### 3.3.1 The Jak/Stat pathway

Following the cloning of cytokine receptors and the various structural and functional studies it was revealed that unlike growth factor receptors, cytokine receptors do not possess a cytoplasmic kinase domain. However, it was observed that the interaction of a cytokine with its receptor induced tyrosine phosphorylation of the receptors themselves as well as a variety of cellular proteins, which suggested that signal transmission by cytokine receptors occurred through cellular tyrosine kinases (Kishimoto et al., 1994). A new family of protein tyrosine kinases was identified by using low-stringency hybridization and PCR approaches, and was called the Jak family of kinases (for Janus kinase) (Wilks et al., 1991; Harpur et al., 1992). Later members of the Jak family of kinases were linked to cytokine receptors signaling where it was observed that these kinases could selectively phosphorylate specific members of the signal transducers and

activators of transcription (Stats), leading to their activation. The identification of Jaks and Stats established the Jak/Stat pathway as an essential pathway in the mediation of cytokine receptors signal transduction. Signaling through the Jak/Stat pathway is initiated when a cytokine binds to its corresponding receptor which leads to conformational changes in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the Jak family of kinases. The Jaks, in turn, mediate phosphorylation on themselves as well as on their perspective receptors that serve as docking sites for Stats and other signaling molecules.

#### 3.3.1.1 Jak Family of Cytoplasmic Tyrosine Kinases

It has been firmly proven now that cytoplasmic Jak kinases are crucial components of all cytokine receptors signal transduction pathways that control vital cellular responses such as survival, proliferation, differentiation and apoptosis (Taga and Kishimoto, 1992; Finidori and Kelly, 1995; Wells and de Vos, 1996; Ihle, 1994; Ihle et al., 1998). The Jak family includes four members, Jak1, Jak2, Jak3, and Tyk2. Cytokine receptors either utilize a single member or a combination of several Jak kinases to transmit the signal within the cell. Optimal Jak kinase activity is a critical determinant for normal transmission of cytokine signals resulting in regulated changes in gene expression. Studies examining Jak kinases expression indicated that Jak1, Jak2 and Tyk2 are expressed ubiquitously, whereas the expression of Jak3 is restricted to cells of the myeloid and lymphoid lineages (Leonard and O'Shea, 1998).

The chromosomal locations of both murine and human Jak kinases have been determined. Human Jak1 was mapped to chromosome 1p13.3 (Pritchard et al., 1992), Jak2 was mapped to chromosome 9p24 (Pritchard et al., 1992). The Jak3 gene was mapped to human chromosome 19p13.1 (Kumar et al., 1996; Riedy et al., 1996) and TYK2 was mapped to chromosome 19p13.2 (Firmbach-Kraft et al., 1990). The

corresponding murine genes were also mapped with JAK1 on chromosome 4, JAK2 on chromosome 19 and JAK3 on chromosome 8 (Kouro et al., 1996) in the mouse.

#### 3.3.1.2 Structure of Jak Kinases

The four mammalian members of the Jak family, Jak1, Jak2, Jak3 and Tyk2 are each around 1000 amino acids in length and range in molecular weight from 120 to 130 kDa. Sequence comparisons among the members of the Jak family revealed seven regions of high homology, and hence were called Jak homology (JH) domains (Figure 1.2). With the exception of the JH1 and JH2 domains, the biological activities of the ramaining domains have not been completely elucidated (Leonard and O'Shea, 1998; Ihle, 2001)

##### 3.3.1.2.1 The JH1 Tyrosine Kinase Domain

The conserved most carboxy terminal JH1 domain contains the typical features of a protein tyrosine kinase (Hubbard and Till, 2000), which include conserved tyrosines that constitute a critical component of the activation loop (Y1038/Y1039 in Jak1, Y1007/Y1008 in Jak2, Y980/Y981 in Jak3, Y1054/ Y1055 in Tyk2) (Leonard and O'Shea, 1998). Phosphorylation of these tyrosines drives the conformational changes that facilitate the binding of substrates (Hubbard and Till, 2000).

###### 3.3.1.2.1.1 General Characteristics of Protein Tyrosine Kinases

Protein tyrosine kinases possess catalytic domains ranging from 250 to 300 amino acids (Hanks et al., 1988). The location of the kinase catalytic domain in most enzymes lies near the carboxy terminus of the molecule, whereas the amino terminus performs a regulatory role. Kinases can be grouped into members of either the Src subfamily, Tec subfamily or one of the three different growth factor receptor subfamilies, the EGF-receptor subfamily, the insulin receptor subfamily or the PDGF-receptor subfamily. In addition to the catalytic kinase domain, tyrosine kinases contain certain modular domains that enable them to interact with diverse signaling molecules. These domains

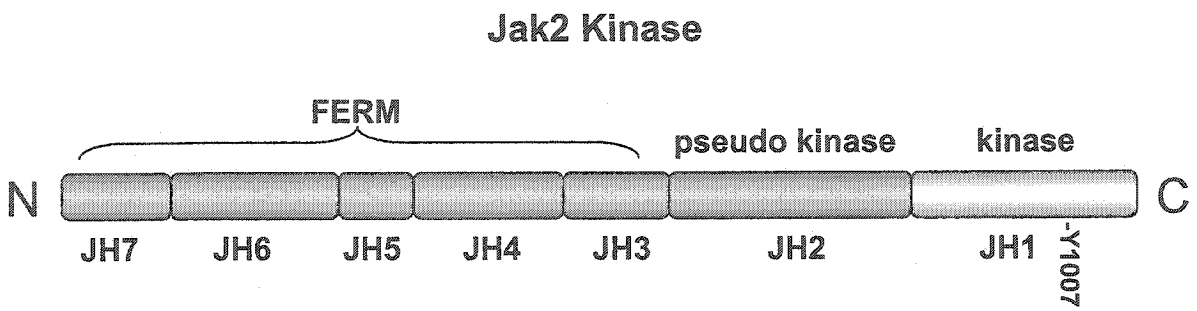


Figure 1.3 Structure of Jak kinases. There are 7 regions of homology among Jak kinases called Jak homology (JH) domains. JH1 is the kinase domain. JH2 is a pseudokinase domain. JH2 has all the features of a kinase domain except a catalytic activity. The N-terminal region (JH3-JH7 domains) is called the FERM domain based on regions of homology to Four-point-one, Ezrin, Radixin, and Moesin proteins and may mediate protein-protein interactions participate in association of Jaks to cytokine receptors. Tyrosine (Y1007) is in the activation loop of Jak2.

could either be Src-homology 2 (SH2), Src-homology 3 (SH3) domains, the pleckstrin homology domain (PH), a negative regulatory tyrosine in the carboxy terminus or myristylation or palmitoylation lipid modification sites at the N-terminus (Cooper and Howell, 1993). The PH domain and the lipid modification sites are considered to be important for attachment of the kinases to membranes. The PH domain may also facilitate association of other signaling proteins (i.e. substrates) to membranes thereby bringing them in close proximity to the kinases. It is worth noting that all these association domains are not unique to kinases but are present in various cellular signaling proteins.

**3.3.1.2.2 The JH2 Pseudokinase Domain**

Jak kinases are distinguished from other protein tyrosine kinases by the presence of a pseudokinase domain (Ihle, 2001). The JH2 domain has most of the structural features of a true tyrosine kinase, however it lacks a catalytic activity. The specific function of the pseudokinase is not clear, however, there is some evidence indicating that it regulates activity of the kinase domain. For example, in the case of Jak2 kinase, deletion of the JH2 domain led to increased activation of the kinase (Leonard and O'Shea, 1998). In the cases of Jak3 and Tyk2, various mutations in the JH2 domain induced modulations (increase/decrease) of the kinase activity (Velazquez et al., 1995; Yeh et al., 2000).

**3.3.1.2.3 The Amino Terminus**

The amino-terminal region of Jak kinases is divergent among various family members. The region was shown to mediate the association to receptors and to play an important role in determining the specificity of the interaction (Leonard and O'Shea, 1998; Ihle, 2001). Studies performed using amino-terminal chimeric Jaks indicated that the JH6 and JH7 regions serve as minimal receptor association elements for Jak2 and Jak3 (Chen et al., 1997; Kohlhuber et al., 1997). However, for the case of Tyk2, an additional JH5

segment is required for association with the IFN-α receptor (Yan et al., 1998). In the case of Jak1, the whole N-terminal region was required for binding to the IFN-α receptor (Kohlhuber et al., 1997). Furthermore, structural studies determined that the JH4–JH7 region shares homology to regions in certain proteins (Four-point-one, Ezrin, Radixin, Moesin) and was therefore called the FERM domain. This domain is known to mediate protein–protein interactions (Girault et al., 1998). For example, studies suggested that the FERM domain of Jak1 was critical for interaction with the cytoplasmic portion of gp130 receptor (Hilkens et al., 2001).

**3.3.1.3 Activation of Jak Kinases**

The precise mechanism of Jaks activation is not fully known. Results obtained from various studies allowed the development of a model to explain the mechanism of Jak kinases activation. The activation of the kinases is normally measured by in vitro kinase assays that measure the tyrosine phosphorylation of substrates. Jak kinase complexed with un-liganded receptor is in a catalytically inactive state. Depending on the specific cytokine receptor type, receptor dimerization or oligomerization due to ligand interaction results in the juxtapositioning of the Jaks either through homo- or heterodimeric interactions resulting in their phosphorylation either through autophosphorylation and/or transphosphorylation by other Jak kinase or possibly by a member of another tyrosine kinase family, such as members of the Src family. This activation results in an increase in Jak kinase activity. The activated Jaks then further phosphorylate themselves and phosphorylate the associated receptors on tyrosine residues. These phosphotyrosine sites on Jaks and on the receptors serve as docking sites that allow the recruitment of SH2-domain-containing signaling molecules such as Stats, Src-kinases, protein phosphatases such as SHP-1 and SHP-2, and adaptor signaling proteins such as Gab1, Gab2, Shc, Grb2 and Cbl (Barber et al., 1994; Zhuang et al., 1994; Frank et al., 1994)

Several investigative studies support this model. For example, ligand stimulation of the EPOR was shown to rapidly induce receptor dimerization leading to Jak2 activation (Watowich et al., 1999). Also, studies with a chimeric receptor that combined the extracellular domain of the EGF receptor with the cytoplasmic domain of the EPOR provided supporting evidence for the role of receptor dimerization/oligomerization in Jak kinase activation (Ihle et al., 1995). Additionally, a chimeric construct containing the Jak2 tyrosine kinase domain linked to the extracellular and transmembrane regions of the EGFR induced tyrosine phosphorylation of the EGFR/Jak2 chimera and Stat5 (Nakamura et al., 1996).

The association between Jak kinases and cytokine receptors occurs through membrane proximal regions that include Box1 and Box2 regions. The Box 1 motif contains approximately eight proline amino acids that were shown to be required for interaction of several cytokine/growth factor receptors with Jak2. The Box 1 region of the receptors for prolactin, growth hormone, erythropoietin, IL-6, and all cytokines that utilize the gp130 signal transducing subunit, is required for association with Jak2 (Witthuhn et al., 1993; Miura et al., 1994; Tanner et al., 1995; VanderKuur et al., 1994; Hackett et al., 1995; DaSilva et al., 1994). The specificity of interaction between Jak kinases and their perspective receptors is controlled primarily by the proline rich Box1 motif. Interestingly however, despite the fact that the Box1 region is believed to adopt the typical folding of SH3-binding domains (Lebrun et al., 1995b), no consensus SH3 domains were identified in Jak kinases (Kouro et al., 1996). Studies were performed where the Box1 regions from different receptors were swapped which led to a change in the specificity of Jak kinase association. For example, IL-2 stimulation leads to the specific activation of Jak3 but not Jak2, whereas EPOR stimulation activates Jak2. By switching the Box 1 region of the EPOR with that of the β-chain of the IL-2R, the associated Jak2 kinase was also replaced accordingly. The IL-2R carrying the EPOR Box1 was able to activate Jak2

rather than the usual Jak3 kinase (Jiang et al., 1996). A similar study demonstrated the importance of the membrane proximal domains of the prolactin receptor in association and activation of Jak2 (DaSilva et al., 1994). Also, the Box1 and Box2 regions of gp130 were shown to be required for association and activation of Jak1 and Jak2 (Narazaki et al., 1994).

#### 3.3.1.4 Biological Functions of Jaks

##### 3.3.1.4.1 Jak1

Various studies have indicated that Jak1 is involved in intracellular signaling by many members of the cytokines receptors superfamily. For example, members of the IL-2 receptor family (IL-2R, IL-7R, IL-9R and IL-15R), the IL-4 receptor family (IL-4R, IL-13R), the gp130 receptor family (IL-6R, IL-11R, LIF-R, OSM-R CT-1R, CNTF-R, NNT-1R/BSF-3R and Leptin-R) and class II cytokine receptors (type I IFN-R, type II IFN-R, IL-10R (Schindler and Strehlow, 2000) all associate with and activate Jak1. These receptors play critical and central roles, therefore, Jak1 knockout mice exhibited an early post-natal lethality. Analysis revealed that these mice suffered from a neurological lesion that made them defective in suckling (Rodig et al., 1998). Since a similar defect was identified in LIFR knockout mice, it was suggested that a loss in LIF function was the reason for this neurological defect (Ware et al., 1995). Similarly, the response to LIF and IL-6 was significantly reduced in Jak1 -/- tissues. Knockout mice for Jak1 were clearly defective in thymocyte and B cell production. This debilitated lymphopoiesis was attributed to defective responses to IL-7, which is important in lymphocyte development (Rodig et al., 1998). In the same manner, Jak1 -/- mice were also defective in their response to IL-10 (von Freeden-Jeffry et al., 1995). Therefore, Jak1 is a critical signaling mediator of biological responses for a number of major cytokine receptor sub-families.

#### 3.3.1.4.2 Jak2

Various studieds identified that Jak2 is a central signaling component for members of the single chain receptors (PRLR, EPOR, TPOR, and GHR), the IL-3 receptor family (IL-3R, IL-5R and GM-CSF-R), the gp130 receptor family and the class II cytokine receptor family (Schindler and Strehlow, 2000). Jak2 gene knockout in mice resulted in an embryonic lethality, where mice died at day 12.5 of gestation. Death was attributed to the lack of erythropoiesis. The same phenotype was observed with EPO -/- knockout mice (Wu et al., 1995; Neubauer et al., 1998; Park et al., 2000). These studies implicated Jak2 as an essential mediator EPOR signaling. The same Jak2 -/- mice were also unresponsive to TPO, IL-3, IFN-γ and GM-CSF (Parganas et al., 1998). Therefore, Jak2 plays a critical role in transducing signals for EPO, IL-3, GM-CSF, IL-5, TPO and IFN-γ.

#### 3.3.1.4.3 Jak3

Based on the pattern of expression, it is suggested that the role of Jak3 in cytokine signal transduction is restricted to hematopoietic cells (Leonard and O'Shea, 1998). Various studies revealed a major role for Jak3 in signaling by the receptors that employ the common γC receptor chain (i.e. IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, and IL-21R (Leonard and O'Shea, 1998). Jak3 knockout mice grow normally in a pathogen free environment (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995). However, they have severe defects in lymphopoiesis, similar what was observed in mice deficient in γC, the partner receptor subunit for Jak3 association (Cao et al., 1995). In addition, Jak3 plays an important role in IL-2 signaling. Jak3 -/- T cells are defective in negative selection (Saijo et al., 1997). In humans, patients that have Jak3 inactivating mutations, have severe combined immuno-deficiency (SCID), which was attributed to a profound T cell defect (Leonard and O'Shea, 1998). Jak3 -/- mice are defective in their response to

IL-2, IL-4 and IL-7, yielding a SCID-like phenotype. Therefore, Jak3 plays a vital role in lymphoid development.

#### 3.3.1.4.4 Tyk2

Tyk2 kinase was the first member of the Jak family to be identified and was the first implicated in cytokine signaling. It was found during screening for mutants in IFN-α signaling (Velazquez et al., 1992). Various studies performed with Tyk2 revealed that it contributed to IL-6, IL-10 and IL-12 signaling (Leonard and O'Shea, 1998). Tyk2 knockout mice unexpectedly gave somewhat relatively subtle defects in IFN-a/β signaling. Tyk2 -/- mouse embryonic fibroblasts exhibited normal antiviral responses with IFN-α. Similarly, the ability of Tyk2 -/- mice to resist viral infection was only modestly different from wild-type mice (Karaghiosoff et al., 2000). Further investigation determined that the mice were defective in their response to IL-12, that was however not completely absent. In contrast, Tyk2 null macrophages exhibited a significant defect in their response to LPS (Bogdan et al., 2000). Therefore, Tyk2 appears to be important in mediating the biological response of primarily IL-12 and LPS.

### 3.3.1.5 Jak kinases and PRLR signaling

Like all other members of the cytokine receptor superfamily, the cytoplasmic tail of the PRLR lacks any enzymatic activity including kinase activity (Kelly et al., 1991b; Boutin et al., 1989). However, it was identified that PRL stimulation of the PRLR leads to tyrosine phosphorylation of several cellular proteins, including the receptor itself (Rui et al., 1992), and tyrosine kinase inhibitors were shown to inhibit the mitogenic effects in the PRL responsive Nb2 cells (Wilks et al., 1991). This led to the identification of Jak2 as the kinase associated with the PRLR (Campbell et al., 1994; Lebrun et al., 1994; Rui et al., 1994).

#### 3.3.1.5.1 Association of JAK2 with the PRLR

Jak2 is the major PRLR-associated Jak kinase. This was shown in a study using mutant cell lines defective in Jak1 or Jak2 (Han et al., 1997). It was determined that milk protein gene induction required Jak2 kinase, whereas Jak1 kinase was dispensable. In fact, Jak2 was shown to be constitutively associated to the PRLR (Lebrun et al., 1994; Goupille et al., 1997). This is in contrast to what was reported for the GHR, where Jak2 association was dependent on ligand stimulation (Argetsinger et al., 1993). The interaction between the PRLR and JAK2 requires the membrane-proximal Box1 region of the PRLR intracellular domain (Lebrun et al., 1995b). As described earlier, the Box1 region includes the motif that is enriched in proline residues (sequence in the rat PRLR, residues 243-250 I-F-**P**-**P**-V-**P**-G-**P**). Mutational studies using alanine substitutions of individual proline residues within Box 1 motif of the rat PRLR showed that the most C-terminal proline (P250) was critical for association of the PRLR with Jak2 and activation of the kinase (Pezet et al., 1997a). Studies showed that for the purpose of activation of Jak2 kinase, an isomerization of the second proline of the Box1 motif of the receptor leads to the regulation of the activation in an On/Off switch manner (O'Neal et al., 1996). The concept agrees with what was reported using mutational studies that the second proline residue of the Box1 motif play a critical role in the activation of Jak2 kinase in both the PRLR and the GHR (Pezet et al., 1997a; Wang and Wood, 1995; Dinerstein et al., 1995). In addition to the Box1 region, residues toward the C terminus are also thought to be important. For example, in the rat short form of the PRLR amino acid 291 associates with Jak2 kinase (Lebrun et al., 1995b). Specific studies examining the region of Jak2 that interacts with the PRLR have not been performed.

#### 3.3.1.5.2 Activation of JAK2 by the PRLR

Homodimerization of the PRLR was reported to be required for the activation of Jak2 (Frank et al., 1995), however, the specific tyrosine residues of JAK2 that are phosphorylated after PRLR activation are not all known. As was expected, box 1-deleted PRLR which was unable to associate with Jak2 was unable to induce tyrosine phosphorylation of the kinase (Lebrun et al., 1995b). In an experiment using chimeras of GM-CSF receptor ($\alpha$ and $\beta$) extracellular domain fused to the intracellular domain of the PRLR, it was demonstrated that heterodimerization of the short and the Nb2 forms of the PRLR results in inactive complexes that are unable to stimulate Jak2 activation (Chang and Clevenger, 1996). This is interesting since both are able to associate with and activate the kinase when they homodimerize (Lebrun et al., 1995b). This indicated that the intracellular region of the PRLR, which is different between the short and Nb2 forms (Kelly et al., 1991b), mediates critical events for the activation of Jak2 kinase. In a different study using chimeras of GM-CSFR extracellular domains fused to the intracellular domain of the PRLR, it was revealed that heterodimerization of either the long or Nb2 forms of the PRLR with the short form of the PRLR was detrimental to Jak2 activation (Chang et al., 1998). In the same study, it was observed that mutation of tyrosines 309 and 382 within the same PRLR chain also prevented Jak2 activation, which suggested that juxtapositioning of identical cytoplasmic domains was required for proper activation of Jak2 kinase. A different study showed that heterodimerization through the expression of the short form of the PRLR with either the long or the Nb2 forms of the PRLR in several cell lines (CHO cells, 293 cells, and bovine mammary gland epithelial cell), resulted in the inhibition of the activation of milk protein gene transcription (Perrot-Applanat et al., 1997; Berlanga et al., 1997). Due to the fact that the activation of Jak2 by the PRLR occurs quite rapidly (1 min) after PRL stimulation

(Lebrun et al., 1995b), Jak2 kinase plays a central and an upstream role in the activation of signaling pathways of the PRLR.

### 3.3.1.2 Signal Transducers and Activators of Transcription (Stat)

The best characterized signaling pathway activated by cytokine receptors is the Jak/Stat pathway. Stat proteins are well known substrates for Jak kinases and activation of all cytokine receptor superfamily members leads to the activation of a single member or a combination of different members of the Stat family. These transcription factors were originally described as ligand-induced transcription factors in interferon-treated cells (Darnell et al., 1994). Further studies showed that Stats play critical roles in signal transduction pathways of cytokines including interleukins, interferons, prolactin, erythropoietin, growth hormone, oncostatin M, and ciliaryneurotrophic factor (Darnell et al., 1994; Darnell, 1997). There are now seven mammalian members identified within the Stat family, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (Darnell, 1997). They all are proteins containing 750-850 amino acids and are characterized by containing a number of conserved domains. In addition to full length members, alternative splicing and post-translational cleavage reactions generate additional forms of Stat1 and Stat3. For example, two isoforms of Stat1 have been described (Stat1$\alpha$, p91 and Stat1$\beta$, p84). These two isoforms were discovered associated with Stat2 and a protein termed p48 that constituted a multi component transcription factor called ISGF3 (Schindler et al., 1992; Muller et al., 1993). Stat3 also has two isoforms that might result from alternative splicing or proteolytic cleavage (Schaefer et al., 1995). Furthermore, there are two forms of Stat5, called Stat5a and Stat5b. These two forms do not result from alternative splicing, rather they are encoded by two separate genes (Lin et al., 1996; Copeland et al., 1995).

#### 3.3.1.2.1 Structure of Stats

The seven identified mammalian members of the Stat family of transcription factors share a number of common features. They all contain six conserved domains connected with each other by large interdomain linkers, indicating that structural changes induced in one domain may induce changes in other domains (Figure 1.3).

##### 3.3.1.2.1.1 The N-terminal Domain

N-terminal domain of Stats is approximately 130 amino acids and is conserved among all the Stats (51% sequence identity between Stat1 and Stat4; 20% between Stat5 and Stat6). By itself, it forms an independently folded and stable domain, which was shown to be cleaved from the full-length molecule by limited proteolysis (Vinkemeier et al., 1996). The crystal structure of the N-terminal 124 amino acids of Stat4 revealed the formation of a dimer of Stat molecules (Vinkemeier et al., 1998). The interface of this dimer is formed by a ring-shaped element consisting of five short helices. Various studies indicated that N-terminal dimerization of Stats promotes cooperativity of binding to tandem GAS elements (Vinkemeier et al., 1996; Xu et al., 1996; Vinkemeier et al., 1998). Studies also demonstrated that the N-terminal domain of Stats promotes association with the transcriptional coactivator CBP/p300 (Horvath, 2000); the PIAS family (refer to section on PIAS proteins) (Shuai, 2000); and association to receptor domains (Leung et al., 1996; Murphy et al., 2000). In addition, it was suggested that the N-terminal domain also plays a role in nuclear translocation of Stats (Strehlow and Schindler, 1998).

##### 3.3.1.2.1.2 Coiled-Coil Domain

The coiled-coil domain consists of four $\alpha$-helices and is linked to the N-terminal domain by a flexible polypeptide chain of approximately 20 amino acids. Crystal structures of Stat1 (Figure 1.3) and Stat3 reveal that this coiled-coil domain extends about 80 A°

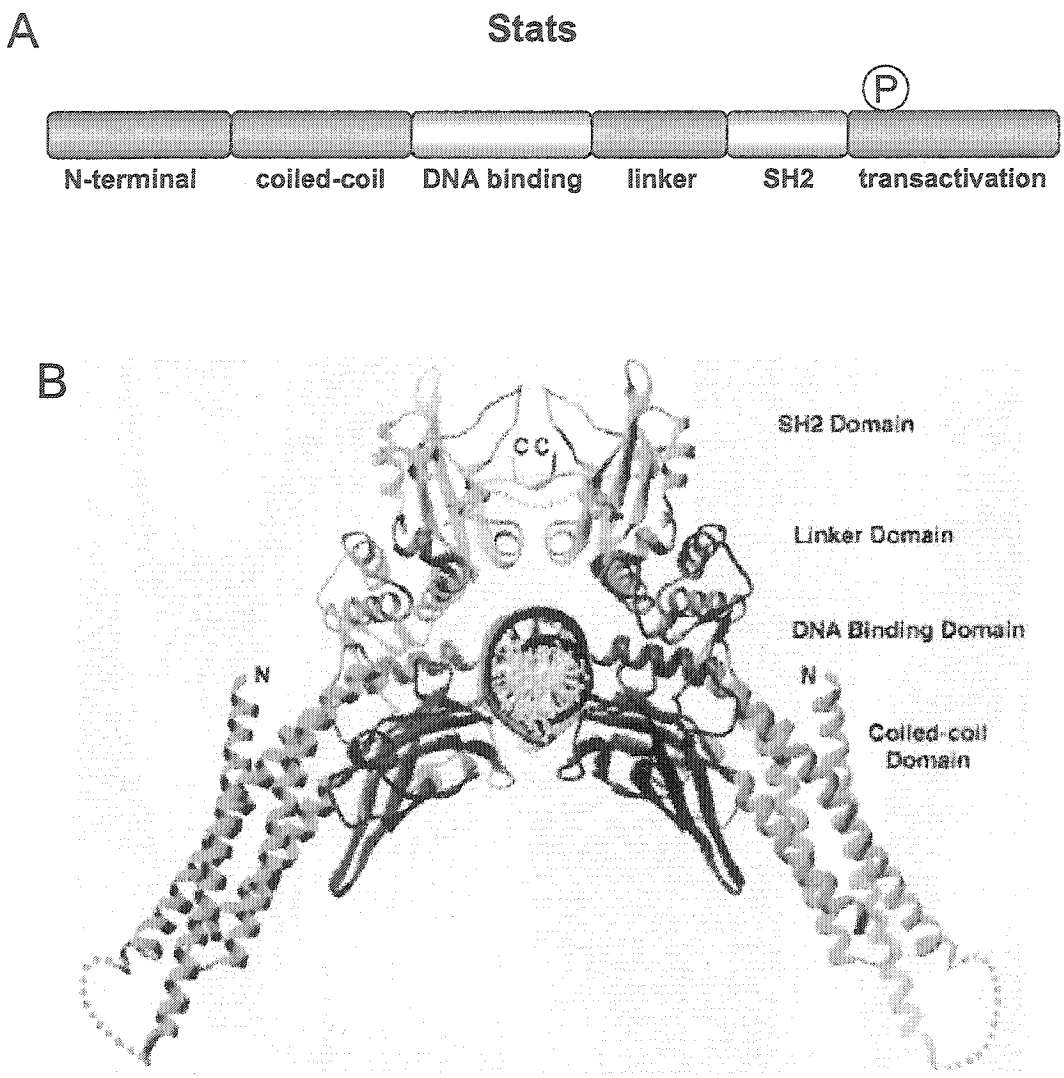


Figure 1.4 Structure of Stats. A. Stat proteins are composed of six domains, the N-terminal domain, the coiled coil domain, the DNA binding domain, the linker domain and the transactivation domain. P, is the conserved tyrosine. B. Crystal structure of Stat1 core dimer (amino acids 136–710) bound to DNA showing the various domains of Stat1 (Chen et al., 1998).

laterally from the core structure which forms a large hydrophilic surface that is available for specific interactions with other helical proteins (Becker et al., 1998; Chen et al., 1998b). Different studies reported that interacting proteins in this domain include p48/IRF9, the transcription factor c-Jun, N-myc interacting protein (Nmi), and StIP (Horvath et al., 1996; Zhang et al., 1999b; Zhu et al., 1999). Various studies have also implicated the coiled-coil domain in association to receptors, in tyrosine phosphorylation and in nuclear export of Stats (Begitt et al., 2000; Zhang et al., 2000).

**3.3.1.2.1.3 DNA-Binding Domain**

The DNA binding domain spans a region of approximately 160 amino acids. It forms a β-barrel with an immunoglobulin-like fold and lies carboxy-terminal to the coiled-coil domain. The structure of the DNA binding domain resembles that of the NF-κB and p53 DNA binding domains (Chen et al., 1998b). Each Stat component in the dimer of Stats recognizes bases in the most proximal half GAS element (see section 3.3.1.2.3, Stats DNA-Binding Sites). Cooperativity in DNA binding is suggested to be important in transcriptional activity. Additionally, since the DNA binding domain is likely to exhibit a different conformation prior to activation, it is possible that it may have additional functions (McBride et al., 2000).

**3.3.1.2.1.4 Linker Domain**

The linker domain is the region that connects the DNA-binding domain to the SH2 domain of Stats. Crystal structure studies of Stat1 (Figure 1.3B) revealed that helix α10 of the linker domain interacts with the SH2 domain below the phosphate binding loop, and that helix α6 interacts with strand β11 of the DNA binding domain which suggested that DNA binding capacity can be regulated by structural changes in the SH2 domain as it binds to a phosphotyrosine (Chen et al., 1998b). Studies have also implicated the linker domain of Stat1 in transcriptional regulation (Yang et al., 1999).

#### 3.3.1.2.1.5 SH2 Domain and Tyrosine Activation Motif

SH2 domains mediate critical functions for Stat proteins in signaling by binding to phosphotyrosine motifs. The domain is the most highly conserved motif in Stat proteins. The Stat SH2 domain spans residues 580–680 and it consists of an anti-parallel β-sheet flanked by two α-helices, which form a pocket. An absolutely conserved arginine, which mediates the interaction with phosphate side chain of the phosphorylated tyrosine, lies at the bottom of this pocket (Arg-602 for Stat1, Arg-609 for Stat3). The essential role of the SH2 domain is the recognition of specific phosphotyrosine motifs and therefore recruitment to the cytokine receptor/Jak complex (Gupta et al., 1996; Barahmand-Pour et al., 1998). The SH2 domain also participates in Stats homo- or heterodimerization (Shuai et al., 1994; Gupta et al., 1996). Crystal structures revealed that Stat dimerization occurs through an interaction between the SH2 domain of one Stat monomer with the tyrosine phosphorylated tail segment of the other monomer. Residues most involved in defining the specificity of the interaction between the SH2 domain and a tyrosine motif are located at positions +1, +3, +5, +6, and +7 C-terminal to the phosphotyrosine. Closely positioned amino acids of the SH2 domain (e.g. Ala-641, Val-642) appear to participate in this interaction (Chen et al., 1998b). All Stats except Stat2 have been shown to form stable homodimers in vitro and in vivo. Additionally, many Stats, including Stat2, can heterodimerize with other STATs through this reciprocal SH2-phosphotyrosine interaction (Schindler et al., 1995; Darnell, 1997).

#### 3.3.1.2.1.5 Transcriptional Activation Domain

The carboxy-terminal transcriptional activation domain is poorly conserved among the Stat family members. The domain regulates unique transcriptional responses for each Stat. The discovery of the transcriptional activation domain came from comparing the full length Stat1α with an alternatively spliced isoform that has a 38 amino acid

truncation at the C-terminus, Stat1β (Schindler et al., 1992). C- terminally truncated isoforms have also been identified for Stat3, Stat4 and Stat5 (Schindler and Strehlow, 2000). In all cases, C-terminal truncations function as dominant-negative regulators. A complete understanding of how the Stat carboxy-terminus regulates transcription remains to be determined. Some evidence indicates that the transcriptional activity of several Stats can be modulated through serine phosphorylation. Serine phosphorylation appears to enhance the transcription of some target genes. It has been suggested that serine phosphorylation may regulate the association with transcriptional regulators like MCM5 and BRCA1, but not CBP/p300 (Decker and Kovarik, 2000).

**3.3.1.2.2 Nucleocytoplasmic Transport of Stats**

Stat proteins in unstimulated cells reside primarily in the cytoplasm and upon stimulation with the perspective ligand translocate to the nucleus and induce gene expression. Stats translocate back to the cytoplasm after the termination of the signal. The bidirectional translocation of Stats is an essential step for signaling and is mediated by the nuclear pore complex (Doye and Hurt, 1997).

**3.3.1.2.2.1 Nuclear Import**

The nuclear import is an active process mediated by a short amino acid sequence termed the nuclear localization signal (NLS). The NLS binds to members of the nuclear receptor family of proteins, called importins (Moroianu et al., 1995). An NLS segment is composed of a short stretch of basic amino acid residues (arginines and lysines). Generally NLS elements are either monopartite (e.g. SV40 large T-antigen (Kalderon et al., 1984)), bipartite (e.g. nucleoplasmin (Robbins et al., 1991)) or tripartite (Pokorska et al., 2000). Bipartite and tripartite NLSs are comprised of clusters of basic amino acid residues separated by variable length spacers.

Importins form a family of proteins called the karyopherins (Moroianu et al., 1995). The NLS is recognized by importin-α and then functions as an adapter that binds to importin-β (Gorlich et al., 1994). Importin-β then interacts with the nuclear pore complex and mediates the transport of cargo into the nucleus. GTP hydrolysis by the Ras-like small GTPase Ran, provides the energy required for cargo translocation through the channel of the NPC (Moore and Blobel, 1993).

**3.3.1.2.2.2 Nuclear Import of Stats**

Stat proteins are known to rapidly translocate to the nucleus following ligand stimulation. This process was found to require tyrosine phosphorylation of Stats on the conserved tyrosine, however, it was independent of association to the cytoskeleton (Lillemeier et al., 2001). Studies performed on Stat1 revealed that nuclear translocation was dependent on Ran GTPase in the IFN-γ system (Sekimoto et al., 1996). A number of studies were done to locate the NLS of Stat proteins through mutagenesis of arginine/lysine-rich motifs. Interestingly, mutagenesis of an arginine/lysine-rich element located in the DNA-binding domain of Stat1 (Lys-410 to Arg-418) and Stat2 (Arg-409 to Lys-415), blocked IFN-induced nuclear import. These mutants also functioned as dominant negative molecules, by inhibiting the nuclear import of endogenous Stats. (Melen et al., 2001). However, this arginine/lysine rich element is not conserved in Stat5a, Stat5b, and Stat6. This suggested that two arginine/lysine-rich elements, one in each STAT monomer, are required for Stat nuclear import. Furthermore, these mutants were not able to bind to DNA elements. In addition to mutants in the arginine/lysine rich sequences, several Stat mutants in the DNA binding domain were found to be defective in nuclear import. Stat1 and Stat5 mutants in DNA binding motifs failed to translocate to the nucleus (Herrington et al., 1999). Similarly, mutants in the amino terminal segment of Stats are also defective in nuclear import (Strehlow and Schindler, 1998; Haspel and

Darnell, 1999). Based on the finding that several cytokine receptors and ligands contain putative NLS elements, a model has been proposed where Stats nuclear translocation is directed through the association with other signaling components (Subramaniam et al., 2000). Therefore, the area of Stats nuclear import remains elusive and much work remains to be done.

**3.3.1.2.2.3 Nuclear Export**

Nuclear export of proteins is directed by specific sequences called the nuclear export signals (NES). These signals are characterized by a leucine rich amino acid sequence (Fornerod et al., 1997). NES sequence comparisons performed on various proteins revealed a loose consensus motif of L-$x_{(1-3)}$-L-$x_{(2-3)}$-L-x-L (L is leucine and x is any amino acid). Substitutions of leucines to isoleucines, valines and methionines is possible (Fornerod et al., 1997). NES signals are recognized by the export receptor CRM1. CRM1 was initially identified in a tightly bound complex to NPC protein interacting with the NES in a Ran-GTP dependent manner (Fornerod et al., 1997). An anti-fungal reagent, Leptomycin B (LMB), associates directly with CRM-1 and inhibits its ability to associate with NES elements (Fornerod et al., 1997; Kudo et al., 1998) and therefore has become an important agent for studying CRM-1 dependent nuclear export. Nuclear export of Stat proteins is better understood than nuclear import. In the case of Stat1, three NES elements have been reported (residues 195–205, 302– 314 and 399–410) (Begitt et al., 2000; McBride et al., 2000; Mowen and David, 2000). A comparison of the NES elements found in Stat1 with other Stats indicate that they are well conserved in the Stat family.

**3.3.1.2.3 Stat DNA-Binding Sites**

Stat proteins interact with specific DNA binding sites and in that sense they act as transcription factors. Early studies of IFN responses identified two different

transcriptional enhancers within the promoter elements of Stat target genes (Decker et al., 1997; Schindler and Brutsaert, 1999). Stat proteins involved in Type I IFNs (α, β, Limitin) signal through the formation of ISGF-3 complexes, which bind to IFN stimulated response elements (ISRE) and initiate gene transcription (Levy et al., 1988). The ISRE response elements have a consensus consisting of two tandem sequences, $AGTTTN_3TTTCC$. The ISGF-3 complex is composed of a number of proteins that include Stat1, Stat2 and IRF-9/p48. Stat1 and p48 make direct contact with the DNA whereas Stat2 does not (Qureshi et al., 1995). In contrast to type I IFNs, type II IFN (IFN-γ) signal through a Stat1 homodimer that bind to the γ-IFN activated sequence (GAS) (Decker et al., 1997). The GAS site is formed by a palindrome that has a consensus sequence of TTTCCNGGAAA. Similar responsive elements were identified in the promoters of other Stat activated genes. The consensus sequence for the optimal binding site for Stats was determined to be $TTCN_{2-4}GAA$. The spacing between palindromic half sites (N) provides an opportunity for most Stat homodimers to have distinct DNA binding preferences. For example, Stat1 binds to an element with a canonical n=3 spacing, while Stat3 favors elements where n=2, and for Stat6, n=4 (Decker et al., 1997; Ehret et al., 2001).

**3.3.1.2.4 Stat Proteins Activated in PRLR Signaling**

Three members of the Stat family have been so far identified as transducer molecules of the PRLR; Stat1, Stat3 and primarily Stat5. Initially Stat5 was identified as a transcription factor in the sheep mammary gland and hence was called the mammary gland factor (MGF) (Wakao et al., 1994). Based on the high structural similarity of MGF with the other Stat proteins, it was later renamed Stat5 (Gouilleux et al., 1994; Darnell et al., 1994). Stat5 cloning from mouse (Liu et al., 1995; Mui et al., 1995) rat (Kazansky et al., 1995)), and human (Silva et al., 1996) revealed the existence of two genes encoding

Stat5a and Stat5b which shows 90–95% homology. The major divergence between Stat5a and Stat5b lies within the C-terminal transactivation domain. The essential tyrosine phosphorylation site in Stat5 (Tyr 694) is conserved in both Stat5a and Stat5b (Gouilleux et al., 1994). It was demonstrated that Stat5a and Stat5b have functional redundancy (Moriggl et al., 1996). However, Stat5a and Stat5b single knockout mice show distinct phenotypes, and the Stat5a/Stat5b double knockout mouse exhibited a combined phenotype. Stat5a single knockout mice were defective in PRL dependent mammary gland development (Liu et al., 1996; Teglund et al., 1998). The Stat5a/Stat5b double knockout mice exhibited a more severe phenotype than Stat5a that was similar to PRLR knockout mice. Stat5a/Stat5b double knockout mice were both infertile and defective in mammary gland development, suggesting that Stat5b can compensate for the loss of Stat5a (Ormandy et al., 1997a; Ormandy et al., 1997b; Teglund et al., 1998). Stat5b single knockout mice exhibited defects similar to GHR deficient mice (Udy et al., 1997; Teglund et al., 1998).

Studies reported that PRLR mutants that were not able to associate and activate Jak2 kinase were similarly unable to activate Stat5 suggesting that Stat5 activation is strictly dependent on Jak2 activation (DaSilva et al., 1996; Pezet et al., 1997b). In addition, if the C-terminal domain of Stat5 is truncated removing the tyrosine phosphorylation site, such mutants function as dominant-negative forms (Miyajima et al., 1997; Mui et al., 1994; Mui et al., 1996; Onishi et al., 1996; Wang et al., 1996). Besides Jak2, both Jak1 and Jak3 were reported to phosphorylate Stat5 on tyrosine 694 (Ferrag et al., 1996).

The requirement of specific tyrosine residues of the PRLR in Stat5 activation remains under investigation. It was reported that tyrosines 580, 479, and 473 of the PRLR were sufficient to activate Stat5, with tyrosine 580 being the most potent (Pezet et al., 1997b). In contrast, others have reported that a C-terminal truncated form of the long PRLR (G328, lacking tyrosine 382) was competent to induce Stat5 tyrosine phosphorylation

(Frank et al., 1995). Other studies determined that the C-terminal tail of the rabbit PRLR was not required for Stat5 activation and for the transcriptional activation of PRL responsive genes such as the β-lactoglobulin gene (Goupille et al., 1997). These studies indicate that Stat5 tyrosine phosphorylation alone is not sufficient for the induction of transcriptional activity.

In addition to tyrosine phosphorylation, serine/threonine phosphorylation may also play a critical role in the activation of Stat5. It was reported that serine/threonine phosphorylation of Stat5 was an absolute requirement for transcriptional activation of Stat5 by the IL-2R (Beadling et al., 1996). Stat1, Stat3, Stat4, and sheep Stat5 contain potential MAP kinase serine phosphorylation sites (P-x-S-P) in their C-termini (Wen et al., 1995). In human, mouse, and rat Stat5a, but not Stat5b, a very similar tetrapeptide is found at the homologous position (R-L-S-P). Although this sequence does not perfectly match the MAP kinase consensus site, it might be a target for another proline-directed kinase (Beadling et al., 1996). PKCα and casein kinase II have been proposed as candidates for serine phosphorylation of Stat5 (Kazansky et al., 1995; Beadling et al., 1996). Although functional distinction between Stat5a and Stat5b remains to be investigated in detail, it might be partly due to differences in serine/threonine phosphorylation sites. In this respect, heterodimerization of Stat5a and Stat5b has been recently reported from several investigations (Quelle et al., 1996; Kirken et al., 1997). Moreover, it was shown that PRL stimulation of Nb2 cells induced phosphorylation of both Stat5a and Stat5b on serine, but not on threonine. The same study also reported that kinetics of serine phosphorylation were markedly different for both Stats (Kirken et al., 1997). Whether this observation explains the functional difference between these two closely related transcription factors requires further investigation.

In addition to Stat5, Stat1 and Stat3 were also reported to be activated by the PRLR (David et al., 1994; Lebrun et al., 1995b; DaSilva et al., 1996). The mechanism of activation of these two Stat members remains under investigation and the requirement of the PRLR and Jak2 kinase in the activation process is not fully known. It was shown that certain membrane-proximal residues were sufficient for tyrosine phosphorylation of both Stats 1 and 3 (DaSilva et al., 1996). In addition, it was reported that phosphotyrosines of Jak2 could bind to Stat3. This is in agreement with the presence of the consensus Stat3-binding site in Jak2 kinase (Sotiropoulos et al., 1996; Smit et al., 1996; Gupta et al., 1996).

Stat5 was shown to interact with a number of proteins which influence the transactivation potential. For example, Stat5 interacts with the glucocorticoid receptor (Stocklin et al., 1996) leading to functional cooperation requiring specific DNA binding of Stat5, but not of the glucocorticoid receptor (Stoecklin et al., 1997; Lechner et al., 1997). The biological significance of this association remains under investigation.

### 3.3.2 Regulators of Cytokine Receptors Signaling

#### 3.3.2.1 SHP-2 Protein Tyrosine Phosphatase

SHP-2 is a member of a family of protein tyrosine phosphatases that includes SHP-1, SHP-2 and a *Drosophila* homologue called the Corkscrew (Csw). Mammalian SHP-2 was cloned independently by several groups and was previously called Syp, SH-PTP2, SH-PTP3, PTP1D, or PTP2C (Adachi et al., 1992; Shen et al., 1991; Vogel et al., 1993). The phosphatase is a ubiquitously expressed cytoplasmic enzyme, in contrast to SHP-1 which is predominantly expressed in hematopoietic cells (Feng and Pawson, 1994; Neel, 1993). The human gene encoding SHP-2 was located to the chromosomal region 12q24.1 and transcription leads to a single, approximately 7 kb mRNA product that was

found in almost all tissues and cell lines investigated. Northern blot analysis revealed that SHP-2 is abundant in brain, heart and skeletal muscle (Adachi et al., 1992).

#### 3.3.2.1.1 Molecular Structure and Regulation of SHP-2

SHP-2 phosphatase, similar to SHP-1 and CSW, is characterized by containing two Src-homology 2 (SH2) domains at the N-terminus and one catalytic phosphatase domain at the C-terminus (Feng and Pawson, 1994; Neel, 1993) (Figure 1.4). After it was cloned, SHP-2 was found to physically associate with a variety of ligand-activated receptor protein tyrosine kinases (Feng et al., 1993; Vogel et al., 1993; Lechleider et al., 1993a). The interaction is mediated through the SH2 domains of the phosphatase and specific phosphotyrosine site(s) on the receptor. It was originally thought that binding of SHP-2 to a phosphotyrosine containing motif on receptors was only to localize the enzyme in the vicinity of substrates. However, studies found that occupation of the SH2 domain by a phosphorylated motif was also responsible for stimulate the phosphatase activity. For example, a peptide comprising a binding site for the SH2 domain of SHP-2 (pTyr1009) on PDGF receptor dramatically stimulated the catalytic activity of the phosphatase (Lechleider et al., 1993b). Therefore, the SH2 domain of SHP-2 has two functions in the modulation of the activity in cells. The first is to mediate the enzyme localization and association with its substrates, and second is to modulate the catalytic phosphatase activity. In addition, it was demonstrated that a peptide containing two phosphorylation sites derived from the insulin receptor substrate-1 (IRS-1) protein had a higher binding affinity for SHP-2 and was much more potent in stimulating the enzymatic activity than monophosphorylated peptides. Therefore, binding of the SH2 domains to two phosphorylated tyrosine containing motif might upregulate SHP-2 catalytic activity (Pluskey et al., 1995).

A study demonstrated that a truncated SHP-2 phosphatase lacking the SH2 domains exhibited enhanced phosphatase activity, compared to the wild-type full-length protein.

