MOLECULAR INTERACTIONS BETWEEN INSULIN-LIKE GROWTH FACTOR SIGNAL TRANSDUCTION AND RETINOIDS IN BREAST CANCER CELLS

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This thesis is dedicated to my parents whom I will miss dearly in SD.

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Abstract

Numerous groups, including ours, have found that retinoids potently inhibit the growth of breast cancer cells, but the mechanisms by which growth regulation is achieved remains unclear. Although several of the effects of retinoids in breast cancer have been linked to the insulin-like growth factor (IGF) system, their effects on key signaling molecules in the IGF type-I receptor (IGF-IR) pathway have not been well characterized. This thesis project examined the hypothesis that retinoids mediate their growth inhibitory effects by targeting specific members of the IGF-IR signal transduction pathway. Although we did not observe regulation of IGF-IR itself, we found that *all-trans* retinoic acid (RA)-mediated growth inhibition is associated with a selective reduction in insulin receptor substrate 1 (IRS-1) protein and activity levels. We also present evidence that decreasing IRS-1 levels results in the selective down-regulation of the PI 3-kinase/AKT pathway in RA-treated MCF-7 cells. The relevance of IRS-1 regulation to the growth inhibitory action of RA is supported by the results showing that forced expression of IRS-1 abrogates the ability of RA to significantly inhibit MCF-7 cell growth.

Several studies have highlighted the importance of IRS-1 in breast cancer pathogenesis. High levels of IRS-1 in human breast tumors correlate with increased disease recurrence and constitutive IRS-1 signaling exists in breast tumors. This suggests that we may develop molecular strategies targeting IRS-1 by understanding the mechanisms controlling its expression and turnover. Since RA decreased IRS-1 protein levels without altering mRNA levels, we examined the hypothesis that RA-mediated regulation of IRS-1 levels was at the posttranslational level. Two proteasome inhibitors rescue the RA-mediated degradation of IRS-1, and RA increases the ubiquitination of

IRS-1. We also found that RA increases the serine phosphorylation of IRS-1 and show that this occurs in a protein kinase C (PKC)-dependant manner, since PKC inhibitors block the RA-induced degradation and serine phosphorylation of IRS-1. We further demonstrate that RA activates PKC-δ in the sensitive, but not in the resistant cells, with a time course that is consistent with the RA-induced decrease of IRS-1. The involvement of PKC in the RA-mediated regulation of IRS-1 is supported by additional data showing that: 1) RA-activated PKC-δ phosphorylates IRS-1 *in vitro*, 2) PKC-δ and IRS-1 interact in RA-treated cells, and 3) mutation of three PKC-δ serine sites in IRS-1 to alanines results in no RA-induced *in vitro* phosphorylation of IRS-1.

Having identified IRS-1 as a novel target of RA and showing that RA regulates this protein via a mechanism involving the ubiquitin-proteasome pathway has contributed to an enhanced understanding of the effect of retinoids in human breast cancer cells.

Résumé

Plusieurs équipes de chercheurs, incluant la nôtre, ont à ce jour démontré que les rétinoides peuvent être utilisés afin de restreindre efficacement la croissance de cellules cancéreuses du sein. Toutefois, les mécanismes régulant cette inhibition ne sont toujours pas clairement élucidés. Bien que des effets répertoriés comme étant causés par les rétinoides aient été reliés à la voie de signalisation de l'IGF-I dans les cellules de cancer du sein, ces effets directs sur les principaux acteurs de la voie de l'IGF-I ne sont toujours pas compris de façon exhaustive. Le projet de cette thèse était donc de vérifier précisément si la voie de transduction de l'IGF-I était précisément une cible d'action des rétinoides pour expliquer leur rôle d'agents inhibiteurs de la croissance cellulaire. Nous n'ayons pu observer la régulation directe du récepteur de l'IGF-I (IGF-IR), mais nous avons découvert que l'inhibition de la croissance cellulaire provoquée par l'administration d'acide rétinoique est clairement associée à la réduction sélective de la protéine IRS-1 ainsi qu'à une diminution marquée de son activité. Nos résultats suggèrent de façon convaincante que, dans la lignée cellulaire MCF-7 traitée avec de l'acide rétinoique, une diminution du niveau de protéine IRS-1 provoque la dérégulation sélective de la voie PI3 kinase/AKT. Ces résultats sont corroborés par l'incapacité de l'acide rétinoique à causer l'inhibition de la croissance cellulaire dans les cellules MCF-7 lorsqu'il y a hausse de l'expression de la protéine IRS-1.

De nombreuses études ont souligné l'implication de la protéine IRS-1 dans la pathogénèse du cancer du sein; l'expression accrue de IRS-1 dans les tissus tumoraux du sein chez l'humain corrèle avec un taux de récurrence augmenté de la maladie et la voie de signalisation propre à IRS-1 est sollicitée dans les tumeurs du sein. Ces informations

permettent d'envisager l'analyse de l'expression d'IRS-1 comme moyen principal dans la recherche d'un potentiel mécanisme moléculaire qui ciblerait directement cette même protéine. Le fait que l'administration d'acide rétinoique provoque une diminution de la quantité de protéine IRS-1, mais ne change en rien la quantité d'ARN messager présent nous a poussé à poser comme hypothèse que la régulation par l'acide rétinoique du niveau d'IRS-1 doit probablement s'effectuer au niveau posttraductionel. En effet, deux inhibiteurs du protéosome sont en mesure de rescaper la dégradation de IRS-1 causée par la présence d'acide rétinoique et l'ubiquitination de IRS-1 est accrue en présence d'acide retinoique. Nous avons aussi découvert que, toujours suite à l'administration d'acide rétinoique, il y a augmentation de la phosphorylation de certaines sérines présentes sur la protéine IRS-1. Cette phosphorylation accrue dépend cependant de la phosphokinase C (PKC) puisque l'inhibition de cette dernière bloque non seulement la phosphorylation des serines de IRS-1, mais prévient aussi la dégradation de IRS-1 induite par l'acide rétinoique. Nous avons par ailleurs fait la démonstration que, dans les lignées cellulaires sensibles seulement, l'acide rétinoique est en mesure d'activer la phosphokinase C-δ (PKC-δ) dans des laps de temps qui correspondent aux délais requis pour la diminution du taux de IRS-1 remarquée lors de l'administration d'acide rétinoique. L'implication de la phosphokinase C (PKC) dans la diminution de IRS-1 suite à l'administration d'acide rétinoique a été démontrée par d'autres résultats : 1) Suite à son activation par l'acide rétinoique, la phosphokinase C phosphoryle IRS-1 *in vitro*, 2) Dans les cellules traitées avec de l'acide rétinoique, la phosphokinase C-δ (PKC-δ) et IRS-1 interagissent ensemble, et 3) En mutant trois des serines présentes dans IRS-1 (servant de sites de

phosphorylation pour PKC-δ) pour l'acide aminé alanine, la phosphorylation *in vitro* de IRS-1 causée par l'administration d'acide rétinoique n'est plus possible.

Notre identification de la protéine IRS-1 comme cible dans le mécanisme d'action de l'acide rétinoique ainsi que l'implication de la voie de l'ubiquitination dans la régulation de cette protéine par l'acide rétinoique permettent une meilleure compréhension des effets causés par les rétinoides sur les cellules du cancer du sein chez l'humain.

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My extreme thanks go first and foremost to my supervisor Dr. Wilson Miller for his guidance and support, both academically and personally...thanks Wilson! I also kindly thank the members of my graduate committee for their helpful suggestions and time: Dr. Antonis Koromilas, Dr. Brian Ward, Dr. Simon Wing, Dr. Constantine Polychronakos, and, Dr. Pnina Brodt. I would like to extend a warm thanks to two people who always made time in their busy schedules for me: Dr. Stéphane Richard and Dr. Eva Surmacz...thanks Stéphane and Eva.

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Thanks to my Mike for his patience and love from the very beginning.

Preface

In accordance with the strict guidelines for thesis preparation from the faculty of graduate studies and research at McGill University ("Guidelines for Thesis Preparations"), I have elected to present my research in a manuscript based format. I have written a general literature review as Chapter 1. The next two chapters include two already published papers in *Oncogene* (Chapters 2 and 3). Each Chapter is connected by a logical bridge and a General Discussion is also included. The manuscripts presented in this thesis are as follows:

1) **del Rincón SV**, Rousseau C, Samanta R, Miller WH Jr. Retinoic acid-induced growth arrest of MCF-7 cells involves the selective regulation of the IRS-1/PI 3-kinase/AKT pathway. *Oncogene.* 22:3353-60, 2003.

2) **del Rincón SV**, Guo Q, Morelli C, Shiu H-Y, Surmacz E, Miller WH Jr. Retinoic acid mediates degradation of IRS-1 by the ubiquitin-proteasome pathway, via a PKC-dependant mechanism. *Oncogene*. 2004 Nov 01, Epub ahead of print.

Two additional published papers that I co-authored but did not include in this thesis are listed below:

1) Morelli C, Garofalo C, Sisci D, **del Rincón SV**, Cascio1 S, Tu X, Vecchione A, Sauter ER, Miller WH Jr and Surmacz E. Nuclear insulin receptor substrate 1 interacts with estrogen receptor-α at ERE promoters. *Oncogene* in press.

2) Côté S, McNamara S, Brambilla D, Bianchini A, Rizzo G, **del Rincón SV**, Grignani F, Nervi C, and Miller WH Jr.. Expression of SMRTβ promotes ligand-induced activation of mutated and wild-type retinoid receptors. *Blood* in press.

Contributions of the authors

All of the research presented in Chapters 2 and 3 was performed by the candidate under the supervision of Dr. W.H. Miller Jr.. Others that have contributed to the work are listed below:

CHAPTER 2:

-Caroline Rousseau provided the growth curve presented in Figure 2.6.

-Ratna Samanta repeated some of the western blots shown in Chapter 2.

CHAPTER 3:

-Eva Surmcaz invited me to her lab to optimize the experiments assessing the ubiquitination of IRS-1.

-Catia Morelli, a doctorate student in Ewa Surmacz's lab gave me the protocol look at IRS-1 levels using immunoflourescence. She also helped troubleshoot the IRS-1/ubiquitin conjugate experiment shown in Figure 3.4a.

-Qi Guo and Hoi-Ying Shiu repeated most of the western blots shown in the Chapter 3.

List of abbreviations

4-HPR	N-(4-hydroxyphenyl)retinamide or fenretinide
9cRA	9-*cis* retinoic acid
13cRA	13-*cis* retinoic acid
α-IR3	monoclonal anti-IGF-IR antibody
AP-1	activating protein-1
APL	acute promyleocytic leukemia
ATP	adenosine triphosphate
CDK	cyclin dependant kinase
CKI	cyclin-dependent kinase inhibitors
CRBP	cellular retinoid-binding protein
CRABP	cellular retinoic acid-binding protein
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E1	ubiquitin-activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERα	estrogen receptor alpha
erk-1 and -2	extracellular signal-regulated kinase -1 and -2

FBS	fetal bovine serum
GSK3	glycogen synthase kinase 3
HDAC	histone deacetylase complexes
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGF-IR	insulin-like growth factor type I receptor
IGF-IIR	mannose-6-phosphate insulin-like growth factor type II receptor
JNK	c-jun N-terminal kinase
kb	kilobase
kDa	kilodalton
IRS-1	insulin receptor substrate 1
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NMU	N-nitroso-N-methylurea
PBS	phosphate buffered saline
PH	pleckstrin homology
PI 3-kinase	phosphoinositide 3-kinase
PKC	protein kinase C
PPAR	peroxisome proliferating associated receptor
PTB	phosphotyrosine-binding
RA	all-trans retinoic acid
RACK	receptors for activated C-kinase

RAR	retinoic acid receptor
RARE	retinoic acid receptor response element
RBP	retinol-binding protein
RTK	receptor tyrosine kinases
RXR	retinoid X receptor
RXRE	retinoid X receptor response element
S	Serine
SFM	serum free media
SH2	Src homology-2
SH3	Src homology-3
SHC	Src-homology collagen
SHP-2	SH2-containing phosphotyrosine phosphatase-2
UBE1L	ubiquitin-activating enzyme-E1-like
WD	tryptophan-aspartic acid
Y	Tyrosine

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CHAPTER 1

LITERATURE REVIEW

Cell line	Type of cancer	Original tissue	ER-α status	Retinoic acid sensitive
MCF-7	Invasive ductal carcinoma	Pleural effusion	+	Yes
T47-D	Invasive ductal carcinoma	Pleural effusion	+	Yes
ZR75.1	Invasive ductal carcinoma	Ascites	+	Yes
SK-BR-3	Invasive ductal carcinoma	Pleural effusion	-	Yes
MDA-MB-231	Invasive ductal carcinoma	Pleural effusion	-	No
MDA-MB-468	Adenocarcinoma	Pleural effusion	-	No

Table 1 – Description of breast cancer cell lines used in this thesis.

1.1 - BREAST CANCER

Breast cancer remains the second leading cause of cancer-related death amongst Canadian women. When a woman is diagnosed with breast cancer, the standard treatment regime involves radiation and chemotherapy that can cause non-specific damage to normal cells in the body. Researchers, oncologists, and pharmaceutical companies alike are in search of therapeutic agents that would exclusively target cancerous cells while having negligible effects on nonmalignant cells. The future of translational research will be aimed at treating women with breast cancer on an individual basis, using the genetic profile of her tumor to tailor a target-specific chemotherapy. With these ideas in mind, basic research attempts to understand the mechanisms by which nonmalignant cells become cancerous.

Many of the advances in our understanding of the biology of breast cancer development and progression have come from *in vivo* and *in vitro* studies performed using a small number of breast cancer cell lines as model systems. The most commonly used cell lines were initially developed in the 1970s (Table 1), and it is estimated that roughly two-thirds of all breast cancer-related studies have used MCF-7, T47-D, and MDA-MB-231 cells [1]. Breast cancer cell lines and tumors are evaluated based on the expression of the estrogen receptor-α (ER), and classified as either steroid hormone-dependent (ER-positive, ER+) or hormone-independent (ER-negative, ER-). The ER has a critical role in mediating the proliferative effects of estrogen and as such has classically been a major target in the treatment of breast cancer. However, with increased research into the pathobiology of breast cancer it has become evident that breast tumors may also use many aspects of growth factor receptor pathways to regulate their growth.

It is hypothesized that cells with increased growth rates are at a higher risk of becoming cancerous[2]. A frequent mechanism leading to enhanced cell proliferation is the amplification of signaling via growth factor receptors, and it is not surprising that several oncogenes encode various components of growth factor receptor signaling pathways. Breast cancer cells can be stimulated to proliferate in response to growth factors arriving on the cell surface from different locations in the body. Once a growth factor binds its cell surface receptor, downstream intracellular factors contribute to the further stimulation of breast cancer cell growth. The overexpression of receptor tyrosine kinases (RTKs) (e.g. type-I insulin-like growth factor receptor (IGF-IR) [3], Her-2/neu receptor [4], and epidermal growth factor receptor (EGFR) [5]), increased levels of intracellular substrates (e.g. IRS-1 [6] and Grb2 [7]), and constitutive activation of kinases (e.g. MAPK and PI3-kinase/AKT) [8] are commonly observed in primary breast tumor specimens, and correlate with poor clinical outcome. The aberration of various aspects of signal transduction pathways does raise the possibility that specific components can be therapeutically targeted in breast cancer. In addition, the knowledge that one woman's breast tumor has an alteration in one component and not another may eventually allow physicians to treat breast cancer on a more individual basis. Increased research into the molecular mechanisms linking specific growth factor receptor signaling pathways and breast carcinogenesis may lead to potential developments in the areas of chemoprevention and therapy.

1.2 - THE INSULIN-LIKE GROWTH FACTOR (IGF) SYSTEM

1.2.1 - IGF Members in the Extracellular Space:

Cellular communication is critical for normal embyrogenesis and development. When cells respond inadequately to external stimuli this could lead to abnormal cellular responses and the development of cancer. Growth factors can stimulate target cells that are located in close proximity (paracrine signal) or at distant locations to the initial site of synthesis (endocrine signal). It is also possible for the same cell to respond to its own growth factors when synthesized and secreted (autocrine signal). Once they reach their target cells, growth factors will initiate signaling by interacting with cell surface receptors to elicit changes in cellular physiology and gene expression that are essential for cell proliferation, differentiation, and cell survival.

