Epidermal growth factor-induced hepatic DNA synthesis: key roles for phosphatidylinositol 3-kinase and the adaptor protein Gab2

by

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Abstract

In primary rat hepatocytes, we found that activation of the PI3-kinase pathway is both necessary and sufficient to account for EGF-induced DNA synthesis. To identify the mechanism of EGF-induced PI3-kinase activation, we demonstrated that three distinct p85-associated complexes were formed following EGF: ErbB3-p85, Shc-p85 and a large complex Gab2-Grb2-SHP2-p85. The latter accounted for > 80% of total PI3-kinase activity. Further experiments showed that these complexes are differentially localized in rat liver following EGF treatment. ErbB3-p85 and Shc-p85 complexes were localized to PM and Endosomes; whereas the multimeric Gab2-Grb2-SHP2-p85 complex was formed rapidly and exclusively in cytosol. A central role for Gab2 in EGF-induced PI3-kinase activation and DNA synthesis was established when we observed that over-expression of wild-type Gab2 augmented these EGF actions, whereas a Gab2 mutant lacking p85 binding sites did not effect such augmentation. Over-expression of the PH-domain of Gab2 did not affect EGF-induced Gab2 phosphorylation, PI3-kinase activation and DNA synthesis, whereas over-expressed Gab2 lacking the PH-domain was comparable to wild-type Gab2 in respect to these EGF-induced signals. These data demonstrated that Gab2 is phosphorylated and mediates EGF signaling in a PH-domain independent manner. We then explored the mechanism of Gab2 phosphorylation by EGF; our results demonstrated that PP1, a selective inhibitor of Src family kinases, blocked EGF-induced Gab2 tyrosine phosphorylation and downstream events. Moreover, Gab2

phosphorylation was increased in Csk knock-out cells in which Src family kinases are constitutively activated. A constitutive association between Gab2 and Src via proline rich sequences on Gab2 was demonstrated since deletion of proline rich sequences in Gab2 prevented EGF-induced association of Src with Gab2, Gab2 phosphorylation, PI3-kinase/Akt activation, and DNA synthesis. The role of SHP2 was defined by our observations that a Gab2 mutant lacking SHP2 binding sites manifested increased EGF-induced Gab2 phosphorylation and PI3-kinase activation but reduced MAPK activation. In summary, our work has identified the different complexes involved in EGF-dependent PI3-kinase activation; and has established a key role for the Gab2-complex in EGF-stimulated DNA synthesis. We further have identified the key role of Src in mediating EGF-induced hepatic mitogenesis through the phosphorylation of Gab2 and the activation of the PI3-kinase cascade.

Résumé

Dans les hépatocytes de rat en culture primaire, nous avons démontré que l'activation des voies de signalisation dépendantes de PI3-kinase est nécessaire et suffisante pour induire la synthèse d'ADN en réponse à l'EGF. L'étude des mécanismes responsables de l'activation de PI3-kinase nous a permis d'identifier la présence de trois différents complexes qui associent p85 en réponse à l'EGF : ErbB3-p85, Shc-p85 et un large complexe Gab2-Grb2-SHP2-p85. 80% de l'activité totale PI3-kinase est issue de ce dernier complexe. En réponse à l'EGF, ces trois entités se forment dans différents compartiments cellulaires. Les complexes ErbB3-p85 et Shc-p85 se forment au niveau de la membrane plasmique puis semblent être internalisés par la suite dans les endosomes. Le complexe Gab2-Grb2-SHP2-p85 quand à lui est exclusivement localisé dans le cytoplasme. Nous avons par la suite confirmé dans ces cellules le rôle central de Gab2 dans l'activation de PI3-kinase et de la synthèse d'ADN en réponse à l'EGF. La sur-expression de Gab2 amplifie l'effet de l'EGF alors que la sur-expression d'un mutant ne contenant plus les sites de fixation de p85 n'a aucun effet. Nous avons aussi démontré que la sur-expression du domaine PH de Gab2 ne modifie en rien la phosphorylation de Gab2 induite par l'EGF, l'activation de PI3-kinase et la synthèse d'ADN. L'effet de la sur-expression d'un mutant Gab2 ne contenant pas de domaine PH est comparable au Gab2 natif venant donc confirmer les observations précédentes. Cela indique que Gab2 est phosphorylé indépendamment de son domaine PH pouvant ainsi transmettre le signal induit par l'EGF. Nous avons par la suite analysé les mécanismes par

lesquels Gab2 est phosphorylé en réponse à l'EGF. PP1, un inhibiteur spécifique de la famille des kinases Src, bloque la phosphorylation de Gab2 induite par l'EGF ainsi que les autres effets en aval. L'utilisation de cellules possédant une activation permanente de Src due à l'absence du gène CSK a permis de confirmer ces résultats. En effet, dans ces cellules Gab2 est phosphorylé en absence de stimulation par l'EGF. Nous avons par ailleurs démontré que Gab2 et Src sont associés de façon constitutive par l'intermédiaire d'une région riche en résidus proline localisée sur Gab2. Aucune association n'est en effet observée en réponse à l'EGF, entre Src et un mutant Gab2 ne contenant pas cette région riche en résidus proline. De plus la sur-expression de ce même mutant ne permet pas détecter après stimulation par l'EGF de phosphorylation de Gab2, d'activation de PI3-kinase, d'Akt1 et de synthèse d'ADN. Ces études nous ont aussi permis de caractériser le rôle de SHP2, une phosphatase présente dans le large complexe multimérique. Nous avons observé que la sur-expression dans les hépatocytes primaires de rat, d'un mutant Gab2 ne contenant plus le site de fixation de SHP2 augmente la phosphorylation de Gab2 et de l'activation de PI3-kinase en réponse à l'EGF mais entraîne une diminution de l'activité MAPK. En résumé, nous avons identifié dans les h`patocytes de rat en culture primaire, trois différents complexes impliqués dans l'activation de PI3-kinase en réponse à l'EGF et avons établit un rôle central pour Gab2 dans la synthèse d'ADN induite par cette même hormone. De plus nous avons aussi identifié Gab2 comme un substrat de la famille des kinases Src et comme un acteur important de la mitogénèse hépatique induite par l'EGF.

To my parents.

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Contributions of Authors

Chapter 2: The work presented in this Chapter are reproduced from

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I contributed most to this paper. Dr. Catherine Mounier was responsible for the generation of the Δp85 mutant and Dr. Jiong Wu helped me with the cloning of Gab2 cDNA.

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I contributed most to this paper. Dr. Catherine Mounier was responsible for the generation of the Gab2ΔSHP2 mutant in pCDNA3 vector; Victor Dumas prepared most of the hepatocytes.

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CHAPTER 1

Literature Review

1.1. Physiological role of EGF in liver

1.1.1. Introduction

Epidermal growth factor (EGF), a small mitogenic polypeptide present in many mammalian species, is distributed throughout a wide number of tissues. It is found in varying concentrations in most body fluids including milk (80ng/ml), saliva (6-17 ng/ml), blood serum (0.18ng/ml) (1), urine (29-272 ng/ml), and plasma (2-4ng/ml) (2). EGF was originally identified in mouse submaxillary gland as a protein that induced incisor eruption and premature eyelid opening in newborn mice (3). It is a single-chain polypeptide consisting of 53 amino acid residues, including six Cys residues that form three intramolecular disulfide bonds (3). EGF is expressed in several adult human tissues, including the submandibular glands, the Brunner glands in the small intestine, and the kidney (4).

The biological activities of EGF are not species-specific. EGF has been shown to inhibit the secretion of gastric acid (5). In vivo biological activities of EGF are best illustrated by its involvement in the embryonic development of mice where it was shown to promote premature lid opening and tooth development (6). EGF acts as a differentiation factor for some embryonic cell types. It also stimulates the proliferation of embryonic cells (reviewed in (3)). EGF strongly influences the synthesis and turn-over of proteins of the extracellular matrix, including fibronectin, collagens, laminin, and glycosaminoglycans; and increases bone resorption through the release of calcium from bone, as does TGF-alpha, a

polypeptide structurally and functionally related to EGF. EGF is also a strong chemoattractant for fibroblasts and epithelial cells. Alone and in combination with other cytokines, EGF mediates wound healing (7). It has also been shown to be a strong mitogen for many cells of ectodermal, mesodermal, and endodermal origin. Thus it controls and stimulates the proliferation of fibroblasts and epidermal and epithelial cells, including, kidney epithelial cells, human glial cells, ovary granulosa cells, hepatocytes, and thyroid cells in culture. Because it is mitogenic for many types of cells, it appears to play essential roles in multiple adult organs, such as in the differentiation and proliferation of mammary gland (8), the healing of gastric ulcers (7), the regeneration of liver (9), and angiogenesis (10). This thesis focuses on the biological role of EGF in liver.

1.1.2. Role of EGF in hepatocyte proliferation

Hepatocytes of normal adult liver are arrested in G_0. They acquire a remarkable capacity to proliferate after partial hepatectomy (PH) or other injuries resulting in liver cell loss. This regeneration of liver is a remarkable process that allows complete restoration of hepatic architecture and tissue specific function after different types of liver injury (11). Important roles have been established for EGF in compensatory liver growth (12, 13). Among the earliest events observed after PH is the rapid and sequential induction of immediate early genes for c-*fos*, c-*jun*, and c-*myc*, all of which are undetectable in normal adult rat liver (13-15). The induction of these genes is a hallmark of the G_0/G_1 transition of the cell cycle and is viewed as necessary to render hepatocytes competent to respond to the proliferative effects of hormonal factors, such as EGF. Indeed, EGF does not

induce liver DNA synthesis when administered to normal rats, whereas EGF mediates hepatic DNA synthesis after PH or chemically induced loss of hepatocytes (13). Studies in primary hepatocyte cultures, where confounding influences of multiple *in vivo* changes are absent, have established that EGF is indeed a hepatic mitogen (16-18). The understanding EGF-induced mitogenesis of hepatocytes should provide understanding of the consequences following hepatic injury by toxins, infectious agents etc.

1.1.3. Other functions of EGF in liver

In addition to its role in liver cell mitogenesis, EGF is involved in the regulation of apoptosis in liver. The pathogenesis of several liver diseases, such as hepatocellular carcinoma and alcoholic liver disease, specifically involves the abnormal regulation of apoptosis (19-22). Furthermore, several studies have reported a marked increase in apoptosis in viral infections such as hepatitis C (23) and B (24). One of the apoptosis-inducing entities that has been linked to human liver disease is the cell-surface 'death receptor' Fas, which is constitutively expressed in hepatocytes (25). Cells undergoing apoptosis after treatment with anti-Fas antibody were protected by EGF treatment (26). It has also been found that EGF is an important survival signal for TGF-beta-induced apoptosis in fetal hepatocytes (27).

Other effects of EGF in liver have also been reported. For example, in the rat liver epithelial cell line, IAR6.1, EGF induced inhibition (~50%) of Gap junction intercellular communication (GJIC), a process involved in intercellular exchange

of low molecular weight substances through pores located in gap junctions. GJIC is an important way for cells to regulate homeostasis, proliferation and differentiation (28). In addition, EGF was found to activate the Na+/H+ antiport in adult but not fetal rat hepatocytes (29).

There have been many studies of EGF signaling designed to understand the mechanism(s) of its physiological effects. These will be described in the next section. However the aim of the research in this thesis was to identify the molecular signaling mechanisms involved in EGF-induced hepatocyte mitogenesis. Therefore this review will focus on EGF signaling involved in its induction of mitogenesis.

1.2. EGF signaling: general introduction

1.2.1. The EGF receptor

1.2.1.1. Introduction

Peptide hormones and growth factors bind to their cell surface receptors to initiate their cellular effects. The cell surface receptors for EGF and other growth factors are receptor tyrosine kinases (RTKs). These are transmembrane proteins containing an intrinsic protein tyrosine kinase activity on the cytosolic domain of the receptor. Binding of EGF to its receptor (EGFR) results in autophosphorylation of EGFR itself as well as the tyrosine phosphorylation of cellular proteins (i.e. RTK substrates). These phosphorylated tyrosines on the EGFR and its substrates play key roles in transducing the signal into the cell (30,

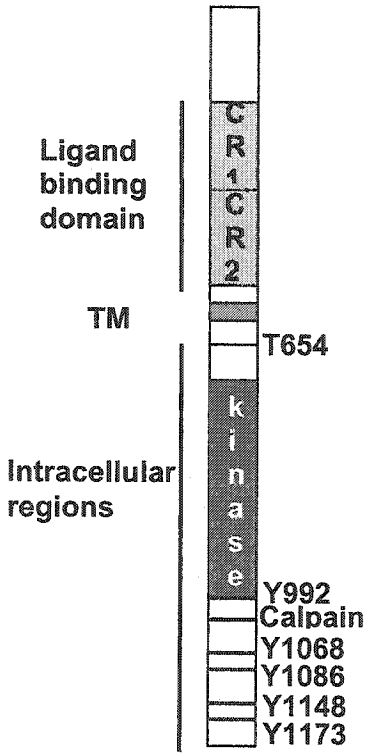
31). Many normal processes such as fertilization, cell proliferation, cell migration, cell differentiation and apoptosis as well as many pathological conditions, including cancer and atherosclerosis, are associated with increased RTK signaling (32).

The EGFR family of RTKs is comprised of four homologous receptors: EGFR/ ErbB1/HER1, which was the first RTK to be molecularly cloned (33), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4 (34,35). EGFR family members are activated by a large group of EGF-related ligands, which all contain a conserved EGF-like domain and are synthesized as transmembrane precursor proteins (36). These include EGF, transforming growth factor-α (TGF-α), epiregulin, betacellulin, heparin-binding EGF-like growth factor, amphiregulin and the large family of alternatively-spliced neuregulins (37). These growth factors bind with different specificities and affinities to EGFR, HER3 and HER4, while no ligand for HER2 has yet been identified. The activity of the EGFR is subject to modulation by multiple positive and negative regulators and is transduced to the nucleus by several conserved signaling cassettes.

1.2.1.2. EGFR structure

All EGFR family members are composed of an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment (Fig.1). The most outstanding feature of the glycosylated extracellular domain of these receptors is the presence of conserved cysteine-rich clusters, CRI and CRII, which comprise

the ligand-binding domain. The juxtamembrane domain serves primarily as a site for feedback attenuation by Ser/Thr protein kinases such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). There is evidence that a motif within this region may link to heterotrimeric G proteins (38). Next comes a tyrosine kinase domain, sometimes referred to as a Src homology domain 1, SH1. This domain is the most conserved region among the EGFR protein family, except in ErbB3, which differs from the other members in that it lacks a tyrosine kinase domain (39). The SH1 kinase region mediates autophosphorylation of the EGFR including five tyrosine residues in its carboxyl-terminal tail. This implies that the carboxyl-terminal tails of EGFRs fulfill important regulatory functions. Phosphorylated tyrosine residues of the EGFR recruit various adapter proteins with Src homology domain 2 (SH2) or phosphotyrosine binding domains (PTB) to defined phosphotyrosine residues in the EGFR leading to the activation of various downstream signals. Moreover, it has been reported that the carboxyl-terminal tail also contains motifs for internalization and degradation of the EGFR (40).



1-Figure1. Structural motifs and regulatory elements in the EGF receptor. The mature EGFR polypeptide is shown. Highlighted structures include the two cysteine-rich (CR) domains, the discontinuous ligand-binding domains, which are different but overlapping for the various ligands. The transmembrane (TM) stretch separates the glycosylated extracellular domain from the intracellular regions. This latter includes the tyrosine kinase domain as well as the autophosphorylated tyrosines (Y), the site of PKC transmodulation on threonine at amino acid 654 (T654), and the calpain cleavage site. Not shown are the three internalization domains (at 973, 996 and 1149).

1.2.1.3. Dimerization of EGFR

As with many other RTKs, EGFR-dependent signal transduction begins with the stabilization of a receptor dimer through ligand binding, which is essential for its activation. The dimerization of EGFR family proteins is not limited to homodimer formation, but also involves heterodimerization of ErbB proteins. It was demonstrated that at least nine different homo- and heterodimers of ErbB proteins exist but their formation displayed a distinct hierarchy (41). Heterodimers are generally more biologically active than homodimers, which is governed by a hierarchy with ErbB2 being the preferred partner of all ErbB proteins (41,42). ErbB2-containing heterodimers display increased ligand affinity due to a decelerated off-rate (43) that can be correlated with prolonged activation of downstream signaling pathways (44,45). Furthermore, biological responses such as proliferation (44,45), morphological differentiation (44) and migration/invasion (46) are enhanced in cells expressing ErbB2.

ErbB heterodimerization is a means not only for signal amplification but also for signal diversification. The subsets of signaling molecules recruited to the SH2- and PTB domains of an activated receptor are defined by the pattern of phosphorylated tyrosine residues in the C-terminus of the receptor. For example, all ErbB proteins apparently activate the Ras-Raf-MAPK signaling route. Nevertheless, each receptor complex may select a distinct set of signaling proteins that collectively specify its unique cellular signature, such as Cbl, a proto-oncogenic adaptor protein, that is recruited by all ErbB-1-containing

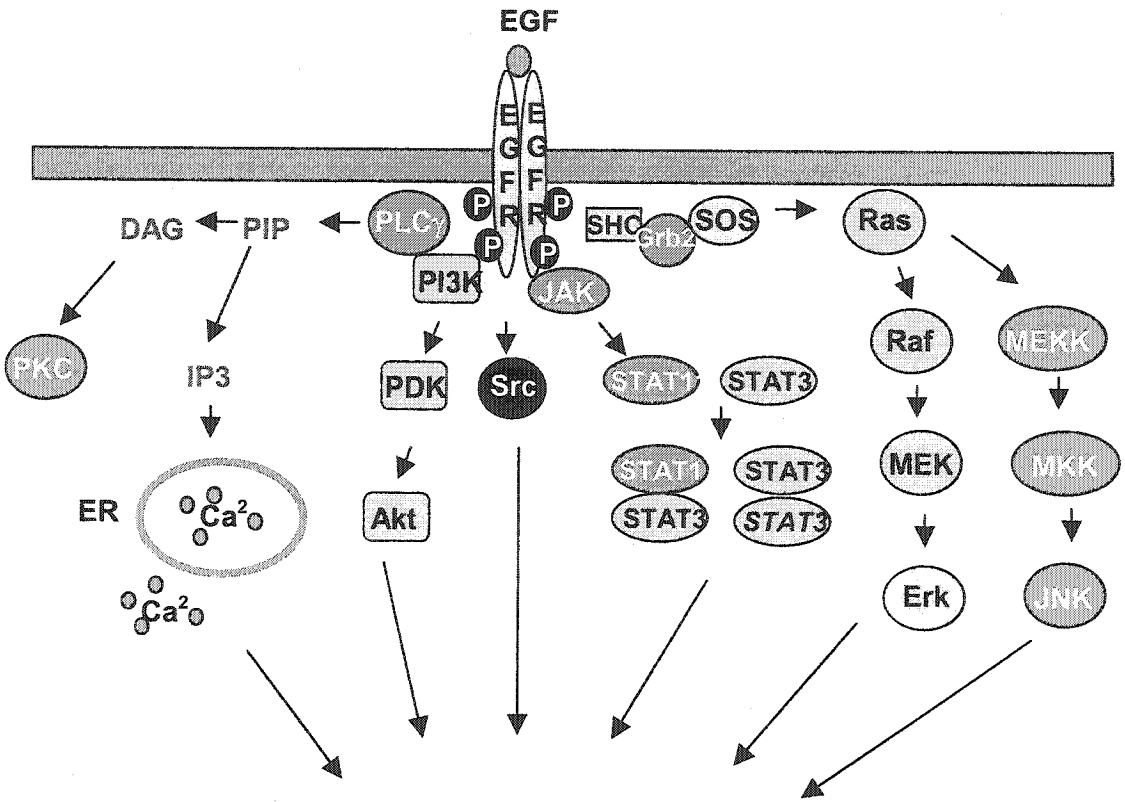
receptor complexes, but not by other dimers (47). Other experiments show that signal diversification arises at one level by differential transphosphorylation of a given receptor in distinct ErbB dimers. By phosphopeptide mapping of activated ErbB1 and ErbB2 from NIH3T3 cells expressing single and pair-wise combinations of ErbB receptors, it was shown that receptor phosphorylation reflects the dimerization partners (48).

Although dimerization of EGFR upon stimulation with monomeric EGF ligand was described more than 10 years ago (49), the exact mechanism of this critical step is still not understood. One of the most favored models suggests a ligand-induced conformational change that leads to dimerization (50), whereas the second assigns a critical role to bivalent ligands with two receptor-binding sites (51). However, a recent study has shown that EGFR can form a ligand-independent inactive dimer and that receptor dimerization and activation are mechanistically distinct and separable events (52).

1.2.1.4. Downstream signaling of EGFR

EGFR kinase triggers numerous downstream signaling pathways similar to other RTKs and tyrosine kinase-linked cytokine receptors (Fig. 2). These pathways include phospholipase C γ (PLCγ) and its downstream calcium- and PKC-mediated cascades, the Ras pathway leading to MAPK activation, GTPases such as Rho and Rac, multiple signal transducer and activator of transcription (STAT) isoforms, heterotrimeric G proteins, and the phospholipid-directed enzymes phosphatidylinositol 3'-OH kinase (PI3-kinase), as well as the non-

receptor tyrosine kinases – Src family kinases. In this review, we focus on the MAPK, PI3-kinase and Src pathways which we have found to be involved in regulation of EGF-induced mitogenesis.



1-Figure 2. Major signaling pathways downstream of EGFR. Upon EGF binding, EGFR dimerizes and autophosphorylates, leading the activation of numerous downstream pathways. These pathway include phospholipase C γ (PLCγ) and its downstream calcium- and PKC-mediated cascades; the Ras-MAPK cascade; multiple signal transducer and activator of transcription (STAT) isoforms; and the phospholipid-directed enzymes phosphatidylinositol 3'-OH kinase (PI3-kinase), as well as the non-receptor tyrosine kinases – Src family kinases.

1.2.2. PI3-kinase pathway

1.2.2.1. Introduction

The PI3-kinase family consists of three classes of enzymes based on different catalytic subunit isoforms and their substrate specificity. In mammals, the class I PI3-kinases is divided into two subclasses, class IA and class IB, based on structural and functional differences (53). The current review and our studies will only focus on the Class IA PI3-kinase.

Class IA PI3-kinase is an enzyme that specifically phosphorylates the D-3 position of the inositol ring of the phosphatidylinositol [PI], phosphatidylinositol 4-phosphate [PI(4)P], and phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] to produce three novel phosphoinositides: phosphatidylinositol 3-monophosphate [PI(3)P], phosphatidylinositol 3,4-biphosphate [$PI(3,4)P_2$], and phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$] (54,55). Several reports have shown that $PI(4,5)P_2$ is the preferred substrate *in vivo* for this class I PI3-kinase thus generating $PI(3,4,5)P_3$ (54,55) .Class IA PI3-kinase is a heterodimer, consisting of an 110kD (p110) catalytic subunit, and an 85kD (p85) regulatory subunit (56). The p85 subunit contains an N-terminal Src homology 3 (SH3) domain which binds proline-rich sequences and two Src homology 2 (SH2) domains that bind to tyrosine phosphorylated sites on receptors or docking proteins (57,58). An inter-SH2 domain (iSH2), which links the two SH2 domains, is involved in the constitutive binding of the p85 to the p110 subunit (reviewed in (59)).

1.2.2.2. Activation of PI3-kinase by EGF

The binding of the SH2 domains of p85 with tyrosine phosphorylated receptors or docking proteins is required for activation of the catalytic subunit (60,61). Several reports have shown that insulin activates PI3-kinase through recruitment of p85 to tyrosine phosphorylated (PY) IRS-1 and IRS-2 (60,62). In the case of EGF, studies in different cell lines have identified several possible mechanisms leading to PI3-kinase activation. Thus in mouse fibroblast cell line over-expressing human EGFR (NRHER5), immunoprecipitates of EGFR were shown to contain PI3-kinase activity (63,64). In A431 cells and MDA-MB-468 breast cancer cell lines, tyrosine phosphorylated ErbB3 was implicated in the activation of PI3-kinase upon EGF stimulation (65,66). In PC12 and A549 cells p120cbl was found to associate with both SH2 and SH3 domain of p85, and with activated PI3-kinase, upon EGF stimulation (67). Other studies in A431 cells demonstrated that the recently cloned docking protein, Grb2 Associated Binder 1 (Gab1), interacts with the p85 subunit of PI3-kinase following EGF (68). However, the mechanism by which EGF activates PI3-kinase in hepatocytes has remained unknown. Our initial study demonstrated three distinct p85-associated complexes: ErbB3-p85, Shc-p85, and a large complex Gab2-Grb2-SHP2-p85 formed upon EGF treatment in primary rat hepatocytes. The latter accounted for > 80% of total EGF-induced PI3-kinase activity (69), indicating a key role for the adaptor protein Gab2 in mediating EGF-induced PI3-kinase activation and DNA synthesis in primary hepatocytes. A detailed review of the Gab family proteins is found in section 1.3.

1.2.2.3. Lipid products mediate PI3-kinase downstream signaling

Activation of PI3-kinase triggers a rapid rise of cellular phospholipids, $PI(3,4)P_2$, and $PI(3,4,5)P_3$. Several molecular targets have been identified for $PI(3,4)P_2$, and $PI(3,4,5)P_3$. They are translocated and activated upon interaction of a pleckstrin homology (PH) domain in the target and the phosphoinositide (70). A well-documented case of a PI3-kinase downstream target is the Ser/Thr protein kinase, Akt/PKB, whose activity depends upon binding to a product of PI-3 kinase activation (71). Akt consists of an amino-terminal PH domain, a central kinase domain, and a carboxyl-terminal region containing critical serine and threonine phosphoacceptor sites. The PH domain of Akt promotes translocation to the plasma membrane by binding to $PI(3,4)P_2$ (72), leading to phosphorylation at Thr308 and Ser473 (73), which is required for full activation of this kinase. Further study identified that the recruitment of Akt to membranes alters its conformation and allows subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK-1) (74), a 63 kDa Ser/Thr kinase ubiquitously expressed in human tissues. PDK1 consists of an N-terminal kinase domain and a C-terminal PH domain. As purified or recombinant PDK1 only phosphorylated Thr308 of PKB (75) and not Ser473, it was assumed that the phosphorylation of Ser473 would be catalyzed by a distinct protein kinase, tentatively termed PDK2 (76). However, the identity of PDK2 has not been established to date.

PKB/Akt phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis, proliferation (increased cell number) and growth (increased cell size) (77). For example, Akt

can regulate cell proliferation through signals to the cell-cycle machinery. The cell cycle is regulated by the coordinated action of cyclin–cyclin-dependent kinase (CDK) complexes and CDK inhibitors (CKIs). Akt has an important role in preventing cyclin D1 degradation by regulating the activity of the cyclin D1 kinase glycogen synthase kinase-3β (GSK3β). After phosphorylation by GSK3β, cyclin D1 is targeted for degradation by the proteasome. AKT directly phosphorylates GSK3β, and blocks its kinase activity, thereby allowing cyclin D1 to accumulate (78). Akt can also negatively influence the expression of CKIs, such as KIP1 (also known as p27) and WAF1 (also known as CIP1 or p21) (79,80). The functional importance of these biochemical connections between Akt and the cell-cycle machinery is supported by experiments showing that inhibition of PI3-kinase or Akt, using pharmacological or dominant-negative strategies, leads to cell-cycle arrest in various systems (81,82).

Dephosphorylation of the PI3-kinase lipid products results in down-regulation of the signaling. Numerous studies have shown that a lipid phosphatase, PTEN, functions as an antagonist of PI3-kinase by specifically dephosphorylating the D3 phosphate of $PI(3,4,5)P_3$, and $PI(3,4)P_2$ (83). Cell lines derived from PTEN knockout mice have increased levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ compared with wild-type cells. Increased lipid levels in these cells correlate with the activation of PKB and other PI3-kinase targets, increased proliferative potential, and decreased sensitivity to cell death, whereas reintroduction of PTEN causes inhibition of PKB, growth suppression, and increased sensitivity to apoptotic

stimuli (for review see (84)). The SHIP phosphatases also act on $PI(3,4,5)P_3$, but remove phosphate from the 5-position rather than the 3-position, creating $PI(3,4)P_2$, which can also function as a second messenger like $PI(3,4,5)P_3$ (85). Thus both PTEN and SHIP reduce the level of $PI(3,4,5)P_3$ in cells, PTEN seems to have primary responsibility for controlling the mitogenic effects of phosphoinositides because it reduces the levels of all those phosphorylated at the D3 position.

1.2.2.4. Biological role of PI3-kinase in EGF signaling

PI3-kinase is activated by a variety of extracellular stimuli and has been implicated in a wide range of cellular processes such as mitogenesis, protein synthesis, membrane ruffling, cell transformation and cell cycle progression (86). Various studies have demonstrated the involvement of PI3-kinase in EGF signaling. For example, several studies suggested that the PI3-kinase pathway is important for EGF-induced mitogenesis in various cell lines (87-89), including our studies in primary rat hepatocytes, which demonstrated that the PI3-kinase, and not MAP kinase, pathway is necessary and sufficient to account for both insulin- and EGF-induced DNA synthesis (69,90). Upon EGF treatment, PI3-kinase was shown to be involved in the regulation of cell mobility in MTLn3 rat adenocarcinoma and MDA-MB-231 breast cancer cells (91,92). In NRK-52E renal epithelial cells, PI3-kinase activity is required for EGF to suppress proteolysis, an important process for controlling cell growth (93). A role for PI3-kinase was demonstrated in EGF-induced ovarian tumor cell metastasis through the modulation of cell surface molecule which binds a migration and matrix

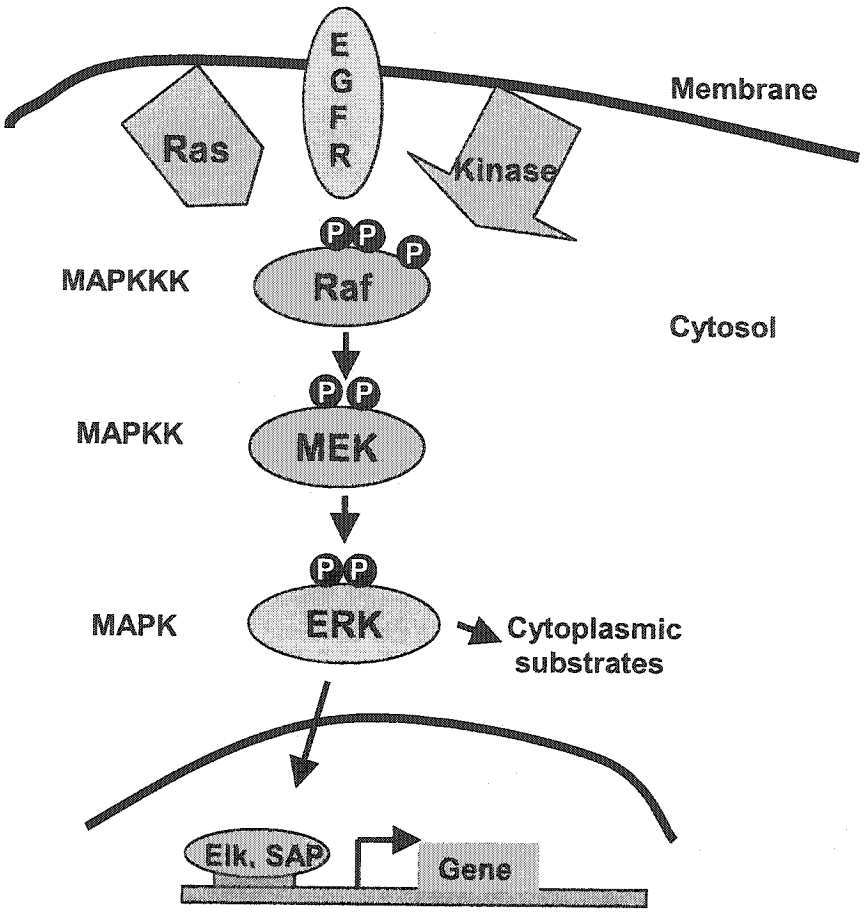
metalloproteinase (MMP)-9 (94). Furthermore, PI3-kinase was also found to play a role in anti-apoptotic effects of EGF signaling by reducing cellular caspase activity (95). EGF signaling via PI3-kinase/Akt has been proposed to enhance the expression of mitochondrial anti-apoptosis proteins such as Bcl-X_L (96). In addition, Akt has been shown to phosphorylate BAD and human pro-caspase 9, thereby rendering these proteins inactive in apoptotic processes (97,98).

1.2.3. MAPK pathway

1.2.3.1. Introduction

The highly conserved MAPK superfamily is one of the major mechanisms by which cells transduce extracellular EGF signals into a cellular response. Three important members of the human MAPK superfamily are extracellular signal-regulated kinase (Erk), p38, and c-Jun N-terminal kinase (JNK). All three MAP kinase families are activated by dual phosphorylation on adjacent threonine and tyrosine residues separated by a single amino acid (i.e. a tripeptide motif (99)). The intervening amino acid for Erk is glutamate (Thr-Glu-Tyr), for the p38 MAPK family, glycine (Thr-Gly-Tyr), and for the JNK family, proline (Thr-Pro-Tyr) (100-102). The classic MAPK cascade consists of three sequential intracellular protein kinase activation steps and is initiated when the first member, MAP kinase kinase kinase (MAPKKK), is activated (Fig. 3). MAPKKK is a Ser/Thr kinase that phosphorylates and activates MAPK kinase (MAPKK). Subsequently, MAPKK activates a MAPK by dual phosphorylation on adjacent threonine and tyrosine residues. MAPKs are proline-directed protein kinases, meaning that they phosphorylate serine or threonine residues that are neighbors to proline (100).

MAPKs then phosphorylate and activate other protein kinases, nuclear proteins, or transcription factors, such as Elk, SAP. Activation of the MAPK cascade is rapid and enables cells to respond to environmental changes in a prompt and regulated fashion. In this review, we focus on the activation of the Erks. This is initiated at the level of Ras and involves a linear cascade of protein kinases: Raf as MAPKKK, MEK as MAPKK and Erk as MAPK.



1-Figure 3. Schematic representation of the structure of MAPK pathways. The classic MAP kinase cascade consists of three sequential intracellular protein kinase activations: MAPKKK, MAPKK and MAPK. The Erk cascade, which is initiated at the level of Ras, consists of a linear cascade of protein kinases: Raf as MAPKKK, MEK (MAPK/Erk kinase) as MAPKK and Erk as MAPK.

1.2.3.2. Regulation of Ras-Raf-MEK-Erk cascade

Ras is a small, membrane-bound, GTP binding protein, whose activity is regulated by cycling between an inactive GDP-bound form and an active GTP-bound form (103). Activation of Ras by EGF is effected by recruiting growth-factor-receptor-bound protein 2 (Grb2) to the EGF receptor. Grb2 exists in the cytoplasm in a preformed complex with a second protein, Son of Sevenless (Sos), which can catalyze Ras GTP/GDP exchange. After EGF stimulation, the tyrosine phosphorylated EGF receptor binds the Grb2/Sos complex, translocating it to both plasma and endosomal membranes, predominantly the latter (104). This translocation is thought to bring Sos into close proximity with Ras, leading to its activation (105). Phosphorylation of Sos by the activated Erk pathway induces disassembly of the Sos complex and the termination of Ras activation.

Mammals possess three Raf proteins: Raf-1, A-Raf and B-Raf. The ubiquitously expressed Raf-1 is certainly the best studied, but probably least understood, isoform. Activated Ras can bind to Raf-1 with high affinity (106) through two domains in the Raf-1 N-terminus: the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) (107). The RBD alone is sufficient for the translocation of Raf-1 from cytosol to cell membrane, while the CRD is dispensable. However, the CRD improves the efficiency of activation (108). The present model postulates that a major function of Ras is the recruitment of Raf to the cellular membrane. Surprisingly, the Ras/Raf interaction is insufficient to activate Raf-1 kinase activity (109). Ras also supplies indirect regulatory signals.