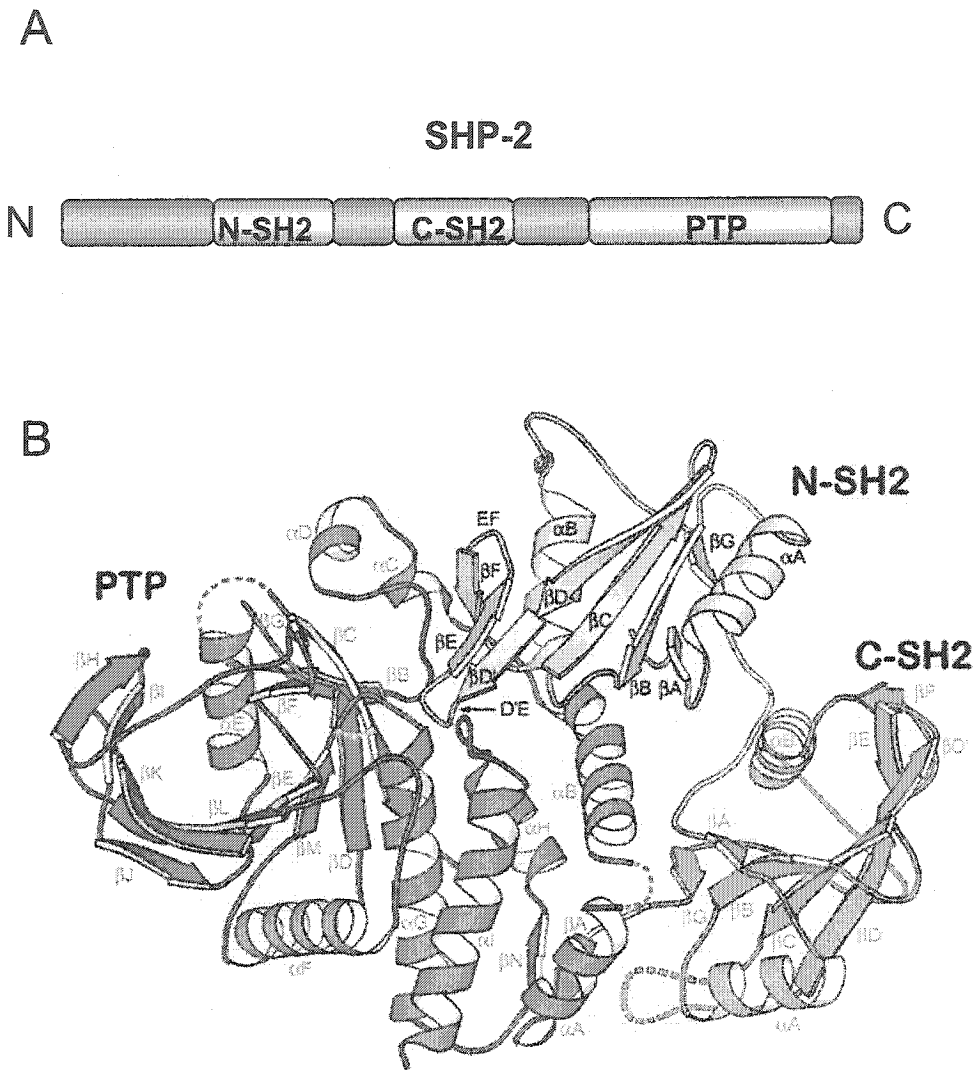


Figure 1.6 Structure of the phosphatase SHP-2. A. SHP-2 has two SH2 domains in the N-treminal part and a phosphatase domain in the C-terminal part of the protein. B. Crystal structure of residues 1–527 (lacking the 66-residue C-terminal tail) of SHP-2 indicated that the N-SH2 domain (D'E loop and the flanking D'E strands) is directly inserted into the catalytic cleft. Several hydrogen bonds between the N-SH2 domain and the phosphatase domain occupy the most critical sites in catalysis (Hof et al., 1998).

The finding suggested that unbound SH2 domains may suppress the enzyme activity through an intramolecular mechanism. The autoinhibitory mechanism is relieved upon association of the SH2 domain with a tyrosine phosphorylated motif (Zhao and Zhao, 1998; Dechert et al., 1994). Crystal structure studies of SHP-2 (residues 1–527, lacking the 66-residue C-terminal tail) (Figure 1.4B) supported the autoinhibitory observation. The structure revealed a broad interaction surface shared by the N-SH2 and the phosphatase domain. In particular, the D'E loop and the flanking D'E strands of N-SH2 domain are directly inserted into the catalytic cleft at the base of the phosphotyrosine-binding pocket. Several hydrogen bonds between the N-SH2 domain and the phosphatase domain occupy the most critical sites in catalysis (Hof et al., 1998). The catalytically essential residue Cysteine 459 is hydrogen bonded via one water molecule with Asparagine 61 in N-SH2 domain. This autoinhibited structure is further stabilized by a number of polar interactions outside the catalytic center. In addition, the EF loop of N-SH2 domain partially blocks its phosphopeptide-binding groove in the inactive "I" state. Intermolecular complexing of N-SH2 domain with a tyrosine phosphorylated motif diminishes the affinity of the intramolecular N-SH2 domain and the phosphatase domain interaction, leading to a switch to the active "A" state. Therefore, targeting of SHP-2 to associate with a target protein is directly coupled to stimulation of catalytic activity. The C-SH2 domain does not have extensive inter-actions with either the N-SH2 or PTP domain and, therefore, may not have significant influence on the activation of catalysis. However, the C-SH2 domain plays a part in the catalytic activity by increasing the local concentration of the phosphatase and presenting the N-SH2 domain to phosphorylated motifs allowing the activation. Thus, at least from this crystal structure study, it appears that the C-SH2 domain contributes to the substrate specificity and binding affinity (Hof et al., 1998).

SHP-2 was found to be phosphorylated on tyrosine residues in cells which were stimulated with different ligands (Feng and Pawson, 1994; Vogel et al., 1993). These tyrosine phosphorylated sequences within SHP-2 generate specific binding sites for other SH2 domain containing molecules that are involved in signal transduction cascades. One of the interacting proteins identified is the adapter protein Grb2 (growth factor receptor binding protein 2) (Bennett et al., 1994; Li et al., 1994). Furthermore, it has been suggested that tyrosine phosphorylation of SHP-2 could also influence the enzymatic activity of the phosphatase (Allard et al., 1996). This concept is difficult to prove since SHP-2 is subject to an autodephosphorylation mechanism resulting in a rapid loss of tyrosine phosphorylation under in vitro phosphatase assay conditions (Sugimoto et al., 1994).

The phosphatase domain of SHP-2 contains all residues required for catalysis including an essential cysteine residue that performs the nucleophilic attack on the phosphate of the substrate. Substitution of this cysteine in, phosphatases results in a substrate-trapping mutant, which is catalytically inactive but unaffected in its substrate affinity. These mutants lack a catalytic turnover which results in constitutive binding, "trapping", of their substrates and therefore they represent important tools to elucidate the biological function of protein tyrosine phosphatases (Timms et al., 1998).

**3.3.2.1.2 Roles of SHP-2 in Signaling**

SHP-2 plays a critical role in intracellular signalling not only from cytokine receptors but also from receptor tyrosine kinases. For example, SHP-2 is involved in signaling by receptors for several cyrokines like PRL (Ali et al., 1996; Berchtold et al., 1998), GH (Kim et al., 1998b); EPO(Tauchi et al., 1995; Tauchi et al., 1996), LIF(Boulton et al., 1994), IFNs (David et al., 1996), IL-2 (Adachi et al., 1997), IL-6(Kim et al., 1998a), and IL-11 (Fuhrer et al., 1995). Similarly, SHP-2 is a mediator of signaling of receptors for EGF (Feng et al., 1993), FGF (Tang et al., 1995), and PDGF (Feng et al., 1993;

Kazlauskas et al., 1993; Bennett et al., 1994). The phosphatase has been described to contribute either in a positive or a negative manner in signaling from these various receptors. It is believed that the specific role(s) in signaling depends on the specific system and cell type. Generally, within RTKs, SHP-2 was shown to play a positive role. As an example, the phosphatase positively mediates the activation ERK through assembling with the adaptor protein Gab1 downstream of the EGFR (Cunnick et al., 2000). It was shown that the catalytic activity and both SH2 domains were required for this function (Deb et al., 1998). Similarly, SHP-2 positively mediates the signaling from the FGFR by forming a complex with Gab1 and FRS2 upon FGF stimulation and leads to a sustained ERK/MAPK activation in PC12 cells (Hadari et al., 1998). A positive role has also been attributed to SHP-2 in PDGFR signaling where it was shown associate to a tyrosine residue on the PDGFR (Kazlauskas et al., 1993), dephosphorylate Ras-Gap recruitment site on the receptor leading to increased activation of ERK (Ronnstrand et al., 1999). In addition to the positive role, SHP-2 may also play a negative role in PDGFR signaling. It was demonstrated that mutating the SHP-2 recruitment site on PDGFR increased tyrosine phosphorylation of the receptor and prolonged the activation of MAPK (Mendez et al., 1996). In the HGF/SF receptors signaling system, SHP-2 was shown to play a positive role in the mediation of the activation of Ras and Rho small G proteins inducing cell scattering and also mediating an increase in ERK activation (Kodama et al., 2000). Similarly, SHP-2 catalytic activity was required for the sustained activation of ERK and epithelial morphogenesis downstream of the Met receptor in MDCK cells (Maroun et al., 2000).

With cytokine receptors, both positive and/or negative functions have been ascribed to SHP-2. For example, the phosphatase was demonstrated to positively mediate GHR signaling to the induction of c-fos gene (Kim et al., 1998b). Interestingly, the phosphatase was also shown to play a negative role in GHR signaling. In a study it was

shown that mutating SHP-2 recruitment site on the GHR led to prolonged duration of phosphorylation of GHR, Jak2 kinase and Stat5b (Stofega et al., 2000). A similar negative regulatory role for SHP-2 was proposed for the gp130 subunit of the IL-6 receptor system. It was reported that mutating the SHP-2 recruitment site on the gp130 subunit led to an increased signaling capacity for the receptor (Kim et al., 1998a). However, the conclusions derived from these studies may not be due to the function of SHP-2 but rather due to another molecule that becomes recruited to the same tyrosine residue. In a study it was shown that both SHP-2 and SOCS-3 are recruited to the same tyrosine residue on the gp130 subunit, with SOCS-3 having a high affinity for the tyrosine residue (Nicholson et al., 2000). Therefore, at least some of the negative roles that were described to SHP-2 might in fact be due the inhibitory actions of SOCS-3.

Some of earliest studies attempting to unravel the contribution of SHP-2 in signaling were performed to examine the role of the phosphatase in PRLR signaling events. It was shown that SHP-2 is a critical positive regulator of PRLR signaling activity. In a study it was demonstrated that SHP-2 is tyrosine-phosphorylated and physically associates with the PRLR-Jak2 complex upon stimulation with prolactin. Additionally, it was shown that using a catalytically inactivated mutant form of SHP-2, resulting from mutating the critical cysteine residue in the catalytic domain, led to the inability of the PRLR to induce the transcription of the β-casein milk protein (Ali et al., 1996). A second study reported a similar outcome where it was shown that using a negative form of the phosphatase, comprised the two SH2 domains without the catalytic domain, resulted in reduced tyrosine phosphorylation, DNA-binding activity and transactivation potential of Stat5a and Stat5b. In addition, the study also reported that the kinase activity of Jak2 was also dependent on a functional SHP-2 phosphatase. The study proposed that SHP-2 relieves an inhibitory tyrosine phosphorylation event in Jak2 required for Jak2 activity, Stat5 phosphorylation, and transcriptional induction (Berchtold et al., 1998). It is not

known how SHP-2 contibutes positively in the activation of Jak2 kinase leading to Stat5 tyrosine phosphoryaltion and induction of trascription of PRL responsive genes.

#### 3.3.2.1.3 Association of SHP-2 with Gab Adaptor Proteins

The Gab proteins form a family of docking proteins that includes Gab1, Gab2, Gab3, the Drosophila homolog DOS, and C. elegans homolog Soc1. Structurally, members of the family are closely related to insulin receptor substrates (IRS) and fibroblast growth factor substrate (FRS2) (reviewed by (Hibi and Hirano, 2000; Huyer and Alexander, 1999)). These proteins function as scaffolding proteins by recruiting signaling molecules through the multiple recruitment motifs that they possess.

#### 3.3.2.1.4 Gab Family Members

Gab1 was the first of three mammalian Gab proteins to be cloned. It was isolated by two groups separately. One group identified the protein as a Grb2-associated protein, hence the name Gab1 (Grb2-associated binder 1) (Holgado-Madruga et al., 1996). The second groups identified Gab1 as a Met-receptor interacting protein in a yeast two-hybrid screen and it was found as the major tyrosine phosphorylated protein in cells transformed by the Tpr-Met (Weidner et al., 1996; Fixman et al., 1997). Gab2 protein was cloned as a p97-p100 tyrosine phosphorylated protein that was associated with SHP2 and the p85 subunit of PI3K (Gu et al., 1998; Zhao et al., 1999). Finally, Gab3 protein was cloned based on sequence similarities to Gab1 (Wolf et al., 2002).

##### 3.3.2.1.4.1 Gab Structure

###### 3.3.2.1.4.1.1 PH domain

The PH domain is present in all Gab family members and it is the most conserved structural domain (Figure 1.5). PH domains can recognize phosphoinositides in cellular membranes and therefore target PH domain containing proteins to those membranes (Lemmon and Ferguson, 1998). PH domain of Gab1 was shown to bind to

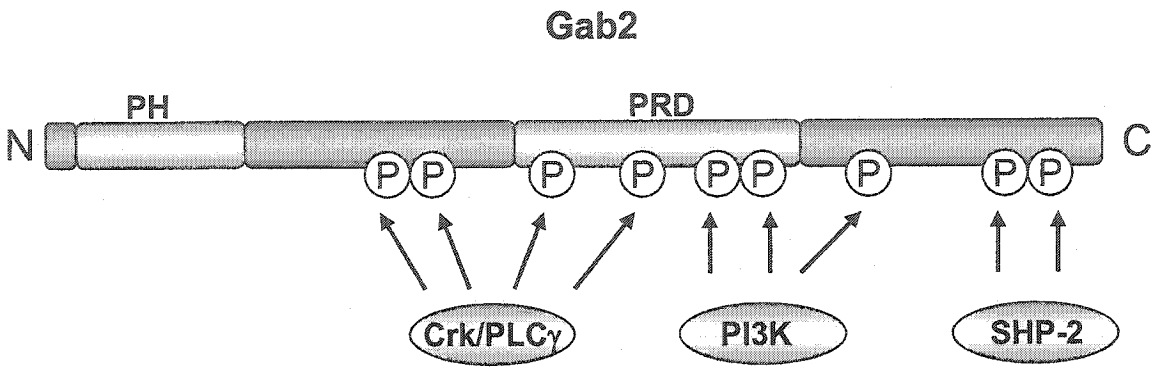


Figure 1.7 Structure of Gab proteins. All Gab family members contain a highly conserved PH domain in the N-terminal part. The central part is proline rich (PRD) and contains consensus binding sites for SH3 domain-containing molecules. Two tyrosine residues in the most C-terminal regions of the Gab family proteins have consensus binding motifs for SHP-2 phopsphatase. Other tyrosines have consensus binding motifs for Crk, PLCγ and p85 subunit of PI3K.

phosphatidylinositol 3,4,5-triphosphate (Rodrigues et al., 2000; Maroun et al., 1999; Isakoff et al., 1998). In addition, the PH domain of Gab1 was reported direct the localization of the protein to the areas of cell-cell contact (Maroun et al., 1999). Mutants of Gab1 with a deletion of the entire PH domain, or point mutations at phospholipid-binding sites, were unable to induce Met-dependent branching tubulogenesis in MDCK cells (Maroun et al., 1999).

**3.3.2.1.4.1.2 Proline-Rich Domain**

All Gab proteins were found to associate with the SH3 domain-containing protein, Grb2 (Holgado-Madruga et al., 1996; Gu et al., 1998; Wolf et al., 2002). The central part of Gab proteins is rich in prolines and contains multiple consensus binding sites for SH3 domain-containing molecules. The binding sites for Grb2 on Gab 1 were found to be two motifs with sequences PXXPXR and PX3RX2KP (Schaeper et al., 2000; Lewitzky et al., 2001). The Met-binding site (MBS) of Gab1, responsible for the binding of Gab1 to the phosphorylated Met receptor, is located within the PRD (Schaeper et al., 2000). Gab2 and Gab3 lack the MBS in their sequences and do not associate with the Met receptor.

**3.3.2.1.4.1.3 Phosphotyrosine Residues**

Gab proteins contain multiple tyrosine residues that undergo phosphorylation following stimulation of receptors. These phosphotyrosines serve as docking sites for interaction with SH2 domain-containing proteins.

**3.3.2.1.5 Gab/SHP2 Association**

Two tyrosine residues in the most C-terminal regions of the Gab family proteins have consensus binding motifs (YXXV/I/L) for SHP2 and all mammalian Gab proteins were shown to bind SHP2 (Gu et al., 1998; Wolf et al., 2002; Lehr et al., 1999) (Figure 1.5). The functional significance of the Gab/SHP2 interaction is still under investigation. Met

dependent morphogenesis and MAPK activation by EGF were defective when a mutant of Gab1 that is unable to bind to SHP-2 was expressed (Schaeper et al., 2000; Cunnick et al., 2000; Maroun et al., 2000). A Gab2 mutant unable to bind SHP2 blocked IL3-induced c-fos promoter activation and M-CSF-induced macrophage differentiation (Gu et al., 2000; Liu et al., 2001). In a study, it was determined that the interaction between Y627 and Y659 of human Gab1 and SHP-2 led to the activation of the phosphatase (Cunnick et al., 2001). Therefore, an important function for Gab proteins appears to be the recruitment and activation of SHP-2 phosphoatase.

**3.3.2.1.6 Other Associations**

In addition to SHP-2, Gab proteins also interact with a number of proteins. An association between Gab proteins and the p85-subunit of the PI3K has been reported. Several studies demonstrated the requirement of the association between p85 and Gab1 or Gab2 in mediating the activation of the PI3K/Akt signaling pathway (Rodrigues et al., 2000; Maroun et al., 1999; Gu et al., 2000).

Gab proteins also associate with members of the Crk family of proteins. Gab1 was shown to bind to CrkI and CrkL (Schaeper et al., 2000; Gual et al., 2000; Lamorte et al., 2000). It was reported that Gab1 associates with Crk in response to Met activation and this association correlates with anchorage-independent growth and JNK activation in cells transformed by the Met receptor (Garcia-Guzman et al., 1999; Lamorte et al., 2000). Gab2 has been found to interact with CrkL in yeast two-hybrid system and also in human T lymphocytes in response to IL2 (Gesbert et al., 1998; Crouin et al., 2001). The functional significance of the interaction between Gab2 and CrkL is not known.

### 3.3.3 Negative Regulators of Cytokine Receptors Signaling

#### 3.3.3.1 SOCS Proteins

The suppressor of cytokine signaling family of proteins plays critical roles in the negative regulation of cytokine signal transduction. These proteins act in a negative feedback loop inhibiting the cytokine-activated Jak/Stat signaling pathway and ultimately modulate cellular responses.

##### 3.3.3.1.1 Discovery of the SCOS Proteins

The founding member of the family, Socs1 was cloned in 1997 by three separate groups using different experimental approaches. One group cloned SOCS-1 as a protein that bound to the catalytic domain of JAK2 in a yeast two-hybrid screen and called it Jak-associated protein (JAB) (Yoshimura et al., 1995). The second group identified SOCS-1 using a functional screen for inhibitors of cytokine signaling (Haque et al., 2000). In that study, a myeloid leukemic cell line (M1) was infected with a retroviral cDNA library, and M1 colonies that failed to undergo interleukin-6 (IL-6)-induced macrophage differentiation were isolated. The cDNA in one IL-6-resistant M1 colony encoded an SH2 domain-containing protein, which was called suppressor of cytokine signaling 1 (SOCS-1). The third group identified SOCS1 as a protein which was recognized by an antibody directed against the Stat3 SH2 domain, and named it Stat-induced Stat-inhibitor (Chen et al., 1998b). Following the initial cloning of SOCS-1, database searches using amino acid sequence of SOCS1 revealed that more than 20 proteins in mice and humans share high sequence homology in a region of 40-residue located at the C-terminal motif, the region was called the SOCS box (Haque et al., 2000, Endo et al., 1997). One of the SOCS-box containing proteins was cloned earlier and was called the cytokine-inducible SH2-containing protein (CIS). This protein was identified as an immediate early gene induced by IL-2, IL-3, and erythropoietin. (Naka et al., 1997).

The proteins identified by database searches which contained the SOCS box were later classified within five subfamilies depending on the domains they contained in their central regions. Proteins containing a central SH2 domain were termed SOCS proteins, proteins containing WD-40 repeats were termed WSB proteins, those containing ankyrin repeats were termed ASB, and those containing SPRY domains were termed SSB. Database searches also revealed a *Drosophila melanogaster* ortholog of SOCS5 (Starr et al., 1997). Little is known about the WSB, ASB, SPRY, and SSB families of proteins. Similarly, within the SOCS family, SOCS-4, SOCS-5, SOCS-6 and SOCS-7 are also poorly understood. In contrast, SOCS-1, SOCS-2, SOCS-3 and CIS have been the subject of intensive research and their roles are fairly well characterized.

In contrast to the highly conserved SH2 domain and the SOCS box, the N-terminal regions of SOCS proteins are variable in length, ranging from 50-380 amino acids and show great divergence in sequence homology (Endo et al., 1997). In addition, the N-terminal regions contains no clearly conserved motifs (Figure 1.7). The exception is SOCS-7 which contains a possible nuclear localization signal and multiple proline-rich sequences (Masuhara et al., 1997).

**3.3.3.1.2 Induction of SOCS mRNA by Cytokines**

Using mRNA screening, transcripts encoding SOCS1, SOCS2, SOCS3, and CIS are often present in cells at low levels. However stimulation by various cytokines was observed to lead to a rapid induction of SOCS mRNAs both in vitro and in vivo. For example, SOCS1, SOCS2, SOCS3, and CIS mRNAs are all induced in response to stimulation with IL-2, IL-3, IL-4, IL-6, IFN-a, EPO, G-CSF, GM-CSF, LIF, and GH, (Gupta et al., 1996; Chen et al., 1998b; Naka et al., 1997; Vasiliauskas et al., 1999; Matsumoto et al., 1999; Ram and Waxman, 1999). PRL stimulation was reported to induce mRNA expression of SOCS-1, SOCS-2, SOCS-3 and CIS (Pezet et al., 1999).

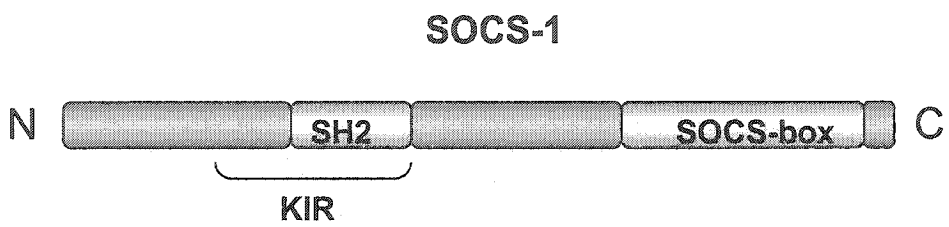


Figure 1.5 Structure of SOCS proteins. SOCS proteins have a variable N-terminal region with little homology among the various members of the family. Centrally, there is an SH2 domain conserved in all SOCS proteins. The 40 amino acid SOCS-box is located In the C-terminal part of the protein. SOCS-1 associates to the kinase domain of Jak2 through the SH2 domain and an additional 24 amino acids region called the kinase inhibitory region (KIR).

The pattern of SOCS induction appears to vary depending on the particular cell line being investigated. For example, IFN-α induces the expression of SOCS1 and SOCS3 mRNAs in the NIH-3T3 cell line, where as only SOCS1 mRNA is induced in the M1 cell line (Adams et al., 1998). In most cases, cytokine stimulation results in the upregulation of several SOCS family members.

SOCS proteins inhibit cytokine receptors signaling through a classical negative feedback loop. Activation of receptors leads to the initiation of signaling events ultimately leading to the induction of expression of responsive genes. SOCS proteins are among those genes activated. SOCS protein levels accumulate with time and then function to attenuate the signal by inhibiting various components of the signaling pathway (Naka et al., 1997).

Studies performed with CIS, SOCS-1 and SOCS-3 illustrated the negative feedback inhibition of SOCS proteins. CIS expression was induced and can inhibit signaling by PRL, (Tomic et al., 1999, Matsumoto et al., 1999, Pezet et al., 1999), EPO (Matsumoto et al., 1997), GH, (Matsumoto et al., 1999; Ram and Waxman, 1999; Adams et al., 1998), IL-2 (Naka et al., 1997; Starr et al., 1997; Matsumoto et al., 1999; Aman et al., 1999) and IL-3 (Starr et al., 1997; Matsumoto et al., 1997) Similarly, SOCS-1 expression is induced and inhibits signaling by PRL (Pezet et al., 1999; Tomic et al., 1999; Helman et al., 1998), GH (Ram and Waxman, 1999; Adams et al., 1998; Favre et al., 1999), IFNs (Nagai and Emi, 2001; Sakamoto et al., 1998; Losman et al., 1999), IL-4 (Endo et al., 1997; Losman et al., 1999; Haque et al., 2000), IL-6 (Endo et al., 1997; Naka et al., 1997) and TPO (Endo et al., 1997). SOCS-3 expression induction inhibits signaling by PRL (Pezet et al., 1999; Tomic et al., 1999; Helman et al., 1998), GH (Matsumoto et al., 1999; Ram and Waxman, 1999; Adams et al., 1998), EPO (Naka et al., 1997; Masuhara et al., 1997), GM-CSF (Masuhara et al., 1997), IFNs (Sakamoto et al., 1998; Song and Shuai, 1998), IL-6 (Naka et al., 1997; Nicholson et al., 1999), leptin (Bjorbaek et al.,

1998) and LIF (Masuhara et al., 1997; Bousquet et al., 1999). In some cases, the induction of SOCS proteins by one cytokine can inhibit signaling by another. For example, IL-6 upregulates SOCS1 expression in CD4+T cells which inhibits IFN-α signaling (Davey et al., 1999). Therefore, SOCS proteins might mediate crosstalk between different cytokine receptors.

**3.3.3.1.2.1 Mediation of SOCS Induction by Stats**

Among the genes activated by Stats are those encoding SOCS proteins. The promoter of the CIS gene contains four Stat5-binding sites, all of which are required for EPO dependent activation of transcription of the CIS promoter in reporter assays (Favre et al., 1999). Similarly, expression of the SOCS-1 gene is also regulated by the Stats. Furthermore, there is evidence that Stat5b regulates the expression of SOCS-3 gene expression. In fact, the GH-induced expression of SOCS-3 mRNA in liver is downregulated in mice lacking STAT5b, and it was found that STAT5b can bind to the Stat1/Stat3 element in the SOCS3 gene promoter (Ram and Waxman, 1999; Helman et al., 1998).

**3.3.3.1.3 SOCS Proteins Modes of Action**

**3.3.3.1.3.1 Inhibition of Jak Kinase Activity**

Despite the fact that SOCS proteins share significant structural similarity, their mechanisms of action seem to be different. For example, SOCS-1 can interact directly with Jak1, Jak2 and Tyk2, leading to the inhibition of their phosphorylation and catalytic activity and downregulation of phosphorylation of receptors and Stats (Yoshimura et al., 1995; Endo et al., 1997; Nicholson et al., 1999). For the purpose of inhibiting the catalytic activity, it was demonstrated that the SH2 domain and the N-terminal region of SOCS-1 are essential (Nicholson et al., 1999; Yasukawa et al., 1999). The SH2 domain interacts with phosphorylated tyrosine (Y1007) residing in the activation loop of Jak2. A

further ~ 30-amino acids N-terminal of the SH2 domain is involved in a high-affinity binding and inhibition of the kinase activity. This region was called the kinase inhibitory region (KIR) (Narazaki et al., 1998). The KIR presents Jak2 with a psuedosubstrate that binds Jak2 blocking the catalytic loop from access to substrates (Nicholson et al., 1999; Yasukawa et al., 1999). It was shown that in LIF signaling, the N-terminal domains of SOCS-1 and SOCS-3 could be interchangeable, but not with those of other SOCS proteins. That illustrated a possible functional overlap between SOCS-1 and SOCS-3 (Nicholson et al., 1999).

The mechanism of action of other SOCS proteins seems to differ. CIS, unlike SOCS-1, does not appear to interact directly with Jak kinases. CIS is induced by Stat5 in response to EPO, and binds EPOR in a phosphorylation-dependent manner (Matsumoto et al., 1997) and inhibits signaling by competing for the phosphorylated receptor residues that act as docking sites for Stats (Matsumoto et al., 1997; Starr and Hilton, 1998). The mechanism of SOCS-3 action seems to share similarities with that of CIS and SOCS-1. SOCS-3 coimmunoprecipitates with Jak2, however, overexpression of SOCS-3 does not inhibit the in vitro kinase activity of the kinase (Nicholson et al., 1999). Similar to CIS, SOCS-3 binds to phosphorylated receptors, including the leptin receptor, GHR, gp130, EPOR and the IL-2 receptor β chain (Hansen et al., 1999; Bjorbaek et al., 1999a; Sasaki et al., 2000; Bjorbaek et al., 1999b). Optimal SOCS-3 inhibition of GH-induced Jak2 activation signaling was shown to require high levels of the GH receptor, suggesting that SOCS-3 inhibits Jak2 when bound to activated receptor, an action which needs the N-terminal domain of SOCS-3 (Kamura et al., 1998). SOCS-3 binds with high affinity to the SHP-2 binding site on the gp130 and leptin receptors (Nicholson et al., 2000). SHP-2 is a known substrate of Jaks mediating positive induction of signaling event and have an influential effects on the activation of Jak2 (refer to section on SHP-2) and the MAPK signaling pathway. Thus, SOCS-3 competes with SHP-2 for a single

recruitment site on the gp130 receptor and hence prevents SHP-2 activation. Therefore, despite their structural similarity, CIS, SOCS-1 and SOCS-3 appear to inhibit signaling via distinct mechanisms.

In contrast to the demonstrated inhibitory roles of SOCS-1, SOCS-3 and CIS, the role of SOCS-2 is not yet clear. In fact, some evidence indicates that it does not effect signaling or might behave as a stimulatory factor. For example, a study illustrated that overexpression of SOCS-2 had no inhibitory effects on the phosphorylation status of the PRLR, Jak2, Stat5 or SHP-2 in response to PRLR activation (Tomic et al., 1999). In other studies, it was illustrated that SOCS-2 could reverse the inhibitory effect by 100% for SOCS-1 and CIS and by 50% for SOCS-3 by restoring Jak2 kinase activity in PRLR signaling [Pezet, 1999 #134. In a separate study, it was observed that at high concentrations, SOCS-2 was able to restore GHR signaling activity (Favre et al., 1999). Therefore, SOCS-2 appears to behave as an antagonist to the inhibitory effects of the other SOCS proteins. Additionally, it was demonstrated that a natural mutation in the SOCS-2 gene results in a larger than normal mice called high-growth. The phenotype is similar to the SOCS-2 gene knockout which was attributed to increased responses to GH/IGF-1 (Dif et al., 2001; Horvat and Medrano, 2001).

**3.3.3.1.3.2 Targeting of Proteins for Ubiquitination and Degradation**

The presence of the SOCS box in five distinct protein families has led to the suggestion that this domain may function as an adaptor via which divergent groups of proteins are linked to a common process. The SOCS-box appears to link SOCS-associated proteins to proteasomal degradation.

**3.3.3.1.3.2.1 Ubiquitination process**

Proteins are targeted for degradation by covalent linkage to chains of ubiquitin through the ubiquitination process. The first step in the process is the activation of free ubiquitin

by an activating enzyme, E1 (Ciechanover et al., 1981). In this step, the C-terminal glycine residue of ubiquitin is adenylated in an ATP-dependent reaction which results in the binding of ubiquitin to a cysteine residue of E1 via a thiolester linkage (Hough and Rechsteiner, 1986). The next step involves the transfer of ubiquitin to a carrier enzyme, E2. This similarly occurs through thiolester linkage to a cysteine residue (Hershko et al., 1983). In the last step, ubiquitin is transferred to a lysine residue on the target substrate in a reaction catalyzed by a third enzyme, E3 (Hershko et al., 1983). More ubiquitin molecules are then added to the first to form a chain, each linked to the next by an isopeptide bond between lysine 48 and glycine 76 (Chau et al., 1989). The critical determinant of the pathway that is responsible for selecting specific targets for ubiquitination and degradation is the E3.

**3.3.3.1.3.2.2 SOCS Proteins Act as E3 Ubiquitin Ligases**

Various studies illustrate that SOCS proteins function by targeting associating proteins to ubiquitination. In fact, this feature is not unique to SOCS proteins, but rather is a general function of all SOCS box containing proteins. Two studies showed that the SOCS box mediated an interaction between the elongin BC complex and various SOCS box-containing proteins including SOCS-1, SOCS-3, ASB-2, WSB-1 and RAR-1 (Kamura et al., 1998; Zhang et al., 1999a). Elongin BC was shown as complex which interacts with the von Hippel-Lindau (VHL) tumor suppressor protein (Kibel et al., 1995). A number of studies illustrated the role of VHL as an E3 ubiquitin ligase protein targeting associated proteins to ubiquitination (Cockman et al., 2000; Kamura et al., 2000; Pause et al., 1997). Additionally, it was demonstrated that elongin BC have an E1- and E2-dependent ubiquitin ligase activity (Kibel et al., 1995) and acts as a linker between VHL and the two proteins Cul2 and Roc1 that are known components of the ubiquitination pathway (Pause et al., 1997).

Studies showed that elongin BC binds the SOCS proteins through a sequence within the SCOS box that was called the BC box. The BC box has the consensus amino acid sequence of (T/S/P-L/M-X-X-X-C/S-X-X-X-V/L/I) (Kamura et al., 1998; Zhang et al., 1999a).

Various studies have demonstrated the role of SOCS proteins in regulating the stability of the associated partners through targeting of associated proteins to proteasomal degradation. A study demonstrated that signaling through the Jak/Stat pathway is sustained in the presence of proteasomal inhibitors (Hansen et al., 1999). In addition, CIS suppresses EPO signaling by binding to the activated EPOR and targeting it for ubiquitin-dependent degradation (Verdier et al., 1998). Similarly, SOCS-1 binds to the guanine nucleotide exchange factor Vav and targets it to ubiquitination and proteasomal degradation (De Sepulveda et al., 2000). Interestingly, a number of studies presented evidence that the SOCS box mediates ubiquitin-proteasome-dependent degradation of the fusion protein TEL-JAK2 (Frantsve et al., 2001; Kamizono et al., 2001; Monni et al., 2001). In one study, it was shown that expression of SOCS-1 in Ba/F3 cells transformed with TEL-JAK2 inhibited their IL-3-dependent growth. However, the inhibition of TEL-JAK2 kinase activity alone was not sufficient to explain the block in cellular transformation. It was found that SOCS-1 expression decreased protein levels of TEL-JAK2. The decrease was associated with SOCS-box-dependent ubiquitination of TEL-JAK2, and is reversed by treatment of cells with proteasomal inhibitors. Interestingly, the SOCS box of CIS can substitute for that of SOCS-1 in mediating TEL-JAK2 degradation, however the SOCS box of SOCS-3 can not (Kamizono et al., 2001).

It is not yet known whether SOCS proteins themselves are targeted for ubiquitinaiton. Studies indicate that CIS and SOCS-3 have both been shown to be unstable proteins that are degraded by the ubiquitin-proteasome system, and removal of the SOCS box from SOCS-3 had a stabilizing effect on the protein (Zhang et al., 1999a; Monni et al.,

2001). Therefore, SOCS proteins are important mediators in targeting associated proteins to proteasomal degradation and hence are critical regulators of the Jak/Stat pathway.

**3.3.3.1.4 Physiological Roles of SOCS Proteins**

Genetic knockout studies of SOCS proteins have provided valuable information about the essential role these proteins play in cytokines action in physiological systems. Gene knockout of the SOCS-1 gene in mice revealed that it is essential for survival beyond the postnatal period. Mice that had SOCS-1 gene deleted were born in good health and in the numbers predicted. However, within 10 days, SOCS-1 -/- mice were considerably smaller than their wild-type littermates, and died before they reached 3 weeks of age (Marine et al., 1999; Starr et al., 1998). The diseases in SOCS-1 -/- mice were complex and were accompanied by fatty degeneration and necrosis of the liver, atrophy of the thymus, marked reduction in lymphocytes in the lymphoid organs and circulation, and many tissues also exhibited inflammatory infiltrates, typically T-lymphocytes, granulocytes and macrophages (Starr et al., 1998). Also, evidence of accelerated apoptosis of lymphocytes within the thymus and spleen with up-regulation of Bax expression was also reported (Naka et al., 1998). Constitutive activation of Stat1 and over-expression of IFN-γ responsive genes in these mice suggested that the lethal disease might have resulted from excessive responses to IFN-γ (Naka et al., 1998; Starr et al., 1998).

In addition to the role of SOCS-1 in IFN-γ responses, an essential role in PRLR signaling was demonstrated. To rescue the SOCS-1 -/- mice from the lethality due to hyper-responsiveness to IFN-γ, a double knockout of SOCS1 and IFN-γ was created. The SOCS-1/ IFN-γ null mice exhibited accelerated lobuloalveolar development of the mammary gland during late pregnancy and precocious lactation. Therefore, the study

established that SOCS1 is a negative regulator of PRLR signaling in the mammary gland and suggest that SOCS1 is required for the prevention of lactation prior to parturition (Lindeman et al., 2001). Mice lacking SOCS-2 could not be distinguished from their normal littermates until weaning, but subsequently display accelerated growth, resulting in adult SOCS-2 -/- mice that are 30 to 40% larger than wild-type mice. The increase in body weight reflects a generalized and uniform increase in the size of most organs, as well as increased muscle mass and skeletal size, with evidence suggesting cell number within organs rather than cell size is increased (Metcalf et al., 2002). These data strongly suggest that SOCS-2 has a key regulatory role in the GH/IGF-I system of growth control without exhibiting hematopoietic abnormalities in SOCS-2 -/- mice (Metcalf et al., 2000).

Mice lacking SOCS-3 have been reported to die at mid-gestation with delayed development and excessive erythropoiesis (Marine et al., 1999). This observation combined with the findings that SOCS-3 associates with the EPOR and can inhibit EPO signaling when overexpressed (Sasaki et al., 2000) suggest that SOCS-3 plays a key role in regulation of EPO signaling in vivo.

Disruption of the CIS gene in mice results in female that fail to lactate and exhibit defective mammary gland differentiation. Similar phenotypes characterize mice lacking Stat5a and/or Stat5b and an important role for CIS in the regulation of these signaling intermediates has been proposed, particularly in the control of prolactin and GH (Starr et al., 1997).

**3.3.3.2 Other Negative Regulators of Cytokine Receptors Signaling**

In addition to SOCS proteins, other intracellular proteins have been identified and were shown to similarly downregulate the signaling capacity of cytokine receptors. Members of the PIAS family are an example.

#### 3.3.3.2.1 PIAS Family

The protein inhibitors of activated Stats (PIAS) proteins family was identified using a yeast two-hybrid screen designed to identify Stat-interacting proteins and the first member was cloned and called PIAS1 (Liu et al., 1998). Database searches using the PIAS1 sequence indicated that it is a member of a family of structurally related proteins. These proteins were found to have a putative zinc-binding domain, a highly acidic region, and approximately 50% homology at the amino acid level (Liu et al., 1998). PIAS proteins are constitutively expressed and associate to activated and dimerized Stats. For example, PIAS1 is constitutively expressed and binds to activated Stat1 dimers and inhibits their DNA-binding activity, whereas the protein does not bind to monomeric Stat1 (Liu et al., 1998). In a study, it was demonstrated that a region in the C-terminal part of PIAS1 interacts with a region in the N-terminal domain of the Stat1 dimer (Liao et al., 2000). PIAS3 is also a constitutively expressed protein that interacts with phosphorylated Stat3 molecules in IL-6-stimulated M1 cells and inhibits all Stat3-mediated gene transcription (Chung et al., 1997). Thus, members of the PIAS family target specific Stat molecules to inhibit their activities. Other members of the PIAS family probably inhibit cytokine signaling through similar mechanisms.

#### 3.3.3.2.2 SHP-1 Phosphatase

SHP-1 is an important negative regulator of a number of cytokine receptors signaling activities. Molecular cloning of SHP-1 revealed that it contains two SH2 domains preceding a phosphatase domain and it is predominantly expressed in hematopoietic cells (Shen et al., 1991). SHP-1 is known to be a regulator of B and T cell receptor signaling it has also been shown to suppress a variety of cytokine signaling systems. *Motheaten* mouse strains have mutations in the SH2 domain of SHP-1 and they suffer from loss of hair, major lymphoid and myeloid abnormalities, immunodeficiency, and

autoimmune diseases (Tsui et al., 1993; Westhoff et al., 1997). SHP-1 has also been shown to suppress the actions of EPO (Klingmuller et al., 1995), IL-3 (Yi et al., 1995), and IFN-γ (David et al., 1995). The mechanism by which SHP-1 inhibits these pathways is not yet fully known. It is thought to involve the deactivation of receptor kinases and Jak kinases. As an example, SHP-1 has been shown to bind to tyrosine-phosphorylated EPOR and to dephosphorylate Jak2 (Klingmuller et al., 1995). Therefore, SHP-1 is thought to function by dephosphorylating critical signaling components such as Jak2.

#### 3.3.3.2.3 CD45 Phosphatase

CD45 is a transmembrane phosphatase that is expressed on all hematopoietic cells and has been shown to be involved in T and B cell antigen receptor signaling and to have a role in regulating adhesion and apoptosis (Alexander, 2000). In addition, CD45 was also found to regulate cytokine receptors signaling. Mast cells lacking CD45 were found to proliferate faster in response to IL-3 compared with wild-type cells, implicating CD45 in regulating IL-3 signaling. Examination of the IL-3-signaling cascade identified JAK2 as being hyperphosphorylated in the absence of CD45 accompanied with an enhanced JAK2 kinase activity (Irie-Sasaki et al., 2001). Other studies confirmed that CD45 could dephosphorylate JAK2 and identified similar roles for CD45 in negatively regulating IFN-γ and EPO signaling (Irie-Sasaki et al., 2001). In a study it was demonstrated that CD45 could dephosphorylate tyrosine 1007 in the activation loop of Jak2 (Irie-Sasaki et al., 2001). An important negative regulatory role for CD45 in regulating cytokine receptors signaling is expected and further investigation is required to fully appreciate the mechanism of action of the phosphatase.

### 3.3.2 The MAP Kinase Pathway

The JAK/Stat pathway is an important signaling mechanism used by cytokine receptors to transduce signals leading to biological activities. However, in addition to the Jak/Stat

pathway, other signaling cascades are also likely to be involved in signal transduction by these receptors. Signaling through MAPK involves the Shc/Grb2/SOS/Ras/Raf/MAPK cascade (Avruch et al., 1994). Various studies have reported the activation of the MAPK pathway following PRL stimulation (Crowe et al., 1991; Das and Vonderhaar, 1996; Albarracin and Gibori, 1991; Buckley et al., 1994; Das and Vonderhaar, 1995; Das and Vonderhaar, 1996). The mechanism of activation of MAPK downstream of the PRLR and the molecular components involved are not well known. Similarly, the biological relevance of activation of the MAPK pathway has not yet been determined. It was reported that the activation of the nucleotide exchange protein Vav takes place following PRL stimulation (Clevenger et al., 1995).

### 3.3.3 Other Signaling Pathways

In addition to the Jak/Stat and MAPK pathways, other pathways and regulators of signaling are also activated/involved in PRLR signaling activities. For example, Fyn, which is a member of the Src kinase family (Erpel and Courtneidge, 1995), becomes associated to the PRLR and is activated in the Nb2 cell line. It was also reposted that Fyn is required for the proliferation of the Nb2 cells (Clevenger and Medaglia, 1994). Also, association of the PRLR with Src was reported in rat hepatocytes (Berlanga et al., 1995). The function(s) of Src kinases and mechanism of activation in signal transduction of the PRLR remain to be clearly determined.

Futhermore, components of the PI3K pathway are also activated with the PRLR. It was reported that PRL induces a rapid tyrosine phosphorylation and association of the IRS-1 and of the p85 subunits of the PI3K pathway (Shirota et al., 1990; al-Sakkaf et al., 1996). In fact, It was proposed that PRL activation of PI3K pathway might be mediated by Fyn in Nb2 cells (al-Sakkaf et al., 1997). Phospholipase-C (PLCγ) and PKC were also suggested to be involved in PRLR signaling, although the role(s) these enzymes play in

PRLR signaling remains unknown and their substrates remain unidentified (Sauro and Zorn, 1991). PRL was also shown to increase the concentration of intracellular calcium in PRLR-transfected CHO cells, similarly, biological consequence of activation remains unresolved (Vacher et al., 1994).

Finally, a study suggested that the PRLR forms a complex with G-proteins (guanine nucleotide- binding proteins) in Nb2 cells (Too et al., 1990b), which may mediate the mitogenic actions of PRL on Nb2 cells (Too et al., 1990a).

## 4 Summary

The anterior pituitary hormone PRL participates in the regulation of a number of essential biological processes in various species. Of particular importance is its role in the regulation of mammary gland development. PRL gene knockout revealed that the hormone is indispensable for the controlled formation of the milk producing lobuloalveolar system of the mammary gland.

The biological roles of PRL are mediated in tissues through the membrane-bound PRLR, a member of the cytokine receptor superfamily. Similar to PRL knockout, null mutation of the PRLR impeded the formation of the lobuloalveolar system in the mammary gland. Association of PRL to the PRLR initiates a sequence of signal transduction events involving multiple cellular factors that belong to defined families of signaling molecules. Interestingly, the signal transduction machinery downstream of the PRLR utilizes common cellular proteins involved in intracellular signaling by other members of the cytokine receptor superfamily.

The primary pathway activated following stimulation of the PRLR is the Jak/Stat pathway. This signaling pathway utilizes a multitude of signaling proteins and various investigative efforts revealed a considerable amount of information about the Jak/Stat pathway and its role in intracellular signaling.

The requirement of Jak2 kinase in PRLR signaling is well documented. Up to date, Jak2 is the principal kinase activated in PRLR signaling. Through the N-terminal region of the kinase, Jak2 associates with a proline-rich box1 region of the PRLR and becomes activated following stimulation of the PRLR. Active Jak2 phosphorylates itself as well as tyrosine residues on the PRLR and hence creates recruitment sites for a number of SH2 containing cellular signaling proteins.

A primary substrate for Jak2 kinase is Stat5 protein. Stat5 is activated by tyrosine phosphorylation, dimerization, translocation to the nucleus and DNA binding to activate

the transcription of PRL responsive genes. The best evidence for the essential role of Stat5 in PRLR signaling came from Stat5a gene knockout studies that illustrated the requirement of the protein for the normal development the lobuloalveolar structures of the mammary gland.

Various molecular components participate in PRLR signaling to produce a balanced and a controlled outcome. The protein tyrosine phosphatase SHP-2 positively modulates PRLR signaling in the induction of PRL responsive genes, with the catalytic activity of the phosphatase being required for the process. On the other hand, SOCS-1 negatively regulates PRLR signaling activity. In fact, the best evidence for the inhibitory role of SOCS-1 in PRLR actions came from mice lacking SOCS-1. These mice display accelerated mammary gland development and precocious lactation.

## 5 Hypothesis and Objectives of this Work

PRL hormone is an essential regulator of key biological processes in the body. Hence, detailed investigative studies are required to realize the mechanisms of involvement of PRL in the modulation of these biological processes both in normal as well as disease states. One major approach to understand the mechanism of participation of PRL in target tissues is to realize the molecular basis behind the physiological responses to PRL through understanding PRLR signal transduction systems. Despite the cumulative knowledge about PRLR signaling, many outstanding issues remained to be solved.

The PRLR is known to be a substrate for Jak2 kinase. In particular, the most C-terminal tyrosine is phosphorylated and critically regulates the activation of transcription of the PRL responsive β-casein gene. The mechanism of participation of the C-terminal tyrosine of the PRLR in signaling was not known.

An important signaling mediator activated downstream from the PRLR is Stat5. Therefore, the first objective for my doctoral project was to **elucidate the role of the C-terminal tyrosine of the PRLR in the activation of Stat5.** The results from this study established that tyrosine phosphorylation of Stat5 was independent of tyrosine phosphorylation of the PRLR. However, the critical processes of Stat5 nuclear translocation and the subsequent DNA binding were dramatically influenced by the C-terminal tyrosine of the PRLR.

The observation that Stat5 tyrosine phosphorylation and nuclear translocation are separately regulated events led to the search for possible candidates that are recruited to the C-terminal tyrosine hence regulating Stat5 activation and PRLR signaling. A second molecular mediator of PRLR signaling is the protein tyrosine phosphatase SHP-2. The phosphatase was shown to play an essential role through positively influencing PRLR signaling activities. The second objective of my project was to **determine the**

**role of the C-terminal tyrosine of the PRLR in the recruitment of SHP-2**. The study determined that indeed the C-terminal tyrosine of the PRLR is a recruitment site for SHP-2. Additionally, the adaptor protein Gab2, but not Gab1, is tyrosine phosphorylated following stimulation of the PRLR and serves as second recruitment site for SHP-2.

The finding that the C-terminal tyrosine recruits SHP-2, plus the previously established positive regulatory role for SHP-2 in PRLR signaling presented the question of what mechanism does the recruitment of SHP-2 to the C-terminal tyrosine regulate to drive the activation of PRLR signaling. The answer was expected to rest in the dephosphorylation of an inhibitory tyrosine residue in a critical signaling molecule.