1.2.1.1 IGFs and IGFBPs:

IGF-I, originally termed somatomedin C, is a single-chain, 7.6 kDa hydrophilic polypeptide molecule with structural similarity to IGF-II and insulin. IGF-I is synthesized primarily in the liver in response to growth hormone, but is also synthesized in a variety of other tissues in a paracrine and autocrine fashion [9]. Once produced, IGF-I travels to the blood where 80-90% of it is bound to one of six high-affinity IGF-binding proteins (IGFBP1-6). The IGFBPs modulate IGF-I activity by regulating the bioavailability of IGF-I for interaction with its receptor, the IGF-IR [10, 11]. In the case of IGFBP-3, the binding of IGF-I can have two opposite effects: 1) It can inhibit IGF-I activity by sequestering it away from the IGF-IR or 2) It can protect IGF-I from degradation thus increasing the concentration of IGF-I in the serum. When IGF-I eventually binds the IGF-IR, this initiates a signal transduction cascade that results in

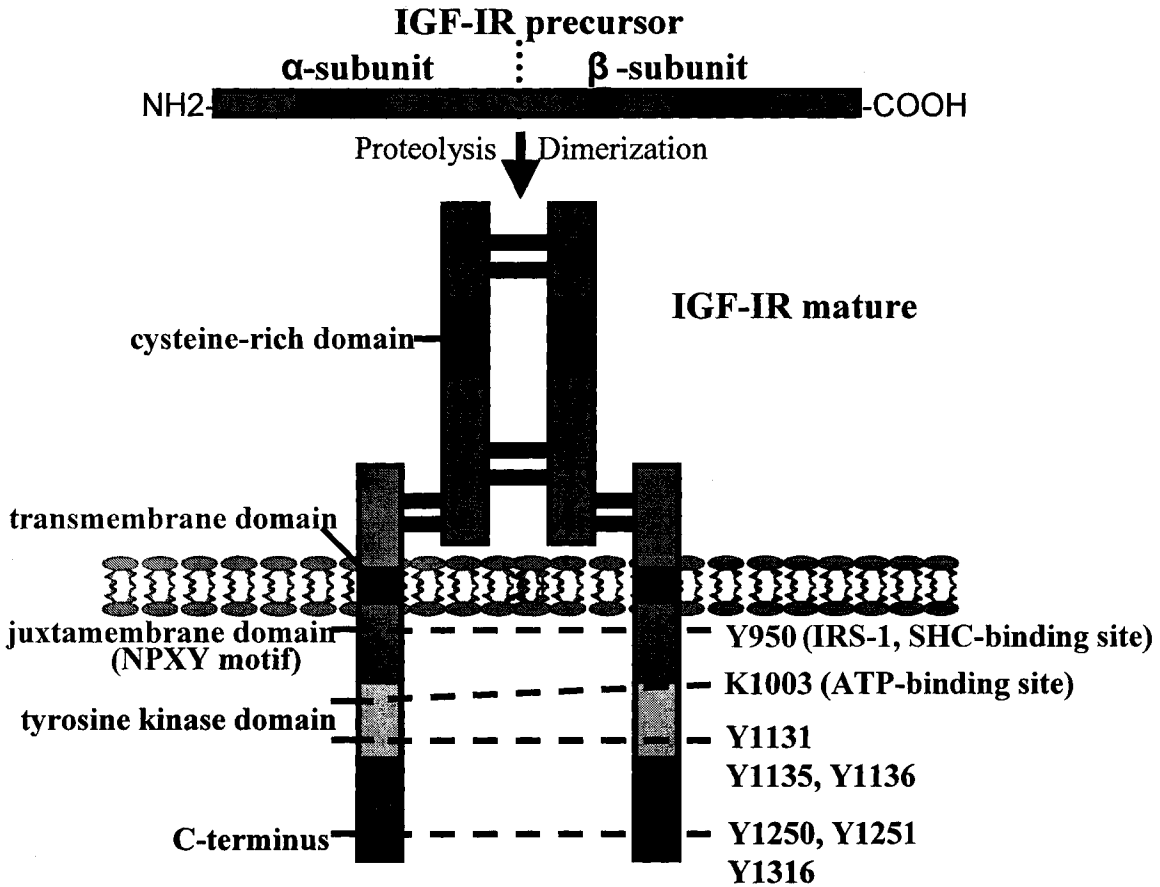

Figure 1.1 – Schematic diagram of the structure of the IGF-IR.

The IGF-IR precursor protein is cleaved into α- and β-subunits which dimerize to form the mature IGF-IR. See text for details of the specific residues and domains detailed in this schematic.

various cellular effects. Although an essential role of the IGF system in regulating normal body growth is highlighted by studies showing that transgenic mice lacking either the IGF-I or IGF-IR are extremely small in stature [12, 13], IGF-I can also regulate differentiation, apoptosis and cellular transformation. How can IGF-I stimulate different cellular responses upon binding to its receptor? This question can be addressed by understanding the domains and function of the IGF-IR and its interaction with downstream substrates.

1.2.2 – IGF Receptors at the Cell Surface:

The receptors for several growth factors, including the IGF-IR, EGFR, fibroblast growth factor, and platelet-derived growth factor, are classified as RTKs. RTKs are transmembrane proteins with an extracellular ligand-binding domain and an intracellular domain containing intrinsic tyrosine kinase activity. The first RTK identified was the EGFR [14] and the link between aberrant RTKs and cancer was made no more than three years later when it was discovered that the EGFR was very similar to the v-erbB oncoprotein [15].

1.2.2.1 IGF-IR:

During embryogenesis IGF-IR mRNA levels are at their highest, and although these levels are significantly decreased by adulthood, most cells in the body express IGF-IR with the exception of the liver [16]. The 11 kb IGF-IR transcript is translated into a 180 kDa immature precursor protein, which is subsequently cleaved to produce an alpha-subunit of 135 kDa and a beta-subunit of 90 kDa. The mature form of the receptor is a heterotetramer containing two extracellular alpha- and two transmembrane beta-subunits linked together by disulfide bonds [17] (Figure 1.1). The cysteine-rich domains within

the alpha-subunits form the IGF-binding domains and the beta-subunits contain three major domains: 1) the juxtamembrane domain (also known as the NPXY motif), 2) the tyrosine kinase domain that becomes active upon ligand binding resulting in receptor autophosphorylation, and 3) the C-terminus. Mutational analysis of these domains has shown that the mitogenic, transforming, and anti-apoptotic functions of the IGF-IR can be mapped to specific regions within the beta-subunits [18]. The results of these mutational analyses can be summarized as follows: 1) mutation of the ATP binding site (lysine 1003) results in a kinase dead, non-signaling IGF-IR, 2) mutation of the C-terminus identified that the transforming domain lies between residues 1245 and 1310 and is dispensable for mitogenesis, and 3) mutation of Tyrosine 950 within the juxtamembrane domain or of the tyrosine cluster (1131, 1135, and 1136) within the kinase domain inhibits transformation and mitogenesis, without affecting cell survival. Tyrosine 950 is the binding site of the two adapter proteins insulin receptor substrate 1 (IRS-1) and Src-Homology Collagen (SHC) that are responsible for propagating the mitogenic IGF-I signal downstream [19]. Since mutation of this site still enables the mutant IGF-IR to elicit anti-apoptotic signals, this suggests that other pathway(s) lie downstream from IGF-IR and this leads to functional selectivity upon IGF-IR activation.

Although the functions of the receptor depend in large part on the presence of these specific domains, the number of IGF-IRs can also influence receptor function. Rubini *et al.* performed the initial experiments in R- cells that are devoid of IGF-IR, showing that the response of these cells to IGF-I in mediating mitogenesis versus transformation depended on the number of receptors expressed on the cell surface after transfection of the IGF-IR [20]. Below a certain level of IGF-IR, R- cells failed to grow

in media containing IGF-I, however, above a certain number the cells grew only in the presence of IGF-I and also acquired the ability to grow in soft agar. These results were consistent with studies in animal models showing that the development of tumors in nude mice depended on the number of IGF-IR's expressed on the surface of xenotransplanted cells [21].

1.2.2.2 IGF-IIR:

The mannose-6-phosphate (M6P)/type-II IGF receptor (IGF-IIR) is a transmembrane glycoprotein initially identified with separate binding sites for M6P and IGF-II [22]. The classic role of this receptor is in binding and trafficking of acidic hydrolases to lysosomes [23]. Like the IGF-IR, the IGF-IIR has a role in regulating cell growth; however, it has no inherent tyrosine kinase activity. Loss or mutation of the IGF-IIR gene is associated with human malignancies [24-26], and overexpression of the receptor *in vitro* and *in vivo* results in growth suppression [27, 28], leading to the proposal that IGF-IIR is a putative tumor suppressor. Although the mechanism by which IGF-IIR regulates growth remains unclear, it is thought to antagonize IGF-induced signal transduction by binding, internalizing, and degrading this growth factor. It has also been proposed to facilitate the activation of the growth inhibitor TGF-beta [29]. In recent years a number of novel ligands, among them retinoic acid [30], and proliferin [31], have been identified for the IGF-IIR, thus complicating our understanding of the mechanisms by which this receptor regulates growth.

1.2.3 - Immediate Substrates of IGF-IR:

The autophosphorylation of RTKs upon binding ligand results in the tyrosine phosphorylation and concomitant association of adapter proteins (also called 'docking

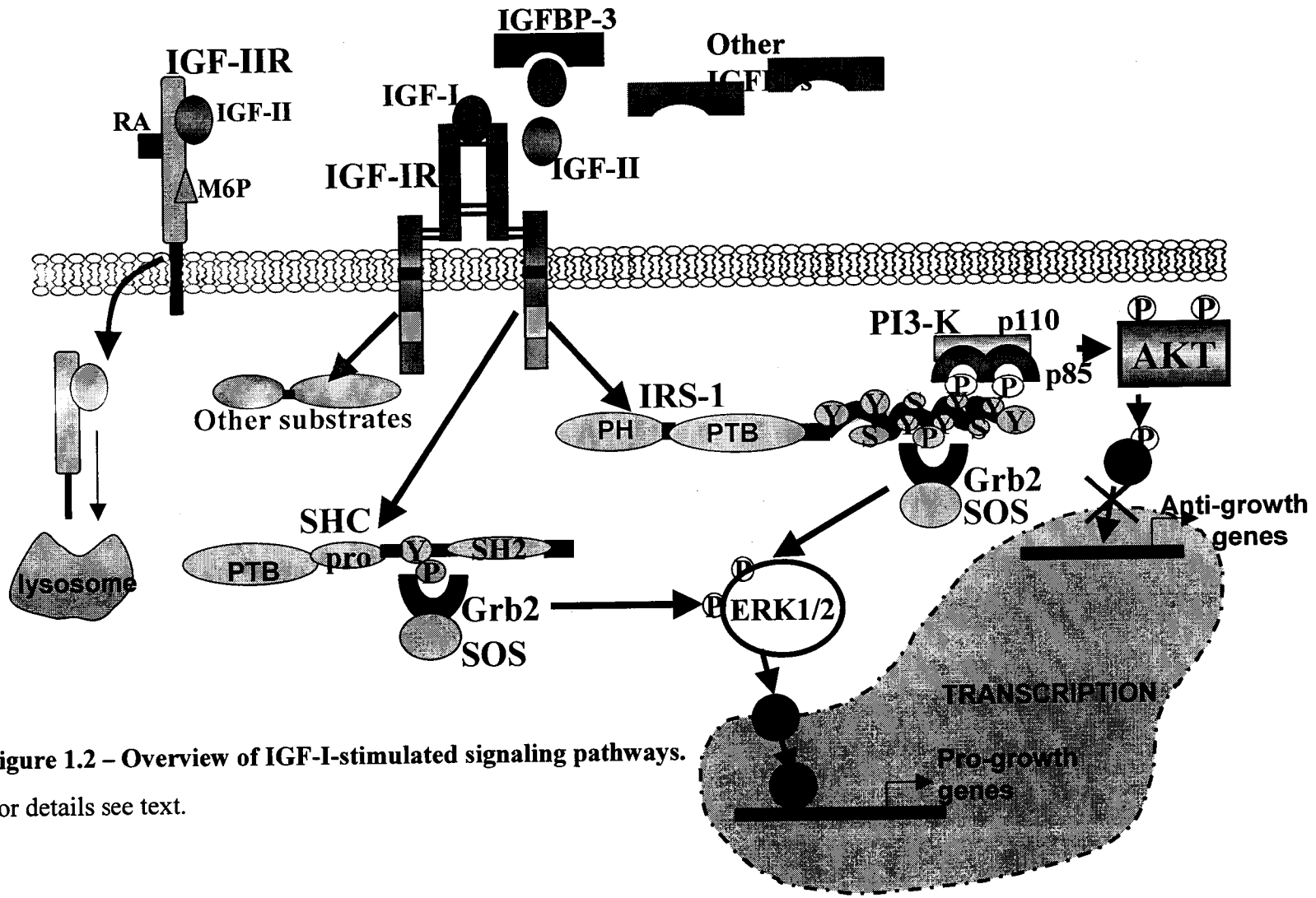

Figure 1.2 – Overview of IGF-I-stimulated signaling pathways.

For details see text.

molecules') to the activated receptor (Figure 1.2). Adapter proteins contain no catalytic domains, but possess modular domains facilitating protein-protein interactions for the recruitment of additional downstream proteins ('the cargo') for the coordinate activation of signaling pathways contributing to a plethora of cellular responses. Some of the most common functional domains include: the PH (pleckstrin homology), PTB (phosphotyrosine-binding), SH2 (Src homology-2), and SH3 (Src homology-3) domains. The amino acids surrounding the phosphorylated tyrosine residues confer specificity for the binding of proteins containing various modular domains. A wealth of data supports a functional role of SHC and IRS-1 in mediating some of the effects of the IGF-IR, however, other IGF-IR substrates have been identified: CrkII [32] and CrkL, FAK [33], RACK1 and Grb10 [34], but their functions remain largely unknown. As such, this section will only briefly describe some of the proteins known to regulate IGF-IR function and examine more thoroughly the impact of regulating IRS-1 levels since the majority of the work presented in this thesis focuses on the effect of retinoic acid on IRS-1 in breast cancer.

1.2.3.1 Grb10:

Grb10 belongs to the superfamily of related adapter proteins that include Grb2, Grb7 and Grb14. These proteins share similar modular domains: a SH2 domain, a central PH domain, and a proline-rich sequence. Grb10 was first identified by its ability to interact with the epidermal growth factor receptor [35] however, it has since been shown to bind other activated RTKs including the platelet-derived growth factor receptor, insulin receptor, and the IGF-IR. Although Grb10 interacts with the kinase domain of the IGF-IR [36], the role of Grb10 in mediating the mitogenic effects of IGF-IR remains under

debate. There is evidence in support of Grb10 as a negative regulator of IGF-I signaling [37, 38], but other reports show that Grb10 has stimulatory effects downstream of the vascular endothelial growth factor receptor [39] and the IGF-IR [40]. One mechanism proposed for the negative regulation of IGF-I signaling by Grb10 is its ability to stimulate ubiquitination and degradation of the IGF-IR [41].

1.2.3.2 RACK1:

RACK proteins were first termed 'receptors for activated C-kinase' for their ability to interact with activated PKC's [42]. More recently RACK1 was shown to bind the IGF-IR in fibroblasts and epithelial cells [43, 44], although its function in IGF-I signaling remains unclear. This protein contains 5-7 WD repeats and potentially can interact with multiple proteins at one time to coordinate the functions of signaling proteins in response to IGF-I. In support of such a role: 1) RACK1 is believed to bridge the integrin and IGF-IR signaling pathways [43] and 2) RACK1 can simultaneously have an activating effect on MAPK signaling while having a negative effect on AKT signaling [44]. Future studies to identify proteins able to bind RACK1 in the presence or absence of IGF-I may further define the function of this scaffolding protein.

1.2.3.3 SHC:

SHC is a unique adapter protein containing both an SH2-domain (C-terminal) and a PTB-domain (N-terminal) [45]. In addition, this protein contains a central region known as the collagen homology domain. SHC is ubiquitously expressed and has three isoforms of 46, 52, and 66 kDa. SHC isoforms have been demonstrated to induce transformation of fibroblasts *in vitro* and *in vivo* [46] and also to function in metastasis [47]. In response to IGF-I, SHC becomes tyrosine phosphorylated upon interaction with Y950 on the

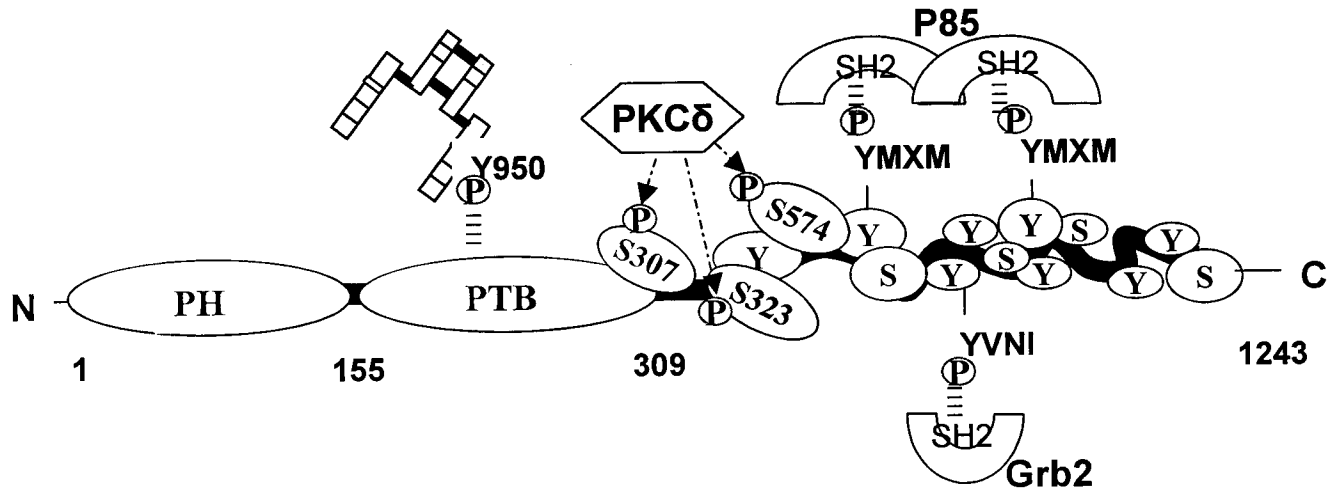

Figure 1.3 – Schematic diagram of the structure of IRS-1 and relevant interacting proteins.