One such signal is provided by PI3-kinase, whose phosphoinositide products can activate Rac, a small G-protein that binds and activates p21cdc42/rac1-activated serine/threonine kinase (PAK) (110). PAK-3 has recently been shown to phosphorylate Raf-1 on serine-338, one of the sites whose phosphorylation is required for activation (111). Furthermore, the Abl (112) and JAK (Janus kinase) (113) family tyrosine kinases effect Raf-1 tyrosine phosphorylation and activation. In addition, PI3-kinase may supply an inhibitory signal via Akt, which has been reported to suppress Raf-1 activity by phosphorylation of serine-259 (114). It has been shown that Raf interaction with 14-3-3 proteins is required to keep the Raf-1 kinase phosphorylated on serine-621, and therefore in its active state. These observations suggest that the 14-3-3 proteins represent other possible regulators of Raf-1 activity (115).

In the mitogen-activated Ras-Raf-MEK-Erk cascade, Raf usually activates the dual-specific kinases (kinases which can phosphorylate both threonine and tyrosine residues) MEK1 and MEK2 in order to activate the MAPKs: Erk1 and Erk2 (p42/p44). Although Raf can activate both MEK-1 and MEK-2 (also called MKK-1 and MKK-2) with similar efficacy *in vitro*, observations on Raf-1 bound to Ras suggest that Raf-1 seems to preferentially interact with, and activate MEK-1 rather than MEK-2 (116). In cells expressing normal MEK1, the kinase appears as a 45-kDa protein (117). The amino terminal end of the kinase has a negative regulatory domain, as deletion of these residues results in constitutive activation of MEK1. The catalytic activity is localized to the carboxyl terminus of the protein

(118). Both MEK1 and MEK2 have unique proline-rich sequences, which have been shown to be required for Raf-1 binding, phosphorylation, and activation of MEK1 (119). Raf-1 activation of MEK1 requires the phosphorylation of Serine residues 218 and 222 (120). Activated MEK then phosphorylates Erk1 and Erk2 on both tyrosine185 and threonine183 residue within the TPY motif (121). No substrates for MEK have been identified other than Erk1 and Erk2 (122). This high selectivity, in addition to the unique ability to phosphorylate both tyrosine and threonine residues, is consistent with a central role for this kinase in the integration of signals in the MAPK pathway. The complexity of this process is illustrated by a recent study showing that Rac and PAK are required for Erk activation by regulating the formation of MEK1-Erk complexes (123).

1.2.3.3. The Biological role of MAPK in EGF signaling

The MAPK pathway is a major mediator for various EGF functions, including cell proliferation, cell survival, differentiation, morphogenesis, and cell mobility. For example, in fetal mouse submandibular glands, Erk1/2 signaling is responsible for the stimulatory effect of EGF on branching morphogenesis (124). NR6 fibroblasts, expressing full-length wild-type EGFR, required both calpain and Erk activation for EGF-induced adhesion and motility (125). In addition, the MAPK downstream effector, p90rsk, phosphorylates the transcription factor CREB, thus activating the promoters of several anti-apoptotic proteins (126). Our work in hepatocytes has shown that the MAPK pathway is required for the augmentation of basal c-*myc* messenger RNA (mRNA) levels by EGF whereas it was not involved in EGF-induced DNA synthesis (90).

The MAPK pathway is generally viewed as the primary effector of the proliferative response of cells to EGF (127). However recent studies suggest that this may not be the case in all cell lines. For example, in a human pancreatic carcinoma cell line (PANC-1) the activation of MAPK alone was not sufficient to induce cell proliferation. On the other hand, treatment with EGF resulted in marked, MEK-dependent increase of directed cell migration (128). Pretreatment of human airway smooth muscle (ASM) cells with the PI3-kinase inhibitors wortmannin and LY-294002 significantly reduced EGF-induced DNA synthesis without affecting MAPK activation (129). Indeed, evidence is accumulating, including work from our laboratory, to implicate PI3-kinase as a key enzyme involved in the EGF-induced mitogenesis in various cell lines including normal hepatocytes (69,89,90,130).

1.2.4. Src family kinases

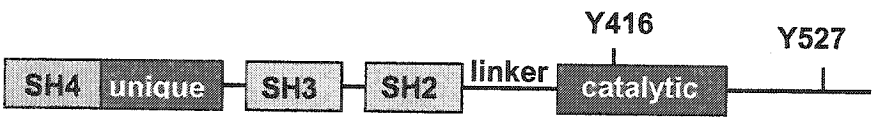
1.2.4.1. Introduction

The prototype member of the Src family of protein tyrosine kinases was first identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous sarcoma virus (RSV) (131). Further investigation into the origin of v-Src revealed that this oncogene was a modified version of a normal cellular gene, c-Src (132). A major breakthrough in understanding the function of the Src protein came from the finding that Src possesses protein tyrosine kinase activity (133). There are presently 8 members of the non-receptor Src family tyrosine kinases, including Src, Lyn, Fyn, Lck, Hck, Fgr, Blk, and Yes. Src, Fyn and Yes are expressed in

most tissues whereas the expression pattern of the other Src family members is mostly restricted to cells of hematopoietic origin (reviewed in (134)). Src family kinases participate in various signaling pathways that control a diverse spectrum of biological activities: proliferation, adhesion, spreading, migration, cell cycle progression, apoptosis, gene transcription and cell differentiation (135).

1.2.4.2. Structural domains and regulation of Src family kinases

Src protein tyrosine kinases (PTKs) are 52–62 kDa proteins which share roughly 60% amino acid identity and possess the same basic modular architecture (Fig. 4). These elements (from N- to C-terminus) are the Src homology (SH) 4 domain, which directs myristylation, resulting in membrane localization, followed by a non-conserved unique region, then an SH3 domain which directs binding to polyproline-rich sequences, an SH2 domain that binds phosphotyrosine, a linker region, a catalytic domain (SH1), and a C-terminal tail which is critical for negative regulation of the kinase (136).



1-Figure 4. Schematic structure of Src family tyrosine kinases. The two inhibitory intramolecular interactions are the SH3-linker region and SH2-Y527. Phosphorylation of Y416 in the catalytic domain activates Src, while phosphorylation at Y527 in the C-terminal tail is inhibitory.

The molecular basis for the tight regulation of Src family kinases recently came into sharp focus with the solution of the crystal structures of Src and Hck (137). The crystal structures include the SH3, SH2, and catalytic domains, and the negative regulatory tail. Both the SH3 and SH2 domains lie on the side of the kinase domain opposite the catalytic cleft. There are two main intramolecular contacts that stabilize the inactive conformation: (i) The SH3 domain interacts with sequences in the catalytic domain, as well as with sequences in the linker region that lie between the SH2 and catalytic domain (137); (ii) The SH2 domain interacts with pTyr 527 (Src) and adjacent residues in the negative regulatory tail (136). Numerous *in vitro* and *in vivo* studies over the past two decades suggest that there are major two mechanisms involved in the activation of src family kinases. The first is by phosphorylation or dephosphorylation of critical tyrosine residues within the molecule. The second is by disruption of repressive intramolecular interactions involving the SH3 and SH2 domain. In Src, there are two tyrosine residues, Y416 in the kinase domain and Y527 in the C-terminal tail (the numbering is based on chicken Src, in human, they are Y419 and Y530 respectively) whose phosphorylation is important in regulating kinase activity. Autophosphorylation at Y416 increases kinase activity, which appears to be an intermolecular process. Phosphorylation of the Y527 which is carried out by another non-receptor tyrosine kinase, CSK (C-terminal Src Kinase) inhibits kinase activity (138). Several lines of evidence indicate that loss of Y527 phosphorylation leads to activation of Src catalytic activity (136): (i) Mutation of Y527 results in constitutive activation of c-Src (139); (ii) Y527 and several amino

acids surrounding this residue are deleted in v-Src and similar truncations of c-Src cause activation of this enzyme (140); (iii) Disruption of the *csk* gene results in activation of at least three Src PTKs (141). These results and others support a model whereby Csk-mediated tyrosine phosphorylation of the C-terminal tail promotes an intramolecular interaction between the SH2 domain and the phosphorylated tail, keeping the kinase in a closed, inactive conformation.

The SH3 and SH2 domains are both involved in stabilizing the catalytic domain in the inactive conformation. Src kinases can be activated by displacing the phosphorylated tail from the SH2 domain, even in the absence of dephosphorylation of Y527. For example, stimulation of cells with PDGF results in activation of Src kinases. Activation is mediated by a PDGF receptor autophosphorylation site, which becomes a high affinity binding-site for the SH2 domain of Src. Preferential binding of the SH2 domain to this autophosphorylation site displaces the C-terminal tail, removing its negative regulatory influence (142). Src kinase can also be activated through displacement of the SH3 domain. In the case of Hck, displacement of the SH3 domain from its catalytic core releases repressive interactions, thus stimulating Hck autophosphorylation at Y416 and subsequent enzymatic activity (143). Similarly, binding of the SH3 domain to a polyproline-containing peptide fragment of Sin (144), FAK (145), or to p130Cas (146) is sufficient to induce activation of Src kinases.

Taken together, these studies show that there are multiple ways to activate Src family kinases, including displacement of the intramolecular interactions of the SH2 or SH3 domains by high-affinity ligands, dephosphorylation of pY527 by a tyrosine phosphatase, and phosphorylation of Y416. More than one mechanism is often involved in Src activation in response to a single stimulus. Individual Src family members may be more sensitive to one kind of regulation depending upon the specific context.

1.2.4.3. Activation of Src by EGF

Although association between Src and EGF receptor has been observed in some cell systems over-expressing the receptor, such as MDA 468 breast tumor cell lines (147) or A431 cervical carcinoma cells (148), the nature of this interaction and its role in the initial activation is unclear. Several groups (147,149,150) have demonstrated that glutathione-S-transferase (GST) fusion proteins containing the Src SH2 domain precipitate activated EGF receptor from crude extracts and bind the receptor in immunoblot overlay assays, suggesting that Src binding is direct and involves the SH2 domain of Src interacting with a tyrosine-phosphorylated site on the receptor. In addition, binding of this SH2 domain to the receptor is blocked by several different synthetic peptides, each encompassing different phosphotyrosine residues of the receptor (147). However, confirmation of SH2 domain binding to these sites *in vivo* is lacking.

To date, there is no evidence for unique phosphorylation(s) on Src following EGF stimulation. However, Src-dependent phosphorylation of several tyrosine residues in EGF receptor was reported (150,151). The initial activation of Src by

EGF has been postulated to be mediated by Src's interaction with the EGFR. Binding of a phosphorylated peptide encompassing Y891 of EGFR to Src increases its catalytic activity; however, since Y891 has been proposed to be phosphorylated by Src, a conundrum exists as to how Src is initially activated (151). One potential explanation is that another EGFR family member (e.g. ErbB2 or Neu), which heterodimerizes with EGFR, could be responsible for Src activation. For example, Neu can heterodimerize with the EGFR and become activated after EGF stimulation. Src can bind to activated Neu *in vivo*. An interaction between the Src SH2 domain and Neu has been shown *in vitro* (152). Thus one possibility is that EGF would induce heterodimerization and activation of Neu and the EGFR. Src could bind to tyrosine-phosphorylated Neu resulting in activation of Src. Src could then phosphorylate the EGFR and provide a binding site for additional Src molecules. However, this model is only applicable in cells expressing Neu.

1.2.4.4. Biological effect of Src in EGF signaling

Several studies have implicated Src family kinases in EGF-induced DNA synthesis. In murine fibroblasts, over-expressing varying levels of c-Src (2-30 times above endogenous) exhibited EGF-induced DNA synthesis that was 2-5 fold higher than in normal cells (153). Furthermore, cell lines over-expressing structurally-altered forms of c-Src failed to potentiate the EGF-induced DNA synthesis as observed with wild-type c-Src (154). Microinjection of DNA encoding a kinase-inactive form of Src or Fyn or a neutralizing antibody that recognizes a conserved sequence in the C-proximal tail of Src, Fyn, and Yes

inhibited the induction of DNA synthesis by EGF or PDGF (155). Further study showed that the SH3 domain of Src family kinases appears to play an important role in PDGF- and EGF-induced DNA synthesis because mutation of the SH3 domain of Src interfered with the induction of DNA synthesis by these growth factors (156). However, the exact mechanism by which Src family kinases mediate EGF-induced DNA synthesis has not yet been identified.

Other functions of Src family kinases in EGF signaling have been identified. For example, the Src family kinase was reported to be involved in EGF-dependent actin cytoskeleton reorganization through the phosphorylation of p190RhoGAP. This protein associates with p120RasGAP through its SH2 domains and displays Rho and Rac GTPase-activating activity. EGF induced a rapid and transient translocation of 190Rho and p120Ras into cytoplasmic, arc-like structures (regions of the greatest stress fiber concentration). This effect was inhibited by over-expressing a kinase-inactive form of Src. In this situation there was a corresponding decrease of p190RhoGAP phosphorylation. Conversely, over-expression of kinase-active Src increased the rate of appearance and number of cells exhibiting EGF-induced arcs as well as tyrosine phosphorylation of p190Rho (157). c-Src is also implicated in EGF-induced migration. c-Src is activated when subconfluent NBT-II rat carcinoma cells are treated with EGF. Under these conditions, the cells undergo an epithelial-to-mesenchyme transition, dissociate from cell clusters, and become motile. Cell dissociation and scattering induced by EGF is inhibited following microinjection of kinase-inactive

Src, and over-expression of c-Src causes a subpopulation of cells to undergo spontaneous cell dissociation and to display increased sensitivity to EGF (158). Finally, Src and EGFR appear to have synergistic roles in tumorigenesis. Thus over-expression of EGFR, Neu and Src was found in a significant proportion of human breast tumors (159,160). Fibroblasts that over-express Src and EGFR show increased tumor formation in nude mice in response to EGF (161).

1.2.5. Endocytosis and intracellular sorting of EGF signaling

Ligand binding to the EGFR initiates dimerization, receptor autophosphorylation, and the activation of signal transduction pathways as well as trafficking events that relocalize the receptor from the cell surface to intracellular compartments. It is now clear that prior to ligand binding, EGFR can be found localized in specialized plasma membrane microdomains known as caveolae (162,163). Following the EGF treatment, EGFR undergoes either homo- or hetero-dimerization. Thereafter EGF receptors migrate out of the caveolae into clathrin-coated pits and coated intracellular vesicles are then formed from the coated pits (164,165). However, this mechanism does not apply to all the cell types. For example, a recent study in A431 cells demonstrates that Grb2 is implicated in the internalization of EGFR in a clathrin-independent manner (166). EGFR, internalized by both clathrin-dependent and clathrin-independent mechanisms, was then delivered to early endosomes, a heterogeneous population of membrane compartments with tubular–vesicular morphology that is located mainly at the cell periphery (167,168). Endosomal EGFRs can either recycle to

the plasma membrane or progress to late endosomes and multivesicular bodies where degradation appears to be initiated (169).

It was first demonstrated that EGF-EGFR endocytosis might function to attenuate signals deriving from the cell surface (170). Thus several studies showed that an increased mitogenic response induced by EGF correlated with a decrease of receptor internalization (171,172). However, it has been established by our work and that of others that activated EGFR accumulates in hepatic endosomes (173-175), and that events associated with signaling occur in this compartment. In liver, EGFR tyrosine phosphorylation was markedly but transiently reduced at the cell surface shortly after the administration of saturating doses of EGF. However, it was augmented and prolonged in the endosomes for up to 30 min after EGF injection (176). Several receptor-interacting proteins are also found in endosomes, which is consistent with the presence of phosphorylated internalized receptors. In particular it was shown that there is an increasing association over time of Shc, Grb2 and Sos with the internalized EGFR whose tyrosine phosphorylation state correspondingly increased during the course of internalization (104).

There has been increasing evidence implicating a role for internalized EGFR in influencing downstream signaling molecules (177). Of particular interest is the well-characterized data linking endocytosis of EGFR with Erk1/2 activation. In fibroblasts, internalized EGFRs participate in the activation of Ras (178) and in

NRK cells, an intact actin cytoskeleton has been reported to be necessary for EGF-mediated transport of caveolin from the cell surface into early endosomes as well as activation of the MAPK pathway (179). Other evidence has been provided by the work of Viera and colleagues in HeLa cells over expressing dominant negative (K44A) dynamin (180). In such cells, which are specifically defective in receptor-mediated endocytosis (181), a decrease in EGFR tyrosine phosphorylation was found, consistent with earlier observations showing the hyperphosphorylation of endosomal versus PM EGFR (104). Furthermore in these cells a decrease in MAPK phosphorylation and activation was seen along with augmented phosphorylation of PLCγ and Shc. Key evidence for the importance of receptor internalization in EGF signaling comes from three recent studies. (i) Molecules involved in the MAPK cascade were localized in highly purified endosomes from rat liver (182). Using the same purified fraction, the authors also showed that administration of EGF led to redistribution of Raf-1 from plasma membrane into endosomes; (ii) In addition, using a combined imaging microscopy and fluorescence resonance energy transfer (FRET), Sorkin et al. elegantly demonstrated that activated EGFR interacts with Shc and Grb2 in membrane ruffles and endosomes (183). These studies provide evidence that EGFR endocytic trafficking is required to trigger MAPK signaling pathways. (iii) the importance of endosomal signaling was recently highlighted by studies of Zhang *et al* in which the endosomal EGFR was exclusively activated. Under these circumstances they observed that endosomal EGFR signaling is sufficient to activate all the major signaling pathways, including the PI3-kinase and MAPK

pathways, with consequences for cell proliferation and survival (184). In this thesis, we detected the association of PI3-kinase activity with Shc or ErbB3 in plasma membrane and endosomes. Therefore, more and more evidence supports the view that there are activated signaling complexes in endosomes, however, the specificity and physiological role of these signaling complexes need to be explored further.

1.3. Gab family proteins

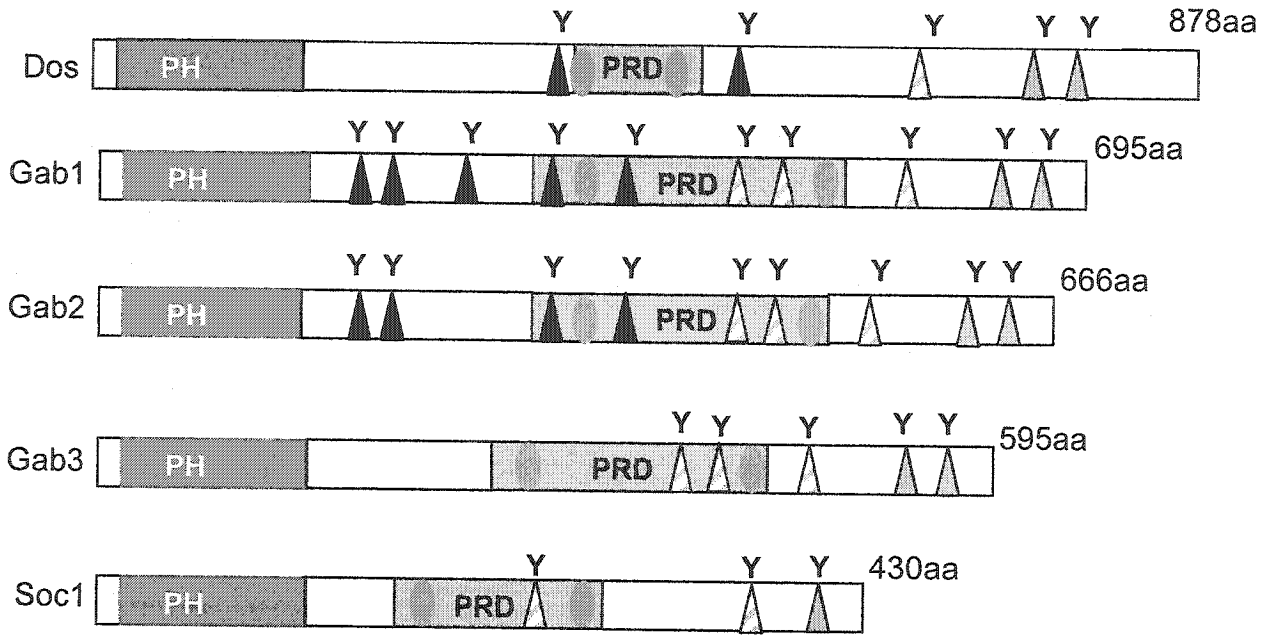
1.3.1. Introduction

The Gab (Grb2-associated binder) proteins, which include mammalian Gab1, Gab2, Gab3, the *Drosophila* homolog DOS (Daughter Of Sevenless), and the *Caenorhabditis elegans* homolog Soc1 (Suppressor-Of Clear), constitute a family of scaffolding proteins closely related to insulin receptor substrates (IRS-1, IRS-2, IRS-3, IRS-4), fibroblast growth factor substrate (FRS2), linker of T cell (LAT) and downstream of kinase (Dok) (reviewed in (185-187)). All Gab family proteins have in common a central proline-rich domain (PRD) and multiple potential binding sites for the SH2 domains of PI3K-p85, SHP2, PLCγ or Crk (Fig. 5). These proteins lack enzymatic activities, but after activation of receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors, they become phosphorylated on tyrosine residues, providing binding sites for multiple proteins involved in signal transduction. In this manner, Gab family proteins act to potentiate and diversify the downstream signals from receptors by virtue of their ability to assemble multiprotein complexes. Further studies in various cell

systems have revealed that Gab family proteins are involved in multiple growth factor and cytokine signaling pathways (Table 1).

Table 1. Role of Gab family proteins in multiple growth factor and cytokine signaling pathways

Ligand	Receptor	Gab family protein involved			Function	Cell type	References
		Gab 1	Gab 2	Gab 3			
EGF	EGF receptor	Yes	Yes	?	Proliferation, neoplastic progression	A431, SHE, hepatocyte	(68,69,188)
Flt3L	Flt3	Yes	Yes	?	?	Baf3	(189)
M-CSF	Fms	?	Yes	Yes	Macrophage differentiation	FD-Fms	(190,191)
Insulin	Insulin receptor	Yes	?	?	?	A431, HepG2	(68,192)
HGF	Met	Yes	Yes	?	Epithelial morphogenesis, cell survival, DNA repair, and branching tubulogenesis	MDCK	(193-195)
PDGF	PDGF receptor	Yes	?	?	?	VSMC	(196)
NGF	TrkA	Yes	Yes	?	Neurite outgrowth, DNA synthesis, and survival	PC12	(197,198)
SCF	Kit	Yes	Yes	?	Mast cell development	BMMC, MO7E	(199,200)
Anti-IgM	B cell receptor	Yes	Yes	?	Differentiation	Ramos, WEHI-231, K562	(201,202)
EPO	EPO receptor	Yes	Yes	?	?	HCD57	(203)
IL-6	Gp130	Yes	?	?	Proliferation	MH60	(204,205)
IFN-α, γ	IFN-α, γ receptor	Yes	?	?	?	Hep3B	(205)
IL-15	IL-15 receptor	?	Yes	?	?	T cells	(206,207)
IL-2	IL-2 receptor	?	Yes	?	?	T cells	(206)
IL-3	IL-3 receptor	Yes	Yes	Yes	?	TF-1, BaF3	(190,205, 208)
TPO	Mpl	Yes	Yes	?	Cell proliferation	BAF3, MK, UT-7	(209,210)
Prolactin	Prolactin receptor	?	Yes	?	?	HC11	(211)



1-Figure 5. Schematic domain structures of Gab family proteins: three mammalian Gab proteins (Gab1, 2 and 3), *Drosophila* DOS and *C. elegans* Soc1. All Gab family proteins consist of a N-terminal pleckstrin homology (PH) domain, a central proline-rich domain (PRD) and multiple potential binding sites favored by various SH2 domain containing proteins (△p85-SH2 binding sites, ▲ Crk/PLCγ-SH2 binding sites, ▲SHP2-SH2 binding sites). Grb2 SH3 potential binding sites are indicated as ●

1.3.2. PH domain

The PH domains were first identified as regions that share homology with an internal repeat of pleckstrin, a major substrate of protein kinase C in platelets

(212). These domains are composed of ≈100 amino acids with seven β strands, which form two antiparallel β sheets with a carboxy-terminal α helix. PH domains are best known for their ability to bind phosphoinositides and therefore contribute to the membrane targeting of proteins involved in downstream signaling from PI3-kinase (reviewed in (213)).

The Gab1 PH domain preferentially binds to $PI(3,4,5)P_3$, which is a product of PI3-kinase (214,215). PI3-kinase activation is sufficient to recruit either full-length Gab1, or the isolated Gab1 PH domain to the plasma membrane (215,216). Moreover, both EGF- and serum-induced membrane translocation of Gab1 can be blocked by PI3-kinase inhibitors (214). To date studies of Gab1 lacking a functional PH domain have indicated that the PH domain of Gab1 is responsible for its membrane localization and function in signaling include the following: (i) failure to play a role in Met-dependent branching tubulogenesis (216,217); (ii) requirement for localizing Gab1 to sites of cell-cell contact (216,217); and (iii) failure to activate Jun kinase (JNK) following EGF stimulation (214). The Gab1 PH domain has also been shown to be able to act as a dominant-negative mutant of Gab1 and inhibited an EGF-stimulated increase in JNK activity (214). Interestingly, expression of Gab1 lacking its PH domain was found to be associated with neoplastic progression of Syrian hamster embryo (SHE) cell cultures. A recent study showed that a form of Gab1 lacking the amino-terminal 103 amino acids ($Gab1^{\Delta 1\text{-}103}$), which corresponds to loss of most of the PH domain, retained the ability to be phosphorylated in an EGF-dependent manner

and to associate with the EGF receptor and SHP-2 upon EGF stimulation. The reason why over-expression of Gab1$^{\Delta 1\text{-}103}$, but not of full-length Gab1, resulted in the up-regulation of soft agar colony formation in response to EGF stimulation remains to be clarified. Together these observations suggest that Gab1 is a key element in EGF signal transduction and that its transforming potential may be activated by the loss of its amino-terminal PH domain (218). Studies on Gab2 have been less abundant and one of our research goals was to define the functional significance of the PH domain of Gab2.

1.3.3. Binding of p85

Gab proteins bind the p85 subunit of PI3-kinase through three consensus YXXM motifs, which are present in all mammalian Gab proteins. Only one such site was found in DOS or Soc1 (Fig. 5). It has been shown that the physical association between p85 and Gab1 or Gab2 is crucial in activating the PI3-kinase/Akt signaling pathway induced by a variety of stimuli (219-222). Mutation of the p85-binding sites of mammalian Gab1 and Gab2 resulted in abrogation of several signaling systems: (i) the inducible expression of a Gab2 mutant unable to bind PI3-kinase markedly impaired IL-3-induced Akt activation and cell growth (223); (ii) the expression of a Gab1 mutant lacking the binding sites for PI 3-kinase enhanced apoptosis and diminished the protective effect of NGF (219); (iii) a Gab1 mutant unable to bind p85 was defective in potentiating EGFR-induced JNK activation (214); (iv) whereas over-expression of Gab1 potentiated FGF-induced Akt activity, over-expression of the p85-binding mutant of Gab1 resulted in decreased Akt activation (221); (v) our work in hepatocytes showed that the

Gab2 mutant (Δp85Gab2), unlike wild-type Gab2, was unable to potentiate PI3-kinase, Akt activation, or DNA synthesis following EGF stimulation. All these results indicate that Gab–p85 interactions play a key role in EGF-induced activation of the PI3-kinase/Akt pathway in mammalian cells. Moreover, since the activation of PI3-kinase leads to the production of $PI(3,4,5)P_3$, which in turn can bind the PH domain of Gab1 and presumably promote further activation of PI3-kinase, a positive feedback loop could be formed to amplify the signals through Gab1 (214).

1.3.4. SHP2 binding

SHP2 binds to Gab family proteins through tyrosine phosphorylated consensus motifs (YXXV/I/L) located in the C-terminal ends of Gab proteins (Fig. 5). Indeed, all mammalian Gab proteins, as well as the *Drosophila* DOS and *C. elegans* Soc1, have been shown to bind SHP2 (or its homologs) (190,224-227), indicating that recruitment of SHP2 is conserved through evolution among all Gab family members. The function of the Gab-SHP2 interaction has been extensively studied using mutants of Gab family proteins lacking SHP2 binding sites. These studies reveal a generally positive role of SHP2 in Gab-mediated signal transduction. For example, mutant DOS bearing a Y to F mutation at either of the two CSW (SHP2 homolog)-binding sites is unable to function during sevenless signaling and fails to rescue the lethality associated with *dos* loss-of-function mutations (228). SHP2 association with Gab1 is required for Met-dependent morphogenesis (229), EGF and lysophosphatidic acid-induced MAPK activation (227,230) and M-CSF-induced macrophage differentiation (191). In our

work we identified a positive role for SHP2 in Gab2-potentiated EGF-induced MAPK activation in primary rat hepatocytes. A recent study showed that epidermal cells, expressing mutant Gab1Y627F, deficient in SHP-2 binding, or the dominant-negative SHP-2C459S, displayed reduced basal levels of active Ras and downstream MAPK proteins while manifesting a blockade of proliferation and the initiation of differentiation (231). It is unclear how the Gab-SHP2 interaction leads to activation of downstream signaling. One hypothesis is that association between the phosphotyrosine(s) of Gab proteins and the SH2 domains of SHP2 is essential for up-regulating the phosphatase activity of SHP2, leading to activation of the MAPK cascade and subsequent biological responses. However, the substrate(s) and downstream effector(s) for the SHP2 phosphatase which lead to MAPK activation have yet to be identified.

Both Gab1 and Gab2 have been identified as substrates for SHP2 using an *in vitro* phosphatase assay (200). Consistent with this are recent reports identifying a negative effect of SHP2 binding on Gab protein tyrosine phosphorylation. Thus two studies have shown that inactive forms of SHP2 markedly increased EGF-stimulated Gab1 tyrosine phosphorylation and PI3-kinase activation (232,233). The negative regulation of EGF-dependent PI3-kinase activation by SHP2 appears to be through the dephosphorylation of Gab1 p85 binding sites (233). In agreement with this study we have found that, in rat hepatocytes, a relatively minor increase of ΔSHP2Gab2 phosphorylation compared to WTGab2 was accompanied by a more noticeable augmentation of PI3-kinase activity and

DNA synthesis, suggesting that SHP2 is involved in the dephosphorylation of specific p85 binding sites on Gab2.

1.3.5. Grb2 binding

All Gab proteins have been found to associate with the SH3 domain-containing protein Grb2 (68,190,224). The binding sites for Grb2 have been mapped on Gab1: a canonical Grb2-binding site with PXXPXR motif and an atypical Grb2-binding site with a PX_3RX_2KP motif (234-236). Both sites are conserved among all Gab members, as well as in several other signaling proteins such as Slp76 and Sos (234-236).

The function of Grb2 binding to Gab family proteins is still not clear. Gab family proteins do not appear to associate directly with various receptor tyrosine kinases (except Gab1, which appears to specifically bind to the Met receptor). (69,235). Previous reports suggest that Grb2 may mediate an indirect association of Gab family proteins with receptor tyrosine kinases to promote their phosphorylation. This hypothesis is supported by the fact that the association between Gab1 and EGF receptor is abolished by mutations either at the Grb2-binding site of Gab1 or that of EGF receptor (214-216). Moreover, the failure of Gab1 to be tyrosine phosphorylated in response to EGF in fibroblasts isolated from mice expressing a null mutant of Grb2 (237), provides genetic evidence that Gab1 is recruited to EGF receptor through a Grb2-dependent mechanism. Interestingly, in IL2 and IL3 receptor signaling, the common β-chain of the receptors does not contain a binding site for Grb2, but does contain a binding site for Shc. Gab2 was found to be recruited to the receptor through a

Gab2-Grb2-Shc pathway (223), a mechanism that is also Grb2-dependent but requires a fourth protein to achieve this goal. It seems that other SH2 and SH3 domain containing proteins may also be able to function as adapters.

1.3.6. Other interactions

Gab1 and Gab2 contain several YXXP motifs which are potential binding sites for the SH2 domain of PLC or Crk family proteins (238). Binding of Gab1 to Crk, CrkL and PLC have been reported (194,234,239-241). Coupling of Gab1 with Crk in response to Met activation correlates with anchorage-independent growth and JNK activation in cells transformed by the Met receptor oncoprotein (239,240). Moreover, binding of Gab1 to CrkII is required for the branching morphogenic program downstream of Met (242). Gab2 has also been found to interact with CrkL in a yeast two-hybrid screen, and also in human T lymphocytes in response to IL2 (243,244). However the functional significance of the Gab2-CrkL association has not yet been addressed. Interestingly, potential Crk-binding sites are completely absent in Gab3 protein, thus providing a possible molecular mechanism for the functional differences among these family members.

1.3.7. Biological function

Various studies showed that Gab family proteins seem to play a non-redundant role in RTK signaling. First, they exhibit overlapping but distinct expression patterns (224). Gab1 appears to exhibit the most widespread expression. It is found in most tissues examined, including brain, heart, lung, kidney, pancreas, spleen, thymus and uterus of the adult mouse, and is expressed at an early

stage (ES cell) during development (68). Gab2 expression is relatively minor in most tissues compared to Gab1. However, it is prominently expressed in a number of hematopoietic progenitor cell lines, such as BAF3, FDC-P1 (224). The expression pattern of Gab3 is also relatively restricted to the hematopoietic system where it is expressed in spleen, thymus, and a number of hematopoietic cell lines (190). These results suggest that the function of each Gab protein may be specialized or restricted to certain signaling pathways or tissues. However at this stage one cannot exclude the possibility that these gene products have functional redundancy and therefore the ability to compensate for defects of other co-expressed Gab proteins.

Second, Gab1 and Gab2 knock-out mice exhibit different phenotypes (245-247). Consistent with early and widespread expression during development, a targeted disruption in Gab1 is embryonic lethal in mice (245). These mice die between E13.5 and E18.5 and display developmental defects in the heart, placenta and skin. Furthermore, $Gab1^{-/-}$ fibroblasts have markedly reduced MAPK activity in response to IL-6, EGF or PDGF stimulation, suggesting a role for Gab1 in mediating MAPK activity via multiple growth factor receptors (245). Among Gab family members, Gab1 is unique in its ability to associate directly with the phosphorylated Met receptor. Interestingly, Gab1-deficient mice have a phenotype similar to those mice harboring mutations in the HGF and c-Met genes. These mice die at a similar embryonic stage as the $Gab1^{-/-}$ animals as a result of insufficient placenta development. They also display a reduced liver size and have defects in the migration of muscle precursor cells (246,248- 250).

These results provide genetic evidence that Gab1 is essential for HGF-Met signaling, and Gab2 and/or Gab3 are unable to compensate for the function of Gab1 *in vivo*.

In contrast to Gab1 knock-out mice, Gab2 knock-out mice develop normally and are generally healthy. Although Gab2 protein is quite abundant in hematopoietic cells and the functional importance of Gab2 in IL2, IL3 and M-CSF signaling pathways has been well established in tissue culture systems (191,224), no apparent defects in the development of hematopoietic lineages were observed in Gab2 knock-out mice (247). However, closer examination demonstrates that these mice have impaired allergic reactions. IgE receptor signaling is defective in bone marrow-derived mast cells from Gab2 $^{-/-}$ mice, mainly due to the inability to induce PI3-kinase activation in these cells. Clearly, Gab2 has some functions distinct from Gab1 *in vivo*. It should be noted that in the Gab2 knock-out mice, the Gab2 targeting vector was constructed to delete the first exon of Gab2, presumably eliminating the gene product of *gab2*. However a truncated protein, possibly arising from a cryptic promoter in the first intron of the Gab2 gene, was found in Gab2 $^{-/-}$ cells (247). It is possible that this truncated protein may partially compensate for full length Gab2. On the other hand, compensation by other Gab family members also might account for the lack of phenotype in the Gab2 knock-out animals.

Recent work showed that Gab family proteins might be involved in oncogenic signaling. A marked over-expression of Gab2 in a subset of breast cancer cell

lines relative to normal breast epithelial strains has been observed (251). Moreover, bone marrow myeloid progenitors from Gab2 (-/-) mice are resistant to transformation by BCR/ABL, whereas lymphoid transformation is diminished as a consequence of markedly increased apoptosis. Also the augmentation of BCR/ABL-evoked PI3-kinase/Akt and Ras/Erk activation is also impaired in Gab2 (-/-) primary myeloid and lymphoid cells, suggesting a key role of Gab2 in the transformation by BCR/ABL (252). Moreover, Gab1 was also required for the efficient transformation of Fr3T3 fibroblasts by the Met oncoprotein (253). All these observations indicate that Gab family proteins act as important mediators in tumorigenesis.