Jak2 kinase is known to associate through tyrosine 1007 that lies in the activation domain to the signaling inhibitory protein SOCS-1. The association between Jak2 and SOCS-1 blocks the kinase activity of Jak2 as well as targets the protein for ubiquitination and proteasomal degradation. Tyrosine 1007 was expected to be a target for the phosphatase activity of SHP-2. The third aim of my doctoral project was to **determine the role of SHP-2 in the activation of Jak2 kinase**. The study illustrated that tyrosine 1007 of Jak2 is a target for the phosphatase activity of SHP-2 and that the dephosphorylation of Jak2 prevents the association to the inhibitory protein SOCS-1 and ultimately prolongs the half-life of Jak2 and maintains the kinase in an active state. Additionally, dephosphorylation of Jak2 prolonged the half-life of the PRLR through preventing the ubiquitination and degradation of the associated PRLR.

Studies performed in this doctoral project establish that tyrosine phosphorylation of the PRLR, particularly on the most C-terminal tyrosine, plays a critical role in PRLR signal transduction leading to the induction of PRL responsive genes. Understanding the mechanisms of PRLR signaling is essential for realizing the molecular basis behind

the physiological responses induced by PRL, such as the regulation of mammary gland development.

# CHAPTER II. PROLACTIN RECEPTOR REGULATES STAT5 TYROSINE PHOSPHORYLATION AND NUCLEAR TRANSLOCATION BY TWO SEPARATE PATHWAYS

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## FORWARD

Tyrosine phosphorylation of the PRLR, in particular phosphorylation of the most C-terminal tyrosine, was shown to critically regulate gene induction of prolactin responsive genes. It was observed that the substituting the C-terminal tyrosine with a phenylalanine significantly reduces the induction of the β-casein gene. The mechanism by which the C-terminal tyrosine of the PRLR regulates induction of gene transcription was not known.

The first objective of my doctoral work was to investigate the molecular basis by which the C-terminal tyrosine controls the intracellular signaling activity of the PRLR.

Stat5 was shown previously to be an important mediator of prolactin receptor signaling. Therefore, the focus was to investigate the effect of inactivating the C-terminal tyrosine on the activation of Stat5 within the context of PRLR signaling.

The work established that the C-terminal tyrosine of the prolactin receptor had no effect on the tyrosine phosphorylation of Stat5, however, the tyrosine had a critical influence on Stat5 activation. Both Stat5 nuclear translocation as well as DNA binding were hindered as a result of mutating the C-terminal tyrosine of the PRLR.

## ABSTRACT

The SH2-domain containing signal transducers and activators of transcription (Stat proteins) are effector molecules downstream of cytokine receptors. Ligand/receptor engagement triggers Stat proteins tyrosine phosphorylation, dimerization and translocation to the nucleus where they regulate gene transcription. Stat5, originally identified as a mammary gland growth factor, is an essential mediator of prolactin (PRL)-induced milk protein gene activation. Prolactin receptor (PRLR), is a member of the cytokine/GH /PRL receptor superfamily. The mechanism through which PRLR modulates Stat5 tyrosine phosphorylation, nuclear translocation and DNA binding was analyzed in HC11 cells, a mammary epithelial cell line, and 293-LA cells, a human kidney cell line stably overexpressing Jak2 kinase. We have found that in HC11 cells, Stat5 is specifically activated by PRL treatment demonstrating that Stat5 is a physiological substrate downstream of PRLR. Furthermore, using different natural forms of the PRLR as well as receptor tyrosine to phenylalanine mutant forms, we determined that tyrosine phosphorylation of Stat5 is independent of PRLR phosphotyrosines. We established, however, that the C-terminal tyrosine of the PRLR Nb2 form, Y382, plays an essential positive role in PRLR-dependent Stat5 nuclear translocation and subsequently DNA binding.. All together, our data propose a new model for activation of Stat5 through the PRLR, suggesting that Stat5 tyrosine phosphorylation and nuclear translocation are two separately regulated events.

## INTRODUCTION

Prolactin (PRL) is a pituitary polypeptide hormone as well as a local growth factor that regulates several physiological functions such as, reproduction, promotion of the growth and differentiation of the mammary gland, and immune function (1). PRL interacts with specific cell surface receptors expressed on different target tissues (Reviewed in ref 2).The receptor for PRL (PRLR) belongs to a large group of receptors known as the cytokine/GH/PRL receptor superfamily, which includes the receptors for GH, EPO, GM-CSF, and several interleukins (2, 3). These receptors share common extracellular structural motifs such as two disulfide loops and intracellular such as the proline rich box1 homology domain. These receptors do not possess intrinsic kinase activity but signal through cytoplasmic protein tyrosine kinases of the Janus kinase family (Jak/Tyk kinases) and the Src-kinase family. Ligand binding to the cytokine/GH/PRL receptor family induces receptor dimerization and activation of the associated kinases. This leads in turn to tyrosine phosphorylation of multiple cellular proteins including the receptors themselves (4).

The downstream signaling molecules activated by this receptor family have not been completely elucidated. However, it has been shown that several of the SH2-domain containing molecules interact with cytokine receptors, e.g. phospholipid metabolizing enzymes, PLC-$\gamma$ and the regulatory unit of PI3 kinase, protein tyrosine phosphoatases, SHP1and SHP2, and adapter proteins, grb2, Shc, IRS1 (5-11). Moreover, a family of SH2-domain containing transcription factors of 85-95 kDa, called signal transducers and activators of trasncription (Stat), have been identified as primary effector molecules for this receptor family (12). Rapidly upon receptor activation, tyrosine phosphorylation of Stat factors occurs leading to their homo- or hetero-dimerization and translocation to the nucleus were they induce transcription of

cytokine responsive genes (13). The complete molecular events leading to Stat proteins activation are not fully understood. It has been suggested that phosphotyrosines on the receptor components may act as docking sites for the SH2 domains of Stat proteins (14, 15) allowing them to be phosphorylated by Jak tyrosine kinase family, a process necessary for Stat proteins activation by cytokine receptors. Recent information, however, indicate that other signaling pathways, might be involved for maximal induction of Stat proteins activity. For example, to fully activate Stat1a, serine phosphorylation by MAPK is required (16).

The rat PRLR, a member of the cytokine/GH/ PRL receptor family, exists in three natural forms; the long form, identified in the mammary epithelial and ovarian cells (17), the short form, characterized in liver cells (18), and a third intermediate form, found in rat T-lymphoma Nb2 cells called PRLR Nb2 form (19). The short form of the receptor results from alternative splicing of the long form, whereas the Nb2 form results from a deletion mutation of the long form. The membrane proximal events following PRLR activation have recently been clarified. PRL binding to its receptor triggers homodimerization of the PRLR and activation of the constitutively associated kinase, Jak2 (20, 21, 22). While this process lead to tyrosine phosphorylation of the PRLR long form and the PRLR Nb2 form, no tyrosine phosphorylation was observed for the PRLR short form (23). Furthermore, studies examining the mechanism through which PRLR regulate gene transcription, have previously demonstrated that while the PRLR long and the Nb2 forms are able to transmit PRL's signal to activate β-casein gene transcription, the short form was inactive in this biological assay system (24). We have further shown that PRLR signals through the coordinated action of Jak2 and a single tyrosine residue present on the PRLR long and Nb2 forms to activate β-casein gene transcription. Indeed,

when the C-terminal tyrosine of the PRLR long form (Y580) or of the PRLR Nb2 form (Y382) was mutated to phenylalanine, signaling of the PRLR to □-casein gene promoter activation was impaired (23, 25).

PRL has recently been shown to activate several Stat proteins such as, Stat1, Stat3 and Stat5 (23, 26, 27). In particular, Stat5, for which two isoforms Stat5a and Stat5b differing in their C-terminal tail were characterized in mouse mammary gland (28), has been shown to be vital for hormonal induction of β-casein gene transcription in mammary gland of lactating animals (29) and in heterologous cell systems(30). Stat5 activation has also recently been shown to correlate with mitogenic signaling in response to PRL (31). Therefore, Stat5 appears to be a key player in PRL-induced gene activation and cell proliferation.

Stat5 has also been shown to be part of the signaling pathway for a number of cytokine receptors such as, GHR, EPOR, IL3-R, GM-CSFR, IL2-R, IL6-gp130, and EGFR (Reviewed in 32). The mechanism of activation of Stat5 through the different cytokine receptors remains elusive and controversial. Indeed, Stat5 tyrosine phosphorylation, DNA binding and induction of transcription via the GHR requires certain phosphotyrosine residues on the receptor (33, 34). Similarly, Stat5 activation downstream of the EPOR was found to be dependent on receptor phosphotyrosines (35-37). However, other studies have reported a mechanism of activation of Stat5 totally independent of receptor phosphotyrosines. For example, GHR phosphotyrosines-independent activation of Stat5 has been documented(38). Separate studies indicated that Stat5 tyrosine phosphorylation and Stat5/DNA binding activity through the gp130 subunit of the IL-6 receptor (39) and the G-CSFR (40) is independent of receptor phosphotyrosines. Furthermore, direct Jak/Stat interactions has recently been implicated as an alternative mechanism for activation

of Stat5 by cytokine receptors (39).Therefore, the mechanism of activation of Stat5 by the cytokine/GH/PRL receptor family and the possible role of receptor phosphotyrosines in this process remains to be elucidated.

In this paper, we examined the significance of tyrosine phosphorylation of the PRLR in activating Stat5 molecules. Our results indicate that while Stat5 tyrosine phosphorylation is regulated by PRLR/Jak2 activation, it is independent of PRLR tyrosine phosphorylation. We further found that the C-terminal tyrosine of the PRLR Nb2 form regulate Stat5 nuclear translocation. Together, our results indicate for the first time that Stat5 tyrosine phosphorylation and nuclear translocation are two separately regulated process.

## Materials and Methods

***Materials, Antibodies and Plasmid Constructs***— Cytomegelo virus based CMV driven expression plasmids , pR/CMV vector (Invitogen), containing cDNAs encoding PRLR Long wild type, LY237F, LY580F, Nb2 wild type, NbY237, NbY382F, Δ296-322, ΔY237F, ΔY382F, were constructed as described previously (25) and were obtained from Dr. P. Kelly (Paris, France). Expression plasmid DNA, pXM-MGF/Stat5, encoding MGF/Stat5 was obtained from Dr. B. Groner (Freiburg, Germany). Ovine PRL (oPRL) used for cell treatment was obtained from Sigma (Mississauga, Canada). Polyclonal antibody to Stat5a and monoclonal antibody to phosphotyrosine (4G10) were purchased from Upstate biotechnology (New York, USA), monoclonal antibody to Stat5 was obtained from Transduction Laboratories (Lexington, USA), and monoclonal-antibodies to PRLR, U5, was provided by Dr. P. Kelly (Paris, France). Protein-A beads used for immunoprecipitations were purchased from Pharmacia (Quebec, Canada).

***HC11 Cell Culture***— HC11, mouse mammary epithelial cells were grown to confluency in RPMI-1640 media containing 10% fetal calf serum (FCS) (Gibco), insulin (5μg/ml), and EGF (10ng/ml). Cells were then induced by incubating them for 3-5 days in RPMI media containing 10%FCS, insulin (5μg/ml), and hydrocortisone (1μM) (41, 42). Cells were then starved in RPMI media containing insulin (5μg/ml), hydrocortisone (1μM), and transferrin (10μg/ml). Cells were then stimulated with oPRL (1.5μg/ml) for the time indicated. Cells were lysed in lysis buffer [10mM Tris-HCl, ph7.5/ 5mM EDTA/ 150mM NaCl / 30mM sodium pyrophosphate/ 50mM sodium floride/ 1mM sodium orthovanadate/ 10% (vol/vol) glycerol/ 0.5% Triton X-100] containing protease inhibitors [1mM phenylmethylsulphonyl floride/ leupeptin

(2μg/ml)/ aprotinin (5μg/ml) for 5 minutes at 4oC. The lysates were then centrifuged at 12,000 x g for 10 min at 4oC to remove insoluble material. Protein concentration was measured using the Bradford technique.

***Transient Transfection***—Transfection was carried out as described earlier (23, 25). Briefly, the human 293 clone stably expressing the tyrosine kinase Jak2 (clone LA) was grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (GIBCO) containing 10% (vol/vol) FCS. Several hours before transfection, cells were plated in a rich medium (2 parts DMEM/F12 and 1 part DMEM containing glucose at 4.5g/liter) containing 10% FCS. Approximately $5x10^6$ cells were then co-transfected with the cDNA encoding the different forms of RPLR (1μg each) and the cDNA for Stat5 (500 ng) by using the calcium phosphate technique. After 24 hours of expression, the cells were starved by serum deprivation over night.

***Immunoprecipitations***— Immunoprecipitations were carried out as described earlier (22). Briefly, protein extracts were immunoprecipitated for two hours using polyclonal antibody to Stat5a and protein-A sepharose beads. This antibody was used since immunoprecipitations were unccusseful using the monoclonal antibody to Stat5. Precipitates were then separated on SDS-PAGE and probed with monoclonal-antibody to phosphotyrosines (4G10) then stripped and re-probed with polyclonal antibody to Stat5a.

***Total Cell Lysis and Western Blotting***— Transiently co-trasfected 293-LA cells were stimulated with oPRL (1.5μg/ml) of for 5 minutes and then lysed in lysis buffer (300μl) [10mM Tris-HCl, ph7.5/ 5mM EDTA/ 150mM NaCl / 30mM sodium pyrophosphate/ 50mM sodium floride/ 1mM sodium orthovanadate/ 10% (vol/vol) glycerol/ 0.5% Triton X-100] containing protease inhibitors [ 1mM

phenylmethylsulphonyl floride/ leupeptin (2μg/ml)/ aprotinin (5μg/ml) for 5 minutes at 4oC. The lysates were then centrifuged at 12,000 x g for 10 min at 4oC to remove insoluble material. Protein concentration was measured using the Bradford technique. Equal amounts of protein obtained by total lysis were loaded and run on an 8% SDS-PAGE. Western analysis was performed using either monoclonal antibodies to phosphotyrosine, PRLR or Stat5. Proteins were revealed using chemiluminescence (ECL kit from Amersham) following the manufacturer's instructions.

***Nuclear Translocation***— Procedure for obtaining nuclear extracts was described previously (30) with some modifications. Briefly, transiently co-transfected 293-LA cells, or HC11 cells were collected by centrifugation, washed with phosphate-buffered saline then lysed in hypotonic buffer [10mM HEPES-KOH, pH 7.9/ 1.5mM $MgCl_2$/ 10mM Kcl/ 0.5mM dithiothreitol/ 1mM $Na_3VO_4$/ 20mM NaF/ 1mM phenylmethylsulfonylfloride/ 5μg/ml aprotinin/ 2μg/ml leupeptin]. Cells were incubated for 15 minutes then vortexed vigorously and centrifuged at 12,000 xg at 4□C. The pellet was washed once with cold phosphate-buffered saline then the nuclear extracts were obtained by adding a high salt buffer [25% glycerol/ 420mM NaCl/ 1.5mM $MgCl_2$ / 0.2mM EDTA/ 1mM $Na_3VO_4$/ 20mM NaF/ 5μg/ml aprotinin/ 2μg/ml leupeptin], shaken for 30 minutes at 4□C, then centrifuged at 12,000g for 5 minutes. Total nuclear proteins were separated on an 8% SDS-PAGE, transferred to nitrocellulose membrane and Western blots were carried out using anti-Stat5 monoclonal antibody and revealed using chemiluminescence (ECL kit, Amersham)

***Electrophoretic Mobility Shift Assay (EMSA)***— EMSA was performed as described elsewhere (30). Briefly, nuclear extracts were prepared as described

above. Binding reactions, performed in binding buffer [10mM EHPES-KOH, pH 7.9/ 0.5mM EDTA/ 0.5mM dithiothreitol/ 10% glycerol], contained, nuclear extract (8-10μg), end labeled double stranded DNA containing the Stat5 response element of the β-casein gene promoter (5pmoles), and non specific competitor polydeoxyinosinic-deoxycyctidylic acid (2μg). For supershifts, protein extracts were incubated on ice for 30 minutes with polyclonal antibody to Stat5a (1μg). We used this antibody, since super-shifts were unsuccessful when the monoclonal-antibody to Stat5 was used. Samples were run on a 0.25X TBE, 5% polyacrylamide gel. The gel was dried and exposed to X-ray film at

-80$^{o}$C (Hyperfilm, Amersham).

## RESULTS

***PRL activates Stat5a in HC11 cells.*** Gene expression of milk proteins in mammary cells is under a complex hormonal control. A combination of insulin, glucocorticoids and PRL is required for maximal hormonal induction of milk proteins, such as β-casein, in mammary organ and epithelial cell cultures (41, 42). Stat5 activation has recently been shown to be the main intracellular mediator for activation of β-casein gene promoter (27). Therefore, we first evaluated the ability of PRL to induce Stat5 activation in HC11 cells, PRL-sensitive mouse mammary epithelial cells (27). Cells were starved in the presence of insulin and hydrocortisone, cells were either left untreated or treated with PRL (1.5 □g/ml) for 10 min. and lysed in lysis buffer. Cell lysates were immunoprecipitated using anti-Stat5a polyclonal antibody. Immune-complexes were run on SDS-PAGE, transferred to nitrocellulose membranes and immunodetected with monoclonal-antibody to phosphotyrosines (4G10). As shown in Figure 2.1A, no detectable tyrosine phosphorylation of Stat5 was observed under basal conditions. However, PRL stimulation of cells (10 min) rapidly induced tyrosine phosphorylation of Stat5. To confirm that equal amounts of Stat5 were immunoprecipitated, membrane was stripped and reprobed with polyclonal-antibodies to Stat5a (Fig. 2.1B). These results indicate that in mammary cells Stat5 tyrosine phosphorylation is induced by PRL treatment. Furthermore, these results indicate that the combinations of insulin and hydrocortisone are not competent to induce Stat5 tyrosine phosphorylation in the absence of PRL.

We then examined the kinetics of Stat5 nuclear translocation in HC11 cells in response to PRL stimulation. Cells were grown and stimulated for the indicated

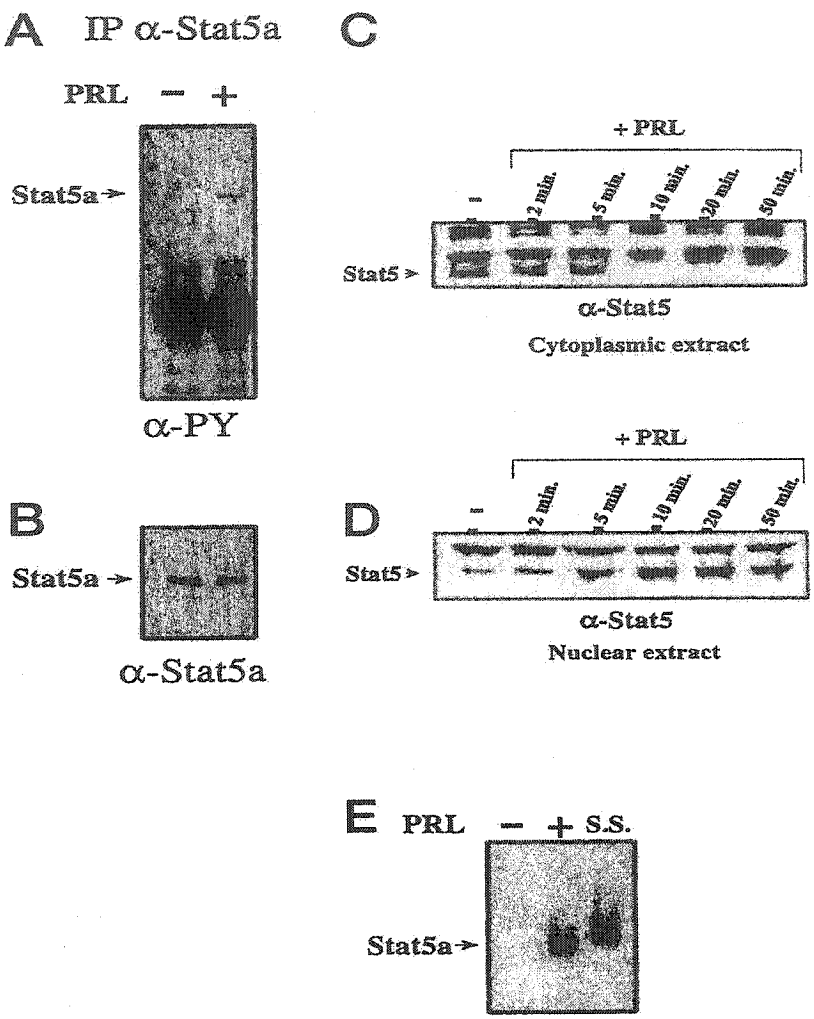


Figure 2.1 PRL stimulation induces Stat5 tyrosine phosphorylation and binding to the GAS sequence of the β-casein gene promoter in HC11 cells. HC11 cells were either non-stimulated (-) or stimulated (+) with oPRL (1.5 μg/ml) for 10 min. Cells were lysed and immunoprecipitations were performed using polyclonal antibody to Stat5a. Immunoprecipitated proteins were separated on SDS-PAGE, and transferred to nitrocellulose membranes. Membrane was probed with monoclonal-antibody to phosphotyrosines (A), then stripped and re-probed with polyclonal antibody to Stat5a (B). Serum starved HC11 cells were stimulated with oPRL (1.5 μg/ml) for 2, 5, 10, 20, and 50 minutes. Total cytoplasmic extracts (C), and nuclear extracts (D), were prepared. Proteins (15μg) from each extract were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with monoclonal-antibody to Stat5. (E) HC11 cells were prepared the same way as above, stimulated with oPRL (1.5 μg/ml) for10 min total nuclear extracts were prepared, and EMSA was performed using Stat5 response element of the β-casein gene promoter. The Stat5/DNA complex was super-shifted (S.S.) with polyclonal antibody to Stat5a.

times, cytoplasmic (Fig. 2.1C) and nuclear (Fig. 2.1D) extracts were then prepared from the same culture. As shown in Figure 2.1D, the amount of Stat5 in the nucleus increases rapidly following PRL stimulation. Meanwhile, the amount of Stat5 present in the cytoplasm (Fig. 2.1C) decreases until eventually no detectable Stat5 was found in the cytoplasm following 10 min of PRL hormone treatment

We next examined the ability of Stat5 to bind to the β-casein gene promoter. It has been shown previously that Stat5 binds to the -75/-104 region of the β-casein gene promoter (27). Electrophoretic mobility shift assay (EMSA) was performed using a primer of the sequence 5'-TGT GGA CTT CTT GGA ATT AAG GGA C-3', and nuclear extracts were prepared from unstimulated or PRL stimulated HC11 cells. As shown in figure 2.1E, PRL stimulation of HC11 cells results in Stat5 binding to the GAS-like element present on the β-casein gene promoter. This complex was supershifted in the presence of anti-Stat5a polyclonal antibody (Lane 3), indicating the specificity of the DNA binding activity. All together, using mammary epithelial cell system we show here that Stat5 activation in mammary cells follows PRL inductive effects.

***Tyrosine phosphorylation of Stat5 is independent of receptor phosphotyrosines of the PRLR Nb2 form and Δ296-322 mutant form.*** Having established that PRL regulates Stat5 tyrosine phosphorylation, nuclear translocation, and DNA binding activity in mammary cells, we were interested in defining the mechanism through which PRLR mediate this effect. We have previously shown that C-terminal tyrosine of the PRLR is necessary for PRL activation of β-casein gene promoter (25). When this tyrosine was mutated to phenylalanine in the PRLR Nb2 form, β-casein gene promoter induction was strongly inhibited. Similar mutation of

the C-terminal tyrosine on the PRLR long form, however, had less notable effects on β-casein gene activation.

To investigate the role of PRLR tyrosine phosphorylation in Stat5 activation, we used a heterologous overexpression system consisting of 293-LA cells, human kidney cell line stably overexpressing Jak2 kinase (23, 25). We have previously shown that transient overexpression of PRLR in these cells leads to ligand-independent constitutive activation of the receptor/kinase complex. Here we have used this system to investigate the role of PRLR tyrosine phosphorylation in activation of Stat5.

The receptor natural and mutant forms used in our studies were described previously (23, 25). The PRLR Nb2 form, found in Nb2 T-lymphoma cells, has an inframe deletion mutation compared to the PRLR long form (19). Three tyrosine residues (Y237, Y309, and Y382) are retained in the PRLR Nb2 form compared to the PRLR long form. Tyrosine 382 of the PRLR Nb2 form corresponds to tyrosine 580 of the PRLR long form. Another biologically active form is the PRLR Nb2 mutant form Δ296-322 (23). This receptor form has a 27 amino acids (aa) internal deletion and lacks tyrosine 309. Finally, the PRLR Nb2 mutant form □243-268 is also used in our studies (23). This mutant form is missing 25 aa region encompassing the Box1 motif, important for PRLR/Jak2 complex formation.(23). Therefore, this receptor form is unable to activate Jak2 and is used here as a negative control for receptor/Jak2 activation.

Point mutation of the C-terminal tyrosine (Y382) in the PRLR Nb2 form and □296-322 mutant form was shown to inhibit receptor tyrosine phosphorylation and □-casein gene promoter activation (25). Consequently, we intended to determine the

role of this tyrosine residue in Stat5 tyrosine phosphorylation. 293-LA cells were co-transfected with cDNA encoding Stat5 (27) and either PRLR Nb2 wild type, (Nb2-WT); PRLR Nb2 form in which tyrosine 237 was substituted by phenylalanine, NbY237F; PRLR Nb2 form in which tyrosine 382 was mutated to phenylalanine, NbY382F; or Δ243-268 as a negative control (Fig. 2.2). In parallel, 293-LA cells were co-transfected with cDNA encoding Stat5 and either with the Δ296-322 receptor form; □296-322 in which tyrosine 237 was exchanged with phenylalanine, ΔY237F; receptor form □296-322 in which tyrosine 382 was mutated to phenylalanine, □Y382F; or □243-268 as a negative control (Fig. 2.2). Cells were then stimulated with PRL for 10 min before lysis. Total protein extracts were separated on SDS-PAGE, transferred to membranes and immunodetected with monoclonal-antibody to phosphotyrosines (Fig. 2.2A). We observed that all natural and mutant forms of the PRLR except mutant Δ243-268, which act as a negative control, are fully capable of inducing Jak2 and Stat5 tyrosine phosphorylation (Fig. 2.2A). However, not all receptor types are themselves tyrosine phosphorylated. The NbY382F and the ΔY382F mutant receptor forms were not tyrosine phosphorylated in this system, similar to what was shown previously (25). To confirm that we have equal expression of receptors and Stat5 in the different samples, the membranes were stripped and reprobed with monoclonal antibodies to PRLR that can recognize all receptor forms (Fig. 2.2B). This was followed by reprobing the membranes with monoclonal antibodies to Stat5 (Fig. 2.2C). Together, these findings indicate that Stat5 is tyrosine phosphorylated following PRLR/Jak2 activation independently of PRLR phosphotyrosines.

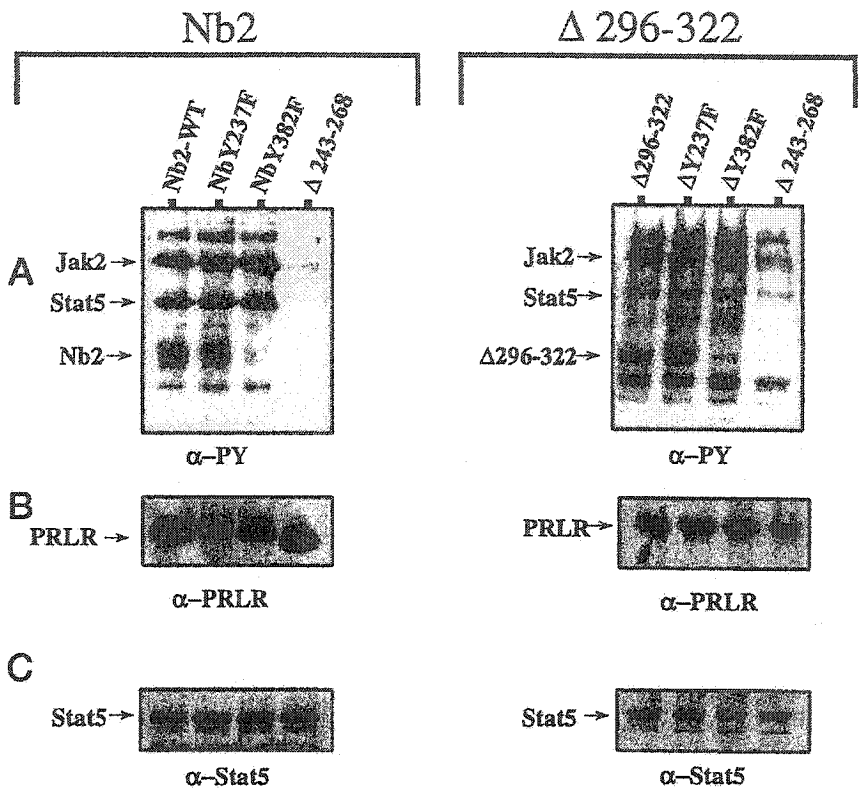


Figure 2.2 Tyrosine phosphorylation of Stat5 is independent of tyrosine 382 of the PRLR Nb2 form and Δ296-322 mutant form. (A) 293-LA cells ($5x10^5$cells) were transiently co-transfected with cDNAs encoding Stat5 (500 ng), and of either Nb2-WT, NbY237F, NbY382F, or the mutant Δ243-268 (1μg).Similarly, 293-LA cells were transiently co-transfected with cDNAs encoding Stat5 (500 ng) and either Δ296-322, ΔY237F, ΔY382F, or the mutant Δ 243-268 (1μg) (B). Cells were stimulated with oPRL (1.5 μg/ml) for five minutes. Total protein extracts were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal-antibody to phosphotyrosines. (B) Membranes were stripped and reprobed with monoclonal-antibody to PRLR. (C) Membranes were stripped and immunodetected with monoclonal antibody to Stat5.

***Stat5 nuclear translocation is regulated by tyrosine 382 of the PRLR Nb2 form and the Δ296-322 mutant form.*** Following Stat protein tyrosine phosphorylation on the conserved tyrosine residue, it is thought that Stat proteins dimerize and translocate to the nucleus where they induce gene transcription (12). Since mutation of tyrosine 382 of the PRLR Nb2 form and of the receptor □296-322 form did not influence Stat5 tyrosine phosphorylation, but it was shown to play a significant role in regulating β-casein gene promoter induction (25), we studied its influence on Stat5 nuclear transloction event. For this purpose, 293-LA cells were co-transfected with the cDNAs encoding the PRLR Nb2 form or one of the YF mutants; NbY237F and NbY382F; along with the cDNA encoding Stat5 (Fig. 2.3A). In Parallel, 293-LA cells were co-transfected with the cDNAs encoding the receptor □296-322 form or one of its YF mutants; ΔY237F or ΔY382F, along with the cDNA encoding Stat5 (Fig. 2.3B). Cells were then stimulated with PRL for 10 min, before lysis and total nuclear extracts were analyzed with monoclonal-antibody to Stat5. As shown in figures 2.3A and 2.3B (upper panel), Y-F mutations of Y237 in the two receptor forms did not affect nuclear translocation of Stat5 compared to wild type receptor forms. However, the amount of nuclear Stat5 immuno-detected in samples overexpressing NbY382F and ΔY382F was dramatically reduced compared to that observed for wild type receptors. Indeed, for the mutant ΔY382F, the level of nuclear Stat5 was similar to that observed in samples in which the inactive mutant form Δ243–268 was overexpressed (Fig. 2.3A and 2.3B, upper panel). This inhibition in Stat5 nuclear translocation was not due to differences in the overexpression of Stat5 as indicated by Western blots with monoclonal antibody to Stat5 of total cell extracts from the same transfections (Fig. 2.3A and 2.3B lower panel). Similarly, receptor expression

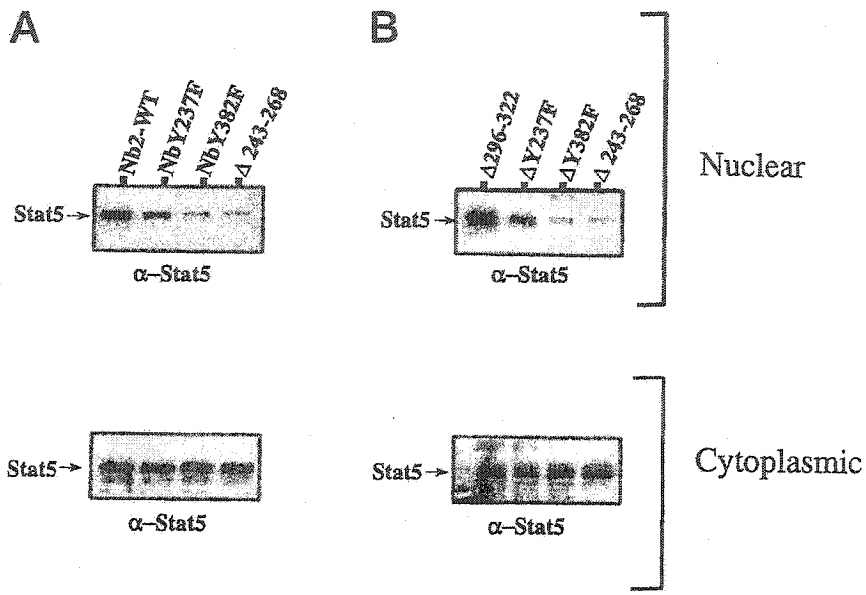


Figure 2.3 Nuclear translocation of Stat5 is significantly influenced by tyrosine 382 of the PRLR Nb2 form and by tyrosine 382 of the Δ296-322 mutant form. (A) 293-LA cells were transiently co-transfected with cDNAs encoding Stat5 and either PRLR Nb2-WT, NbY237F, NbY382F, or the mutant Δ243-268. (B) 293-LA cells were transiently co-transfected with cDNA encoding Stat5 and either Δ296-322, ΔY237F, ΔY382F, or the mutant Δ243-268. Cells were stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts or total cell extracts were prepared from two stets of cells that were simultaneously transfected. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal-antibody to Stat5.

was equal in the different samples (data not shown). These results indicate that the C-terminal tyrosine residue of the PRLR Nb2 form is required for Stat5 nuclear translocation.

***Tyrosine 382 of the PRLR Nb2 form and the Δ296-322 mutant form inhibits Stat5 binding to the GAS response element of the β-casein gene promoter.*** To confirm the role of tyrosine 382 of the PRLR Nb2 form in regulating Stat5 nuclear entry, we next studied the importance of this tyrosine in regulating Stat5 DNA binding activity. We co-transfected 293-LA cells with the cDNA encoding Stat5 and with the cDNAs encoding either PRLR Nb2 wild type or its YF mutants (Fig. 2.4A). Similarly, we co-overexpressed in 293-LA cells Stat5 with either □296-322 receptor form or its YF mutants (Fig. 2.4B). Nuclear extracts were then prepared and an EMSA binding reaction containing the β-casein gene promoter GAS sequence was performed. We found (Figure 2.4A and 2.4B) that overexpression of PRLR Nb2 wild type, Δ296-322 receptor form and their Y237F mutant forms lead to the appearance of Stat5 DNA binding activity to the β-casein gene promoter in gel shift assays. However, Stat5/DNA interactions were greatly reduced in samples overexpressing the mutant receptor forms, NbY382F and ΔY382F. This is judged from the absence of DNA bound Stat5 compared to the wild type PRLR Nb2 form and Δ296-322 mutant form. Therefore, tyrosine 382 of the PRLR Nb2 form is important and necessary for Stat5 activation.

***Tyrosine 580 of the PRLR long form is not required for Stat5 tyrosine phosphorylation.*** The PRLR long form contains 9 tyrosine residues within its cytoplasmic domain. It was shown previously that individual Y-F mutations, Y237 and Y580, in the PRLR long form had no effect on the level of tyrosine phosphorylation of

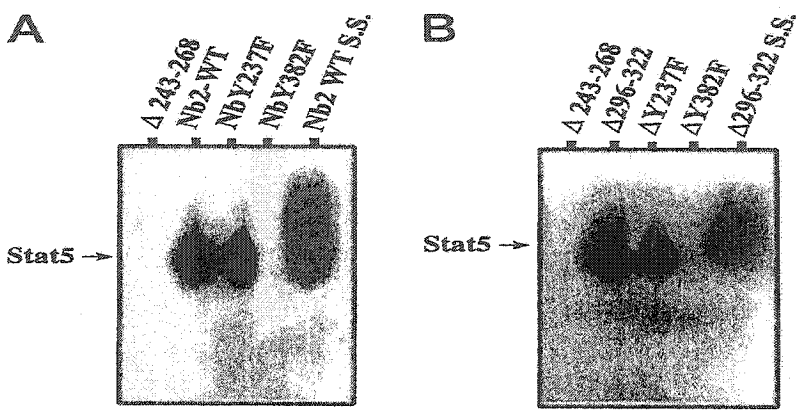


Figure 2.4. Tyrosine 382 of the PRLR Nb2 form inhibits Stat5 binding to the GAS sequence of the β-casein gene promoter. (A) 293-LA cells ($5x10^5$ cells) overexpressing Stat5, and of either Nb2-WT, NbY237F, NbY382F, or the mutant Δ243-268. (B) 293-LA cells were transiently co-transfected with cDNA encoding Stat5 and either Δ296-322, ΔY237F, ΔY382F, or the mutant Δ 243-268 (B). Cells were stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts were prepared and EMSA was performed using Stat5 response element of the β-casein gene promoter. The Stat5/DNA complex was super-shifted (S.S.) with polyclonal-antibody to Stat5a

the PRLR (25). This suggests that other tyrosine residues of the PRLR may undergo tyrosine phosphorylation following PRLR/Jak2 activation. Functional analysis of these mutant receptors indicate that Y580 is required for β-casein gene promoter activation, albeit less apparent than that observed for NbY382F mutant (25). To assess the role of Y580 of PRLR long form in mediating Stat5 tyrosine phosphorylation, 293-LA cells were co-transfected with cDNA encoding Stat5 and cDNAs encoding either PRLR long wild type (Long-WT); PRLR long in which tyrosine 237 was exchanged with phenylalanine, LY237F; PRLR long in which tyrosine 580 was mutated to phenylalanine, LY580F; or Δ243-268 as a negative control (Fig. 2.5). Cells were starved for an overnight period before being stimulated with PRL for 10 min. Total cellular proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Immunodetections with antibodies to phosphotyrosine indicate that the PRLR long form as well as the two YF mutants were able to mediate both Jak2 and Stat5 tyrosine phosphorylation compared to the negative control samples in which Δ243-268 were overexpressed (Fig. 2.5A). This is consistent with what we observed for the PRLR Nb2 form and Δ296-322 mutant form (Fig. 2.2A). Furthermore, Figure 2.5A also indicates that PRLR long form as well as the two Y-F mutant forms, LY273F and LY580F, are themselves tyrosine phosphorylated as shown previously (25). To confirm that receptor expression was equal in all samples, the membrane was stripped and reprobed with monoclonal-antibodies to the extracellular domain of the PRLR (Fig. 2.5B). Similarly, to verify that Stat5 was equally expressed in the different samples, the membrane was stripped and immunodetected by monoclonal antibodies to Stat5

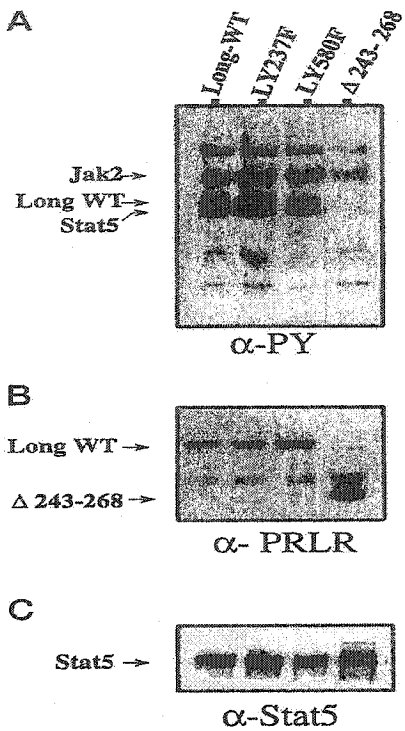


Figure 2.5. Tyrosine phosphorylation of Stat5 is independent of tyrosine 580 of the PLRL long form. 293-LA cells ($5x10^5$ cells) were transiently co-transfected with cDNAs encoding Stat5 (500 ng) and either Long-WT, LY237F, LY382F, or the mutant Δ243-268. Cells were stimulated with oPRL (1.5 μg/ml) for 5 min. Total protein lysates were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal-antibody to phosphotyrosines (A). Membrane was stripped and reprobed with monoclonal-antibody to PRLR (B). Membrane was then stripped and immunodetected with monoclonal-antibody to Stat5 (C).

(Fig. 2.5C). These results demonstrate that Stat5 tyrosine phosphorylation is not modulated by either tyrosine 237 or tyrosine 580 of the PRLR long form.

***Tyrosine 580 of the PRLR long form does not significantly influence Stat5 nuclear translocation.*** We next investigated the influence of the same YF mutants of PRLR long form on Stat5 nuclear translocation. 293-LA cells co-transfections were carried out with cDNAs encoding Stat5 and either PRLR long wild type (Long-WT), LY237F, LY580F; or Δ243-268 (Fig. 2.6). Cells were starved for an overnight period before being stimulated by PRL for 10 min. Total nuclear extracts were prepared then loaded on an SDS-PAGE, transferred to membranes and Western blot analysis was performed using anti-Stat5 monoclonal antibody. As shown in Figure 2.6, mutation of either Y237 or Y580 in the PRLR long form, had no or very little effect on Stat5 nuclear translocation (Fig. 2.6 Upper Panel). These results are in contrast to that observed for the PRLR Nb2 form and Δ296-322 mutant form (Fig. 2.3) and suggest that additional tyrosine residues specific to the PRLR long form might substitute the C-terminal tyrosine in mediating Stat5 nuclear translocation. Indeed, our data is consistent with the fact that mutation of the last tyrosine in the long form only partially inhibited β-casein gene promoter activation (25).

***Tyrosine 580 of the PRLR long form does not influence Stat5 binding to the response element of the β-casein gene promoter.*** We then used the PRLR long form and its Y-F mutants, LY237F and LY580F, to study their effects on the binding activity of Stat5 to the β-casein gene promotor. For this reason, 293-LA cells were co-transfected with cDNA encoding Stat5 and cDNAs encoding either the PRLR long wild type, LY237F, or LY580F. Nuclear extracts were prepared, and

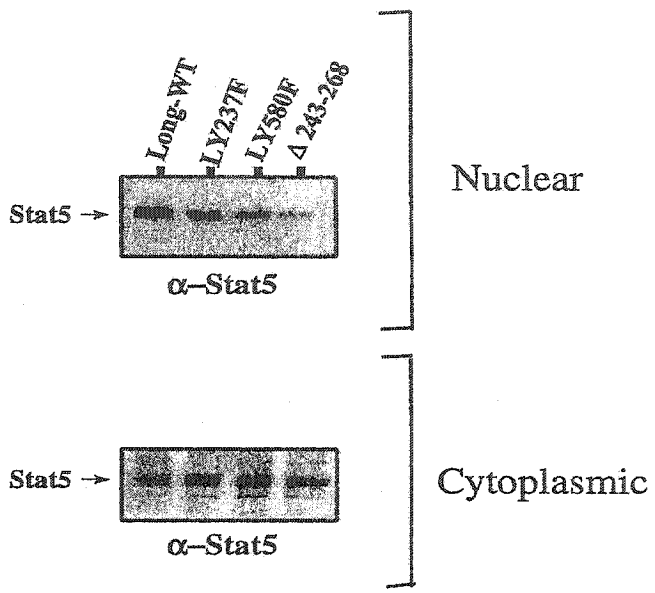


Figure 2.6. Tyrosine 580 of the PRLR long form does not influence Stat5 nuclear translocation. Stat5 and either Long-WT, LY237F, LY382F, or the mutant Δ243-268 were co-overexpressed in 293-LA cells as described in legend to Fig. 5. Cultures were then stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts or total cell extracts were prepared from two stets of cells that were simultaneously transfected. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes. Both membranes were probed with monoclonal antibody to Stat5

electrophoretic mobility shift assay was performed using the GAS sequence of the □-casein gene promoter. As shown in figure 2.7, we did not detect any Stat5 DNA binding activity in samples overexpressing the negative control mutant Δ243-268. However, Stat5 DNA binding activity was detected at similar levels in samples overexpressing the PRLR long form and the two tyrosine mutant forms LY237F and LY580F. These results indicate that mutation of tyrosine 580 of the PRLR long form does not affect PRLR-regulated Stat5/DNA interactions.

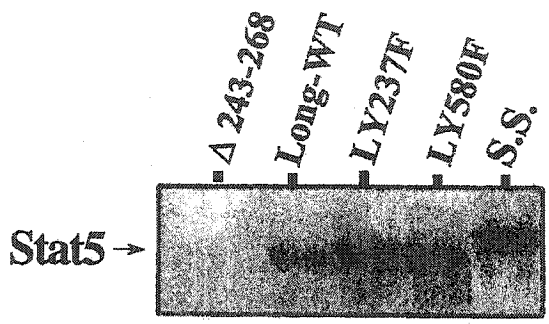


Figure 2.7. Tyrosine 580 of the PRLR long form does not affect significantly Stat5 binding to the GAS sequence of the b-casein gene promoter. 293-LA cells ($5x10^5$ cells) were transiently co-transfected with cDNAs encoding Stat5 and either Long-WT, LY237F, LY382F, or the mutant D243-268 as described in legend to figure 6. Cells were stimulated with oPRL (1.5 mg/ml) for 10 min. nuclear extracts were prepared and EMSA was performed using Stat5 response element of the b-casein gene promoter. The Stat5/DNA complex was super-shifted (S.S.) with polyclonal antibody to Stat5a.

## DISCUSSION

The initial steps in the signaling mechanisms of the PRLR, a member of the cytokine receptor superfamily, have recently been elucidated. Following PRL/receptor engagement there is activation of cytoplasmic tyrosine kinases of the Jak and Src families. This process leads to activation and tyrosine phosphorylation of the kinase, the receptor and cellular effector molecules resulting in the transmission of PRL biological responses.

Here we investigated the mechanism through which PRLR regulate the activation of the signal transducer and activator of transcription, Stat5. In the HC11 mammary epithelial model system which we used in this study, we find that PRL is able to induce Stat5 tyrosine phosphorylation, nuclear translocation, and DNA binding to the GAS-like element of the β-casein gene promoter. This clearly demonstrate that Stat5 is a physiological downstream mediator in PRLR intracellular signaling pathway in mammary epithelial cells. This is consistent with the fact that PRL induces activation of Stat5 in overexpression system (30) and in the Nb2 rat T-lymphoma cell line (43).

Stat5 is a member of the Stat family of transcription factors downstream of cytokine receptors. Our understanding of the mechanism of activation of these factors in the signaling relay of cytokine receptors is limited. It has been shown in different systems, however, that these factors undergo tyrosine phosphorylation on a conserved C-terminal tyrosine residue necessary for their homo- or hetero-dimerization. This process leads to their translocation to the nucleus where they bind to specific response elements on target genes to regulate gene expression (4, 12).

Stat5 is activated by a number of cytokines and growth factors (32). Studies on the role of the cytokine receptors to mediate Stat5 protein activation focused on

events like Stat5 association to the receptor, tyrosine phosphorylation and DNA binding. These studies indicated that Stat5 tyrosine phosphorylation and DNA binding via the GHR, EPOR and IL-2Rβ chain requires receptor phosphotyrosines (33-37, 44-46). While, similar events mediated through the IL-6-gp130 receptor system and G-CSFR was found to be independent of receptor phosphotyrosines (38-39). Limited attempts, however, have been performed to study the effect of receptor phosphotyrosine residues on Stat proteins nuclear translocation event and its regulation mechanism.

We report here for the first time that the C-terminal tyrosine residue of the PRLR does not influence Stat5 tyrosine phosphorylation, but it is required for regulating Stat5 nuclear translocation and DNA binding. Our data suggest a possible direct Jak2-Stat5 interaction or through an adapter protein may be taking place allowing Stat5 tyrosine phosphorylation by the kinase Jak2 itself independently of PRLR tyrosine phosphorylation. This would be consistent with what was demonstrated for the Il-6-gp130 receptor system (38). We observed that Stat5 tyrosine phosphorylation did not grant its nuclear translocation. This implies that Stat5 activation via the PRLR involves at least two separately regulated events; tyrosine phosphorylation and nuclear translocation. Whether other members of the cytokine receptor superfamily share the same feature remains to be determined.