PH: pleckstrin homology domain, PTB: protein tyrosine binding domain

Y950: residue in IGF-IR that becomes phosphorylated upon IGF-I treatment. Site of interaction with IRS-1.

S307, S323, S574: serine residues phosphorylated by PKC-δ.

YMXM: when the tyrosine (Y) within this motif becomes phosphorylated it interacts with the SH2 domain of p85.

YVNI: when the Y within this motif becomes phosphorylated it interacts with the SH2 domain of Grb2.

activated IGF-IR [19]. This phosphorylation event then facilitates the association of SHC with the Grb2/Sos complex, resulting in Ras activation, and subsequent activation of the MAPK pathway. There are several studies suggesting that the survival and differentiating functions of the IGF-IR are mediated through the activation of MAPK pathway via SHC [48, 49]. However mutation of Y950 suggests that other pathways can take part in MAPK activation since cells expressing these mutant receptors still have detectable MAPK activity [50]. In addition, MAPK activation may be only one of the downstream functions of SHC, since this adapter can also be tyrosine phosphorylated by a number of non-RTK receptors, including the T-cell receptor [51], granulocyte colony-stimulating factor receptor [52], and the polyoma middle T antigen [53]. In support of this hypothesis, SHC has been shown to activate c-Myc by a yet unidentified mechanism [54].

1.2.3.4 IRS-1:

a - Structure and Function: IRS-1 is a member of the insulin receptor substrate family of proteins (IRS 1-4) containing the following modular domains: N-terminal PH and PTB domains and C-terminal tails of variable lengths containing putative tyrosine and serine phosphorylation sites [55] (Figure 1.3). Despite their structural similarity, the family members can differ in their tissue distribution, subcellular localization, and recruitment of SH2 domain-containing proteins, thus impacting the specific functions of each family member [56-58]. Specifically, IRS-1 has 21 potential tyrosine phosphorylation sites within its C-terminus, many of which are located within specific motifs that can interact with SH2 domain-containing proteins when phosphorylated. It is this phosphorylation event that enables IRS-1 to interact with such proteins as the p85 subunit of

phosphoinositide 3-kinase (PI 3-kinase), Grb2, and the SH2-containing phosphotyrosine phosphatase-2 (SHP-2) thus functioning as a critical scaffolding protein between the activated IGF-IR and various downstream signaling cascades [59, 60].

Using a variety of cellular models the function of IRS-1 has been studied extensively *in vitro* and found to regulate several cellular processes (reviewed in [61]). It is well established that IRS-1 plays a predominant role in mediating mitogenesis, while other IRS family members are less involved in growth regulation [62-66]. Several groups agree that the main pathway controlling cellular proliferation/survival downstream of the IGF-IR/IRS-1 interaction is the PI 3-kinase/AKT pathway [49, 50, 67-69], however, given that signaling pathways are not linear, a role of the MAPK pathway in IGF-I-stimulated mitogenesis cannot be excluded [49, 70, 71]. Perhaps the most impressive study showing that altering the levels of IRS-1 can affect the cellular response to IGF-I stimulation was done using 32D-IGF-IR cells that lack IRS-1 [72]. Briefly, these cells normally differentiate in response to IGF-I, however when transfected to overexpress IRS-1 these cells adopt a proliferative program that overrides IGF-I-stimulated differentiation. In fact, these cells can grow indefinitely, and when injected into nude mice, form tumors. The proliferative response of 32D-IGF-IR/IRS-1 cells was accompanied by a sustained increase in PI 3-kinase/AKT/$p70^{S6K}$ activity supporting an essential role of this pathway in cell growth and survival. Incidentally, this effect was not observed when cells were forced to overexpress SHC, indicating that in addition to the signaling specificity incurred by the functional domains of the IGF-IR, further specificity occurs at the level of its immediate downstream signaling substrates.

A question that remained unanswered from the *in vitro* models of IRS-1 function was whether all mitogenic signals emanating from the IGF-IR required the presence of IRS-1. In an attempt to answer this question, two independent groups generated the IRS-1 knockout mice (IRS-$1^{-/-}$). These mice were born but had slowed embryonal and postnatal growth as well as resistance to the glucose-lowering effects of insulin and IGF-I, revealing the importance of this substrate for normal growth and glucose metabolism [73, 74]. In general the IRS-$1^{-/-}$ mice had milder phenotypes than IGF-$IR^{-/-}$ mice, suggesting that not all signaling downstream from the IGF-IR requires IRS-1. Indeed, initial studies characterizing signaling in IRS-$1^{-/-}$ mice showed that 1) the MAPK pathway was not significantly affected [73] perhaps due to the compensatory action of SHC and 2) PI 3-kinase activity remained high due to the ability of IRS-2 to co-precipitate with the p85 subunit of PI 3-kinase [74]. More detailed studies of the individual organs and tissues from IRS-$1^{-/-}$ mice demonstrated that the function of IRS-1 in mediating the mitogenic effects of IGF-I depends on the organ examined [75]. Overall, the results generated from *in vitro* and *in vivo* studies to date suggest that some functional redundancy exists among IRS family members and that IRS-1 has a predominant role in regulating growth.

b – Regulation of IRS-1 Activity: Given the importance of IRS-1 in proliferation and transformation, one can envision that uncontrolled signaling through this substrate might lead to uncontrolled cell growth, and so cells must have mechanisms in place for the tight regulation of IRS-1 signaling. Altering the steady-state phosphorylation of IRS-1 or the amount of IRS-1 present can alter its signaling. In order for IRS-1 to propagate the IGF signal downstream of the activated IGF-IR, it must remain phosphorylated on specific

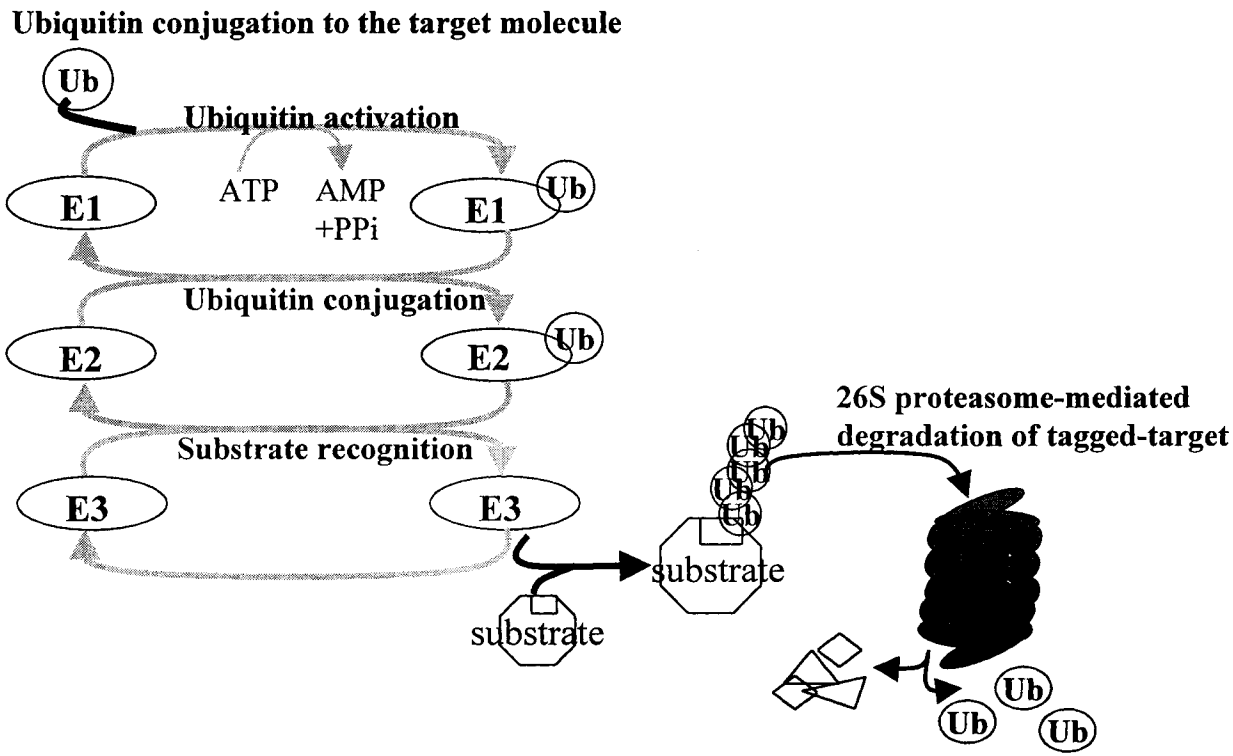

Figure1. 4 – Description of the ubiquitin-proteasome pathway.

E1: ubiquitin activating enzyme, E2: ubiquitin conjugating enzyme,

E3: ubiquitin ligase, Ub: ubiquitin

tyrosine residues within the C-terminal domain. One mechanism negatively regulating IRS-1 activity involves its dephosphorylation by protein tyrosine phosphatases, such as protein-tyrosine phosphatase-1B and SHP-2, which have been shown to dephosphorylate IRS-1, suppress its association with Grb2, and inhibit MAPK activation [76, 77].

In addition to the tyrosine residues located in the C-terminal domain of IRS-1, there are more than 30 serine/threonine sites throughout the protein that can become phosphorylated in response to a variety of stimuli. Many of the studies examining the serine phosphorylation of IRS-1 have linked this modification to insulin resistance [78], since in this state IRS-1's ability to propagate mitogenic signals downstream is hindered by at least two known mechanisms: 1) its tyrosine phosphorylation is abrogated and 2) it is degraded via the ubiquitin-proteasome pathway. Several studies have shown that in the first option, serine phosphorylation of IRS-1 reduces its interaction with the insulin receptor and IGF-IR [79]. In the second option, numerous serine/threonine kinases, glycogen synthase kinase 3 (GSK3) [80], protein kinase C (PKC) [81, 82], PI 3-kinase [83, 84], c-jun N-terminal kinase (JNK) [85] , mammalian target of rapamycin (mTOR) [86], when activated can phosphorylate IRS-1 on serines, and in most cases this is correlated with the subsequent degradation of IRS-1. It is generally well accepted that phosphorylation of proteins can result in their subsequent recognition by the ubiquitin-proteasome machinery. Since proteasome inhibitors are able to rescue the degradation of IRS-1, this has given rise to the model that serine phosphorylation provides the signal for subsequent tagging of IRS-1 by ubiquitin. The classical pathway used for the proteasomal degradation of proteins requires prior binding of ubiquitin to the target protein via three successive reactions (Figure 1.4). First, the ubiquitin-activating enzyme,

E1, activates ubiquitin in an ATP-dependant reaction. Secondly, the ubiquitin molecule is transferred from the E1 to the ubiquitin conjugating enzyme, E2. Thirdly, ubiquitin is transferred from the E2 to the ubiquitin ligase, E3, some of which recognize substrates in a phosphorylation dependent manner [87]. The formation of a polyubiquitin chain on the target protein is recognized by the 26S proteasomal machinery where the protein is degraded into small peptides and the ubiquitin molecules are recycled [88]. Several groups have linked insulin and IGF-I intracellular signaling to the ubiquitin system [89-92]. For example, longterm exposure of cells to IGF-I induces PI 3-kinase activity and this activation correlates with reduced IRS-1 protein levels [90]. Using chemical inhibitors of PI 3-kinase, as well as inhibitors of the proteasome, the levels of IRS-1 could be rescued in the presence of IGF-I.

The effects of numerous agents (including hormones, growth factors, and cytokines) on growth, differentiation, and apoptosis have been associated with changes in IRS-1 expression at the level of transcription and/or proteolysis. Agents stimulating the proliferation of breast cancer cells such as 17-β-estradiol and the phytoestrogen genistein enhance IGF-I signaling by increasing the mRNA and protein levels of IRS-1 [93-96], while compounds that inhibit cell growth (anti-estrogens) or induce apoptosis decrease IRS-1 mRNA and/or protein levels [97-99]. Taken together, the regulation of IRS-1 levels can directly influence cellular growth rates, and thus further defining the mechanisms involved in the phosphorylation, degradation and subsequent silencing of IRS-1 signaling should prove to be directly relevant to our understanding of cancer cell growth since this component of the IGF pathway is frequently altered in cancer. This will allow the development of more targeted therapeutic strategies that impair the

proliferative and/or anti-apoptotic activities that are mediated by this branch of IGF-IR signaling.

1.2.4 - Linking the Cell Surface and Nucleus - MAPK and PI 3-kinase/AKT:

In response to IGF-I stimulation of cells, the activation of the IGF-1R is associated with the activation of two highly conserved signal transduction cascades: the MAPK and PI 3-kinase/AKT signaling pathways, these in turn, regulate the activity of nuclear transcription factors and alter IGF target gene expression.

1.2.4.1 MAPK:

The MAP kinases are a family of serine-threonine kinases with essential roles in regulating cell proliferation, differentiation, and apoptosis. Three main categories of MAP kinases exist: extracellular signal-regulated kinase (erk-1 and -2 with molecular weights of 42 and 44 kDa, respectively), JNK, and p38 MAP kinase [100]. Erk1/2 are generally stimulated by growth-related signals, whereas the JNK and p38 MAP kinase pathways are activated in response to cellular stress. Hyper-activated MAPK signaling is frequently observed in breast cancer, as determined by the increased phosphorylation of erk1/2 in malignant versus nonmalignant breast epithelial cells and tissues [71, 101, 102]. In response to IGF-I stimulation, SHC interacts with the Grb2/Sos complex, resulting in the activation of the Ras-Raf-Erk1/2 pathway that ultimately induces cell differentiation and migration [72]. Although IRS-1 also associates with the Grb2/Sos complex and induces MAPK activation, it is generally believed that the main pathway activated by IRS-1, at least in IGF-I stimulated breast cancer cells, is the PI 3-kinase signal transduction cascade.

1.2.4.2 PI 3-kinase/AKT:

PI 3-kinase is a lipid kinase composed of a catalytic subunit of 110 kDa (p110) and a regulatory subunit of 85 kDa (p85). When phosphorylated, two of the nine YMXM motifs within the C-terminal tail of IRS-1 interact with the SH2 domains of p85 and this in turn results in the activation of PI 3-kinase [55]. Activated PI 3-kinase phosphorylates the D3 position of phosphoinositides to generate PI-3,4-P_2 and PI-3,4,5-P_3, which recruit the serine/threonine kinase AKT, via its PH domain, to the cell membrane. At the cell membrane AKT becomes activated by PDK1 and regulates a number of cellular processes including cell death, cell cycle progression, and protein synthesis. AKT suppresses cell death by phosphorylating and inhibiting the activity of the forkhead family of transcription factors (FKHR and FKHRL1), which normally induce the transcription of pro-apoptotic target genes such as FAS ligand, p27, and IGFBP1 [103]. A role for AKT in cell cycle progression can be mediated via mechanisms including: 1) stabilizing cyclin D1 levels through the phosphorylation and inactivation of GSK3, and 2) destabilizing p27 levels by increasing the levels of Skp2 (reviewed in [104]). Finally, AKT also promotes protein synthesis by phosphorylating and activating the serine/threonine kinase mTOR [105]. The PI 3-kinase pathway is frequently hyperactivated in breast cancer causing the overproduction of phosphoinositides and increased AKT activity. Although the tumor suppressor gene PTEN normally negatively regulates the activation of AKT, it is often mutated in breast cancer and results in the constitutive activation of AKT seen in more aggressive breast tumors and cancer cell lines. For these reasons, AKT is a potential therapeutic target in breast cancer, as its

inhibition would block the proliferative and anti-apoptotic signals emanating from the activated PI 3-kinase.

1.2.5 - IGF Signaling in Breast Cancer:

Having introduced the IGF signal transduction system in the previous section, we can envision how the deregulation of various components of the signaling cascade could potentially be involved in carcinogenesis. Initial studies with IGFs *in vitro* showed that these growth factors are potent mitogens of normal and malignant cells in a variety of cell models. Researchers at McGill and Harvard universities extended these laboratory results to patients in a landmark study showing a tight correlation between elevated levels of circulating IGF-I and an increased incidence of prostate cancer [106]. The study sparked a surge of interest into the role of IGFs in cancer and has been since supported by a series of epidemiologic studies. This section will give a brief history of the specific components of the IGF signaling cascade that are often deregulated in breast cancer.