In summary, EGF signals by binding to and activating its cell surface receptors which undergo a variety of ligand-stimulated receptor homo- and heterodimerization events, as well as internalization. These in turn allow the generation of a broad range of intracellular signals and thus a variety of cellular responses. The activated EGFR leads to the activation of a number of downstream signaling pathways, including the PI3-kinase and Ras-Raf-MAPK pathways, as well as the activation of Src family kinases. Numerous studies have linked EGF action to organ repair as evidenced by EGF-induced mitogenesis in adult liver. The aim of the research in this thesis was to identify the signaling mechanisms involved in EGF-induced hepatic mitogenesis.

1.4. References

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CHAPTER 2

EGF-induced PI3-kinase activation and DNA synthesis:

Identification of Gab2 as the major mediator in rat hepatocytes

2.1. Preface to Chapter 2

EGF has been identified as a hepatic mitogen and can induce DNA synthesis in cultured primary hepatocytes. Previous work from our laboratory demonstrated that PI3-kinase, not MAPK, is necessary and sufficient for EGF-induced DNA synthesis in primary rat hepatoctyes. Thus understanding the mechanism of EGF-induced PI3-kinase activation is key to understanding EGF-induced mitogenesis. It has been shown that PI3-kinase is activated by recruiting its p85 subunit to tyrosine phosphorylated (PY) proteins. The PY-proteins which recruit p85 and activate PI3-kinase following EGF treatment vary in different cell types. Up to the time of our work the identities of the PY-proteins, produced by EGF treatment of primary hepatocytes, were unknown. The aim of our work, presented in this Chapter, was to identify the EGF-dependent PY-proteins of primary rat hepatocytes, which recruit and activate PI3-kinase and are hence crucial to the mechanism of EGF-induced mitogenesis in these cells.

2.2. Summary

In previous work we showed that the PI3-kinase, not the MAP kinase, pathway is necessary and sufficient to account for insulin- and EGF-induced DNA synthesis in rat hepatocytes. Here, using a dominant-negative p85, we confirm the key role of EGF-induced PI3-kinase activation, and sought to identify the mechanism by which this is effected. Our results show that EGF activates PI3-kinase with a time course similar to that of the association of p85 with 3 principal phosphotyrosine proteins (i.e. PY180, PY105, and PY52). We demonstrated that each formed a distinct p85-associated complex. PY180 and PY52 each constituted about 10% of EGF-activated PI3-kinase whereas PY105 was responsible for 80%. PY105 associated with Grb2 and SHP-2, and although it behaved like Gab1 none of the latter was detected in rat liver. We therefore cloned a cDNA from rat liver which was found to be 95% homologous to the mouse Gab2 cDNA sequence. Using a specific Gab2 antibody we demonstrated its expression in and association with p85, SHP-2 and Grb2 upon EGF treatment of rat hepatocytes. Gab2 accounted for most if not all of the PY105 species since immunoprecipitation of Gab2 with specific antibodies demonstrated parallel immunodepletion of Gab2 and PY105 from the residual supernatants. We also found that the PI3-kinase activity associated with Gab2 was totally abolished by dominant-negative p85. Thus Gab2 appears to be the principal EGF-induced PY-protein recruiting and activating PI3-kinase and mitogenesis.

2.3. Introduction

Phosphatidylinositol 3-kinase (PI3-kinase) is an enzyme that phosphorylates the D-3 position of the inositol ring of PI to produce three novel phosphoinositides: phosphatidylinositol 3-monophosphate [PI(3)P], phosphatidylinositol 3,4-biphosphate [$PI(3,4)P_2$] and phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$] (1, 2). This enzyme is a heterodimer of a 110kD (p110) catalytic subunit and an 85kD (p85) regulatory subunit (3). p85 has two SH2 domains that bind to tyrosine-phosphorylated sites and an SH3 domain which binds proline-rich sequences on receptors or docking proteins (4, 5). It has been demonstrated that p110 requires the binding of p85 to achieve full activation (6).

Several studies, in various cell lines, suggest that the PI3-kinase pathway is important for both insulin (7, 8) and Epidermal Growth factor (EGF)-induced mitogenesis (9, 10). In primary rat hepatocytes we previously demonstrated that the PI3-kinase, and not MAP (Mitogen Activated Protein) kinase, pathway is necessary and sufficient to account for both insulin- and EGF-induced DNA synthesis (11).

Several reports show that insulin achieves activation of PI3-kinase through the recruitment of p85 to tyrosine phosphorylated (PY) IRS-1 and IRS-2 (12-14). In the case of EGF, studies in different cell lines have identified several possible mechanisms leading to PI3-kinase activation. Thus in mouse fibroblast cell line over-expressing human EGFR (NRHER5), immunoprecipitates of EGFR were

shown to contain PI3-kinase activity (15,16). In A431 cells and MDA-MB-468 breast cancer cell lines, tyrosine phosphorylated ErbB3, a member of the EGFR family, was implicated in the activation of PI3-kinase upon EGF stimulation (17, 18). In PC12 and A549 cells p120cbl was found to associate with both SH2 and SH3 domain of p85, leading to activation of PI3-kinase, upon EGF stimulation (19). Other studies have demonstrated that, in A431 cells, the recently cloned docking protein "Grb2 Associated Binder 1" (Gab1) interacts with the p85 subunit of PI3-kinase following EGF (20). Another member of Gab family, Gab2, was recently described to associate with p85 after treatment of hemopoietic cells with erythropoietin (21) and IL-3 (22, 23).

The aim of this work was to clarify the mechanism of PI3-kinase activation following EGF stimulation of primary rat hepatocytes, a relevant physiological system. By over-expressing a dominant-negative p85 we confirmed the key role of PI3-kinase in EGF-induced DNA-synthesis. We next characterized three different p85-associated complexes generated by EGF treatment, and identified Gab2 as a key molecule responsible for EGF-induced PI3-kinase activation in rat hepatocytes.

2.4. Experimental Procedures

2.4.1. Materials

Porcine insulin was a gift from Lilly Research Laboratories (Indianapolis, IN) and mouse EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Collagenase was from Worthington Biochemical Corporation (Halls Mills Road, NJ). Cell culture medium and antibiotics were from Gibco-BRL (Life Technologies, Burlington, Ont., Canada). Vitrogen-100 was from Collagen Corporation (Toronto, Canada). [^{3}H]-methylthymidine, [^{125}I]-labeled goat anti-rabbit antibody ([^{125}I]-GAR) and [^{125}I]-labeled goat anti-mouse antibody ([^{125}I]-GAM) were from ICN Biomedicals Canada Ltd. (Mississauga, Ontario, Canada). [γ-^{32}P]ATP was purchased from NEN-DuPont (Wilmington, DE). Protein A-sepharose (PAS) was from Pharmacia (Montreal, Quebec, Canada). The P-Tyr (PY99), EGFR (for immunoblotting), SHP-2, Grb2 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The PI3-kinase p85 and Gab2 (for immunoblotting) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Gab2 antibodies (for immunoprecipitation studies) were generous gifts of Dr. Gen-Sheng Feng (Burnham Institute, La Jolla, CA) (22) and Dr. Haihua Gu (Harvard Medical School, Boston, MA)(24). The anti-EGFR antibody (for immunoprecipitation studies) was kindly provided by Dr. John J. M. Bergeron (McGill University, Montreal, PQ). The pShuttle-CMV and pAdEasy-2 vectors were generously provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest grade available.

2.4.2. Preparation of a dominant negative p85 mutant

Previous work has shown that the p85 deletion mutant lacking the p110 binding site works as a dominant negative regulator for wild-type p85 in competing for upstream docking proteins (25). The mutant p85 (Δ478-513) was constructed as previously described (25). Briefly, the 478-513 coding region, corresponding to the p110 binding domain, was deleted from the wild-type p85 (a generous gift from Dr. Thomas Franke, Columbia University, NY) using PCR. Two PCR fragments (P1 and P2) were generated. P1 encompassed nucleotides 1-1431 and had a BglII site introduced at the 5' end. P2 encompassed nucleotides 1542-2172 and had 30 bases complementary to the 3' end of P1 product introduced at the 5' end. After purification, the two PCR fragments were mixed, denaturated and re-annealed. Using primers at each end, the mutated p85 was then re-amplified by PCR and subcloned into pShuttle-CMV vector digested by BglII and EcoRV. The pShuttle-CMV-Δp85 plasmid was then mixed with pAdEasy-1 vector to prepare recombinant adenovirus as previously described (26).

2.4.3. Cell culture

Primary hepatocytes, isolated from 160-180g male Sprague Dawley rats (Charles River, St. Constant, Quebec, Canada) by *in situ* liver perfusion with collagenase (protocol (# 4110) approved by McGill), were plated on a collagen matrix (Vitrogen-100). Cultures were prepared by seeding 1 x 10^6 cells onto 9.6 cm^2 x 6-well plates (Corning Costar Corporation, Cambridge, MA), or 5 x 10^6 cells, onto 78-cm^2 culture dishes (Starstedt Canada, St. Laurent, Quebec, Canada). Cells were bathed for 24 h in seeding medium (Dulbecco's modified

Eagle's medium/Ham's F-12 (DMEM/F-12) containing 10% FBS, 10 mM HEPES, 20 mM $NaHCO_3$, 500 IU/ml penicillin, and 500 μg/ml streptomycin), and then for 48 h in serum-free medium (SFM) that differed from the seeding medium in that it lacked FBS and contained 1.25 μg/ml fungizone, 0.4 mM ornithine, 2.25 μg/ml L-lactic acid, 2.5×10^{-8} M selenium, and 1×10^{-8} M ethanolamine. In some experiments, treatment with adenovirus was performed after cell attachment. Cells were infected with stocks of either recombinant (AdΔp85) or wild-type (AdΔE1/ΔE3) adenovirus for 4 h at 37°C. After viral exposure, wide type and Δp85- infected cells were serum-starved for 20 h in serum-free medium (SFM).

2.4.4. [^{3}H]-thymidine incorporation assay

After viral exposure, **wide type and Δp85-** infected cells were serum-starved for 20 h in serum-free medium (SFM), then 100nM insulin or EGF and [^{3}H]-thymidine (5 μCi/ml) were added to the medium. After an 18 h incubation cells were rinsed twice with 3 ml cold PBS, incubated for 15 min at 4°C in 10% trichloro-acetic acid (TCA), solubilized at room temperature in 1 ml 1 N NaOH, and then transferred to scintillation vials and counted for [^{3}H].

2.4.5. Preparation of cell lysates

After treatment with the test agents for the time and concentration indicated in the figure legends, primary rat hepatocytes were rinsed twice with ice-cold phosphate-buffered saline (pH 7.4) and solubilized with lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10%

glycerol, and 1% Triton X-100). Cell lysates were clarified by centrifugation at 10,000 × g for 20 min at 4°C, and protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (27).

2.4.6. PI3-kinase activity assay

Lysates (500 μg protein) from EGF-treated (100 nM EGF for 1 min) or non-treated cells were immunoprecipitated in the presence of protein A-sepharose, using different antibodies as indicated in figure legends. Immunoprecipitates were extensively washed and the PAS-pellets were resuspended in 50 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 0.5 mg/ml L-α-phosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster, AL), and assayed for PI3-kinase activity as previously described (11).

2.4.7. Immunoprecipitation and immunoblotting

Before immunoprecipitation, lysates (1 mg protein) from EGF-treated (100 nM EGF for 1 min) or non-treated cells were pre-cleared using Rabbit IgG (Sigma St. Louis, MO) in the presence of protein A-sepharose for 1 h at 4°C. After centrifugation, the resulting supernatants were incubated for 2 h at 4°C with the indicated antibody. Protein A-sepharose was then added to each sample and incubated for an additional hour. The beads were collected by centrifugation, washed three times in lysis buffer and boiled in Laemmli sample buffer. After separation on SDS-PAGE, immunoprecipitated proteins were transferred to Immobilon-P membranes (Millipore Ltd., Mississauga, Ontario, Canada). For immunoblotting, membranes were probed with the indicated first antibody for 90 min followed by 1 h incubation with [^{125}I] or HRP-labeled goat anti-rabbit IgG

except for the use of anti-PY where the second antibody was [^{125}I] or HRP-labeled goat anti-mouse IgG. Immunoreactive proteins were detected by autoradiography or by ECL system (Amersham pharmacia biotech, Montreal Quebec). Densitometric quantifications of the signals were performed using the BioRad densitometer model GS-700.

2.4.8. Immunodepletion studies

Lysates (500 μg protein) from EGF-treated cells (100 nM EGF for 1 min) were pre-cleared using Rabbit IgG in the presence of protein A-sepharose for 1 h at 4°C. After centrifugation, the resulting supernatants were incubated overnight at 4°C with the indicated antibody. Protein A-sepharose was then added to each sample and incubated for an additional hour. After centrifugation, the immunodepleted-supernatants were equally divided into two fractions. One fraction was immunoprecipitated in the presence of protein A-sepharose with the antibody used for the immunodepletion. After separation on SDS-PAGE, immunoprecipitated proteins were transferred to Immobilon-P membranes and immunoblotted with the same antibody. The other fraction was immunoprecipitated with anti-p85 antibody in the presence of protein A-sepharose, run on SDS-PAGE, transferred to membranes and blotted with anti-PY antibody. As control for non-immunodepleted samples, lysates (150 μg protein) from EGF-treated (100 nM EGF for 1 min) or non-treated cells were immunoprecipitated with anti-p85 antibody after preclearing with Rabbit IgG. Immunoprecipitated proteins were then detected by immunoblot with anti-PY antibody.

2.5. Results

2.5.1. Dominant-negative PI3-kinase (Δp85) blocks insulin and EGF induced DNA synthesis in primary rat hepatocytes

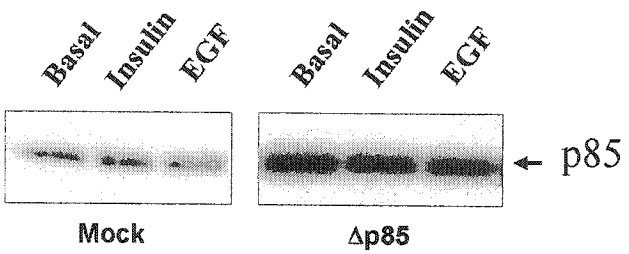
PI3-kinase activated by growth factors, including insulin and EGF, has been implicated in DNA synthesis in various cell lines (28). We have previously shown that the PI3-kinase pathway regulates DNA synthesis in response to insulin and EGF in primary rat hepatocyte cultures by using PI3-kinase inhibitors, wortmannin and LY294002 (11). To further confirm this result, we used a recombinant adenovirus containing a cDNA encoding the p85 regulatory subunit, whose p110 binding region was deleted (25, 29, 30). Infection efficiency was assessed in cells transiently infected with recombinant adenovirus by measuring Δp85 expression using a p85 antibody (Fig. 1A, *right panel*). The average expression of p85 was increased 9.3 ± 2.7 fold (mean ± S.E.) in cells infected with Δp85 recombinant adenovirus compared to cells infected only with the wild-type adenovirus. The effect of Δp85 expression was, in accordance with our study using the PI3-kinase pharmaceutical inhibitors (11), to inhibit completely basal as well as insulin- and EGF-stimulated DNA synthesis (Fig. 1B). This confirmed the critical role for PI3-kinase in mediating DNA synthesis in these cells.

2.5.2. Tyrosine phosphorylated (PY) proteins play a key Role in EGF-induced PI3-kinase activity

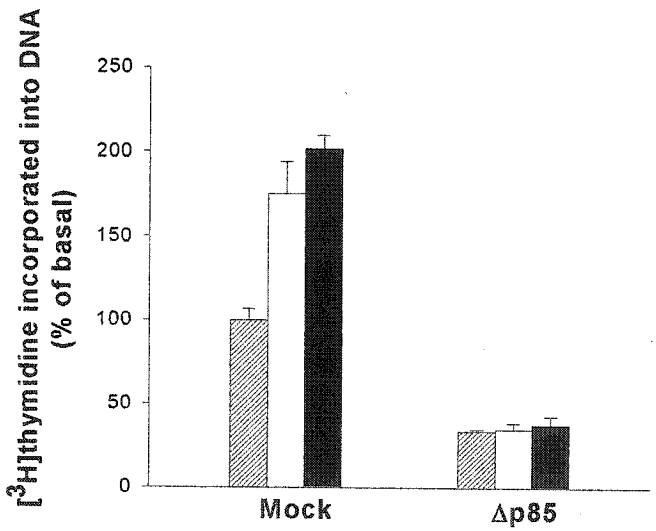
The mechanism by which insulin stimulates PI3-kinase activity is well known (12-14). Since the mechanism for EGF is less clear we sought to evaluate it in

2-Figure 1. Dominant-negative PI3-kinase (Δp85) blocks insulin and EGF-induced DNA synthesis in primary rat hepatocytes. A. Cell lysates, prepared from wild-type (Mock) or Adeno-Δp85 infected primary rat hepatocytes, were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with an anti-p85 (αp85) antibody. B. Hepatocytes were infected with either wide-type (Mock) or Adeno-Δp85 for 3 h, then starved for 24 h before a 20h incubation in SFM containing 5μCi [^{3}H]methylthymidine without (*hatched bars*) or with 100 nM insulin (*empty bars*) or EGF (*black bars*). Incorporation of [^{3}H]thymidine into DNA was determined as described in "Experimental Procedures". Results are expressed as the percentage of basal (wide-type cells) (mean ±S.E., three separate experiments).

A


Basal
Insulin
EGF
Basal
Insulin
EGF
p85
Mock
Δp85

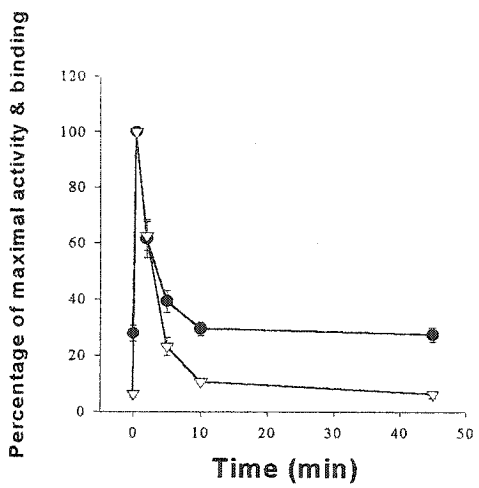
B


[3H]thymidine incorporated into DNA
(% of basal)
250
200
150
100
50
0
Mock
Δp85

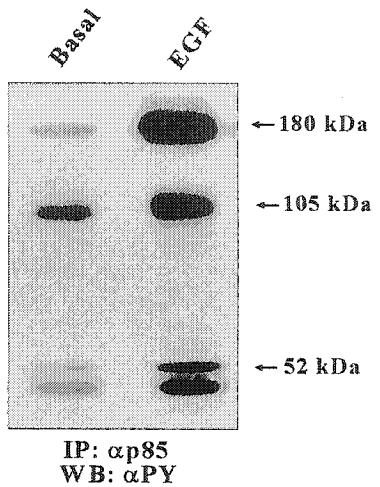
primary rat hepatocytes. Cells were stimulated at different times with 100nM EGF and tyrosine phosphorylated proteins were immunoprecipitated using anti-PY antibody (Fig. 2A). After immunoprecipitation, PI3-kinase activity (Fig. 2A, *black circles*) was measured as well as the amount of p85 protein (Fig. 2A, *open triangles*) present in the PY pellets. As shown EGF augmented PI3-kinase activity in PY immunoprecipitates, and the association of p85 with particular PY - proteins. The activation of PI3-kinase activity and the association of p85 with PY-proteins were maximum at 30 sec and then declined to basal levels by 10 min after EGF. To identify the PY- proteins associated with p85 we subjected the immunoprecipitate generated with anti-p85 antibody to SDS-PAGE followed by immunoblotting with anti-PY antibody as shown in Figure 2B. Following EGF stimulation three major PY-protein bands were evident at 180kDa, 105kDa and 52kDa (a minor band at 46 kDa was also seen) as indicated in Figure 2B. The time course of p85 association with these PY-proteins was assessed (Fig. 2C). Our results show that the tyrosine phosphorylation of each band reached a maximum level at 30 sec, then declined to basal. Only the tyrosine phosphorylation of the 105kDa protein (Fig. 2C, *middle panel*) followed the same pattern as total EGF-induced PI3-kinase activity (anti-PY immunoprecipitates; Fig. 2A) by declining to basal levels 10 min after exposure to EGF. These results demonstrate that EGF-induced PI3-kinase activation correlates with the association of p85 with three PY-proteins amongst which the one migrating at 105kDa showed the best correlation.

2-Figure 2. EGF-stimulated PI3-kinase activity correlates with the association of p85 with tyrosine phosphorylated proteins. A. Serum-deprived hepatocytes were treated with 100nM EGF for the indicated times. Cell lysates were immunoprecipitated with an anti-PY (αPY) antibody. The immunoprecipitated proteins were analyzed for PI3-kinase activity (*black circle*) or for p85 content using immunoblot analysis with αp85 antibody (*open triangle*). Results are expressed as percentage of maximum values obtained for each analysis (mean ± ½ the range, two separate experiments). B. Hepatocytes were treated with or without 100nM EGF for 1 min. Cell lysates were immunoprecipitated with αp85, resolved by 7.5% SDS-PAGE, and subjected to immunoblotting with αPY. The three major bands (PY180, PY105, PY52) are indicated. C. Hepatocytes were treated with 100nM EGF for the indicated times. Cell lysates were processed as described in A. The graphs represent quantification of the three major bands as described in "Experimental Procedures". Results are expressed as percent maximum tyrosine phosphorylation (mean ± S.E., three separate experiments).

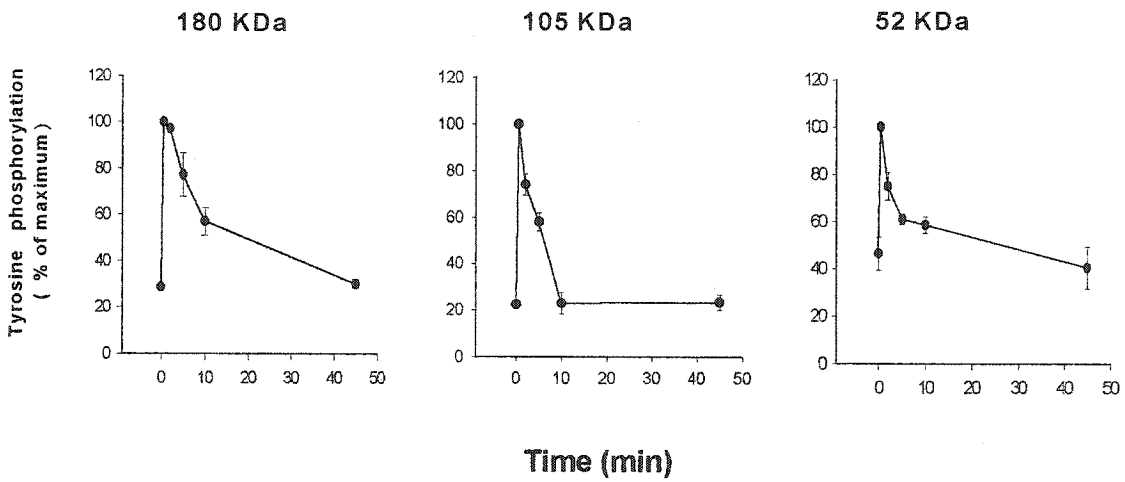
A


Percentage of maximal activity & binding
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (min)

B


Basal
EGF
←180 kDa
←105 kDa
←52 kDa
IP: αp85
WB: αPY

C


180 KDa
105 KDa
52 KDa
Tyrosine phosphorylation
(% of maximum)
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (min)

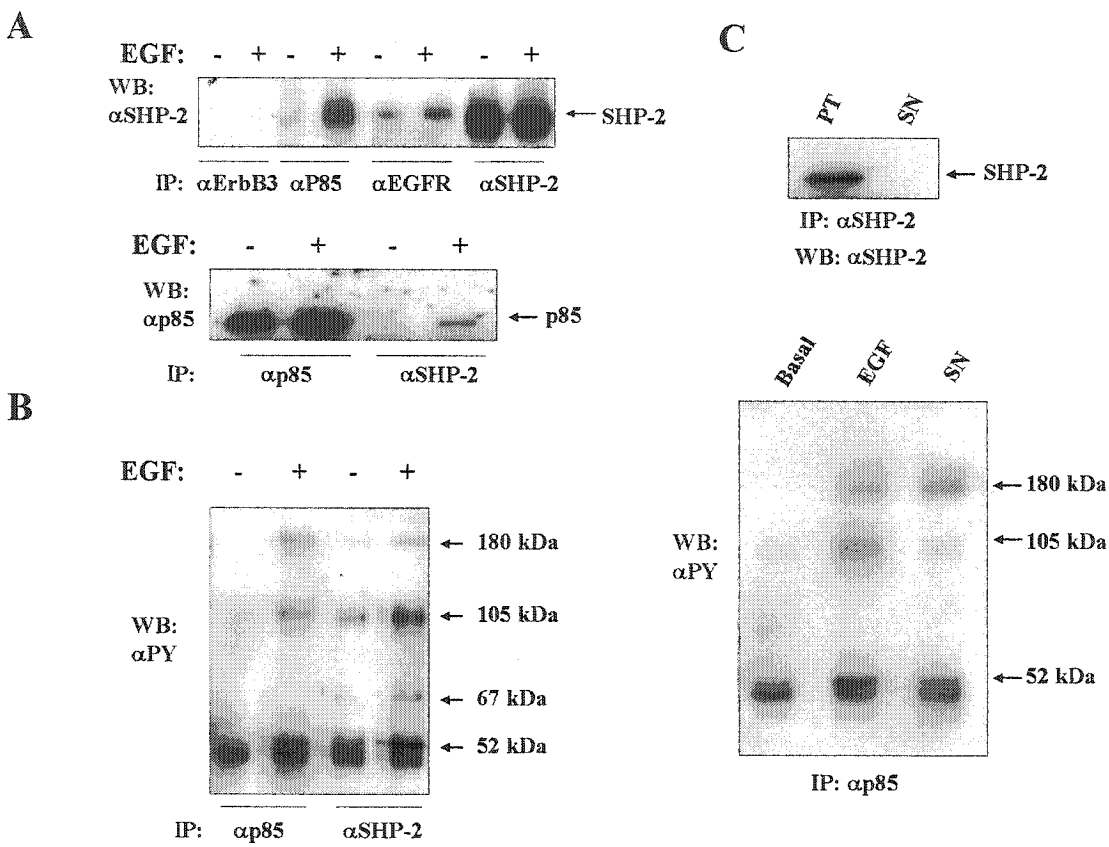
2.5.3. The association of p105 with p85, SHP-2 and Grb2 following EGF treatment

Using specific antibodies, we found that the PY180 species was largely accounted for by ErbB3 a member of the EGFR protein family (31) and that PY52 was accounted for by Shc, an adapter protein tyrosine phosphorylated in response to EGF (32,33). Neither of these species associated with p105 (Appendices Fig.1 &2). Since the time course of tyrosine phosphorylation of p105 best correlated with the time course of EGF-induced PI-3 kinase activation (Fig. 2C), we examined in detail the EGF-induced complex formed by p105 and p85 in primary rat hepatocytes. In agreement with an earlier report (34) we found that the association of SHP-2 with p85 increased 4-5 fold after EGF stimulation (Fig. 3A, *top and bottom panel, compare lane 3 to lane 4*). Interestingly, we also found that SHP-2 associated with EGFR (*top panel, lanes 5,6*), but not with ErbB3 (*top panel, lanes 1,2*). Since SHP-2 associated with p85 we determined if it was part of the complex formed by p85 and p105 by assessing which EGF-induced PY-proteins are present in SHP-2 immunoprecipitates. Following EGF treatment (Fig. 3B, *lane 4*) four PY- proteins were detected in SHP-2 immunoprecipitates with molecular weights ~ 180kDa, 105kDa, 67kDa and 52kDa. It is clear that SHP-2 associates with the EGFR (Fig. 3A, *lanes 5 & 6*). The band detected at 180kDa in the SHP-2 immunoprecipitates (Fig. 3B, *lane 4*) thus corresponds to PY-EGFR. The 67 kDa protein corresponds to PY- SHP-2 (data not shown) which was not readily detected in p85 immunoprecipitates (Fig 3B, *lane 2*) due to the smaller quantity of SHP-2 present in the latter than in

SHP-2 immunoprecipitates (Fig. 3A, *compare top panel, lanes 7,8 to bottom panel, lanes 3,4*). A band migrating at 105 kDa, as in anti-p85 immunoprecipitates, is observed in both control and EGF- stimulated hepatocytes (Fig. 3B, *compare lane 2 to 3 and 4*). Since SHP-2 associates with Shc upon EGF treatment of hepatocytes (data not shown) the 52kDa protein is likely PY- Shc.

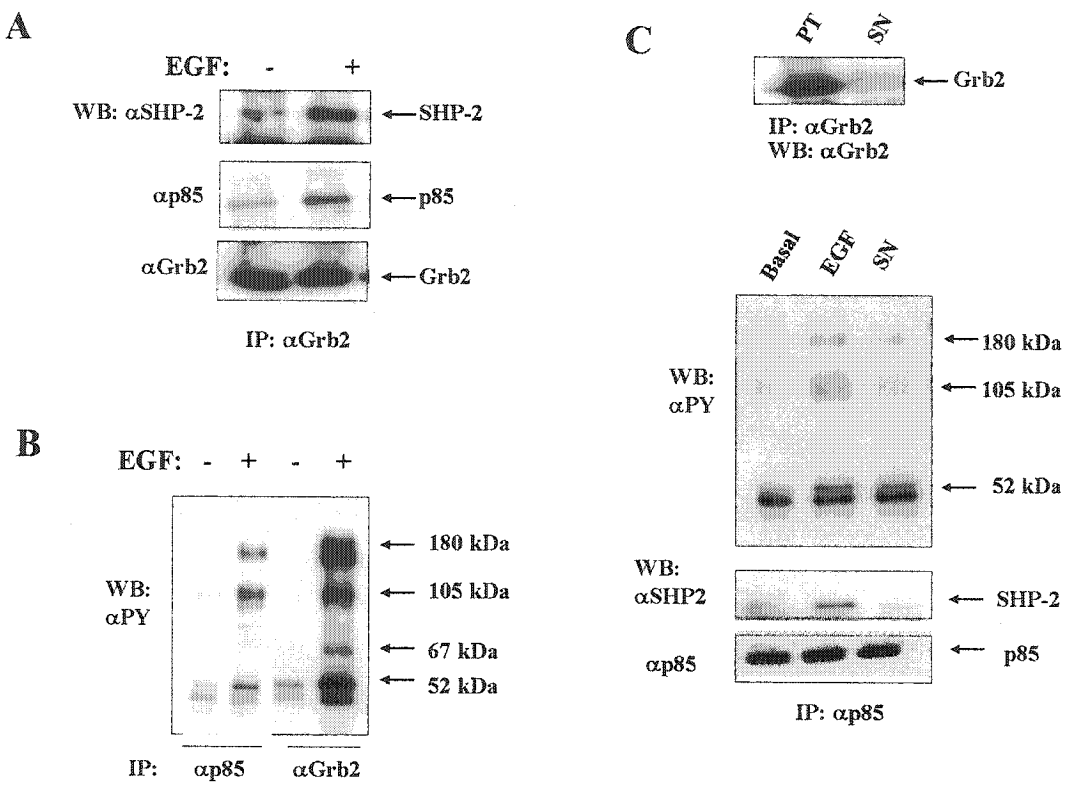
The association of SHP-2 with the 105kDa protein led us to determine whether this protein was the same p105 as that associated with p85. We therefore immunodepleted SHP-2 molecules by preadsorbing lysates from EGF-treated cells with SHP-2 antibody and then tested for the presence of PY-p105 in anti-p85 immunoprecipitates. The efficiency of the SHP-2 immunodepletion was confirmed by demonstrating the full removal of SHP-2 proteins from the supernatant of SHP-2 immunoprecipitates (Fig. 3C, *top panel*). Using the immunodepleted SHP-2 supernatants (Fig. 3C, *bottom panel*) we showed that after anti-p85 immunoprecipitation and immunoblotting with PY antibody the intensity of the p105 band decreased to essentially the same level as observed in lysates from control non-immunodepleted cells (*compare lanes "Basal" and "SN"*). It is of interest to note that the intensity of the 180 kDa (ErbB3) and 52kDa (Shc) bands were not affected by the SHP-2 immunodepletion. These results demonstrate that in primary rat hepatocytes, EGF treatment effects the association of p85 with SHP-2 and p105.

2-Figure 3. SHP-2 is a part of the complex formed by p85 and PY 105. Hepatocytes were treated with (+) or without (-) 100nM EGF for 1min. A. Proteins, immunoprecipitated with anti-ErbB3 (αErbB3), αp85, anti-EGFR (αEGFR) or anti-SHP-2 (αSHP-2) were resolved on 7.5% SDS-PAGE and immunoblotted with αSHP-2 (*top panel*) or αp85 (*bottom panel).* B. Proteins, immunoprecipitated with αp85 or αSHP-2 and resolved on 7.5% SDS-PAGE were subjected to immunoblotting with αPY. C. EGF-treated cell lysates were preabsorbed on PAS beads with αSHP-2. The pellet (PT) and supernatant (SN) were prepared by centrifugation, and the latter was subjected to immunoprecipitation with αSHP-2. The pellet and the SN immunoprecipitate were resolved on 7.5% SDS-PAGE, and subjected to immunoblotting with αSHP-2 (*top panel*). The immunodepleted SN and lysates of cells ± EGF were immunoprecipitated with αp85, run on 7.5% SDS-PAGE and immunoblotted with αPY (*lower panel*).


A
EGF: - + - + - + - +
WB: αSHP-2
SHP-2
IP: αErbB3 αP85 αEGFR αSHP-2
EGF: - + - +
WB: αp85
p85
IP: αp85 αSHP-2
B
EGF: - + - +
WB: αPY
180 kDa
105 kDa
67 kDa
52 kDa
IP: αp85 αSHP-2
C
PT SN
SHP-2
IP: αSHP-2
WB: αSHP-2
Basal EGF SN
WB: αPY
180 kDa
105 kDa
52 kDa
IP: αp85

EGF has been shown to induce the coupling of SHP-2 to Grb2 via the COOH-terminal SH3 domain of Grb2 (35). In addition, a direct association of Grb2 and p85, mediated by the SH3 domains of Grb2 and the proline-rich motifs of p85, has also been reported (36). We therefore evaluated the extent of Grb2 association with SHP-2 and p85. Lysates from control and EGF-stimulated cells were immunoprecipitated with anti-Grb2 antibody and subjected to anti-SHP-2, anti-p85 and anti-Grb2 immunoblotting. Grb2 was shown to associate with SHP-2 (Fig. 4A, *top panel*) and p85 (Fig. 4A, *middle panel*) and this was increased following EGF stimulation. These results indicated that Grb2 might engage in the complex of p85 with SHP-2 and p105. We thus assessed which PY-proteins are present in Grb2 immunoprecipitates after EGF treatment. With anti-PY immunoblotting we detected 4 PY- bands (Fig. 4B, *lane 4*), and identified the 180kDa protein as PY-EGFR, the 67kDa protein as PY- SHP-2 and the 52 and 46kDa proteins as PY-Shc (data not shown). A PY- protein, migrating at 105 kDa, was also detected in Grb2 immunoprecipitates of lysate from EGF-stimulated cells. Immunodepletion of Grb2 from the lysates was performed to determine if the p105 protein present in the anti-p85 immunoprecipitates was the same as that associated with Grb2. As with the SHP-2 study, the efficiency of the immunodepletion was confirmed by immunoblotting the anti-Grb2 supernatants (Fig. 4C, *top panel*). It can be seen that the level of p105 is barely detectable in anti-p85 immunoprecipitates after Grb2 immunodepletion whereas the level of the 46-52 kDa band (corresponding to Shc) was unaffected (Fig. 4C, second *panel*).