We also report that tyrosine 580 of the PRLR long, which is homologous to tyrosine 382 of the PRLR Nb2 form, to have no effect on Stat5 nuclear translocation or DNA binding. A redundancy may be implicated in this tyrosine activity since the PRLR long form has six other tyrosine residues that can potentially substitute for the mutated tyrosine 580. Indeed, in contrast to the PRLR Nb2 form, mutation of tyrosine 580 of the PRLR long form does not lead to inhibition of receptor tyrosine

phosphorylation indicating that other tyrosines on the PRLR long form may undergo phosphorylation following receptor activation. In addition, mutation of the C-terminal tyrosine in the PRLR long form only partially inhibited β-casein gene promoter activation, suggesting again that other alternative tyrosine residues specific to the receptor long form might mediate PRL effects. Moreover, it has been recently suggested that tyrosines 509 and 496 of the PRLR long form in the context of gp130 truncation mutant to be potential sites for mediating Stat5 activation and DNA binding (47). Using myeloid cell system, however, a previous report suggested a mechanism for activation of Stat5 independent of PRLR phosphotyrosines (26). These seemingly controversial observations might be due to differences in the GAS-like element used in the study. In addition, it is possible that the PRL-induced DNA binding activity observed is that of Stat5 related proteins in myeloid cells (48)

The mechanism by which tyrosine 382 of the PRLR Nb2 form exert its effect on Stat5 nuclear translocation is not known. We speculate that this phosphotyrosine residue is involved in the activation of a certain cellular component that modulates Stat5 complexes in the cytoplasm controlling their nuclear entry.

Accumulating evidence suggest that Stat proteins do not act in seclusion, rather they are a part of complex interactions with a number of other cellular components. For example, Stats1 and 3 are shown to be phosphorylated by mitogen-activated protein kinase (MAPK) on a serine residue adjacent to the conserved tyrosine phosphorylation site (49, 50). Furthermore, Stat5 has been shown to be serine phosphorylated following PRL stimulation (43), however, the physiological significance of this phosphorylation is not known. Potentially it can play a role in Stat5 activation including nuclear translocation. Stat proteins have also been shown to interact with other cellular components besides MAPK, examples are, PI-3

kinase (51), glucocorticoid receptor (52), and a number of nuclear transcription factors (53). Any one of these molecules, or even possibly a new cellular protein, can potentially influence Stat5 activation by the PRLR. Further investigation is required to determine how receptor phosphotyrosines are affecting Stat proteins nuclear translocation.

In conclusion, we demonstrate in this study for the first time that Stat5 activation involves two separately regulated events, Stat5 tyrosine phosphorylation and nuclear translocation. We further report that Stat5 tyrosine phosphorylation induced by the PRLR is independent of receptor phosphotyrosines; and we established that tyrosine 382 of the PRLR Nb2 form positively regulates Stat5 nuclear translocation.

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# CHAPTER III. RECRUITMENT OF THE PROTEIN TYROSINE PHOSPHATASE SHP-2 TO THE CARBOXY-TERMINAL TYROSINE OF THE PROLACTIN RECEPTOR AND TO THE ADAPTOR PROTEIN GAB2

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## FORWARD

The C-terminal tyrosine of the PRLR plays a critical role in regulating the signaling capacity of the receptor through modulating the activation of Stat5. The mechanism by which the C-terminal tyrosine of the receptor participated in signaling was not fully known.

The protein tyrosine phosphatase SHP-2 was reported to play an essential positive role in the activation and signaling of the PRLR. Therefore, the second objective was to investigate the role of the C-terminal tyrosine in the function of SHP-2 within PRLR signaling.

The work established that the C-terminal tyrosine of the PRLR is a site for the recruitment of SHP-2, and shows that the recruitment of SHP-2 to the C-terminal tyrosine occurs through the C-terminal SH2 domain of the phosphatase. Furthermore, the study determines that the adaptor protein Gab2, but not Gab1, is tyrosine phosphorylated in response to stimulation of the PRLR. Also the study indicates that in addition to recruitment to the PRLR, SHP-2 is also recruited to the phosphorylated Gab2 adaptor protein.

## ABSTRACT

The protein tyrosine phosphatase SHP-2 modulates signaling events through receptor tyrosine kinases and cytokine receptors including the receptor for prolactin (PRLR). Here we investigated mechanisms of SHP-2 recruitment within the PRLR signaling complex. Using SHP-2 and PRLR immunoprecipitation studies in 293 cells and in the mouse mammary epithelial cell line HC11, we found that SHP-2 co-immunoprecipitates with the PRLR and that the carboxy (C)-terminal tyrosine of the PRLR plays a regulatory role in both the tyrosine phosphorylation and the recruitment of SHP-2. Our results further indicate that SHP-2 association to the PRLR occurs via the C-terminal SH2 domain of the phosphatase. In addition, we determined that the newly identified adaptor protein Gab2, but not Gab1, is specifically tyrosine phosphorylated and is able to recruit SHP-2 and PI3-K in response to PRLR activation. Together, these studies suggest the presence of dual recruitment sites for SHP-2, the first is to the C-terminal tyrosine of the PRLR and the second is to the adaptor protein Gab2.

## INTRODUCTION

The prolactin receptor (PRLR) is a member of the large cytokine receptor superfamily. Ligand stimulation induces receptor dimerization which leads to the activation of the constitutively associated Jak2 kinase as well as members of the Src family of cytoplasmic tyrosine kinases (1,2). Activated Jak2 molecules phosphorylate themselves as well as receptor subunits on tyrosine residues creating docking sites for SH2 domain containing proteins belonging to various signaling pathways. Activated PRLR can induce the activation of the signal transducer and activator of transcription 5 (Stat5), MAPK and PI3-K pathways and the activation of several signaling regulatory proteins including the protein tyrosine phosphatase SHP-2.

The Src homology 2 domain-containing protein tyrosine phosphatase, SHP-2, is a ubiquitously expressed protein that is characterized by having two amino (N)-terminally located SH2 domains and a carboxy (C)-terminal catalytic domain. The phosphatase plays a critical role in cell growth and differentiation as evidenced by the early embryonic lethality in SHP-2$^{-/-}$ mice (3). SHP-2 plays an essential regulatory role in signaling from certain receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) and insulin receptor (IR) (4) as well as cytokine receptors such as the α/βinterferon receptors (IFN α/βreceptor (5), (6) and the PRLR (7). We have previously shown that SHP-2 is tyrosine phosphorylated in response to PRL stimulation and physically associates with the PRLR/Jak2 complex. We also determined that SHP-2 is a positive mediator of PRLR signaling leading to the activation of β-casein gene promoter (7). However, the mechanism and site(s) of SHP-2 recruitment within the PRLR signaling complex remain unknown.

SHP-2 phosphatase can be directly recruited via its two SH2 domains to membrane receptors, cell surface proteins as well as a number of intracellular signaling

proteins. Specific tyrosine residues on the β chain of the granulocyte macrophage-colony stimulating factor (GM-CSF) receptor (8), the platelet derived growth factor receptor (PDGF) (9), the gp130 subunit of the interleukin-6 receptor (IL-6R) (10) and the carboxy-terminal portion of the growth hormone receptor (GHR) (11) are sites for SHP-2 recruitment. Point mutations of these residues eliminated SHP-2 association, and tyrosine phosphorylation. Furthermore, through the SH2 domains, SHP-2 interacts with a number of cellular proteins such as signal regulatory protein (SIRP/SHPS) (12), SH2-containing inositol phosphatase (SHIP) (13), insulin receptor substrates (IRS) -1 and –2 (14), Jak2 (15) and importantly, members of the Grb2 associate binder (Gab) family.

The Gab family of proteins, p110 Gab1 (16), p97 Gab2 (17) and the Drosophila DOS protein (18), contain a number of structurally conserved regions. These proteins contain an N-terminal PH domain, proline rich motifs and multiple similarly situated tyrosine phosphorylation residues. In addition, these proteins have unique tissue distributions (17) suggesting that they serve non-redundant functions. These proteins appear to function primarily as adaptor proteins linking activated receptor tyrosine kinase and cytokine receptors to several signaling molecules. EGF, Insulin (19), hepatocyte growth factor (HGF) (20), IL-3, IL-6, IFN α/β (21) and erythropoietin (EPO) (22), (23) have been reported to induce the tyrosine phosphorylation of Gab1 and its association with the SH2 domain containing protein SHP-2 (22), (23), (24), (25), the p85 subunit of PI3-K (26), (27), SHIP (23), Shc (23) and Grb2 (16), (21). Other studies have shown that IL-2, colony stimulating factor-1 (CSF-1) (17), IL-3, IL-6, and the activation of the T-cell receptor (TCR), B-cell receptor (BCR) (28) can induce tyrosine phosphorylation of Gab2 and its association with SHP-2, the p85 subunit of PI3-K, CrkL and Grb-2. Therefore, it appears that certain cytokines are able to induce the tyrosine phosphorylation of both Gab1 and Gab2 whereas others may utilize one member only.

In this study, we investigated the sites and mechanisms of SHP-2 recruitment to the PRLR. We determined that the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment. In addition, we determined that Gab2 but not Gab1 is tyrosine phosphorylated following PRL stimulation of mammary cells and that Gab2 presents a second site for SHP-2 recruitment to the PRLR signaling complex.

## MATERIALS AND METHODS

***Materials, Antibodies and Plasmid Constructs***— expression plasmids encoding the PRLR mutants LY237F, LY580F, NY237F, NY382F, Δ296-322, ΔY237F, ΔY382F and Δ243-268 were described previously (29). Expression plasmids encoding SHP-2, the SHP-2 R32K and SHP-2 R138K and the polyclonal antibody for p110 subunit of the PI3-K were obtained from Axel Ullrich (Max-Plank Institute, Germany). Monoclonal antibody to phosphotyrosine (4G10) was from Upstate Biotechnology, monoclonal antibody to SHP-2 was form Transduction Laboratories, and polyclonal antibody to SHP-2 was from Santa Cruz. Polyclonal Gab1 antibodies and HA-tagged Gab1 expression plasmid were kindly provided by Toshio Hirano (Osaka University, Osaka, Japan). Polyclonal Gab2 antibodies and HA-tagged Gab2 and Gab2DM expression plasmids were generously supplied by Benjamin Neel and Haihua Gu (Harvard Medical School, Boston, USA). The U6 monoclonal antibody to the PRLR was provided by Paul Kelly (Paris, France). Hemagluttinin antibodies were from Santa Cruz. Protein-A sepharose beads used for immunoprecipitations were from Pharmacia. Ovine PRL (oPRL) used for treatment of cells was obtained from Sigma.

***HC11 Cell Culture***— HC11, mouse mammary epithelial cells, obtained from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Bernd Groner (Georg Speyer Haus, Frankfurt, Germany), were grown to confluency in RPMI-1640 media containing 10% fetal calf serum (FCS) (Gibco), insulin (5μg/ml), and EGF (10ng/ml). Cells were then induced by incubating them for 3 days in RPMI media containing 10% FCS, insulin (5μg/ml), and hydrocortisone (1μM) (30,31). Depending on the specific experiment (see results section), cells were either starved in RPMI media containing insulin (5μg/ml), hydrocortisone (1μM) or only in RPMI media without insulin and hydrocortisone. Cells were then stimulated with oPRL (1.5μg/ml) for the time

indicated. Cells were lysed in lysis buffer [10mM Tris-HCl, ph7.5/ 5mM EDTA/ 150mM NaCl / 30mM sodium pyrophosphate/ 50mM sodium floride/ 1mM sodium orthovanadate/ 10% (vol/vol) glycerol/ 0.5% Triton X-100] containing protease inhibitors [1mM phenylmethylsulphonyl floride/ leupeptin (2μg/ml)/ aprotinin (5μg/ml) for 5 minutes at 4°C. The lysates were then centrifuged at 12,000 x g for 10 min at 4°C to remove insoluble material. Protein concentration was measured using the Bradford technique.

***Transient Transfection***— the human embryonic 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM 4.5 g/liter glucose) (Bio Media) containing 10% (vol/vol) FCS. Approximately $5x10^5$ cells were plated then co-transfected with expression plasmids encoding the different forms of RPLR (1μg each), SHP-2 (1μg) and the kinase Jak2 (0.25μg) by the calcium phosphate technique. After 24 hours of expression, the cells were starved by serum deprivation over night.

***Total Cell Lysis Immunoprecipitations and Western Blotting***—Transiently co-transfected 293 cells were stimulated with oPRL (1.5μg/ml) for 5 minutes and then lysed in lysis buffer [10mM Tris-HCl, ph7.5/ 5mM EDTA/ 150mM NaCl / 30mM sodium pyrophosphate/ 50mM sodium floride/ 1mM sodium orthovanadate/ 10% (vol/vol) glycerol/ 0.5% Triton X-100] containing protease inhibitors [1mM phenylmethylsulphonyl floride/ leupeptin (2μg/ml)/ aprotinin (5μg/ml) for 5 minutes at 4°C. The lysates were then centrifuged at 12,000 x g for 10 min at 4°C to remove insoluble material. Protein concentration was measured using the Bradford technique. Equal amounts of protein obtained by total lysis were loaded and run on an 8% SDS-PAGE. Immunoprecipitations were performed on endogenous or overexpressed cell lysates. Extracts were immunoprecipitated for two hours (or overnight for endogenous proteins) using specific antibodies (SHP-2, PRLR, HA, Gab1 and Gab2) and protein-A sepharose beads. Precipitates were then washed with HNTG buffer [20mM HEPES pH 7.5, 150 mM NaCl,

0.1% Triton X-100, 10% glycerol], separated on SDS-PAGE. Western blotting analysis was performed using the indicated antibodies. Proteins were revealed using chemiluminescence (Super Signal kit from Pierce) following the manufacturer's instructions.

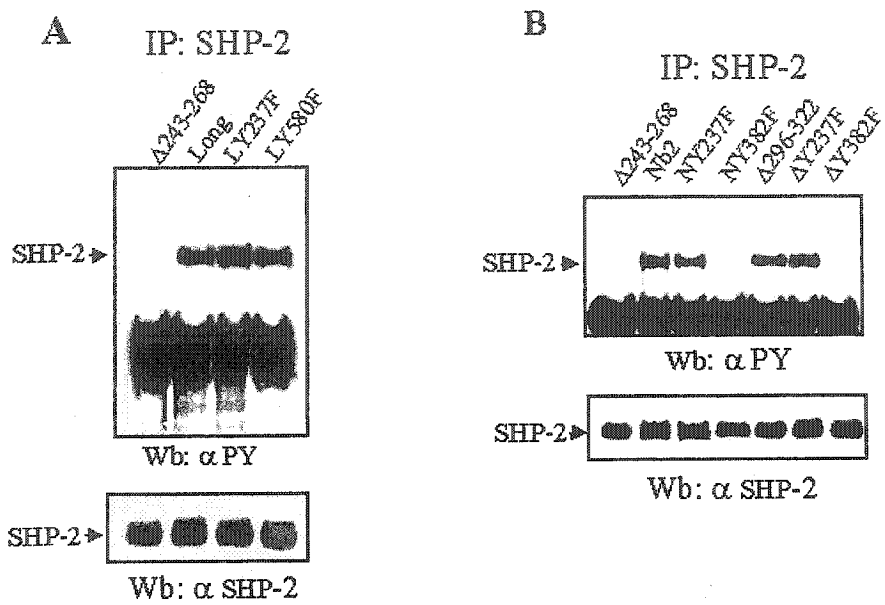


Figure 3.1 The C-terminal tyrosine of the PRLR regulates the state of SHP-2 tyrosine phosphorylation. A, 293 cells were transiently transfected with expression plasmids encoding SHP-2 along with the Δ243-268, the long form of the PRLR, LY237F or LY580F. B, 293 cells were transfected with expression plasmids encoding SHP-2 and the Nb2 form of the PRLR, NY237F, NY382F, Δ293-322, ΔY237F or ΔY382F. Cells were lysed and SHP-2 was immunoprecipitated with a polyclonal antibody to SHP-2. Immunoprecipitates were run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with monoclonal antibodies against phosphotyrosine (upper panel) then stripped and reprobed with monoclonal antibodies to SHP-2 (lower panel).

## RESULTS

**The C-terminal tyrosine of the PRLR regulates the state of SHP-2 tyrosine phosphorylation**—We have previously reported that the C-terminal tyrosine of the PRLR to be critical for the induction of the prolactin responsive β-casein gene (29) and the activation of Stat5 (32). In addition, a positive role for SHP-2 in the activation of Stat5 and the induction of early genes has also been reported (7), (33). Therefore, we hypothesized a regulatory role for the C-terminal tyrosine of the PRLR in the activation of SHP-2. In order to test this hypothesis, we used various natural, mutant and deletion forms of the PRLR for the purpose of focusing on the C-terminal tyrosine of the PRLR. The long form of the PRLR has nine intracellular tyrosine residues. The PRLR Nb2 form, found in Nb2 T-lymphoma cells, has an inframe deletion mutation that removes six of the nine intracellular tyrosine residues found in the long form leaving tyrosine residues 237, 309 and 382 (34). The C-terminal tyrosine 382 of the PRLR Nb2 form corresponds to tyrosine 580 of the PRLR long form. Another deletion form that is capable of inducing β-casein gene expression is the PRLR Nb2 mutant form Δ296-322 (29). This receptor form has a 27 amino acids internal deletion and lacks tyrosine 309. Tyrosine to phenylalanine point mutants of the membrane proximal (LY237F, NY237F, ΔY237F) and the C-terminal tyrosines (LY580F, NY382F, ΔY382F) in the long form, Nb2 form and the Δ296-322 mutant form were described previously (29), (32). Finally, the PRLR Nb2 mutant form Δ243-268 is also used in our studies as a negative control (29). This mutant receptor is unable to associate with Jak2 kinase due to the deletion of the proline rich interaction domain and is incapable of signaling in response to PRL stimulation (35), (29).

To investigate the effect of the C-terminal tyrosine of the PRLR on SHP-2 tyrosine phosphorylation, the human embryonic kidney 293 cells were transiently

transfected with expression plasmids that encode the phosphatase SHP-2 and either the wild type long form PRLR, the tyrosine to phenylalanine point mutants of the membrane proximal tyrosine LY237F or the C-terminal tyrosine LY580F (Fig. 3.1A). Another set of 293 cells was transfected with expression plasmids encoding SHP-2 and either the wild type intermediate Nb2 form of the PRLR, tyrosine to phenylalanine mutant of the membrane proximal tyrosine NY237F or the C-terminal tyrosine NY382F. In addition, expression plasmids encoding the Δ296-322 mutant form of the Nb2 receptor, and tyrosine to phenylalanine mutant of the membrane proximal tyrosine ΔY237F or the C-terminal tyrosine mutant ΔY382F were co-transfected into 293 cells (Fig. 3.1B). In addition to the PRLR and SHP-2, 293 cells were transfected with a limited amount of an expression plasmid for Jak2 tyrosine kinase (see Materials and Methods).

SHP-2 was immunoprecipitated from cellular lysates using polyclonal antibodies for SHP-2. Precipitates were run on SDS-PAGE, transferred to a nitrocellulose membrane and probed with monoclonal antibodies for phosphotyrosine in a Western Blot. As expected no SHP-2 phosphorylation was detectable in samples expressing the deletion mutant Δ243-268 (Fig. 3.1A). In contrast, a strong phosphorylation of SHP-2 was detected in the samples expressing the wild type long form of the PRLR, and also in samples expressing both the membrane proximal mutant LY237F and the C-terminal mutant LY580F (Fig. 3.1A). Moreover, SHP-2 phosphorylation was also detectable in the samples expressing the wild type Nb2 form of the PRLR and the membrane proximal mutant NY237F (Fig. 3.1B). Interestingly, however, no SHP-2 phosphorylation was detected in samples expressing the C-terminal mutant of the Nb2 form of the PRLR receptor NY382F (Fig. 3.1B). Similarly, tyrosine phosphorylation levels of SHP-2 were comparable in samples expressing the Δ296-322 form as well as its corresponding tyrosine to phenylalanine mutants ΔY237F (Fig. 3.1B). In agreement with the results

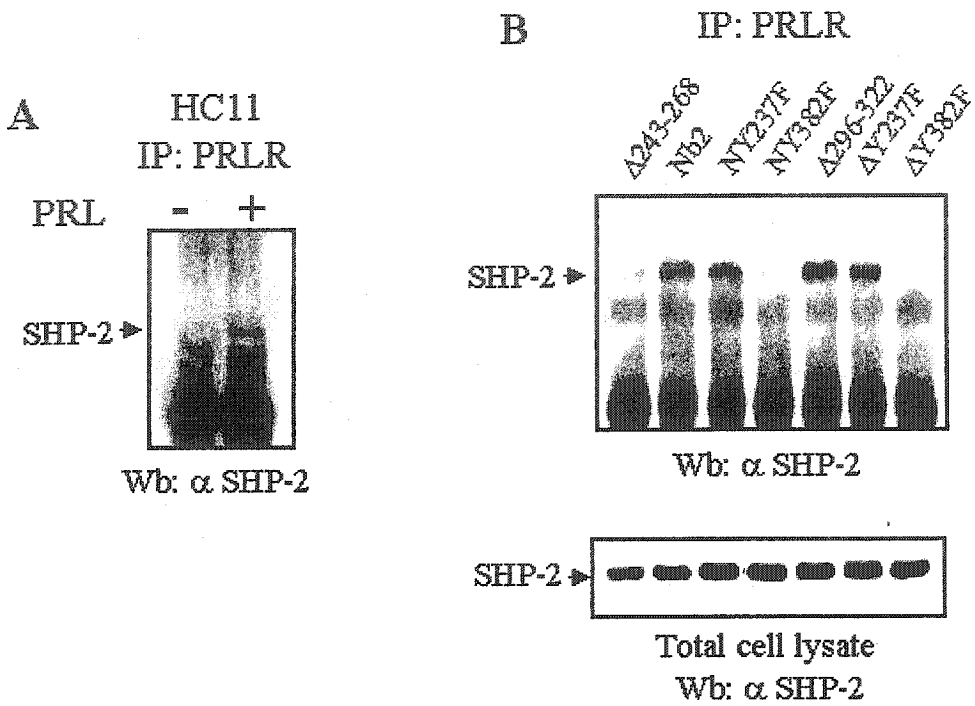


Figure 3.2 SHP-2 is recruited to the C-terminal tyrosine of the PRLR. A, HC11 cells were either non stimulated or stimulated with PRLR for 10 minutes. B, 293 cells were transfected with expression plasmids encoding SHP-2 and the Δ243-268, the Nb2 form of the PRLR, NY237F, NY382F, Δ293-322 , ΔY237F or ΔY382F. Cells were lysed and the PRLR was immunoprecipitated using the U6 monoclonal antibody against the PRLR. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes then blotted with monoclonal antibodies against SHP-2. In the lower panel, total cellular lysates obtained from the same transfection were run on SDS-PAGE, transferred to a nitrocellulose membrane and blotted with monoclonal antibodies to SHP-2.

obtained for the PRLR Nb2 form, SHP-2 tyrosine phosphorylation was lost in the sample expressing the C-terminal mutant of the Δ296-322 form, ΔY382F (Fig. 3.1 B). The loss of SHP-2 tyrosine phosphorylation in samples expressing the C-terminal tyrosine to phenylalanine mutants of the PRLR suggests that this tyrosine plays a regulatory role in SHP-2 tyrosine phosphorylation. The sustenance of SHP-2 phosphorylation in the samples expressing the C-terminal tyrosine mutation in the long form of the PRLR suggests that in addition to the C-terminal tyrosine, one or more of the cytoplasmic tyrosine residues that are present in the long form of the PRLR but absent form the Nb2 form of the PRLR may play a role in SHP-2 phosphorylation. The membranes were stripped and reprobed with a monoclonal antibody to SHP-2 in a Western blot analysis to confirm equal expression of the phosphatase (Fig. 3.1A & 3.1B lower panels).

**The carboxy terminal tyrosine of the PRLR is a site for SHP-2 recruitment**—the loss of SHP-2 tyrosine phosphorylation in samples expressing the C-terminal tyrosine to phenylalanine mutant of the PRLR suggests that this tyrosine may act as a recruitment site for SHP-2. To determine whether SHP-2 associates to the PRLR, we initially examined this association in the PRL responsive mammary epithelial cell line HC11. The PRLR was immunoprecipitated using the U6 monoclonal antibody against the PRLR from the mouse mammary epithelial cell line HC11 that were induced and starved then were either non treated or treated with PRL for 10 minutes (see Materials and Methods). Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes and blotted with monoclonal antibody against SHP-2 in a Western blot analysis. PRL stimulation of HC11 cells induces a specific association between SHP-2 and the PRLR (Fig. 3.2A).

To confirm the association of SHP-2 to the PRLR and to determine whether the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment, the receptor was immunoprecipitated from 293 cells transiently transfected with expression plasmids encoding SHP-2, the Nb2 form of the PRLR, NY237F, NY382F, Δ296-322,ΔY237F, ΔY382F, and a limited amount of an expression vector encoding Jak2 kinase. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membrane and blotted with a monoclonal antibody against SHP-2 in Western blotting. No SHP-2 was detectable in samples expressing the control Δ243-268 deletion form of the PRLR (Fig. 3.2B). A clear SHP-2 co-immunoprecipitation with the PRLR was detectable in samples expressing the wild type Nb2 form, NY237F, Δ296-322 and ΔY237F (Fig. 3.2B). However, no co-immunoprecipitating SHP-2 was detectable with the PRLR complexes in the samples expressing the C-terminal tyrosine to phenylalanine mutants NY382F and ΔY382F (Fig. 3.2B). Altogether, these results indicate that the C-terminal tyrosine of the PRLR is a site for SHP-2 association.

No detectable decrease in the association between SHP-2 and the PRLR was observed in samples expressing the C-terminal tyrosine to phenylalanine mutant of the long form of the PRLR LY580F (data not shown). The sustenance of SHP-2 tyrosine phosphorylation and association to the PRLR with the C-terminal mutant of the long form of the PRLR suggests that there is redundancy in SHP-2 association to the PRLR long form where one or more of the tyrosine residues that are only present in the long form of the PRLR can provide additional association sites for SHP-2.

**SHP-2 utilizes the C-terminal SH2 domain for association to the PRLR**—The protein tyrosine phosphatase SHP-2 has two N-terminally located SH2 domains. To determine the mechanism of association between SHP-2 and the PRLR, the ability of the two SH2

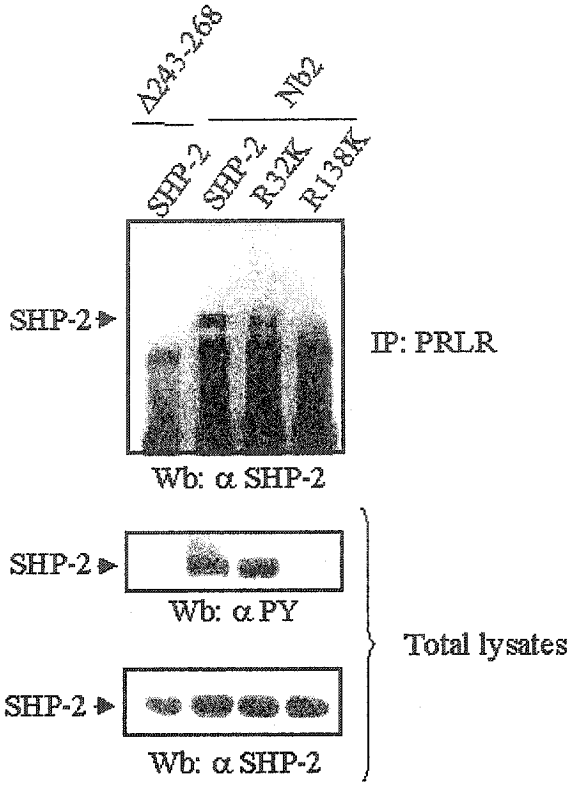


Figure 3.3 SHP-2 utilizes the C-terminal SH2 domain for association to the PRLR. A, 293 cells were transfected with expression plasmids encoding either the Δ243-268 mutant form along with wild type SHP-2 or the Nb2 form along with the either the wild type SHP-2, the N-terminal or the C-terminal SH2 domains arginine to lysine point mutants, R32K and R138K respectively. Total cellular lysates were run on SDS-PAGE, transferred to nitrocellulose membranes and blotted with monoclonal antibodies to phosphotyrosine or monoclonal antibodies to SHP-2. The same cellular lysates were prepared for immunoprecipitation using U6 monoclonal antibodies against the PRLR. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes and blotted with monoclonal antibodies against SHP-2.

domains to bind to phosphotyrosine motifs was interrupted by point mutations that substitute the critical arginine in the SH2 domain to lysine. 293 cells were co-transfected with expression plasmids for either the Δ243-268 deletion form of the PRLR along with SHP-2 wild type or the Nb2 form of the PRLR with SHP-2 wild type, the arginine to lysine mutant of the N-terminal SH2 domain, SHP-2 R32K mutant, or the C-terminal SH2 domain SHP-2 R138K mutant (7). In addition, cells were co-transfected with an expression plasmid for the kinase Jak2. Cellular lysates were prepared for immunoprecipitation of the PRLR using the U6 monoclonal antibody. The immunecomplexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane then blotted with monoclonal antibodies to SHP-2 (Fig. 3.3). Both wild type SHP-2 and the SHP-2 K32R mutant forms can be detected co-immunoprecipitating with the PRLR. In contrast, SHP-2 did not co-immunoprecipitate with the PRLR in samples expressing the SHP-2 R138K mutant. Therefore, SHP-2 interacts with the C-terminal tyrosine of the PRLR through the C-terminal SH2 domain.

To investigate the individual roles of the N- and C-terminal SH2 domains of SHP-2 in regulating tyrosine phosphorylation levels of the phosphatase, total cellular lysates obtained from the same transfection described above were run on SDS-PAGE, transferred to a nitrocellulose membrane then blotted with antibodies to phosphotyrosine. As shown in Figure 3.3 (middle panel) tyrosine phosphorylation of SHP-2 was observed in samples expressing wild type SHP-2 as well as SHP-2 R32K. In contrast, tyrosine phosphorylation of SHP-2 was lost in the sample expressing SHP-2 R138K (Fig. 3.3 middle panel) further indicating the importance of the C-terminal SH2 domain of SHP2 for recruitment to the PRLR and tyrosine phosphorylation of SHP-2. The membrane was stripped then re-blotted with a monoclonal antibody against SHP-2 to confirm equal expression of the protein (Fig. 3.3 lower panel) .

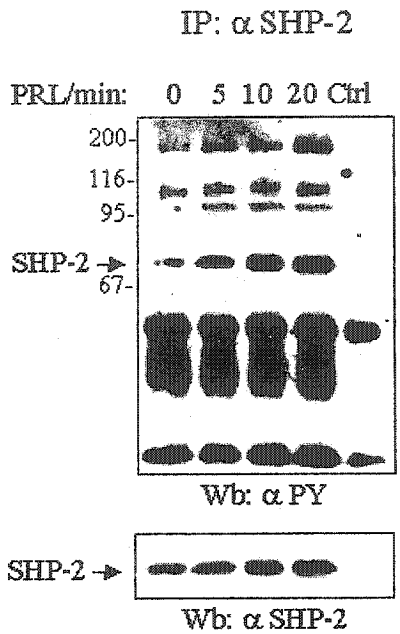


Figure 3.4 Multiple tyrosine phosphorylated proteins associate to SHP-2 following PRL stimulation of HC11 cells. HC11 cells were either nonstimulated or stimulated with PRL for 5, 10 or 20 minutes. Cells were lysed and SHP-2 was immunoprecipitated using polyclonal antibodies to SHP-2. The complexes were separated on SDS-PAGE then transferred to a nitrocellulose membrane and blotted with monoclonal antibodies to phosphotyrosine. The membrane was stripped and reprobed with monoclonal antibodies against SHP-2. Ctrl sample contains antibody and beads but no lysates.

**95-110 kDa Tyrosine phosphorylated proteins associate to SHP-2 in HC11 cells—** The protein tyrosine phosphatase SHP-2 is known to associate with multiple tyrosine phosphorylated proteins. In order to examine the different possible associations of SHP-2 with tyrosine phosphorylated proteins in response to PRLR activation, we performed SHP-2 immunoprecipitations in the mouse mammary epithelial cell line HC11. These cells were grown to confluency, induced to differentiate and starved for 48 hours. Then the cells were either nonstimulated or stimulated with PRL for 5, 10 and 20 minutes. Cellular lysates were prepared for immunoprecipitation with polyclonal antibody to SHP-2. The immunecomplexes were run on SDS-PAGE, transferred to a nitrocellulose membrane then Western blotted with a monoclonal antibody to phosphotyrosine. As shown in Fig. 3.4, SHP2 is tyrosine phosphorylated in the basal condition in HC11 cells. However, there is a clear increase in its phosphorylation state following PRL stimulation. Furthermore, in these immunoprecipitates of SHP2 we observe a number of tyrosine phosphorylated proteins, including proteins in the range of 95-110kDa, co-precipitating with SHP2. Two of these proteins having a molecular weight of 180 kDa and 110 kDa are constitutively tyrosine phosphorylated. However, a protein of 95 kDa undergoes tyrosine phosphorylation in response to PRL stimulation. Together, this data indicates that SHP2 interacts with multiple tyrosine phosphorylated proteins in mammary epithelial cells.

**Gab2 is specifically tyrosine phosphorylated and recruits SHP-2 and PI3-K in response to PRL stimulation in HC11 cells—**Recently a family of proteins that have molecular sizes in the range of 95-110 kDa called the Gab (Gab1 and Gab2) family of adaptor proteins was identified. These proteins were reported to be tyrosine phosphorylated and to recruit SHP-2 within the signaling complexes for several receptor

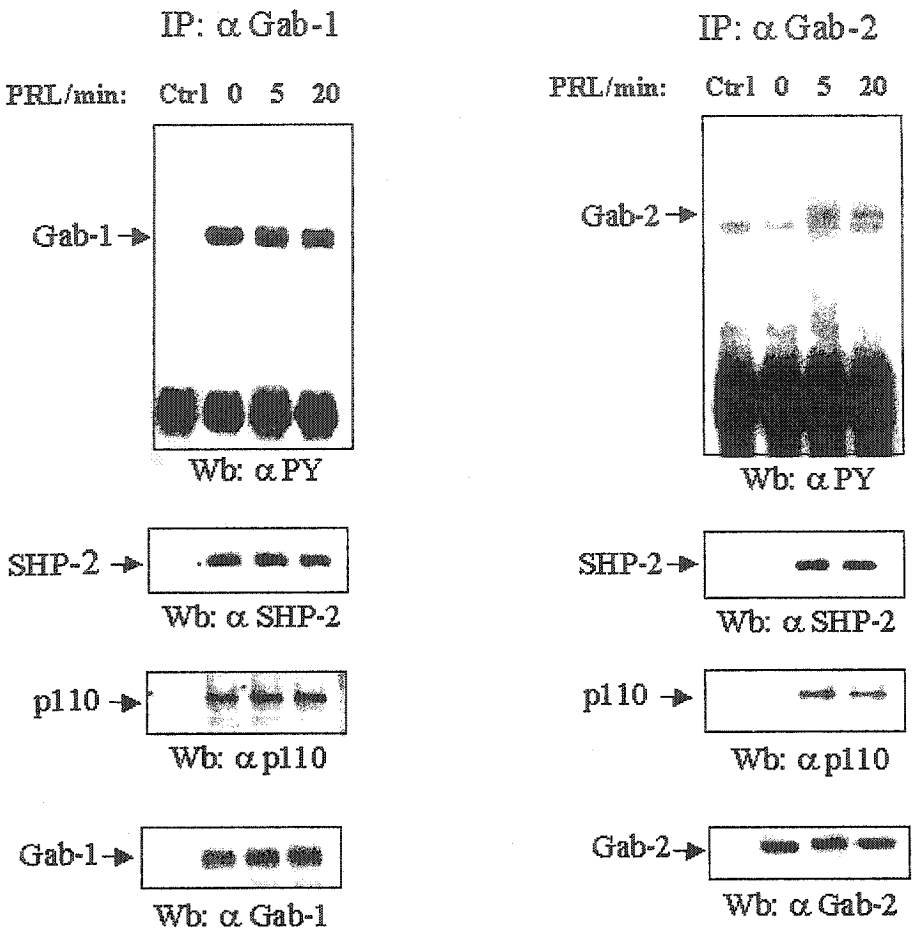


Figure 3.5 Gab2 is specifically tyrosine phosphorylated and recruits SHP-2 and PI3-K in response to PRL stimulation of HC11 cells. HC11 cells were either nonstimulated or stimulated with PRL for 5 and 20 minutes. Cells were lysed and lysates were immunoprecipitated with polyclonal antibodies to either Gab1 or Gab2. Sample (Ctrl) contains antibodies and beads but no lysates was used as a control. Immunecomplexes were run on SDS-PAGE, transferred to nitrocellulose membranes and blotted with monoclonal antibodies to phosphotyrosine. The Membranes were stripped then reprobed with monoclonal antibodies against SHP-2, p110 subunit of the PI3-K, and with polyclonal antibodies to Gab1 or Gab2.

tyrosine kinases and cytokine receptors. Therefore, we intended to determine whether members of the Gab family of proteins are involved in PRLR mediated signaling and whether they are able to recruit SHP-2 in response to PRLR activation.

Two sets of the mouse mammary epithelial HC11 cells were grown to confluency, induced to differentiate and starved for 48 hours. The cells were then either non-stimulated or stimulated with PRL for 5 or 20 minutes. One set of cells was immunoprecipitated with a polyclonal antibody to Gab1 (Fig. 3.5A) and the second with a polyclonal antibody to Gab2 (Fig. 3.5B). Samples (Ctrl) containing antibody and protein A-sepharose beads were used as controls. Both sets of immunoprecipitates were run on SDS-PAGE then transferred to nitrocellulose membranes and probed with monoclonal antibodies to phosphotyrosine in a Western blot. Interestingly, in immunoprecipitates of Gab1, constitutive levels of tyrosine phosphorylation of the protein were detected irrespective of PRL stimulation (Fig. 3.5A). In contrast, in immunoprecipitates of Gab2, tyrosine phosphorylation of the protein was significantly increased following 5 minutes of PRL stimulation and remained elevated after 20 minutes (Fig. 3.5B). This suggests that Gab2 but not Gab1 is specifically tyrosine phosphorylated following PRLR activation in HC11 mammary epithelial cells. Equal levels of Gab1 and Gab2 immunoprecipitation were confirmed by stripping the membranes and reprobing with polyclonal antibodies to Gab1 and Gab2 (Fig. 3.5A and Fig. 3.5B lower panels, respectively). To confirm that Gab1 is not tyrosine phosphorylated in response to PRL stimulation, we examined the tyrosine phosphorylation state of the protein in 293 cells transiently transfected with the long form of the PRLR along with HA-tagged Gab1 and Jak2 kinase. Under these conditions, we also did not detect Gab1 tyrosine phosphorylation (data not shown). Altogether, our results suggest that gab2 but not Gab1 is a substrate for the PRLR.

To determine whether SHP-2 recruitment to Gab1 or Gab2 might be regulated by PRLR activation in HC11 cells, the membranes were stripped and reprobed with

monoclonal antibodies to SHP-2. Similar to tyrosine phosphorylation pattern of Gab1, SHP-2 recruitment to Gab1 was constitutive and was not affected by PRL stimulation (Fig. 3.5A). On the other hand, SHP-2 recruitment to Gab2 showed clear responsiveness to PRLR activation. Stimulation of HC11 cells for 5 or 20 minutes led to a significant increase in SHP-2/Gab2 complex formation (Fig. 3.5B). Therefore, SHP-2 association to Gab2 is regulated by PRL stimulation in the mammary epithelial cell line HC11.

Gab family members were also reported to associate with the p85 subunit of the PI3-K. To determine whether PRL stimulation can induce the association of PI3-K to Gab members, the membranes used were stripped and reprobed with polyclonal antibodies to the p110 subunit of the PI3-K. Gab1 associated to p110 irrespective of PRL stimulation (Fig. 3.5A), in contrast, Gab2 showed selective recruitment of p110 following PRLR activation (Fig. 3.5B). Altogether, our results indicate that Gab2, but not Gab1, is a specific target for PRLR activation and that the adaptor protein is capable of selectively recruiting SHP-2 and PI3-K in response to PRL stimulation.

**The three natural forms of the PRLR; long, intermediate Nb2 and short forms can induce Gab2 tyrosine phosphorylation and association to SHP-2 in 293 cells—** The rat PRLR has three forms; the long, the intermediate Nb2 and the short form. Only the long and Nb2 forms are tyrosine phosphorylated following PRL stimulation, and in biological assays testing β-casein gene promoter induction, only the long and Nb2 forms were able to activate expression of the PRLR responsive milk protein (34), (36). To determine whether stimulation of these three forms leads to tyrosine phosphorylation of Gab2 and its association to SHP-2. 293 cells were transfected with plasmids expressing HA-tagged Gab2 along with SHP-2 and the long, Nb2 or the short forms of the PRLR.

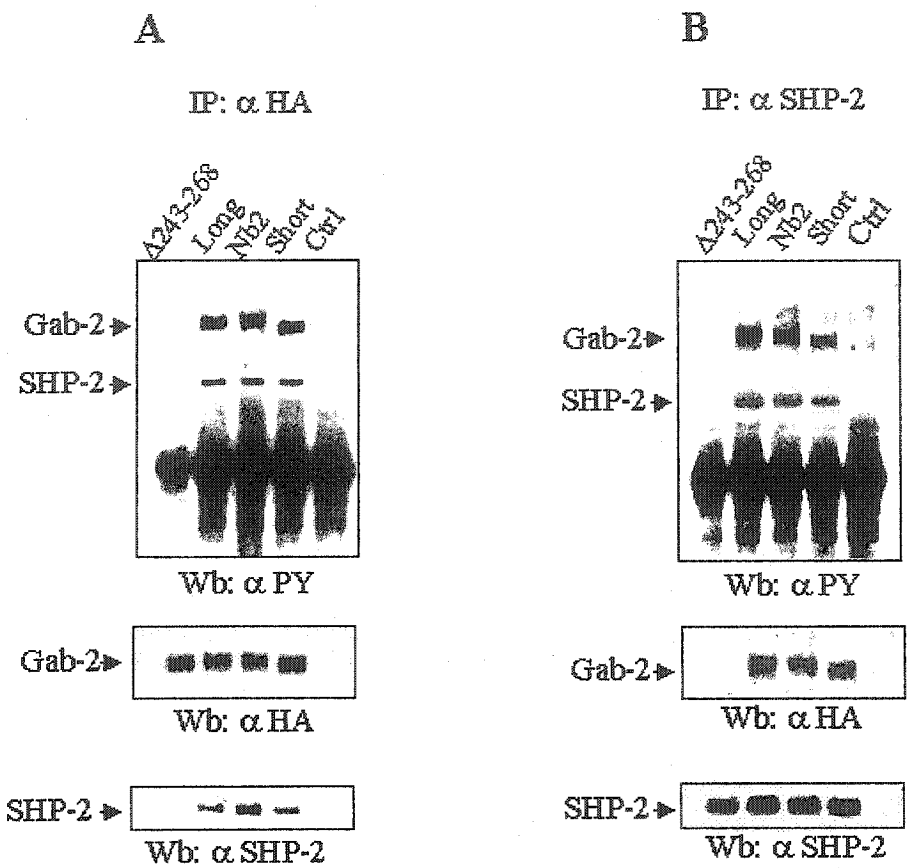


Figure 3.6 All three natural forms of the PRLR can induce Gab2 tyrosine phosphorylation and association to SHP-2—293 cells were transfected with expression plasmids encoding HA-tagged Gab2, SHP-2 and either the Δ243-268 deletion form, the long form, the Nb2 form or the short form of the PRLR. Cells were lysed and immunoprecipitations were carried out using either polyclonal antibody against HA tag (A) or using polyclonal antibody to SHP-2 (B). In both sets, sample (Ctrl) contains beads and the respective antibody was used as a control. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes and then HA immunoprecipitates were blotted with monoclonal antibodies against SHP-2 and SHP-2 immunoprecipitates were blotted with monoclonal antibodies for HA tag. The membranes were stripped the blotted with the indicated antibodies.

In addition, the deletion mutant form of the PRLR, Δ243-268, was used as a control. The cells were also transfected with a limited amount of an expression plasmid for the kinase Jak2. Cellular lysates were separated into two parts. The first part was used for immunoprecipitation with polyclonal antibodies to the HA-tag. Precipitates were run on SDS-PAGE, transferred to nitrocellulose membranes and probed with monoclonal antibodies to phosphotyrosine in Western blot analysis (Fig. 3.6A). The second part of the lysates was used for immunoprecipitation using polyclonal antibodies to SHP-2. Samples (Ctrl) containing antibody and beads were used as controls. Immunoprecipitates were run on SDS-PAGE then transferred to a nitrocellulose membrane and probed with monoclonal antibodies to phosphotyrosine (Fig. 3.6B). A strong PRL responsive phosphorylation of SHP-2 and a significant association with Gab2 adaptor protein was detected with all three forms of the PRLR (Fig. 3.6A). Similarly, in the reverse immunoprecipitation, both tyrosine phosphorylation and association of SHP-2 to Gab2 were detectable in samples expressing all three forms of the PRLR (Fig. 3.6B). Interestingly however, in both immunoprecipitated sets Gab2 migrated at a faster rate in samples expressing the short form of the PRLR compared to samples expressing the long and Nb2 forms (Fig. 3.6A and 3.6B) suggesting that a different pattern of phosphorylation for Gab2 when expressed with the short form of the PRLR. The identities of these proteins were confirmed by stripping and reprobing the membrane with monoclonal antibodies to the HA tag and to SHP-2 (Fig. 3.6A and 3.6B middle and lower panels respectively). Together, the data indicate that all three forms of the PRLR can induce the phosphorylation of Gab2 and its association to the phosphatase SHP-2. Taking into consideration that the short form of the PRLR does not become tyrosine phosphorylated in response to PRL stimulation (35), suggests that Gab2 tyrosine phosphorylation and association to SHP-2 is independent of PRLR tyrosine phosphorylation.

**SHP-2 association to Gab2 requires tyrosines 604 and 633 on the C-terminal part of Gab2**—SHP-2 binding to Gab2 in the IL-3 receptor signaling system requires tyrosines 604 and 633 located in the C-terminal portion of Gab2 (17). We intended to determine the mechanism of association between SHP-2 and Gab2 in the PRLR signaling system. 293 cells were co-transfected with expression plasmid encoding the Nb2 form of the PRLR alone, sample denoted by (-), or with expression plasmids for HA-tagged Gab2 or the double tyrosine to phenylalanine mutant of Gab2 (Gab2DM). The Gab2DM mutant was shown to lose the ability to associate to SHP-2 (17). As a negative control, the Δ243-268 deletion form was co-transfected. All transfections contained limited amounts of an expression plasmid encoding the Jak2 kinase. Cell lysates were obtained and SHP-2 was immunoprecipitated with polyclonal antibody to SHP-2, the immunecomplex was separated on SDS-PAGE, transferred to a nitrocellulose membrane and Western blotted with monoclonal antibodies to phosphotyrosine (Fig. 3.7). As expected, tyrosine phosphorylation was not observed in the samples expressing the Δ243-268 deletion form of the PRLR whereas SHP-2 was tyrosine phosphorylated in samples expressing the Nb2 form of the PRLR. In samples expressing Gab2, the protein became tyrosine phosphorylated and co-immunoprecipitated with SHP-2. Interestingly, however, the level of SHP-2 tyrosine phosphorylation was not significantly enhanced in the presence of Gab2 over-expression. The association between SHP-2 and Gab2 was eliminated in the samples expressing the Gab2DM mutant suggesting that the association between SHP-2 and Gab2 is mediated through either one or two of the tyrosines in the C-terminal portion of Gab2. The tyrosine phosphorylation level of SHP-2 was not affected by the lack of association between SHP-2 and Gab2 suggesting that SHP-2 phosphorylation can take

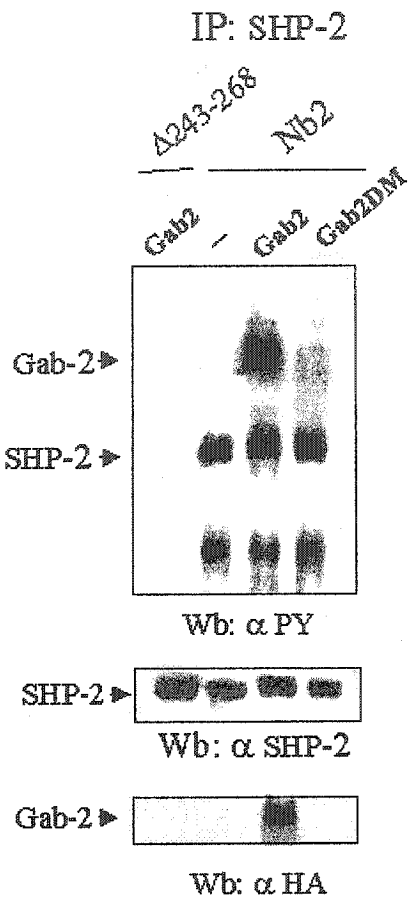


Figure 3.7 SHP-2 association to Gab2 requires tyrosines 604 and 633 on the C-terminal part of Gab2. 293 cells were transfected with expression plasmids encoding the Nb2 form of the PRLR alone (-), or along with HA-tagged Gab2 or HA-tagged Gab2DM. In addition, expression plasmid encoding the Δ243-268 mutant form of the PRLR was also transfected along with Gab2 and SHP2 as a control. Cells were lysed and SHP-2 was immunoprecipitated with polyclonal antibodies against SHP-2. The immunecomplexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane and then blotted with monoclonal antibodies against phosphotyrosine. The membrane was then stripped then re-blotted with monoclonal antibodies to SHP-2 then with monoclonal antibodies to the HA tag.

place through the interaction of SHP-2 with the C-terminal tyrosine of the PRLR (Fig. 3.7). The membranes were stripped then reprobed with a monoclonal antibody to SHP-2 then with monoclonal antibody to the HA tag (Fig. 3.7 middle and lower panels respectively).