1.2.5.1 IGFs and IGFBPs:

Although it is clear that IGFs are essential for normal mammary gland development, past findings also support a role of IGFs in breast tumorigenesis (reviewed in [107]). Twenty years ago, when IGF-I was still referred to as somatomedin C, it was shown that this growth factor, acting via binding to the IGF-IR, had mitogenic effects on a variety of human breast cancer cells [108, 109]. Similarly, the impact of IGF-II in breast carcinogenesis is supported by studies showing that its expression is mainly in breast stromal tissue, thus it can act in a paracrine manner to stimulate the growth of malignant epithelia, and its expression is often higher in tumors with a more metastatic phenotype [110, 111]. IGFs can potentiate their mitogenic effects on breast cancer cells by

synergizing with other growth promoting factors, in particular 17-β-estradiol [112-114]. Consistent with these *in vitro* studies, high circulating levels of IGF-I in women have been associated with increased risk of breast cancer [115, 116]. Furthermore, a recent study shows that intravenous injection of mice with IGF-I leads to increased activation of IGF-IR downstream signaling events, such as increased IRS-1 activation and enhanced AKT and MAPK activity in normal and tumorous mammary tissue [117]. The mitogenic effects of IGF-I can be altered by the IGFBPs; specifically IGFBP-3 can inhibit breast cancer cell growth, is pro-apoptotic, and is induced by a number of cytostatic agents [118]. These *in vitro* data has led to the theory that IGFBP-3 is a putative tumor suppressor, however, in recent years this has been challenged by *in vivo* studies. Forced expression of IGFBP-3 in T47-D cells was associated with enhanced tumor growth *in vivo* [119] and high IGFBP-3 levels in breast cancer tissue is associated with a more malignant phenotype [6]. Furthermore, unlike the clear relationship existing between high serum IGF-I levels and breast cancer risk, the *in vivo* data in support of a relationship between low IGFBP-3 levels and increased cancer risk has not been consistently observed.

1.2.5.2 IGF-IR:

As is the case with many other receptor tyrosine kinases, aberrant signaling through the IGF-IR is a common oncogenic event in breast cancer. The key studies cementing a role of IGF-IR in malignancy were made over a decade ago when cells lacking the IGF-IR could not be transformed by viral and cellular oncogenes [120, 121]. In breast cancer, malignant epithelial cells and primary tumors generally overexpress the IGF-IR as compared to normal cells and benign tumors [18]. The overexpression of the receptor

has been correlated with an increased sensitivity to IGF-I and an increased ability to grow in serum-free media. Consistent with this, the kinase activity of the receptor is also significantly higher in primary breast tumor tissue [122]. The importance of this receptor in breast cancer is often demonstrated by studies showing that inhibiting binding of IGF-I to the IGF-IR inhibits breast cancer cell proliferation and, conversely, that activating the IGF-IR protects cells from death induced by radiation and anti-cancer agents [3, 123].

1.2.5.3 IRS-1:

In recent years, researchers in the IGF field have attempted to clarify the selective signaling pathways that become deregulated downstream of the IGF-IR. Several studies have shown that the amount and/or activity of adaptor proteins downstream of receptor tyrosine kinases may also be important factors in carcinogenesis. The function of IRS-1 in regulating cell growth was clear from the growth retardation observed in IRS-1 knockout mice [73], and the decreased cell proliferation of mouse embryonic fibroblasts isolated from these knockout mice [124]. In ER+ breast cancer cell lines (MCF-7, ZR75.1 and T47-D), IRS-1 is the main substrate recruited to the IGF-IR, resulting in the activation of downstream signaling cascades and ultimately in cell proliferation [70]. As stated above, estradiol synergizes with IGF-I to stimulate breast cancer cell proliferation. To achieve this synergistic effect, it is thought that estradiol regulates numerous components of the IGF system. Apart from up-regulating the levels of the IGF-IR [113], estradiol increases the expression of IRS-1 in *in vitro* and *in vivo* models of breast cancer [94]. Consistent with this synergy is the observed estrogen independence and prolonged cell survival under serum free media conditions when MCF-7 cells are transfected to overexpress IRS-1 [125]. Several studies support an essential role of IRS-1 in breast

cancer cell proliferation and survival. Decreasing the abundance of IRS-1 in breast cancer cells by antisense strategies inhibits cell growth, while augmenting the levels potentiates cell growth and induces transformation [125, 126]. *In vivo* high IRS-1 levels are associated with increased tumor size and recurrence of the disease [6, 94], and the signaling of this substrate is frequently hyperactivated in several tumor tissues including primary breast tumors [127].

1.2.6 - Therapeutic Intervention:

Collectively, the studies *in vitro*, in animals, and in humans strongly implicate the IGF system in breast tumorigenesis, and thus provide a clear rationale for future studies aimed at inhibiting IGF-mediated signaling in breast cancer. Some of the strategies employed to date, specifically in breast cancer, will be summarized in the following section.

1.2.6.1 Antisense Strategies:

Several components of growth factor receptor pathways are often overexpressed in cancer, thus an obvious strategy to inhibit their function is to decrease their expression. It is possible to interfere with target gene expression by using antisense oligonucleotides, and several members of the IGF system have been downregulated using this method. The role of IGF-IR in cell growth and transformation has made this receptor the center of such therapeutic strategies aimed at inhibiting malignant growth [128]. Antisense RNA to IGF-IR decreases IGF-I-induced proliferation of MCF-7 cells [129] and reduces the growth and transformation of the more aggressive breast cancer cell line MDA-MB-435 [130]. In addition, there is a significant reduction in tumor growth when mammary carcinoma cells expressing antisense IGF-IR RNA are injected into syngeneic mice [131] or when antisense oligodeoxynucleotides to IGF-IR are injected directly into already

established mammary tumors [132]. Since tumor growth can not be completely abrogated by downregulating IGF-IR in this manner, this has given rise to a need for targeting other components of the IGF system. The expression of IGF-IR downstream substrates has been the target of such antisense approaches to regulate breast tumorigenesis. Downregulating IRS-1 results in an inhibition of anchorage-independent and -dependent breast cancer cell growth and an induction of apoptosis under serum free or IGF-I-stimulated culture conditions [126, 133]. Likewise, decreasing IRS-2 using antisense constructs results in a dampening of the metastatic potential of the MDA-MB-231 breast cancer cell line via mechanisms involving reduced cell motility, adhesion, and anchorage-independent growth [134]. MCF-7 cells expressing antisense RNA to SHC show reduced cell motility in addition to defects in cell-cell interactions [126]. Thus far, no one has determined the efficacy of targeting these substrates using antisense in animal models. Clinically, the usefulness of such antisense strategies will likely depend on the development of efficient and site-specific delivery of antisense approaches to the tumor tissue.

1.2.6.2 Dominant-negative Strategies:

Several dominant-negative IGF-IR mutants have been shown to block the many functions of the IGF-IR *in vitro*, albeit with less effective results *in vivo* (reviewed in [135]). One dominant-negative strategy that has shown the most consistent anti-tumor effects *in vitro* and *in vivo* has been the mutant IGF-IR (486/STOP) [136, 137]. The 486/STOP mutant does not contain a transmembrane domain, and thus can be secreted from cells where it exerts its dominant-negative activity by competing with the wild-type IGF-IR for available IGFs. Until recently this was thought to be the mechanism of action of the

486/STOP, however, more recent data suggests that this mutant receptor can exist intracellularly where it interacts with the wild-type receptor to send a pro-apoptotic signal [138]. Since IRS-1 is constitutively active in breast tumors, the dominant-negative strategy has also been employed to decrease its function. Expression of a dominant negative IRS-1 in breast cancer cells inhibited cellular proliferation and transformation, thus highlighting the significance of this constitutive activity in breast tumorigenesis [127].

1.2.6.3 Blocking IGF-IR Activation:

With the promise of targeted therapies, several groups have tried developing human monoclonal antibodies to RTKs to abrogate the interaction between ligand and growth factor receptor, thus inhibiting receptor activation and subsequent downstream signaling. In breast cancer the well known success story is the monoclonal antibody to the Her-2/neu receptor trastuzumab (Herceptin) [139]. Herceptin appears to be one of the most effective treatments of women whose breast tumors overexpress the Her-2/neu protein. However, well before the development of Herceptin, Rohlik *et al.* [140] and Arteaga *et al.* [141] explored the potential use of a monoclonal antibody-based therapy directed against the IGF-IR protein. These two groups showed that the monoclonal anti-IGF-IR antibody, α-IR3 could inhibit the binding of IGF-I to the IGF-IR and was effective at inhibiting the *in vitro* and *in vivo* growth of some breast cancer cells. More recently α-IR3 was shown to induce degradation of the IGF-IR in MCF-7 cells and inhibit growth in soft agar [142]. The current drawback to employing monoclonal antibodies to IGF-IR *in vivo* is predominantly the cross reactivity toward the insulin receptor.

1.2.6.4 Small Molecule Therapy:

Several groups have tried blocking IGF-IR activation by using small molecule inhibitors that target the ATP- or substrate-binding sites within the kinase domain of the receptor. Such a strategy has received recent attention in lung cancer where the EGFR tyrosine kinase inhibitor Iressa seems to be therapeutically beneficial to patients harbour activating mutations in the kinase domain of the EGFR [143, 144]. Although initial studies with these small molecules showed that they were not selective for the IGF-IR [145, 146], recently two groups have shown that small molecule kinase inhibitors can be used to selectively inhibit IGF-IR signaling *in vitro* and inhibit tumor growth *in vivo* [147, 148].

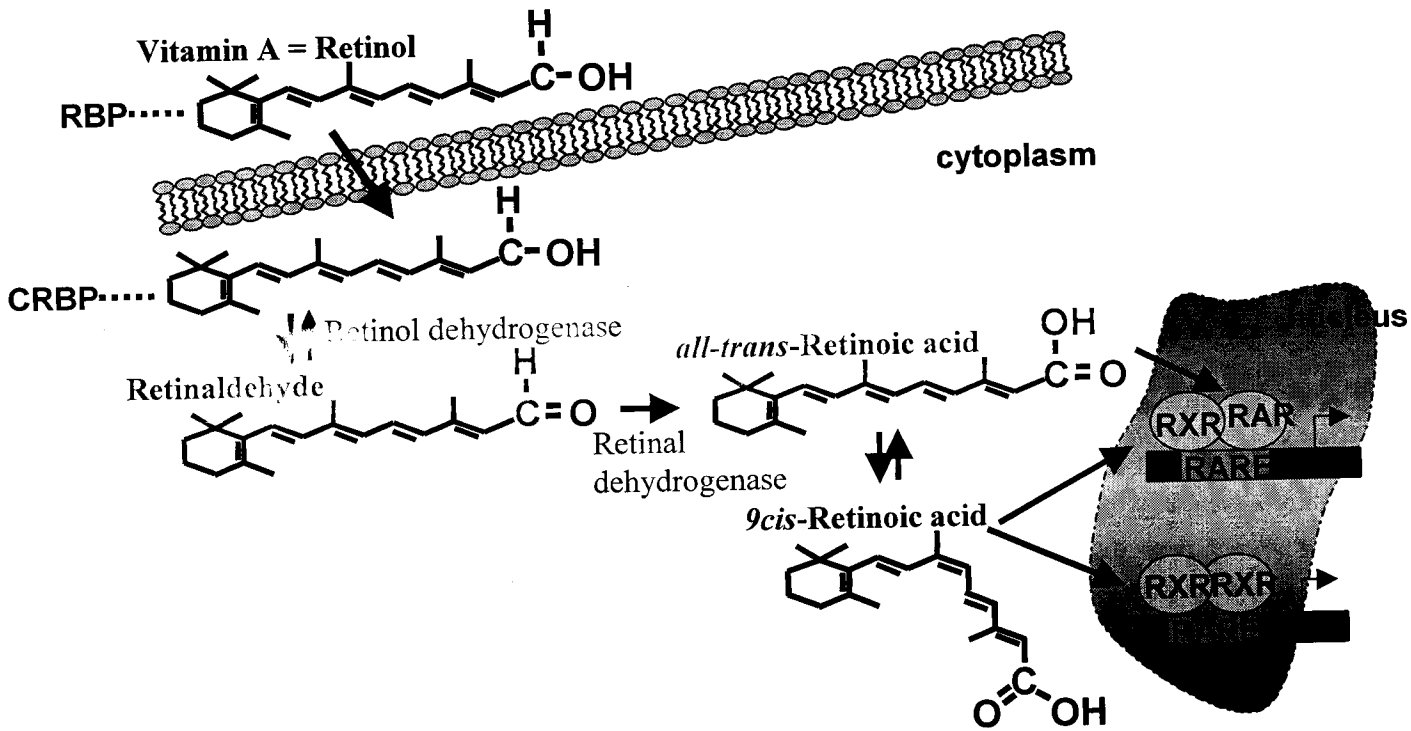

Figure1.5 – Biosynthesis of retinoic acid

RBP: retinol binding protein, CRBP: cellular retinol binding protein

1.3 - THE RETINOIDS

1.3.1 - Retinoids and their Nuclear Receptors:

From the initial studies showing that abnormal levels of vitamin A (deficient or excessive) could destroy the integrity of epithelial tissues, this vitamin has been greatly studied for its potential as a regulator of carcinogenesis. Vitamin A (retinol), its natural metabolites, and also its synthetic analogues are collectively termed retinoids. These lipid-based compounds are involved in several critical functions during embryogenesis and in adulthood: 1) limb development, 2) growth and differentiation of epithelia and bone, 3) vision, and 4) reproduction. Retinol travels to target tissues complexed with serum retinol-binding protein (RBP) (Figure 1.5). Historically liver was used as a therapy for night blindness, and so it is of no surprise that it is the main storage depot of retinoids and is also the principal site of serum RBP synthesis. Once retinol reaches the target epithelial tissue, it enters the cell and binds cytoplasmic cellular retinoid-binding proteins (CRBP-I and -II, and CRABP-I and -II). CRBP-I is thought to promote the conversion of retinol to retinoic acid, while CRABP-I functions in retinoic acid metabolism. The most potent naturally occurring retinoid is retinoic acid, existing as stereoisomers: all-trans retinoic acid (RA) and 9-cis retinoic acid (9cRA). Retinoids can mediate a number of their effects by regulating gene expression through binding to nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each comprising three subtypes (α, β and γ), and each subtype includes different isoforms [149]. 9cRA binds with great affinity to both RAR and RXR, but RA selectively binds RAR. Upon retinoid/nuclear receptor interaction, RAR/RXR heterodimers or RXR/RXR homodimers form and these complexes then bind their cognate response elements, RARE

or RXRE respectively, located in the promoters of many retinoid target genes [150]. Although knockouts of individual RAR isoforms showed no phenotypic malformations, knockouts of entire RAR subtypes and compound knockout mice exhibited developmental abnormalities, at least in some instances, consistent with vitamin A deficiency syndrome, and these could be prevented by treating the mice with RA [151].

1.3.2 - Disruption of Retinoid Signaling in Breast Cancer:

Many investigators studying the role of retinoids in cancer have adhered to the hypothesis that proper retinoid homeostasis can safeguard against the onset of cancer. It has been shown that animals deficient in vitamin A are more susceptible to cancer-causing agents and have a higher incidence of cancer [152]. In humans, low circulating levels of retinoids also correlates with increased risk of malignancy [152]. A tight link between carcinogenesis and the disruption of retinoid action in target cells was first observed in patients with acute promyleocytic leukemia (APL). APL is characterized by the chromosomal translocation of the RAR-α locus to the PML gene, resulting in the formation of the oncogenic PML-RARα fusion protein acting as a dominant negative over the RAR-α, and causing the repression of RA target genes. Remarkably, a large percentage of patients with APL receiving RA as a chemotherapeutic undergo complete remission. At the molecular level, the response to pharmacologic doses of RA is associated with the ability of RA to 're-activate' target gene transcription, overriding the dominant negative activity of the PML-RARα oncoprotein. Further support for the role of RA in cancer has come from numerous studies showing that various components of RA signaling are often disrupted in tumorigenesis. This section will briefly describe the components that, when disrupted, can have an impact on breast carcinogenesis.

1.3.2.1 RARs and RXRs:

Although little is known of the physiological role of retinoids and their receptors in the adult breast, evidence exists for their function in normal breast cell proliferation and differentiation and in mammary duct differentiation [153-156]. Most of the RAR and RXR isoforms are expressed in breast epithelia, albeit to differing degrees [157]. Specifically, RAR-α, RXR-α, -β and -γ are expressed in ductal epithelial and myoepithelial cells, while RAR-β is detectable only in myoepithelia [158]. Since retinoids exert a number of their effects by binding to and activating retinoid receptors, it is logical to imagine that alterations in the expression and/or activity of these receptors would affect a cell's response to retinoids, result in aberrant cell growth, and ultimately cause cancer. Support for this line of thought comes from examination of retinoid receptor status in breast cancer cell lines and primary tumor samples. Several groups have reported the loss of RAR-β gene expression in malignant versus normal breast tissue [159-161]. Although RAR-β is one of the most extensively studied retinoid receptors in breast cancer, the mechanism for the lack of RAR-β present in cancerous breast tissue and its role as a putative tumor suppressor are still under debate. A common event in carcinogenesis is the methylation of tumor suppressor genes. Several groups have examined the methylation status of the RAR-β gene in breast cancer cell lines and primary breast tumors and found that it is silenced by this mechanism [162, 163]. Further evidence in support of this mechanism of RAR-β repression comes from studies in which demethylating agents such as 5-aza-2-deoxycytidine, which inhibits breast cancer cell growth, can induce RAR-β expression [164, 165]. Several studies have examined the importance of RAR-β in regulating the proliferation of breast cancer cells: 1) expression

vectors transfected into breast cancer cells sensitize them to chemotherapeutic agents, 2) expression of RAR-β retroviral vectors inhibit metastasis in a xenograft model [166], and 3) activation of RAR-β by numerous cytostatic agents correlates with their ability to growth arrest breast cancer cells [167]. Although RAR-β is a strong negative regulator of growth, the importance of other retinoid receptors in breast carcinogenesis cannot be excluded since: 1) RAR-α levels are reduced in hormone-independent breast cancers that generally exhibit more malignant phenotypes [160], 2) RXR-α is frequently overexpressed in invasive carcinoma [168], and 3) RXR-α localization is altered in RA-resistant breast cancer cells [169]. Further studies will be needed to address the extent to which these aberrations collectively impact breast tumorigenesis.