2-Figure 4. Grb2 is part of a complex formed by p85, PY 105 and SHP-2. Hepatocytes were treated with (+) or without (-) 100nM EGF for 1min. A. Proteins immunoprecipitated with anti-Grb2 (αGrb2) were resolved on 10% SDS-PAGE and immunoblotted with αSHP-2 (*top panel*), αp85 (*middle panel)* or αGrb2 (*bottom panel). B.* Proteins immunoprecipitated with αp85 or αGrb2 were resolved on 7.5% SDS-PAGE and immunoblotted with αPY. C. EGF-treated cell lysates were preabsorbed on PAS beads with αGrb2. The pellet (PT) and supernatant (SN) were prepared by centrifugation, and the latter was subjected to immunoprecipitation with αGrb2. The pellet and the SN immunoprecipitate were resolved on 10% SDS-PAGE, and subjected to immunoblotting with αGrb2 (*top panel*). The immunodepleted SN and lysates of cells ± EGF were immunoprecipitated with αp85, run on 7.5% SDS-PAGE and immunoblotted with αPY (*middle panel*) and the stripped membranes were reblotted with αSHP-2 or αp85 (*bottom panel*).

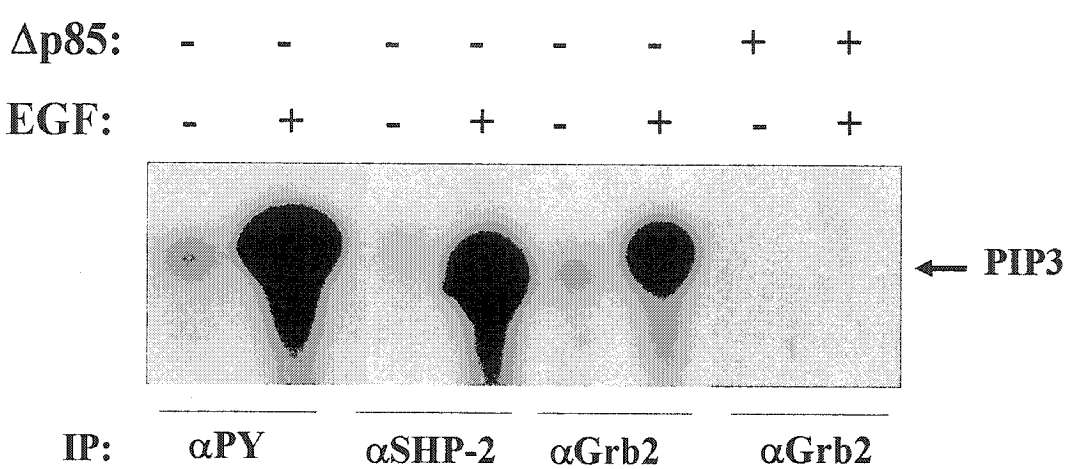

A
EGF: - +
WB: αSHP-2
←SHP-2
αp85
←p85
αGrb2
←Grb2
IP: αGrb2
B
EGF: - + - +
WB:
αPY
← 180 kDa
← 105 kDa
← 67 kDa
← 52 kDa
IP: αp85 αGrb2
C
PT SN
← Grb2
IP: αGrb2
WB: αGrb2
Basal EGF SN
WB:
αPY
← 180 kDa
← 105 kDa
← 52 kDa
WB:
αSHP2
← SHP-2
αp85
← p85
IP: αp85

To determine if SHP-2 is part of the same complex with Grb2, the membranes used to detect PY- proteins after Grb2 immunodepletion were stripped and reblotted with either anti-SHP2 or anti-p85. It can be seen that immunodepletion of Grb2 leads to the disappearance of SHP-2 (Figure 4C, *third panel*) indicating association between them. Our results show that EGF stimulation of hepatocytes leads to the formation of a unique complex containing Grb2, p85, SHP-2 and p105.

2.5.4. EGF-induced PI3-kinase activity associated with p105

We next sought to evaluate the proportion of total EGF-activated PI3-kinase which associated with the p105-containing complex. Hepatocytes were treated with or without 100 nM EGF for 1 min, and the lysates were subjected to immunoprecipitation with the appropriate antibodies to generate the above noted complexes in which PI3-kinase activity was measured (Fig 5). Quantitation by scanning densitometry revealed that ErbB3 and Shc associated PI3-kinase activity represented less than 10% of the PI3-kinase activity in PY immunoprecipitates (Appendices Fig.3). More than 80% of total EGF-induced PI3-kinase activity of rat hepatocytes was found in anti-SHP2 or anti-Grb2 immunoprecipitates, indicating that the complex formed by p105, p85, SHP-2, and Grb2 is the major one in which EGF-induced PI3-kinase activity is concentrated. To establish the significance of the p105 complex for EGF-induced DNA synthesis we measured Grb2-associated PI3-kinase activity in lysates from cells infected with Δp85. As seen in Fig. 5 Grb2-associated PI3-kinase activity was totally abolished in hepatocytes over-expressing Δp85. Taken together, our

2-Figure 5. A complex of p105 with p85, SHP-2, and Grb2 is the major species responsible for EGF-induced PI3-kinase activation. Hepatocytes were infected with (+) or without (-) Adeno-Δp85 for 3h, serum-deprived for 48h, then treated with (+) or without (-) 100nM EGF for 1min. Cell lysate proteins were immunoprecipitated with αSHP-2, αGrb2 or αPY and analyzed for PI3-kinase activity as described under "Experimental Procedures".


Δp85: - - - - - - + +
EGF: - + - + - + - +
PIP3
IP: αPY αSHP-2 αGrb2 αGrb2

data demonstrate the important role of p105 as a docking protein for EGF-induced PI3-kinase activation.

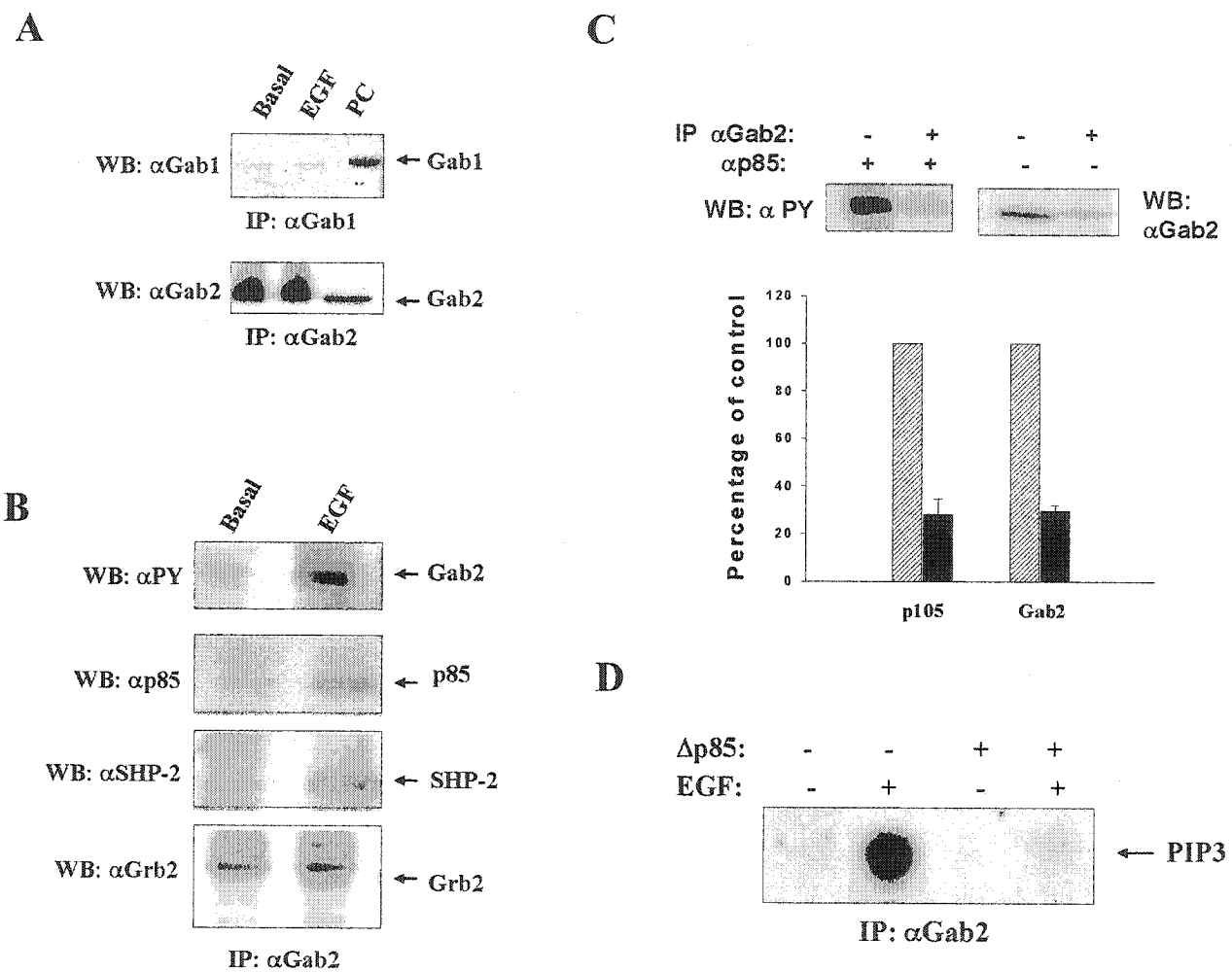
2.5.5. Identification of the p105 protein as Gab2

p105 behaves like a member of the Gab family of adapter proteins, which have been shown to become tyrosine phosphorylated and associated with p85, SHP-2 and Grb2 in response to several kinds of cytokines or growth factors. (20,24,37). Using immunoblotting we showed that Gab1 is not expressed in primary rat hepatocytes. (Fig. 6A, *top panel*), consistent with previous determinations of Gab1 distribution using Northern blotting to measure mRNA levels (20). Recently Gab2, a Gab1 isoform, was cloned from human and mouse cDNA libraries (23,24). Using degenerate oligonucleotides, whose sequences corresponded to conserved regions between mouse and human (one in the PH domain and other in the MBD domain), we performed RT-PCR using rat hepatocyte mRNA and cloned the full-length rat cDNA (Genbank accession number AF230367). At the amino acid level, the rat clone exhibited 96.5% and 93% identity with the mouse and human Gab2 respectively indicating that the isolated clone encodes a rat Gab2 protein. As with human and mouse, rat Gab2 has many functional domains, in particular the binding motifs for Grb2, Crk, PI3-kinase and SHP-2, the PH domain (respectively 97% and 95% with mouse and human respectively) and also a region similar to the c-Met binding domain (MBD) of Gab1 (23).

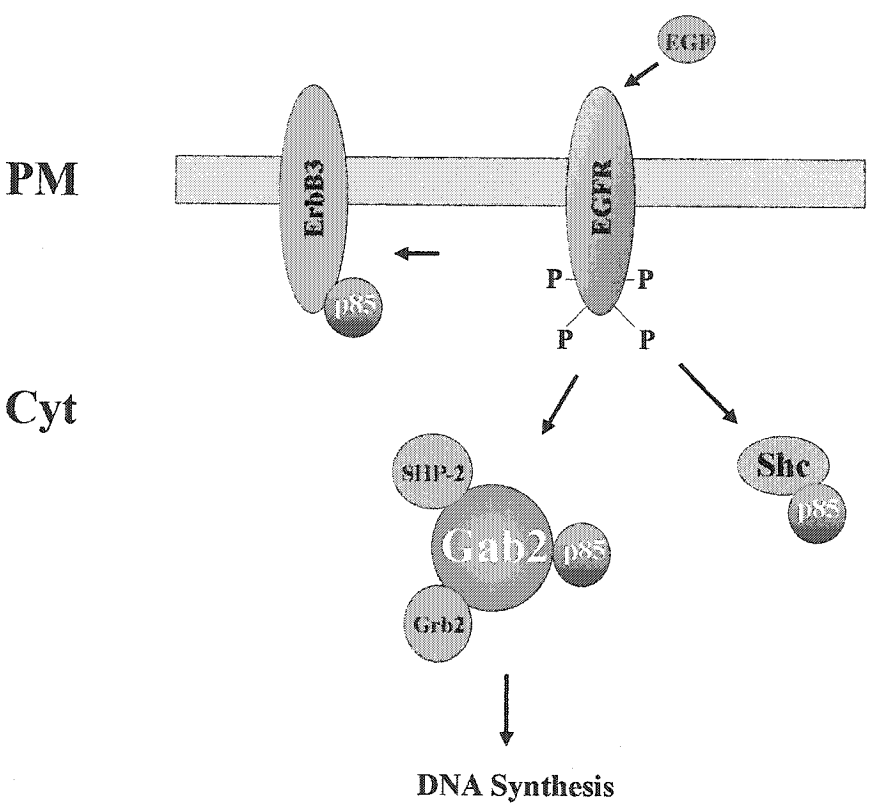
By the time we had completed the cloning, a commercial antibody raised against the human Gab2 protein became available. Using this antibody we showed that Gab2 is indeed expressed in primary rat hepatocytes (Fig. 6A, *lower panel*), and confirmed that EGF stimulation produces PY- Gab2 (Fig. 6B, *first panel*). We also demonstrated that Gab2 associates with p85 (Fig. 6B, *second panel*), SHP-2 (Fig. 6B, *third panel)* and Grb2 (Fig. 6B, *fourth panel*) in an EGF dependent manner. Immunodepletion studies confirmed that p105 is Gab2. EGF-treated cell lysates were preadsorbed by Gab2 antibody and the amount of p105 and Gab2 associated with p85 in the residual supernatants was tested. Both Gab2 and p105 decreased by about 70% after an initial immunoprecipitation with Gab2 (Fig. 6C) indicating that the p105 protein is largely if not completely identical to tyrosine phosphorylated Gab2. In addition we found that Gab2-associated PI3-kinase activity was totally abolished by Δp85 (Fig. 6D).

Taken together our data suggest an important role for PI3-kinase in EGF-induced DNA synthesis in primary rat hepatocytes. Activated PI3-kinase was shown to be largely associated with a complex constituted by Gab2, SHP-2 and Grb2. We also demonstrated the presence of two other complexes in which PI3-kinase associated with ErbB3 and Shc respectively (Fig. 7).

2-Figure 6. Identification of Gab2 as the PY 105 protein. A. Hepatocytes were treated with (+) or without (-) 100nM EGF for 1min. Proteins, immunoprecipitated with anti-Gab1 (αGab1) or anti-Gab2 (αGab2) were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αGab1 (*top panel*) or αGab2 (*bottom panel*). Lysate (20 μg protein) from non-stimulated A431 or Hela cells was a positive control (PC) for Gab1 or Gab2 respectively. B. Proteins, immunoprecipitated with αGab2, were resolved on 10% SDS-PAGE and immunoblotted with αPY (*first panel*), αp85 (*second panel*), αSHP-2 (*third panel*) or αGrb2 (*fourth panel*). C. Lysates from EGF-treated cells were incubated with αGab2 (to effect immunodepletion) or with normal IgG as described in "Experimental Procedures". The supernatants from these incubations were further incubated with *(upper left panel)* or without (*upper right panel*) αp85. The supernatants from this latter incubation along with the original supernatants were subjected to 7.5% SDS-PAGE followed by immunoblotting with αPY (*upper left panel*) or αGab2 (*upper right panel*). The bar graph (*bottom panel*) represents quantification of the upper bands. Control refers to the bands obtained from supernatants not immunoprecipitated with αGab2. The data are expressed as percentage of control values obtained for each analysis (mean ± S.E., 4 separate experiments). D. Hepatocytes were infected with (+) or without (-) Adeno-Δp85 for 3h, serum-deprived for 48h, then treated with (+) or without (-) 100nM EGF for 1min. Cell lysate proteins were immunoprecipitated with αGab2 and analyzed for PI3-kinase activity as described under "Experimental Procedures".


A
Basal
EGF
PC
WB: αGab1
Gab1
IP: αGab1
WB: αGab2
Gab2
IP: αGab2
B
Basal
EGF
WB: αPY
Gab2
WB: αp85
p85
WB: αSHP-2
SHP-2
WB: αGrb2
Grb2
IP: αGab2
C
IP αGab2:
αp85:
WB: α PY
WB: αGab2
Percentage of control
120
100
80
60
40
20
0
p105
Gab2
D
Δp85:
EGF:
PIP3
IP: αGab2

2-Figure 7. Scheme depicting PY-protein complexes formed in rat hepatocytes following EGF treatment. PM indicates plasma membrane and Cyt the cytosol.


PM
Cyt
EGF
ErbB3
EGFR
p85
P
P
P
P
SHP-2
Gab2
p85
Grb2
Shc
p85
DNA Synthesis

2.6. Discussion

Using pharmacologic inhibitors of PI3-kinase and MAPK activation we and others have demonstrated that the former and not latter pathway is both necessary and sufficient for insulin and EGF-induced DNA synthesis (11,38-40). However, inhibitors may not always be sufficiently specific to warrant precise conclusions. Hence wortmannin has also been shown to inhibit PI4-kinase as well as PI3-kinase (41). Using a dominant negative p85, in which the p110 binding site was deleted, we showed that both insulin and EGF-induced DNA synthesis is totally abrogated (Fig. 1), confirming the essential role of PI3-kinase in mediating this effect in rat hepatocytes.

Two general mechanisms for the recruitment and activation of PI3-kinase by growth factor receptors have been described in different transformed cell lines. The first involves the direct binding of the p85 regulatory subunit of PI3-kinase to PY-RTKs such as the PDGF (42), CSF-1 (43) and c-met receptors (44). The second mechanism comprises the recruitment to and activation of p85 by substrates of RTKs (i.e. PY- docking proteins) such as the IRS (12-14) and Gab protein families (20). The mechanism by which PI3-kinase becomes activated upon insulin stimulation has been well characterized (12-14); however the events consequent to EGF stimulation have been less clearly defined. As noted above EGF activates PI3-kinase through recruitment to a range of PY-proteins in a cell line specific manner. In this paper we demonstrate that PY- proteins, especially that migrating at 105kDa, play a key role in EGF-induced PI3-kinase activity in

rat hepatocytes, a physiologically relevant system. Further analysis demonstrated that, upon EGF treatment, three distinct p85-associated complexes were formed with the one containing the PY-105kDa protein (identified as Gab2, Fig. 6) accounting for a large proportion of the activated PI3-kinase generated by EGF stimulation (Fig. 5).

This study is the first to demonstrate that in rat hepatocytes EGF stimulates the formation of a complex comprised of SHP-2, p105, p85 and Grb2 which accounts for over 80% of total EGF-induced PI3-kinase activity (Fig. 5).

Based on our immunodepletion studies (Fig. 6) we conclude that PY-105 is largely if not completely accounted for by Gab2 (Grb2 Associated Binder 2). Gab2, a 100 kDa protein, has recently been cloned from mouse and human tissues (22,23). It shows high homology with Gab1, a previously identified docking protein involved in growth factor signaling (45-47). Gab2 contains an N-terminal PH domain and proline-rich sequences as well as consensus PI 3-kinase, SHP-2, Grb2 and Crk tyrosine binding sites (23,24). The direct association of Grb2 and p85, mediated by the SH3 domains of Grb2 and the proline-rich motifs of p85, has also been reported (36). However the direct association of Grb2 and p85 in rat hepatocytes still needs to be established. Although SHP-2 was observed in anti-p85 immunoprecipitates in various cells, no direct association of SHP-2 and p85 has been reported. Rather this association was probably mediated via a docking protein(s) (48), such as Gab1 (49) and Gab2 (24). Indeed on the basis of its similarity to Gab1 and homology

with other docking molecules such as the IRSs (50) it is likely that Gab2 works as a platform for the signaling complex consisting of p85, SHP-2 and Grb2. Our data also showed that dominant-negative p85 fully inhibited both Grb2 and Gab2-associated PI3-kinase as well as the EGF-induced DNA synthesis. Taken together these results argue for a critical role for the Gab2 protein in EGF-induced PI3-kinase activation and DNA synthesis in primary rat hepatocytes.

Several reports have shown that, in hematopoetic cells, different stimuli (viz. interleukin (IL)-2, IL-3 and M-CSF) promote the association of PY - proteins (95-110 kD) with SHP-2, p85 and Grb2 (34, 48, 51-54). These reports are consistent with the possibility that the 95-110 kDa protein(s) is Gab2 which associates with p85, Grb2, and SHP-2 to play an important role in cytokine- and growth factor-regulated cell proliferation and differentiation.

The function of ErbB3 / p85-containing complex is unclear. Analysis of PI3-kinase activity in ErbB3 immunoprecipitates shows that it is less than 10% of the total EGF-induced PI3-kinase activity present in the PY immunoprecipitates (Appendices Fig.3). As the association of p85 to ErbB3 does not lead to activation of a significant proportion of the PI3-kinase pool we suggest its main role might be to recruit p85 to the PM in which compartment it may play a selective role. PY - 52 and 46kDa isoforms of Shc also associate with p85 in response to EGF in primary rat hepatocytes. As with ErbB3 this p85/Shc association, measured in Shc immunoprecipitates, represents less than 10% of

the total PI3-kinase activation effected by EGF (Appendices Fig.3). It also remains to determine whether this association is direct or is via other proteins. However, we cannot see the association of ErbB3 with Shc, which suggest that p85 formed a complex with Shc distinct from that formed with ErbB3.

In summary we have demonstrated the critical role played by the recruitment of p85 to PY- proteins in mediating EGF-induced PI3-kinase activity. We have identified three distinct p85-associated complexes which form in primary rat hepatocytes in response to EGF (Fig. 7). One complex contained ErbB3 and p85; the second, p85 and Shc; and the third p85, Gab2, SHP-2 and Grb2. The latter complex accounted for most EGF-induced PI3-kinase activation in rat hepatocytes. These findings point to a key role for Gab2 in effecting EGF-dependent biological functions such as mitogenesis.

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2.8. Footnotes

[1]The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; MAP kinase, mitogen activated protein kinase; Gab1, Grb2 associated binder 1; Gab2, Grb2 associated binder 2; Grb2, growth factor receptor bound 2; SHP-2, src homology 2 domain-containing protein tyrosine phosphatase-2.

CHAPTER 3

Gab2 tyrosine phosphorylation by a PH-domain independent mechanism: Role in EGF-induced mitogenesis

3.1. Preface to Chapter 3

In Chapter 2, we demonstrated that PI3-kinase is important for EGF-induced DNA synthesis by using the dominant-negative PI3-kinase. This established the validity of our previous data using PI3-kinase inhibitors. To study further the mechanism of EGF-induced PI3-kinase activation, we identified three distinct p85-associated complexes: ErbB3-p85, Shc-p85 and a large complex Gab2-Grb2-SHP2-p85. The latter accounted for > 80% of total EGF-induced PI3-kinase activity, indicating that it is a key mediator of EGF-induced PI3-kinase activation.

Since the specific intracellular localization of different activated signaling molecules might be a determinant of signaling specificity we sought, in this Chapter, to investigate the subcellular localization of these three different p85-associated complexes. Our work will focus on the Gab2 complex, which accounts for > 80% of the total PI3-kinase activity. We thus generated various recombinant adenovirus-Gab2 mutant constructs and over-expressed them in primary hepatocytes. The role of Gab2 in EGF-induced PI3-kinase activation and DNA synthesis was investigated by over-expressing and comparing the consequences of both wild-type Gab2 and a Gab2 mutant lacking p85 binding sites. Since the multimeric Gab2 complex was only found in cytosol and no translocation of Gab2 was observed either by cell fractionation studies or immunofluorescence, we examined the role of the PH-domain of Gab2 in its phosphorylation and EGF signaling by over-expressing either the PH-domain of Gab2 or Gab2 mutant lacking the PH-domain.

3.2. Summary

In primary rat hepatocyte cultures activation of PI3-kinase is both necessary and sufficient to account for EGF-induced DNA synthesis. In these cells three major p85-containing complexes were formed following EGF treatment: ErbB3-p85, Shc-p85 and a multimeric Gab2-Grb2-SHP2-p85, which accounted for > 80% of total EGF-induced PI3-kinase activity [Kong, et al, J. Biol. Chem. (2000) 275:36035]. Here we show that, following EGF treatment ErbB3-p85 and Shc-p85 complexes were localized to PM and endosomes (ENs), whereas the multimeric Gab2-Grb2-SHP2-p85 complex was formed rapidly (peak at 30 secs) and exclusively in cytosol. To study the mechanism of Gab2 tyrosine phosphorylation and its role in EGF-induced mitogenesis in liver, we infected primary rat hepatocytes with various recombinant adenoviruses - Gab2 mutants. Over-expression of wild-type Gab2 (WTGab2) augmented EGF-induced PI3-kinase activity and DNA synthesis whereas the Gab2 mutant lacking p85 binding sites (Δp85Gab2) did not effect such augmentation. This established the central role of Gab2 in EGF-induced PI3-kinase activation and DNA synthesis. Over-expression of the PH-domain of Gab2 did not affect EGF-induced Gab2 phosphorylation, PI3-kinase activation and DNA synthesis. Over-expressed Gab2 lacking the PH-domain (ΔPHGab2) was comparable to WTGab2 in respect to EGF-induced tyrosine phosphorylation, recruitment of p85, and DNA synthesis. Immunofluorescence analyses in cultured hepatocytes demonstrated that EGF did not promote the association of cytosolic Gab2 with cell membranes. In summary, following EGF stimulation, ErbB3, Shc and Gab2 are differentially

compartmentalized in rat liver where they associate with and activate PI3-kinase. Our data demonstrate that Gab2 mediates EGF-induced PI3-kinase activation and DNA synthesis in a PH-domain independent manner.

3.3. Introduction

The EGF-like growth factor family, encompassing over a dozen different growth factor ligands, signals through four receptors of the ErbB family: ErbB1/EGFR, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. ErbB receptors undergo a variety of ligand-stimulated receptor homo- and heterodimerization events (1), allowing the generation of a broad range of intracellular signals and thus a variety of cellular responses (2). In adult liver, numerous studies have linked EGF action to organ repair through increased mitogenesis (3). The activated EGFR leads to a cascade of intracellular signaling, including activation of the phosphoinositide 3-kinase (PI3-kinase) and Ras-Raf-MAPK pathways.

PI3-kinase is an important modulator of cell survival, mitogenesis, cytoskeletal remodeling, metabolic control, and vesicular trafficking in various cell systems (4). Class I PI3-kinase consists of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit. Binding of the p85 subunit to phosphotyrosine residues activates the p110 subunit (5). In previous work we demonstrated, in primary hepatocyte cultures, that the PI3-kinase is necessary and sufficient for EGF-induced DNA synthesis (6,7); and that p85 was recruited to Gab2, ErbB3, and Shc in an EGF-dependent manner (7). Our study showed that phosphotyrosine Gab2 was responsible for over 80% of EGF-induced p85 recruitment and PI3-kinase activation.

Gab2 belongs to a super family of docking proteins, including Gab1, Gab2, Gab3, insulin receptor substrates (IRS-1, IRS-2, IRS-3), fibroblast growth factor substrate (FRS2), linker of T cell (LAT) and downstream of kinase (Dok) (for review (8,9)). All Gab family proteins contain binding sites for various signaling molecules, such as the adaptor molecule Grb2, the phosphotyrosine phosphatase SHP2, as well as the p85 subunit of PI3-kinase (10,11). In addition all family members possess an N-terminal pleckstrin homology (PH) domain, best known for its ability to bind phosphoinositides and thus contribute to the membrane targeting of the protein (reviewed in (12)). The Gab1 PH-domain has been shown preferentially to bind phosphatidylinositol 3,4,5-triphosphate, a product of PI3-kinase (13), and has been shown to be responsible for the membrane localization and signaling function of Gab1 (14,15).

Although various hormones and growth factors, such as insulin and PDGF, are able to activate PI3-kinase (16,17), each elicits specific biological effects. In primary rat hepatocytes, both insulin and EGF activate PI3-kinase but only insulin is able to down-regulate the expression of the IGFBP-1 mRNA (18). In adipocytes, insulin and PDGF activate PI3-kinase, but only insulin is able to promote translocation of glucose transporters (GLUT4) from intracellular vesicles to the cell surface (19). Previous data showed that insulin and PDGF recruit PI3-kinase to distinct intracellular locations. With PDGF, the major increase was observed in PM, whereas with insulin, PI3-kinase was recruited to a high speed pellet fraction (HSP) that also contained IRS-1 and the intracellular pool of

GLUT4, thus suggesting that the specific intracellular localization of different activated signaling molecules is a determinant of signaling specificity (20).

In the present study, we evaluated the subcellular distribution in liver of p85-containing complexes formed upon EGF treatment. We observed that the cytosolic Gab2-Grb2-SHP2-p85 complex was initially involved in and responsible for ~80% of total EGF-induced PI3-kinase activation in liver. Our data clearly demonstrated that the PH-domain of Gab2 is not required for Gab2 tyrosine phosphorylation or its mediation of EGF-induced mitogenesis.

3.4. Experimental Procedures

3.4.1. Animals

For the in vivo studies, 160-180 g female Sprague Dawley rats (Charles River, St Constant, Quebec, Canada) were housed at 25°C in an animal facility with a 12 h light cycle and fed ad libitum. All animals were fasted 16 to 18 h before use.

3.4.2. Materials

Mouse EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Collagenase was from Worthington Biochemical Corporation (Halls Mills Road, NJ). Cell culture medium and antibiotics were from Gibco-BRL (Life Technologies, Burlington, Ont., Canada). Vitrogen-100 was from Collagen Corporation (Toronto, Canada). [^{3}H]-methylthymidine, [^{125}I]-labeled goat anti-rabbit antibody ([^{125}I]-GAR) was from ICN Biomedicals Canada Ltd. (Mississauga, Ontario, Canada). [γ-^{32}P]ATP was purchased from NEN-DuPont (Wilmington, DE). Protein A-Sepharose (PAS) was from Pharmacia (Montréal, Québec, Canada). The anti-phosphotyrosine antibody (PY99), Grb2, SHP2 and HA antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The p85, ErbB3, Shc, Myc and Gab2 (for immunoblotting) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Phospho-Akt473, Akt, phospho-Erk1/2, Erk1/2 were from New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest grade available.

3.4.3. Generation of a Gab2 antibody

A GST-Gab2 fusion construct containing the amino acids 376 to 552 of the rat Gab2 cDNA sequence (7) was generated by PCR with 5'-ACTGGGATCCAGATCTGTAGCTGCTACTATCC as the sense primer and 5' CCCTTAGTACTGGGAGGAACTGGAG as the antisense primer. The amplified product was digested with SmaI and BamHI and then subcloned into the corresponding sites of the pGEX 4T-1 vector (Pharmacia). Ligated DNAs were transformed into *E. coli* strain DH5α, and colonies were screened for the correct insert by restriction digestion. GST fusion proteins were prepared from DH5α lysates by adsorption to glutathione-agarose as described in the Pharmacia GST Gene Fusion System manual and were used to immunize rabbits. The anti-Gab2 antibody generated was used for immunoprecipitation studies.

3.4.4. Preparation of subcellular fractions

Rats were anaesthetized and killed by decapitation after intrajugular injections of EGF at the indicated times in the text (1μg/100g BW). Livers were rapidly excised, and minced at scissor point in ice-cold buffer (5 mM Tris-HCL buffer, pH 7.4, containing 0.25 M sucrose, 1mM benzamidine, 1mM PMSF, 1mM $MgCl_2$, 2mM NaF, and 2mM sodium orthovanadate). Plasma membrane (PM), endosomes (ENs), and cytosol were prepared as previously described (21). Plasma membranes/mitochondria/nuclei (PMN) and cytosol (Cyt) were prepared from primary hepatocytes as previously described (22). The protein content of these fractions was measured using a modification of Bradford's method with BSA as standard (23).

3.4.5. Preparation of the Gab2 mutants

The Gab2Δp85 was generated by replacing Tyr-461, Tyr-465 and Tyr-573 by phenylalanine using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The PH-domain of Gab2 and ΔPH Gab2 constructs were generated by PCR amplification of cDNA sequences encoding amino acids 1-119 and 120-666 of rat Gab2 respectively. All constructs were verified by DNA sequencing. In order to produce the adenoviruses, mutants as well as the full-length cDNA were first subcloned into pShuttle between the NheI and NotI sites using 5'-oligonucleotides containing an NheI- site and the sequences encoding different tags (HA for WTGab2, Δp85Gab2 and ΔPH Gab2, Myc for PHGab2) and 3'-oligonucleotides containing a NotI site. The adenoviral DNAs were further generated using the Adeno-X expression system (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

3.4.6. Adenovirus production and titration

Large-scale production of recombinant viral particles was performed by infecting 293A cells. The titer of viral particles was determined using the Tissue Culture Infectious Dose 50 ($TCID_{50}$) method as described in the protocol of the Ad-easy vector system (Q.Biogene, Carlsbad, CA).

3.4.7. Cell culture and viral infection

Primary hepatocytes were prepared as previously described (7). Cells were first bathed for 24 h in seeding medium (Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) containing 10% FBS, 10 mM HEPES, 20 mM $NaHCO_3$, 500

IU/ml penicillin, and 500 μg/ml streptomycin) and then infected with different adenoviruses for 3 h at 37°C. The infected cells were serum-starved for 48 h in serum-free medium (SFM) before harvesting.

3.4.8. Immunoprecipitation and immunoblotting

Cell fractions were incubated in 1% (v/v) Triton X-100 and 0.5% (w/v) sodium deoxycholate at 4°C for 1 h. Cell lysates, prepared as previously described (7), from EGF-treated (100 ng/ml EGF) or non-treated cells were pre-cleared with non-immune rabbit IgG (Sigma St. Louis, MO) and protein A-Sepharose for 1 h at 4°C. After centrifugation, the resulting supernatants were incubated for 2 h at 4°C with the antibody indicated in the figure legends. Protein A-Sepharose (50 μl of a 50% slurry) was added to each sample and the incubation was continued for an additional hour. Immune complexes were isolated by centrifugation, washed three times in PBS, and boiled in Laemmli sample buffer. Immunoprecipitates or intact samples where subjected to SDS-PAGE, transferred to Immobilon-P membranes (Millipore Ltd., Mississauga, Ontario, Canada), and immunoblotted with the indicated first antibody for 90 min followed by 1 h incubation with HRP-, or [^{125}I]-labeled GAR or GAM IgG. Immunoreactive proteins were detected by autoradiography or by ECL system (Amersham Pharmacia biotech, Montréal, Québec). Densitometric quantification of the signals was performed using a BioRad densitometer, Model GS-700.

3.4.9. PI3-kinase activity assay

Cell fractions or cell lysates (500 μg protein) were subjected to immunoprecipitation with Protein A-Sepharose and the antibodies indicated in

the figure legends. Immunoprecipitates were extensively washed and the immune complexes were resuspended in 50 µl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 0.5 mg/ml L-α-phosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster, AL), and assayed for PI3-kinase activity as previously described (6).

3.4.10. [^{3}H]-thymidine incorporation assay

Infected cells were serum-starved for 20 h in SFM, then 100ng/ml EGF and [^{3}H]methylthymidine (5 µCi/ml) were added to the medium. After 18 h incubation cells were rinsed three times with 3 ml cold PBS, incubated for 15 min at 4°C in 10% trichloro-acetic acid (TCA), solubilized at room temperature in 1 ml 1 N NaOH, neutralized with 1ml 1N HCl, and transferred to scintillation vials for counting of [^{3}H].

3.4.11. Immunofluorescence

Primary hepatocytes were plated on glass coverslips (Fisher, Ottawa, ONT) in a 6-well dish. Cells were infected with an HA epitope-tagged Gab2-expressing adenovirus. After 48 h of starvation, cells treated with or without 100 ng/ml EGF were fixed in 4% paraformaldehyde for 10 min at room temperature, washed 5 times in PBS, and incubated for 5 min in 0.5% Triton to permeabilize the cells. Following washing the cells were treated with PBS containing 0.5 mg/ml $NaBH_4$ for 10 min at room temperature. After the wash, αHA antibody, at a 1:200 dilution in PBS containing 5% BSA, was added to the cells for 1 h (RT). After washing, the secondary antibody (anti-GAR IgG FITC-conjugated, Sigma, St. Louis, MO), at a 1:300 dilution in the same buffer, was added for 1 h (RT). After

4 to 5 final washes, the glass coverslips were mounted onto slides (Gold Seal®, Portsmouth, NH) in moviol medium (Calbiochem, San Diego, California) and visualized using a Zeiss Axiovert 135 confocal microscope. Photographs were taken using a Zeiss LSM microsystem.