## DISCUSSION

The protein tyrosine phosphatase SHP-2 plays a critical role in signaling downstream from several receptor tyrosine kinases and cytokine receptors including the PRLR (37). We have reported earlier that tyrosine phosphorylated SHP-2 was found in a complex containing the PRLR and Jak2 (7). In this study we investigated the mechanisms of SHP-2 recruitment and activation within the PRLR signaling complex. We identified SHP-2 in PRLR immunoprecipitates from PRL stimulated mouse mammary epithelial HC11 cells suggesting that the PRLR is able to recruit SHP-2 leading to its tyrosine phosphorylation, an event that is known to regulate the activation of the phosphatase. In addition, we determined that the C-terminal tyrosine of the PRLR has a regulatory role in the recruitment and tyrosine phosphorylation of SHP-2. SHP-2 tyrosine phosphorylation as well as association to the PRLR was lost in samples expressing the C-terminal tyrosine mutant of the Nb2 form of the PRLR. However, we did not observe a loss of SHP-2 association to the PRLR with the C-terminal tyrosine mutant of the long form of the PRLR, suggesting that possibly one or more of the other tyrosine residues present in the long form of the PRLR but absent from the Nb2 form may act as sites for SHP-2 recruitment. Together, our results indicate that the C-terminal tyrosine residue of the PRLR is important for the recruitment of SHP2 to the PRLR signaling complex.

We have previously reported that this C-terminal tyrosine of the PRLR plays a critical role in the induction of PRL responsive genes such as the β-casein gene (29). Furthermore, we determined that this tyrosine also regulates the activation of Stat5 (32). In addition, other studies have shown that the C-terminal tyrosine of the receptor regulates Stat5 recruitment to the PRLR (38). The association of SHP-2 or Stat5 to the C-terminal tyrosine might be two sequential events during the activation process.

Alternatively, both molecules can associate to the C-terminal tyrosine through a third intermediate adaptor protein. Therefore, it is possible to speculate that the C-terminal tyrosine residue of the PRLR may coordinate multiple pathways downstream from the PRLR.

The association between cytokine receptors and SHP-2 has been previously reported for various systems. Two specific tyrosine residues in the intracellular domains of the PDGF-R were shown to be sites for SHP-2 direct recruitment (39), (9). Similarly, three tyrosine residues in GM-CSF receptor were also shown to be sites for SHP-2 association (8). In addition, SHP-2 was found in a complex containing GHR, Jak2 and SIRP$\alpha$and the interaction between SHP-2 and GHR was mapped to the carboxy terminal tail of the receptor (11). Besides SHP-2 recruitment to phosphorylated receptors, receptor tyrosine phosphorylation independent recruitment of SHP-2 has also been suggested. The association of SHP-2 to activated IFN $\alpha/\beta$ receptors was found to be independent of receptor tyrosine phosphorylation (40).

The association between SHP-2 and the PDGF receptor was mapped to the N-terminal SH2 domain of the phosphatase (41), (42). Similarly, SHP-2 associates to the IRS-1 subunit through the N-terminal SH-2 domain. The optimal phosphotyrosine motif that associates to the N-terminal SH2 domain of SHP-2 was determined to be Y (I/V) X (V/I/L/P) (43). In our study, we determined that SHP-2 associates to the C-terminal tyrosine of the PRLR through the C-terminal SH2 domain of SHP2. The sequence of the tyrosine of the PRLR that binds to SHP-2 is DYLDP. To our knowledge, an optimal phosphotyrosine motif for the C-terminal SH2 domain of SHP-2 has not been determined.

In immunoprecipitates of SHP-2 from HC11 cells, we normally find multiple tyrosine phosphorylated proteins in the range of 95-100 kDa that co-immunoprecipitate

with SHP2 in response to PRL stimulation. SHP-2 has been reported to interact with members of the Gab family of adaptor proteins that range in size between 95-110 kDa. Therefore, we investigated the possible PRL-mediated interactions of SHP2 to members of the Gab family of proteins. Our investigation revealed that Gab2, is specifically tyrosine phosphorylated in response to PRL stimulation in mammary epithelial cells. In addition, we found that PRL stimulation also led to Gab2/SHP-2 as well as Gab2/PI3-K complex formation. This is in contrast to Gab1 tyrosine phosphorylation and interaction with SHP-2 and PI3-K which was constitutive and independent of PRL stimulation in HC11 cells. Gab1 and Gab2 proteins were shown to be tyrosine phosphorylated and to associate to a number of cellular signaling mediators including SHP-2, p85 subunit of PI3-K, Grb2 and CrkL in various systems including those for several cytokines such as IL-2, IL-6, EPO, and TPO and growth factors such as HGF and Insulin (22), (23), (28), (17), (25), (19), (21), (44). The two proteins share a similar structure (up to 73% within their PH domain), however, they appear to function in a non-redundant manner. Future studies will focus on the contribution of Gab2 to PRLR signaling.

Our identification of at least two potential recruitment sites for SHP-2 in PRLR signaling only partially explains its complex interactions and role in signaling. It is not clear whether two different SHP-2 molecules can independently bind to the two different sites (i.e. the PRLR and Gab2) or it is the same SHP-2 molecule that concurrently binds these two sites. We identified that SHP-2 associates to the PRLR through its C-terminal SH2 domain leaving the N-terminal SH2 domain to possibly bind to Gab2. This finding favors that one SHP-2 molecule is binding simultaneously to the two sites. On the other hand, we found SHP-2 to be tyrosine phosphorylated in samples expressing the short form of the PRLR that itself does not become tyrosine phosphorylated (35). This suggests that the interaction of SHP-2 with Gab2 is independent of PRLR tyrosine phosphorylation state. Therefore, possibly two different molecules of SHP-2 are binding

independently to the PRLR and to Gab2. Further investigation is required to elucidate the effects of SHP-2 recruitment to the PRLR and to Gab2 on the modulation of its functions in signaling downstream of the PRLR.

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## CHAPTER IV. SHP-2 Positively Modulates the Jak/Stat Pathway Downstream of the ProlactinReceptor by Preventing SOCS-1 Mediated Ubiquitination/Degradation Pathway

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**FORWARD**

The C-terminal tyrosine of the PRLR positively regulates the signaling activity of the receptor. The identification of the C-terminal tyrosine of the PRLR as a site for recruitment of the protein tyrosine phosphatase SHP-2 combined with the established positive role of the phosphatase in the activation of the PRLR suggested that recruitment of SHP-2 to the C-terminal tyrosine of the receptor has a critical influence on the signaling capacity of the PRLR.

The third objective for my doctoral work was to investigate the mechanism by which the C-terminal tyrosine/SHP-2 regulate PRLR signaling.

Jak2 kinase was shown as an essential activator and mediator of PRLR signaling. The kinase is known to associate with the inhibitory protein SOCS-1 which leads to downregulation of the kinase activity of Jak2 and to the uniquitination and degradation of the protein.

Work in this study established that SHP-2 phosphatase dephosphorylates tyrosine 1007 in the activation loop of Jak2. This tyrosine residue was reported to be the recruitment site of the SH2 domain of SOCS-1. The dephosphorylation of Jak2 leads to the inhibition of association between Jak2 and SOCS-1 and to the prolongation of the life time of Jak2 as well as the PRLR.

## ABSTRACT

The protein tyrosine phosphatase SHP-2 is an important regulator of the Jak/Stat pathway downstream of cytokine/prolactin receptor family. We report that SHP-2 dephosphorylates tyrosine (Y1007) of Jak2 kinase, a critical recruitment site for the ubiquitin ligase-associated inhibitory protein SOCS-1, thereby contributing to Jak2 and prolactin receptor stability. Inactivation of SHP-2 function by blocking receptor/SHP-2 association or by using a catalytically inactive mutant of SHP-2 lead to a marked increase in Jak2/receptor ubiquitination/degradation, Jak2 phosphorylation on Y1007 and Jak2/SOCS-1 association. Furthermore, functional studies indicate that modulation of Jak2/SOCS-1 interaction by SHP-2 is essential for prolactin/Stat5-mediated signaling. Together our results provide a novel function for SHP-2 as a positive regulator of cytokine receptor signaling by inhibiting ubiquitination/degradation pathways.

## INTRODUCTION

Prolactin (PRL) is a polypeptide neuroendocrine hormone that exerts a broad range of biological effects on diverse target tissues. PRL regulates different processes such as those involved in mammary gland development, reproduction and immune regulation (reviewed by Goffin et al., 2002). The hormone generates its multiple biological functions by interacting with the PRL receptor (PRLR), a member of the class I cytokine receptor superfamily (Bole-Feysot et al., 1998). Ligand binding to the PRLR induces receptor dimerization leading to activation of the constitutively associated Janus kinase-2 (Jak2), resulting in Jak2 and receptor tyrosine phosphorylation (Lebrun et al., 1994). These membrane proximal signaling events play a critical role in signal propagation by recruiting effector molecules, such as the signal transducer and activator of transcription-5 (Stat-5). The Jak2/Stat5 pathway has been shown to be indispensable in mediating PRL signals leading to various physiological responses such as terminal differentiation of mammary epithelial cells (reviewed by Groner and Hennighausen, 2000).

SHP-2, a ubiquitously expressed cytoplasmic protein tyrosine phosphatase, plays a central role in signaling downstream of receptor tyrosine kinases, G-protein coupled receptors and cytokine receptors, including the PRLR (Stein-Gerlach et al., 1998). Following PRLR activation, SHP-2 is tyrosine phosphorylated, physically associates to the carboxy (C)-terminal tyrosine of the PRLR, and is critical for activation of Stat5 and induction of the β-casein gene promoter (Lebrun et al., 1995; Ali et al., 1996; Ali and Ali, 2000; Berchtold et al., 1998). The precise function of SHP-2 in PRLR signaling is thus of vital importance and is yet to be fully characterized.

Modulation of Jak kinase activity has appeared as an important mechanism of regulation of cytokine receptor signaling. Studies have indicated that protein tyrosine

phosphatases and adaptor/proteaosmal degradation machinery are two means by which Jak2 kinase activity is regulated downstream of cytokine receptors. The protein tyrosine phosphatases SHP-1 (Klingmuller et al., 1995; Yi et al., 1993), CD45 (Irie-Sasaki et al., 2001) and PTP1B (Aoki and Matsuda, 2000; Myers et al., 2000; Zabolotny et al., 2002; Cheng et al., 2002) were shown to dephosphorylate Jak2 and block cytokine receptor signaling. In the case of CD45 and PTP1B, it was demonstrated that these phosphatases inhibit the activation of Jak2 via dephosphoraylating Y1007 within the activation loop of the kinase (Feng et al., 1997).

The suppressor of cytokine signaling-1 (SOCS-1, also referred to as JAB) (Starr et al., 1997; Endo et al., 1997) has been established as an important negative-feedback inhibitor of cytokine-activated Jak/Stat signaling pathway (reviewed by Alexander et al., 1999; O'Shea et al., 2002). SOCS-1 is a member of the SOCS family of proteins that includes eight members, SOCS-1 to SOCS-7 and CIS. Like other members of the family, SOCS-1 contains a central SH2 domain followed by a conserved carboxy-terminal region called the SOCS-box. The inhibitory role of SOCS-1 is due in part to the fact that Jak2/SOCS-1 interaction blocks substrate access to Jak2 kinase through a 24 amino acid segment denoted as the kinase inhibitory region (KIR) (Sasaki et al., 1999; Yasukawa et al., 1999). Recruitment of SOCS-1 through its SH2 domain to phosphorylated Y1007 in the activation loop of Jak2 leads to inhibition of the catalytic activity of Jak2. A second and a critical role for SOCS-1 in down regulating signaling is through targeting associated proteins to proteasomal degradation. Various studies have indicated that this function of SOCS-1 is attributed to the highly conserved SOCS-box (Zhang et al., 1999; Nicholson et al., 1999; Zhang et al., 2001). The SOCS-box of SOCS-1 was shown to target both the fusion product TEL-Jak2 (Frantsve et al., 2001; Kamizono et al., 2001) and the full-length wild type Jak2 (Ungureanu et al., 2002) to

ubiquitination and proteasomal degradation. This degradation process was dependent on Jak2 phosphorylation on Y1007.

In this study, we investigated the hypothesis that SHP-2 contributes to PRL-mediated activation of the Jak2/Stat5 pathway by modulating complex formation between Jak2 and SOCS-1 thus maintaining the activity and stability of Jak2. We determined that abrogating SHP-2 activity downstream of the PRLR by either using a mutant form of the PRLR, deficient in SHP-2 recruitment site, or by using a phosphatase inactive mutant of SHP-2, resulted in a significant increase in Jak2 phosphorylation on Y1007 and in Jak2/SOCS-1 complex formation, in turn leading to ubiquitination and degradation of Jak2. Thus, SHP-2 critically regulates PRLR signaling by dephosphorylating SOCS-1 recruitment site within Jak2 consequently allowing signal propagation/maintenance.

## MATERIALS AND METHODS

### Materials, Plasmid Constructs and Antibodies

Expression plasmids encoding the Nb2 form of the PRLR, and the corresponding C-terminal tyrosine to phenylalanine mutant form, Nb2PRLRY382F, were described previously (Lebrun et al., 1995). Expression plasmids encoding the wild type form of SHP-2 (SHP-2WT) and the catalytically inactive cysteine 463 to alanine mutant SHP-2CA were obtained from Axel Ullrich (Max-Plank Institute, Germany). Expression plasmids encoding myc-SOCS-1 and an SH2 domain inactivating mutant form of SOCS-1, SOCS-1R105E, was kindly provided by Akihiko Yashimura (Life Sciences Institute, Kurume, Japan). Monoclonal antibody to phosphotyrosine (4G10) and polyclonal antibody to Jak2 were purcahsed from Upstate Biotechnology (NY, USA). Polyclonal antibody to phosphorylated Y1007/Y1008 of Jak2 was purchased from Biosource International (CA, USA). Polyclonal antibody to ubiquitin was purchased from Novocastra Laboratoris (Newcastle, UK). Monoclonal antibodies to Stat5 and SHP-2 were obtained from BD Transduction Laboratories (Ontario, Canada). Polyclonal antibody to SOCS-1 (along with mouse thymus extracts as a positive control) and monoclonal antibodies to phosphorylated Y694 of Stat5 were purchased from Zymed Laboratories (CA, USA). HRP-tagged monoclonal antibody to myc tag was purchased from Roche (Laval, Canada). Monoclonal antibody (U6) to the PRLR was provided by Paul Kelly (Paris, France). Phosphorothioated SOCS-1 antisense oligonucleotides were synthesized by alpha-DNA Inc. (Montreal, Canada). Protein-A sepharose beads used for immunoprecipitations were from Pharmacia (Montreal, Canada). Ovine PRL (PRL) used for treatment of cells was obtained from Sigma (MO, USA).

### Nb2 Cell Culture and SOCS-1 Antisense Oligonucleotides Treatment

The Nb2 rat pre T-lymphoma cells dependent on PRL for growth were provided by Peter Gout (BC Cancer Agency, Vancouver, Canada) were grown to confluence in growth media (RPMI media containing 10% fetal bovine serum (FBS), 10% horse serum, 7.5% sodium bicarbonate, 5 mM β-mercaptoethanol, 200 mM glutamine) then starved for 18h in starvation media (RPMI media containing 10% horse serum, 7.5% sodium bicarbonate, 200 mM glutamine). For blocking SOCS-1 expression, a phosphorothioated antisense oligonucleotide to SOCS-1 mRNA (sequence: 5'-CACCTGGTTGTGTGCTACCAT-3') was designed to recognize the initiation site of SOCS-1 protein. Oligonucleotides were incubated with the cells at a concentration of 15 μM for a 16h period.

HC11 Cell Culture

HC11, mouse mammary epithelial cells, obtained from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Bernd Groner (Georg Speyer Haus, Frankfurt, Germany), were grown to confluence in RPMI-1640 media containing 10% FBS, insulin (5μg/ml), and EGF (10ng/ml). Cells were then induced to differentiate by incubating them for 3 days in RPMI media containing 10% FBS, insulin (5μg/ml) and hydrocortisone (1μM). Cells were starved in RPMI media containing insulin (5μg/ml) and hydrocortisone (1μM).

**Transient Transfection**

The human embryonic 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM 4.5 g/liter glucose) (Bio Media) containing 10% FBS (vol/vol). Approximately $5x10^5$ cells were plated then co-transfected (by calcium phosphate precipitation method) with expression plasmids encoding the Nb2 form of the PRLR, PRLRY382F, SHP-2WT, SHP-2CA, SOCS-1, SOCS-1R105E, Stat5 and Jak2 as described for each experiment. After 18h of expression, the cells were starved by serum

deprivation and then stimulated by PRL for different periods of time as indicated for each experiment.

**Cell Lysis**

Cells were lysed in RIPA lysis buffer (50mM Tris-HCl, PH7.4, 1% NP-40, 0.25% sodium deoxyxholate, 150 mM NaCl, 1mM EDTA, 1mM PMSF, 1 μg/ml (aprotinin, leupeptin and pepstatin), 1 mM sodium orthovanadate, 1 mM NaF). The lysates were cleared by centrifugation at 12,000x g for 10 min at 4°C to remove insoluble material. Protein concentrations were measured using the Bradford technique.

**Immunoprecipitation**

Immunoprecipitations were performed on cellular lysates obtained from either Nb2, HC11or 293 cells that were transiently transfected as indicated for each specific experiment. Immunoprecipitations were carried out on cell lysates for 3h at 4°C using the specified antibodies and protein-A sepharose beads. Immunoprecipitates were then washed with HNTG buffer (20mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), and separated on SDS-PAGE.

**Western Blotting**

Standardized amounts of protein obtained from either whole cell lysates or immunoprecipitations were loaded and separated on an appropriate concentration of SDS-PAGE. Western blotting analysis was performed using the specified antibodies for each experiment. Proteins were revealed using chemiluminescence (Roche Diagnostics, Laval, Canada) following the manufacturer's instructions.

**Luciferase Assay**

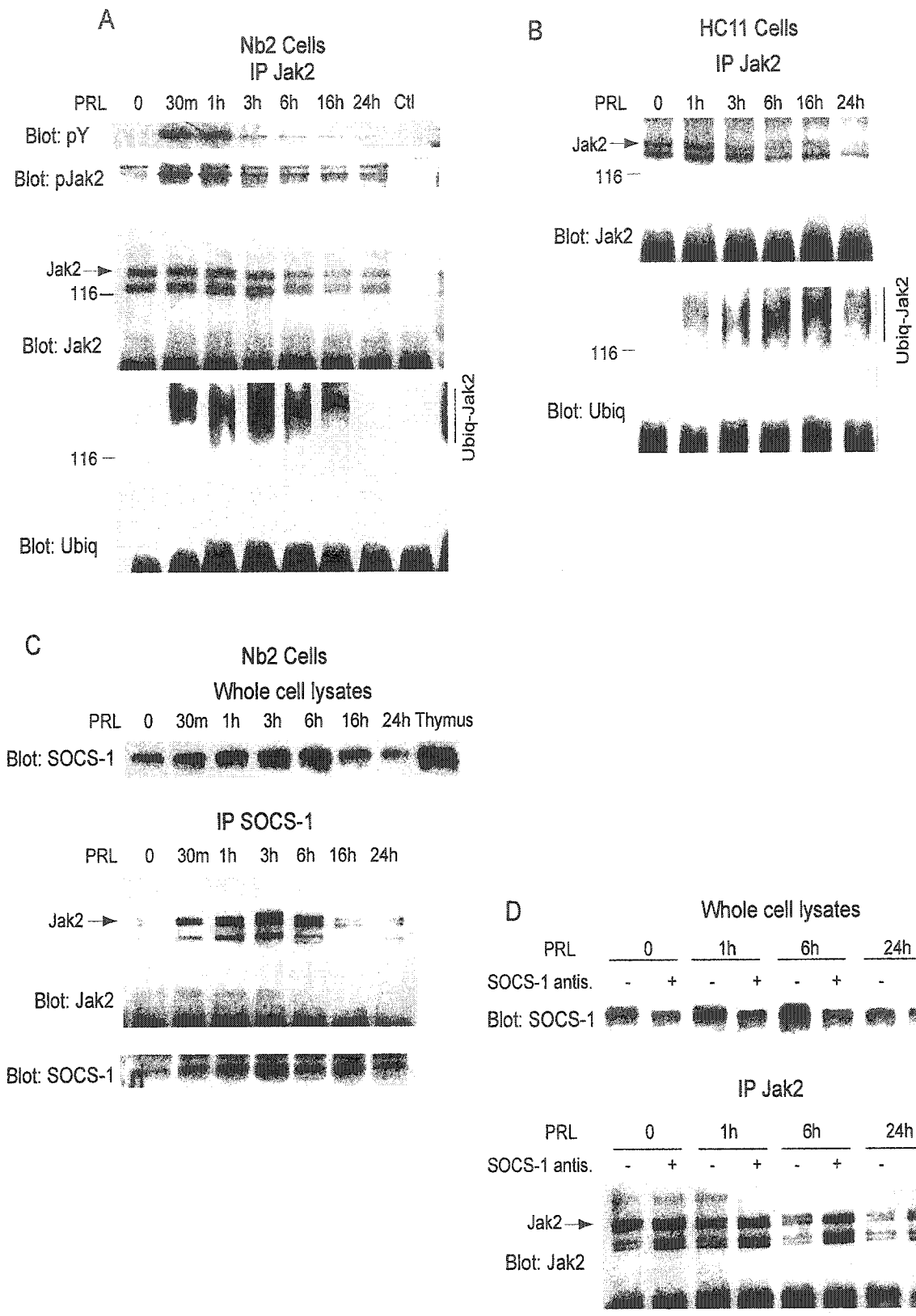
The assay was carried out as described previously (Lebrun et al., 1995). Briefly, 293 cells were transiently co-transfected with expression vectors as described for each experiment along with the βcasein gene promoter/luciferase reporter construct (Ali et al.,

1992) and the internal control expression vector encoding the β-galactosidase, Pharmacia (Montreal, Canada). Cells were lysed in lysis buffer (1% Triton-X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) then centrifuged at 12,000x to clear the lysates. Lysates were mixed with ATP buffer (1 mM ATP, 15 mM $KH_2PO_4$ pH 7.8, 15 mM $MgCl_2$) and luciferase assay was carried out following the addition of luciferin substrate (Roche Diagnostic, Laval, Canada) using the Dynex luminometer (VA, USA). Relative luciferase units were normalized against β-galactosidase values and a representative of five different experiments is presented as relative light units.

## RESULTS

### Prolactin Dependent Jak2/SOCS-1 Association Leads to Ubiquitination and Degradation of Jak2

SOCS-1 protein negatively regulates the signaling of various cytokine receptors. It was shown to inhibit Jak2 kinase activity through an interaction between the SH2 domain of SOCS-1 and phosphorylated Y1007 of Jak2 (Yasukawa et al., 1999) leading to the proteasomal degradation of Jak2 (Kamizono et al., 2001; Frantsve et al., 2001; Ungureanu et al., 2002). Similar to other cytokines the negative feedback loop of SOCS-1 in PRL signaling has been previously established as PRLR activation leads to induction of SOCS-1 mRNA (Pezet et al., 1999), Jak2/SOCS-1 interaction, inhibition of activation of gene transcription (Tomic et al., 1999) and prevention of lactation (Lindeman et al., 2001). We initially intended to determine whether the association of Jak2/SOCS-1 would lead to the ubiquitination and degradation of Jak2 kinase in response to PRL stimulation. The pre-T lymphoma Nb2 cells were grown to confluence, starved by serum deprivation for 18h then stimulated with PRL for the time points indicated in Figure 4.1A. Cells were then collected, lysed and immunoprecipitations using a polyclonal antibody to Jak2 were carried out as well as a sample was included containing the antibody and lysis buffer as a negative control. Proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Initially Western blotting analysis using either a monoclonal antibody to phosphotyrosine or a polyclonal antibody to phosphorylated Y1007/Y1008 of Jak2 indicated that indeed PRL induces transient tyrosine phosphorylation of Jak2 at tyrosines 1007/1008 returning to basal level by 3h post-treatment. Western blotting analysis using a polyclonal antibody to Jak2 revealed that there was a time dependent decrease in Jak2 protein level starting at 3h post-treatment and continued to decrease reaching a stable low levels of the protein by 6h (Figure 4.1A, middle panel). Blotting with the polyclonal


A
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IP Jak2
PRL 0 30m 1h 3h 6h 16h 24h Ctl
Blot: pY
Blot: pJak2
Jak2
116
Blot: Jak2
Ubiq-Jak2
116
Blot: Ubiq
B
HC11 Cells
IP Jak2
PRL 0 1h 3h 6h 16h 24h
Jak2
116
Blot: Jak2
Ubiq-Jak2
116
Blot: Ubiq
C
Nb2 Cells
Whole cell lysates
PRL 0 30m 1h 3h 6h 16h 24h Thymus
Blot: SOCS-1
IP SOCS-1
PRL 0 30m 1h 3h 6h 16h 24h
Jak2
Blot: Jak2
Blot: SOCS-1
D
Whole cell lysates
PRL 0 1h 6h 24h
SOCS-1 antis. - + - + - + - +
Blot: SOCS-1
IP Jak2
PRL 0 1h 6h 24h
SOCS-1 antis. - + - + - + - +
Jak2
Blot: Jak2

Figure 4.1 Prolactin induces a time dependent phosphorylation, ubiquitination and degradation of Jak2 in PRL dependent Nb2 and HC11 cells. (A) Nb2 cells were starved by serum depravation for 18h then stimulated with PRL for the indicated times followed by immunoprecipitation of Jak2, separation on SDS-PAGE and an immunoblotting with phospho-tyrosine antibody (pY), phopsho 1007/1008 Jak2 (pJak2), Jak2 or ubiquitin (Ubiq). (B) HC11 cells were grown to confluency, starved by serum depravation then stimulated with PRL for the indicated times. Jak2 was immunoprecipitated using a Jak2 polyclonal antibody and immonoblots were performed using Jak2 then ubiquitin antibodies sequentially. Nb2 cells were either untreated (-) or treated (+) with SOCS-1 phosphorothioated oligos then were immunoblotted using SOCS-1 antibodies on whole cell lysates (upper panel) or Jak2 antibodies on Jak2 immunoprecipitates (lower panel). (C) Whole cell lysates obtained from PRL stimulated Nb2 cells for the time periods indicated were immunoblotted with SOCS-1 polyclonal antibody (upper pannel) or SOCS-1 immunoprecipitates obtained from same cell lysates were immunoblotted with Jak2 or SOCS-1 (middle and lower panels respectively).

antibody to Jak2 revealed two bands of which the upper one corresponded to Jak2 observed in the phosphotyrosine and phospho-Jak2 immunoblots. The nature of the lower band detected by the antibody to Jak2 is not yet clear. To determine whether the decrease in Jak2 protein level is due to ubiquitination, the membrane was stripped and reprobed with a polyclonal antibody to ubiquitin. As shown in Figure 4.1A lower panel, ubiquitinated Jak2, migrating as a smear, could be detected starting at 1h, reaching a maximum level by 3h and gradually decreasing thereafter up to 24h time point examined. We also examined PRL-mediated Jak2 ubiquitination/degredation in the PRL responsive mouse mammary epithelial HC11 cells, and observed that it followed a similar pattern to that seen in Nb2 cells (Figure 4.1B). Together the data indicate that PRL stimulation leads to a time dependent ubiquitination and degradation of Jak2.

SOCS-1 is known to act in a negative feedback loop to inhibit cytokine signaling. Using Nb2 cells we next investigated whether PRL stimulation would lead to increase in SOCS-1 protein level and consequently Jak2/SOCS-1 interaction. As shown in Figure 4.1C upper panel, PRL stimulation led to a time dependent increase in SOCS-1 protein level as detected by Western blotting using a polyclonal antibody to SOCS-1. As a positive control for SOCS-1 detection, extracts of mouse thymus were used as described in Experimental Procedures. Interestingly, there was a detectable amount of SOCS-1 protein present under basal conditions and the highest level detected was at 3h-6h post-treatment. SOCS-1 protein level returned to basal at 16h-24h following PRL stimulation. In agreement with the increase in SOCS-1 protein, SOCS-1 immunoprecipitations followed by Western blotting using a polyclonal antibody to Jak2 revealed a time dependent increase in Jak2/SOCS-1 complex formation following PRL stimulation. Jak2/SOCS-1 association was detected at 30min and peaked at 3h-6h following treatment (Figure 4.1C, middle panel). When the membrane was stripped and

reprobed with a polyclonal antibody to SOCS-1 revealed again the time dependent increase in SOCS-1 protein level (Figure 4.1C, lower panel).

To determine the contribution of PRL-induced SOCS-1 expression in Jak2 degradation, SOCS-1 protein expression was blocked in Nb2 cells using antisense oligonucleotides to SOCS-1 mRNA. Nb2 cells were grown to confluence then starved and either left untreated or treated with the antisense oligonucleotides to SOCS-1 for an overnight period. As shown in Figure 4.1D, the presence of the antisense oligonucleotides prevented the increase in SOCS-1 expression at all time points examined. The effect was particularly obvious at the 6h time point. This correlated with increased Jak2 stability observed at the different time points in samples pretreated with the antisense oligonucleotides to SOCS-1 (Figure 4.1D, lower panel), at the 6h time point the attenuation in degradation of Jak2 was most apparent. Thus, blocking SOCS-1 protein expression impedes the time dependent degradation of Jak2. Taken together, PRLR stimulation induces a time dependent increase in SOCS-1 expression accompanied by an increase in Jak2/SOCS-1 association leading ultimately to a time dependent ubiquitination and degradation of Jak2.

**The Carboxy-Terminal Tyrosine of the PRLR Regulates the Kinetics of Jak2 Ubiquitination/Degradation**

We have previously reported that the C-terminal tyrosine of the PRLR plays a vital role in the regulation of Jak2/Stat5 signaling pathway leading to gene activation (Lebrun et al., 1995; Ali and Ali, 1998) and is a recruitment site for SHP-2 (Ali and Ali, 2000). Here we examined initially the role of the C-terminal tyrosine of the PRLR in regulating Jak2 stability. For these studies, the human embryonic kidney 293 cells were transiently co-transfected with expression vectors encoding the wild type Nb2 form of the PRLR, or the C-terminal tyrosine mutant form of the receptor in which tyrosine 382 was

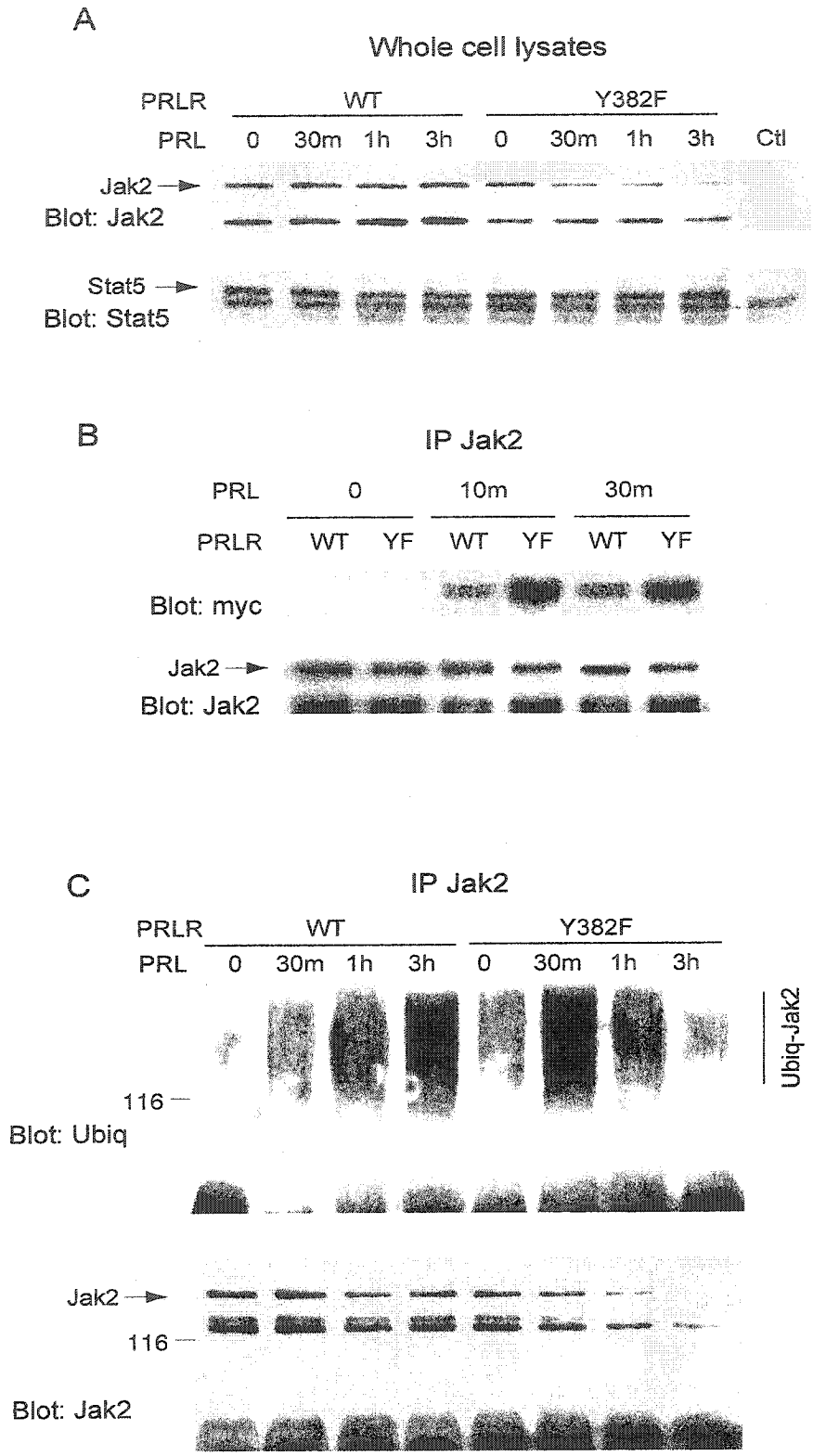

A
Whole cell lysates
PRLR
WT
Y382F
PRL
0
30m
1h
3h
0
30m
1h
3h
Ctl
Jak2
Blot: Jak2
Stat5
Blot: Stat5
B
IP Jak2
PRL
0
10m
30m
PRLR
WT
YF
WT
YF
WT
YF
Blot: myc
Jak2
Blot: Jak2
C
IP Jak2
PRLR
WT
Y382F
PRL
0
30m
1h
3h
0
30m
1h
3h
Ubiq-Jak2
116
Blot: Ubiq
Jak2
116
Blot: Jak2

Figure 4.2 C-terminal tyrosine of the PRLR regulates Jak2/SOCS-1 association and ubiquitination and degradation of Jak2. (A) 293 cells that were transiently transfected with expression vectors for PRLR WT or C-terminal tyrosine mutant Y382F of the PRLR were starved by serum depravation then stimulated with PRL for the times indicated. Whole cell lysates were separated on SDS-PAGE then immunoblotted with polyclonal antibody for Jak2 (upper panel) or Stat5 (lower panel). (B) Cellular lysates obtained from 293 cells that were transiently transfected with expression vectors for PRLR WT or Y382F mutant were immunoprecipitated with Jak2 polyclonal antibody followed by immunoblotting with the same antibody (upper panel) or HRP-myc tagged monolconal antibody (lower panel). (C) Immunoprecipitations for Jak2 were carried out on cellular lysates obtained from 293 cells that were transfected and starved as above in (A). Immunoblotting was performed on the precipitates using Jak2 (upper panel) or ubiquitin (lower panel) antibodies

mutated to phenylalanine, PRLRY382F, along with expression vectors for Jak2 and SOCS-1. We specifically used the Nb2 form of the PRLR (Ali et al., 1992) as we have previously shown that this form is biologically active and that the C-terminal tyrosine Y382 is the major tyrosine phosphorylation site in this receptor form (Lebrun et al., 1995) and is a recruitment site for SHP-2 (Ali and Ali, 2000). Cells were starved for 18h, stimulated with PRL for 30min, 1h or 3h then collected and lysed. Cellular lysates were separated on an SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a polyclonal antibody to Jak2. As shown in Figure 4.2A, Jak2 protein level remained relatively constant over the 3h time course of PRL stimulation in samples overexpressing the Nb2 wild type receptor. In contrast, in samples overexpressing the C-terminal tyrosine mutant form of the receptor there was a rapid decrease in Jak2 protein level starting at 30min and clearly visible at 1h post treatment. After 3h of PRL stimulation there was little detectable Jak2 present. Therefore, the C-terminal tyrosine of the PRLR plays an important role in Jak2 stability. Next we sought to determine if protein degradation was specific to Jak2 and for that reason, 293 cells were transiently co-transfected with expression vectors encoding either the Nb2 PRLR or Nb2 PRLRY382F mutant along with expression vectors for Jak2, SOCS-1 and Stat5. As shown in Figure 4.2A lower panel, unlike the pattern for Jak2 degradation, Stat5 protein level did not change over the time course of PRL stimulation in the different samples. Therefore, Stat5 protein does not undergo a similar protein degradation pattern as that observed for Jak2. Together, the results indicate that the C-terminal tyrosine of the PRLR regulates the stability of Jak2 kinase in response to PRL stimulation.

Next we examined whether the C-terminal tyrosine of the PRLR regulates Jak2/SOCS-1 interaction leading to the observed differential stability of Jak2. Cells transiently co-transfected as in above were starved for 18h and stimulated with PRL for 10min or 30min. These time points of stimulation were selected since there was minimal

degradation of Jak2. Immunoprecipitations were carried out using a polyclonal antibody to Jak2 and immune complexes were separated on an SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a monoclonal antibody to myc, recognizing myc-tagged SOCS-1 protein (Figure 4.2B). As expected, PRL stimulation led to Jak2/SOCS-1 interaction in all samples. Interestingly, more SOCS-1 protein was found to co-immunoprecipitate with Jak2 in samples overexpressing the C-terminal mutant form of the PRLR compared to samples overexpressing wild type PRLR. Reprobing the membrane with a polyclonal antibody to Jak2 showed equal immunoprecipitiations of Jak2 (Figure 4.2B, lower panel). This data indicates that a mechanism(s) operating downstream of the C-terminal tyrosine of the PRLR regulates Jak2 and SOCS-1 complex formation in response to PRL stimulation.

Since SOCS-1-mediated degradation of associated proteins is through ubiquitination, we next examined the role of the C-terminal tyrosine of the PRLR in Jak2 ubiquitination. 293 cells were transiently co-transfected with expression vectors for the wild type Nb2 form of the PRLR or the C-terminal tyrosine mutant of the Nb2 form, PRLRY382F, along with expression vectors for Jak2 and SOCS-1. Cells were starved, stimulated with PRL for different time points, and immunoprecipitations were carried out using a polyclonal antibody to Jak2. Immunoprecipitates were separated on SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a polyclonal antibody to ubiquitin (Figure 4.2C). As can be observed, PRLR activation led to Jak2 ubiqiutination. Interestingly, the rate of Jak2 ubiquitination was faster, reaching a maximal level within 1h of stimulation in samples overexpressing the C-terminal tyrosine mutant form of the PRLR compared to samples overexpressing the wild type PRLR where Jak2 ubiquitination became apparent only following 3h of stimulation. Reprobing the membrane with a polyclonal antibody to Jak2 showed similar pattern of Jak2 degradation to that observed in Figure 4.2A. Thus, the C-terminal tyrosine of the PRLR

regulates the kinetics of Jak2 and SOCS-1 association and ultimately Jak2 ubiquitination and degradation.

**The C-Terminal Tyrosine of the PRLR Controls Receptor Ubiquitination and Degradation**

The cytoplasmic tyrosine kinase Jak2 forms a complex with the PRLR independent of ligand stimulation (Lebrun et al., 1994). Since we observed that the C-terminal tyrosine of the PRLR is important in regulating Jak2 ubiquitination/degradation following PRL stimulation, we sought to determine whether PRLR ubiquitination/degradation itself is regulated by receptor tyrosine phosphorylation and might parallel that of Jak2. For that reason, we co-transfected 293 cells with expression vectors for the wild type or the C-terminal tyrosine mutant form of the Nb2 PRLR along with expression vectors for Jak2 and SOCS-1. Cells were lysed and PRLR was immunoprecipitated using a monoclonal antibody to the PRLR. Immunoprecipitates were separated using SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a polyclonal antibody to ubiquitin. As shown in Figure 4.3, PRL stimulation led to an increased rate of PRLR ubiquitination in samples overexpressing the C-terminal tyrosine mutant form of the PRLR, with highest level after 1h of stimulation, compared to samples overexpressing the wild type PRLR where PRLR ubiquitination was elevated only 3h following stimulation. Western blotting with a monoclonal antibody to the PRLR revealed accelerated degradation of the receptor harbouring the mutation compared to the wild type receptor form (Figure 4.3, lower panel). Taken together, the results indicate that the C-terminal tyrosine of the PRLR regulates both receptor and Jak2 ubiqiutination and stability.

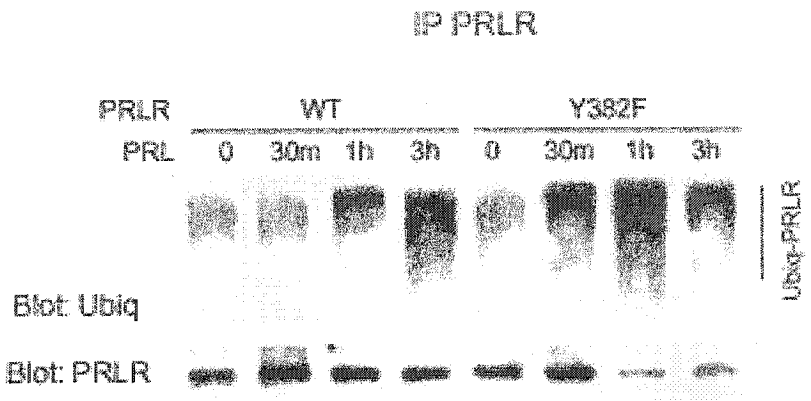
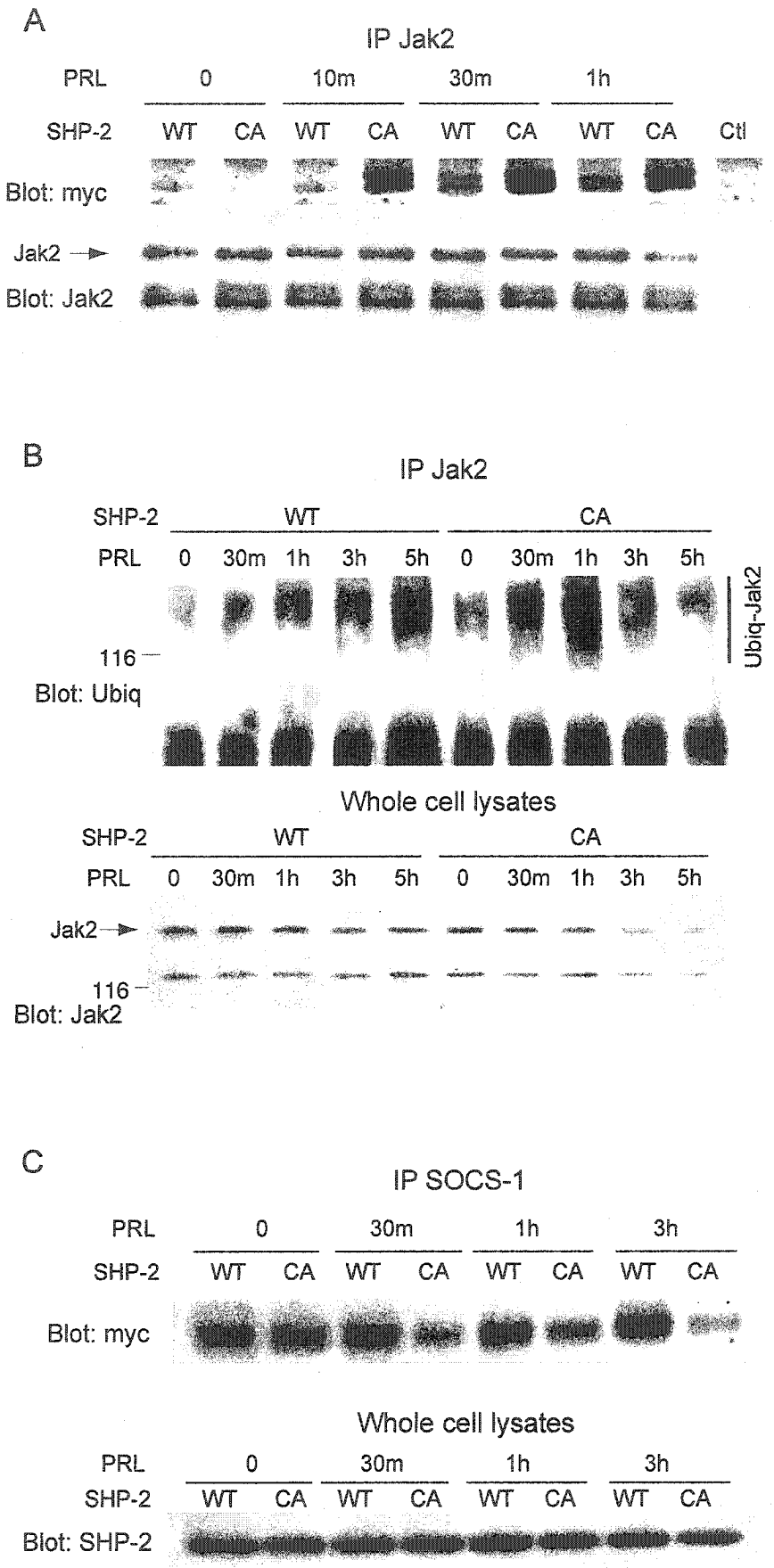



Figure 4.3 C-terminal tyrosine of the PRLR regulates the receptor's own rate of ubiquitination and degradation. 293 cells were transiently transfected with expression vectors for the wild type PRLR or the C-terminal mutant, cells were then lysed and the PRLR was immunoprecipitated with a monoclonal antibody to the PRLR and immunoblotting was performed using a polyclonal antibody to ubiquitin (upper panel) then membrane was stripped then reblotted with a monoclonal antibody to PRLR (lower panel)

**SHP-2 Modulates Jak2/SOCS-1 Complex Formation and Jak2 Protein Stability**

We have previously reported that the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment (Ali and Ali, 2000). Therefore, we sought to determine whether the regulation of Jak2 ubiquitination and degradation by the C-terminal tyrosine of the PRLR is due to the function of SHP-2. 293 cells were transiently co-transfected with expression vectors for either the wild type SHP-2 (SHP-2WT) or a catalytically inactive mutant of SHP-2 where cysteine 463 is mutated to alanine (SHP-2CA), along with expression vectors for the Nb2 form of the PRLR, Jak2 and SOCS-1 (Figure 4.4A). Cells were starved for an overnight period, and stimulated with PRL for 10min, 30min or 1h. Immunoprecipitations were carried out on cell lysates using a polyclonal antibody to Jak2. Immuno-complexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a monoclonal antibody to the myc tag, recognizing SOCS-1 (Figure 4.4A, upper panel). As can be observed SOCS-1 protein co-immunoprecipitated with Jak2 following PRL stimulation in the different samples. However, in samples overexpressing the catalytically inactive mutant of SHP-2 (SHP-2CA), a notably higher association between Jak2 and SOCS-1 was observed at all time points examined compared to samples overexpressing SHP-2 wild type. When the membrane was probed with a polyclonal antibody to Jak2 similar levels of Jak2 protein were seen in the different samples (Figure 4.4A, lower panel). This data indicate that SHP-2 regulates Jak2/SOCS-1 complex formation downstream of the PRLR.