1.3.2.2 Retinoid Binding Proteins:

Retinol travels within the plasma bound to RBPs and enters the cell to interact with CRBPs and CRABPs, which aid in maintaining the homeostatic intracellular levels of retinoic acid. It can be proposed that alterations in the expression of these retinoid binding proteins would alter the intracellular level of retinoic acid and thus lead to changes in cellular proliferation. Indeed, CRBP-I was found to be silenced in malignant breast epithelial tissues and primary tumors [170, 171]. Interestingly, it appears that the mechanism behind the repression of CRBP-I in malignant breast tissue is once again methylation, and 5-aza-2-deoxycytidine can activate CRBP-I gene expression [172].

1.3.2.3 Retinoid Dehydrogenases:

The ability of the retinoic acids to regulate target gene expression in a coordinated manner depends on the tight regulation of their levels and distribution, a function that is mediated in large part through the enzymes controlling the synthesis and catabolism of

retinoids. The alcohol dehydrogenases or short-chain dehydrogenases/reductases reversibly convert retinol to retinal, while the aldehyde dehydrogenases participate in the irreversible oxidation of retinal to RA (Figure 1.5). There is increasing evidence to suggest that the effectiveness of retinoids in the treatment of breast cancer depends on the proper functioning of these catalytic reactions, and moreover that defects in RA synthesizing enzymes might have a role in tumorigenesis. Retinol is not an effective growth inhibitor of all breast cancer cell lines, and this has been hypothesized to be due to the inability of some cell lines to convert retinol to RA [173]. In support of this hypothesis, a few groups have shown that normal and malignant breast epithelial cells differ in their ability to convert retinol to RA, due to the lack of expression of specific dehydrogenases in the breast cancer cell lines [174, 175].

Future work will be needed to obtain a more complete picture of how the alterations of multiple components in the retinoid signaling pathway might lead to the development and/or progression of breast cancer.

1.3.3 - Retinoid Action *In vitro* and *In vivo*:

One of the earliest studies performed to test the growth inhibitory effects of retinol was done in a mouse fibroblast tumor cell line (L-929). When L-929 cells were treated with retinol their proliferation decreased, and this was not due to an overall cytotoxic effect of the vitamin [176]. Subsequent investigations using various cell lines, including breast, reported that retinoids were effective growth inhibitors and had great potential as chemotherapeutic agents [177]. This section will summarize the studies examining the effects of retinoids *in vitro* (i.e. breast cancer cell lines), *in vivo* (i.e. animal models of mammary carcinogenesis), and in clinical trials of breast cancer.

1.3.3.1 Retinoid Action in In Vitro Models:

a – RA: In examining the potency of various retinoids in regulating the growth of MCF-7 cells, RA was found to be the most effective of the retinoic acid stereoisomers at inducing growth arrest in the G1 phase of the cell cycle [178-180]. The response of breast cancer cell lines to RA is correlated to the presence or absence of the ER, such that RA inhibits the proliferation of ER+ (e.g. MCF-7, T47-D, ZR75.1) but not of ER- (e.g. MDA-MB-231, MDA-MB-468, BT-20) breast cancer cells [181, 182]. However this theory can be challenged by reports showing that the ER- breast cancer cell lines, SK-BR3 and Hs578T, are growth inhibited by RA [183]. This group and others have proposed that retinoid sensitivity correlates better with the expression of RAR-α than with ER expression, and this was supported by studies showing that resistance coincides with decreased RAR-α levels and that stable expression of RAR-α in RA-resistant cell lines sensitizes them to RA [183-185].

b – N-(4-hydroxyphenyl)retinamide/fenretinide/4-HPR: Although earlier studies showing the growth inhibitory effects of retinoids focused on the natural retinoids, chemists have been developing synthetic derivatives of RA, such as 4-HPR, with increased organ specificity and decreased toxicity. Unlike RA, 4-HPR does not induce cell cycle arrest but is a potent inducer of apoptosis in breast cancer cells that are sensitive or resistant to RA [186, 187]. This difference in cellular regulation may be attributed to the fact that 4-HPR regulates growth by RAR-independent mechanisms since it is a low affinity ligand for RARs [188] and only minimally activates RAREs and RXREs [189]. The pro-apoptotic effects of 4-HPR may be mediated via mechanisms involving the production of ceramide [190], or alternatively by increasing nitric oxide levels [191-193], however, a

definitive mechanism remains unknown. Another possible explanation for the increased potency of this retinoid in comparison with RA is due to its tissue-specific accumulation in the mammary gland [194].

c – Bexarotene/LGD1069: Similar to the rationale behind the development of 4-HPR, LGD1069 is a synthetic analogue of 9cRA that exhibits reduced toxicity and increased potency. Unlike 9cRA, LGD1069 preferentially binds to RXRs and thus was termed a 'rexinoid' [195]. *In vitro*, LGD1069 suppresses the growth of cells derived from N-nitroso-N-methylurea- (NMU) induced mammary tumors, and this was associated with changes in the expression of adipocyte-related genes [196]. It has been proposed that LGD1069 regulates growth by inducing terminal differentiation of tumor cells into adipocytes and subsequent cell death.

1.3.3.2 Retinoid Action in Animal Models:

To validate the studies showing the effectiveness of retinoids as chemotherapeutic agents using *in vitro* models of breast cancer, researchers quickly tested the efficacy of these compounds *in vivo* using a variety of animal models, including xenotransplanted cancers, carcinogen-induced tumors, and transgenic models of mammary carcinoma. In reviewing the literature it is clear that the *in vitro* results obtained with retinoids are not always reproducible *in vivo*. Although retinol and RA inhibited tumor growth of transplanted tumorigenic epithelial cells, the stereoisomer 13-cis-retinoic acid (13cRA), which is also known to inhibit *in vitro* cell growth showed no such effect [197-199]. Rat chemical carcinogenesis models have been quite useful in examining the effects of retinoids in tumor initiation versus tumor progression. When administered to rats, 7,12-dimethylbenz[a]anthracene (DMBA) and NMU will induce mammary tumors within a

time frame of several months. One criterion that a chemotherapeutic must comply with is minimal toxicity; unfortunately RA did not meet this criterion *in vivo* as it was found to be greatly toxic to rats at doses capable of inhibiting tumor growth [200]. Studies with 9cRA and 4-HPR have shown that these agents are less toxic and more effective in their tumor suppression than RA [201, 202]. From these studies, it seems that retinoids are more effective in tumor prevention than in tumor regression, that is, retinoids are less effective against established tumors than when they are given before or immediately after DMBA or NMU [200]. There is hope, however, with the continued development of RAR- and RXR-selective retinoids. The rexinoid LGD1069 has proven its effectiveness in the NMU model of mammary carcinogenesis by preventing tumor onset [203] and regressing existing tumors [204]. LGD1069 also has proven chemotherapeutic action in MMTV-c-Neu and C3(1)/SV40 T-antigen transgenic mice, which are both more physiologically relevant models of breast cancer [205, 206]. Interestingly, LGD1069 can also inhibit tumor progression in MMTV-c-neu transgenics that fail to respond to the well known breast cancer therapeutic Tamoxifen [207]. Other retinoids such as 9cRA have also been shown to be effective in suppressing tumor development in the C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer [208]. Of interest is that the tumors formed in these transgenic mice are hormone-independent [209], and the ability of retinoids/rexinoids to inhibit the onset of tumors in these mice suggests that these agents could be used in breast tumors that have lost the ER and consequently would be anti-estrogen insensitive and highly metastatic. Taken as a whole, the examination of retinoids in experimental animals has lead to the proposal that these agents are effective in blocking the onset of breast cancer and inhibiting the progression of existing breast

tumors. For this reason, retinoids/rexinoids made a relatively swift transition into Phase I clinical trials of breast cancer.

1.3.3.3 Retinoid Action in Clinical Trials:

For several decades retinoids have been examined in clinical trials for the treatment of malignant and nonmalignant disease. The success of RA in the treatment of APL led to an overall excitement by oncologists and researchers alike to determine the effects of RA in patients with solid tumors. It should also be mentioned that the promising effects of 13cRA in the prevention of second primary tumors in patients with head and neck squamous cell carcinoma further sparked an interest in examining the effectiveness of RA in patients with breast cancer [210, 211]. The first phase II clinical trial employing RA in the treatment of metastatic breast cancer was in 1997 [212]. Despite the wealth of data supporting the role of RA in suppressing carcinogenesis, this trial revealed that at tolerable doses, RA was not effective in reducing the size of already established tumors. Perhaps this first trial with RA in breast cancer was doomed from the beginning since the data obtained from retinoid use in animal experiments had already suggested that perhaps retinoids, including RA [200], are more effective as chemopreventive agents than chemotherapeutics. In addition, toxicity issues dampened the hope of using RA as a preventive agent in breast cancer, since the dose of RA employed in the phase II clinical trial would not serve as a tolerable dose if used in chemoprevention [212]. In keeping with the idea that retinoids may be useful in chemoprevention, treatment of C3(1)/SV40 T-antigen transgenic mice with 9cRA, prior to the appearance of tumors, resulted in a significant delay in the development and multiplicity of tumors [208]. Although 13cRA made it to a Phase II trial in metastatic breast cancer, the unfortunate reality was that it

too had no effect on tumor regression and caused severe side effects [213]. Although the use of these 'retinoic acids' in clinical trials has been limited by their adverse side effects and inability to reduce tumor size, recently several studies have assessed the outcome of combining them with other chemotherapeutic agents (e.g. 9cisRA+Tamoxifen [214]) in an attempt to find an adequate dose (low toxicity/high potency) of retinoid that can be used in chemoprevention.

A major interest in the use of the synthetic retinoid 4-HPR in breast cancer came from early studies in rats showing that it was far superior as a chemopreventive agent than the 'retinoic acids'. These studies agreed that 4-HPR was less toxic [215], more potent, and accumulated in breast tissue [216]. Data from a phase II and III clinical trials of 4-HPR in breast cancer supported these initial findings in rat models of breast carcinogenesis, with increased tolerability and breast tissue specificity [194, 217]. Although 4-HPR was not found to decrease tumor progression, these trials provided data in support of the use of the synthetic retinoid in the chemoprevention of second breast cancers, but only in premenopausal women (reviewed in [218]). Clinical trials are ongoing to determine the effectiveness of combining 4-HPR with the anti-estrogen Tamoxifen, and to date suggest that this combination is promising only for the treatment of ER+ tumors [219]. A variety of agents are being studied to examine whether they can potentiate the actions of 4-HPR in ER+ and ER- tumors. Based on the current understanding of the mechanism of action of 4-HPR, some of the agents proposed for use in combination with 4-HPR include: 1) modulators of ceramide-related pathways [220], 2) modulators of nitric oxide production [221, 222].

The strong pre-clinical data, especially the impressive results showing almost complete regression of tumors in the NMU-model of breast tumorigenesis [204], provided great optimism for the use of bexarotene in metastatic breast cancer. Unfortunately, the wealth of positive data in animal experiments did not translate into similar positive anti-tumor effects in patients participating in a phase II clinical trial of bexarotene with metastatic breast cancer [223]. Given its proposed mechanism of action, future studies using bexarotene will surely assess its effectiveness in combination with agents that induce terminal differentiation of breast epithelial cells [196].

Although the data to date point to negligible effects of retinoids in established breast tumors, there is still optimism in these agents being used in chemoprevention alone or in combination with other agents. The development of more effective treatment strategies using retinoids in breast cancer will rely on our ability to first understand the molecular events regulated by compounds.

1.3.4 - Mechanisms of Retinoid-mediated Regulation of Breast Cancer Cell Growth:

Several cellular events contribute to the development and progression of breast cancer, and retinoids have been shown to block carcinogenesis by regulating many aspects of these events at the molecular level. Some of the mechanisms proposed for the anti-proliferative effects of RA will be discussed in the following section.

1.3.4.1 Transcriptional Regulation of Growth Inhibitory Genes:

It is within the nucleus where RA inhibits breast cancer cell growth through a mechanism involving the regulation of target gene expression. The RAR/RXR heterodimers function as RA-inducible transcription factors when bound to RAREs within the promoter of target genes. In the absence of RA these receptor dimers recruit co-repressors that inhibit

transcription by recruiting histone deacetylase complexes (HDACs). HDACs deacetylate histones resulting in a closed chromatin conformation such that the DNA is not accessible to the transcriptional machinery. The binding of RA causes a conformational change in the ligand-binding domain of the RAR that allows the release of co-repressors and subsequent recruitment of co-activators to the AF-2 region of the receptor. Some co-activators possess histone acetyl transferase activity that acetylates histones, resulting in a more open chromatin conformation that ultimately results in transcriptional activation [150]. RA-induced growth arrest of breast cancer cells has been associated with the upregulation of multiple genes with putative tumor suppressor function including; IGFBP-3 [224], SOX-9 [225], RAR-β [226], and ubiquitin-activating enzyme-E1-like (UBE1L) (del Rincón *et al.* unpublished results). A common feature of these genes is that their forced expression in breast cancer cell lines results in cell cycle changes similar to those changes observed when these cells are treated with RA alone, although the underlying mechanisms in this process remain unknown. To identify changes in gene expression occurring when MCF-7 cells growth arrest in response to treatment with RA, Dokmanovic and co-workers performed a cDNA microarray [227]. Of the thirteen genes strongly induced by RA, some are known to have roles in regulating cell growth, while others are functionally related to the ubiquitin-proteasome pathway. Since recent studies have elucidated that RA treatment of various cellular models results in the posttranslational modification of a growing number of proteins, it is of potentially great importance to our understanding of the mechanism of retinoid-mediated growth inhibition to consider that RA regulates genes whose products function in proteasomal degradation. Although the identification of RA target genes has shed some light into the

mechanism of RA-mediated growth inhibition, direct transcriptional targets of RA are usually regulated within 12 hours of treatment, while growth inhibition normally arises only after 72 hours. This suggests that mechanisms other than direct transcriptional activation of RA target genes are required for the growth inhibition of breast cancer cells. Therefore, future work should be aimed at: 1) identifying the downstream events regulated by the proteins encoded by these RA-target genes so as to better define the functional relationship of the multiple genes regulated by RA, and 2) identifying the additional mechanisms regulated by RA that are essential for the growth arrest of cancer cells.

1.3.4.2 Post-translational Modification of Proteins:

Although the past two decades have focused on examining the transcriptional mechanisms linked to the pleiotropic effects of RA, the importance of posttranslational modification of various proteins, namely ubiquitination, has been recently highlighted. The proteasomal degradation of cyclins and cyclin-dependent kinase inhibitors (CKIs) (e.g. p27) is crucial for the proper functioning of the cell cycle. If the proteasomal machinery malfunctions, this could lead to the altered expression of regulatory proteins that enable cells to proceed through the G1/S phase in an untimely manner, thus promoting tumorigenesis. This is supported by studies showing that cyclins are frequently overexpressed in human malignancies [228], including breast cancer [229]. Further highlighting the essential role of the ubiquitin-proteasome pathway in regulating the levels of cell cycle regulatory proteins are the recent clinical trials using proteasome inhibitors as a chemotherapeutics [230, 231]. One could postulate that a chemotherapeutic that induces G1 arrest may do so by regulating the levels of cyclins and

CKIs. This seems to be true of RA, since it promotes the ubiquitination of cyclin D1 [232] and cyclin E [233], and also stabilizes p27 levels through the ubiquitination of Skp2 [234, 235]. In addition, the anti-proliferative and differentiating effects of RA have been associated with its ability to ubiquitinate a growing list of proteins, including, p300 [236], cyclin dependant kinase 4 (CDK4) [237], RAR [238, 239], and IRS-1 [240]. The sequence of events leading to the ubiquitination of these proteins in cells treated with RA remains largely unknown. It could be hypothesized that perhaps RA regulates protein ubiquitination by first regulating the expression of a component(s) of the ubiquitin-proteasome pathway. One such candidate gene is UBE1L which is involved in the RA-mediated degradation of PML/RARα [241]. Future work is clearly needed to explore at which step in the ubiquitin-proteasome pathway RA is acting.