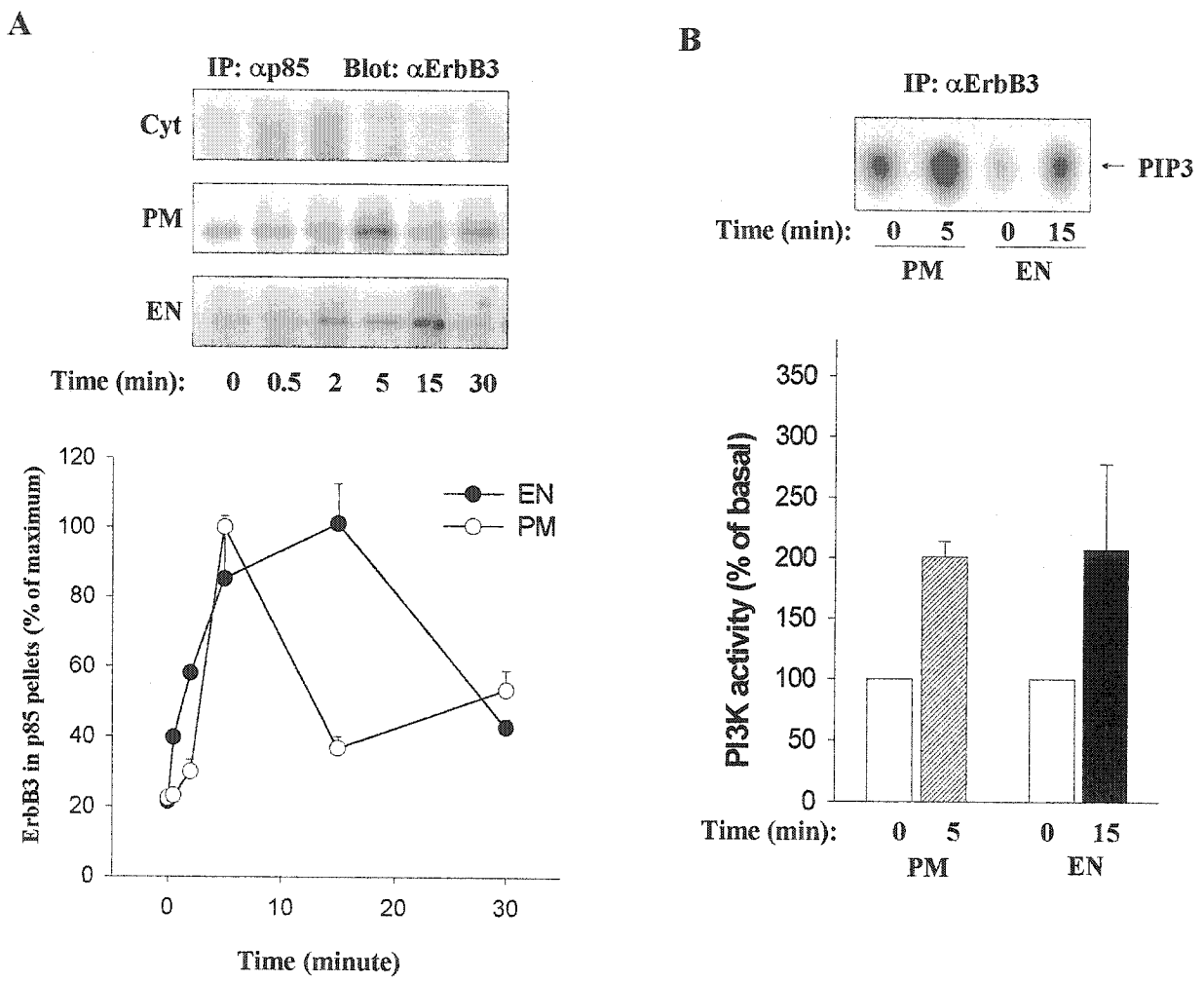
3.5. Results

We recently demonstrated the critical role played by the recruitment of p85 to PY proteins in mediating EGF-induced PI3-kinase activity (7) and identified three distinct p85-containing complexes in primary rat hepatocytes in response to EGF. One complex contained ErbB3, a second Shc, and a third Gab2, SHP-2, and Grb2. In the present study we evaluated the relationship between compartmentalization of these complexes and signaling, and hence isolated hepatic subcellular fractions from EGF-treated rats to evaluate them for the presence of the different complexes following ligand administration.

3.5.1. Effect of EGF on the subcellular localization of the p85-ErbB3 complex

We analyzed the distribution of the ErbB3 protein in rat liver after EGF injection. The ErbB3 protein, which belongs to the EGFR family, was found both in PM and ENs, but not in the cytosol (*data not shown*). We subsequently investigated the EGF-induced association of ErbB3 with p85 in these different fractions. Equal amounts of protein from each fraction were subjected to immunoprecipitation with anti-p85 antibody and were subsequently immunoblotted with an anti-ErbB3 antibody (Fig. 1A). No signal for ErbB3 was detected in the cytosol (Fig. 1A, *top panel*). However, EGF induced the association of p85 with ErbB3 in PM and ENs with a maximum reached at 5 and 15 mins respectively; and a return of the latter to basal at 30 mins (Fig. 1A, *bottom panel*). These data suggest that upon EGF treatment, a complex containing ErbB3 and p85 is formed at the PM and then internalized into ENs. To confirm that the association with p85 reflected activated

3-Figure 1. Subcellular distribution and associated PI3-kinase activity of the ErbB3-p85 complex in rat liver. After fasting overnight rats received a single i.j. dose of EGF (1μg/100g BW), and were killed at the noted times thereafter. PM (○), EN (●), and cytosol (Cyt) were prepared as described in Experimental Procedures. A. Aliquots of each fraction (500 μg protein) were immunoprecipitated with αp85. Immunoprecipitates were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αErbB3. *Upper panel*, representative immunoblots at different times after injection of EGF. *Lower panel*, levels of ErbB3 in αp85 immunoprecipitates were quantified using scanning densitometry and the results plotted as a percentage of the maximum value obtained (PM, 5 mins after EGF treatment). Each point is the mean ± S. E. of three separate experiments. B. Aliquots (500 μg protein) from PM and ENs were immunoprecipitated with αErbB3 and analyzed for PI3-kinase activity as described under Experimental Procedures. *Upper panel*, autoradiograph of a representative experiment indicating the location of the reaction product, phosphatidylinositol-3-phosphate (PIP_3). *Lower panel*, quantification of the PIP_3s generated in PM and ENs fractions 5 or 15 mins post EGF injection respectively. Results are expressed as the percentage of basal activity (that in non EGF-treated rats) in each fraction (mean ± S. E., three separate experiments).


A
IP: αp85 Blot: αErbB3
Cyt
PM
EN
Time (min): 0 0.5 2 5 15 30
ErbB3 in p85 pellets (% of maximum)
120
100
80
60
40
20
0
EN
PM
0
10
20
30
Time (minute)
B
IP: αErbB3
PIP3
Time (min): 0 5 0 15
PM EN
PI3K activity (% of basal)
350
300
250
200
150
100
50
0
Time (min): 0 5 0 15
PM EN

PI3-kinase we measured this activity, at the time of maximal association in PM and ENs, in an anti-ErbB3 immunoprecipitate. As shown in Fig. 1B, there was a two fold increase in the ErbB3-associated PI3-kinase activity in both PM and ENs 5 and 15 mins after EGF injection, respectively.

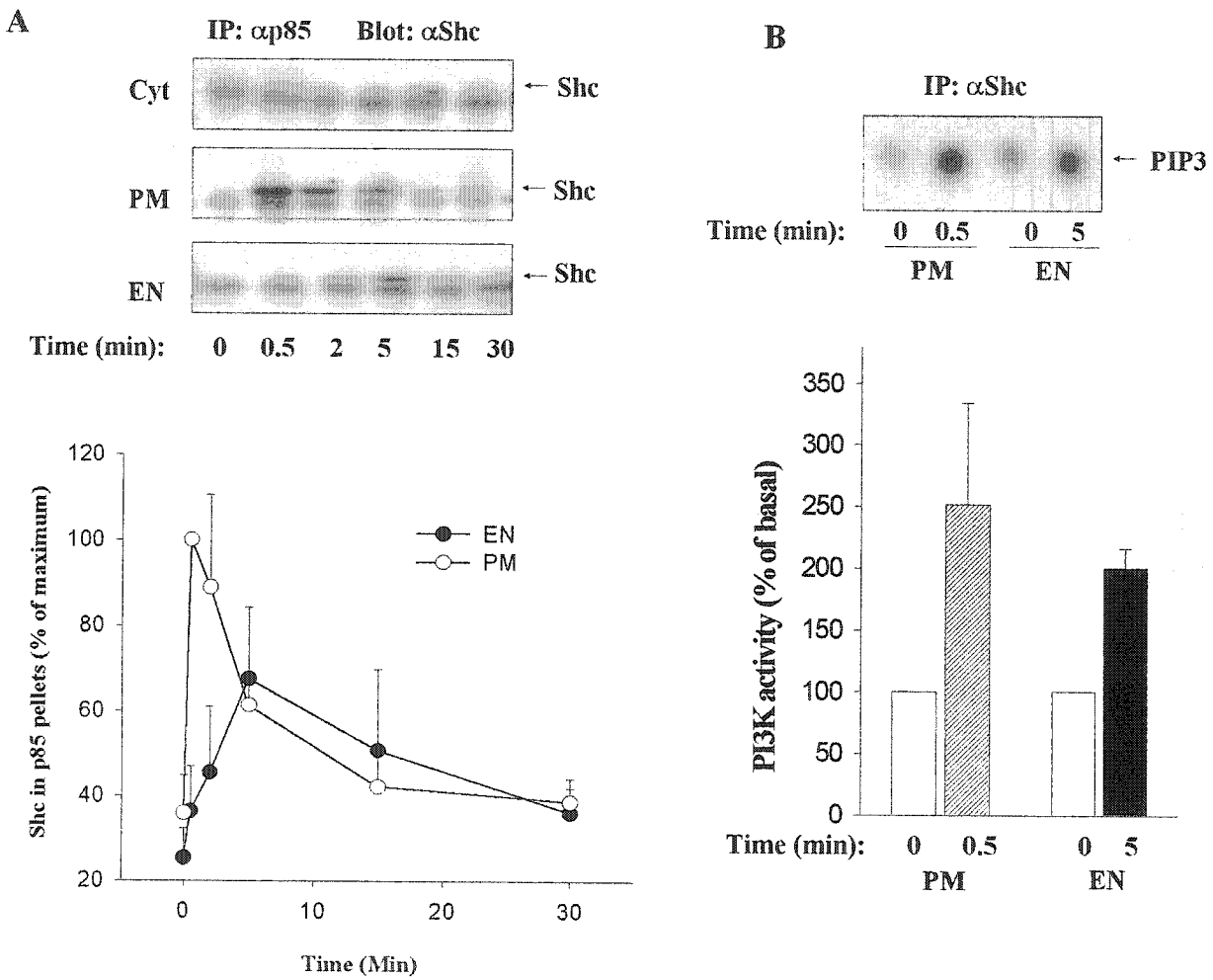
3.5.2. Effect of EGF on the subcellular localization of the p85-Shc complex

To investigate the subcellular localization of the p85-Shc complex in rat liver, we performed the same experiments as described for the p85-ErbB3 complex. Cytosol, PM and ENs fractions were subjected to immunoprecipitation with anti-p85 antibody and subsequently immunoblotted with an anti-Shc antibody (Fig. 2A). Although Shc proteins were detected in all subcellular fractions (*data not shown*), we could not observe any association of Shc with p85 in cytosol (Fig. 2A, *top panel*). In PM, Shc association with p85 reached a maximum at 30 secs and declined rapidly thereafter (Fig. 2A, *middle panel*). In ENs, its association with p85 increased from 30 secs, reached a maximum at 5 mins, and then declined (Fig. 2A, *bottom panel*). These data suggest that, upon EGF treatment, a complex of the adaptor protein Shc and p85 is formed at the PM and undergoes rapid internalization into ENs. As shown in Fig. 2B, Shc-associated PI3-kinase activity increased about 2.5 fold in PM and 2 fold in ENs at 30 secs and 5 mins respectively following EGF injection.

3.5.3. Effect of EGF on the subcellular distribution of the Gab2-SHP2-Grb2-p85 complex

We have previously shown that a multimeric Gab2-SHP2-Grb2-p85 complex accounts for 80% of the total EGF-induced PI3-kinase activity in rat primary

3-Figure 2. Subcellular distribution and associated PI3-kinase activity of the Shc-p85 complex in rat liver. After fasting overnight rats received a single i.j. dose of EGF (1μg/100g BW), and were killed at the noted times thereafter. PM (○), EN (●), and cytosol (Cyt) were prepared as described in Experimental Procedures. A. Aliquots (500 μg protein) were immunoprecipitated with αp85. Immunoprecipitates were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αShc. *Upper panel*, representative immunoblot at different times following EGF. *Lower panel*, levels of Shc in αp85 immunoprecipitates were quantified using scanning densitometry and the results plotted as a percentage of the maximum value obtained (PM, 0.5 min after EGF treatment). Each point is the mean ± S. E. of three separate experiments. B. Aliquots (500 μg protein) immunoprecipitated with αShc were analyzed for PI3-kinase activity as described under Experimental Procedures. *Upper panel*, autoradiograph of a representative experiment indicating the location of the reaction product (PIP3). *Lower panel*, quantification of the PIP3s in PM and ENs 0.5 or 5 mins respectively after EGF injection. Results are expressed as the percentage of basal values (non EGF-treated rats) for each fraction (mean ± S. E., three separate experiments).


A
IP: αp85
Blot: αShc
Cyt
PM
EN
Shc
Shc
Shc
Time (min): 0 0.5 2 5 15 30
Shc in p85 pellets (% of maximum)
120
100
80
60
40
20
0
10
20
30
Time (Min)
EN
PM
B
IP: αShc
PIP3
Time (min): 0 0.5 0 5
PM
EN
PI3K activity (% of basal)
350
300
250
200
150
100
50
0
Time (min): 0 0.5 0 5
PM
EN

hepatocytes (7). First we assessed the subcellular localization of Gab2 upon EGF stimulation, using Western blot analyses on intact samples isolated from cytosol, PM and ENs. As shown in Fig. 3A, Gab2 is detected in each subcellular fraction however, with different intensities. It is barely detectable in ENs but seems to be more concentrated in the PM. Even though the Gab2 protein bands in cytosol are weaker than in PM, the main bulk of the Gab2 protein is localized in the cytosolic fraction, since the amount of cytosolic proteins extracted in these experimental conditions, is about 100 times higher than protein from the PM fraction (*data not shown*). The presence of a PH-domain in the Gab2 sequence suggests that Gab2 might be recruited to membranes (10,11). However, we were unable to detect any significant translocation of Gab2 from the cytosol to either PM or ENs upon EGF stimulation.

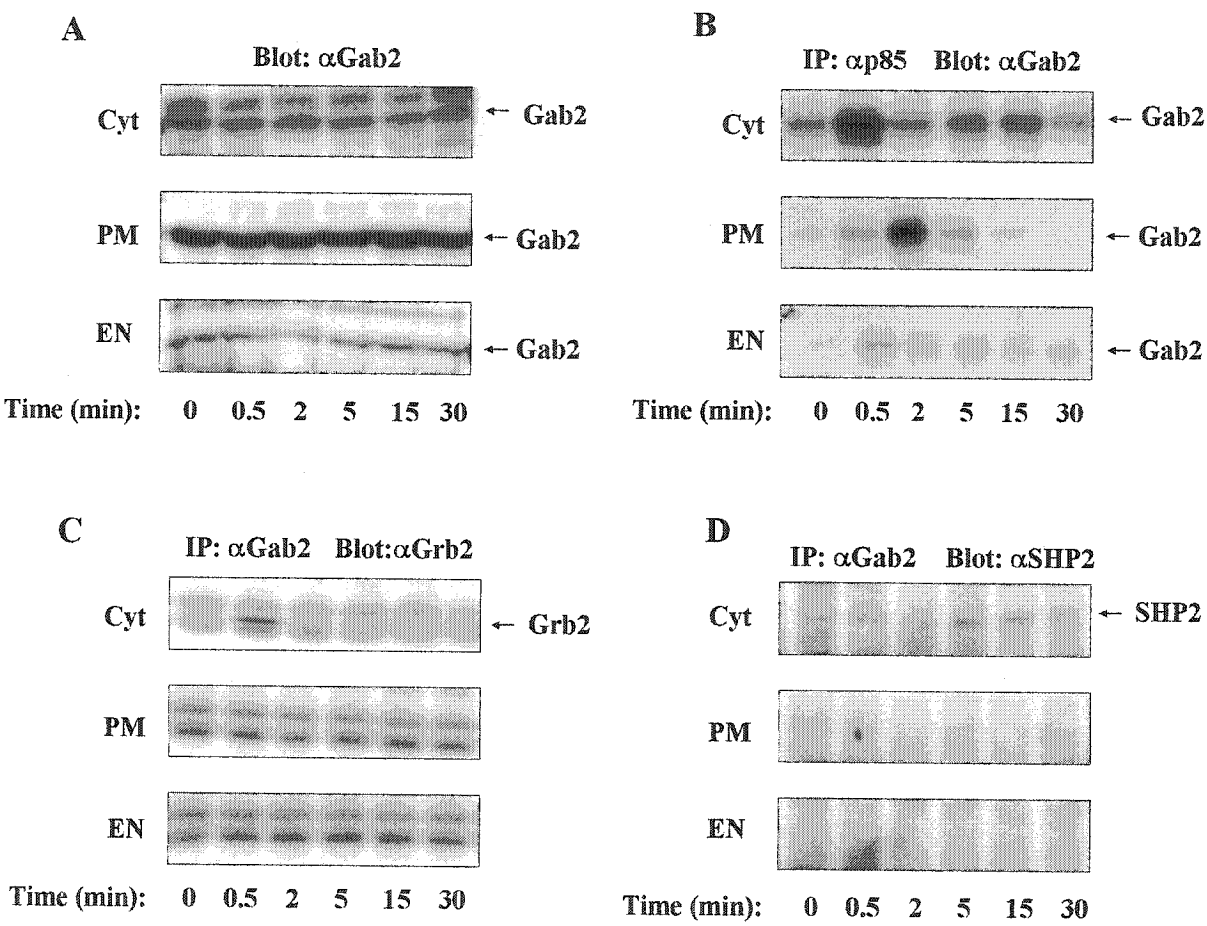
We then assessed the subcellular distribution of Gab2-p85 complexes following EGF treatment. Protein (500 μg) from cytosol, PM, and ENs was subjected to immunoprecipitation with αp85 and immunoblotted with αGab2. As seen in Fig. 3B, EGF treatment induced a rapid association of Gab2 with p85 in all 3 subcellular fractions. In ENs, we detected an increased association at 30 secs after EGF treatment. However, the intensity of the detected complex was much lower than that observed in PM and cytosol. In PM, EGF also induced the association of Gab2 to p85 at 30 secs after EGF treatment reaching a maximum at 2 mins and returning to basal levels by 15 mins post injection. In cytosol, the maximal association was observed at 30 secs and was followed by a rapid

decrease to basal levels by 15 mins post injection. Thus Gab2 is associated with p85 principally in PM and cytosol reaching a maximal association at 2 mins and 30 secs respectively. This time course suggests that the activation of PI3-kinase is initiated by its association with cytosolic Gab2.

In further studies we analyzed the association of the two other Gab2 binding partners, Grb2 and SHP2, in each subcellular fraction. Proteins were first immunoprecipitated with anti-Gab2 antibody and then immunoblotted with either αGrb2 or αSHP2 (Figs. 3C and 3D, respectively). Grb2 and SHP2 were found in all 3 subcellular fractions. However, following EGF treatment Grb2 was found to associate with Gab2 only in the cytosol. Furthermore, this EGF-induced association was rapid and transient (detected only after 30 secs of EGF treatment) (Fig. 3C). SHP2 and Gab2 were also found to associate only in the cytosol after EGF treatment (Fig. 3D). However, in contrast to Grb2, this interaction, which was detectable at 30 secs, was sustained up to 30 mins after EGF treatment.

Taken together, our data show that ErbB3-p85 and Shc-p85 complexes were localized to PM and ENs; whereas the multimeric Gab2-Grb2-SHP2-p85 complex was detected exclusively in cytosol. The association of Gab2 with p85 in PM might reflect an EGF-dependent translocation of Gab2 and p85 from cytosol to PM, or the independent direct phosphorylation of the membrane-associated Gab2.

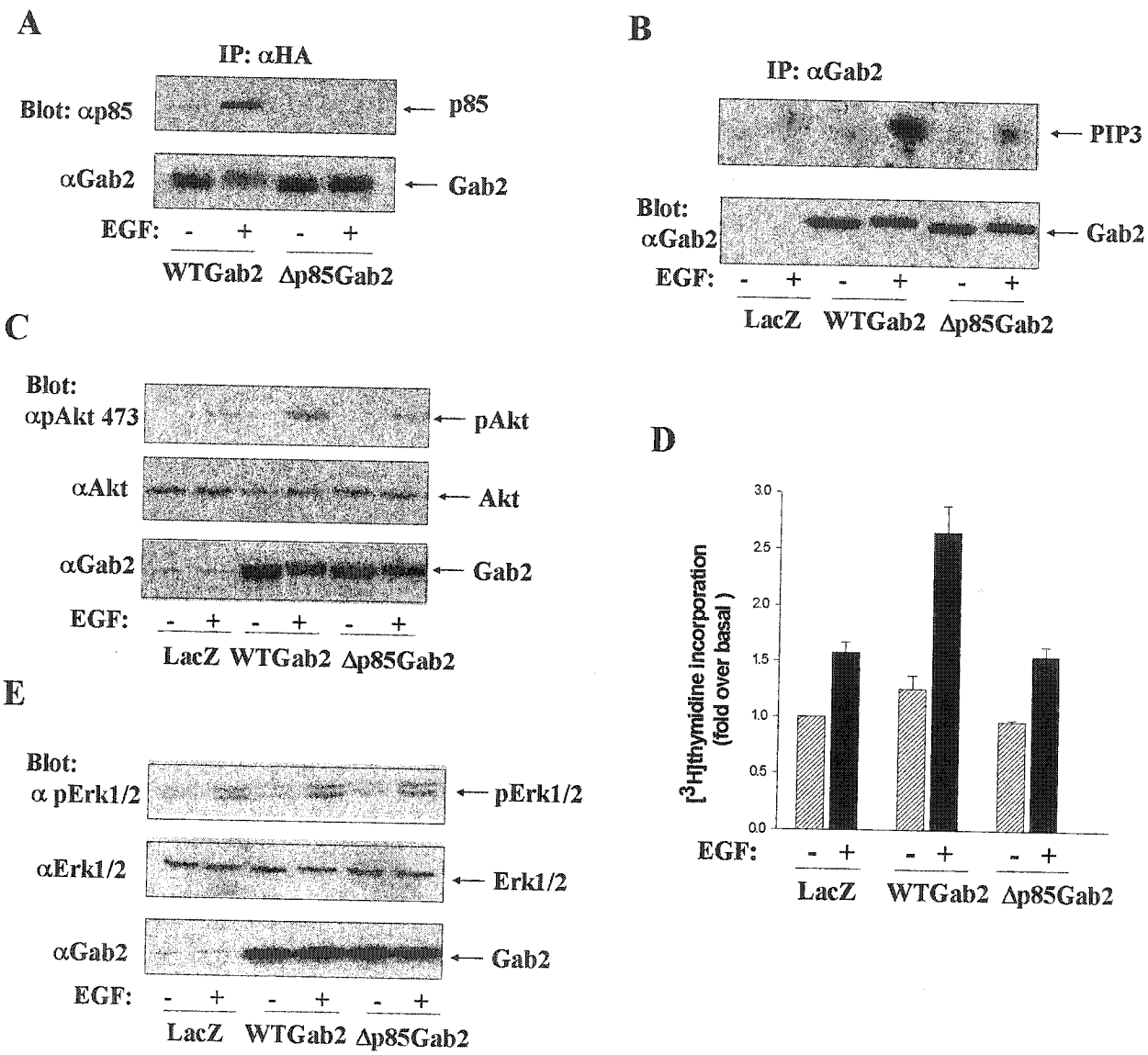
3-Figure 3. Subcellular localization of the Gab2-Grb2-SHP2-p85 complex in rat liver. After fasting overnight rats received a single i.j. dose of EGF (1μg/100g BW), and were killed at the noted times thereafter. PM, EN, and Cyt were prepared as described in Experimental Procedures. Panels show representative immunoblots of three separate experiments performed in each case, with the times after EGF injection indicated in minutes. A. 100 μg protein of each fraction was subjected to SDS-PAGE, transferred to Immobilon-P membranes and immunoblotted with αGab2. B. Aliquots of each fraction (500 μg protein) were immunoprecipitated with αp85, resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αGab2. For C and D, aliquots of each fraction (500 μg protein) were immunoprecipitated with αGab2, resolved on 12.5% SDS-PAGE and then subjected to immunoblotting with either αGrb2 or αSHP2 respectively.


A
Blot: αGab2
Cyt
PM
EN
← Gab2
Time (min): 0 0.5 2 5 15 30
B
IP: αp85 Blot: αGab2
Cyt
PM
EN
← Gab2
Time (min): 0 0.5 2 5 15 30
C
IP: αGab2 Blot:αGrb2
Cyt
PM
EN
← Grb2
Time (min): 0 0.5 2 5 15 30
D
IP: αGab2 Blot: αSHP2
Cyt
PM
EN
← SHP2
Time (min): 0 0.5 2 5 15 30

3.5.4. Role of Gab2 in EGF-induced PI3-kinase activation and DNA synthesis

Our previous study in primary rat hepatocyte cultures pointed to a role for EGF-induced PI3-kinase activity in DNA synthesis (7). To establish the importance of Gab2 we over-expressed in primary hepatocytes both wild-type Gab2 (WTGab2) and Δp85Gab2 (mutant lacking the p85-binding sites, see *Experimental Procedures*), using adenovirus technology. Cells were infected with either control recombinant adenovirus (LacZ) or HA-tagged constructs (WTGab2 or Δp85Gab2). Infected-cells expressed the HA-Gab2 constructs 20 fold over the endogenous Gab2 (Fig. 4A, *lower panel).* As expected, deletion of the p85 binding sites totally abolished p85 binding to HA-Gab2 (Fig. 4A, *upper panel*, lanes 3 and 4). Over-expression of WTGab2 augmented the effect of EGF on PI3-kinase activity by 25 fold (Fig. 4B, *upper panel*, compare lanes 2 and 4); whereas over-expression of Δp85Gab2 had no effect beyond that observed with control cells (Fig. 4B, *upper panel*, compare lanes 2 and 6). Interestingly Δp85Gab2 did not exert a dominant negative effect in that it did not reduce PI3-kinase activity to levels below control despite a 20 fold greater level of expression than endogenous Gab2. Similar results were obtained when we measured EGF-induced Akt phosphorylation on Ser 473 and DNA synthesis (Fig. 4C and 4D, respectively).

3-Figure 4. Effect of over-expression of Gab2 constructs on EGF-induced PI3-kinase, Akt activation and DNA synthesis. Hepatocytes were infected with 10 moi LacZ, wild-type Gab2 (WTGab2) or Δp85Gab2 recombinant adenoviruses for 3 h and then starved for 48 h. A. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. Cell lysates were equally divided into two tubes and immunoprecipitated with anti-HA antibody. The immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αp85 (*upper panel*) or αGab2 (*lower panel*). B. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. Cell lysates were equally divided into two tubes and immunoprecipitated with αGab2. The immunoprecipitated proteins were subjected either to the PI3-kinase activity assay (*upper panel*) or to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αGab2 (*lower panel*). C. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 5 mins. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblot analysis with an anti-phospho-Akt473 (*top panel*), αAkt (*middle panel*) or αGab2 (*bottom panel*). D. Virus-infected hepatocytes were starved for 24 h before an 18 h incubation in serum-free medium containing 5μCi of [^{3}H]methylthymidine without (*hatched bars*) or with 100 ng/ml EGF (*black bars*). Incorporation of [^{3}H]methylthymidine into DNA was determined as described under *Experimental Procedures*. Results are expressed as fold over basal level (LacZ cells) (mean ± S.E., three separate experiments). E- Serum-deprived hepatocytes were treated with (+) or without (-) 0.2 ng/ml EGF for 5 mins. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblot analysis with an anti-phospho-Erk1/2 (*top panel*), αErk1/2 (*middle panel*) or αGab2 (*bottom panel*).


A
IP: αHA
Blot: αp85
p85
αGab2
Gab2
EGF: - + - +
WTGab2 Δp85Gab2
B
IP: αGab2
PIP3
Blot:
αGab2
Gab2
EGF: - + - + - +
LacZ WTGab2 Δp85Gab2
C
Blot:
αpAkt 473
pAkt
αAkt
Akt
αGab2
Gab2
EGF: - + - + - +
LacZ WTGab2 Δp85Gab2
D
[3H]thymidine incorporation
(fold over basal)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EGF: - + - + - +
LacZ WTGab2 Δp85Gab2
E
Blot:
α pErk1/2
pErk1/2
αErk1/2
Erk1/2
αGab2
Gab2
EGF: - + - + - +
LacZ WTGab2 Δp85Gab2

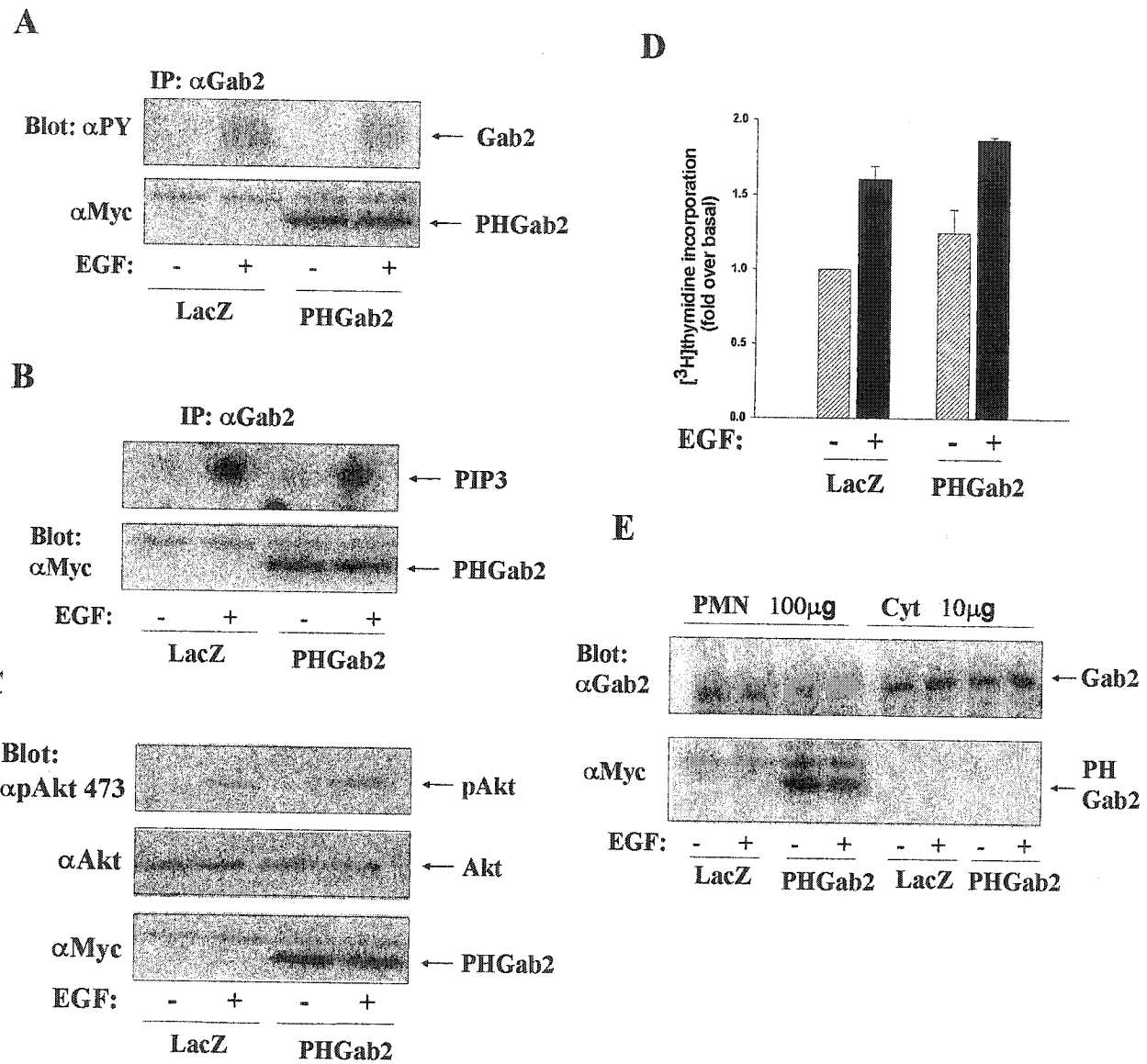
As shown in Fig. 4E, both WTGab2 and Δp85Gab2 equally potentiated EGF-induced Erk1/2 activation[1] indicating that MAPK activation is independent of a PI3-kinase mechanism. In summary Gab2 plays a key role in EGF-induced PI3-kinase activation and DNA synthesis and is also clearly implicated in EGF-induced MAPK activation though not through the activation of PI3-kinase.

3.5.5. Role of the PH-domain in Gab2 activation

Various studies have demonstrated that the PH-domain in IRSs and Gab1 facilitate phosphorylation and activation of these molecules by directing their recruitment to membranes through binding to PIP3 (9,24,25) Surprisingly we found that, following *in vivo* EGF treatment, Gab2 did not undergo net translocation to membranes but rather formed a multimeric complex with p85 in the cytosol (Fig. 3). We sought to examine the role of the PH-domain of Gab2 on its phosphorylation and downstream signaling. We therefore constructed two recombinant adenoviruses (see *Experimental Procedures*), one containing the entire PH-domain of Gab2 linked to a Myc epitope (PHGab2) and one where the PH-domain was deleted from the full length Gab2 sequence (ΔPHGab2). Hepatocytes infected with PHGab2 containing recombinant adenovirus readily expressed the Myc tagged molecule (Fig. 5A, *lower panel*, lanes 3 and 4). Over-expression of PHGab2 did not interfere with EGF-induced phosphorylation of endogenous Gab2 (Fig. 5A, *upper panel*, compare lanes 2 and 4),

[1]This is observed at an EGF dose of 0.2 ng/ml reflecting the greater sensitivity of the MAPK pathway to EGF than the PI3-kinase pathway, where a 100 ng/ml EGF dose was used.

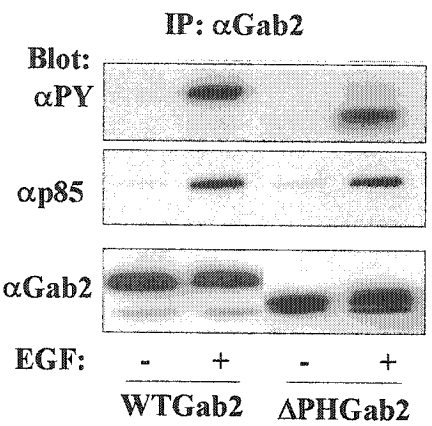
3-Figure 5. Effect of over-expression of PHGab2 on EGF-induced Gab2 phosphorylation and activation of downstream responses. Hepatocytes were infected with 10 moi LacZ, wild-type Gab2 (WTGab2) or PHGab2 recombinant adenoviruses for 3 h and then starved for 48 h. A. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. *Upper Panel*: Cell lysates were immunoprecipitated with αGab2, subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αPY. *Lower panel*: Cell lysates were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αMyc. B. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. *Upper Panel*: Cell lysates were immunoprecipitated with αGab2 and immunoprecipitated proteins were subjected to PI3-kinase activity assay. *Lower panel*: Cell lysates were subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αMyc. C. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 5 mins. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblot analysis with an anti-phospho-Akt473 (*top panel*), αAkt (*middle panel*) or αMyc (*bottom panel*). D. Virus-infected hepatocytes were starved for 24 h before a 18 h incubation in serum-free medium containing 5 μCi of [^{3}H] methylthymidine without (*hatched bars*) or with 100 ng/ml EGF (*black bars*). Incorporation of [^{3}H]methylthymidine into DNA was determined as described under *Experimental Procedures*. Results are expressed as fold over basal level (LacZ cells) (mean ± S.E., three separate experiments). E. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. PMN and Cyt were prepared as described in *Experimental Procedures*. 100 μg protein of PMN and 10 μg protein of Cyt were subjected to 10% SDS-PAGE, transferred to immobilon-P membranes and immunoblotted with αGab2 (*Upper Panel)* or αMyc (*Lower panel*).