The association between Jak2 and SOCS-1 leads to the ubiquitination and degradation of Jak2. We next sought to determine if the increased association between Jak2 and SOCS-1 observed in the presence of catalytically inactive mutant of SHP-2 would translate into a faster rate of ubiquitination and degradation of Jak2. For that reason, we transiently co-transfected 293 cells with expression vectors for either SHP-2WT or SHP-2CA along with expression vectors for the Nb2 form of the PRLR, Jak2 and


A
IP Jak2
PRL
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10m
30m
1h
SHP-2
WT
CA
WT
CA
WT
CA
WT
CA
Ctl
Blot: myc
Jak2
Blot: Jak2
B
IP Jak2
SHP-2
WT
CA
PRL
0
30m
1h
3h
5h
0
30m
1h
3h
5h
Ubiq-Jak2
116
Blot: Ubiq
Whole cell lysates
SHP-2
WT
CA
PRL
0
30m
1h
3h
5h
0
30m
1h
3h
5h
Jak2
116
Blot: Jak2
C
IP SOCS-1
PRL
0
30m
1h
3h
SHP-2
WT
CA
WT
CA
WT
CA
WT
CA
Blot: myc
Whole cell lysates
PRL
0
30m
1h
3h
SHP-2
WT
CA
WT
CA
WT
CA
WT
CA
Blot: SHP-2

Figure 4.4 Catalytically inactive SHP-2 increases the rate of Jak2 and SOCS-1 proteins degradation. (A) Jak2 was immunoprecipitated using a polyclonal antibody from 293 cells transiently co-transfected with expression vectors for either SHP-2WT or the catalytically inactive mutant SHP-2CA then starved by serum depravation and stimulated with PRL for the times indicated. Immunecomplexes were separated on SDS-PAGE and then immunodetected using a polyclonal antibody to Jak2 (upper panel) or a monoclonal antibody to myc detecting SOCS-1 (lower panel). (B) Whole cell lysates obtained from 293 cells that were transiently transfected with either SHP-2WT or SHP-2CA were separated on SDS-PAGE and immunodetected using a polyclonal antibody to Jak2 (upper panel). Immunoprecipitates of Jak2 cells obtained using a polyclonal antibody to Jak2 from 293 cells transiently expressing SHP-2WT or SHP-CA were immunodetected using a polyclonal antibody to ubiquitin (lower panel). (C) Cellular lysates collected from 293 cells that were transiently transfected with SHP-2WT or SHP-2CA were used for immunoprecipitating SOCS-1 using a polyclonal antibody to SOCS-1, immune-complexes were separated on SDS-PAGE, and immune-detected using a monoclonal antibody to myc tag recognizing SOCS-1 (upper panel), or whole lysates were separated on SDS-PAGE and immunoblotted using a monoclona antibody to SHP-2 (lower panel).

SOCS-1. Cells were starved for an overnight period, then stimulated with PRL for 30 min, 1h or 3h. Cellular lysates were used for immunoprecipitations of Jak2 utilizing a polyclonal antibody to Jak2. Immunoprecipitates were separated on SDS-PAGE, transferred to a nitrocellulose membrane and blotted with a polyclonal antibody to ubiquitin (Figure 4.4B). As can be seen, following PRL stimulation the rate of Jak2 ubiquitination was faster in samples overexpressing the inactive form of SHP-2, peaking at 1h following stimulation, compared to samples overexpressing SHP-2WT, where elevated levels of ubiquitination was observed following 3h-5h of stimulation. To examine whether the increased rate of ubiquitination of Jak2 was associated with a decrease in the amount of Jak2, whole cell lysates from the above transfection were separated on SDS-PAGE and immuno-detected with a polyclonal antibody to Jak2 (Figure 4.4B, lower panel). Following PRL stimulation there was an accelerated decrease in the amount of Jak2 in samples overexpressing SHP-2CA compared with samples overexpressing SHP-2 wild type. Amounts of detected Jak2 were significantly lower after 3h-5h of PRL stimulation. Therefore, the phosphatase SHP-2 regulates Jak2/SOCS-1 interaction and hence controlling the processes of ubiquitination and degradation of Jak2.

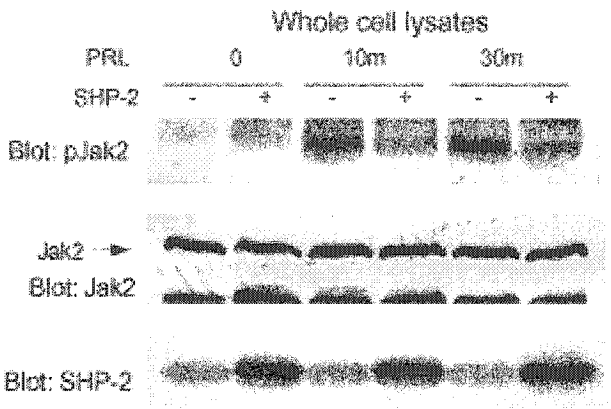
The association between SOCS-1 and Jak2 results in the formation of a complex targeted for degradation. We sought next to investigate whether SHP-2 function leads to modulation in SOCS-1 protein degradation in a manner similar to the changes observed for Jak2 protein. We obtained cellular lysates from 293 cells that were co-transfected with expression vectors for either SHP-2WT or SHP-2CA, along with expression vectors for the Nb2 form of the PRLR, Jak2 and SOCS-1. As shown in Figure 4.4C, immunoprecipitations of SOCS-1 followed by Western blotting with a monoclonal antibody to the myc-tag, recognizing SOCS-1 indicate that following PRL stimulation there is a time dependent loss in the amount of total SOCS-1 protein in

samples overexpressing SHP-2CA compared to samples overexpressing SHP-2WT (Figure 4.4C, upper panel). This indicates that inactivation of SHP-2 leads to SOCS-1 protein degradation in a similar manner to that observed for Jak2 and implicate degradation of SOCS-1 in a complex with Jak2. Reprobing the membrane with a monoclonal antibody to SHP-2 revealed equivalent overexpression of SHP-2 and the catalytically inactive mutant form in the different samples (Figure 4.4C, lower panel).

**Y1007 of Jak2 is a Target for Dephosphorylation by SHP-2**

Y1007 of Jak2 is located within the activation loop in the kinase domain of Jak2 and its phosphorylation is important for the kinase activity of Jak2 (Feng et al., 1997). However, phosphorylation of this tyrosine serves as a recruitment site for the SH2 domain of SOCS-1 leading to Jak2 kinase inhibition and ultimately ubiquitination and degradation (Yasukawa et al., 1999). We investigated whether SHP-2 regulation of Jak2/SOCS-1 association, ubiquitination/degradation of Jak2 is due to dephosphorylation of Y1007 by the phosphatase. 293 cells were transiently co-transfected with expression vectors for the Nb2 form of the PRLR and Jak2 either in the absence or the presence of overexpression of SHP-2. Cells were starved then stimulated with PRL for either 10min or 30min. Whole cell lysates were separated on SDS-PAGE and immunodetected with a polyclonal antibody recognizing phosphorylated Y1007/Y1008 of Jak2. As presented in Figure 4.5A, PRL stimulation led to the phosphorylation of Jak2 on Y1007/Y1008, however, the amount of detectable phosphorylation was notably lower in samples overexpressing SHP-2, suggesting that Y1007 is a target for SHP-2 dephosphorylation. Reblotting the membrane with a polyclonal antibody to Jak2 revealed similar level of overexpression of Jak2 and with a monoclonal antibody to SHP-2 displayed SHP-2 overexpression in the appropriate samples. Hence, SHP-2 phosphatase dephosphorylates Jak2 on Y1007/Y1008.

A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10m
30m
SHP-2
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+
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+
-
+
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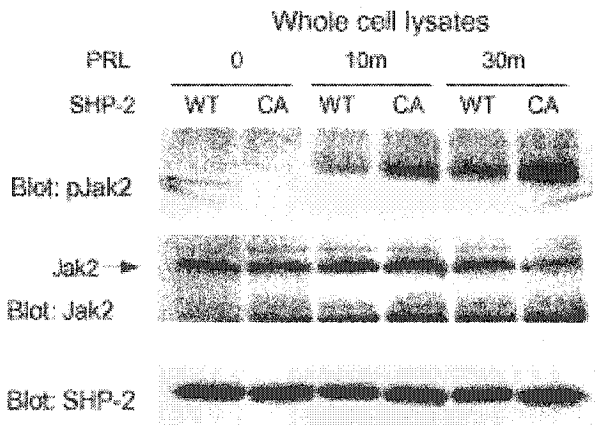
Jak2
Blot: Jak2
Blot: SHP-2

B


Whole cell lysates
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0
10m
30m
SHP-2
WT
CA
WT
CA
WT
CA
Blot: pJak2
Jak2
Blot: Jak2
Blot: SHP-2

C

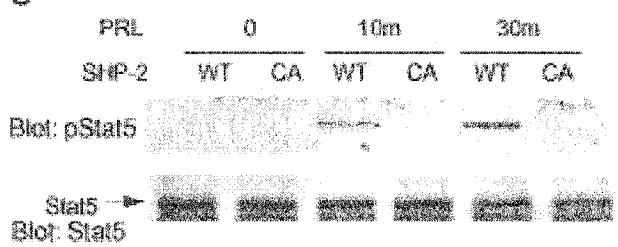

PRL
0
10m
30m
SHP-2
WT
CA
WT
CA
WT
CA
Blot: pStat5
Stat5
Blot: Stat5

Figure 4.5 SHP-2 dephosphorylates tyrosine 1007 of Jak2 enhancing tyrosine Stat5 phosphorylation. (A) 293 cells were either non-transfected (-) or transfected (+) with SHP-2 expression vector and whole cell lysates were separated on SDS-PAGE then immunoblotted with a polyclonal antibody to phosphorylated tyrosine 1007/1008 of Jak2 (upper panel), membrane was stripped then reblotted with a polyclonal antibody to Jak2 (middle panel) then with a monoclonal antibody to SHP-2 (lower panel). (B) Whole cell lysates obtained from 293 cells that were transiently transfected with either SHP-2WT or SHP-2CA expression vectors, starved by serum withdrawal and stimulated with PRL for the times indicated, were separated on SDS-PAGE and immuno-detected with a polyclonal antibody to phosphorylated tyrosines 1007/1008 of Jak2 (upper panel), membrane was stripped then reprobed with a polyclonal antibody to Jak2 (middle panel) then with a monoclonal antibody to SHP-2 (lower panel). (C) 293 cells were transiently transfected with expression vectors for either SHP-2WT or SHP-2CA along with an expression vector for Stat5 then cells were starved by serum depravation and stimulated with PRL for the times indicated. Whole cell lysates were separated on SDS-PAGE then immunoblotted with a monoclonal antibody to phosphorylated Stat5 (upper panel), the membrane was tripped then reblotted with a monoclonal antibody to Stat5 (lower panel).

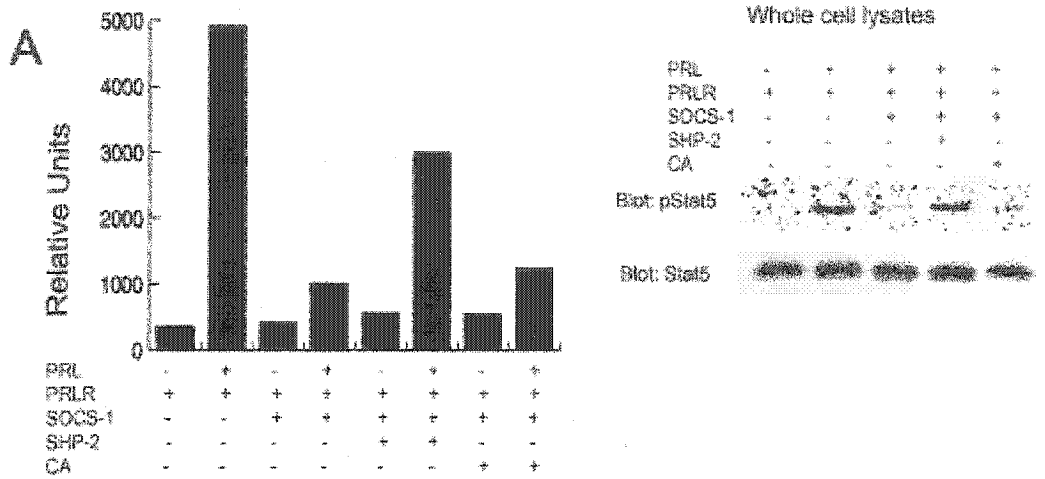
To confirm that phosphorylated Y1007 is a substrate for the catalytic activity of SHP-2 we transiently co-transfected 293 cells with expression vectors for either SHP-2WT or the inactive mutant, SHP-2CA, along with expression vectors for the Nb2 form of the PRLR and Jak2. Cells were starved by serum deprivation then stimulated with PRL for either 10min or 30min then whole cellular lysates were separated on SDS-PAGE and immunoblotted with a polyclonal antibody to phosphorylated Y1007/Y1008 of Jak2. As can be seen in Figure 4.5B, Jak2 phosphorylation was observed upon PRL stimulation in all samples, however, the level of phosphorylation was clearly higher in samples overexpressing the catalytically inactive form of SHP-2. The membrane was reblotted with a polyclonal antibody to Jak2 then with a monoclonal antibody to SHP-2 to confirm similar levels of protein expression in all samples. These results further confirm that Y1007 of Jak2 is a substrate for SHP-2.

Since dephosphorylation of Y1007 of Jak2 by different phosphatases such as CD45 (Irie-Sasaki et al., 2001) and PTP1B (Aoki and Matsuda, 2000; Myers et al., 2001; Zabolotny et al., 2002; Cheng et al., 2002) have been linked to negative regulation of the kinase, we next examined the role of dephosphorylation of Jak2 by SHP-2 on the tyrosine phosphorylation of Stat5 as an indicator of the signaling activity of Jak2 downstream of the PRLR. 293 cells were transiently co-transfected with expression vectors for either SHP-2WT or the catalytically inactive mutant form, SHP-2CA, along with expression vectors for Jak2 and Stat5. Cells were starved then stimulated with PRL for 10min or 30min. Whole cell lysates were separated on SDS-PAGE and immuno-detected with a monoclonal antibody to phospho-Y694 of Stat5. As can be observed in Figure 4.5C, following PRL stimulation tyrosine phosphorylation of Stat5 was only detected in samples overexpressing SHP-2WT but not in samples overexpressing the catalytically inactive form of the phosphatase. This data confirms previous studies indicating that SHP-2 is required for PRLR signaling (Ali et al., 1996; Ali and Ali, 2000;

Berchtold et al., 1998; Chughtai et al., 2002) and correlates the dephosphorylation of Y1007/Y1008 of Jak2 by SHP-2 to Jak2 activation. Taken together, the phosphatase SHP-2 dephopshorylates Jak2 on Y1007/Y1008, encompassing the recruitment site for SOCS-1, and this dephosphorylation plays a positive role in the regulation of Jak2 kinase activity.

**SHP-2 Regulates SOCS-1 Mediated Inhibition of PRLR Signaling**

Activation of the PRLR leads to the induction of gene expression of various milk proteins. Measuring gene promoter activation of the milk protein β-casein, by employing luciferase assays, is used as a marker of PRLR activation of signaling. To investigate SHP-2-mediated regulation of Jak2/SOCS-1 interaction in PRLR signaling to gene activation, we co-transfected 293 cells with expression plasmids encoding either SHP-2WT or the catalytically inactive mutant of the phosphatase, SHP-2CA, along with the Nb2 form of the PRLR, Stat5, SOCS-1, β-galactosidase and the reporter construct β-casein/lux. Following transfection cells were starved and stimulated with PRL for an overnight period, lysed and luciferase assays were performed. As shown in Figure 4.6A (a representative experiment), PRL stimulation led to the activation of the β-casein gene promoter (15 fold). As expected, overexpressing SOCS-1 potently inhibited this activation. Interestingly, overexpressing SHP-2 led to the recovery of PRL signals (approximately 80%). In contrast no recovery of PRLR signaling was observed in the presence of the catalytically inactive form of SHP-2. This indicates that SHP-2 catalytic activity attenuates SOCS-1 mediated inhibition of PRLR signaling to milk protein gene promoter activation. As a second measure of signaling activity of the PRLR, we examined the tyrosine phosphorylation level of Stat5. 293 cells transiently co-transfected with expression vectors for SHP-2WT or SHP-2 CA along with the Nb2 form


A
Relative Units
5000
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PRL
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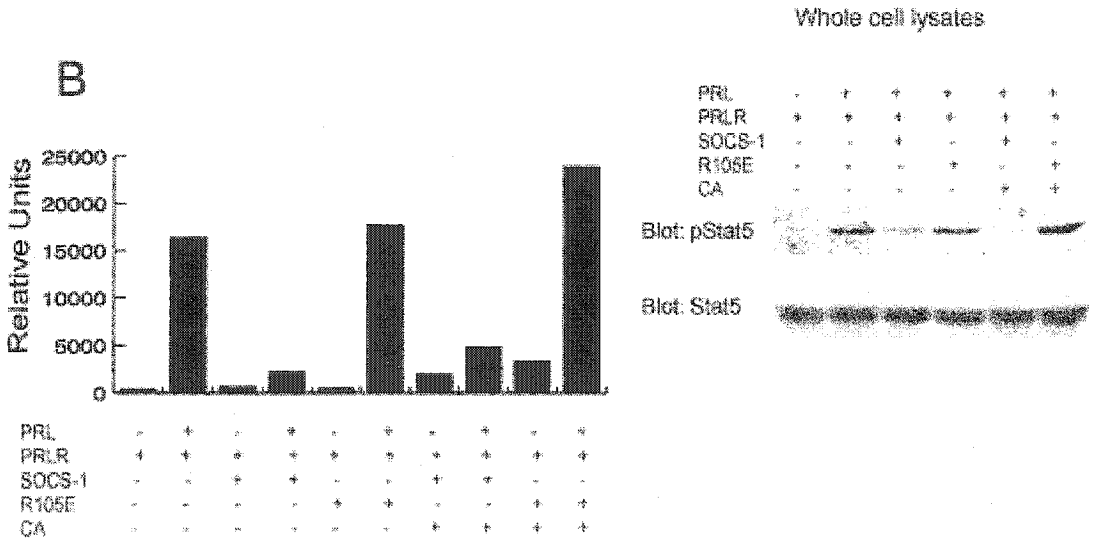
SOCS-1
SHP-2
CA
Whole cell lysates
Blot: pStat5
Blot: Stat5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PRL
PRLR
SOCS-1
R105E
CA
Whole cell lysates
Blot: pStat5
Blot: Stat5

Figure 4.6 SHP-2 phosphatase recovers SOCS-1 mediated inhibition of PRLR signalling. (A) Luciferase assay performed on 293 cells that were transfected with expression vectors for PRLR, SOCS-1, SHP-2 or the catalytically inactive SHP-2 mutant C436A as indicated. One representative experiment was chosen from five independent experiments and data is presented as relative light units (left panel). Whole cell lysates obtained from 293 cells that were transiently transfected with expression vectors for PRLR, SOCS-1, SHP-2 or SHP-2CA as indicated and then separated on SDS-PAGE and immunoblotted with a monoclonal antibody to phosphorylated Stat5, the membrane was stripped then reprobed with a monoclonal antibody to Stat5 (right panel). (B) Luciferase assay was conducted using 293 cells that were transfected with expression vectors for the PRLR, SOCS-1, the SH2 domain mutant of SOCS-1, R105E or the SHP-2CA mutant C436A. Data presented as relative light units and was chosen as a representative from five independent experiments (left panel). Whole cell lysates obtained from 293 cells that were transiently transfected with expression vectors for PRLR, SOCS-1, R105E, or SHP-2CA mutant were separated on SDS-PAGE then immunodetected using a monoclonal antibody to phosphorylated Stat5, the membrane was stripped and reprobed with a monoclonal antibody to Stat5 (right panel).

of the PRLR, SOCS-1 and Stat5. Cells were then starved and stimulated with PRL for 10 min. Cellular lysates were separated on SDS-PAGE, and prepared for Western blotting with a polyclonal antibody to phospho-Y694 of Stat5. As shown in Figure 4.6 right panel, PRL stimulation led to tyrosine phosphorylation of Stat5, which was inhibited by overexpressing SOCS-1. However, tyrosine phosphorylation of Stat5 was clearly restored in samples overexpressing SHP-2WT but not in samples overexpressing SHP-2CA mutant. Therefore, the phosphatase SHP-2, through its catalytic phosphatase activity is able to block the inhibitory effects of SOCS-1 in PRLR signaling leading to Stat5 phosphorylation and gene promoter activation.

To further confirm that SHP-2 contribution to PRLR signaling is mediated through the regulation of Jak2/ SOCS-1 interaction, we used an SH2-domain inactivating mutant form of SOCS-1, SOCS-1R105E (Kamizono et al., 2001) thereby blocking the recruitment of SOCS-1 to Jak2 in the functional luciferase assay system. We co-transfected 293 cells with expression plasmids encoding either SOCS-1 or SOCS-1R105E along with the Nb2 form of the PRLR, Stat5, catalytically inactive mutant form of SHP-2, SHP-2CA, β-galactosidase and the reporter construct β-casein/lux. Cells were starved and stimulated with PRL for an overnight period, lysed and luciferase assay was performed. As shown in Figure 4.6B (a representative experiment), the data revealed that stimulation with PRL led to activation of the β-casein gene promoter and as expected overexpression of SOCS-1 blocked this effect. Furthermore, overexpression of SOCS-1R105E had on effects on PRL-mediated gene promoter activation indicating that SOCS-1 mediated inhibition of PRLR signaling requires its SH2 domain as has been reported for other cytokine receptor systems (Kamizono et al., 2001). Co-overexpressing SHP-2CA mutant with SOCS-1 as expected inhibited PRLR signaling. Significantly, co-overexpressing SHP-2CA with the SH2 domain inactive mutant form of SOCS-1, SOCS-

1R105E restored luciferase activity indicating that the ability of catalytically inactive form of SHP-2 to inhibit PRLR signaling requires a functional SH2 domain of SOCS-1. Together, that data indicate that the positive effect of SHP-2 in PRLR signaling is a result of the inhibition in Jak2/SOCS-1 interaction.

We next investigated whether blocking SOCS-1 recruitment to Jak2 would modulate the inhibitory effects of catalytically inactive SHP-2 on Stat5 tyrosine phosphorylation. 293 cells were transiently co-transfected with expression vectors for the Nb2 form of the PRLR, SHP-2CA with either SOCS-1 or SOCS-1R105E as indicated in Figure 4.6B, right panel. Cellular lysates were separated on SDS-PAGE, transferred to a nitrocellulose membrane and then blotted with antibodies to phosphorylated Y694 of Stat5. As expected tyrosine phosphorylation of Stat5 was induced by PRL, and inhibited by SOCS-1 overexpression. No inhibition of Stat5 tyrosine phosphorylation was observed in samples overexpressing SOCS-1R105E indicating the essential role of the SH2 domain of SOCS-1 in mediating the inhibitory effects of SOCS-1. Moreover, overexpressing SHP-2CA mutant along with SOCS-1 as expected blocked PRL-mediated Stat5 phosphorylation. In contrast, co-overexpressing the catalytically inactive form of SHP-2, together with SOCS-1R105E relieved the inhibitory effects of SHP-2CA on PRL-mediated Stat5 tyrosine phosphorylation (Figure 4.6B, right panel). These results taken together indicate that SHP-2 modulates the signaling activity of the PRLR by regulating Jak2/SOCS-1 interaction and hence SOCS-1 mediated inhibition.

## DISCUSSION

Strict regulation of intracellular signaling is critical for obtaining accurate biological and physiological responses. Both protein kinases and phosphatases are part of an elaborate network of pathways that control the outcome of cellular signaling activities. It is clear that kinases and phosphatases act to regulate and fine-tune each other's activities in dynamic temporaly and spatialy dependent mechanisms.

The protein tyrosine phosphatase SHP-2 has emerged as an important physiological regulator of signaling of a wide variety of cytokines and growth factors. However, the molecular mechanism of action and target(s) of SHP-2, particularly related to cytokine receptor signaling are not well characterized. Here we report a new mechanism of action for SHP-2 in regulating cytokine receptor signaling leading to signal propagation. We determined that the protein tyrosine phosphatase SHP-2 plays an essential role in regulating PRLR signaling by dephosphorylating Y1007 within the activation loop of Jak2 kinase, a known recruitment site for SOCS-1, thereby contributing to PRLR/Jak2 protein stability hence enhancing their signaling capacity.

Our results indicate that blocking SHP-2 function downstream of the PRLR by utilizing the C-terminal tyrosine mutant form of the PRLR, lacking the association site for SHP-2, as well as a catalytically inactive form of SHP-2 leads to increased rate of ubiquitination and degradation of Jak2. Significantly, we determined that all these events are regulated by SHP-2 through modulation of the phosphorylation state of Y1007 of Jak2 thus influencing Jak2/SOCS-1 interaction. Moreover, our results also indicate that loss of SHP-2 function leads to an increase in the degradation of the PRLR as well as SOCS-1 proteins suggesting that SOCS-1 binding to Jak2 will target PRLR/Jak2/SOCS-1 as a complex to the proteasomal degradation machinery associated with SOCS-1.

Phosphorylation of Y1007 of Jak2 is a requirement for the activity of the kinase (Feng et al., 1997). Our results indicate that following PRL stimulation there is an

increase in the phosphorylation of Y1007 of Jak2, interestingly however, we observed that the same tyrosine is also a target for dephosphorylation by SHP-2. In fact, we observed that less phosphorylation of Y1007 of Jak2 is linked to a higher signaling capacity of the PRLR as indicated by increased phosphorylation of Stat5 and activation of the β-casein gene promoter. Furthermore, the fact that the inhibitory effects of the catalytically inactive mutant of SHP-2 on PRLR signaling were blocked in the presence of an SH2-domain inactivating mutant of SOCS-1, unable to bind Jak2, indicates that blocking Jak2/SOCS-1 interaction is a major mechanism by which SHP-2 regulates PRLR signaling. Together, our results indicate that dephosphorylation of Y1007 of Jak2 by SHP-2 is a necessary event for PRLR signal propagation.

The seemingly paradoxical role of phosphorylation of Y1007 of Jak2 could be clarified by adopting a dynamic vision of the signaling process as depicted in Figure 4.7. Following activation of cytokine receptors, if Jak2 phosphorylation on Y1007 was to be maintained, this would create an association site for the increasingly accumulating inhibitory SOCS-1 protein and would ultimately lead to the degradation of Jak2 and termination of the signal. However, in the presence of the phosphatase SHP-2, Y1007 of Jak2 is dephosphorylated preventing SOCS-1/Jak2 interaction and impeding ubiquitination and degradation of Jak2 consequently maintaining the signaling capacity of the receptor/Jak2 complex. The dephosphorylated Jak2 is then rephosphorylated and reactivated by auto/transphosphorylation. It is conceivable that the cycle of phosphorylation/dephosphorylation of Jak2 could repeat itself a number of times. The cycle is terminated when SOCS-1 levels are elevated to a certain threshold where the equilibrium is shifted towards degradation of Jak2 rather than dephosphorylation by SHP-2 and reactivation. Intriguingly, immunoblotting with SOCS-1 antibodies performed in Nb2 cells (Figure 4.1C) and HC11 cells (data not shown), revealed the presence of high basal amounts of SOCS-1 prior to PRL stimulation, suggesting that the process of

phosphorylation/dephosphorylation of Jak2 starts early following PRLR activation. Our results implicate the duration of phosphorylation of Y1007 of Jak2 as a critical determinant in the final outcome of signaling following cytokine receptor activation.

The transmembrane phosphatase CD45 and the cytoplasmic phosphatase PTP1B were both shown to dephosphorylate Y1007 of Jak2. However, these two phosphatases differ from SHP-2 in that they negatively regulate cytokine signaling (Irie-Sasaki et al., 2001; Myers et al., 2001; Zabolotny et al., 2002). The reason for the divergence in the function of SHP-2 compared to CD45 and PTP-1B is not yet known. It is possible to speculate that the differences might be due to local concentrations of these phosphatases at the receptor/Jak2 complex. Alternatively, it may be explained by the possibility that CD45 and PTP1B possess higher catalytic activities compared to SHP-2. In such a case Jak2 would be in a continuous dephosphorylated state, which would lead to its permanent inhibition. Further investigation is required to clarify these mechanisms.

This study provides the first example of a phosphatase that acts in a positive manner via maintaining the signaling capacity of Jak2 through dephosphorylation of Y1007 of the kinase. The possibility exists that other phosphatases may act in a similar manner or that SHP-2 could modulate other kinases in a mode similar to its regulation of Jak2. Interestingly, our data presented here is in agreement with recently published mathematical models employed to describe signal transduction events (Heinrich et al., 2002). It was concluded that signal amplitude is controlled primarily by kinases, whereas phosphatases have a more profound effect than kinases on the rate and duration of signaling.

In this report we demonstrated that the protein tyrosine phosphatase SHP-2 plays a positive role in signaling through delaying the proteasomal degradation of the kinase Jak2 as well as the PRLR. This function of SHP-2 allows signal generation and

maintenance by preventing the association of Jak2 with the inhibitory protein SOCS-1. Further exploration should reveal that this function of SHP-2 is not unique to the PRLR signaling system, but rather is a general mechanism of regulation of Jak2 kinase by SHP-2 phosphatase.

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## DISCUSSION

Phosphorylation of specific tyrosine residues on the PRLR is regarded as an essential step in the process of signal transduction. This conclusion was initially based on observations made with signalling from other cytokine receptors as well as receptor tyrosine kinases. It was observed that one or more of the tyrosines present in the intracellular portions of those receptors becomes phosphorylated and acts as a recruitment site for one or a number of the cellular signalling mediators. Subsequently, a study aimed at examining the participation of PRLR tyrosine phosphorylation in signalling determined that a single tyrosine residue present at the most carboxy-terminal section of the PRLR critically controlled PRLR signalling activity. In that study, the signalling capacity of the PRLR was examined using manipulated forms of the PRLR trough exchanging specific PRLR tyrosines into alanines, and accordingly examined the ability of the receptor to activate the transcription of the PRL responsive β-casein gene (Lebrun et al., 1995a). The finding that the C-terminal tyrosine plays such a critical role in PRLR was quite significant, however, the mechanism(s) regulated by this C-terminal tyrosine was completely unknown.

### The C-terminal Tyrosine of the PRLR Regulates Stat5 Activation

The activation process for Stat5 is similar to the activation of other members of the signal transducers and activators of transcription family members. Stat5 is recruited to a phosphorylated tyrosine residue present in the PRLR/Jak2 complex an event that is followed by tyrosine phosphorylation of Stat5, dissociation from the complex, dimerization of two Stat5 molecules, translocation to the nucleus and finally binding to DNA and activating transcription of PRL responsive gene which contain Stat5 responsive elements (GAS). It is not yet known how Stat5 is recruited to the PRLR/Jak2 complex and what are the molecular requirements for recruitment. In a study examining

the role of PRLR tyrosines in Stat5 activation reported that tyrosines 580, 479, and 473 of the PRLR were all sufficient to activate Stat5, with Tyrosine 580 being the most potent (Pezet et al., 1997b). However, in our study we observed that tyrosine phosphorylation of the PRLR was not required for tyrosine phosphorylation of Stat5 (Ali, 1998). We observed that Stat5 became tyrosine phosphorylated under conditions when the PRLR itself was not phosphorylated. In addition to our report, similar observations were made by other studies. It was reported that a truncated form of the PRLR lacking some or all of the intracellular tyrosines was competent in inducing Stat5 tyrosine phopshorylation and activation (Frank et al., 1995; Goupille et al., 1997). The recruitment site for Stat5 in the PRLR/Jak2 complex is not yet known. We propose that Stat5 could directly associate through its SH2 domain to a phosphorylated tyrosine residue on Jak2. This association would then bring Stat5 in close proximity allowing it to be phosphorylated by the kinase. There is some evidence suggesting that in fact there is a direct association between Stat5 and Jak2. Using the two-hybrid screening approach, a direct association between Stat5 and Jak2 was observed (ref).

**The C-terminal Tyrosine of the PRLR Regulates Stat5 Nuclear Translocation**

Little is known about the mechanism that regulates Stat protein nuclear translocation. We present data that Stat5 tyrosine phosphorylation and nuclear translocation processes are separate and independent of each other. We report that Stat5 tyrosine phosphorylation is required but not sufficient to cause Stat5 nuclear translocation. We observed that when the C-terminal tyrosine mutant of the PRLR was expressed, Stat5 phosphorylation was maintained, however, under those conditions, Stat5 failed to translocate to the nucleus. Therefore, the C-terminal tyrosine regulates a process that influences Stat5 nuclear translocation. An explanation of how the C-terminal tyrosine regulates Stat5 nuclear translocation is not yet available. We propose that a certain factor present in the cells might be involved in the regulation process. In a study

performed in our laboratory, it was demonstrated that Stat5 and the protein tyrosine phosphatase SHP-2 associate and translocate together to the nucleus following stimulation with PRL (Chughtai et al., 2002). And since we reported that SHP-2 is recruited to the C-terminal tyrosine in a PRL dependent manner, it seems possible that SHP-2 recruitment to the C-terminal tyrosine is the determinant that participates in Stat5 nuclear translocation following its tyrosine phosphorylation. Further investigation is required to determine if SHP-2 plays a role in Stat5 nuclear translocation and by what mechanism.

The picture of how Stat proteins translocate to the nucleus is just beginning to immerge. It has been suggested that Stat proteins translocate to the nucleus through the Importin/RanGTPase system. An arginine and lysine-rich NLSs motif was identified in Stat1 and was shown to mediate interactions between a homodimer of Stat1, or a heterodimer of Stat1 and Stat2, and importin-α5. Additionally, it was demonstrated that two intact NLS elements, one in each monomer, were required for binding to importin-α5. (Fagerlund et al., 2002). However, unlike Stat1, Stat5 does not contain a putative nuclear localization sequence (NLS) and the mechanism of translocation remains unknown.

**SHP-2 is Recruited to the C-terminal Tyrosine of the PRLR**

The protein tyrosine phosphatase SHP-2 plays a central role in mediating the signalling activities of the PRLR. The consequences of inactivating the phosphatase through either mutating or deleting the phosphatase domain were a complete inhibition of PRLR signalling manifested by loss of Jak2 activity, loss of phosphorylation of Stat5, inability of Stat5 to bind DNA, and most significantly, inability to activate transcription of PRL responsive genes (Ali et al., 1996; Berchtold et al., 1998). Therefore, it is of high

relevance to understand the specific mechanism of activation as well as determine the cellular substrates of SHP-2 phosphatase.

We detected SHP-2 in immunoprecipitations of the PRLR indicating the association between SHP-2 and the PRLR. When the C-terminal tyrosine of the PRLR was mutated to phenylalanine, SHP-2 was no longer tyrosine phosphorylated nor did it associate with the PRLR. Therefore, the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment. We further illustrated that the association of SHP-2 to the PRLR occurs through the C-terminal SH2 domain of the phosphatase. Taking into consideration previously described experiments indicating that occupation of the SH2 domain by a phosphotyrosine as well as tyrosine phosphorylation of SHP-2 leads to the activation of the phosphatase, it is possible to conclude that recruitment of SHP-2 to the C-terminal tyrosine of the PRLR leads to the stimulation of the catalytic activity of SHP-2.

Similar to recruitment to the PRLR, SHP-2 is recruited to a number of cytokine receptors. For example, SHP-2 is recruited to intracellular tyrosine residues in the GHR, EPOR and the gp130 subunit of the IL-6 receptor (Kim et al., 1998b; Tauchi et al., 1996; Kim et al., 1998a). In a similar manner, SHP-2 also is recruited to the PDGF receptor, a member of the RTK family (Kazlauskas et al., 1993).

It is not fully clear whether SHP-2 recruitment to the PRLR is a direct one-to-one association, or it occurs through a third adaptor protein. In a study, it was reported that the C-terminal tyrosine of the PRLR is one of a number of sites for recruiting Stat5 (Pezet et al., 1997b). Therefore, either SHP-2 and Stat5 could individually associate to the same tyrosine residue, and hence compete for the same recruitment site, or alternatively, they could both associate utilizing an adaptor protein. A third explanation could also be that one of the proteins is an adaptor that recruits the other. A possible approach to tackle the question is to perform Far-Western blots using the PRLR and SHP-2 or Stat5 as associating partners.

**SHP-2 Associates with Gab2 Following PRLR Activation**

In addition to recruitment to the PRLR, we detected SHP-2 in immunoprecipitations of Gab2, the adaptor protein belonging to the Gab family. The mechanism of association of SHP-2 to Gab2 is not yet known. SHP-2 contains two SH2 domains, N-SH2 and C-SH2 domains. We observed in our studies that SHP-2 recruitment to the PRLR occurs through the C-SH2 domain. Therefore, the N-SH2 domain is free to associate with a third molecule such as Gab2. In support of this concept is that Gab2 has two consensus SHP-2 recruitment sites both sites fit the reported consensus for N-SH2 recruitment **Y** (I/V) X (V/I/L/P) [Songyang, 1993 #1332]. Alternatively, rather than one SHP-2 molecule simultaneously associating with the PRLR and Gab2, two different pools of the phosphatase are independently recruited to the PRLR and to Gab2 and are spatially segregated.

Not only is the mechanism of recruitment of SHP-2 to Gab2 not known, but also the method of recruitment of Gab2 to the PRLR remains to be determined. In fact, little information is available on the method of recruitment of Gab2 to any cytokine receptor. Some information could be inferred from the literature available on the mechanism of recruitment of Gab1 to RTK such as the Met receptor. The PH domain of Gab1 was reported to bind to the product of PI3K, phosphatidylinositol 3,4,5-triphosphate, in the cellular membrane (Rodrigues et al., 2000; Maroun et al., 1999; Isakoff et al., 1998). Gab2 recruitment to the PRLR could occur in a similar manner. However, there should be some activation of the PI3K prior to recruitment of Gab2, meaning that the role of Gab2 in signaling needed at a later time point in signalling. A second possible method of recruitment of Gab2 to the PRLR is through the interaction between proline-rich sequences and SH3 containing proteins. A possible candidate for this association is Grb2, which is known to be involved in PRLR signalling (Das and Vonderhaar, 1996).

Hence, the method of recruitment of Gab2 to the PRLR remains unknown and further investigation is required. To understand the mechanism of complex formation between the PRLR, Gab2 and SHP-2, a combination of point mutations in tyrosine recruitment sites, deletions or mutations of SH2 domains, and deletions of the PH domain are required for use in a number of co-immunoprecipitation studies. A complementary approach which will allow the determination of molecular interactions among these various proteins is the use of GST-pulldown experiments.

**Biological Significance of Gab2 Recruitment**

The consequence for Gab2 involvement in PRLR signalling is not yet known. It was reported that Gab2 involvement has a positive effect on signalling as measured by the activation of Stat5 induced genes downstream of IL-2 receptor activation (Gu et al., 1998). In another study, it was shown that a Gab2 mutant which was unable to bind SHP2 blocked IL3-induced c-fos promoter activation and M-CSF-induced macrophage differentiation (Gu et al., 2000; Liu et al., 2001). In the case of Gab1, a study demonstrated that the interaction between Y627 and Y659 of human Gab1 and SHP-2 leads to the activation of the catalytic activity of the phosphatase (Cunnick et al., 2001). Similarly, in a different study it was shown that MAPK activation and subsequent morphogenesis induces by EGF were defective when a mutant of Gab1 that is unable to bind to SHP-2 was expressed (Schaeper et al., 2000; Cunnick et al., 2000; Maroun et al., 2000). Therefore, it was suggested that a major function for Gab proteins is the recruitment and activation of SHP-2 phosphatase.

In addition to SHP-2, Gab proteins also associate with other cellular proteins. In our study we observed that Gab2 could recruit the p85 subunit of the PI3K. Therefore, it is conceivable that Gab2 is a main mediator of PI3K activation downstream of the PRLR. In fact, the activation of the PI3K pathway was reported previously in PRLR signalling (Shirota et al., 1990; al-Sakkaf et al., 1996). However, the biological significance of

PI3K activation in PRLR signalling is not known. The most likely consequence on the PI3K activation is the activation of Akt/PKB which is a known mediator of cellular survival. This concurs with the agreed upon role of PRL as a survival and differentiation factor (Flint and Knight, 1997). Indeed, data resulting from work performed in our laboratory confirms that activation of the PI3K/Akt downstream of the PRLR induces cell survival (unpublished results). Similarly, other studies that were conducted elsewhere using various signalling systems reported that both Gab2 and Gab1 associate to the p85 subunit and mediate the activation of the PI3K/Akt signaling pathway (Rodrigues et al., 2000; Maroun et al., 1999; Gu et al., 2000).

Gab proteins also associate with members of Crk family of adaptor proteins. Preliminary experiments conducted in our laboratory demonstrate that Gab2 associates to CrkI and CrkII in response to stimulation of the PRLR (unpublished data). In other studies, it was shown that Gab2 interacts with CrkL in a yeast two-hybrid screen (Gesbert et al., 1998). A similar association was reported in human T lymphocytes in response to IL2 (Crouin et al., 2001). The influence on signalling of the association between Gab2 and Crk proteins is not known. Several studies indicated that CrkII and CrkL form a complex with Stat5 as a result of Bcr/Abl transformation and EPOR activation (Grumbach et al., 2001). Therefore, the association between Gab2 and Crk proteins may play a role in the activation of Stat5 in the signalling scheme of the PRLR.

In a manner similar to Gab2, other reports using different signalling systems indicated that Gab1 binds to both CrkI and CrkL (Schaeper et al., 2000; Gual et al., 2000; Lamorte et al., 2000). For example it was shown that Gab1 associates with Crk in response to Met activation and this association correlates with anchorage-independent growth and JNK activation in cells transformed by the Met receptor (Garcia-Guzman et al., 1999; Lamorte et al., 2000).

These various studies illustrate that Gab proteins act as focal points for recruitment of various signalling proteins and hence act as integration factors for a number of signalling pathways in the cell.

**Jak2 is a Target for Dephosphorylation by SHP-2**

All cytokine receptors examined to date activate one or more members of the Jak family of kinases. The PRLR is known to activate Jak2 kinase and it was firmly demonstrated that Jak2 activation is an essential event in PRLR signalling (Lebrun et al., 1994; Lebrun et al., 1995b). Prior to our studies, there were some indications that SHP-2 could positively regulate Jak2 kinase activation within PRLR signalling cascades, however, the mechanism of contribution of SHP-2 in the activation process was not known. Furthermore, prior to our studies, little was known about the physiological targets of SHP-2. We demonstrate that Jak2 is a target for dephosphorylation by the protein tyrosine phosphatase SHP-2. In particular, a specific tyrosine, Y1007, located in the activation loop of Jak2 is dephosphorylated by SHP-2. This tyrosine was shown to be a recruitment site for SOCS-1, which was previously shown to be a potent inhibitor of Jak2.

SOCS-1 inhibition of Jak2 kinase activity is two fold. First, through the SH2 domain, SOCS-1 is recruited to Y1007 of Jak2 and this association brings in a kinase inhibitory motif composed of a portion of the SH2 domain and a further 24 amino acids N-terminal to the SH2 domain (called KIR) which blocks the kinase domain of Jak2 from access of substrates (Sasaki et al., 1999). Second, various studies demonstrated that recruitment of SOCS-1 to Jak2 leads to the ubiquitination and proteasomal degradation of Jak2, a function which was dependent on the SOCS-box of SOCS-1. It was shown that the SOCS-box forms a complex with elongin BC and Cullin2, which acts as an E3 ubquitin ligase (Kamura et al., 2000; Kamizono et al., 2001). Therefore, dephosphorylation of Y1007 of Jak2 would prevent SOCS-1 from associating to Jak2 and hence would

alleviate the inhibition of Jak2 kinase activation and also prevents Jak2 kinase from becoming ubiquitinated and degraded.

**SHP-2 Prolongs Half-Life of Jak2**

We observed that the event of dephosphorylation of Jak2 by SHP-2 was linked to a longer half-life of Jak2 and we demonstrated that the extension in the life of Jak2 was due to the inhibition in the association between Jak2 and SOCS-1. We could not precisely pinpoint the amount of increase in Jak2 half-life due to variability among experiments. However, there was on average an increase between 3-6 fold in the presence of Jak2 when SHP-2 was dephosphorylating Jak2. Under conditions when SHP-2 was inactive, either with the C-terminal tyrosine mutant of the PRLR that is incapable of recruiting SHP-2, or with the CA mutant in the catalytic phosphatase domain of SHP-2, we observed that Jak2 ubiquitination peaks at 30min-1h post PRL stimulation. In contrast, Jak2 ubiquitination only peaks after 3-6h following stimulation with PRL when SHP-2 was present and functional. Therefore, dephosphorylation of Jak2 by SHP-2 permits Jak2 to continue to signal through allowing a window in time where Jak2 does not associate with SOCS-1 and therefore would be able to phosphorylate and activate its physiological targets in signalling.

**Dephosphorylation of Jak2 by SHP-2 Positively Modulates PRLR signalling**

In addition to the fact that dephosphorylation Jak2 by SHP-2 inhibits Jak2 ubiquitination and degradation, the dephosphorylation and prevention of association between Jak2 and SOCS-1 should also influence the kinase activity of Jak2. We were able to demonstrate the positive effect of Jak2 dephosphorylation on PRLR signalling through using two approaches. First, using luciferase assays we observed that as predicted, the inhibition of association between Jak2 and SOCS-1 had a positive outcome on signalling. Under the conditions where SHP-2 phosphatase was present, we observed a

significantly decreased phosphorylation of Y1007 of Jak2 accompanied by less association of SOCS-1 to Jak2 and combined with restoration of SOCS-1 inhibited PRLR signalling activity. With the second approach, we used Stat5 tyrosine phosphorylation as an indication of PRLR signalling capacity. Similar to the luciferase data, we observed that the inhibition of Stat5 tyrosine phosphorylation by SOCS-1 was recovered in the presence of SHP-2 phosphatase. The recovery of PRLR signalling required the catalytic activity of the phosphatase, where catalytically inactive SHP-2 was unable to reverse the inhibition of signalling by SOCS-1.

**Partial Inhibition of Jak2 Kinase activity through Dephosphorylation by SHP-2**

Our studies indicate that dephosphorylation of Jak2 by SHP-2 has an overall positive outcome on the signalling capacity of the PRLR. However, at least some inhibition of Jak2 kinase activity might be associated with the dephosphorylation event by SHP-2. The phosphorylation of Y1007 of Jak2 was shown to be required for the activation of the kinase (Feng et al., 1997). Therefore, dephosphorylation by SHP-2 is predicted to have some inhibitory effect on Jak2 kinase activation. We have some data to support this conclusion. In the luciferase assay study (Figure 4.6), we observed that a catalytically inactive SHP-2, SHP-2CA, resulted in a higher activation of Jak2 kinase. However, this only occurred when the inhibitory effect of SOCS-1 was no longer a consideration when the association between SOCS-1 and Jak2 was prevented via an SH2 domain mutation in the SOCS-1 SH2 association domain. Therefore, under circumstances where SOCS-1 is completely absent from the cell, dephosphorylation of Jak2 by SHP-2 has some inhibitory effects on Jak2 kinase activity. However, in real cellular environments, SOCS-1 is present and the inhibitory effect of SOCS-1 on Jak2 activation appears to be more significant that dephosphorylation by SHP-2. The dephosphorylation event of Jak2 is reversible where a dephosphorylated Jak2 could be rephosphorylated and reactivated through auto- or transphosphorylation event in the PRLR complex. In contrast,

association of Jak2 to SOCS-1 leads to an irreversible ubiquitination and degradation of the protein. Therefore, under conditions where SOCS-1 is at low concentrations in the cell at the start of stimulation of the PRLR, dephopshorylaiton of Jak2 by SHP-2 and the inhibition of SOCS-1 recruitment has a positive outcome on PRLR signalling. Simply, a dephosphorylated Jak2 goes through a second round of rephosphorylation and reactivation and therefore continue to signal.