1.3.4.3 Modulation of Signal Transduction Pathways:

Breast tumors are often characterized by hyperactivated RTK signaling that can be further potentiated by the actions of steroid hormones. A well studied example of this is the increased IGF-I produced in breast cancer cells treated with estradiol, which in turn activates the ER. In addition, estradiol also induces the expression of IGF-IR, and synergizes with IGFs to stimulate cell proliferation and survival, probably via the upregulation of IRS-1 [94, 95]. This has given rise to the concept of cross-talk between the steroid hormone pathways and mitogenic signaling pathways including the: EGFR, Her2/neu receptor, and IGF-IR [242-244]. Agents used in the treatment of breast cancer, retinoids and anti-estrogens, have been shown not only to inhibit estrogen-induced growth and block ER-mediated transcription, but also to abrogate the growth promoting effects of mitogens such as EGF and IGF [245-248]. Given what we know about the

cross-talk between the IGF and ER pathways, it is reasonable to propose that RA antagonizes IGF-I- and estrogen-stimulated growth by interfering with some component(s) of the signaling cascades initiated by these mitogens. In support of this hypothesis, a number of studies have shown considerable cross-talk between the retinoid and IGF pathways:

a – IGFBP-3: It was found in the early 90's that the anti-proliferative effects of RA were associated with the increased production of IGFBP-3 in breast cancer cells [245]. This initial report was supported by studies showing that if the induction was blocked using antisense oligodeoxynucleotides to IGFBP-3 or IGFBP-3 blocking antibodies the anti-proliferative effects of RA were lost [224, 249]. Since it had been reported that high serum IGFBP-3 levels reduces cancer risk as predicted by high IGF-I levels [115], researchers wondered what the circulating IGFBP-3 levels were in patients undergoing treatment regimes with retinoids. Interestingly, breast cancer patients receiving 4-HPR treatment had reduced plasma IGF-I levels and increased serum IGFBP-3 levels [250, 251]. In further support of a role of retinoids in regulating IGFBP-3 levels, a positive correlation between IGFBP-3 plasma levels and serum retinol levels was found in individuals participating in a Physicians' Health Study [252]. More recently, IGFBP-3 has been implicated in mediating the resistance of breast cancer cells to the anti-proliferative effects of RA. The latter builds on initial observations that IGFBP-3 interacts with RXRα, antagonizes the effects of RA on an RARE, and synergizes with rexinoids to induce apoptosis in prostate cancer cells [253]. In their study Schedlich and co-workers test the hypothesis that MDA-MB-231 cells are resistant to RA because they

have constitutively high levels of IGFBP-3, which can sequester RXR or RAR and thus hinder the ability of RA to activate RARE-mediated transcription [118].

b – AP-1: The transcription factor activating protein-1 (AP-1) is a heterodimer of the proteins Jun and Fos, functioning as a link between extracellular signals and the transcription of genes associated with cell growth. IGF-I-stimulated mitogenesis is associated with the induction of the c-fos gene, as exemplified by the observed decrease in c-fos expression and inhibition of breast cancer cell growth when IGF-IR levels are reduced using antisense [129]. Some of the anti-proliferative effects of retinoids are attributed to the inhibition of AP-1 activity by a yet undefined mechanism. It has been shown that RA-mediated inhibition of IGF-stimulated breast cancer cell growth is associated with blocking IGF-stimulated c-fos expression, that would presumably lead to a decrease in the transcription of genes involved in cell proliferation [248]. Interestingly, the RA-mediated inhibition of AP-1 activity may only depend on the action of RARα and RARγ since 1) RARβ represses AP-1 activity in an RA-independent manner [254] and 2) c-jun overexpression in MCF-7 cells causes RA resistance with a concomitant decline in RARα and RARγ levels [255].

c - IGF-IIR: The IGF-IIR is thought to have tumor suppressive function in part through its ability to regulate circulating IGF-II levels. This receptor is often absent in breast tumors due to a loss of heterozygosity and this has been linked to the development of breast cancer [256]. In support of a role of this receptor in breast tumorigenesis, MCF-7 cells that have been manipulated to express lower levels of IGF-IIR have an increased proliferation rate and are more resistant to apoptosis [257], while the overexpression of IGF-IIR in the MDA-MB-231 breast cancer cell line renders these cells less tumorigenic

in vitro and *in vivo* [258]. The IGF-IIR has been shown to bind RA with high affinity and one functional consequence of this interaction is an increase in internalization of IGF-II [30]. It has been hypothesized that the IGF-IIR mediates the anti-proliferative effects of RA in neonatal-rat cardiac fibroblasts [259]. To determine if the IGF-IIR is indeed involved in RA-induced growth inhibition, it may help to determine if the MCF-7 cells with reduced IGF-IIR levels are more resistant to RA and if the MDA-MB-231 cells with increased IGF-IIR are rendered more sensitive to RA. It may also be important to examine the functional consequence of mutating the RA binding site on the IGF-IIR.

d - IGF-I, IGF-II, insulin: Although RA can inhibit IGF-I-stimulated growth, conversely the growth inhibitory effects of RA in MCF-7 cells can be antagonized by incubating the cells with IGF-I, insulin, or overexpressing IGF-II [260]. Bentel and co-workers further showed that the α-IR3 blocking antibody reverses the IGF-I and IGF-II antagonism while having no effect on insulin-stimulated cell growth, indicating the mitogenic effects of IGF-I and IGF-II are via IGF-IR activation. In addition, the RA derivative 4-HPR not only blocks IGF-I-stimulated growth of ER+ cells, but also of ER- cells by a mechanism that may involve the downregulation of IGF-IR levels [261]. Consistent with 4-HPR's ability to modulate the IGF system *in vitro*, 4-HPR treatment of patients with breast cancer results in decreased circulating IGF-I levels in pre-menopausal women [250].

The cross-talk between RA and growth factor receptor pathways is not limited to the IGF system. Numerous studies have demonstrated that the anti-proliferative effects of RA are associated with decreases in EGFR levels [262], and decreased EGF-EGFR interaction [263]. More recently, RA resistance has been associated with the amplification of signaling via the Her2/neu receptor pathway [264]. Although several

groups have examined the effect of RA on the initial steps of growth factor receptor signaling, its effects on the downstream intracellular signaling intermediates have not been well characterized. This deserves attention since there may be a signaling intermediate that is regulated by RA and that is common to multiple RTK pathways.

1.3.5 - Enhancing the Effectiveness of Retinoids.

Although significant contributions to understanding the mechanism of retinoid action have been made in the last twenty years, the pitfalls of using retinoids as single agents in the treatment of cancer have been their limited effectiveness as chemotherapeutics and their toxicity. A possible solution to this has been to combine retinoids with other agents used in the treatment of cancer. Potential approaches to identify agents that could synergize with retinoids are to: 1) identify the pathways regulated by retinoids, and 2) determine what factors are present in breast tumors that may hinder the activity of retinoids. It is well documented that RA elicits its anti-proliferative effects by inducing G1 phase arrest, while IGF-I induces its mitogenic effects by positively regulating this phase of the cell cycle. Since aberrations in the cell cycle and amplification in IGF-I signaling are frequently observed in breast cancer, it is reasonable then to propose to examine the usefulness of using agents that target the cell cycle and IGF-I signal transduction cascades in combination with RA.

1.3.5.1 Targeting the Cell Cycle:

The improper functioning of the cell cycle is a potent contributor to the onset of cancer. Transition through the G1 to S phase of the cell cycle requires the coordinate activity of the serine/threonine CDKs, CDK4/6 and CDK2 that interact with cyclin D and cyclin E, respectively. Negative regulators of these interactions include the CKIs p21 and p27 that

prevent inappropriate entry into the S phase. In MCF-7 cells it is well documented that IGF-I stimulates the expression of cyclin D1 at the mRNA and protein level via activation of the PI 3-kinase/AKT pathway [69, 112, 265]. RA on the other hand represses cyclin D1 expression via its ubiquitination and degradation [266, 267]. Since components of the G1 phase of the cell cycle are common targets of RA and IGF signaling pathways, combining inhibitors of the IGF-IR with RA may increase the chemopreventive action of either agent alone.

The timely expression of cyclins and CKIs is regulated by the ubiquitin-proteasome machinery. If this machinery malfunctions, then this may potentiate the effects of IGF-I and at the same time hinder the effects of RA by stabilizing the levels of proteins that should be degraded. Indeed, the overexpression of cyclin D1, loss of p27, and overexpression of Skp2 have been linked to breast carcinogenesis and RA resistance, further highlighting the importance of targeting the cell cycle as a strategy to enhance the actions of RA. Since RA stimulates the ubiquitin-proteasome mediated degradation of Skp2 [234], a protein known to be involved in the degradation of p27 [268], it is possible that perhaps agents that stabilize p27 levels would potentiate the anti-proliferative effects of RA. A nice candidate may be the proteasome inhibitor Bortezomib (PS-341) that is currently used in clinical trials and induces anti-tumor effects by a mechanism that involves the stabilization of p27 levels [269].

1.3.5.2 Targeting Signaling Pathways:

Signal transduction pathways convey growth regulatory signals from the extracellular space to the nucleus via a cascade of events involving the action of signaling intermediates that activate downstream kinases such as the MAPK and PI 3-kinase/AKT

that regulate changes in the expression of growth-promoting genes. Although RA can antagonize the mitogenic effects of IGFs, its resistance has been associated with increased AKT activity which often results from the overexpression of RTKs. It would thus be reasonable to propose the use inhibitors of the IGF-IR that would block AKT activation, in an attempt to re-sensitize breast cancer cells to RA. The feasibility of such a strategy is supported by studies showing that RA-resistance in some breast cancer cell lines is associated with Her2/neu receptor overexpression and can be overcome by pre-incubation with the blocking antibody Herceptin and subsequent treatment with RA [264].

Unfortunately, increased AKT activity does not always arise as a result of overexpressed RTKs, there are breast cancer cells lacking the tumor suppressor gene PTEN and this leads to the constitutive activation of AKT. The question then arises: Is it possible to sensitize breast cancer cells lacking PTEN (e.g. MDA-MB-468) to RA by using inhibitors of PI 3-kinase or AKT? Several groups have attempted to address this question by using pharmacological inhibitors of PI 3-kinase (e.g. LY294002 and wortmannin) [270-272] and AKT (e.g. 1L-6-hydroxymethyl-chiro-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) [270, 273] in RA-resistant leukemic cell lines. The inhibition of both kinases re-sensitized the cells to RA, and supported the hypothesis that the PI 3-kinase/AKT pathway is involved in RA resistance. Although to date no such studies have been done using models of breast cancer, one study did use a dominant negative AKT construct to sensitize Her2/neu-overexpressing cells to the anti-proliferative effects of RA [264]. Taken together, these studies have provided a

conceptual rationale for combining agents that inhibit PI 3-kinase/AKT activity with RA for therapeutic use in breast cancer.

Continuing to examine the role of retinoids in breast cancer is essential for the generation of novel combination therapies that will improve the effectiveness and tolerability of retinoids as chemopreventive agents in breast cancer.

SPECIFIC AIMS and PREFACE to CHAPTER 2

Retinoids have been used successfully to treat patients with acute promyelocytic leukemia and to prevent certain solid tumors. Although retinoids inhibit breast cancer cell proliferation *in vitro* and show promise as chemopreventive agents in animal models of breast cancer, their effectiveness in clinical trials has been limited. It may be possible to improve efficacy without excessive toxicity by using retinoids at lower doses in combination with other agents used in the treatment of breast cancer. Choosing the appropriate agent for use in combination with retinoids will ultimately depend on our understanding of the mechanisms by which retinoids act to regulate breast cancer cell growth.

Numerous studies have linked the retinoid-responsiveness in tumor cells, including breast cancer cells, to IGF signaling. In breast cancer cells (e.g. MCF-7) RA inhibits IGF-induced growth stimulation, and in patients with breast cancer, retinoids have been reported to reduce circulating IGF-1 levels. However, the effects of RA on key signaling molecules in the IGF-IR pathway have not been well characterized. The hypothesis I examined was that retinoids, in part, mediate their growth inhibitory effects by targeting specific members implicated in the growth stimulatory pathway mediated by the IGF-IR.

Examine the effects of RA on the IGF-IR signal transduction pathway in the MCF-7 breast cancer cell line.

1.1 Determine the expression or activation state of IGF-IR in response to RA.

1.2 Determine the effect of overexpression of IGF-IR and IRS-1 on RA-mediated growth inhibition of MCF-7 cells.

1.3 Determine the expression or activation state of IGF-IR downstream substrates: IRS-1 and SHC in response to RA.

1.4 Determine if IGF-stimulated AKT or MAPK activity are altered in response to RA treatment.

CHAPTER 2

Retinoic acid-Induced Growth Arrest of MCF-7 Cells Involves the Selective Regulation of the IRS-1/PI 3-kinase/AKT pathway.

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2.1 ABSTRACT

In the MCF-7 breast cancer cell line, insulin-like growth factors (IGFs) are known to elicit pro-proliferative actions via the IRS-1/PI 3-kinase/AKT pathway. All-trans retinoic acid (RA) is a potent inhibitor of MCF-7 cell proliferation, but the mechanism by which growth regulation is achieved remains unclear. We investigated the effects of RA on the regulation of the IGF-IR and its key signaling elements: IRS-1, IRS-2, and SHC. Treatment of MCF-7 cells with RA caused a significant reduction in IRS-1 protein and tyrosine phosphorylation levels at a concentration and time consistent with RA-mediated growth inhibition. IRS-1 regulation is selective, as RA did not influence IRS-2 or SHC levels. Downstream signaling events were also selectively reduced, as RA abrogated IGF-I-stimulated AKT activation but did not alter erk1/2 activation. To confirm the importance of IRS-1 regulation by RA, we examined the response to RA in MCF-7 cells over-expressing IGF-IR and IRS-1. RA resistance was observed in MCF-7 cells over-expressing IRS-1 but not IGF-IR. This suggests that RA-mediated growth inhibition requires the selective down regulation of IRS-1 and AKT. Therapeutic agents targeting the IRS-1/PI 3-kinase/AKT pathway may enhance the cytostatic effects of RA in breast cancer, since over-expression of IRS-1 and AKT have been reported in primary breast tumors.

2.2 INTRODUCTION

Several studies have reported either the direct or indirect involvement of insulin-like growth factor-I and IGF-II in the development of the normal breast epithelium and in the progression of breast carcinoma [274, 275]. It is generally accepted that the mitogenic actions of IGFs are mediated through binding to and activation of the type I IGF receptor [276]. The IGF-IR is a transmembrane tyrosine kinase with the ability to regulate mitogenesis, transformation, and survival [277, 278]. The activation of IGF-IR leads to the subsequent phosphorylation and activation of various adaptor proteins involved in IGF signal transduction, such as insulin receptor substrate-1, IRS-2, SHC, and Crk [55, 279-281].

Phosphorylated IRS-1 serves as a docking molecule for a number of Src homology (SH) 2 domain containing proteins such as Grb2, Syp, and the regulatory subunit of PI 3-kinase (p85) [282, 283]. The interaction of IRS-1 with p85 activates PI 3-kinase activity and subsequent activation of the serine/threonine protein kinase AKT, which mediates anti-apoptotic pathways [284]. The association of IRS-1 with the Grb2-Sos complex results in the activation of Ras and subsequent activation of mitogen-activated protein (MAP) kinases, which are critical regulators of breast cancer cell growth. IRS-1 has been shown to be the main adaptor molecule phosphorylated by IGF-I in estrogen receptor-positive breast cancer cell lines, and this activation was associated with increased activation of the PI 3-kinase and MAP kinase pathways [70]. Although increasing evidence in breast cancer cell models suggests a major role of IRS-2 and SHC in regulating cell motility, the predominant role of IRS-1 appears to be in transmitting proliferative signals [126, 134, 285]. Recent studies have correlated high levels of IRS-1

in human breast tumors with increased recurrence of the disease [6, 94]. The knowledge that IRS-1 can be over-expressed in breast cancer makes this component of the IGF signal transduction pathway a potential target for agents used in the treatment of breast cancer.

All-trans-retinoic acid (RA) is a vitamin A derivative that regulates cell growth, differentiation and apoptosis [286, 287]. Various synthetic retinoids, including RA, have shown promise for the treatment and prevention of several cancers, including carcinoma of the breast [288-290]. Numerous groups, including ours, have found that retinoids potently inhibit the growth of breast cancer cell lines [179, 182, 291], and others have reported the capacity of retinoids to inhibit mammary carcinogenesis in animal models [292, 293]. Many mechanisms have been proposed to explain the inhibition of breast cancer cell growth by retinoids including: cyclin D degradation, RARβ induction, and inhibition of AP-1 activity [294-296]. Another possible mechanism of RA-mediated regulation of breast cancer cell growth may be through interference with the IGF signal transduction pathway. A number of the known effects of retinoids on the IGF system in breast cancer cells include: abrogation of IGF-I stimulated growth [245], increased production of the growth inhibitory IGF binding protein-3 in breast cancer cell models [297], and down-regulation of plasma IGF-I levels in patients with breast cancer [298]. However, the effects of retinoids on key signaling molecules in the IGF-I pathway have not been well characterized [181, 248]. Thus, we examined the effect of RA on the IGF-IR and its main intracellular substrates, IRS-1, IRS-2, and SHC. Although we did not observe regulation of IGF-IR itself, we found that RA-mediated growth inhibition is associated with a selective reduction in IRS-1 protein and activity levels. We propose

that the abrogation of IRS-1 protein signaling acts as a novel mechanism of RA-mediated growth inhibition of MCF-7 cells. We present evidence that decreasing IRS-1 levels may result in the selective down-regulation of the PI 3-kinase/AKT pathway in MCF-7 cells treated with RA. The relevance of IRS-1 regulation to the growth inhibitory action of RA is supported by our finding that forced expression of IRS-1 abrogates RAs ability to significantly inhibit MCF-7 cell growth.