A
IP: αGab2
Blot: αPY
Gab2
αMyc
PHGab2
EGF: - + - +
LacZ
PHGab2
B
IP: αGab2
PIP3
Blot: αMyc
PHGab2
EGF: - + - +
LacZ
PHGab2
C
Blot: αpAkt 473
pAkt
αAkt
Akt
αMyc
PHGab2
EGF: - + - +
LacZ
PHGab2
D
[3H]thymidine incorporation (fold over basal)
2.0
1.5
1.0
0.5
0.0
EGF: - + - +
LacZ
PHGab2
E
PMN 100μg
Cyt 10μg
Blot: αGab2
Gab2
αMyc
PH Gab2
EGF: - + - + - + - +
LacZ
PHGab2
LacZ
PHGab2

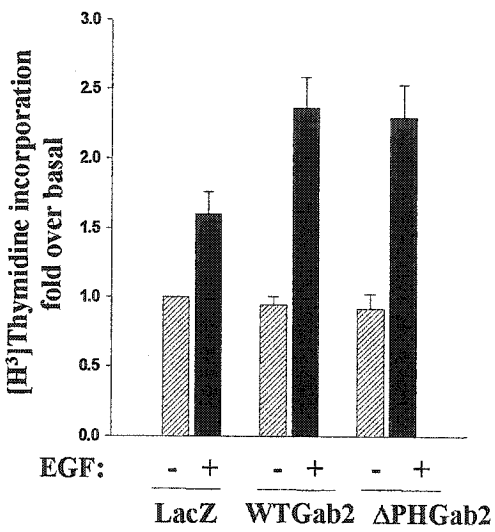
EGF-induced PI3-kinase activation (Fig. 5B, *upper panel*, compare lanes 2 and 4), Akt phosphorylation (Fig. 5C, *upper panel*, compare lanes 2 and 4), or DNA synthesis (Fig. 5D, compare black bars). Western blot analyses of plasma membrane/mitochondria/nuclei (PMN) and cytosolic fractions from LacZ or PHGab2 infected primary hepatocytes showed that over-expressed PHGab2 was exclusively detected in PMN independent of EGF treatment (Fig. 5E). However, the expression of PHGab2 minimally affected the amount of endogenous Gab2 present in membrane fractions (Fig. 5E, *top panel*, compare lanes 1 & 2 vs. 3 & 4). In addition we showed that over-expressed ΔPHGab2, which lacks the entire PH-domain, is tyrosine phosphorylated upon EGF treatment to a similar extent as over-expressed WTGab2 and can similarly recruit p85 (Fig. 6A), and potentiate EGF-induced thymidine incorporation into DNA (Fig. 6B). To evaluate the effect of the PH-domain deletion on Gab2 distribution, we performed Western blot analyses of PMN and cytosolic fractions from primary hepatocytes infected with WTGab2 or ΔPHGab2. As shown in Fig. 6C, both over-expressed WTGab2 and ΔPHGab2 were detected in cytosol and to a lesser extent in the PMN. Interestingly, compared to WTGab2, there was less ΔPHGab2 bound to membranes even though ΔPHGab2 equally potentiated EGF-induced DNA synthesis with the WTGab2. Surprisingly, EGF treatment further reduced membrane-associated ΔPH Gab2 (48.3$\pm$0.25%, mean ± ½ range, N = 2) compared to non-EGF treated. Taken together, these observations indicate that the PH-domain of Gab2 is not required for its EGF-induced phosphorylation and downstream signaling.

3-Figure 6. Effect of over-expression of ΔPHGab2 on EGF-induced Gab2 phosphorylation and DNA synthesis. A. Hepatocytes were infected with 10 moi WTGab2 or ΔPHGab2 recombinant adenoviruses for 3 h and then starved for 48 h. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. Cell lysates were immunoprecipitated with αGab2, subjected to SDS-PAGE (7.5% gel) followed by immunoblot analysis with αPY (*top panel*) or αp85 (*middle panel*). The membrane was then striped and reblotted with αGab2 (*bottom panel*). B. Hepatocytes were infected with 10 moi LacZ, WTGab2 or ΔPHGab2 recombinant adenoviruses for 3 h. Virus-infected hepatocytes were starved for 24 h before a 18 h incubation in serum-free medium containing 5 μCi of [^{3}H] methylthymidine without (*hatched bars*) or with 100 ng/ml EGF (*black bars*). Incorporation of [^{3}H]methylthymidine into DNA was determined as described under *Experimental Procedures*. Results are expressed as fold over basal level (LacZ cells) (mean ± S.E., four separate experiments). C. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. PMN and Cyt were prepared as described in *Experimental Procedures*. 100 μg protein of PMN and 10 μg protein of Cyt were subjected to 7.5% SDS-PAGE, transferred to Immobilon-P membranes and immunoblotted with αHA.

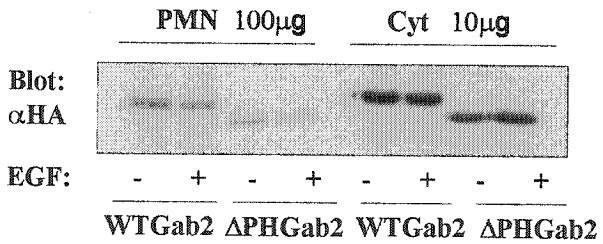
A


IP: αGab2
Blot:
αPY
αp85
αGab2
EGF: - + - +
WTGab2 ΔPHGab2

B

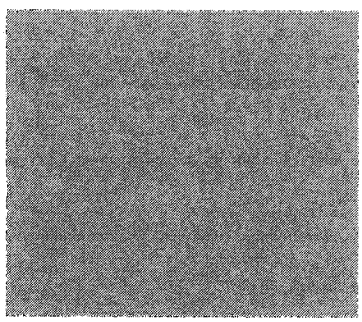

[H³]Thymidine incorporation
fold over basal
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EGF: - + - + - +
LacZ WTGab2 ΔPHGab2

C

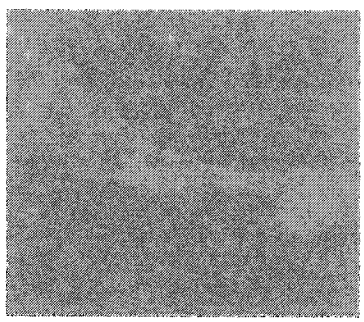

PMN 100μg
Cyt 10μg
Blot:
αHA
EGF: - + - + - + - +
WTGab2 ΔPHGab2 WTGab2 ΔPHGab2

To evaluate these findings further we studied the EGF effect on the cellular distribution of over-expressed Gab2 using confocal immunofluorescence microscopy (Fig. 7). As expected, no fluorescence was detected in LacZ infected cells, but strong cytosolic staining was visualized in HA-WTGab2 infected cells. EGF treatment for 1 or 5 mins did not alter the pattern of cytosolic staining, indicating that this treatment had no effect on recruitment of Gab2 to the PM. Taken together these data demonstrate that despite the presence of a PH-domain, Gab2 does not undergo net translocation to the PM in response to EGF.

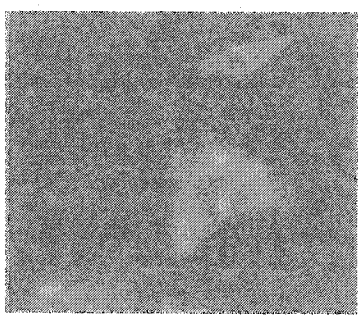
3-Figure 7. Immunolocalization of WTGab2 in hepatocytes after EGF treatment. Primary hepatocytes were infected with either LacZ or WTGab2 adenoviruses. Cell were grown for 48 h on glass coverslips and then stimulated or not by 100 ng/ml EGF for the indicated times. Cells were then treated as described in *Experimental Procedures* and labeled with anti-HA followed by FITC conjugated anti-rabbit. Cells were visualized with a confocal microscope and photographs were taken at a magnification of X60.



LacZ



HA-WT Gab2



HA-WT Gab2
1 min EGF

HA-WT Gab2
5 min EGF

3.6. Discussion

We previously demonstrated that EGF-induced PI3-kinase activation is critical for its ability to promote mitogenesis in rat hepatocytes. We found that EGF stimulated the association of p85 with 3 different tyrosine phosphorylated molecules and determined these to be ErbB3, Shc and Gab2 (7). A number of studies have now shown that ligand-induced compartmentalization of key signaling molecules is critical for transducing at least some aspects of the downstream response (26). In the present study we delineated the subcellular localization of the three p85-containing complexes formed upon EGF stimulation, the key role of Gab2 in mediating EGF action in rat liver, and the mechanism of Gab2 tyrosine phosphorylation.

3.6.1. PM and endosomal localization of ErbB3 and Shc-containing complexes

We found that ErbB3 associates with p85 after EGF treatment leading to PI3-kinase activation both in PM and ENs. The time course of association was consistent with translocation of ErbB3-p85 from PM to ENs. ErbB3 has been previously described as the most potent activator of PI3-kinase among the proteins of the ErbB family. Several analyses revealed the presence of multiple binding sites for p85 on the ErbB3 receptor (27-30). Like the other ErbB family members, the ErbB3 protein contains a consensus motif for the binding of Shc. Previous studies have documented an association of ErbB3 with Shc upon EGF treatment (27,31). Although we detected an association between p85 and Shc in

PM and ENs, we were unable to detect any association between ErbB3 and Shc arguing against the formation of an ErbB3-Shc-p85 complex in liver (7).

Prior work has defined a role for Shc principally in the regulation of the Ras-MAPK pathway via its association with Grb2 and Sos (32,33). Although a role for Shc in the regulation of PI3-kinase activity has been previously noted, this appeared to be through its association with the docking proteins Gab1 (34) and Gab2 (35). However, Gab1 is not expressed in liver and following EGF-treatment we found that Shc did not associate with Gab2 (7). Rather, in liver Shc seems to be directly involved in EGF-induced activation of PI3-kinase.

3.6.2. The cytosolic Gab2-p85-Grb2-SHP2 complex

In the present study, Gab2-p85 association was detected in cytosol, PM and ENs. In previous work in primary hepatocyte cultures we demonstrated that EGF induced the formation of a Gab2-SHP2-Grb2-p85 complex without evidence for other Gab2-containing complexes (7). This probably reflected the fact that in hepatocyte lysates, cytosolic proteins predominate and less abundant complexes are difficult to observe. In the present *in vivo* studies the isolated PM and ENs were enriched in their associated proteins allowing identification of the lower abundance Gab2-p85 complexes in these cellular compartments. Our data support the view that the majority of EGF-induced PI3-kinase activation is initiated in cytosol since: (i) the association of Gab2 with p85 reaches peak activity at 30 secs in cytosol but only by 2 mins in PM, and (ii) the multimeric

complex Gab2-SHP2-Grb2-p85, which accounts for over 80% of the total PI3-kinase, is exclusively found in cytosol.

3.6.3. Gab2 phosphorylation and membrane localization are PH-domain independent

Our data demonstrate that Gab2 membrane concentration does not change following EGF treatment. This is based on our immunofluorescence studies showing no recruitment of HA-tagged WTGab2 to the PM (Fig. 7), the absence of EGF-induced net translocation of Gab2 to PM *in vivo* (Fig. 3A), the cytosolic location of the bulk of the Gab2-p85 complex (Fig. 3 C&D), and the lack of Gab2 association with the EGFR (7) following EGF treatment. Various studies have established the essential role of the PH-domain for Gab1 tyrosine phosphorylation and membrane translocation (13-15,36). The PH-domain of IRS1 is also critical for insulin-induced tyrosine phosphorylation and association of IRS1 with the IR (24,25). In contrast to what has been observed with these docking proteins over-expressing PHGab2 did not inhibit EGF-induced Gab2 phosphorylation, PI3-kinase or Akt activation, or DNA synthesis in primary rat hepatocytes although it was completely bound to cell membranes (Fig. 5). On further investigation we found that ΔPHGab2 was comparable to WTGab2 in respect to the extent of tyrosine phosphorylation, association with p85, and potentiation of DNA synthesis (Fig. 6). Thus the PH domain of Gab2 is not necessary for EGF-induced tyrosine phosphorylation and function of Gab2.

In these studies over-expressed PHGab2 did not significantly decrease the membrane association of endogenous Gab2, and ΔPHGab2 was found to associate with membranes, suggesting that the PH-domain of Gab2 is not required for its membrane targeting. Analysis of the Gab2 sequence suggests that the association of Gab2 with membranes may be mediated by two myristoylation sites located at amino acids 74-79 (GLTFNK) and 412-417(GSGESA) of the Gab2 protein. Interestingly, compared to WTGab2, a smaller proportion of ΔPHGab2 was found associated with membranes. This may reflect the loss of one of the myristoylation sites within the deleted PH-domain. Despite the fact that the amount of membrane bound ΔPHGab2 in PM was decreased, ΔPHGab2 equally potentiated EGF-induced signaling as WTGab2. This suggests that the cytosolic form of Gab2 might be key for EGF induced PI3-kinase activation and DNA synthesis.

The present work is compatible with the study of others showing that, in BAF3 cells, IL-3 induced comparable tyrosine phosphorylation of Gab2 and ΔPHGab2 (35). By contrast, following EGF treatment Gab1 recruitment to PM has been clearly demonstrated (13,14). Therefore although Gab1 and Gab2 are closely related they appear to be activated by different mechanisms. These two docking proteins also seem to play a non-redundant role in RTK signaling since they exhibit overlapping but distinct expression patterns (10), and reciprocal effects in mediating Elk-1 induction (37). Furthermore, Gab1 and Gab2 knock-out mice exhibit different phenotypes (38-40). Our data raise the possibility that Gab2 is

phosphorylated and activated by a tyrosine kinase, perhaps a cytosolic entity, other than EGFR.

3.6.4. Positive role of Gab2 in EGF signaling in liver

Gab1 has been clearly defined as a positive effector of various signaling pathways (13,41,42). In contrast the physiological significance of Gab2 appears to vary with the cellular context. Thus Gab2 acts as a positive mediator for G-CSF-induced MAPK activation in 293T cells (11,43), but as a negative mediator for TCR signaling in Jurkat cells (44), EGF-induced Elk-1 transcription (37), T-cell receptor signaling (45) and M-CSF-induced proliferation (43). In order to establish the role of Gab2 in EGF signaling in liver, we over-expressed both WTGab2 and a mutant of Gab2 lacking p85 binding sites. Over-expressing the WTGab2 construct clearly demonstrated that Gab2 acts as a positive mediator for EGF-induced PI3-kinase activation and DNA synthesis (Fig. 4). This observation was confirmed by the demonstration that over-expression of Δp85Gab2 (Gab2 with p85-binding sites mutated) failed to effect an augmentation of PI3-kinase and Akt activation, and DNA synthesis without affecting the augmentation of MAPK activation (Fig. 4). Interestingly Δp85Gab2, though expressed at a level of 10 – 20-fold over endogenous Gab2, failed to prevent EGF induced recruitment and activation of PI3-kinase to the latter (Fig. 4). Our results indicate a key role for Gab2-associated PI3-kinase activity in EGF-induced Akt activation and DNA synthesis but not for MAPK activation in liver (Fig. 4). It further confirms our earlier observations indicating that activation of the MAPK pathway does not play a significant role in EGF-induced DNA

synthesis in rat liver (6). The failure of Δp85Gab2 to act as a dominant negative mutant in rat hepatocytes raises questions of how activation of the EGFR leads to Gab2 tyrosine phosphorylation.

An important question is how activated PI3-kinase associated with Gab2 accesses its membrane substrate(s). One possibility is that the cytosolic Gab2 complex loosely associates with membranes *in vivo* in a dynamic manner, permitting a catalytic interaction between PI3-kinase and its lipid substrates. It is also possible that membrane associated Gab2 plays a critical role in signaling. One possibility is EGF induced tyrosine phosphorylation of membrane-associated Gab2, and the recruitment of PI3-kinase to this pool of Gab2, occurs independently from the effect of EGF on cytosolic Gab2. A second possibility is that, following EGF treatment, PI3-kinase is activated first in the cytosol and then translocated to the PM. The fact that tyrosine phosphorylated Gab2-p85 was maximal in the cytosol at 30 secs and in PM at 2 mins (Fig. 3B) is consistent with this. However our failure to observe net translocation of Gab2 to PM under these circumstances raises questions about this possibility. Our current data do not permit us to distinguish amongst these different possibilities.

In conclusion, we show that in normal rat hepatocytes EGF action involves the formation of 3 distinct p85-containing complexes. The most abundant multimeric Gab2-p85-Grb2-SHP2 complex is detected exclusively in cytosol whereas the other two complexes are found in the PM and endosomes. In contrast to what

has been found with Gab1 and IRS1, EGF-induced Gab2 phosphorylation is PH-domain independent, and occurs in the absence of net membrane recruitment.

3.7. References

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3.8. Footnotes

[1]The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; MAPK, mitogen activated protein kinase; Gab1, Grb2 associated binder 1; Gab2, Grb2 associated binder 2; Grb2, growth factor receptor bound 2; SHP2, src homology 2 domain-containing protein tyrosine phosphatase-2; PH-domain, pleckstrin homology domain; moi, multiplicity of infection; FITC, Fluorescein isothiocyanate form.

CHAPTER 4

EGF-induced DNA synthesis: Key role for Src phosphorylation of the docking protein Gab2

4.1. Preface to Chapter 4

In Chapter 3, we demonstrated that tyrosine phosphorylated Gab2 is largely localized in the cytosol. Further experiments in primary hepatocytes with over-expressed wild-type Gab2 and a Gab2 mutant lacking p85 binding sites established the central role of Gab2 in EGF-induced PI3-kinase activation and DNA synthesis. This stimulated us to investigate further the mechanism of Gab2 phosphorylation evoked by EGF. Our data showed EGF did not promote the net translocation of cytosolic Gab2 to cell membranes using either Western blotting analyses of purified subcellular fractions or immunofluorescence analyses in cultured hepatocytes. Moreover, we demonstrated that Gab2 could not associate with the EGFR, and is phosphorylated in a PH-domain independent manner. Together these data suggested that Gab2 might be phosphorylated by tyrosine kinase(s) other than the EGFR.

The Src family kinases are non-receptor protein tyrosine kinases which have been shown to be activated by EGF. Moreover, a key role for Src family kinases in EGF-induced mitogenesis has been previously demonstrated. However, the mechanism by which Src kinases lead to EGF-induced mitogenesis has hitherto remained obscure. In this Chapter, we have examined the role of Src family kinases in EGF-induced Gab2 phosphorylation and downstream signaling. In addition, we evaluated the role of the PTPase SHP2 which associates with phosphorylated Gab2 in modulating the function of Gab2 and downstream signaling events.

4.2. Summary

We have previously demonstrated that phosphatidylinositol 3-kinase (PI3-kinase) is necessary and sufficient to account for EGF-induced mitogenesis in rat primary hepatocytes. A cytosolic Gab2-containing complex accounts for > 80% of the total EGF-induced PI3-kinase activity [Kong, et al, J. Biol. Chem. (2000) 275:36035], suggesting a key role for Gab2 in EGF-induced mitogenesis. Here, we demonstrate that PP1, a selective inhibitor of Src family kinases, blocks the EGF-induced Gab2 tyrosine phosphorylation without inhibiting EGF-induced phosphorylation of the EGFR, ErbB3 or Shc. We also show that Gab2 phosphorylation is increased in Csk knock out cells in which Src family kinases are constitutively activated. Furthermore, PP1 blocks Gab2-associated downstream events including EGF-induced PI3-kinase activation, Akt phosphorylation and DNA synthesis. We demonstrate that Gab2 and Src are constitutively associated. Since this association involves the proline rich sequences of Gab2 it likely involves the SH3 domain of Src kinase. Mutation of the proline rich sequences in Gab2 prevented EGF-induced Gab2 phosphorylation, PI3-kinase/Akt activation, and DNA synthesis demonstrating that Gab2 phosphorylation is critical for EGF-induce mitogenesis and is not complemented by ErbB3 or Shc phosphorylation. We also found that over-expression of a Gab2 mutant lacking SHP2 binding sites increased EGF-induced Gab2 phosphorylation and the activation of PI3-kinase but blocked activation of MAPK. In addition, we demonstrated that the Src-induced response was down-regulated by Gab2-associated SHP2. In summary our results have defined the

role for Src activation in EGF-induced hepatic mitogenesis through the phosphorylation of Gab2 and the activation of the PI3-kinase cascade.

4.3. Introduction

Upon ligand binding, the activated epidermal growth factor receptor (EGFR) mediates a number of important biological responses, including the stimulation of cell proliferation, migration and differentiation (1-3). Src family kinases are cytosolic non-receptor tyrosine kinases, and have been described as essential mediators of EGF signaling (4). Like most non-receptor tyrosine kinases, Src family kinases contain a Src homology 2 (SH2) domain, which binds phosphotyrosine residues, and a Src homology 3 (SH3) domain, which binds proline rich sequences (reviewed in (5)). Several studies have established that Src kinases are required for growth factor-induced mitogenesis such as that effected via receptors for EGF (6-8), the platelet-derived growth factor (PDGF) (9,10), and colony stimulation factor-1 (CSF-1) (6). To date the manner in which Src family kinases participate in effecting the mitogenic response is unclear.

Several reports have identified Gab family proteins as key molecules for EGF-induced mitogenesis (11,12). Gab proteins, which include mammalian Gab1, Gab2, Gab3, the *Drosophila* homolog DOS (Daughter Of Sevenless), and the *Caenorhabditis elegans* homolog Soc1 (Suppressor-Of Clear), belong to a family of scaffolding proteins closely related to insulin receptor substrates (IRS-1, IRS-2, IRS-3), fibroblast growth factor substrate (FRS2), linker of T cell (LAT) and downstream of kinase (Dok) (reviewed in (13-15)). They have in common a central proline-rich domain (PRD) and multiple potential binding sites for the SH2 domains of p85, SHP2, PLCγ or Crk. Gab2 is tyrosine phosphorylated upon

stimulation of hepatocytes by EGF (12), T cells by cytokines (16), and following activation of T- and B-cell antigen receptors (17,18). Phosphorylated Gab2 has been shown to bind PI3-kinase (p110) via its 85-kDa (p85) regulatory subunit (19), as well as Grb2, and SHP-2.

In previous studies we demonstrated that the activation of PI3-kinase and not mitogen-activated protein kinase (MAPK), is necessary and sufficient to account for EGF-induced mitogenesis (12,20). Although activated PI3-kinase was shown to associate with 3 phosphotyrosine phosphorylated proteins (ErbB3, Shc and Gab2), over 80% was found in a multimeric complex consisting of Gab2-p85-SHP2-Grb2 (12). Confirming the key role of Gab2 was our finding that over-expression of wild-type Gab2 (WTGab2) augmented EGF-induced PI3-kinase activity and DNA synthesis, whereas the Gab2 mutant (Gab2Δp85) lacking p85 binding sites (pYXXM motifs as reviewed in (21)) effected no such augmentation. Furthermore, we showed that following EGF treatment, the phosphorylated multimeric Gab2 complex was exclusively cytosolic and did not associate with membranes. Nor did over-expression of the PH domain of Gab2 interfere with EGF induced Gab2 phosphorylation or mitogenesis (Chapter 3).

In the present study we considered the possibility that Gab2 is phosphorylated by a tyrosine kinase other than the EGFR; and thus sought to elucidate a link between Src family kinases and Gab2 in the regulation of EGF-induced mitogenesis in primary hepatocytes. Our study demonstrates that Src kinase(s)

promote EGF-induced PI3-kinase activation and DNA synthesis through effecting the tyrosine phosphorylation of Gab2. In addition, we found that the proline rich domains of Gab2 are essential for constitutive Src association with and tyrosine phosphorylation of Gab2. Finally we demonstrate that these Src-dependent responses are down-regulated by the association of SHP2 with Gab2 and that this latter association is critical to EGF-induced MAPK activation.

4.4. Experimental Procedures

4.4.1. Materials

Mouse EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Collagenase was from Worthington Biochemical Corporation (Halls Mills Road, NJ). Cell culture medium and antibiotics were from Gibco-BRL (Life Technologies, Burlington, Ont., Canada). Vitrogen-100 was from Collagen Corporation (Toronto, Canada). [^{3}H]-methylthymidine, [^{125}I]-labeled goat anti-rabbit antibody ([^{125}I]-GAR), [^{125}I]-labeled goat anti-mouse antibody ([^{125}I]-GAM) and [γ-^{32}P] ATP were purchased from NEN-DuPont (Wilmington, DE). Protein A-Sepharose (PAS) was from Pharmacia (Montréal, Québec, Canada). PP1 was purchased from Calbiochem (San Diego, CA). The anti-phosphotyrosine antibody (PY99), and antibodies to SHP-2, Src, IRS-2 and HA were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-p85, IRS-1, Shc, ErbB3, Myc and Gab2 (for immunoblotting) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phospho-Akt473, Akt, phospho-Src416, phospho-Erk1/2, and Erk1/2 antibodies were from New England Biolabs (Beverly, MA). Anti-Gab2 antibody (for immunoprecipitation) was raised by immunizing rabbits with a GST-Gab2 fusion protein containing the amino acids 376 to 552 of the rat Gab2 sequence. All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest grade available.

4.4.2. Preparation of the Gab2 mutants

The ΔSHP2Gab2 was generated by replacing Tyr-603 and Tyr-632 by phenylalanine using the Chameleon double-stranded site-directed mutagenesis

kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutant was verified by DNA sequencing. A plasmid containing ΔProGab2 with a deletion of 348-355 amino acids and mutations of P500A and R504A was kindly provided by Dr. Morag Park (McGill University, Montréal, Québec, Canada). In order to produce the adenoviruses, Gab2 mutants as well as the full-length cDNA (to generate the WTGab2 construct) were subcloned into pShuttle between the *NheI* and *NotI* sites using 5'-oligonucleotides containing an *NheI*-site and the sequences encoding different tags (HA for WTGab2, Flag for Δ ProGab2 and Myc for ΔSHP2Gab2) and 3'-oligonucleotides containing a *NotI* site. The recombinants of adenoviral DNA were further generated using the Adeno-X Expression System (Clontech, Palo Alto, CA) according to the kit's user manual.

4.4.3. Adenovirus preparation and titration

Large-scale production of recombinant viral particles was performed by infecting 293A cells. The titer of viral particles was determined using the Tissue Culture Infectious Dose 50 ($TCID_{50}$) method as described in the protocol of the Ad-easy vector system (Q.Biogene, Carlsbad, CA).

4.4.4. Cell culture and viral infection

Primary hepatocytes were prepared as previously described (12). Before infection, cells were bathed for 24 h in DMEM/F-12 medium containing 10% FBS, 10 mM HEPES, 20 mM $NaHCO_3$, 500 IU/ml penicillin, and 500 μg/ml streptomycin. Wild type and $Csk^{-/-}$ cells, kindly provided by Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) (22) were maintained

in DMEM medium containing 10% FBS. All cells were then infected with 10 moi of different recombinant adenoviruses for 3 h at 37°C. The infected cells were then serum-starved for 48 h before harvesting.

4.4.5. Immunoprecipitation and immunoblotting

Cell lysates, prepared as previously described (12), from EGF-treated (100 ng/ml EGF) or non-treated cells were pre-cleared with non-immune rabbit IgG (Sigma St. Louis, MO) and protein A-Sepharose for 1 h at 4°C. After centrifugation, the resulting supernatants were incubated for 2 h at 4°C with the antibody indicated in the figure legend. Protein A-Sepharose (50 µl of a 50% slurry) was added to each sample and the incubation was continued for an additional hour. Immune complexes were isolated by centrifugation, washed three times in PBS, and boiled in Laemmli sample buffer. Immunoprecipitates or intact samples were subjected to SDS-PAGE, transferred to Immobilon-P membranes (Millipore Ltd., Mississauga, Ontario, Canada), and immunoblotted with the indicated first antibody for 90 min followed by 1 h incubation with HRP-, or [^{125}I]-labeled GAR or GAM IgG. Immunoreactive proteins were detected by autoradiography or by ECL system (Amersham Pharmacia biotech, Montréal, Québec, Canada). Densitometric quantification of the signals was performed using a BioRad densitometer, Model GS-700.

4.4.6. [^{3}H]-thymidine incorporation assay

After viral exposure, infected cells were serum-starved for 20 h in serum-free medium (SFM), then 100ng/ml EGF and [^{3}H]-methylthymidine (5 µCi/ml) were added to the medium. After an 18 h incubation cells were rinsed three times with

3 ml cold PBS, incubated for 15 min at 4°C in 10% trichloro-acetic acid (TCA), solubilized at room temperature in 1 ml 1N NaOH and neutralized with 1ml 1N HCl, and then transferred to scintillation vials and counted for [^{3}H].

4.4.7. PI3-kinase activity assay

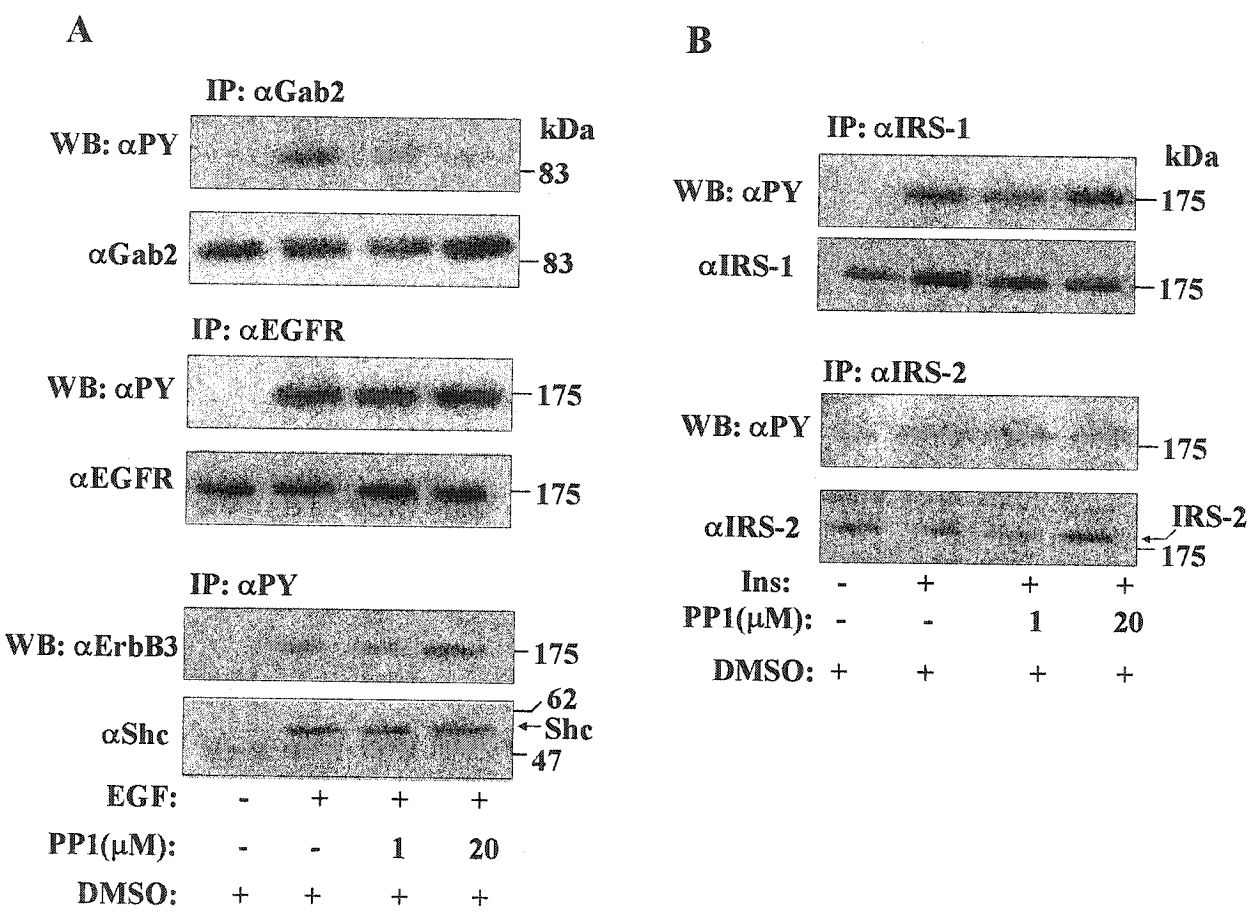
Lysates (500 μg protein) from EGF-treated (100 ng/ml EGF for 1 min) or non-treated cells were immunoprecipitated in the presence of protein A-sepharose, using different antibodies as indicated in figure legends. Immunoprecipitates were extensively washed and the PAS-pellets were resuspended in 50 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 0.5 mg/ml L-α-phosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster, AL), and assayed for PI3-kinase activity as previously described (20).

4.5. Results

4.5.1. A key role for Src kinase in EGF-induced Gab2 phosphorylation

In liver tyrosine phosphorylated Gab2 is largely localized in the cytosol, could not be demonstrated to associate with the EGFR, and is phosphorylated in a PH-domain independent manner (Chapter 3). EGF has been shown to activate tyrosine kinases of the Src family (4); and studies have linked their activation to cell proliferation (6-10). We tested the hypothesis that Gab2 is a Src substrate by examining the effect of PP1, a selective inhibitor of Src family kinases (23), on EGF-induced Gab2 tyrosine phosphorylation in rat primary hepatocytes. As shown in Fig. 1A (*top panels*), PP1 reduced EGF-induced tyrosine phosphorylation of Gab2 in a dose-dependent manner. Compared with cells incubated with vehicle only, 1μM PP1 decreased Gab2 phosphorylation by 50% and 20μM PP1 by more than 90%. In contrast, the same doses of PP1 had no effect on EGF-induced tyrosine phosphorylation of EGFR (Fig. 1A, *middle panels*), ErbB3 or Shc (Fig. 1A, *bottom panels*). Nor did these doses of PP1 affect insulin-induced tyrosine phosphorylation of IRS-1 or IRS-2 (Fig.1B). PP2, another selective inhibitor of Src family kinases, also inhibited the stimulatory effect of EGF on Gab2 tyrosine phosphorylation (*data not shown*). The specificity of inhibition by PP1 and PP2 of EGF-induced tyrosine phosphorylation of Gab2 suggests that, in rat hepatocytes, this molecule, but not other tyrosine phosphorylated docking proteins, is a substrate for Src family kinase(s).

4-Figure 1. Src kinase inhibitor PP1 specifically inhibits Gab2 phosphorylation. Serum-deprived hepatocytes were pre-incubated with either vehicle (DMSO) or PP1 (1 or 20 μM) for 30 mins followed by treatment with (+) or without (-) 100 ng/ml EGF (**A**) or 100nM insulin (**B**) for 1 min. Cell lysates were incubated with different antibodies and the immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with the indicated antibodies as described in "Experimental Procedures".