Early reports on SOCS proteins indicated that there were no detectable SOCS proteins in the basal state of the cell. However, those studies at the time examined the presence of SOCS mRNA and not SOCS proteins. In our studies, we used the recently available competent SOCS-1 antibody and observed a significant amount of SOCS-1 protein present in the cell prior to stimulation with PRL. In all cell lines that we examined, Nb2, HC11 and 293, we detected high levels of SOCS-1 protein present in basal conditions. Therefore, the inhibition by SHP-2 of the association between SOCS-1 and Jak2 is predicted to take place immediately following stimulation with PRL. At later time points after the initial signal leads to the induction of SOCS-1 gene and the production of the protein, SOCS-1 protein increasingly accumulates and SHP-2 no longer could compete with SOCS-1 protein. So the preservation of Jak2 association to SOCS-1 by SHP-2 is overcome by the increasingly present SOCS-1, and therefore Jak2 is directed to ubiquitination and degradation rather than dephosphorylation and reactivation by SHP-2. The end result is the termination of PRLR signal.

Taken all together, SHP-2 plays a positive role in PRLR through dephosphorylating Y1007 of Jak2 and inhibiting the association of SOCS-1, the blocking of the kinase activity, and preventing the ubiquitination and degradation of Jak2.

**Inhibition of Jak2 Kinase through Dephopshorylation by CD45, PTP1B and SHP-1**

The positive role that SHP-2 plays in PRLR, mediated through dephosphorylation of Jak2, is unlike the roles that CD45, SHP-1 and PTP1B play in the regulation of Jak2

signalling. These phosphatases were shown to similarly dephosphorylate Jak2 on Y1007 but to negatively regulate signalling activity of the kinase (Irie-Sasaki et al., 2001; Klingmuller et al., 1995; Yi et al., 1993; Aoki and Matsuda, 2000). The reason behind the divergence in actions between SHP-2 and these other phosphatases is not known. A possible explanation is that the difference could be due to a higher catalytic phosphatase activity for CD45, SHP-1 and PTP1B compared to SHP-2. As a result, in the presence of these phosphatases, Jak2 is continuously in the dephosphorylated inactive state and it not allowed to rephosphorylate itself, unlike SHP-2. An alternative explanation is that these phosphatases are present at higher concentrations in the cells, or are more densely localized around Jak2 kinase compared to SHP-2. Further investigation should reveal the rationale behind the difference in actions between SHP-2 and CD45, SHP-1, and PTP1B.

To determine whether the variation in actions is due to higher catalytic activity for these other phosphatases compared to SHP-2, *in vitro* catalytic phosphatase assays should reveal whether these phosphatases behave differently. It is also possible to examine the kinetics of association between Jak2 and the various phosphatase using immunoprecipitation studies. Therefore, more studies are needed to understand the intriguing differences in actions among various phosphatases.

**The C-terminal Tyrosine of the PRLR Partially Contributes to PRLR Signalling**

We demonstrated that the C-terminal tyrosine of the PRLR controls essential events in the signalling scheme of the PRLR. The effect was most obvious when using the Nb2 form of the PRLR, which contains only three intracellular tyrosines. It was previously illustrated that the C-terminal tyrosine is the major phosphorylation site on the receptor (Lebrun et al., 1995a). However, with the long form of the PRLR, the effect of mutating the C-terminal tyrosine had only a partial (50%) inhibition on the various signalling events examined. □-casein gene activation (Lebrun et al., 1995a), Stat5 nuclear

translocation (Ali, 1998), and SHP-2 recruitment were only partially inhibited by the loss of the C- terminal tyrosine, tyrosine 580, of the long form of the PRLR. Therefore, due to the fact that the long form of the PRLR contains six intracellular tyrosine, compared to the Nb2 form which contains three, it is predicted that one or more of the other tyrosines residues present only in the long form of the PRLR could substitute for the C-terminal tyrosine mutation and act in a redundant manner.

We reported that the C-terminal tyrosine of the PRLR is a recruitment site for the protein tyrosine phosphatase SHP-2. Therefore, we expect that the redundant second tyrosine in the long form of the PRLR would also be a recruitment site for SHP-2. For that reason, we conducted sequence comparisons and identified tyrosine 479 (Y479) in the long form of the PRLR as a possible association site for SHP-2. Our conclusion was based on the observation that Y479 motif fits the consensus sequence **Y**(I/V)X(V/I/L/P) reported for the N-SH2 domain of SHP-2 (Songyang et al., 1993) . Therefore, we selected Y479 for functional and association studies to investigate the possible recruitment of SHP-2 to this tyrosine residue and the tyrosine residues was substituted to phenylalanine (Y479F). Preliminary data suggest that Y479 is a recruitment site for SHP-2. Using co-immunoprecipitaions, we determined that with single mutations Y479F and the C-terminal Y580F mutations, there was a partial loss of SHP-2 recruitment to the long form of the PRLR. However, a complete loss of SHP-2 association was observed with the combined mutant Y479F/Y580F (unpublished data). When we examined the effect on the induction of the □-casein gene, we similarly observed that mutating Y479F or Y580F individually had a partial effect, and the combined mutations Y479/Y580F led to a complete inhibition of long form of the PRLR signalling capacity (unpublished data). Therefore, it appears that Y479 is a second site for SHP-2 recruitment in the PRLR. This data further confirms the positive effect of SHP-2 phosphatase in PRLR signalling.

**Summary and Future Prospects**

Tyrosine phosphorylation of the PRLR, particularly of the C-terminal tyrosine modulates critical signaling events in intracellular signaling to activate the transcription of PRL responsive genes. The tyrosine residue participates in the activation of Stat5, recruits SHP-2, modulates the association of Jak2 to SOCS-1, and protects Jak2 from SOCS-1 mediated proteasomal dagradation. Therefore, the C-terminal tyrosine is a focal point in PRLR signaling.

Results presented in this thesis reveal the role of the C-terminal tyrosine of the PRLR in modulating signaling events. The studies demonstrated a critical role and revealed the mechanisms of action of the C-terminal tyrosine of the PRLR in intracellular signaling. Further investigation into the other tyrosine residues present in the PRLR, particularly in the long from of the PRLR, is required to understand what role they play in signaling.

The PRLR and other members of the cytokine receptors superfamily share many of signalling components. Therefore, to understand the molecular basis of prolactin receptor signal transduction is not only important for understanding the mechanisms of action of PRL in target tissues, such as its role in mammary gland development, but also important for understanding the intracellular signaling basis of other members of the family of cytokine receptors.

## CONTRIBUTIONS TO ORIGINAL RESEARCH

1. Stat5 tyrosine phosphorylation is independent of tyrosine phosphorylation of the PRLR.
2. The C-terminal tyrosine of the PRLR regulates the process of Stat5 nuclear translocation.
3. The C-terminal tyrosine of the PRLR is a site for recruitment of the protein tyrosine phosphatase SHP-2.
4. Recruitment of SHP-2 to the PRLR occurs through the C-terminal SH2 domain of the phosphatase.
5. Stimulation of the prolactin receptor induces the tyrosine phosphorylation of the adaptor protein Gab2.
6. SHP-2 associates with Gab2 adaptor protein in response to stimulation of the PRLR.
7. The C-terminal tyrosine of the PRLR modulates the tyrosine phosphorylation state of Jak2.
8. SHP-2 dephosphorylates tyrosine 1007 of Jak2 and prevents the association of the inhibitory protein SOCS-1.
9. The prevention of Jak2 association with SOCS-1 prolongs the life time of Jak2 and the prolactin receptor.

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# APPENDIX

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# Prolactin Receptor Regulates Stat5 Tyrosine Phosphorylation and Nuclear Translocation by Two Separate Pathways*

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**The SH2 domain containing signal transducers and activators of transcription (Stat proteins) are effector molecules downstream of cytokine receptors. Ligand/receptor engagement triggers Stat proteins tyrosine phosphorylation, dimerization, and translocation to the nucleus where they regulate gene transcription. Stat5, originally identified as a mammary gland growth factor, is an essential mediator of prolactin (PRL)-induced milk protein gene activation. Prolactin receptor (PRLR) is a member of the cytokine/growth hormone/PRL receptor superfamily. The mechanism through which PRLR modulates Stat5 tyrosine phosphorylation, nuclear translocation, and DNA binding was analyzed in HC11 cells, a mammary epithelial cell line, and 293-LA cells, a human kidney cell line stably overexpressing Jak2 kinase. We have found that in HC11 cells, Stat5 is specifically activated by PRL treatment, demonstrating that Stat5 is a physiological substrate downstream of PRLR. Furthermore, using different forms natural forms of the PRLR as well as receptor tyrosine to phenylalanine mutant forms, we determined that tyrosine phosphorylation of Stat5 is independent of PRLR phosphotyrosines. We established, however, that the C-terminal tyrosine of the PRLR Nb2 form, $Tyr^{382}$, plays an essential positive role in PRLR-dependent Stat5 nuclear translocation and subsequently DNA binding. All together, our data propose a new model for activation of Stat5 through the PRLR, suggesting that Stat5 tyrosine phosphorylation and nuclear translocation are two separately regulated events.**

Prolactin (PRL)[1] is a pituitary polypeptide hormone as well as a local growth factor that regulates several physiological functions such as reproduction, promotion of the growth and differentiation of the mammary gland, and immune function (1). PRL interacts with specific cell surface receptors expressed on different target tissues (reviewed in Ref. 2).The receptor for PRL (PRLR) belongs to a large group of receptors known as the cytokine/GH/PRL receptor superfamily, which includes the receptors for GH, EPO, granulocyte-macrophage colony-stimulating factor, and several interleukins (2, 3). These receptors share common extracellular structural motifs such as two disulfide loops and intracellular such as the proline-rich Box1 homology domain. These receptors do not possess intrinsic kinase activity but signal through cytoplasmic protein tyrosine kinases of the Janus kinase family (Jak/Tyk kinases) and the Src-kinase family. Ligand binding to the cytokine/GH/PRL receptor family induces receptor dimerization and activation of the associated kinases. This leads in turn to tyrosine phosphorylation of multiple cellular proteins including the receptors themselves (4).

The downstream signaling molecules activated by this receptor family have not been completely elucidated. However, it has been shown that several of the SH2 domain containing molecules interact with cytokine receptors, *e.g.* phospholipid metabolizing enzymes, phospholipase C-γ, and the regulatory unit of phosphatidylinositol 3-kinase, protein tyrosine phosphatases SHP1 and SHP2, and adapter proteins Grb2, Shc, and IRS1 (5–11). Moreover, a family of SH2 domain containing transcription factors of 85–95 kDa, called signal transducers and activators of trasncription (Stat), have been identified as primary effector molecules for this receptor family (12). Rapidly upon receptor activation, tyrosine phosphorylation of Stat factors occurs leading to their homo- or heterodimerization and translocation to the nucleus where they induce transcription of cytokine responsive genes (13). The complete molecular events leading to Stat proteins activation are not fully understood. It has been suggested that phosphotyrosines on the receptor components may act as docking sites for the SH2 domains of Stat proteins (14, 15), allowing them to be phosphorylated by Jak tyrosine kinase family, a process necessary for Stat proteins activation by cytokine receptors. Recent information, however, indicate that other signaling pathways might be involved for maximal induction of Stat proteins activity. For example, to fully activate Stat1α, serine phosphorylation by mitogen-activated protein kinase is required (16).

The rat PRLR, a member of the cytokine/GH/PRL receptor family, exists in three natural forms; the long form, identified in the mammary epithelial and ovarian cells (17), the short form, characterized in liver cells (18), and a third intermediate form, found in rat T-lymphoma Nb2 cells called PRLR Nb2 form (19). The short form of the receptor results from alternative splicing of the long form, whereas the Nb2 form results from a deletion mutation of the long form. The membrane-proximal events following PRLR activation have recently been clarified. PRL binding to its receptor triggers homodimerization of the PRLR and activation of the constitutively associated kinase, Jak2 (20–22). Although this process lead to tyrosine phosphorylation of the PRLR long form and the PRLR Nb2 form, no tyrosine phosphorylation was observed for the PRLR

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[1] The abbreviations used are: PRL, prolactin; PRLR, PRL receptor(s); IL, interleukin; oPRL, ovine PRL; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; GAS, γ-interferon-activated sequence; GH, growth hormone; GHR, GH receptor; EPO, erythropoietin; EPOR, EPO receptor.

short form (23). Furthermore, studies examining the mechanism through which PRLR regulate gene transcription have previously demonstrated that although the PRLR long and the Nb2 forms are able to transmit the signal of PRL to activate β-casein gene transcription, the short form was inactive in this biological assay system (24). We have further shown that PRLR signals through the coordinated action of Jak2 and a single tyrosine residue present on the PRLR long and Nb2 forms to activate β-casein gene transcription. Indeed, when the C-terminal tyrosine of the PRLR long form ($Tyr^{580}$) or of the PRLR Nb2 form ($Tyr^{382}$) was mutated to phenylalanine, signaling of the PRLR to β-casein gene promoter activation was impaired (23, 25).

PRL has recently been shown to activate several Stat proteins such as Stat1, Stat3, and Stat5 (23, 26, 27). In particular, Stat5, for which two isoforms Stat5a and Stat5b differing in their C-terminal tail were characterized in mouse mammary gland (28), has been shown to be vital for hormonal induction of β-casein gene transcription in mammary gland of lactating animals (29) and in heterologous cell systems (30). Stat5 activation has also recently been shown to correlate with mitogenic signaling in response to PRL (31). Therefore, Stat5 appears to be a key player in PRL-induced gene activation and cell proliferation.

Stat5 has also been shown to be part of the signaling pathway for a number of cytokine receptors such as GHR, EPOR, IL3-R, granulocyte-macrophage colony-stimulating factor receptor, IL2-R, IL6-gp130, and epidermal growth factor receptor (reviewed in Ref. 32). The mechanism of activation of Stat5 through the different cytokine receptors remains elusive and controversial. Indeed, Stat5 tyrosine phosphorylation, DNA binding, and induction of transcription via the GHR requires certain phosphotyrosine residues on the receptor (33, 34). Similarly, Stat5 activation downstream of the EPOR was found to be dependent on receptor phosphotyrosines (35–37). However, other studies have reported a mechanism of activation of Stat5 totally independent of receptor phosphotyrosines. For example, GHR phosphotyrosines-independent activation of Stat5 has been documented (38). Separate studies indicated that Stat5 tyrosine phosphorylation and Stat5-DNA binding activity through the gp130 subunit of the IL-6 receptor (39) and the granulocyte colony-stimulating factor receptor (40) is independent of receptor phosphotyrosines. Furthermore, direct Jak-Stat interaction has recently been implicated as an alternative mechanism for activation of Stat5 by cytokine receptors (39).Therefore, the mechanism of activation of Stat5 by the cytokine/GH/PRL receptor family and the possible role of receptor phosphotyrosines in this process remains to be elucidated.

In this paper, we examined the significance of tyrosine phosphorylation of the PRLR in activating Stat5 molecules. Our results indicate that although Stat5 tyrosine phosphorylation is regulated by PRLR-Jak2 activation, it is independent of PRLR tyrosine phosphorylation. We further found that the C-terminal tyrosine of the PRLR Nb2 form regulates Stat5 nuclear translocation. Together, our results indicate for the first time that Stat5 tyrosine phosphorylation and nuclear translocation are two separately regulated process.

## EXPERIMENTAL PROCEDURES

*Materials, Antibodies, and Plasmid Constructs*—Cytomegelovirus-based expression plasmids, pR/CMV vector (Invitogen), containing cDNAs encoding PRLR long wild type, LY237F, LY580F, Nb2 wild type, NbY237, NbY382F, Δ296–322, ΔY237F, and ΔY382F, were constructed as described previously (25) and were obtained from Dr. P. Kelly (Paris, France). Expression plasmid DNA, pXM-MGF/Stat5, encoding MGF/Stat5 was obtained from Dr. B. Groner (Freiburg, Germany). Ovine PRL (oPRL) used for cell treatment was obtained from Sigma (Mississauga, ON, Canada). Polyclonal antibody to Stat5a and monoclonal antibody to phosphotyrosine (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), monoclonal antibody to Stat5 was obtained from Transduction Laboratories (Lexington, KY), and monoclonal antibody to PRLR, U5, was provided by Dr. P. Kelly (Paris, France). Protein A beads used for immunoprecipitations were purchased from Pharmacia (Montreal, Quebec, Canada).

*HC11 Cell Culture*—HC11, mouse mammary epithelial cells, were grown to confluency in RPMI 1640 medium containing 10% fetal calf serum (Life Technologies, Inc.), insulin (5 μg/ml), and epidermal growth factor (10 ng/ml). Cells were then induced by incubating them for 3–5 days in RPMI medium containing 10% fetal calf serum, insulin (5 μg/ml), and hydrocortisone (1 μM) (41, 42). Cells were then starved in RPMI medium containing insulin (5 μg/ml), hydrocortisone (1 μM), and transferrin (10 μg/ml). Cells were then stimulated with oPRL (1.5 μg/ml) for the time indicated. Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 μg/ml aprotinin) for 5 min at 4 °C. The lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C to remove insoluble material. Protein concentration was measured using the Bradford technique.

*Transient Transfection*—Transfection was carried out as described earlier (23, 25). Briefly, the human 293 clone stably expressing the tyrosine kinase Jak2 (clone LA) was grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium medium (Life Technologies, Inc.) containing 10% (v/v) fetal calf serum. Several hours before transfection, cells were plated in a rich medium (two parts Dulbecco's modified Eagle's medium/Ham's F-12 medium and one part Dulbecco's modified Eagle's medium containing glucose at 4.5 g/liter) containing 10% fetal calf serum. Approximately 5 × $10^6$ cells were then co-transfected with the cDNA encoding the different forms of RPLR (1 μg each) and the cDNA for Stat5 (500 ng) by using the calcium phosphate technique. After 24 h of expression, the cells were starved by serum deprivation overnight.

*Immunoprecipitations*—Immunoprecipitations were carried out as described earlier (22). Briefly, protein extracts were immunoprecipitated for 2 h using polyclonal antibody to Stat5a and protein A-Sepharose beads. This antibody was used because immunoprecipitations were unsuccessful using the monoclonal antibody to Stat5. Precipitates were then separated on SDS-PAGE and probed with monoclonal antibody to phosphotyrosines (4G10) then stripped and reprobed with polyclonal antibody to Stat5a.

*Total Cell Lysis and Western Blotting*—Transiently co-trasfected 293-LA cells were stimulated with oPRL (1.5 μg/ml) of for 5 min and then lysed in 300 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 μg/ml aprotinin) for 5 min at 4 °C. The lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C to remove insoluble material. Protein concentration was measured using the Bradford technique. Equal amounts of protein obtained by total lysis were loaded and run on an 8% SDS-PAGE. Western analysis was performed using monoclonal antibodies to phosphotyrosine, PRLR, or Stat5. Proteins were revealed using chemiluminescence (ECL kit from Amersham Corp.) following the manufacturer's instructions.

*Nuclear Translocation*—Procedure for obtaining nuclear extracts was described previously (30) with some modifications. Briefly, transiently co-transfected 293-LA cells or HC11 cells were collected by centrifugation, washed with phosphate-buffered saline, and then lysed in hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM $Na_3VO_4$, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin). Cells were incubated for 15 min and then vortexed vigorously and centrifuged at 12,000 × *g* at 4 °C. The pellet was washed once with cold phosphate-buffered saline, and then the nuclear extracts were obtained by adding a high salt buffer (25% glycerol, 420 mM NaCl,1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM $Na_3VO_4$, 20 mM NaF, 5 μg/ml aprotinin, 2 μg/ml leupeptin), shaken for 30 min at 4 °C, and then centrifuged at 12,000 × *g* for 5 min. Total nuclear proteins were separated on an 8% SDS-PAGE and transferred to nitrocellulose membrane, and Western blots were carried out using anti-Stat5 monoclonal antibody and revealed using chemiluminescence (ECL kit, Amersham Corp.).

*Electrophoretic Mobility Shift Assay*—EMSA was performed as described elsewhere (30). Briefly, nuclear extracts were prepared as described above. Binding reactions performed in binding buffer (10 mM

HEPES-KOH, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol) contained nuclear extract (8–10 μg), end labeled double stranded DNA containing the Stat5 response element of the β-casein gene promoter (5 pmol) and nonspecific competitor polydeoxyinosinic-deoxycytidylic acid (2 μg). For supershifts, protein extracts were incubated on ice for 30 min with polyclonal antibody to Stat5a (1 μg). We used this antibody because supershifts were unsuccessful when the monoclonal antibody to Stat5 was used. Samples were run on a 0.25× TBE (45 mM Tris-borate, 1 mM EDTA), 5% polyacrylamide gel. The gel was dried and exposed to x-ray film at −80 °C (Hyperfilm, Amersham Corp.).

## RESULTS

*PRL Activates Stat5a in HC11 Cells*—Gene expression of milk proteins in mammary cells is under a complex hormonal control. A combination of insulin, glucocorticoids, and PRL is required for maximal hormonal induction of milk proteins, such as β-casein, in mammary organ and epithelial cell cultures (41, 42). Stat5 activation has recently been shown to be the main intracellular mediator for activation of β-casein gene promoter (27). Therefore, we first evaluated the ability of PRL to induce Stat5 activation in HC11 cells, PRL-sensitive mouse mammary epithelial cells (27). Cells were starved in the presence of insulin and hydrocortisone, were either left untreated or treated with PRL (1.5 μg/ml) for 10 min, and were lysed in lysis buffer. Cell lysates were immunoprecipitated using anti-Stat5a polyclonal antibody. Immune complexes were run on SDS-PAGE, transferred to nitrocellulose membranes, and immunodetected with monoclonal antibody to phosphotyrosines (4G10). As shown in Fig. 1*A*, no detectable tyrosine phosphorylation of Stat5 was observed under basal conditions. However, PRL stimulation of cells (10 min) rapidly induced tyrosine phosphorylation of Stat5. To confirm that equal amounts of Stat5 were immunoprecipitated, membrane was stripped and reprobed with polyclonal antibodies to Stat5a (Fig. 1*B*). These results indicate that in mammary cells Stat5 tyrosine phosphorylation is induced by PRL treatment. Furthermore, these results indicate that the combinations of insulin and hydrocortisone are not competent to induce Stat5 tyrosine phosphorylation in the absence of PRL.

We then examined the kinetics of Stat5 nuclear translocation in HC11 cells in response to PRL stimulation. Cells were grown and stimulated for the indicated times. Cytoplasmic (Fig. 1*C*) and nuclear (Fig. 1*D*) extracts were then prepared from the same culture. As shown in Fig. 1*D*, the amount of Stat5 in the nucleus increases rapidly following PRL stimulation. Meanwhile, the amount of Stat5 present in the cytoplasm (Fig. 1*C*) decreases until eventually no detectable Stat5 was found in the cytoplasm following 10 min of PRL hormone treatment.

We next examined the ability of Stat5 to bind to the β-casein gene promoter. It has been shown previously that Stat5 binds to the −75/−104 region of the β-casein gene promoter (27). EMSA was performed using a primer of the sequence 5′-TGT GGA CTT CTT GGA ATT AAG GGA C-3′, and nuclear extracts were prepared from unstimulated or PRL-stimulated HC11 cells. As shown in Fig. 1*E*, PRL stimulation of HC11 cells results in Stat5 binding to the GAS-like element present on the β-casein gene promoter. This complex was supershifted in the presence of anti-Stat5a polyclonal antibody (*third lane*), indicating the specificity of the DNA binding activity. All together, using the mammary epithelial cell system, we show here that Stat5 activation in mammary cells follows PRL inductive effects.

*Tyrosine Phosphorylation of Stat5 Is Independent of Receptor Phosphotyrosines of the PRLR Nb2 Form and Δ296–322 Mutant Form*—Having established that PRL regulates Stat5 tyrosine phosphorylation, nuclear translocation, and DNA binding activity in mammary cells, we were interested in defining the mechanism through which PRLR mediate this effect. We have

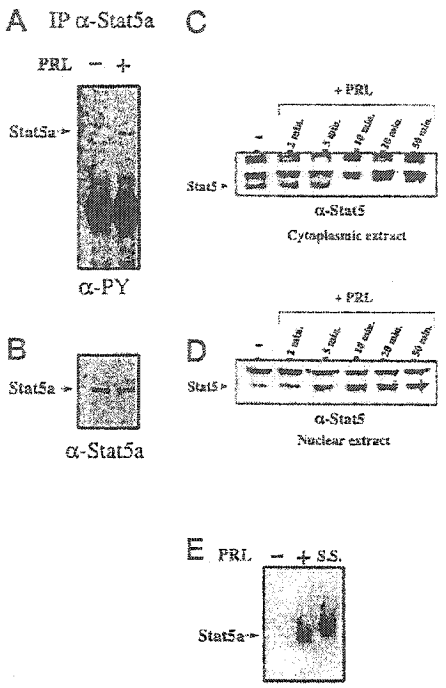


FIG. 1. **PRL stimulation induces Stat5 tyrosine phosphorylation and binding to the GAS sequence of the β-casein gene promoter in HC11 cells.** HC11 cells were either nonstimulated (−) or stimulated (+) with oPRL (1.5 μg/ml) for 10 min. Cells were lysed, and immunoprecipitations were performed using polyclonal antibody to Stat5a. Immunoprecipitated (*IP*) proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Membrane was probed with monoclonal antibody to phosphotyrosines (*A*) and then stripped and reprobed with polyclonal antibody to Stat5a (*B*). Serum-starved HC11 cells were stimulated with oPRL (1.5 μg/ml) for 2, 5, 10, 20, and 50 min. Total cytoplasmic extracts (*C*) and nuclear extracts (*D*) were prepared. Proteins (15 μg) from each extract were separated on SDS-PAGE, transferred to nitrocelluiose membranes, and probed with monoclonal antibody to Stat5. *E*, HC11 cells were prepared the same way as above and stimulated with oPRL (1.5 μg/ml) for 10 min. Total nuclear extracts were prepared, and EMSA was performed using Stat5 response element of the β-casein gene promoter. The Stat5-DNA complex was supershifted (*S.S.*) with polyclonal antibody to Stat5a.

previously shown that C-terminal tyrosine of the PRLR is necessary for PRL activation of β-casein gene promoter (25). When this tyrosine was mutated to phenylalanine in the PRLR Nb2 form, β-casein gene promoter induction was strongly inhibited. Similar mutation of the C-terminal tyrosine on the PRLR long form, however, had less notable effects on β-casein gene activation.

To investigate the role of PRLR tyrosine phosphorylation in Stat5 activation, we used a heterologous overexpression system consisting of 293-LA cells, human kidney cell line stably overexpressing Jak2 kinase (23, 25). We have previously shown that transient overexpression of PRLR in these cells leads to ligand-independent constitutive activation of the receptor-kinase complex. Here we have used this system to investigate the role of PRLR tyrosine phosphorylation in activation of Stat5.

The receptor natural and mutant forms used in our studies were described previously (23, 25). The PRLR Nb2 form, found in Nb2 T-lymphoma cells, has an inframe deletion mutation compared with the PRLR long form (19). Three tyrosine residues ($Tyr^{237}$, $Tyr^{309}$, and $Tyr^{382}$) are retained in the PRLR Nb2 form compared with the PRLR long form. Tyrosine 382 of the PRLR Nb2 form corresponds to tyrosine 580 of the PRLR long form. Another biologically active form is the PRLR Nb2 mutant form Δ296–322 (23). This receptor form has a 27-amino acid internal deletion and lacks tyrosine 309. Finally, the PRLR

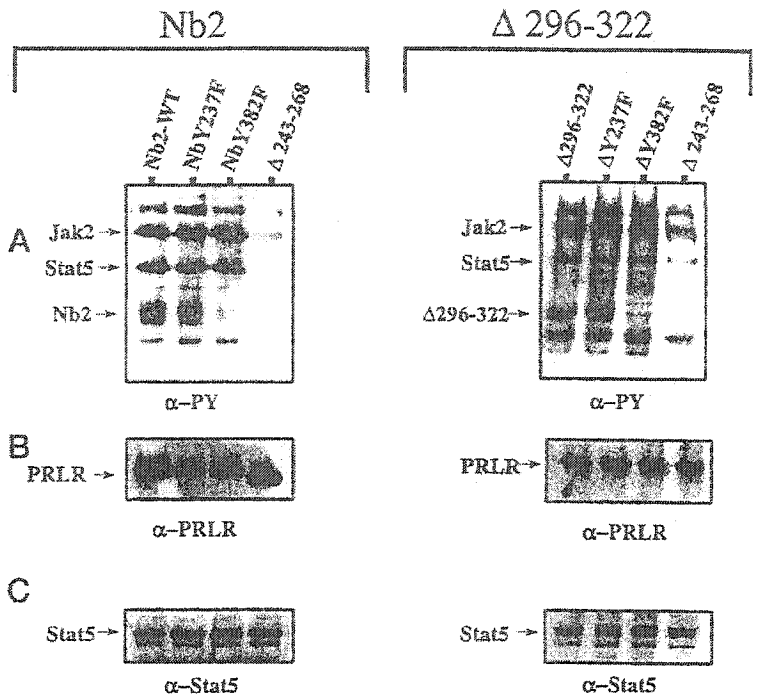


FIG. 2. **Tyrosine phosphorylation of Stat5 is independent of tyrosine 382 of the PRLR Nb2 form and Δ296–322 mutant form.** *A*, 293-LA cells ($5 \times 10^5$ cells) were transiently co-transfected with cDNAs encoding Stat5 (500 ng) and with Nb2-WT, NbY237F, NbY382F, or the mutant Δ243–268 (1 μg). Similarly, 293-LA cells were transiently co-transfected with cDNAs encoding Stat5 (500 ng) and with Δ296–322, ΔY237F, ΔY382F, or the mutant Δ 243–268 (1 μg) (*B*). Cells were stimulated with oPRL (1.5 μg/ml) for 5 min. Total protein extracts were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibody to phosphotyrosines. *B*, membranes were stripped and reprobed with monoclonal antibody to PRLR. *C*, membranes were stripped and immunodetected with monoclonal antibody to Stat5.

Nb2 mutant form Δ243–268 is also used in our studies (23). This mutant form is missing a 25-amino acid region encompassing the Box1 motif, important for PRLR-Jak2 complex formation (23). Therefore, this receptor form is unable to activate Jak2 and is used here as a negative control for receptor/Jak2 activation.

Point mutation of the C-terminal tyrosine ($Tyr^{382}$) in the PRLR Nb2 form and Δ296–322 mutant form was shown to inhibit receptor tyrosine phosphorylation and β-casein gene promoter activation (25). Consequently, we intended to determine the role of this tyrosine residue in Stat5 tyrosine phosphorylation. 293-LA cells were co-transfected with cDNA encoding Stat5 (27) and the PRLR Nb2 wild type (Nb2-WT); the PRLR Nb2 form in which tyrosine 237 was substituted by phenylalanine, NbY237F; the PRLR Nb2 form in which tyrosine 382 was mutated to phenylalanine, NbY382F; or Δ243–268 as a negative control (Fig. 2). In parallel, 293-LA cells were co-transfected with cDNA encoding Stat5 and with the Δ296–322 receptor form; Δ296–322 in which tyrosine 237 was exchanged with phenylalanine, ΔY237F; receptor form Δ296–322 in which tyrosine 382 was mutated to phenylalanine, ΔY382F; or Δ243–268 as a negative control (Fig. 2). Cells were then stimulated with PRL for 10 min before lysis. Total protein extracts were separated on SDS-PAGE, transferred to membranes, and immunodetected with monoclonal antibody to phosphotyrosines (Fig. 2*A*). We observed that all natural and mutant forms of the PRLR except mutant Δ243–268, which act as a negative control, are fully capable of inducing Jak2 and Stat5 tyrosine phosphorylation (Fig. 2*A*). However, not all receptor types are themselves tyrosine phosphorylated. The NbY382F and the ΔY382F mutant receptor forms were not tyrosine phosphorylated in this system, similar to what was shown previously (25). To confirm that we have equal expression of receptors and Stat5 in the different samples, the membranes were stripped and reprobed with monoclonal antibodies to PRLR that can recognize all receptor forms (Fig. 2*B*). This was followed by reprobing the membranes with monoclonal antibodies to Stat5 (Fig. 2*C*). Together, these findings indicate that Stat5 is tyrosine phosphorylated following PRLR/Jak2 activation independently of PRLR phosphotyrosines.

*Stat5 Nuclear Translocation Is Regulated by Tyrosine 382 of the PRLR Nb2 Form and the Δ296–322 Mutant Form*—Following Stat protein tyrosine phosphorylation on the conserved tyrosine residue, it is thought that Stat proteins dimerize and translocate to the nucleus where they induce gene transcription (12). Because mutation of tyrosine 382 of the PRLR Nb2 form and of the receptor Δ296–322 form did not influence Stat5 tyrosine phosphorylation but it was shown to play a significant role in regulating β-casein gene promoter induction (25), we studied its influence on Stat5 nuclear transloction event. For this purpose, 293-LA cells were co-transfected with the cDNAs encoding the PRLR Nb2 form or one of the Tyr → Phe mutants, NbY237F and NbY382F, along with the cDNA encoding Stat5 (Fig. 3*A*). In parallel, 293-LA cells were co-transfected with the cDNAs encoding the receptor Δ296–322 form or one of its Tyr → Phe mutants, ΔY237F or ΔY382F, along with the cDNA encoding Stat5 (Fig. 3*B*). Cells were then stimulated with PRL for 10 min before lysis and total nuclear extracts were analyzed with monoclonal antibody to Stat5. As shown in Figs. 3 (*A* and *B*, *upper panels*), Tyr → Phe mutations of $Tyr^{237}$ in the two receptor forms did not affect nuclear translocation of Stat5 compared with wild type receptor forms. However, the amount of nuclear Stat5 immunodetected in samples overexpressing NbY382F and ΔY382F was dramatically reduced compared with that observed for wild type receptors. Indeed, for the mutant ΔY382F, the level of nuclear Stat5 was similar to that observed in samples in which the inactive mutant form Δ243–268 was overexpressed (Fig. 3, *A* and *B*, *upper panels*). This inhibition in Stat5 nuclear translocation was not due to differences in the overexpression of Stat5 as indicated by Western blots with monoclonal antibody to Stat5 of total cell extracts from the same transfections (Fig. 3, *A* and *B*, *lower panels*). Similarly, receptor expression was equal in the different samples (data not shown). These results indicate that the C-terminal tyrosine residue of the PRLR Nb2 form is required for Stat5 nuclear translocation.

*Tyrosine 382 of the PRLR Nb2 Form and the Δ296–322 Mutant Form Inhibits Stat5 Binding to the GAS Response*

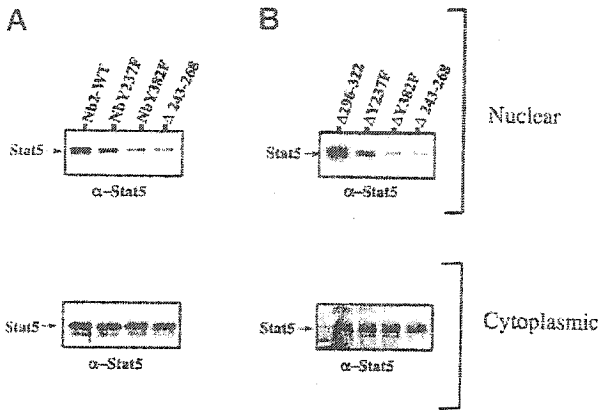


FIG. 3. **Nuclear translocation of Stat5 is significantly influenced by tyrosine 382 of the PRLR Nb2 form and by tyrosine 382 of the Δ296–322 mutant form.** *A*, 293-LA cells were transiently co-transfected with cDNAs encoding Stat5 and with PRLR Nb2-WT, NbY237F, NbY382F, or the mutant Δ243–268. *B*, 293-LA cells were transiently co-transfected with cDNA encoding Stat5 and with Δ296–322, ΔY237F, ΔY382F, or the mutant Δ243–268. Cells were stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts or total cell extracts were prepared from two sets of cells that were simultaneously transfected. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibody to Stat5.

*Element of the β-Casein Gene Promoter*—To confirm the role of tyrosine 382 of the PRLR Nb2 form in regulating Stat5 nuclear entry, we next studied the importance of this tyrosine in regulating Stat5 DNA binding activity. We co-transfected 293-LA cells with the cDNA encoding Stat5 and with the cDNAs encoding either PRLR Nb2 wild type or its Tyr → Phe mutants (Fig. 4*A*). Similarly, we co-overexpressed in 293-LA cells Stat5 with either Δ296–322 receptor form or its Tyr → Phe mutants (Fig. 4*B*). Nuclear extracts were then prepared, and an EMSA binding reaction containing the β-casein gene promoter GAS sequence was performed. We found (Fig. 4, *A* and *B*) that overexpression of PRLR Nb2 wild type, Δ296–322 receptor form, and their Y237F mutant forms lead to the appearance of Stat5 DNA binding activity to the β-casein gene promoter in gel shift assays. However, Stat5-DNA interactions were greatly reduced in samples overexpressing the mutant receptor forms, NbY382F and ΔY382F. This is judged from the absence of DNA bound Stat5 compared with the wild type PRLR Nb2 form and Δ296–322 mutant form. Therefore, tyrosine 382 of the PRLR Nb2 form is important and necessary for Stat5 activation.

*Tyrosine 580 of the PRLR Long Form Is Not Required for Stat5 Tyrosine Phosphorylation*—The PRLR long form contains nine tyrosine residues within its cytoplasmic domain. It was shown previously that individual Tyr → Phe mutations, $Tyr^{237}$ and $Tyr^{580}$, in the PRLR long form had no effect on the level of tyrosine phosphorylation of the PRLR (25). This suggests that other tyrosine residues of the PRLR may undergo tyrosine phosphorylation following PRLR/Jak2 activation. Functional analysis of these mutant receptors indicate that $Tyr^{580}$ is required for β-casein gene promoter activation, albeit less apparent than that observed for NbY382F mutant (25). To assess the role of $Tyr^{580}$ of PRLR long form in mediating Stat5 tyrosine phosphorylation, 293-LA cells were co-transfected with cDNA encoding Stat5 and cDNAs encoding PRLR long wild type; PRLR long in which tyrosine 237 was exchanged with phenylalanine, LY237F; PRLR long in which tyrosine 580 was mutated to phenylalanine, LY580F; or Δ243–268 as a negative control (Fig. 5). Cells were starved overnight before being stimulated with PRL for 10 min. Total cellular proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Immunodetections with

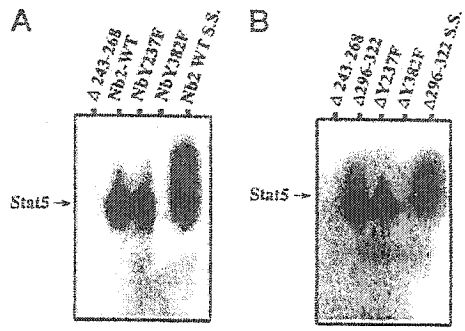


FIG. 4. **Tyrosine 382 of the PRLR Nb2 form inhibits Stat5 binding to the GAS sequence of the β-casein gene promoter.** *A*, 293-LA cells ($5 \times 10^5$ cells) overexpressing Stat5 and Nb2-WT, NbY237F, NbY382F, or the mutant Δ243–268. *B*, 293-LA cells were transiently co-transfected with cDNA encoding Stat5 and with Δ296–322, ΔY237F, ΔY382F, or the mutant Δ 243–268 (*B*). Cells were stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts were prepared, and EMSA was performed using Stat5 response element of the β-casein gene promoter. The Stat5-DNA complex was supershifted (*S.S.*) with polyclonal antibody to Stat5a.

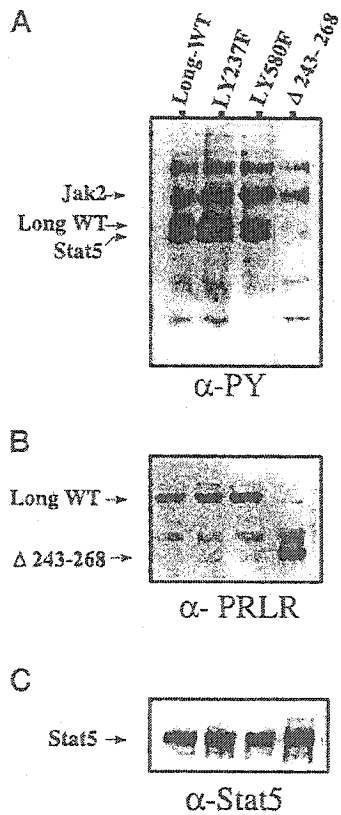


FIG. 5. **Tyrosine phosphorylation of Stat5 is independent of tyrosine 580 of the PLRL long form.** 293-LA cells ($5 \times 10^5$ cells) were transiently co-transfected with cDNAs encoding Stat5 (500 ng) and with long wild type (*Long WT*), LY237F, LY382F, or the mutant Δ243–268. Cells were stimulated with oPRL (1.5 μg/ml) for 5 min. Total protein lysates were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibody to phosphotyrosines (*A*). Membrane was stripped and reprobed with monoclonal antibody to PRLR (*B*). Membrane was then stripped and immunodetected with monoclonal antibody to Stat5 (*C*).

antibodies to phosphotyrosine indicate that the PRLR long form as well as the two Tyr → Phe mutants were able to mediate both Jak2 and Stat5 tyrosine phosphorylation compared with the negative control samples in which Δ243–268 were overexpressed (Fig. 5*A*). This is consistent with what we

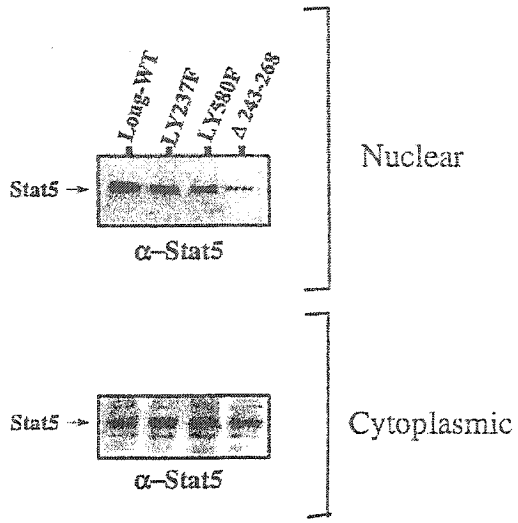


FIG. 6. **Tyrosine 580 of the PRLR long form does not influence Stat5 nuclear translocation.** Stat5 and long wild type (*Long-WT*), LY237F, LY382F, or the mutant Δ243–268 were co-overexpressed in 293-LA cells as described in legend to Fig. 5. Cultures were then stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts or total cell extracts were prepared from two sets+ of cells that were simultaneously transfected. Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Both membranes were probed with monoclonal antibody to Stat5.

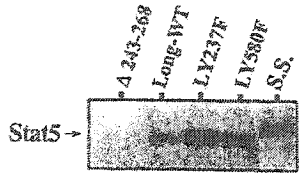


FIG. 7. **Tyrosine 580 of the PRLR long form does not affect significantly Stat5 binding to the GAS sequence of the β-casein gene promoter.** 293-LA cells ($5 \times 10^5$ cells) were transiently co-transfected with cDNAs encoding Stat5 and long wild type (*Long-WT*), LY237F, LY382F, or the mutant Δ243–268 as described in legend to Fig. 6. Cells were stimulated with oPRL (1.5 μg/ml) for 10 min. Nuclear extracts were prepared, and EMSA was performed using Stat5 response element of the β-casein gene promoter. The Stat5-DNA complex was supershifted (*S.S.*) with polyclonal antibody to Stat5a.

observed for the PRLR Nb2 form and Δ296–322 mutant form (Fig. 2*A*). Furthermore, Fig. 5*A* also indicates that PRLR long form as well as the two Tyr → Phe mutant forms, LY273F and LY580F, are themselves tyrosine phosphorylated as shown previously (25). To confirm that receptor expression was equal in all samples, the membrane was stripped and reprobed with monoclonal antibodies to the extracellular domain of the PRLR (Fig. 5*B*). Similarly, to verify that Stat5 was equally expressed in the different samples, the membrane was stripped and immunodetected by monoclonal antibodies to Stat5 (Fig. 5*C*). These results demonstrate that Stat5 tyrosine phosphorylation is not modulated by either tyrosine 237 or tyrosine 580 of the PRLR long form.

*Tyrosine 580 of the PRLR Long Form Does Not Significantly Influence Stat5 Nuclear Translocation*—We next investigated the influence of the same Tyr → Phe mutants of PRLR long form on Stat5 nuclear translocation. 293-LA cell co-transfections were carried out with cDNAs encoding Stat5 and PRLR long wild type, LY237F, LY580F, or Δ243–268 (Fig. 6). Cells were starved overnight before being stimulated by PRL for 10 min. Total nuclear extracts were prepared, then loaded on an SDS-PAGE, and transferred to membranes, and Western blot analysis was performed using anti-Stat5 monoclonal antibody. As shown in Fig. 6, mutation of either $Tyr^{237}$ or $Tyr^{580}$ in the PRLR long form, had no or very little effect on Stat5 nuclear translocation (Fig. 6, *upper panel*). These results are in contrast to that observed for the PRLR Nb2 form and Δ296–322 mutant form (Fig. 3) and suggest that additional tyrosine residues specific to the PRLR long form might substitute the C-terminal tyrosine in mediating Stat5 nuclear translocation. Indeed, our data are consistent with the fact that mutation of the last tyrosine in the long form only partially inhibited β-casein gene promoter activation (25).

*Tyrosine 580 of the PRLR Long Form Does Not Influence Stat5 Binding to the Response Element of the β-Casein Gene Promoter*—We then used the PRLR long form and its Tyr → Phe mutants, LY237F and LY580F, to study their effects on the binding activity of Stat5 to the β-casein gene promotor. For this reason, 293-LA cells were co-transfected with cDNA encoding Stat5 and cDNAs encoding the PRLR long wild type, LY237F, or LY580F. Nuclear extracts were prepared, and electrophoretic mobility shift assay was performed using the GAS sequence of the β-casein gene promoter. As shown in Fig. 7, we did not detect any Stat5 DNA binding activity in samples overexpressing the negative control mutant Δ243–268. However, Stat5 DNA binding activity was detected at similar levels in samples overexpressing the PRLR long form and the two tyrosine mutant forms LY237F and LY580F. These results indicate that mutation of tyrosine 580 of the PRLR long form does not affect PRLR-regulated Stat5-DNA interactions.

## DISCUSSION

The initial steps in the signaling mechanisms of the PRLR, a member of the cytokine receptor superfamily, have recently been elucidated. Following PRL/receptor engagement there is activation of cytoplasmic tyrosine kinases of the Jak and Src families. This process leads to activation and tyrosine phosphorylation of the kinase, the receptor and cellular effector molecules resulting in the transmission of PRL biological responses.

Here we investigated the mechanism through which PRLR regulate the activation of the signal transducer and activator of transcription, Stat5. In the HC11 mammary epithelial model system that we used in this study, we find that PRL is able to induce Stat5 tyrosine phosphorylation, nuclear translocation, and DNA binding to the GAS-like element of the β-casein gene promoter. This clearly demonstrate that Stat5 is a physiological downstream mediator in PRLR intracellular signaling pathway in mammary epithelial cells. This is consistent with the fact that PRL induces activation of Stat5 in overexpression system (30) and in the Nb2 rat T-lymphoma cell line (43).

Stat5 is a member of the Stat family of transcription factors downstream of cytokine receptors. Our understanding of the mechanism of activation of these factors in the signaling relay of cytokine receptors is limited. It has been shown in different systems, however, that these factors undergo tyrosine phosphorylation on a conserved C-terminal tyrosine residue necessary for their homo- or heterodimerization. This process leads to their translocation to the nucleus where they bind to specific response elements on target genes to regulate gene expression (4, 12).

Stat5 is activated by a number of cytokines and growth factors (32). Studies on the role of the cytokine receptors to mediate Stat5 protein activation focused on events like Stat5 association to the receptor, tyrosine phosphorylation, and DNA binding. These studies indicated that Stat5 tyrosine phosphorylation and DNA binding via the GHR, EPOR, and IL-2Rβ chain requires receptor phosphotyrosines (33–37, 44–46), whereas similar events mediated through the IL-6-gp130 receptor system and granulocyte colony-stimulating factor receptor were found to be independent of receptor phosphotyrosines

(38–39). Limited attempts, however, have been performed to study the effect of receptor phosphotyrosine residues on Stat protein nuclear translocation event and its regulation mechanism.