2.3 MATERIALS AND METHODS

Reagents

All-trans-retinoic acid (RA) was purchased from Sigma. Recombinant human IGF-I was purchased from PeproTech (Princeton NJ). Protein G-agarose, and Nonidet P40 were purchased from Sigma (Oakville, Canada). Enhanced chemiluminescence (ECL) detection system was purchased from Amersham Pharmacia Biotech. The following antibodies (Abs) were used for immunoprecipitations: for the IGF-IR: anti-IGF-IR mAb alpha-IR3 (Oncogene Science); for IRS-1: anti-C-terminal IRS-1 pAb (Upstate Biotechnology); for SHC: anti-SHC pAb (Transduction Laboratories); for GRB2: anti-GRB2 mAb (Transduction Laboratories); for the p85 subunit of PI3K: anti-PI3K-p85 pAb (Upstate Biotechnology). Tyrosine phosphorylation was detected with an antiphosphotyrosine mAb PY100 (Cell Signaling Technology). The following antibodies were used for western blotting: for IRS-1 and IRS-2: anti-IRS-1 pAb and anti-IRS-2 pAb (Upstate Biotechnology); for IGF-IR: anti-IGF-IR pAb (Santa Cruz Biotechnology); for SHC and GRB2: anti-SHC mAb and anti-GRB2 mAb (Transduction Laboratories); for total AKT levels and active AKT: anti-AKT pAb and anti-phospho-Akt (on Ser-473) pAb

(Cell Signaling Technology); and for active ERK1/2: anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) pAb (Cell Signaling Technology).

Cell stimulation and cell lysate preparation

MCF-7 cells (ATCC) were plated in phenol red containing α-MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (FBS). At 70% confluence cells were washed twice with phosphate-buffered saline and changed to phenol red-free α-MEM supplemented with BSA and holo-transferrin (serum free media - SFM) in the presence or absence of 1 μM RA or DMSO vehicle control for the times indicated. MCF-7 cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet® P40, 0.05% sodium deoxycholate, 0.1% SDS). The extracts were centrifuged at 13,000 *g* at 4° C for 30 min to remove insoluble material. After centrifugation, the protein content was measured by the Bradford assay using Bio-Rad reagents and BSA as standard. For IRS-1, IRS-2, and SHC western blotting, cells were immediately lysed as described above. For experiments requiring IGF-I stimulation, prior to harvesting cells, they were incubated at 37 °C for 10 minutes with 5nM IGF-I.

Immunoprecipitation and Western Blotting

For western blotting, cell lysates were boiled for 3 minutes in 2X SDS sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% β-mercapto-ethanol, Bromophenol Blue) and resolved in 8-10% SDS-polyacrylamide gels (SDS-PAGE). For immunoprecipitation, after IGF-I stimulation, 500 μg – 1mg of pre-cleared cell lysates were incubated with the indicated antibody overnight at 4 °C, followed by the addition of protein G agarose overnight to collect immune complexes. The immunoprecipitates were

washed in RIPA buffer, resuspended in SDS sample buffer, and boiled for five minutes. The solubilized proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to nitrocellulose membrane (Bio-Rad) and detected by immunoblotting with the indicated antibody using ECL. Some membranes were stripped to prepare them for a second round of immunoblotting.

Cell proliferation assay

Exponentially growing cells were plated at sub-confluent densities in 24-well plates. MCF-7 cells over-expressing IGF-IR (clones 12 and 17) or IRS-1 (clone 18) were incubated in phenol red containing DMEM-F12 (Life Technologies, Inc.) supplemented with 5% FBS for five days in the presence of 1 μM (RA) or DMSO (vehicle control). MDA-MB-231 (ATCC) and the ERα-positive subclone of MDA-MB-231, S30 (courtesy of Dr. V C Jordan) were maintained in phenol red free α-MEM (Life Technologies, Inc.) supplemented with 5% charcoal stripped serum. Cells in triplicate wells were counted by a hemacytometer. The Student's t-test was used to analyze the significance of the results obtained in cell growth curves. Dr. Ewa Surmacz provided the MCF-7 cells stably transfected with various IGF signaling components.

2.4 RESULTS

RA regulates a specific IGF-IR downstream substrate in MCF-7 Cells

Although previous studies of regulation of IGF-IR mRNA by RA have yielded conflicting results [181, 248], in the ATCC clones of MCF-7 currently growing in our laboratory, we observe no change in the level of IGF-IR protein expression (Figure 2.1a) or mRNA expression (data not shown) in cells treated with 1 μM RA for 24, 48, and 72 hours. We also observed no change in the level of IGF-I-stimulated tyrosine

phosphorylation of the IGF-IR in cells pre-treated with 1 μM RA for 24, 48 or 72 hours (Figure 2.1b).

Since we did not observe any change on IGF-IR levels or phosphorylation, we proceeded to look at key IGF-IR downstream substrates: IRS-1, IRS-2 and SHC. The expression of IRS-1 mRNA was found to be unchanged in MCF-7 cells treated with 1 μM RA (data not shown). However, we found the expression of IRS-1 protein decreased after exposure of MCF-7 cells to 1 μM RA for 48 and 72 hours (Figure 2.1c, panel 1). The protein expression of IRS-2 (Figure 2.1c, panel 2) and SHC (isoforms p46, p52) (Figure 2.1c, panel 3) were unaffected by treatment of MCF-7 cells with 1 μM RA, suggesting that IRS-1 is a novel and specific target for RA in the IGF-I signaling pathway.

RA modulates IGF-I-stimulated IRS-1 tyrosine phosphorylation in MCF-7 cells

IRS-1 is a key regulator of cell proliferation, and so we hypothesize that the lower IRS-1 protein levels in RA-treated MCF-7 cells may contribute to an inhibition of proliferation. Since IGF-I uses IRS-1 to transmit its mitogenic signal via a signal transduction pathway that begins with IRS-1 tyrosine phosphorylation; we examined the effect of RA on the level of IRS-1 tyrosine-phosphorylation. MCF-7 cells were pre-treated with 1 μM RA for the times indicated, treated with IGF-I for ten minutes, and then tyrosine phosphorylated IRS-1 was assessed. MCF-7 cells have barely detectable phosphorylated IRS-1, which increases dramatically by treatment with IGF-I (Figure 2.2a, lane 2). This stimulation was decreased in MCF-7 cells pre-treated with RA for 24 hours and more

significantly in cells pre-treated with RA for 48 and 72 hours (lanes 4 and 5, respectively).

In addition to inducing phosphorylation of IRS-1, the activated IGF-IR can recruit and phosphorylate another downstream substrate, SHC. Although RA selectively decreases the levels and phosphorylation status of IRS-1 (Figure 2.2a), we found that RA does not have any effect on IGF-I-induced tyrosine phosphorylation of SHC (Figure 2.2b) or on the ability of SHC to associate with GRB2, as detected by immunoprecipitation studies (Figure 2.2c). We conclude that RA selectively regulates IRS-1 and that RA-mediated growth inhibition does not require the regulation of the IGF-I-stimulated SHC/Grb2 pathway.

RA inhibits IGF-I-stimulated downstream signaling in MCF-7 cells

The phosphorylated IRS-1 serves as a docking protein to recruit other molecules, including the p85 subunit of PI 3-kinase, tyrosine phosphatase Syp and the adaptor protein Grb2 [61]. Grb2 interacts with Sos, the guanylnucleotide exchange factor for Ras, and the IRS-1/Grb2/Sos complex subsequently activates the MAPK pathway that has been shown to regulate breast cancer cell growth [299]. In MCF-7 cells, the PI 3-kinase pathway has been reported to be the specific pathway transmitting IGF-I mitogenic signals [69]. We examined the possibility that the decreased IRS-1 expression and phosphorylation after RA treatment might alter IRS-1/Grb-2 and IRS-1/p85 binding and thus regulate MAPK and PI 3-kinase activities, respectively. IGF-I stimulation increased the amounts of Grb2 co-immunoprecipitated with IRS-1 from control cells (Figure 2.3a, lane 2), but the levels of co-immunoprecipitated Grb2 were reduced in cells pre-treated

with 1 μM RA for 48 and 72 hours (lanes 4 and 5, respectively), and this was not due to an effect of RA on total levels of Grb2 (Figure 2.3b). Similarly, the levels of IRS-1 co-immunoprecipitated with p85 from MCF-7 cells pre-treated with 1 μM RA for 48 and 72 hours (Figure 2.3c, lanes 4 and 5, respectively) were reduced compared with controls. This decrease in p85 associated with IRS-1 was not due to a reduction in the level of total p85 (Figure 2.3d). The finding that RA inhibits the association of IRS-1 with Grb2 and p85 confirms the observed down-regulation of IRS-1 protein levels (Figure 2.1c, panel 1) and tyrosine phosphorylation (Figure 2.2a).

RA impairs IGF-I-stimulated AKT activity but not ERK1/2 activation in MCF-7 cells

We sought to verify that the RA-mediated decrease in associations of IRS-1 with GRB2 and p85 correspond to reductions in the activation of downstream targets of these complexes. We looked at the effect of RA on phosphorylation of the p44 and p42 (erk1 and erk2) members of the MAPK pathway and phosphorylation of AKT, a serine/threonine kinase activated downstream of PI 3-kinase. Phosphorylated IRS-1 can activate erk1 and erk2 by binding Grb2 and activating the ras/MAPK pathway [300]. In spite of decreased levels of phosphorylated IRS-1, we found that IGF-I-stimulated erk1 and erk2 phosphorylation (Figure 2.4a, lane 2) were not changed by pre-treatment of MCF-7 cells with 1 μM RA. In contrast IGF-I-stimulated AKT activation (Figure 2.4b, lane 2), as assessed by phosphorylation of the AKT kinase in Ser473, was markedly decreased in MCF-7 cells that were pre-treated with 1 μM RA for 24 and 48 hours (Figure 2.4b, lanes 3 and 4, respectively). In MCF-7 cells that had been pre-treated for

72 hours with RA, a slight recovery of AKT kinase activation was observed (Figure 2.4b, lane 5). The effects of RA on the serine phosphorylation status of AKT kinase were not due to a change in the level of total AKT (Figure 2.4c). To strengthen our hypothesis that RA-mediated growth inhibition involves the selective down-regulation of the PI 3-kinase/AKT pathway, we modified the experimental conditions used in Figures 2.4a and 4b, using culture conditions more related to RA-mediated growth inhibition. To this end, in Figures 2.4d and 2.4e, we did not stimulate cells with IGF-I, we cultured MCF-7 cells in medium containing 5% FBS, which is known to contain IGF-I, to determine whether RA could still regulate AKT activity under these culture conditions. Indeed, RA decreases AKT kinase activation (Figure 2.4d) but not erk1/2 activity (Figure 2.4e) in medium containing 5% FBS, that is, using conditions similar to those employed in the proliferation assays showing RA-mediated growth arrest (see Figure 2.5a).

Over-expression of specific components of the IGF-I signaling pathway decreases the growth inhibitory action of RA

Although both IGF-IR and IRS-1 have been implicated in the control of breast cancer cell growth [133], our data suggest that IRS-1 plays a more important role in the growth inhibitory response to RA. To confirm the importance IRS-1 regulation by RA, we examined the response to RA in two previously characterized MCF-7 cell lines over-expressing IGF-IR (clone 17) [301] and IRS-1 (clone 18) [125]. On days three and five of the growth curve, MCF-7 cells over-expressing IGF-IR were significantly growth inhibited by 1 μM RA (Figure 2.5a). However, MCF-7 cells over-expressing IRS-1 (clone 18) were significantly less responsive to the growth inhibitory effects of 1 μM RA

in a representative five-day growth curve than the parental MCF-7 cells. This supports our data that the effect of retinoids on the IGF signaling pathway is not mediated at the level of IGF-IR, but at the level of IRS-1. We next examined whether down-regulation of IRS-1 signaling by RA is blocked by the over-expression of IRS-1 in clone 18. We found that RA does not decrease IRS-1 protein expression (Figure 2.5b), does not inhibit IGF-I-stimulated IRS-1 tyrosine phosphorylation (Figure 2.5c), and is unable to inhibit serine phosphorylation of AKT kinase in these cells (Figure 2.5d). This supports our hypothesis that the selective down-regulation of IRS-1 signaling by RA mediates its growth inhibitory effect.

RA-mediated growth arrest involves IRS-1 regulation.

We have previously reported, and include here (Figure 2.6a), that RA can inhibit the growth of the ER-negative breast cancer cell line, MDA-MB-231 stably transfected to express wild-type ER (S30), but not of MDA-MB-231 [182]. To provide additional evidence that RA-mediated growth arrest involves the regulation of IRS-1, we assessed the regulation of IRS-1 in the S30 retinoid sensitive cell versus its regulation in the RA-resistant MDA-MB-231 cell line. The western blot shown in Figure 2.6b shows that RA decreases IRS-1 levels in the S30 cell line but not in the parental MDA-MB-231. The lack of IRS-1 regulation in MDA-MB-231 is consistent with the data obtained in the RA-resistant IRS-1 over-expressing MCF-7 cell line (clone 18) (see Figure 2.5a), and supports our hypothesis that RA-mediated growth arrest involves decreasing IRS-1 levels.

2.5 DISCUSSION

To our knowledge, this is the first study elucidating the effect of RA on signaling elements downstream of the IGF-IR, and from the results presented herein; we propose a novel mechanism by which RA inhibits the growth of MCF-7 cells. Although RA-mediated growth inhibition of MCF-7 cells is not directly mediated via alterations in IGF-IR, we show a significant reduction in IRS-1 protein levels when MCF-7 cells are treated with RA. This reduction in IRS-1 protein corresponds to a decrease in IGF-I-stimulated IRS-1 tyrosine phosphorylation at a concentration and time that is consistent with RA-mediated growth inhibition of these cells. The findings presented in this report are directly relevant to RA-mediated regulation of breast cancer cell growth, since previous studies down-regulating IRS-1 expression using antisense strategies in several cellular systems implicate IRS-1 as a key mediator of cellular growth [66, 126, 302].

Our hypothesis that reducing IRS-1 protein levels and tyrosine phosphorylation may be a key step in RA-mediated growth arrest of MCF-7 cells is supported by our observations in MCF-7 cells that stably over-express IRS-1 (clone 18). In these cells, RA does not reduce IRS-1 protein levels or IRS-1 tyrosine phosphorylation, nor does it lead to a significant inhibition of cell proliferation. The study of other anticancer agents has revealed similar findings. Specifically, the anti-estrogen ICI 182,780 can decrease IRS-1 levels in MCF-7 cells, however this agent is not effective in reducing IRS-1 levels or inhibiting the growth of MCF-7 cells over-expressing IRS-1 [98]. Although additional compounds such as 12-O-tetradecanoylphorbol-13-acetate [303] and endothelin-1 [304] have been reported to decrease IRS-1 levels, not all growth regulatory agents are capable of doing so. For example, like RA, vitamin D3 derivatives have potent differentiating

and cell growth inhibitory activity and can effectively inhibit IGF-I-stimulated cell growth [305] and reduce IGF-IR levels [306], but they exert no changes on IRS-1 expression [307]. To further support the observation that RA-mediated growth arrest of breast cancer cells involves IRS-1 down-regulation, we show in the RA-resistant cell line MDA-MB-231, that IRS-1 levels remain unchanged, while in the RA-sensitive MDA-MB-231 subclone, S30, RA does decrease IRS-1 protein levels. These results suggest that resistance of breast cancer cells to the growth inhibitory effects of RA is accompanied by an inability of RA to decrease IRS-1 levels.

To extend our hypothesis that IRS-1 regulation may mediate RA-induced growth inhibition, we looked at the ability of IRS-1 to transmit the IGF-I-stimulated mitogenic signal downstream. IRS-1 possesses 20-22 potential tyrosine phosphorylation sites and interacts with many SH2 domain-containing proteins, including Grb2 and p85. Thus, tyrosine phosphorylation of IRS-1 represents a key step in activating distinct downstream IGF-I signaling pathways, including the MAP kinase and PI 3-kinase pathways. Although we observe less association of Grb2 with IRS-1 in RA-treated MCF-7 cells, MAP kinase activity was not abolished, as assessed by ERK1/2 phosphorylation. Both IRS-1/Grb2 and SHC/Grb2 lie upstream of ERK1/2, thus it is feasible that the SHC branch of the IGF-I signaling pathway can directly activate Erk1/2. Consistent with this possibility, decreasing IRS-1 expression using antisense IRS-1 has been reported to increase SHC levels and SHC tyrosine phosphorylation [302]. It has also been shown that blocking IRS-1 function, using IRS-1 deletion mutants, has no compensatory effect on the SHC/MAP kinase pathway [308]. In this study, RA did not affect SHC protein levels, SHC tyrosine phosphorylation status, or the complex formation between SHC and

Grb2. Thus, we show that the SHC/Grb2/MAP kinase pathway is unaffected by the RA-induced decrease in IRS-1 levels.