A
IP: αGab2
WB: αPY
kDa
83
αGab2
83
IP: αEGFR
WB: αPY
175
αEGFR
175
IP: αPY
WB: αErbB3
175
αShc
62
Shc
47
EGF: - + + +
PP1(μM): - - 1 20
DMSO: + + + +
B
IP: αIRS-1
WB: αPY
kDa
175
αIRS-1
175
IP: αIRS-2
WB: αPY
175
αIRS-2
IRS-2
175
Ins: - + + +
PP1(μM): - - 1 20
DMSO: + + + +

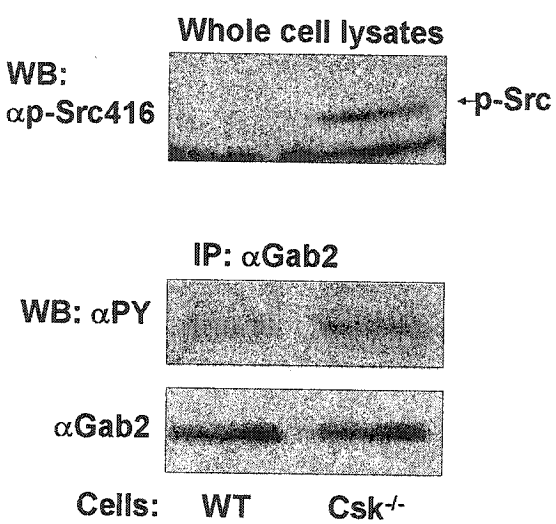
All Src family tyrosine kinases are negatively regulated by phosphorylation at a carboxyl-terminal tyrosine (Tyr 527 in Src kinase) carried out by another non-receptor tyrosine kinase, Csk (C-terminal Src kinase) (24). Cells derived from Csk-deficient embryos exhibit an order of magnitude increase in activity of Src and the related Fyn kinase (22,25). To further confirm the role of Src family kinases on Gab2 phosphorylation, we infected Csk-deficient and wild-type (WT) mouse embryo fibroblasts with recombinant Gab2 adenovirus and starved the cells for 48 hours. Western blot analysis, using a specific phospho-Src antibody (p-Src416) (26), demonstrated that Src kinase is constitutively activated in $Csk^{-/-}$ cells (Fig. 2A *top panel*). Gab2 tyrosine phosphorylation was then investigated in absence of EGF stimulation. Immunoprecipitation with αGab2 and Western blotting with αPY demonstrated that Gab2 phosphorylation (Fig. 2A, *middle panel*) is increased 1.8-fold in $Csk^{-/-}$ compared to WT fibroblasts (Fig. 2B). This study supports the view that Gab2 is a substrate for Src family kinases *in vivo*.

4.5.2. Gab2 tyrosine phosphorylation by Src family kinases is essential for EGF-dependent downstream signaling events

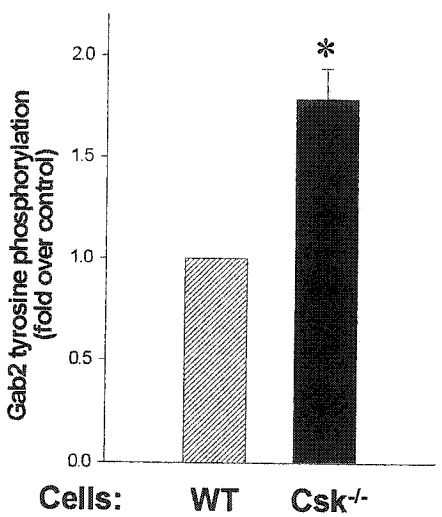
In previous work we showed that over 80% of PI3-kinase, activated following EGF treatment, was associated with tyrosine phosphorylated Gab2 (12); and that over-expression of Gab2 was sufficient to augment EGF-induced DNA synthesis (Chapter 3). In this study we pretreated hepatocytes with either vehicle (DMSO) or 20 μM PP1 for 30 mins followed by a stimulation with 100ng/ml EGF. As shown in Figure 3A, EGF induced a 20-fold increase in Gab2-associated PI3-kinase activity in control cells. Pretreatment with PP1 abolished this activation,

4-Figure 2. Increased tyrosine phosphorylation of Gab2 in Csk$^{-/-}$ cells. A. Wild type (WT) or Csk knock out (Csk$^{-/-}$) cells were infected with 10 moi of recombinant Gab2 adenovirus for 3 h and then incubated for 48 h in serum-free medium. Cell lysates were prepared and aliquots (50 μg protein) were subjected to 7.5% SDS-PAGE followed by immunoblot analysis with anti-phospho-Src416 antibody (*top panel*). Other aliquots were incubated with anti-Gab2 antibody, and the immunoprecipitates were subjected to 7.5% SDS-PAGE followed by immunoblot analysis with anti-PY antibody (*middle panel*) or anti-Gab2 antibody (*bottom panel*). **B**. The level of Gab2 tyrosine phosphorylation, quantified as described in "Experimental Procedures", is expressed in Csk $^{-/-}$ cells (solid bar) as fold over WT cells (hatched bar) (mean ± S.E., n=3, *p<0.01).

A

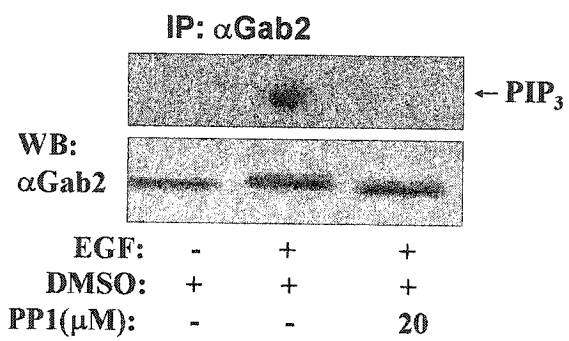

Whole cell lysates
WB:
αp-Src416
←p-Src
IP: αGab2
WB: αPY
αGab2
Cells: WT Csk-/-

B

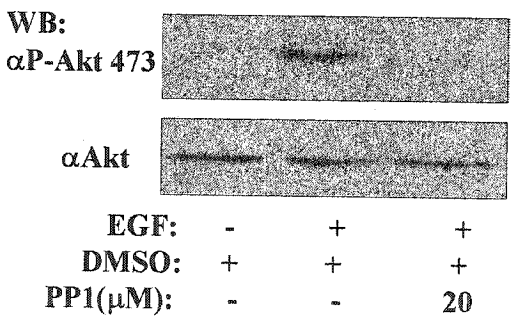

Gab2 tyrosine phosphorylation
(fold over control)
2.0
1.5
1.0
0.5
0.0
*
Cells: WT Csk-/-

4-Figure 3. Src family kinases are required for Gab2-mediated downstream events. A. Serum-deprived hepatocytes were pre-incubated with 20 μM PP1 or vehicle (DMSO) for 30 mins followed with (+) or without (-) EGF (100 ng/ml) for 1 min. Cell lysates were incubated with anti-Gab2 antibody, and immunoprecipitated proteins were either assayed for PI3-kinase activity (*top panel*) or subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-Gab2 antibody (*bottom panel*). **B.** Serum-deprived hepatocytes were pre-incubated with 20 μM PP1 or vehicle (DMSO) for 30 mins and then treated with (+) or without (-) EGF (100 ng/ml) for 5 min. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with either anti-phospho-Akt473 (*top panel*) or Akt antibody (*bottom panel*). **C.** Hepatocytes were incubated in serum-free medium for 24 h followed by incubation with either vehicle (DMSO) (blank and hatched bars) or PP1 (20 μM)(solid bar). After 30 mins. 5 μCi of [^{3}H] methylthymidine was added and the incubation was continued with (hatched and solid bars) or without (blank bar) EGF (100 ng/ml) for 18 h in serum-free medium as described under "Experimental Procedures". Results are expressed as fold over control (non-EGF treated cells). The inhibitory effect of PP1 on EGF stimulation of DNA synthesis was assessed in 3 separate experiments (mean ± S.E. *$p<0.01$).

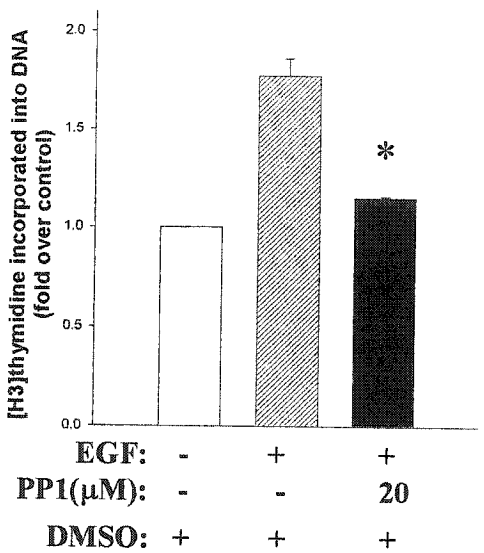
A


IP: αGab2
← PIP3
WB:
αGab2
EGF: - + +
DMSO: + + +
PP1(μM): - - 20

B


WB:
αP-Akt 473
αAkt
EGF: - + +
DMSO: + + +
PP1(μM): - - 20

C

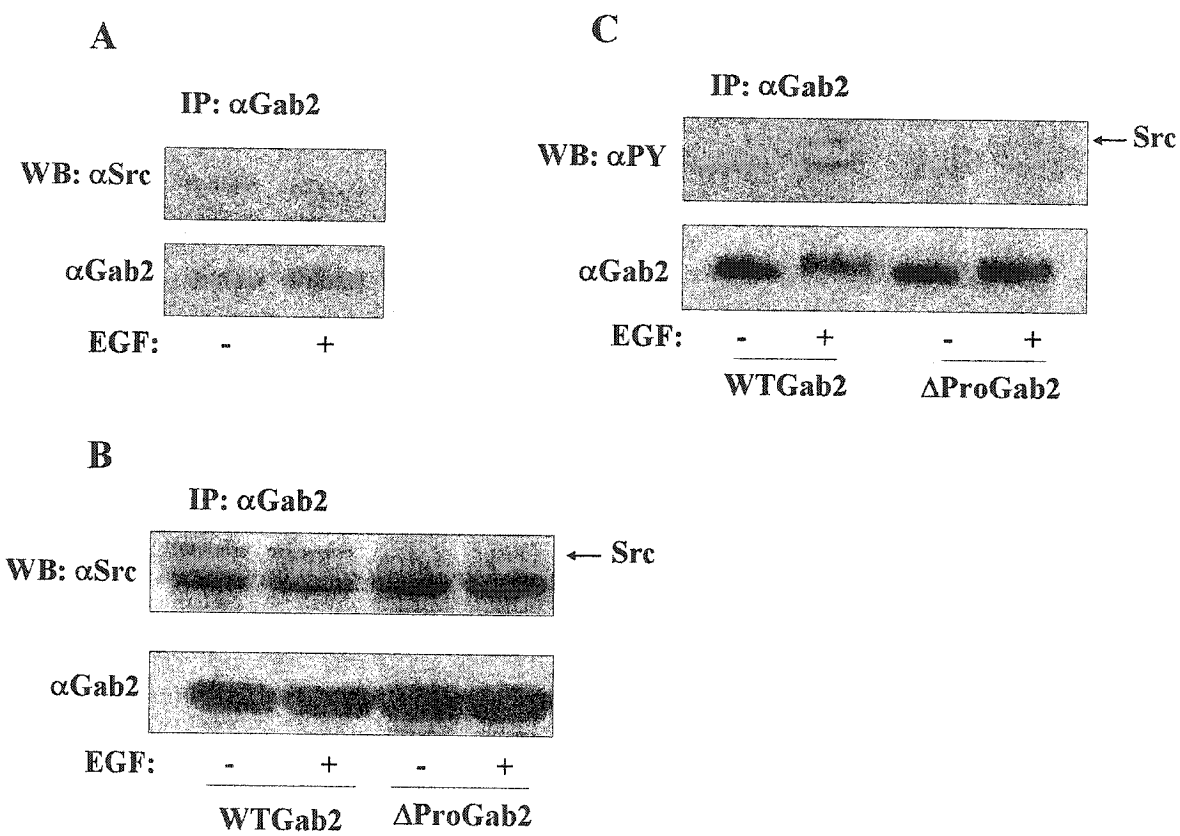

[H3]thymidine incorporated into DNA
(fold over control)
2.0
1.5
1.0
0.5
0.0
*
EGF: - + +
PP1(μM): - - 20
DMSO: + + +

that of Akt, as reflected in Akt-Ser 473 phosphorylation (Fig. 3B), and DNA synthesis (Fig. 3C). Similar results were obtained by treatment of the cells with PP2 (*data not shown*). Our results demonstrate that EGF-induced tyrosine phosphorylation of Gab2, mediated by Src family kinases, is necessary for EGF-induced DNA synthesis in primary rat hepatocytes; and that the tyrosine phosphorylation of ErbB3 and Shc, which are unaffected by PP1 (Fig. 1), cannot supplant the critical requirement for tyrosine phosphorylated Gab2 for this response.

4.5.3. Association of Src with Gab2: significance for Gab2 tyrosine phosphorylation and downstream signaling

In our previous experiments, demonstrating that Gab2 is a substrate for Src family kinases, we observed a 60kDa band in anti-Gab2 immunoprecipitates (*data not shown*). We therefore examined the possibility that Src kinase was associated with its substrate, Gab2. As shown in Fig. 4A (*top panel*), Src family kinases are constitutively associated with endogenous Gab2; and EGF treatment did not appear to augment this association. This suggested that the association might result from the binding of the SH3 domain of Src with proline rich sequences in Gab2. To test this hypothesis, we generated a recombinant adenovirus containing a Gab2 mutant in which key proline rich sequences were mutated (ΔProGab2) (*See Experimental Procedures).* Hepatocytes were infected with either WTGab2 or ΔProGab2 recombinant adenovirus and starved for 48 h before EGF treatment. In contrast to WTGab2 over-expressed ΔProGab2

4-Figure 4. Constitutive association of Src with Gab2. A. Serum-deprived hepatocytes were treated with (+) or without (-) 100 ng/ml EGF for 1 min. Cell lysates were incubated with anti-Gab2 antibody and the immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-Src antibody (*top panel*) or anti-Gab2 antibody (*bottom panel*). **B&C.** Hepatocytes were infected with 10 moi of either recombinant WTGab2 or ΔProGab2 adenovirus for 3 h followed by incubation in serum-free medium for 48 h. The hepatocytes were then treated with (+) or without (-) EGF (100 ng/ml) for 1min, and cell lysates were prepared and incubated with anti-Gab2 antibody. The immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-Src antibody (**B**, *top panel*), anti-PY antibody (**C**, *top panel*) or anti-Gab2 antibody (**B** and **C**, *bottom panel*).

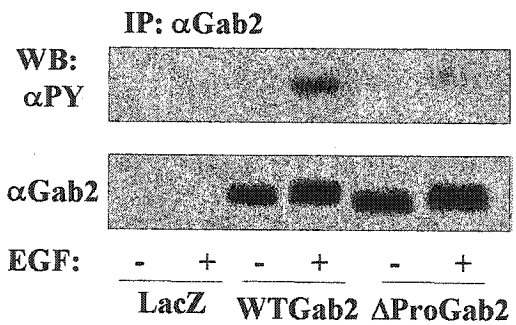

A
IP: αGab2
WB: αSrc
αGab2
EGF: - +
B
IP: αGab2
WB: αSrc
← Src
αGab2
EGF: - + - +
WTGab2
ΔProGab2
C
IP: αGab2
WB: αPY
← Src
αGab2
EGF: - + - +
WTGab2
ΔProGab2

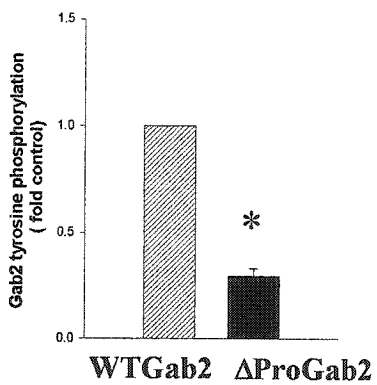
manifested no association with Src kinase (Fig. 4B, *top panel*, lanes 1 & 2 vs. lanes 3 & 4), indicating that the proline rich sequences are essential for mediating the binding of Src family kinases to Gab2. Src kinase phosphorylation was analyzed in anti-Gab2 immunoprecipitates. A tyrosine phosphorylated band migrating at the same position as Src kinase (60kDa) was detected in EGF-treated cells infected with WTGab2 but not in EGF-treated cells infected with ΔProGab2 (Fig. 4C, *top panel*). These results suggest that Src kinase is activated upon EGF treatment although it is constitutively associated with Gab2.

We sought to determine the extent to which the loss of constitutive Src binding influenced Gab2 phosphorylation by Src family kinases. Primary hepatocytes were infected with recombinant WTGab2 or ΔProGab2 adenoviruses. As shown in Fig. 5 (*top panel*), EGF treatment resulted in substantial tyrosine phosphorylation of WTGab2 whereas the phosphorylation of ΔProGab2 is reduced by more than 70% (Fig.5, *bottom panel*). These results demonstrate that, in hepatocytes, the proline rich sequences of Gab2 are important for Src kinase-mediated Gab2 phosphorylation.

Since over-expressing ΔProGab2 dramatically reduced EGF-induced Gab2 tyrosine phosphorylation, we examined whether this resulted in a decrease of Gab2-dependent downstream signaling. Cells were infected with either control recombinant adenovirus (LacZ) or Gab2 constructs (WTGab2 or ΔProGab2). As previously observed over-expression of WTGab2 potentiated EGF-induced PI3-

4-Figure 5. The proline rich sequences of Gab2 are required for EGF-induced Gab2 phosphorylation. Hepatocytes were infected with 10 moi of either recombinant WTGab2 or ΔProGab2 adenovirus for 3 h and then incubated in serum-deprived medium for 48 h. Cell lysates were incubated with anti-Gab2 antibody and the immunoprecipitates were subjected to 7.5% SDS-PAGE followed by immunoblotting with either anti-PY antibody or anti-Gab2 antibody (*top panel*). The bar graph (*bottom panel*) represents quantification of Gab2 tyrosine phosphorylation in EGF-treated cells. The extent of Gab2 phosphorylation in ΔProGab2-infected cells is expressed as a fraction of that in control (WTGab2 infected) hepatocytes (mean ± S.E., n=3, *$p<0.005$).

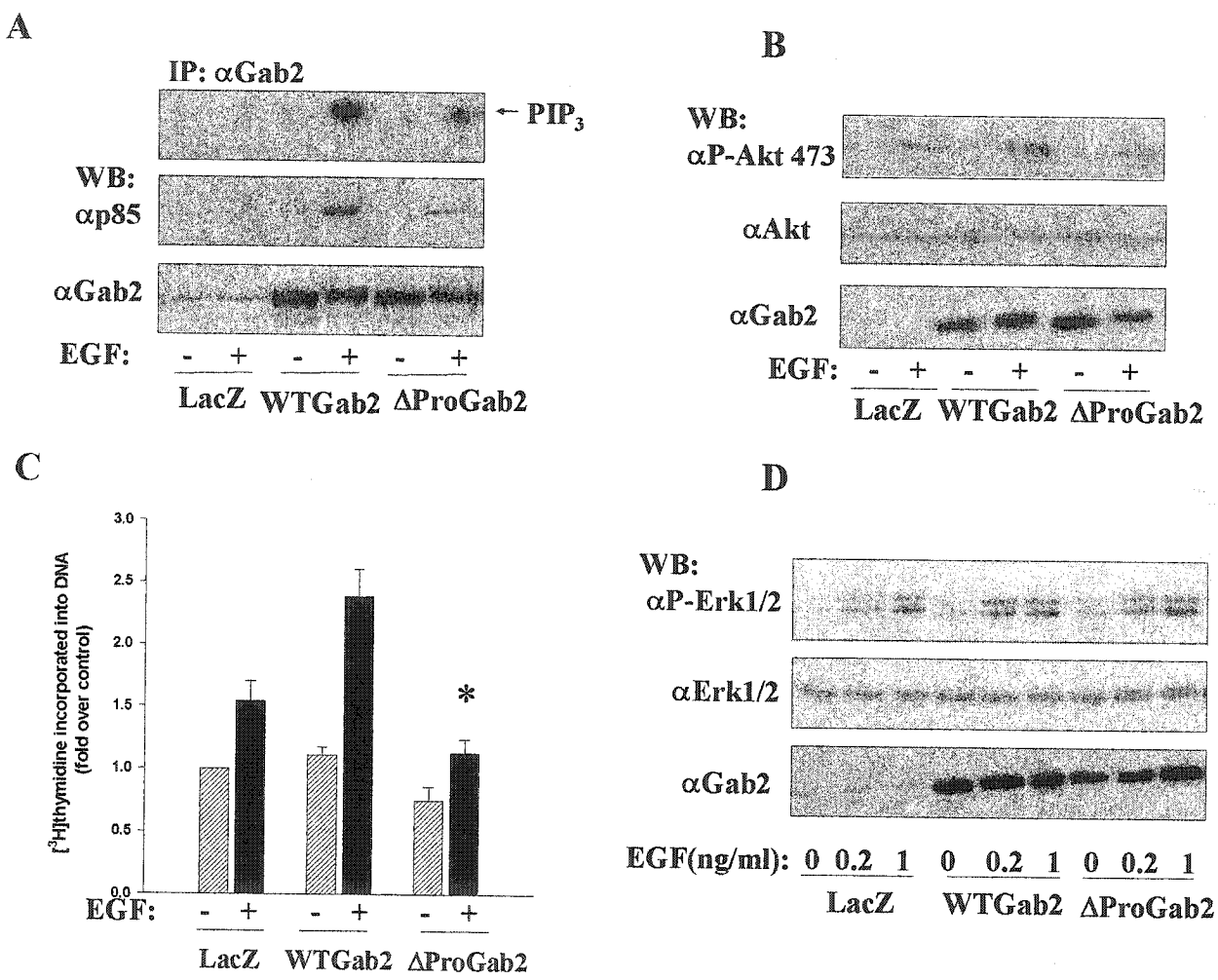

IP: αGab2
WB:
αPY
αGab2
EGF:
-
+
-
+
-
+
LacZ
WTGab2
ΔProGab2


Gab2 tyrosine phosphorylation
(fold control)
1.5
1.0
0.5
0.0
*
WTGab2
ΔProGab2

kinase activation by 25-fold (Fig. 6A, *top panel*, lane 2 vs. lane 4) whereas over-expression of ΔProGab2 reduced EGF-induced PI3-kinase activity by more than 70% (Fig. 6A, *top panel*, lane 4 vs. 6). This parallels the association of Gab2 with p85 (Fig. 6A, *middle panel*). Interestingly ΔProGab2 did not exert a dominant negative effect in that it did not reduce PI3 kinase activity to less than control levels despite being expressed at 20 fold the level of endogenous Gab2. Corresponding to the PI3-kinase activity, we found that EGF treatment augmented Akt serine 473 phosphorylation in cells over-expressing WTGab2 but not in those over-expressing ΔProGab2 [WTGab2 vs. ΔProGab2 infected cells, expressed as a percent of LacZ infected cells was 146 ± 4.7 vs. 110 ± 4.1 (n=3, mean ± SE, $p<0.01$)] (Fig. 6B). Parallel results were obtained when we measured EGF-induced DNA synthesis (Fig. 6C). The observations in Fig. 6 are consistent with a greater sensitivity to EGF of PI3-kinase activation and p85 recruitment than are Akt phosphorylation and DNA synthesis. This could explain the closer correlation between Akt activation and the extent of stimulation of DNA synthesis. These findings indicate that the proline rich sequences in Gab2, which mediate Src binding, are important for EGF-induced PI3-kinase activation and DNA synthesis.

We then examined the effect of WTGab2 and ΔProGab2 on Erk phosphorylation. As shown in Fig. 6D, Erk phosphorylation was minimally observed with 0.2 ng/ml EGF in LacZ infected cells whereas, at this dose, there was clear phosphorylation of Erk in cells infected with WTGab2. We also

4-Figure 6. Influence of Gab2 proline rich sequences on EGF-induced activation of PI3-kinase/Akt, MAPK and DNA synthesis. Hepatocytes were infected with 10 moi of either recombinant LacZ, WTGab2 or ΔProGab2 adenovirus for 3 h followed by incubation in serum-free medium for 48 h. **A.** Serum-deprived hepatocytes were treated with (+) or without (-) EGF (100 ng/ml) for 1min., and cell lysates were prepared, equally divided, and incubated with anti-Gab2 antibody. Immunoprecipitated proteins were assayed for PI3-kinase activity (*top panel*) or subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-p85 antibody (*middle panel*). The membrane was stripped and reblotted with an anti-Gab2 antibody (*bottom panel*). **B.** Serum-deprived hepatocytes were treated with (+) or without (-) EGF (100 ng/ml) for 5 min. Cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with an anti-phospho-Akt473 (*top panel*), anti-Akt (*middle panel*) or anti-Gab2 (*bottom panel*) antibody. **C.** Virus infected hepatocytes were incubated in serum-free medium for 24 h followed by the addition of 5 μCi [^{3}H]methylthymidine without (*hatched bars*) or with (*solid bars*) EGF (100 ng/ml) for 18 h. Incorporation of [^{3}H]methylthymidine into DNA was determined as described under "Experimental Procedures". Results are expressed as fold over control (LacZ adenovirus infected cells without EGF) cells. The difference between EGF-treated hepatocytes infected with WTGab2 vs. ΔProGab2 was determined in 3 separate experiments (mean ± S.E., *$p<0.005$). **D.** Hepatocytes were treated with the indicated doses of EGF for 5 min. and cell lysates were prepared and subjected to SDS-PAGE (10% gel) followed by immunoblotting with anti-phospho-Erk1/2 (*top panel*), anti-Erk1/2 (*middle panel*) or anti-Gab2 (*bottom panel*) antibodies.


A
IP: αGab2
← PIP3
WB:
αp85
αGab2
EGF: - + - + - +
LacZ WTGab2 ΔProGab2
B
WB:
αP-Akt 473
αAkt
αGab2
EGF: - + - + - +
LacZ WTGab2 ΔProGab2
C
[3H]thymidine incorporated into DNA
(fold over control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
EGF: - + - + - +
LacZ WTGab2 ΔProGab2
D
WB:
αP-Erk1/2
αErk1/2
αGab2
EGF(ng/ml): 0 0.2 1 0 0.2 1 0 0.2 1
LacZ WTGab2 ΔProGab2

demonstrated that Erk1/2 phosphorylation at higher doses of EGF (10 or 100 ng/ml) is much greater than that at 0.2 and 1.0 ng/ml. At these concentrations we did not observe any effect of over-expressed Gab2 (data not shown). This suggests that over-expressed Gab2 increases the sensitivity of Erk to EGF which correlated with previous studies showing that at a low dose of EGF (0.25 ng/ml), over-expression of Gab1 potentiated EGF-induced Erk activation in HEK293 cells (27). These results may indicate that, at higher doses of EGF, the influences of Gab2 could be obscured by input(s) from Gab2-independent signaling pathways. Alternatively, a high sensitivity of Erk phosphorylation to EGF may result in its saturation at the 10 ng/ml dose of EGF. As shown in Fig. 6D, ΔProGab2 comparably potentiated EGF-induced Erk1/2 activation as WTGab2 at the low dose of EGF [i.e. following EGF (0.2 ng/ml) treatment, Erk activation in WTGab2 and ΔProGab2 were 218 ± 4.2 and 236 ± 12 respectively when expressed as percent of values in LacZ infected cells (n=3, mean ± SE, $p<0.05$ LacZ vs. either construct)]. Thus the reduced level of tyrosine phosphorylation of ΔProGab2 would appear to be sufficient to activate Erk1/2 but not PI3-kinase.

4.5.4. SHP2 is a negative effector for PI3-kinase/Akt activation but positive for MAPK activation

Upon EGF treatment, Src activation leads to substantial tyrosine phosphorylation of Gab2 and activation of DNA synthesis. We previously demonstrated that phosphorylated Gab2 associates with the SH2-containing PTP, SHP2 (12). We considered that this binding might effect dephosphorylation of Gab2 and

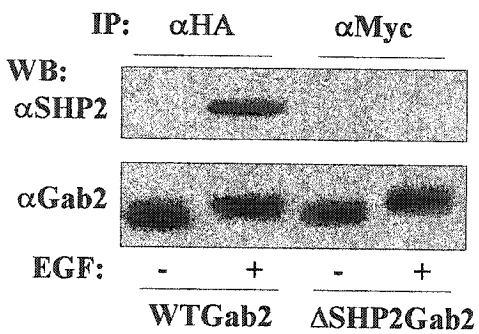
therefore generated a Gab2 mutant lacking the SHP2 binding sites (ΔSHP2Gab2, see *Experimental Procedures*). Over-expression of ΔSHP2Gab2 in hepatocytes totally abolished SHP2 binding to Gab2 (Fig. 7A, *top panel*, lane 2 vs. lane 4), and was tyrosine phosphorylated (Fig. 7B, lane 4 vs. lane 6) to an extent 25% greater than WTGab2 (Fig. 7C).

Of interest is the finding that over-expression of ΔSHP2Gab2 potentiated EGF-induced PI3-kinase activation 2.6 fold compared to that observed in cells over expressing comparable levels of WTGab2 (Figure 8A, *top panel*, lane 4 vs. lane 6). As expected this correlated with augmented p85-Gab2 association (Figure8A, *middle panel,* lane 4 vs. lane 6). In parallel we found that following EGF treatment Akt serine 473 phosphorylation, expressed as percent LacZ infected cells, was 146 ± 4.7 vs. 201 ± 25, in WTGab2 vs. ΔSHP2Gab2 (n=3, mean±SE, $p<0.01$) (Fig.8B, *top panel,* lane 4 vs. lane 6). Similar results were observed when we looked at the EGF-induced DNA synthesis by fold (Fig. 8C). Thus SHP2 binding to tyrosine phosphorylated Gab2 acts has a negative regulator of EGF-induced PI3-kinase activation and DNA synthesis.

In contrast to these findings, whereas over-expression of WTGab2 increased EGF-induced Erk1/2 (Fig. 6D and Fig. 8D, lanes 4 to 6), over-expression of ΔSHP2Gab2 had no capacity to effect EGF-induced activation of Erk1/2 (Fig.8D, lanes 7 to 9). [i.e. following EGF treatment (0.2 ng/ml) Erk activity in WTGab2 vs.

4-Figure 7. SHP2 binding negatively regulates EGF-induced Gab2 phosphorylation. A. Hepatocytes were infected with 10 moi of either recombinant WTGab2 or ΔSHP2Gab2 adenovirus for 3 h followed by incubation in serum-free medium for 48 h. Cells were then treated with (+) or without (-) EGF (100 ng/ml) for 1min., and cell lysates were incubated with either anti-HA or anti-Myc antibody as described in ""Experimental Procedures"". Immunoprecipitated proteins were subjected to 7.5% SDS-PAGE followed by immunoblotting with anti-SHP2 (*top panel*), or anti-Gab2 (*bottom panel*) antibody. **B.** Hepatocytes were infected with 10 moi of either recombinant WTGab2 or ΔSHP2Gab2 adenovirus for 3 h followed by incubation for 48 h in serum-free medium. The cells were treated with (+) or without (-) EGF (100 ng/ml) for 1min. Cell lysates were incubated with anti-Gab2 antibody and immunoprecipitated proteins were subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-PY antibody (*top panel*). The membrane was stripped and reblotted with anti-Gab2 antibody (*bottom panel*). The bar graph represents determination of Gab2 tyrosine phosphorylation in EGF-treated cells infected with ΔSHP2Gab2 versus that in cells infected with WTGab2 (mean ± S.E., n=3, *$p<0.005$ *versus*).

A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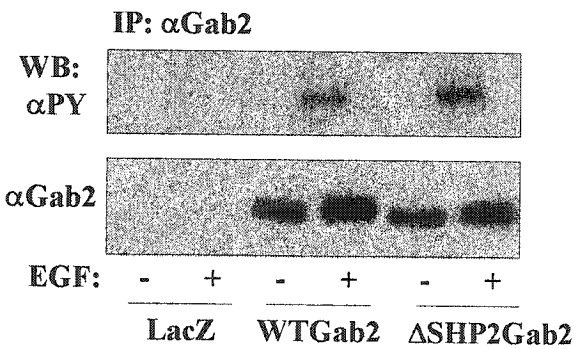
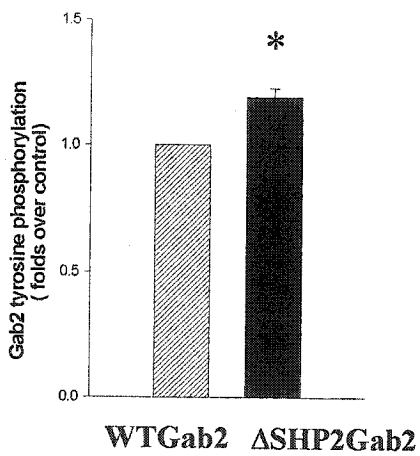
αMyc
WB:
αSHP2
αGab2
EGF: - + - +
WTGab2
ΔSHP2Gab2

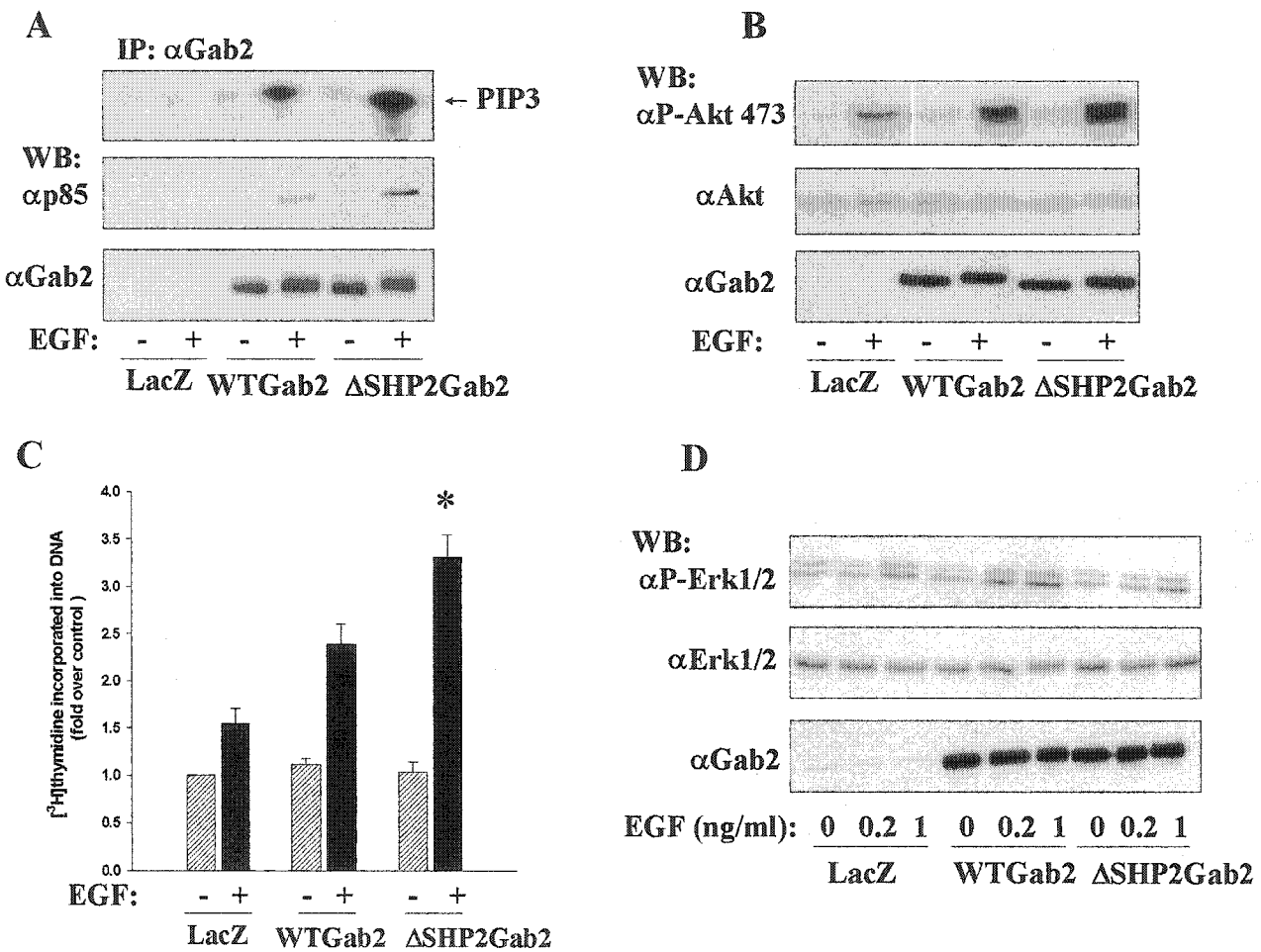
B


IP: αGab2
WB:
αPY
αGab2
EGF: - + - + - +
LacZ
WTGab2
ΔSHP2Gab2

C


Gab2 tyrosine phosphorylation
(folds over control)
1.5
1.0
0.5
0.0
*
WTGab2
ΔSHP2Gab2

4-Figure 8. SHP2 binding negatively affects EGF-induced PI3-kinase/Akt activation and DNA synthesis, but positively affects MAPK activation. Hepatocytes were infected with 10 moi of either recombinant LacZ, WTGab2 or ΔSHP2Gab2 adenovirus for 3 h followed by incubation in serum-free medium for 48 h. **A.** Serum-deprived hepatocytes were treated with (+) or without (-) EGF (100 ng/ml) for 1min., and cell lysates were equally divided and incubated with anti-Gab2 antibody. The immunoprecipitated proteins were either assayed for PI3-kinase activity (*top panel*) or subjected to SDS-PAGE (7.5% gel) followed by immunoblotting with anti-p85 antibody (*middle panel*). The membrane was then stripped and re-blotted with anti-Gab2 antibody (*bottom panel*). **B.** Serum-deprived hepatocytes were treated with (+) or without (-) EGF (100 ng/ml) for 5 min., and cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with anti-phospho-Akt473 (*upper panel*), anti-Akt (*middle panel*) or anti-Gab2 (*bottom panel*) antibodies. **C.** Virus infected hepatocytes were incubated in serum-free medium for 24 h followed by the addition of 5 μCi [^{3}H]methylthymidine without (*hatched bars*) or with (*solid bars*) EGF (100 ng/ml) for 18 h. Incorporation of [^{3}H]methylthymidine into DNA was determined as described under "Experimental Procedures". Results are expressed as fold over control (LacZ adenovirus infected cells without EGF). The difference between EGF-treated hepatocytes infected with WTGab2 vs. ΔSHP2Gab2 was determined in 3 separate experiments (mean ± S.E., *$p<0.005$). **D.** Serum-deprived hepatocytes were treated with the indicated dose of EGF for 5 min., and cell lysates were subjected to SDS-PAGE (10% gel) followed by immunoblotting with anti-phospho-Erk1/2 (*top panel*), anti-Erk1/2 (*middle panel*) or anti-Gab2 (*bottom panel*) antibodies.