We report here for the first time that the C-terminal tyrosine residue of the PRLR does not influence Stat5 tyrosine phosphorylation, but it is required for regulating Stat5 nuclear translocation and DNA binding. Our data suggest a possible Jak2-Stat5 interaction that is direct or that is through an adapter protein may be taking place, allowing Stat5 tyrosine phosphorylation by the kinase Jak2 itself independently of PRLR tyrosine phosphorylation. This would be consistent with what was demonstrated for the Il-6-gp130 receptor system (38). We observed that Stat5 tyrosine phosphorylation did not grant its nuclear translocation. This implies that Stat5 activation via the PRLR involves at least two separately regulated events; tyrosine phosphorylation and nuclear translocation. Whether other members of the cytokine receptor superfamily share the same feature remains to be determined.

We also report that tyrosine 580 of the PRLR long, which is homologous to tyrosine 382 of the PRLR Nb2 form, has no effect on Stat5 nuclear translocation or DNA binding. A redundancy may be implicated in this tyrosine activity because the PRLR long form has six other tyrosine residues that can potentially substitute for the mutated tyrosine 580. Indeed, in contrast to the PRLR Nb2 form, mutation of tyrosine 580 of the PRLR long form does not lead to inhibition of receptor tyrosine phosphorylation, indicating that other tyrosines on the PRLR long form may undergo phosphorylation following receptor activation. In addition, mutation of the C-terminal tyrosine in the PRLR long form only partially inhibited β-casein gene promoter activation, suggesting again that other alternative tyrosine residues specific to the receptor long form might mediate PRL effects. Moreover, it has been recently suggested that tyrosines 509 and 496 of the PRLR long form in the context of gp130 truncation mutant to be potential sites for mediating Stat5 activation and DNA binding (47). Using myeloid cell system, however, a previous report suggested a mechanism for activation of Stat5 independent of PRLR phosphotyrosines (26). These seemingly controversial observations might be due to differences in the GAS-like element used in the study. In addition, it is possible that the PRL-induced DNA binding activity observed is that of Stat5-related proteins in myeloid cells (48).

The mechanism by which tyrosine 382 of the PRLR Nb2 form exerts its effect on Stat5 nuclear translocation is not known. We speculate that this phosphotyrosine residue is involved in the activation of a certain cellular component that modulates Stat5 complexes in the cytoplasm controlling their nuclear entry.

Accumulating evidence suggests that Stat proteins do not act in seclusion; rather they are a part of complex interactions with a number of other cellular components. For example, Stats1 and 3 are shown to be phosphorylated by mitogen-activated protein kinase on a serine residue adjacent to the conserved tyrosine phosphorylation site (49, 50). Furthermore, Stat5 has been shown to be serine phosphorylated following PRL stimulation (43); however, the physiological significance of this phosphorylation is not known. Potentially it can play a role in Stat5 activation including nuclear translocation. Stat proteins have also been shown to interact with other cellular components besides mitogen-activated protein kinase; examples are phosphatidylinositol 3-kinase (51), glucocorticoid receptor (52), and a number of nuclear transcription factors (53). Any one of these molecules, or even possibly a new cellular protein, potentially influence Stat5 activation by the PRLR. Further investigation is required to determine how receptor phosphotyrosines are affecting Stat proteins nuclear translocation.

In conclusion, we demonstrate in this study for the first time that Stat5 activation involves two separately regulated events, Stat5 tyrosine phosphorylation and nuclear translocation. We further report that Stat5 tyrosine phosphorylation induced by the PRLR is independent of receptor phosphotyrosines, and we establish that tyrosine 382 of the PRLR Nb2 form positively regulates Stat5 nuclear translocation.

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# Recruitment of the Protein-tyrosine Phosphatase SHP-2 to the C-terminal Tyrosine of the Prolactin Receptor and to the Adaptor Protein Gab2*

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**The protein-tyrosine phosphatase SHP-2 modulates signaling events through receptor tyrosine kinases and cytokine receptors including the receptor for prolactin (PRLR). Here we investigated mechanisms of SHP-2 recruitment within the PRLR signaling complex. Using SHP-2 and PRLR immunoprecipitation studies in 293 cells and in the mouse mammary epithelial cell line HC11, we found that SHP-2 co-immunoprecipitates with the PRLR and that the C-terminal tyrosine of the PRLR plays a regulatory role in both the tyrosine phosphorylation and the recruitment of SHP-2. Our results further indicate that SHP-2 association to the PRLR occurs via the C-terminal SH2 domain of the phosphatase. In addition, we determined that the newly identified adaptor protein Gab2, but not Gab1, is specifically tyrosine phosphorylated and is able to recruit SHP-2 and phosphatidyinositol 3-kinase in response to PRLR activation. Together, these studies suggest the presence of dual recruitment sites for SHP-2; the first is to the C-terminal tyrosine of the PRLR and the second is to the adaptor protein Gab2.**

The prolactin receptor (PRLR)[1] is a member of the large cytokine receptors superfamily. Ligand stimulation induces receptor dimerization, which leads to the activation of the constitutively associated Jak2 kinase as well as members of the Src family of cytoplasmic tyrosine kinases (1, 2). Activated Jak2 molecules phosphorylate themselves as well as receptor subunits on tyrosine residues creating docking sites for SH2 domain containing proteins belonging to various signaling pathways. Activated PRLR can induce the activation of the Stat5 (signal transducer and activator of transcription 5), mitogen-activated protein kinase, and PI3-K pathways and the activation of several signaling regulatory proteins including the SHP-2 (the SH2 domain containing protein-tyrosine phosphatase-2).

SHP-2 is a ubiquitously expressed protein that is characterized by having two N-terminally located SH2 domains and a C-terminal catalytic domain. The phosphatase plays a critical role in cell growth and differentiation as evidenced by the early embryonic lethality in SHP-2$^{-/-}$ mice (3). SHP-2 plays an essential regulatory role in signaling from certain receptor tyrosine kinases like the epidermal growth factor receptor and insulin receptor (4) as well as cytokine receptors such as the $\alpha/\beta$ interferon receptors (5, 6) and the PRLR (7). We have previously shown that SHP-2 is tyrosine phosphorylated in response to PRL stimulation and physically associates with the PRLR·Jak2 complex. We also determined that SHP-2 is a positive mediator of PRLR signaling leading to the activation of $\beta$-casein gene promoter (7). However, the mechanism and site(s) of SHP-2 recruitment within the PRLR signaling complex remain unknown.

SHP-2 phosphatase can be directly recruited via its two SH2 domains to membrane receptors, cell surface proteins, and a number of intracellular signaling proteins. Specific tyrosine residues on the $\beta$ chain of the granulocyte macrophage colony-stimulating factor receptor (8), the platelet-derived growth factor receptor (9), the gp130 subunit of the interleukin-6 receptor (IL-6R) (10), and the C-terminal portion of the growth hormone receptor (11) are sites for SHP-2 recruitment. Point mutations of these residues eliminated SHP-2 association and tyrosine phosphorylation. Furthermore, through the SH2 domains, SHP-2 interacts with a number of cellular proteins such as signal regulatory protein (12), SH2-containing inositol phosphatase (13), insulin receptor substrates 1 and 2 (14), and Jak2 (15) and, importantly, members of the Gab (Grb2 associate binder) family.

The Gab family of proteins, p110 Gab1 (16), p97 Gab2 (17), and the *Drosophila* DOS protein (18), contain a number of structurally conserved regions. These proteins contain an N-terminal pleckstrin homology domain, proline-rich motifs, and multiple similarly situated tyrosine phosphorylation residues. In addition, these proteins have unique tissue distributions (17), suggesting that they serve nonredundant functions. These proteins appear to function primarily as adaptor proteins linking activated receptor tyrosine kinase and cytokine receptors to several signaling molecules. Epidermal growth factor, insulin (19), hepatocyte growth factor (20), IL-3, IL-6, interferon $\alpha/\beta$ (21), and erythropoietin (22, 23) have been reported to induce the tyrosine phosphorylation of Gab1 and its association with the SH2 domain containing protein SHP-2 (22–25), the p85 subunit of PI3-K (26, 27), SH2-containing inositol phosphatase (23), Shc (23), and Grb2 (16, 21). Other studies have shown that IL-2, colony stimulating factor-1 (17), IL-3, IL-6, and the activation of the T-cell receptor and B-cell receptor (28) can induce tyrosine phosphorylation of Gab2 and its association with

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[1] The abbreviations used are: PRL, prolactin; PRLR, PRL receptor; IL, interleukins; Jak2, Janus kinase-2; PI3-K, phosphatidyinositol 3-kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.

SHP-2, the p85 subunit of PI3-K, CrkL, and Grb-2. Therefore, it appears that certain cytokines are able to induce the tyrosine phosphorylation of both Gab1 and Gab2, whereas others may utilize one member only.

In this study, we investigated the sites and mechanisms of SHP-2 recruitment to the PRLR. We determined that the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment. In addition, we determined that Gab2 but not Gab1 is tyrosine phosphorylated following PRL stimulation of mammary cells and that Gab2 presents a second site for SHP-2 recruitment to the PRLR signaling complex.

## EXPERIMENTAL PROCEDURES

*Materials, Antibodies, and Plasmid Constructs*—Expression plasmids encoding the PRLR mutants LY237F, LY580F, NY237F, NY382F, Δ296–322, ΔY237F, ΔY382F, and Δ243–268 were described previously (29). Expression plasmids encoding SHP-2, the SHP-2 R32K and SHP-2 R138K, and the polyclonal antibody for p110 subunit of the PI3-K were obtained from Axel Ullrich (Max-Plank Institute, Germany). Monoclonal antibody to phosphotyrosine (4G10) was from Upstate Biotechnology, monoclonal antibody to SHP-2 was form Transduction Laboratories, and polyclonal antibody to SHP-2 was from Santa Cruz. Polyclonal Gab1 antibodies and HA-tagged Gab1 expression plasmid were kindly provided by Toshio Hirano (Osaka University, Osaka, Japan). Polyclonal Gab2 antibodies and HA-tagged Gab2 and Gab2DM expression plasmids were generously supplied by Benjamin Neel and Haihua Gu (Harvard Medical School, Boston, MA). The U6 monoclonal antibody to the PRLR was provided by Paul Kelly (Paris, France). Hemagluttinin antibodies were from Santa Cruz. Protein A-Sepharose beads used for immunoprecipitations were from Amersham Pharmacia Biotech. Ovine PRL used for treatment of cells was obtained from Sigma.

*HC11 Cell Culture*—HC11, mouse mammary epithelial cells obtained from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland) and Bernd Groner (Georg Speyer Haus, Frankfurt, Germany) were grown to confluency in RPMI 1640 medium containing 10% fetal calf serum (Life Technologies, Inc.), insulin (5 μg/ml), and epidermal growth factor (10 ng/ml). Cells were then induced by incubating them for 3 days in RPMI medium containing 10% fetal calf serum, insulin (5 μg/ml), and hydrocortisone (1 μM) (30, 31). Depending on the specific experiment (see "Results"), cells were starved either in RPMI medium containing insulin (5 μg/ml) and hydrocortisone (1 μM) or in RPMI medium alone without insulin and hydrocortisone. Cells were then stimulated with ovine PRL (1.5 μg/ml) for the time indicated. Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium floride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulphonyl florid, 2 μg/ml leupeptin, 5 μg/ml aprotinin) for 5 min at 4 °C. The lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C to remove insoluble material. Protein concentration was measured using the Bradford technique.

*Transient Transfection*—The human embryonic 293 cells were grown in Dulbecco's modified Eagle's medium (4.5 g/liter glucose) (Bio Media) containing 10% (v/v) fetal calf serum. Approximately $5 \times 10^6$ cells were plated then co-transfected with expression plasmids encoding the different forms of PRLR (1 μg each), SHP-2 (1 μg), and the kinase Jak2 (0.25 μg) by the calcium phosphate technique. After 24 h of expression, the cells were starved by serum deprivation overnight.

*Total Cell Lysis Immunoprecipitations and Western Blotting*—Transiently co-transfected 293 cells were stimulated with ovine PRL (1.5 μg/ml) for 5 min and then lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium floride, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulphonyl floride, 2 μg/ml leupeptin, 5 μg/ml aprotinin) for 5 min at 4 °C. The lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C to remove insoluble material. Protein concentration was measured using the Bradford technique. Equal amounts of protein obtained by total lysis were loaded and run on an 8% SDS-PAGE. Immunoprecipitations were performed on endogenous or overexpressed cell lysates. Extracts were immunoprecipitated for 2 h (or overnight for endogenous proteins) using specific antibodies (SHP-2, PRLR, HA, Gab1, and Gab2) and protein A-Sepharose beads. Precipitates were then washed with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and separated on SDS-PAGE. Western blotting analysis was performed using the indicated antibodies. Proteins were revealed using chemiluminescence (Super Signal kit from Pierce) following the manufacturer's instructions.

## RESULTS

*The C-terminal Tyrosine of the PRLR Regulates the State of SHP-2 Tyrosine Phosphorylation*—We have previously reported that the C-terminal tyrosine of the PRLR is critical for the induction of the prolactin responsive β-casein gene (29) and the activation of Stat5 (32). In addition, a positive role for SHP-2 in the activation of Stat5 and the induction of early genes has also been reported (7, 33). Therefore, we hypothesized a regulatory role for the C-terminal tyrosine of the PRLR in the activation of SHP-2. To test this hypothesis, we used various natural, mutant, and deletion forms of the PRLR for the purpose of focusing on the C-terminal tyrosine of the PRLR. The long form of the PRLR has nine intracellular tyrosine residues. The PRLR Nb2 form, found in Nb2 T-lymphoma cells, has an in-frame deletion mutation that removes six of the nine intracellular tyrosine residues found in the long form leaving tyrosine residues 237, 309, and 382 (34). The C-terminal tyrosine 382 of the PRLR Nb2 form corresponds to tyrosine 580 of the PRLR long form. Another deletion form that is capable of inducing β-casein gene expression is the PRLR Nb2 mutant form Δ296–322 (29). This receptor form has a 27-amino acid internal deletion and lacks tyrosine 309. Tyrosine to phenylalanine point mutants of the membrane proximal (LY237F, NY237F, and ΔY237F) and the C-terminal tyrosines (LY580F, NY382F, and ΔY382F) in the long form, Nb2 form, and the Δ296–322 mutant form were described previously (29, 32). Finally, the PRLR Nb2 mutant form Δ243–268 is also used in our studies as a negative control (29). This mutant receptor is unable to associate with Jak2 kinase because of the deletion of the proline-rich interaction domain and is incapable of signaling in response to PRL stimulation (29, 35).

To investigate the effect of the C-terminal tyrosine of the PRLR on SHP-2 tyrosine phosphorylation, the human embryonic kidney 293 cells were transiently transfected with expression plasmids that encode the phosphatase SHP-2 and either the wild type long form PRLR, the tyrosine to phenylalanine point mutants of the membrane proximal tyrosine LY237F, or the C-terminal tyrosine LY580F (Fig. 1*A*). Another set of 293 cells was transfected with expression plasmids encoding SHP-2 and either the wild type intermediate Nb2 form of the PRLR, tyrosine to phenylalanine mutant of the membrane proximal tyrosine NY237F or the C-terminal tyrosine NY382F. In addition, expression plasmids encoding the Δ296–322 mutant form of the Nb2 receptor and tyrosine to phenylalanine mutant of the membrane proximal tyrosine ΔY237F or the C-terminal tyrosine mutant ΔY382F were co-transfected into 293 cells (Fig. 1*B*). In addition to the PRLR and SHP-2, 293 cells were transfected with a limited amount of an expression plasmid for Jak2 tyrosine kinase (see "Experimental Procedures").

SHP-2 was immunoprecipitated from cellular lysates using polyclonal antibodies for SHP-2. Precipitates were run on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with monoclonal antibodies for phosphotyrosine in a Western blot. As expected, no SHP-2 phosphorylation was detectable in samples expressing the deletion mutant Δ243–268 (Fig. 1*A*). In contrast, a strong phosphorylation of SHP-2 was detected in the samples expressing the wild type long form of the PRLR and also in samples expressing both the membrane proximal mutant LY237F and the C-terminal mutant LY580F (Fig. 1*A*). Moreover, SHP-2 phosphorylation was also detectable in the samples expressing the wild type Nb2 form of the PRLR and the membrane proximal mutant NY237F (Fig. 1*B*). Interestingly, however, no SHP-2 phosphorylation was detected in

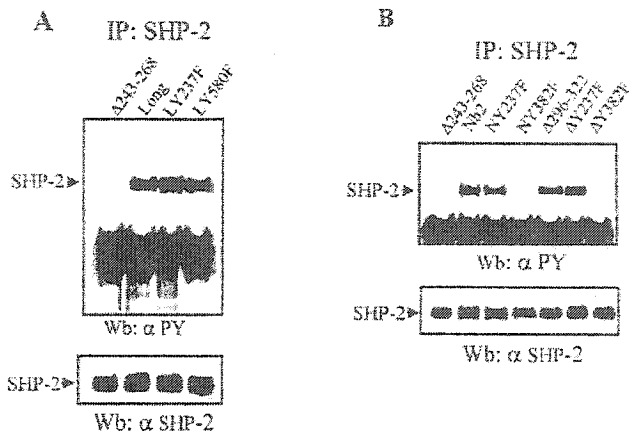


FIG. 1. **The C-terminal tyrosine of the PRLR regulates the state of SHP-2 tyrosine phosphorylation.** *A*, 293 cells were transiently transfected with expression plasmids encoding SHP-2 along with the Δ243–268, the long form of the PRLR, LY237F, or LY580F. *B*, 293 cells were transfected with expression plasmids encoding SHP-2 and the Nb2 form of the PRLR, NY237F, NY382F, Δ293–322, ΔY237F, or ΔY382F. Cells were lysed, and SHP-2 was immunoprecipitated with a polyclonal antibody to SHP-2. Immunoprecipitates were run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with monoclonal antibodies against phosphotyrosine (*upper panel*), then stripped, and reprobed with monoclonal antibodies to SHP-2 (*lower panel*). *IP*, immunoprecipitation; *Wb*, Western blot.

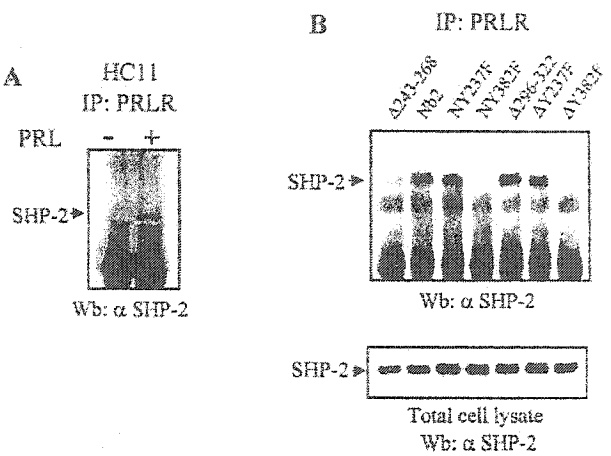


FIG. 2. **SHP-2 is recruited to the C-terminal tyrosine of the PRLR.** *A*, HC11 cells were either unstimulated or stimulated with PRLR for 10 min. *B*, 293 cells were transfected with expression plasmids encoding SHP-2 and the Δ243–268, the Nb2 form of the PRLR, NY237F, NY382F, Δ293–322, ΔY237F, or ΔY382F. Cells were lysed, and the PRLR was immunoprecipitated using the U6 monoclonal antibody against the PRLR. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes, and then blotted with monoclonal antibodies against SHP-2. In the *lower panel*, total cellular lysates obtained from the same transfection were run on SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with monoclonal antibodies to SHP-2. *IP*, immunoprecipitation; *Wb*, Western blot.

samples expressing the C-terminal mutant of the Nb2 form of the PRLR receptor NY382F (Fig. 1*B*). Similarly, tyrosine phosphorylation levels of SHP-2 were comparable in samples expressing the Δ296–322 form as well as its corresponding tyrosine to phenylalanine mutants ΔY237F (Fig. 1*B*). In agreement with the results obtained for the PRLR Nb2 form, SHP-2 tyrosine phosphorylation was lost in the sample expressing the C-terminal mutant of the Δ296–322 form, ΔY382F (Fig. 1*B*). The loss of SHP-2 tyrosine phosphorylation in samples expressing the C-terminal tyrosine to phenylalanine mutants of the PRLR suggests that this tyrosine plays a regulatory role in SHP-2 tyrosine phosphorylation. The sustenance of SHP-2 phosphorylation in the samples expressing the C-terminal tyrosine mutation in the long form of the PRLR suggests that in addition to the C-terminal tyrosine, one or more of the cytoplasmic tyrosine residues that are present in the long form of the PRLR but absent form the Nb2 form of the PRLR may play a role in SHP-2 phosphorylation. The membranes were stripped and reprobed with a monoclonal antibody to SHP-2 in a Western blot analysis to confirm equal expression of the phosphatase (Fig. 1, *A* and 1*B*, *lower panels*).

*The C-terminal Tyrosine of the PRLR Is a Site for SHP-2 Recruitment*—the loss of SHP-2 tyrosine phosphorylation in samples expressing the C-terminal tyrosine to phenylalanine mutant of the PRLR suggests that this tyrosine may act as a recruitment site for SHP-2. To determine whether SHP-2 associates to the PRLR, we initially examined this association in the PRL-responsive mammary epithelial cell line HC11. The PRLR was immunoprecipitated using the U6 monoclonal antibody against the PRLR from the mouse mammary epithelial cell line HC11 that were induced and starved then were either left untreated or treated with PRL for 10 min (see "Experimental Procedures"). Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with monoclonal antibody against SHP-2 in a Western blot analysis. PRL stimulation of HC11 cells induces a specific association between SHP-2 and the PRLR (Fig. 2*A*).

To confirm the association of SHP-2 to the PRLR and to determine whether the C-terminal tyrosine of the PRLR is a site for SHP-2 recruitment, the receptor was immunoprecipitated from 293 cells transiently transfected with expression plasmids encoding SHP-2, the Nb2 form of the PRLR, NY237F, NY382F, Δ296–322, ΔY237F, and ΔY382F and a limited amount of an expression vector encoding Jak2 kinase. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membrane, and blotted with a monoclonal antibody against SHP-2 in Western blotting. No SHP-2 was detectable in samples expressing the control Δ243–268 deletion form of the PRLR (Fig. 2*B*). A clear SHP-2 co-immunoprecipitation with the PRLR was detectable in samples expressing the wild type Nb2 form, NY237F, Δ296–322, and ΔY237F (Fig. 2*B*). However, no co-immunoprecipitating SHP-2 was detectable with the PRLR complexes in the samples expressing the C-terminal tyrosine to phenylalanine mutants NY382F and ΔY382F (Fig. 2*B*). Altogether, these results indicate that the C-terminal tyrosine of the PRLR is a site for SHP-2 association.

No detectable decrease in the association between SHP-2 and the PRLR was observed in samples expressing the C-terminal tyrosine to phenylalanine mutant of the long form of the PRLR LY580F (data not shown). The sustenance of SHP-2 tyrosine phosphorylation and association to the PRLR with the C-terminal mutant of the long form of the PRLR suggests that there is redundancy in SHP-2 association to the PRLR long form where one or more of the tyrosine residues that are only present in the long form of the PRLR can provide additional association sites for SHP-2.

*SHP-2 Utilizes the C-terminal SH2 Domain for Association to the PRLR*—The protein-tyrosine phosphatase SHP-2 has two N-terminally located SH2 domains. To determine the mechanism of association between SHP-2 and the PRLR, the ability of the two SH2 domains to bind to phosphotyrosine motifs was interrupted by point mutations that substitute the critical arginine in the SH2 domain to lysine. 293 cells were co-transfected with expression plasmids for either the Δ243–268 deletion form of the PRLR along with SHP-2 wild type or the Nb2 form of the PRLR with SHP-2 wild type, the arginine to lysine mutant of the N-terminal SH2 domain, SHP-2 R32K mutant, or the C-terminal SH2 domain SHP-2 R138K mutant (7). In

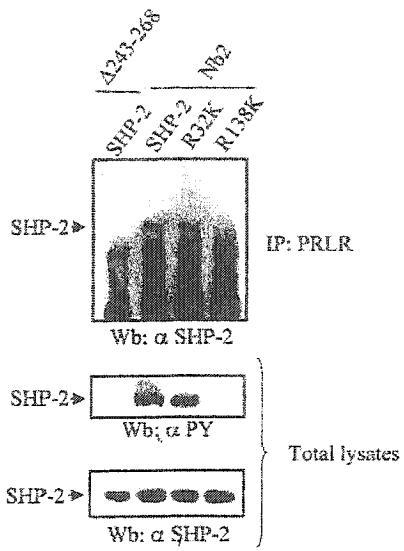


FIG. 3. **SHP-2 utilizes the C-terminal SH2 domain for association to the PRLR.** *A*, 293 cells were transfected with expression plasmids encoding either the Δ243–268 mutant form along with wild type SHP-2 or the Nb2 form along with the either the wild type SHP-2, the N-terminal, or the C-terminal SH2 domains arginine to lysine point mutants R32K and R138K, respectively. Total cellular lysates were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with monoclonal antibodies to phosphotyrosine or monoclonal antibodies to SHP-2. The same cellular lysates were prepared for immunoprecipitation using U6 monoclonal antibodies against the PRLR. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with monoclonal antibodies against SHP-2. *IP*, immunoprecipitation; *Wb*, Western blot.

addition, cells were co-transfected with an expression plasmid for the kinase Jak2. Cellular lysates were prepared for immunoprecipitation of the PRLR using the U6 monoclonal antibody. The immune complexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with monoclonal antibodies to SHP-2 (Fig. 3). Both wild type SHP-2 and the SHP-2 K32R mutant forms can be detected co-immunoprecipitating with the PRLR. In contrast, SHP-2 did not co-immunoprecipitate with the PRLR in samples expressing the SHP-2 R138K mutant. Therefore, SHP-2 interacts with the C-terminal tyrosine of the PRLR through the C-terminal SH2 domain.

To investigate the individual roles of the N- and C-terminal SH2 domains of SHP-2 in regulating tyrosine phosphorylation levels of the phosphatase, total cellular lysates obtained from the same transfection described above were run on SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with antibodies to phosphotyrosine. As shown in Fig. 3 (*middle panel*) tyrosine phosphorylation of SHP-2 was observed in samples expressing wild type SHP-2 as well as SHP-2 R32K. In contrast, tyrosine phosphorylation of SHP-2 was lost in the sample expressing SHP-2 R138K (Fig. 3, *middle panel*), further indicating the importance of the C-terminal SH2 domain of SHP-2 for recruitment to the PRLR and tyrosine phosphorylation of SHP-2. The membrane was stripped and then reblotted with a monoclonal antibody against SHP-2 to confirm equal expression of the protein (Fig. 3, *lower panel*).

*95–110-kDa Tyrosine Phosphorylated Proteins Associate to SHP-2 in HC11 Cells*—The protein-tyrosine phosphatase SHP-2 is known to associate with multiple tyrosine phosphorylated proteins. To examine the different possible associations of SHP-2 with tyrosine phosphorylated proteins in response to PRLR activation, we performed SHP-2 immunoprecipitations in the mouse mammary epithelial cell line HC11. These cells

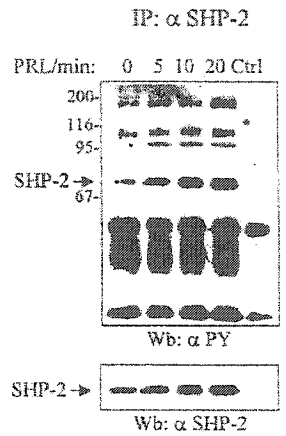


FIG. 4. **Multiple tyrosine phosphorylated proteins associate to SHP-2 following PRL stimulation of HC11 cells.** HC11 cells were either unstimulated or stimulated with PRL for 5, 10, or 20 min. Cells were lysed, and SHP-2 was immunoprecipitated using polyclonal antibodies to SHP-2. The complexes were separated on SDS-PAGE and then transferred to a nitrocellulose membrane and blotted with monoclonal antibodies to phosphotyrosine. The membrane was stripped and reprobed with monoclonal antibodies against SHP-2. Control sample contains antibody and beads but no lysates. *IP*, immunoprecipitation; *Wb*, Western blot.

were grown to confluency, induced to differentiate, and starved for 48 h. Then the cells were either unstimulated or stimulated with PRL for 5, 10, and 20 min. Cellular lysates were prepared for immunoprecipitation with polyclonal antibody to SHP-2. The immune complexes were run on SDS-PAGE, transferred to a nitrocellulose membrane, and then Western blotted with a monoclonal antibody to phosphotyrosine. As shown in Fig. 4, SHP-2 is tyrosine phosphorylated in the basal condition in HC11 cells. However, there is a clear increase in its phosphorylation state following PRL stimulation. Furthermore, in these immunoprecipitates of SHP-2, we observe a number of tyrosine phosphorylated proteins, including proteins in the range of 95–110 kDa, co-precipitating with SHP-2. Two of these proteins having molecular masses of 180 and 110 kDa are constitutively tyrosine phosphorylated. However, a protein of 95 kDa undergoes tyrosine phosphorylation in response to PRL stimulation. Together, these data indicate that SHP-2 interacts with multiple tyrosine phosphorylated proteins in mammary epithelial cells.

*Gab2 Is Specifically Tyrosine Phosphorylated and Recruits SHP-2 and PI3-K in Response to PRL Stimulation in HC11 Cells*—Recently a family of proteins that have molecular sizes in the range of 95–110 kDa called the Gab (Gab1 and Gab2) family of adaptor proteins was identified. These proteins were reported to be tyrosine phosphorylated and to recruit SHP-2 within the signaling complexes for several receptor tyrosine kinases and cytokine receptors. Therefore, we intended to determine whether members of the Gab family of proteins are involved in PRLR-mediated signaling and whether they are able to recruit SHP-2 in response to PRLR activation.

Two sets of the mouse mammary epithelial HC11 cells were grown to confluency, induced to differentiate, and starved for 48 h. The cells were then either unstimulated or stimulated with PRL for 5 or 20 min. One set of cells was immunoprecipitated with a polyclonal antibody to Gab1 (Fig. 5*A*) and the second with a polyclonal antibody to Gab2 (Fig. 5*B*). Samples containing antibody and protein A-Sepharose beads were used as controls. Both sets of immunoprecipitates were run on SDS-PAGE and then transferred to nitrocellulose membranes and probed with monoclonal antibodies to phosphotyrosine in a Western blot. Interestingly, in immunoprecipitates of Gab1,

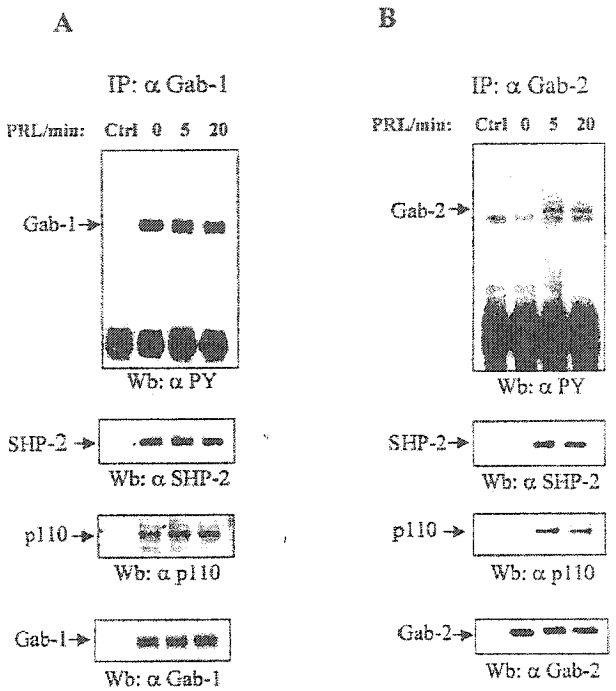


FIG. 5. **Gab2 is specifically tyrosine phosphorylated and recruits SHP-2 and PI3-K in response to PRL stimulation of HC11 cells.** HC11 cells were either unstimulated or stimulated with PRL for 5 and 20 min. Cells were lysed, and lysates were immunoprecipitated with polyclonal antibodies to either Gab1 or Gab2. Sample (*Ctrl*) contains antibodies and beads, but no lysates was used as a control. Immune complexes were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with monoclonal antibodies to phosphotyrosine. The membranes were stripped and then reprobed with monoclonal antibodies against SHP-2, p110 subunit of the PI3-K, and with polyclonal antibodies to Gab1 or Gab2. *IP*, immunoprecipitation; *Wb*, Western blot.

constitutive levels of tyrosine phosphorylation of the protein were detected irrespective of PRL stimulation (Fig. 5*A*). In contrast, in immunoprecipitates of Gab2, tyrosine phosphorylation of the protein was significantly increased following 5 min of PRL stimulation and remained elevated after 20 min (Fig. 5*B*). This suggests that Gab2 but not Gab1 is specifically tyrosine phosphorylated following PRLR activation in HC11 mammary epithelial cells. Equal levels of Gab1 and Gab2 immunoprecipitations were confirmed by stripping the membranes and reprobing with polyclonal antibodies to Gab1 and Gab2 (Fig. 5, *A* and *B*, *bottom panels*, respectively). To confirm that Gab1 is not tyrosine phosphorylated in response to PRL stimulation, we examined the tyrosine phosphorylation state of the protein in 293 cells transiently transfected with the long form of the PRLR along with HA-tagged Gab1 and Jak2 kinase. Under these conditions, we also did not detect Gab1 tyrosine phosphorylation (data not shown). Altogether, our results suggest that Gab2 but not Gab1 is a substrate for the PRLR.

To determine whether SHP-2 recruitment to Gab1 or Gab2 might be regulated by PRLR activation in HC11 cells, the membranes were stripped and reprobed with monoclonal antibodies to SHP-2. Similar to the tyrosine phosphorylation pattern of Gab1, SHP-2 recruitment to Gab1 was constitutive and was not affected by PRL stimulation (Fig. 5*A*). On the other hand, SHP-2 recruitment to Gab2 showed clear responsiveness to PRLR activation. Stimulation of HC11 cells for 5 or 20 min led to a significant increase in SHP-2·Gab2 complex formation (Fig. 5*B*). Therefore, SHP-2 association to Gab2 is regulated by PRL stimulation in the mammary epithelial cell line HC11.

Gab family members were also reported to associate with the p85 subunit of the PI3-K. To determine whether PRL stimulation can induce the association of PI3-K to Gab members, the membranes used were stripped and reprobed with polyclonal antibodies to the p110 subunit of the PI3-K. Gab1 associated to p110 irrespective of PRL stimulation (Fig. 5*A*), in contrast, Gab2 showed selective recruitment of p110 following PRLR activation (Fig. 5*B*). Altogether, our results indicate that Gab2, but not Gab1, is a specific target for PRLR activation and that the adaptor protein is capable of selectively recruiting SHP-2 and PI3-K in response to PRL stimulation.

*The Three Natural Forms of the PRLR, Long, Intermediate Nb2m, and Short Forms, Can Induce Gab2 Tyrosine Phosphorylation and Association to SHP-2 in 293 Cells*—The rat PRLR has three forms: the long, the intermediate Nb2, and the short form. Only the long and Nb2 forms are tyrosine phosphorylated following PRL stimulation, and in biological assays testing β-casein gene promoter induction, only the long and Nb2 forms were able to activate expression of the PRLR responsive milk protein (34, 36). To determine whether stimulation of these three forms leads to tyrosine phosphorylation of Gab2 and its association to SHP-2, 293 cells were transfected with plasmids expressing HA-tagged Gab2 along with SHP-2 and the long, Nb2, or the short forms of the PRLR. In addition, the deletion mutant form of the PRLR, Δ243–268, was used as a control. The cells were also transfected with a limited amount of an expression plasmid for the kinase Jak2. Cellular lysates were separated into two parts. The first part was used for immunoprecipitation with polyclonal antibodies to the HA tag. Immunoprecipitates were run on SDS-PAGE, transferred to nitrocellulose membranes, and probed with monoclonal antibodies to phosphotyrosine in Western blot analysis (Fig. 6*A*). The second part of the lysates was used for immunoprecipitation using polyclonal antibodies to SHP-2. Immunoprecipitates were run on SDS-PAGE and then transferred to a nitrocellulose membrane and probed with monoclonal antibodies to phosphotyrosine (Fig. 6*B*). In both immunoprecipitations, samples containing antibody and beads were used as controls. A strong PRL responsive phosphorylation of SHP-2 and a significant association with Gab2 adaptor protein was detected with all three forms of the PRLR (Fig. 6*A*). Similarly, in the reverse immunoprecipitation, both tyrosine phosphorylation and association of SHP-2 to Gab2 were detectable in samples expressing all three forms of the PRLR (Fig. 6*B*). Interestingly however, in both immunoprecipitated sets Gab2 migrated at a faster rate in samples expressing the short form of the PRLR compared with samples expressing the long and Nb2 forms (Fig. 6), suggesting a different pattern of phosphorylation for Gab2 when expressed with the short form of the PRLR. The identities of these proteins were confirmed by stripping and reprobing the membrane with monoclonal antibodies to the HA tag and to SHP-2 (Fig. 6, *A* and *B*, *middle* and *lower panels*, respectively). Together, the data indicate that all three forms of the PRLR can induce the phosphorylation of Gab2 and its association to the phosphatase SHP-2. Taking into consideration that the short form of the PRLR does not become tyrosine phosphorylated in response to PRL stimulation (35) suggests that Gab2 tyrosine phosphorylation and association to SHP-2 is independent of PRLR tyrosine phosphorylation.

*SHP-2 Association to Gab2 Requires Tyrosines 604 and 633 on the C-terminal Part of Gab2*—SHP-2 binding to Gab2 in the IL-3 receptor signaling system requires tyrosines 604 and 633 located in the C-terminal portion of Gab2 (17). We intended to determine the mechanism of association between SHP-2 and Gab2 in the PRLR signaling system. 293 cells were co-trans-

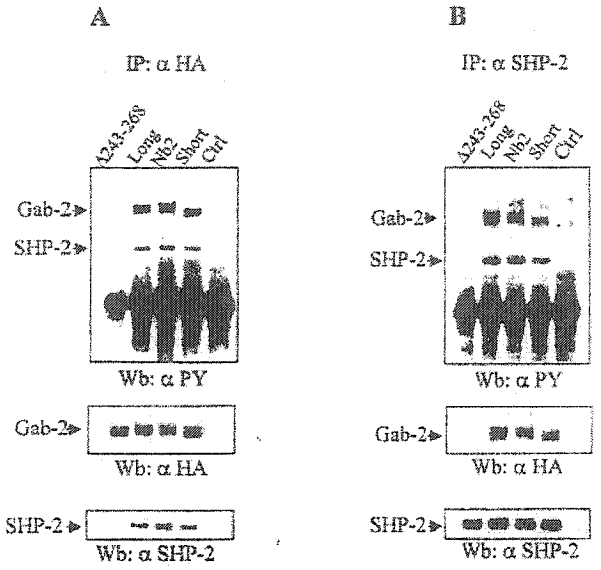


FIG. 6. **All three natural forms of the PRLR can induce Gab2 tyrosine phosphorylation and association to SHP-2.** 293 cells were transfected with expression plasmids encoding HA-tagged Gab2, SHP-2, and either the Δ243–268 deletion form, the long form, the Nb2 form, or the short form of the PRLR. Cells were lysed, and immunoprecipitations were carried out either using polyclonal antibody against HA tag (*A*) or using polyclonal antibody to SHP-2 (*B*). In both sets, sample (*Ctrl*) contains beads, and the respective antibody was used as a control. Immunoprecipitates were run on SDS-PAGE and transferred to nitrocellulose membranes, and then HA immunoprecipitates were blotted with monoclonal antibodies against SHP-2 and SHP-2 immunoprecipitates were blotted with monoclonal antibodies for HA tag. The membranes were stripped and then blotted with the indicated antibodies. *IP*, immunoprecipitation; *Wb*, Western blot.

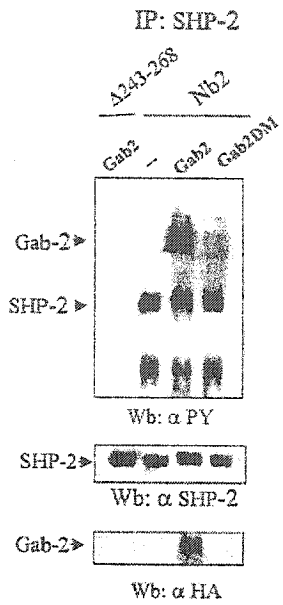


FIG. 7. **SHP-2 association to Gab2 requires tyrosines 604 and 633 on the C-terminal part of Gab2.** 293 cells were transfected with expression plasmids encoding the Nb2 form of the PRLR alone (–) or along with HA-tagged Gab2 or HA-tagged Gab2DM. In addition, expression plasmid encoding the Δ243–268 mutant form of the PRLR was also transfected along with Gab2 and SHP-2 as a control. Cells were lysed, and SHP-2 was immunoprecipitated with polyclonal antibodies against SHP-2. The immune complexes were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with monoclonal antibodies against phosphotyrosine. The membrane was then stripped and then reblotted with monoclonal antibodies to SHP-2 and then with monoclonal antibodies to the HA tag. *IP*, immunoprecipitation; *Wb*, Western blot.

fected with an expression plasmid encoding the Nb2 form of the PRLR alone, sample denoted by (–), or with expression plasmids for HA-tagged Gab2 or the double tyrosine to phenylalanine mutant of Gab2 (Gab2DM). The Gab2DM mutant was shown to lose the ability to associate to SHP-2 (17). As a negative control, the Δ243–268 deletion form was co-transfected. All transfections contained limited amounts of an expression plasmid encoding the Jak2 kinase. Cell lysates were obtained, and SHP-2 was immunoprecipitated with polyclonal antibody to SHP-2; the immune complex was separated on SDS-PAGE, transferred to a nitrocellulose membrane, and Western blotted with monoclonal antibodies to phosphotyrosine (Fig. 7). As expected, tyrosine phosphorylation was not observed in the samples expressing the Δ243–268 deletion form of the PRLR, whereas SHP-2 was tyrosine phosphorylated in samples expressing the Nb2 form of the PRLR. In samples expressing Gab2, the protein became tyrosine phosphorylated and co-immunoprecipitated with SHP-2. Interestingly, however, the level of SHP-2 tyrosine phosphorylation was not significantly enhanced in the presence of Gab2 overexpression. The association between SHP-2 and Gab2 was eliminated in the samples expressing the Gab2DM mutant, suggesting that the association between SHP-2 and Gab2 is mediated through either one or two of the tyrosines in the C-terminal portion of Gab2. The tyrosine phosphorylation level of SHP-2 was not affected by the lack of association between SHP-2 and Gab2, suggesting that SHP-2 phosphorylation can take place through the interaction of SHP-2 with the C-terminal tyrosine of the PRLR (Fig. 7). The membranes were stripped and then reprobed with a monoclonal antibody to SHP-2 and then with monoclonal antibody to the HA tag (Fig. 7, *middle* and *lower panels*, respectively).

## DISCUSSION

The protein-tyrosine phosphatase SHP-2 plays a critical role in signaling downstream from several receptor tyrosine kinases and cytokine receptors including the PRLR (7). We have reported earlier that tyrosine phosphorylated SHP-2 was found in a complex containing the PRLR and Jak2 (7). In this study we investigated the mechanisms of SHP-2 recruitment and activation within the PRLR signaling complex. We identified SHP-2 in PRLR immunoprecipitates from PRL-stimulated mouse mammary epithelial HC11 cells, suggesting that the PRLR is able to recruit SHP-2 leading to its tyrosine phosphorylation, an event that is known to regulate the activation of the phosphatase. In addition, we determined that the C-terminal tyrosine of the PRLR has a regulatory role in the recruitment and tyrosine phosphorylation of SHP-2. SHP-2 tyrosine phosphorylation as well as association to the PRLR was lost in samples expressing the C-terminal tyrosine mutant of the Nb2 form of the PRLR. However, we did not observe a loss of SHP-2 association to the PRLR with the C-terminal tyrosine mutant of the long form of the PRLR, suggesting that possibly one or more of the other tyrosine residues present in the long form of the PRLR but absent from the Nb2 form may act as sites for SHP-2 recruitment. Together, our results indicate that the C-terminal tyrosine residue of the PRLR is important for the recruitment of SHP-2 to the PRLR signaling complex.

We have previously reported that this C-terminal tyrosine of the PRLR plays a critical role in the induction of PRL responsive genes such as the β-casein gene (29). Furthermore, we determined that this tyrosine also regulates the activation of Stat5 (32). In addition, other studies have shown that the C-terminal tyrosine of the receptor regulates Stat5 recruitment to the PRLR (38). The association of SHP-2 or Stat5 to the

C-terminal tyrosine might be two sequential events during the activation process. Alternatively, both molecules can associate to the C-terminal tyrosine through a third intermediate adaptor protein. Therefore, it is possible to speculate that the C-terminal tyrosine residue of the PRLR may coordinate multiple pathways downstream from the PRLR.

The association between cytokine receptors and SHP-2 has been previously reported for various systems. Two specific tyrosine residues in the intracellular domains of the platelet-derived growth factor receptor were shown to be sites for SHP-2 direct recruitment (9, 39). Similarly, three tyrosine residues in granulocyte macrophage colony-stimulating factor receptor were also shown to be sites for SHP-2 association (8). In addition, SHP-2 was found in a complex containing growth hormone receptor, Jak2, and signal regulatory protein $\alpha$, and the interaction between SHP-2 and growth hormone receptor was mapped to the C-terminal tail of the receptor (11). Besides SHP-2 recruitment to phosphorylated receptors, receptor tyrosine phosphorylation-independent recruitment of SHP-2 has also been suggested. The association of SHP-2 to activated interferon $\alpha/\beta$ receptors was found to be independent of receptor tyrosine phosphorylation (40).

The association between SHP-2 and the platelet-derived growth factor receptor was mapped to the N-terminal SH2 domain of the phosphatase (41, 42). Similarly, SHP-2 associates to the insulin receptor substrate 1 subunit through the N-terminal SH-2 domain. The optimal phosphotyrosine motif that associates to the N-terminal SH2 domain of SHP-2 was determined to be Y(I/V)X(V/I/L/P) (43). In our study, we determined that SHP-2 associates to the C-terminal tyrosine of the PRLR through the C-terminal SH2 domain of SHP-2. The sequence of the tyrosine of the PRLR that binds to SHP-2 is DYLDP. To our knowledge, an optimal phosphotyrosine motif for the C-terminal SH2 domain of SHP-2 has not been determined.

In immunoprecipitates of SHP-2 from HC11 cells, we normally find multiple tyrosine phosphorylated proteins in the range of 95–100 kDa that co-immunoprecipitate with SHP-2 in response to PRL stimulation. SHP-2 has been reported to interact with members of the Gab family of adaptor proteins that range in size between 95 and 110 kDa. Therefore, we investigated the possible PRL-mediated interactions of SHP-2 to members of the Gab family of proteins. Our investigation revealed that Gab2 is specifically tyrosine phosphorylated in response to PRL stimulation in mammary epithelial cells. In addition, we found that PRL stimulation also led to Gab2·SHP-2 as well as Gab2·PI3-K complex formation. This is in contrast to Gab1 tyrosine phosphorylation and interaction with SHP-2 and PI3-K that was constitutive and independent of PRL stimulation in HC11 cells. Gab1 and Gab2 proteins were shown to be tyrosine phosphorylated and to associate to a number of cellular signaling mediators including SHP-2, p85 subunit of PI3-K, Grb2, and CrkL in various systems including those for several cytokines such as IL-2, IL-6, erythropoietin, and TPO and growth factors such as hepatocyte growth factor and insulin (17, 19, 21–23, 25, 28, 37). The two proteins share a similar structure (up to 73% within their pleckstrin homology domain); however, they appear to function in a nonredundant manner. Future studies will focus on the contribution of Gab2 to PRLR signaling.

Our identification of at least two potential recruitment sites for SHP-2 in PRLR signaling only partially explains its complex interactions and role in signaling. It is not clear whether two different SHP-2 molecules can independently bind to the two different sites (*i.e.* the PRLR and Gab2) or whether it is the same SHP-2 molecule that concurrently binds these two sites. We identified that SHP-2 associates to the PRLR through its C-terminal SH2 domain leaving the N-terminal SH2 domain to possibly bind to Gab2. This finding favors that one SHP-2 molecule is binding simultaneously to the two sites. On the other hand, we found SHP-2 to be tyrosine phosphorylated in samples expressing the short form of the PRLR that itself does not become tyrosine phosphorylated (35). This suggests that the interaction of SHP-2 with Gab2 is independent of PRLR tyrosine phosphorylation state. Therefore, possibly two different molecules of SHP-2 are binding independently to the PRLR and to Gab2. Further investigation is required to elucidate the effects of SHP-2 recruitment to the PRLR and to Gab2 on the modulation of its functions in signaling downstream of the PRLR.

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