A
IP: αGab2
← PIP3
WB:
αp85
αGab2
EGF: - + - + - +
LacZ WTGab2 ΔSHP2Gab2
B
WB:
αP-Akt 473
αAkt
αGab2
EGF: - + - + - +
LacZ WTGab2 ΔSHP2Gab2
C
[3H]thymidine incorporated into DNA (fold over control)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
EGF: - + - + - +
LacZ WTGab2 ΔSHP2Gab2
D
WB:
αP-Erk1/2
αErk1/2
αGab2
EGF (ng/ml): 0 0.2 1 0 0.2 1 0 0.2 1
LacZ WTGab2 ΔSHP2Gab2

ΔSHP2Gab2 infected cells, expressed as percent LacZ infected cells, was 218 ± 4.2 vs. 130 ± 9 (n=3, mean±SE, $p<0.05$)] (Fig. 8D). These data indicate that SHP2 binding to tyrosine phosphorylated Gab2 influences EGF downstream signaling by negatively affecting EGF-dependent PI3-kinase activation and positively affecting activation of the MAPK pathway.

The above observations identify the mechanism by which Src regulates EGF-induced mitogenesis in hepatocytes. Upon EGF-binding Gab2 associated Src is activated leading to activation of the PI3-kinase cascade and eventually DNA synthesis. This signal appears to be attenuated by SHP2 through dephosphorylation of Gab2.

4.6. Discussion

In response to the binding of EGF to its receptor Gab proteins become tyrosine-phosphorylated and bind SH2 domain containing proteins, including p85, the PI3-kinase subunit, and SHP2 (12,27-30). The mechanism by which EGF induces the phosphorylation of Gab docking proteins is key to understanding EGF-induced signaling. Several reports indicate that EGFR is the kinase which phosphorylates Gab1 (31-33). Furthermore studies have demonstrated that the PH domain of Gab1 is required for its localization to membranes thus facilitating its phosphorylation by the EGFR kinase (31,34,35). However, the process involved in phosphorylating Gab2 appears to be different. Thus, as we have previously found, Gab2 is phosphorylated in a PH-domain independent manner nor was it observed to associate with the EGFR. Moreover the major multimeric Gab2-complex was found exclusively in the cytosol of rat liver (Chapter 3). For these reasons we examined the possibility that Gab2 is tyrosine phosphorylated by a cytosolic kinase and not the EGFR.

Though previous reports have established that Src family kinases are required for EGF-induced mitogenesis (6-8) the mechanism by which Src family kinases act has remained unclear. In the present study we demonstrate that EGF treatment of rat hepatocytes activates Src leading to Gab2 tyrosine phosphorylation, after which Gab2 recruits SHP2, Grb2 and p85 thus forming a multimeric cytosolic complex (Chapter 3). The evidence that Src family kinases are involved in Gab2 tyrosine phosphorylation is based on the inhibitory effects

of PPI and PP2, two widely used specific inhibitors of Src family kinases (36,37). Thus, in the present study, we observed that the inhibition of Gab2 tyrosine phosphorylation was dose dependent and occurred in the absence of inhibition of tyrosine phosphorylation of EGFR, ErbB3, or the IRS proteins (Fig.1). Furthermore, using $CSK^{-/-}$ mouse embryonic cells, in which Src family kinases are constitutively activated, we confirmed a key role for these kinase(s) in the phosphorylation of Gab2. Consistent with our results are previous studies demonstrating that the target involved in Src-dependent mitogenic activity is a cytosolic molecule accessible to unmyristoylated Src. Thus Src deletion mutants, lacking the amino-terminal one-third of the molecule, including the membrane binding domain, were shown to still induce cell proliferation (38). It has been known for some time that EGF signaling involves c-Src substrates with molecular sizes of 120-130, 100 and 75 kDa (39). Further studies have identified p75 as cortactin (40), and p120-130 as comprising several proteins, including p125FAK (41) and p130CAS (42). However, the identity of p100 has hitherto remained unknown. Our results appear to establish the p100 Src kinase substrate as Gab2.

To elucidate the mechanism by which Gab2 is phosphorylated by Src family kinases, we analyzed the association between these two proteins. In most cases, substrate recognition is dictated primarily by interactions with non-catalytic regions of the Src family kinases such as SH2 and SH3 domains (reviewed in (5)). A constitutive association of Src family kinases with Gab2 was

observed and this association disappeared when the two key proline rich regions on Gab2 were mutated (Fig. 4), indicating that the SH3 domain of Src is likely involved in this interaction. This is consistent with two previous reports demonstrating that the SH3 domain of Src is required for EGF mitogenic signaling (9,10), and that this domain can bind to Gab2 in an *in vitro* binding assay (43). The SH3 domains of Src family kinases recognize proline-rich sequences found in a large number of substrates of Src family kinases (44-46). The SH2 domains of Src family kinase selectively recognize the sequence pYEEI, with a hydrophobic residue at position +3 being an important determinant of binding (47). Interestingly, the Gab2 sequence does not contain a pYEEI motif suggesting that Src does not bind to Gab2 through its SH2 domain. Although Src family kinases are constitutively associated with Gab2, the activation of Src kinase is only observed after EGF treatment (Fig. 4B). The mechanism by which Src kinase activation is effected by the binding of EGF to the EGFR remains to be elucidated. In hepatocytes, Src, Fyn and Yes are the three Src family members expressed (48). However, in the present analysis, we could not identify which isoform(s) phosphorylate Gab2. Indeed, the dose of PP1 we used inhibits all three Src isoforms. Furthermore, the Src antibody we used for Western blots recognizes Fyn and Yes as well as Src.

In this paper we show that in rat hepatocytes inhibition of Src family kinases prevents EGF-induced phosphorylation of Gab2 and activation of PI3-kinase/Akt as well as DNA synthesis (Figs. 1 and 3). Several recent studies have shown that activation of Src family kinases is involved in signal transduction by

regulating PI3-kinase activity. Thus erythropoietin appears to effect erythroid differentiation by effecting Src dependent tyrosine phosphorylation of and the recruitment plus activation of PI3-kinase to the erythropoietin receptor (48). Also in MCF-7 cells oestradiol was shown to promote cell cycle progression via a Src-dependent activation of PI3-kinase (49). Finally in T47D cells EGF activation of Akt was blocked in parallel with inhibition of Src activation (36). In rat hepatocytes EGF-dependent Gab2 phosphorylation leads to downstream PI3-kinase/Akt activation and DNA synthesis; and is effected by the activation of Src constitutively associated with Gab2 (Figs. 4 and 6).

SHP2 binds with Gab family proteins through a consensus binding motifs (YXXV/I/L) located in the C-terminal ends of Gab proteins (15). The function of the Gab-SHP2 interaction has been extensively studied using mutants of Gab family proteins lacking SHP2 binding sites. These studies reveal a general positive role of SHP2 in Gab-mediated signal transduction. For example, SHP2 association with Gab1 is required for Met-dependent morphogenesis (50) as well as EGF and lysophosphatidic acid-induced MAPK activation (27,30). It was also found that SHP2 association with Gab2 is essential for M-CSF-induced macrophage differentiation (51). In the present study we identified a positive role for SHP2 in Gab2-potentiated EGF-induced MAPK activation (Fig. 8D). Recent reports have also identified a negative effect of SHP2 binding on Gab protein tyrosine phosphorylation. Thus both Gab1 and Gab2 have been identified as substrates for SHP2 using an *in vitro* phosphatase assay (18). More recently,

two studies have shown that inactive forms of SHP2 markedly increased EGF-stimulated Gab1 tyrosine phosphorylation and PI3-kinase activity (28,52). The negative regulation of EGF-dependent PI3-kinase activation by SHP2 appears to be through the dephosphorylation of Gab1 p85 binding sites (52). In agreement with this study we have found that, in rat hepatocytes, a relatively minor increase of ΔSHP2Gab2 phosphorylation compared to WTGab2 (Fig. 7B) was accompanied by a more noticeable augmentation of PI3-kinase activity and DNA synthesis, suggesting that SHP2 is involved in the dephosphorylation of specific p85 binding sites on Gab2 (Fig. 8A, B, C). Moreover, the fact that the ΔSHP2Gab2 mutant blocks EGF-induced MAPK activation and augments PI3-kinase and DNA synthesis, further confirms, in primary hepatocytes, the key role of PI3-kinase and not MAPK in EGF-induced DNA synthesis.

In summary we have found that, in rat hepatocytes, Src family kinases regulate EGF-induced mitogenesis through association to and phosphorylation of the major cytosolic docking protein, Gab2. Furthermore, we demonstrate that the association of SHP2 with tyrosine phosphorylated Gab2 leads to dephosphorylation of the latter and a corresponding decrease in EGF-induced DNA synthesis.

4.7. References

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4.8. Footnotes

[1]The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; MAPK, mitogen activated protein kinase; Gab1, Grb2 associated binder 1; Gab2, Grb2 associated binder 2; Grb2, growth factor receptor bound 2; SHP2, src homology 2 domain-containing protein tyrosine phosphatase-2; PH-domain, pleckstrin homology domain; moi, multiplicity of infection.

CHAPTER 5

General Discussion and Original Contribution

5.1. EGF-induced mitogenesis in liver

EGF is an important mitogen during liver regeneration a remarkable process that allows complete restoration of hepatic architecture and tissue specific function after different types of liver injury (1). Understanding the mechanism of EGF-induced hepatocyte mitogenesis is key for understanding both normal liver cell growth and the diseases related to liver cell injury or excessive proliferation.

EGF activates both PI3-kinase and MAPK cascade in primary hepatocytes. Although the MAPK pathway is generally viewed as the primary effector of the proliferative response of cells to EGF (2,3), in hepatoctyes, our data clearly demonstrated that PI3-kinase, not the MAPK is required for EGF-induced DNA synthesis by either using specific inhibitors or over-expressing dominant-negative forms of PI3-kinase (4,5). EGF-induced MAPK activation does not seem to be required for DNA synthesis but is required for the augmentation of basal c-*myc* messenger RNA (mRNA) levels by EGF in hepatocytes (5). C-*myc*, together with c-*fos* and c-*jun* are growth-related immediate early genes, which are known markers of the G_0/G_1 transition preceding DNA synthesis. Thus our data indicate that either the PI3-kinase induced DNA synthesis is c-*myc* independent or the c-myc level, perhaps induced by the collagen matrix on which the primary hepatocytes are growing, is sufficient for the G_0/G_1 transition. This may have been effected by activation of MAPK during the early adaptation of these cells to culture. Although EGF has the ability to activate several STAT

proteins (such as Stat1, Stat3, and, Stat5b), this does not seem to be part of the mitogenic mechanisms used by the EGF receptor in hepatocytes (6).

In this thesis, we further explored the mechanism of EGF-induced PI3-kinase activation and DNA synthesis. Our data support the model that, following EGF treatment, Src family kinases are activated and phosphorylate Gab2 on multiple tyrosines. The phosphorylated Gab2 recruits p85 leading to PI3-kinase activation and DNA synthesis. Though previous reports have noted that Src family kinases are required for EGF-induced mitogenesis (7,8) none have provided a mechanism by which Src family kinases act. Our data reveal that in liver, Src family kinases regulate EGF-induced DNA synthesis through phosphorylation of the docking protein, Gab2. It is noteworthy that previous studies have identified the SH3 domain of Src kinase as being required for EGF mitogenic signaling (9). The SH3 domains of Src family kinases recognize proline-rich sequences found in a large number of substrates of Src family kinases. Fully compatible with this are our findings that it is the proline-rich sequences in Gab2, which mediate the interaction between Gab2 and Src presumably via the SH3 domain of Src family kinases.

The steps downstream of PI3-kinase by which EGF effects an augmentation of DNA synthesis remain unclear. Our data indicate that Akt phosphorylation correlates well with EGF-induced DNA synthesis, suggesting that Akt is a key downstream mediator of PI3-kinase leading to DNA synthesis. It will be

interesting to determine whether this EGFR-Src-Gab2-PI3-kinase mechanism underlies EGF-induced mitogenesis in some cancers such as hepatoma or breast cancer since both Src kinase and Gab2 have been reported to be involved in tumorigenesis (10,11).

5.2. Mechanism of EGF-induced PI3-kinase activation

Two general mechanisms for the recruitment and activation of PI3-kinase by growth factor receptors have been described in different cell lines. The first involves the direct binding of the p85 regulatory subunit of PI3-kinase to PY-RTKs such as the PDGF (12), CSF-1 (13) and c-met receptors (14). The second mechanism comprises the recruitment to and activation of p85 by tyrosine phosphorylated substrates of RTKs (i.e. PY- docking proteins) such as the IRS (15,16) and Gab family proteins (17). The mechanism by which PI3-kinase becomes activated by different ligand in different tissues seems to vary. For example, it has been clearly demonstrated that insulin activates PI3-kinase through recruiting p85 to IRSs (15) whereas PDGF activates PI3-kinase via a direct recruitment to the PDGFR of p85 (12). However the events following EGF stimulation have been less clearly defined. Numerous studies indicate that EGF activates PI3-kinase through recruitment to a range of PY-proteins in a cell line specific manner. In this thesis I have demonstrated that, upon EGF treatment, three distinct p85-associated complexes are formed: ErbB3-p85, Shc-p85 and Grb2-SHP2-Gab2-p85. The latter complex accounts for the bulk (>80%) of the activated PI3-kinase generated by EGF stimulation. Our study is the first to show

that the docking protein Gab2 is a major mediator in EGF-induced PI3-kinase activation in rat primary hepatocytes.

5.3. EGF receptor signaling specificity

One central and enduring question regarding signaling by receptor tyrosine kinases is: how is the specificity of each receptor tyrosine kinase generated? For example, although various hormones and growth factors, such as insulin and PDGF, are able to activate PI3-kinase, each elicits specific biological effects. In primary rat hepatocytes, both insulin and EGF activate PI3-kinase but only insulin is able to down-regulate the expression of the IGFBP-1 mRNA (18). In adipocytes, insulin and PDGF activate PI3-kinase, but only insulin is able to promote translocation of glucose transporters (GLUT4) from intracellular vesicles to the cell surface (19). A number of studies have now shown that ligand-induced compartmentalization of key signaling molecules is critical for transducing key aspects of the downstream response (20) and that this compartmentalization likely contributes to signaling specificity. For example it was shown that in adipocytes PDGF recruited PI3-kinase to PM whereas insulin recruited PI3-kinase to a high speed pellet fraction (HSP) containing IRS-1 and the intracellular pool of GLUT4, thus suggesting that the intracellular localization of activated signaling molecules is a major determinant of signaling specificity (21). In this thesis we found different subcellular localization of the p85-associated complexes. Our data suggest that ErbB3-p85 and Shc-p85 were initially formed in the plasma membrane followed by internalization to endosomes, whereas a multimeric Gab2 complex was mainly found in the cytosol. The specific

subcellular localization of these complexes might contribute to the specificity of EGF signaling.

The discovery of docking proteins provides another means for generating signaling specificity since different receptors appear to use different docking proteins. Thus the insulin receptor phosphorylates IRSs which activate PI3-kinase by recruiting p85 whereas the EGFR uses Gab family proteins or Cbl as docking protein substrates for the activation of PI3-kinase through p85 recruitment. The same receptor might use several docking proteins in the same cell to effect PI3-kinase activation and hence perhaps lead to individual and unique downstream effects. This is exemplified in our own work where we identified three different proteins which recruit and activate PI3-kinase: ErbB3, Shc and Gab2. Only the latter was engaged in EGF-induced DNA synthesis since PP1 blocked EGF-induced Gab2 tyrosine phosphorylation and DNA synthesis without inhibiting the phosphorylation of ErbB3 or Shc. The specific functions of ErbB3 and Shc-associated PI3-kinase in EGF signaling remain to be identified. Finally, the integration of signals generated by an activated docking protein and other activators could produce specific biological effects. For example, signals via Gab-SHP2 and Grb2-Sos-Ras pathways could conjoin to activate MAPK and promote other specific biological responses via additive or synergistic effects. Therefore, the simultaneous or sequential activation of receptors and Gab proteins might lead to qualitatively and quantitatively different

signals, thus conferring specific messages for the control of cell growth and differentiation during the development of multicellular organisms.

5.4. Mechanism of Gab2 phosphorylation by EGF

Most docking proteins are phosphorylated directly by RTKs following their recruitment to the phosphorylated RTK by either PTB or SH2 domains – a process frequently facilitated by a PH domain (22). Surprisingly we found that following *in vivo* EGF treatment, Gab2 did not undergo net translocation to membranes but rather formed a multimeric complex with p85 in the cytosol (Chapter 3). This interpretation was supported by immunofluorescence analyses in cultured hepatocytes demonstrating that EGF did not promote the association of dispersed cytosolic Gab2 with cell membranes. These observations corresponded to our finding that the PH-domain of Gab2 was not required for Gab2 tyrosine phosphorylation or localization to membranes. For these reasons we examined the possibility that Gab2 is tyrosine phosphorylated by a cytosolic kinase rather than the EGFR.

In Chapter 4, we demonstrate that EGF treatment of rat hepatocytes activates Src leading to Gab2 tyrosine phosphorylation, after which Gab2 recruits SHP2, Grb2 and p85 thus forming a multimeric cytosolic complex. We also showed that the proline-rich sequences on Gab2 are important for mediating the association with and phosphorylation by Src family kinases. The phosphorylation of Gab2 by the non-receptor protein kinases, Src family kinases, not the EGFR, explains why Gab2 is phosphorylated in a PH-domain independent manner without

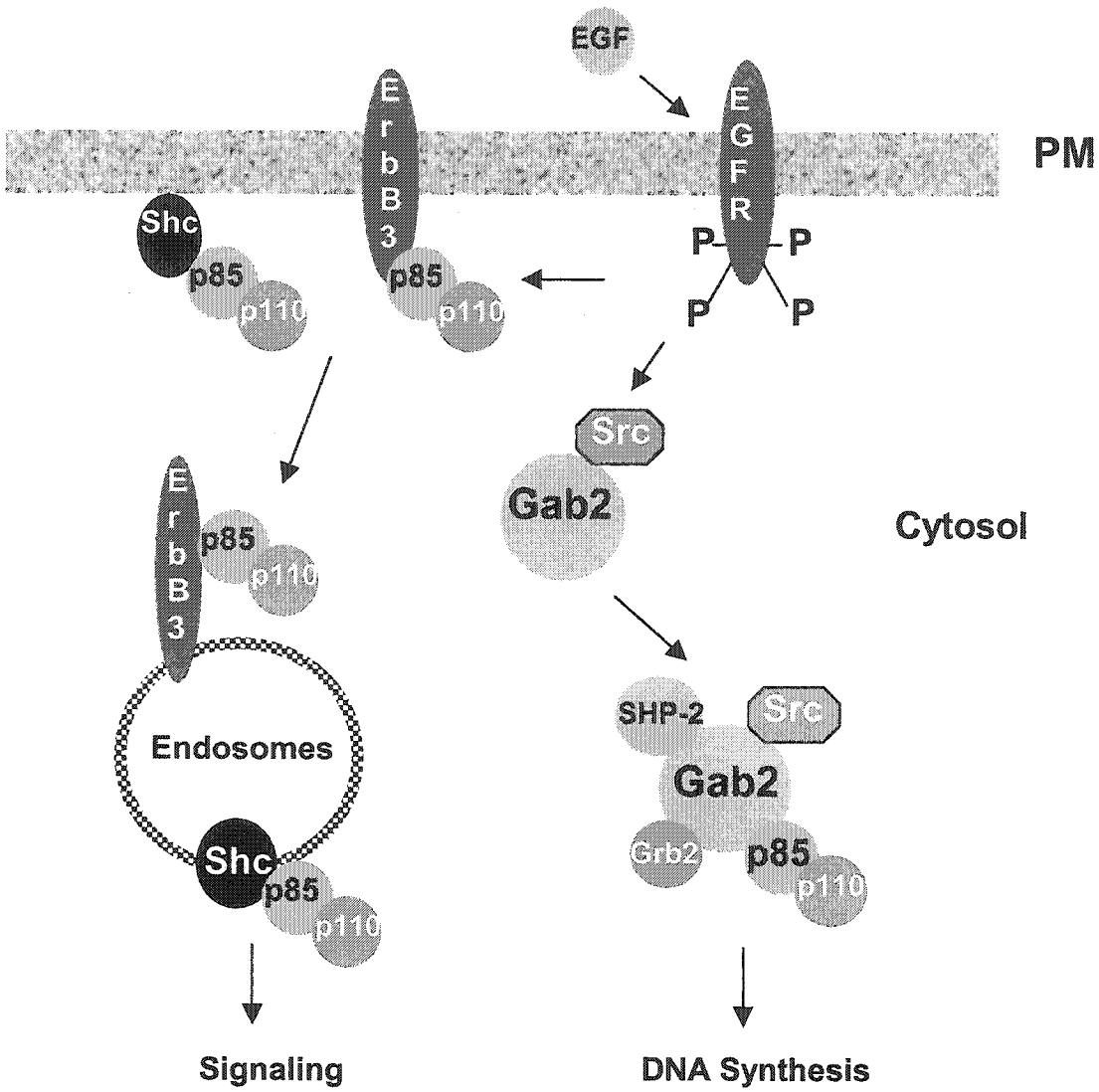
translocation to the membrane. However, how EGF activates Src leading to phosphorylation of Gab2 despite a constitutive association of Src with Gab2 needs to be elucidated. In contrast, EGF-dependent Gab1 phosphorylation is PH-domain dependent and correlates with the recruitment of Gab1 to PM and its association with EGFR as clearly demonstrated (23). Therefore although Gab1 and Gab2 are closely related they appear to be activated by different mechanisms.

We also found that there was negative regulation of Gab2 phosphorylation via the recruitment of SHP2. Our data suggest that the binding of SHP2 to Gab2 results in down-regulation of EGF-induced PI3-kinase activation and DNA synthesis through dephosphorylation of the p85 binding sites on Gab2. The negative role of SHP2 on Gab2 phosphorylation is correlated with two recent studies showing that inactive forms of SHP2 markedly increased EGF-stimulated Gab1 tyrosine phosphorylation and PI3-kinase activity (24,25). Furthermore, in another study it was found that phosphorylation of Gab2 on Ser 159, a consensus site for Akt, resulted in reduced tyrosine phosphorylation of Gab2 (26). Further work is needed to establish the presence of this negative regulatory process in primary rat hepatoctyes,

5.5. Original contribution

This thesis has investigated the molecular signaling pathway involved in EGF-induced DNA synthesis in primary hepatocytes. This work has generated several novel findings: (i) the activation of the PI3-kinase pathway is both necessary and

sufficient to account for EGF-induced DNA synthesis; (ii) three distinct p85-associated complexes: ErbB3-p85, Shc-p85 and a large complex Gab2-Grb2-SHP2-p85 are formed following EGF treatment. The latter accounts for > 80% of total EGF-induced PI3-kinase activity; (iii) these complexes are differentially localized in rat liver following EGF treatment. ErbB3-p85 and Shc-p85 complexes are localized to PM and endosomes, whereas the multimeric Gab2-Grb2-SHP2-p85 complex is formed rapidly and exclusively in cytosol. (iv) the Gab2 complex plays the critical role in EGF-induced PI3-kinase activation and DNA synthesis ; (v) Gab2 is phosphorylated in a PH-domain independent manner without net translocation from cytosol to plasma membrane; (vi) Gab2 is phosphorylated by Src family kinases which are constitutively bound to Gab2 via proline rich sequences; (vii) there is negative regulation of Gab2 phosphorylation and DNA synthesis through SHP2 binding. All these results are summarized and presented in Figure 1.



5- Figure1: EGF induced-PI3-kinase activation and DNA synthesis in primary rat hepatocytes. Upon EGF treatment, three distinct p85-associated complexes: ErbB3-p85, Shc-p85 and a large complex Gab2-Grb2-SHP2-p85 are formed. These complexes are differentially localized following EGF treatment. ErbB3-p85 and Shc-p85 complexes were localized to PM and Endosomes, whereas the multimeric Gab2-Grb2-SHP2-p85 complex was formed rapidly and exclusively in cytosol. The latter is formed through Gab2 phosphorylation mediated by Src family kinases, which constitutively associate with Gab2. The multimeric Gab2 complex accounts for over 80% of the PI3-kinase activity and is essential for EGF- induced DNA synthesis.

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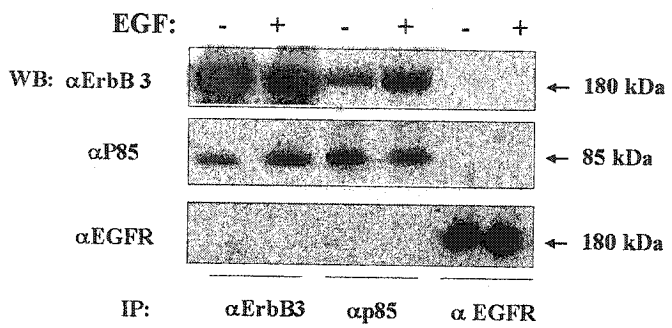
APPENDICES

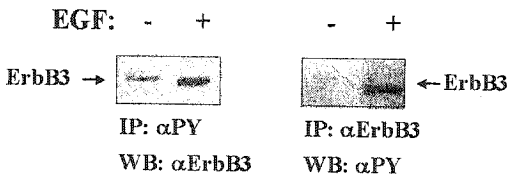
1. Supplemented Figures

A-Figure 1. Characterization of the PY180. Hepatocytes were treated with (+) or without (-) 100nM EGF for 1min. A. Proteins, immunoprecipitated with anti-ErbB3 (αErbB3), αp85, or anti-EGFR (αEGFR), and resolved on 7.5% SDS-PAGE were subjected to immunoblotting with αErbB3 (*top panel*), αp85 (*middle panel*) or αEGFR (*bottom panel).* B. Proteins, immunoprecipitated with αPY (*left panel*) or αErbB3 (*right panel)* and resolved on 7.5% SDS-PAGE, were subjected to immunoblotting with αErbB3 or αPY as indicated. C. Proteins, immunoprecipitated with αp85 or αErbB3 and resolved on 7.5% SDS-PAGE, were immunoblotted with αPY. The three major tyrosine phosphorylated bands are noted by the arrows.

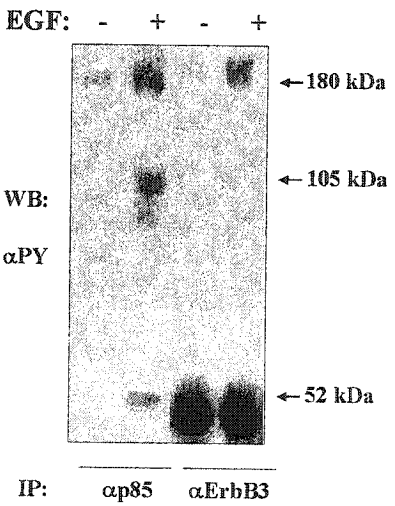
A


EGF: - + - + - +
WB: αErbB 3
← 180 kDa
αP85
← 85 kDa
αEGFR
← 180 kDa
IP: αErbB3 αp85 α EGFR

B

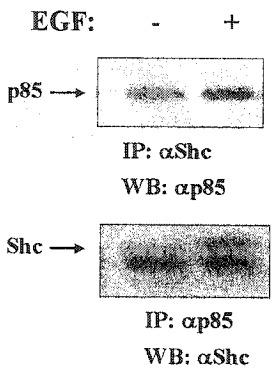

EGF: - + - +
ErbB3 →
←ErbB3
IP: αPY
WB: αErbB3
IP: αErbB3
WB: αPY

C

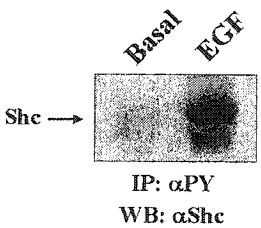

EGF: - + - +
←180 kDa
←105 kDa
WB:
αPY
←52 kDa
IP: αp85 αErbB3

A-Figure 2. Characterization of PY 52. A. Cell lysates from hepatocytes treated. (+) or not (-) with 100 nM EGF for 1 min, were immunoprecipitated using either anti-Shc (αShc) (*top panel*) or αp85 (*bottom panel*). Immunoprecipitates were resolved on 7.5% SDS-PAGE, and subjected to immunoblotting with either αp85 or αShc as indicated. B. Cell lysates from hepatocytes treated with or without 100 nM insulin or EGF for 1 min, were immunoprecipitated with αPY, resolved on 7.5% SDS-PAGE, and immunoblotted with αShc. C. Cell lysates from hepatocytes treated with (+) or without (-) 100 nM EGF for 1 min, were immunoprecipitated with αShc or αp85 antibodies. Immunoprecipitates were resolved on 7.5% SDS-PAGE and subjected to immunoblotting with αPY. The major tyrosine phosphorylated bands are noted.

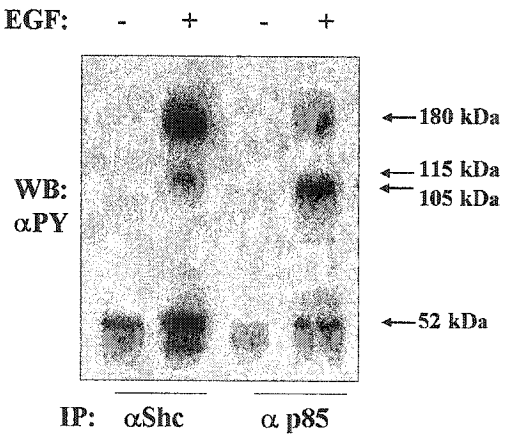
A


EGF: - +
p85
IP: αShc
WB: αp85
Shc
IP: αp85
WB: αShc

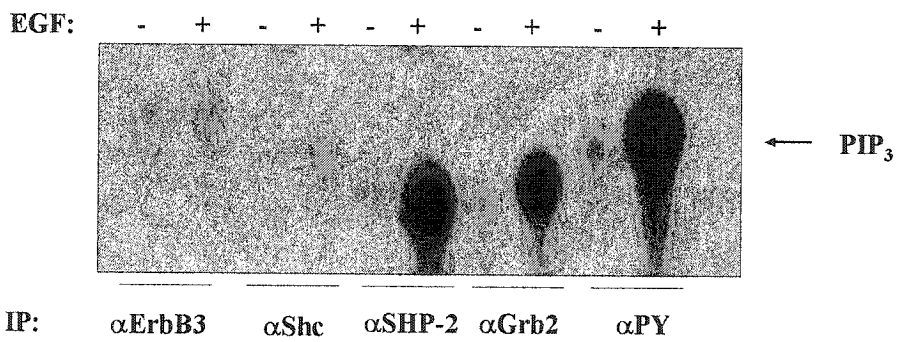
B


Basal
EGF
Shc
IP: αPY
WB: αShc

C


EGF: - + - +
WB: αPY
180 kDa
115 kDa
105 kDa
52 kDa
IP: αShc
α p85

A-Figure 3. SHP-2, Grb2, PY 105 and p85 form the major complex responsible for EGF-induced PI3-kinase activation. Hepatocytes were treated with (+) or without (-) 100nM EGF for 1min. Cell lysate proteins immunoprecipitated with αErbB3, αSHC, αSHP-2, αGrb2 or αPY were analyzed for PI3-kinase activity as described under "Experimental Procedures" in Chapter 1.


EGF: - + - + - + - + - +
PIP3
IP: αErbB3 αShc αSHP-2 αGrb2 αPY

2. Abbreviations

CSF, colony-stimulating factor

CSK, c-terminal Src Kinase;

DNA, deoxyribonucleic acid;

EGF, epidermal growth factor;

EPO, erythropoietin;

Erk1/2, extracellular signal-regulated kinase 1 and 2;

FITC, fluorescein isothiocyanate form;

Gab1, Grb2 associated binder 1;

Gab2, Grb2 associated binder 2;

Grb2, growth factor receptor bound 2;

GST, glutathione-S-transferase;

HGF, hepatocyte growth factor

IL, Interleukin;

MAPK, mitogen activated protein kinase;

moi, multiplicity of infection;

NGF, nerve growth factor;

PH-domain, pleckstrin homology domain;

PDGF, platelet-derived growth factor;

PDK-1, phosphoinositide-dependent kinase-1

PI3-kinase, phosphatidylinositol 3-kinase;

PKB, protein kinase B;

PKC, protein kinase C;

PRD, proline-rich domain;

RTK, receptor tyrosine kinase;

SCF, stem cell factor;

SH2, Src homology domain 2 ;

SH3, Src homology domain 3;

SHP-2, src homology 2 domain-containing protein tyrosine phosphatase-2.

3. Ethics Certificates

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR

ADDRESS: Polypeptide Laboratory, TELEPHONE:

FAX NUMBER:

DEPARTMENT: Medicine E-MAIL:

PROJECT TITLE: Insulin and growth factors in growth, development and disease.

---

2. FUNDING SOURCE: MRC X NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify)

Grant No.: MT-4182 Beginning date: Oct.1, 2000 End date: Sept. 30, 2005

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3. Indicate if this is

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date July 31, 2001.

New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

X New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: SIGNATURE date: 04 - 05 - 2000. (day month year)

Chairperson, Biohazards Committee: SIGNATURE date: 24 - 05 - 2000 (day month year) (1)

Approved period: beginning 01 day 10 mo. 2000 year ending day 30 month 09 year 2005

X → C

McGill University
Animal Use Protocol – Research
Guidelines for completing the form are available at
www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4110
Investigator #: 192
Approval End Date: Sept. 30, 2003
Facility Committee

☐ **Pilot** ☐ **New Application** ☒ **Renewal of Protocol # 4110**

Title (must match the title of the funding source application): Insulin and growth factors in growth, development and disease.

1. Investigator Data:

Principal Investigator: Dr. Barry Posner **Office #:**
Department: Polypeptide Laboratory **Fax#:**
Address: **Email:**

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Mr. Gerry Baquiran **Work #:** **Emergency #:**
Name: Mr. Victor Dumas **Work #:** **Emergency #:**

3. Funding Source:

External ☒	Internal ☐
Source (s): CIHR 8837	Source (s):
Peer Reviewed: ☒ YES ☐ NO**	Peer Reviewed: ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	Status: ☐ Awarded ☐ Pending
Funding period: Oct.1,2000 - Sept.30,2005	Funding period:

ACTION	✓	DATE
CCs	✓	Nov.6'02
DEI	✓	"
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed. e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	or ongoing ☒
Expected Date of Completion of Animal Use (d/m/y):	or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: **Date:** Aug. 26/02

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:** 1/11/02
University Veterinarian:		**Date:** 1/11/02
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:** Oct. 1, 2002	**Ending:** Sept. 30, 2003

☐ This protocol has been approved with the modifications noted in section 13.

NOV 06 2002