In MCF-7 cells, the mitogenic action of IGF-I is not via the MAP kinase pathway, but through PI 3-kinase activation [69]. Furthermore, the serine/threonine kinase AKT is one of the major downstream effectors of PI 3-kinase reported to mediate the effects of IGF-I [309-311]. AKT acts to regulate a number of cellular processes involved in transmitting key survival signals [312] and can be found over-expressed in a variety of cancer cell lines, including MCF-7 cells [313, 314]. The observed abrogation of IRS-1/p85 association in cells that had been pre-treated with RA for 48 hours correlated well with a decrease in the phosphorylation status of AKT in MCF-7 cells. It remains unclear why we observe a partial recovery of AKT activation in MCF-7 cells that had been pre-treated with RA for 72 hours (Figure 2.4b, lane 5). We are currently using biological inhibitors of the PI 3-kinase and MAP kinase pathways in attempt to elucidate if there is a feedback mechanism involved in activating AKT. It is also of interest that in the presence of normal cell culture conditions, that is in 5% FBS, that RA is still able to decrease AKT activity, albeit at a later time point than in the absence of serum growth factors (Figure 2.4d). This is the first study reporting an effect of RA in down-regulating AKT kinase activation in MCF-7 cells, and from recent studies, it appears that RA can now be added to a growing list of chemotherapeutic agents, such as Herceptin, Genistein, and CI-1033, that inhibit cancer cell growth via a mechanism involving the regulation of PI 3-kinase and AKT activity [315-317].

Our finding that RA-mediated growth inhibition involves the regulation of the PI 3-kinase/AKT pathway is in accordance with a recent paper showing that RA-mediated

degradation of RAR gamma requires the down-regulation of the PI 3-kinase/AKT pathway [318]. Furthermore, the observed abrogation of AKT activation may be a requirement for RA-mediated growth inhibition, as we do not observe AKT regulation in RA-resistant MCF-7 cells over-expressing IRS-1 (clone 18). Consistent with the data obtained in clone 18, another group has recently shown that the transfection of a dominant negative AKT construct in RA-resistant, Her2/neu-over-expressing cells can overcome RA resistance [264]. Additional work will be needed to elucidate the molecules involved in RA-mediated down-regulation of the IRS-1/PI 3-kinase/AKT pathway. To this end, we are currently elucidating the mechanism of RA-mediated regulation of IRS-1. We speculate that RA regulates IRS-1 via a post-translational modification, due to our findings that RA has no effect on IRS-1 mRNA levels (data not shown), the RA-mediated decrease in IRS-1 protein expression was not altered by either actinomycin D (an inhibitor of transcription) or cycloheximide (an inhibitor of translation) (data not shown), and RA affects IRS-1 protein levels at later time points (Figure 2.1c). Based on previous reports showing that IRS-1 undergoes ubiquitin-dependent degradation [90-92, 319] and unpublished preliminary data from our laboratory, we hypothesize that IRS-1 is degraded by the ubiquitin-proteasome pathway during RA treatment. There is indeed a growing list of proteins known to be regulated by RA via mechanisms involving the ubiquitin-proteasome pathway; cyclin D1 [232], PML/RAR [320], RAR alpha and RAR gamma [238], CDK-4 [237], Skp2 [234], and future experiments will elucidate whether IRS-1 can be added to this list.

In conclusion, we find that RA-mediated growth inhibition of MCF-7 cells involves alterations in specific downstream IGF signaling elements. We have shown that

RA induces a significant reduction in the expression and tyrosine phosphorylation of IRS-1 protein in MCF-7 cells treated with RA. These data emphasize growth factor receptor adaptor molecules, such as insulin receptor substrate-1, rather than the cell surface IGF-IR as targets for antitumor therapeutic strategies. In fact, a recent study demonstrates that constitutive activation of IRS-1 is not restricted to breast tumors, thus presenting IRS-1 as a potential drug target in other human malignancies [127]. In addition, our data suggest that a possible functional outcome of reduced IRS-1 protein levels is the selective down-regulation of the PI 3-kinase/AKT pathway, which may be important for RA-mediated growth inhibition. These novel targets of RA could lead to the development of novel combination therapies in the treatment of breast cancer, making use of known biological inhibitors of the PI 3-kinase/AKT [321, 322] pathway in combination with retinoids.

2.6 ACKNOWLEDGEMENTS

We thank Dr. Eva Surmacz for helpful discussion and for providing the MCF-7 cells stably transfected with various IGF signaling components. This work was supported by a pre-doctoral fellowship award from the US Army Medical Research and Materiel Command Breast Cancer Research Program (award number DAMD1701-1-0320 to SV del Rincón) and a grant from the Canadian Breast Cancer Research Initiative. Wilson H. Miller Jr. is an Investigator of the Canadian Institutes of Health Research.

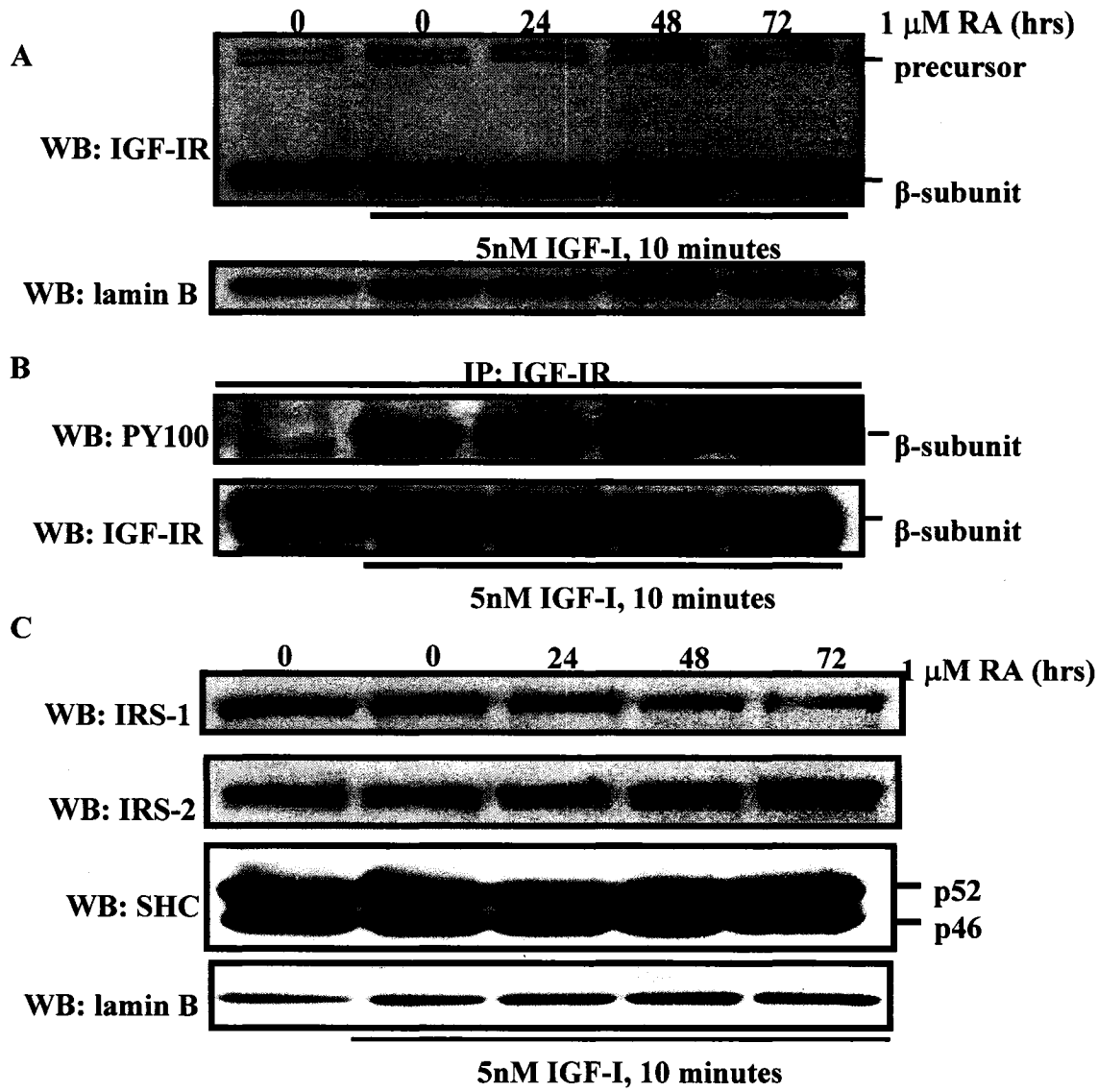


Figure 2.1 - RA selectively decreases IRS-1 levels in MCF-7 cells.

(A) Cells were treated with 1 μM RA for 24, 48 and 72 hours in serum free media (SFM). Western blot (WB) was used to determine the expression level of IGF-IR. Lamin B was used as a loading control. (B) Cells were pre-treated with 1 μM RA for 24, 48 and 72 hours in SFM. Prior to protein extraction, cells were stimulated with 5 nM IGF-I for 10 minutes. Phosphorylated IGF-IR was detected by immunoprecipitating IGF-IR and immunoblotting using a phosphotyrosine antibody (PY100). Upper panel: the phosphorylation of the IGF-IR β-subunit. Lower panel: the immunoblot in (B) stripped and immunoblotted with an anti-IGF-IR antibody. (C) Cells were treated as in (B). WB was used to determine the expression levels of IRS-1, IRS-2, SHC.

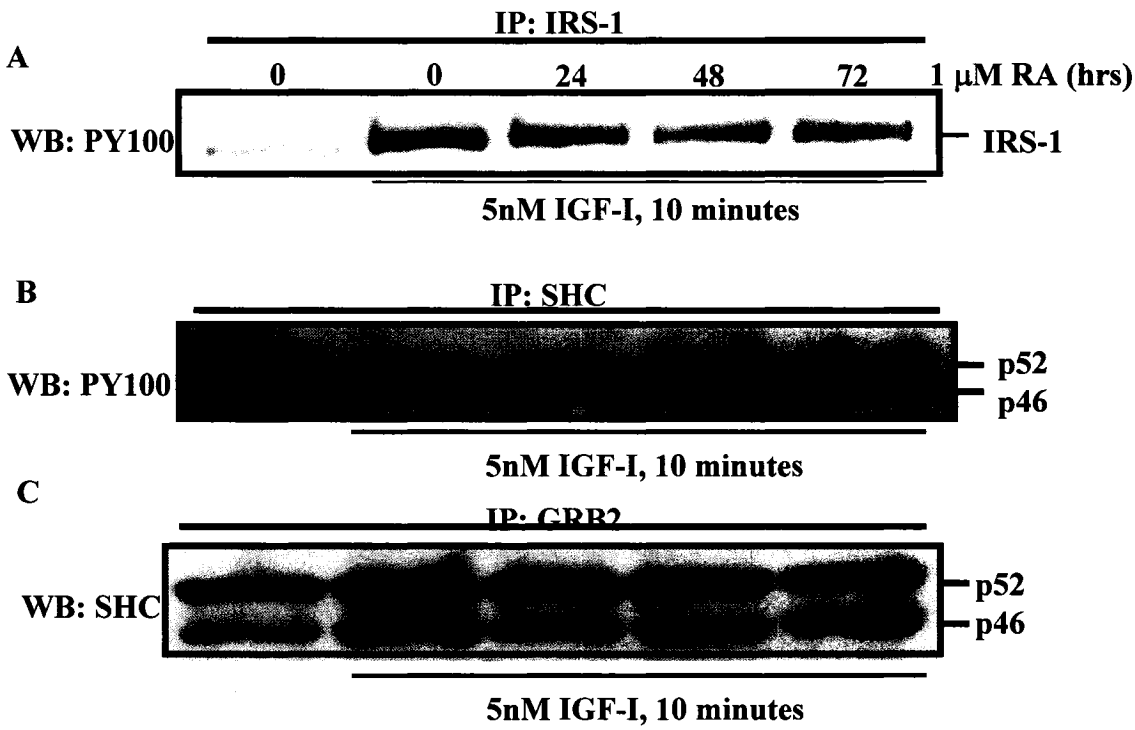


Figure 2.2 - RA modulates IGF-I-stimulated IRS-1 tyrosine phosphorylation in MCF-7 cells.

MCF-7 cells were treated as in Figure 1 (B). (A) Phosphorylated IRS-1 was detected by immunoprecipitating cell lysates with an anti-IRS-1 pAb and immunoblotting using a phosphotyrosine antibody (PY100). (B) Phosphorylated SHC was detected by immunoprecipitating cell lysate with an anti-SHC pAb and immunoblotting using PY100. (C) To detect SHC/Grb2 association, cell lysate was immunoprecipitated with an anti-Grb2 mAb and immunoblotted with an anti-SHC pAb. All data are representative of three independent experiments.

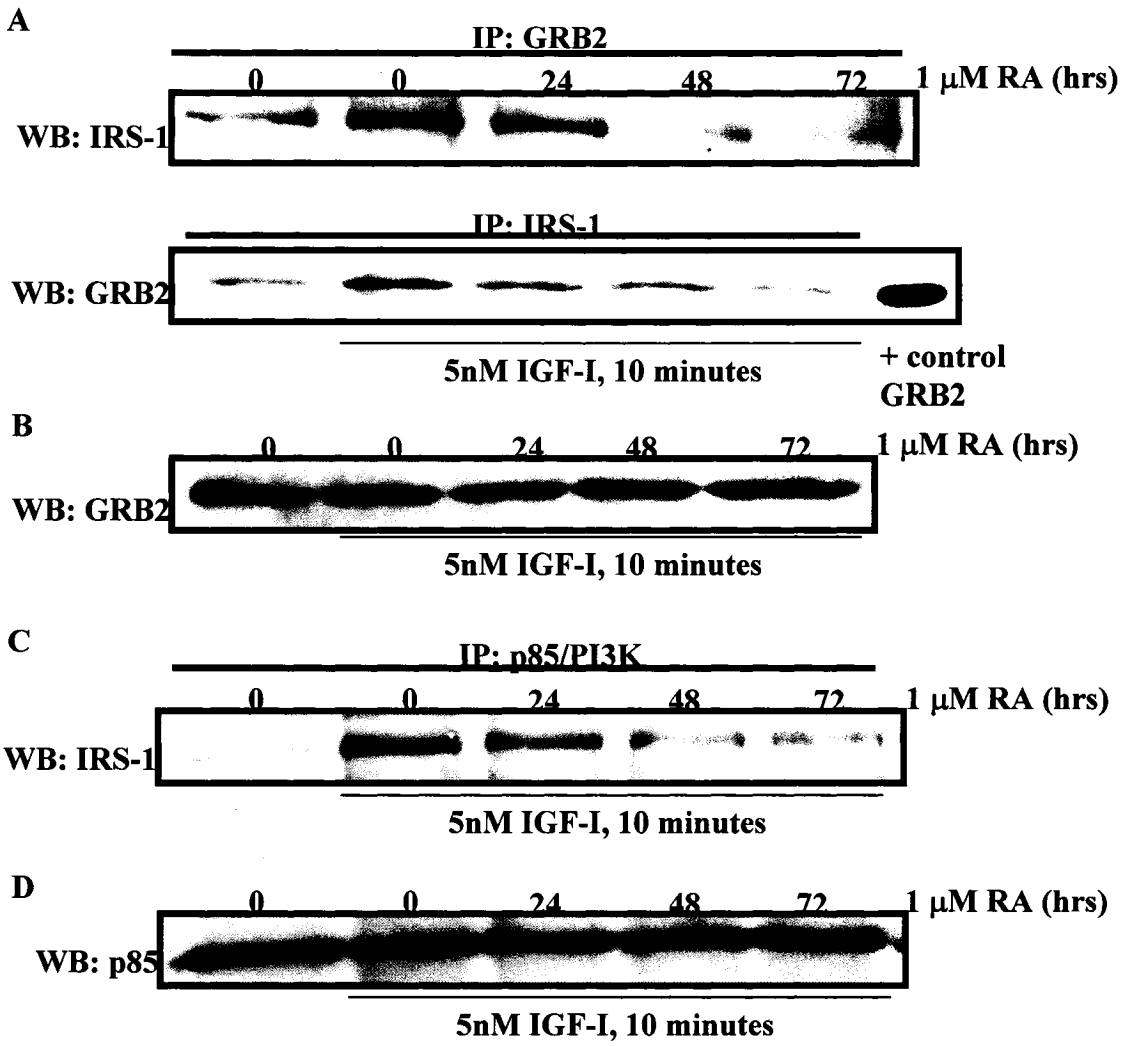


Figure 2.3 - RA suppresses IGF-I-stimulated IRS-1 /GRB2 and IRS-1/p85 complex formation.

MCF-7 cells were treated as in Figure 1 (B). (A) To detect IRS-1/Grb2 association, cell lysate was immunoprecipitated with an anti-GRB2 mAb and immunoblotted with an anti-IRS-1 pAb (upper panel). To confirm IRS-1/Grb2 binding, cell lysate was immunoprecipitated with an anti-IRS-1 pAb and immunoblotted with an anti-GRB2 mAb (lower panel). (B) Western blot showing equal levels of GRB2. (C) To detect IRS-1/p85 binding, cell lysate was immunoprecipitated with an anti-p85 pAb and immunoblotted with an anti-IRS-1 pAb. (D) Western blot showing equal levels of p85. Data depicted are representative of at least three independent experiments.

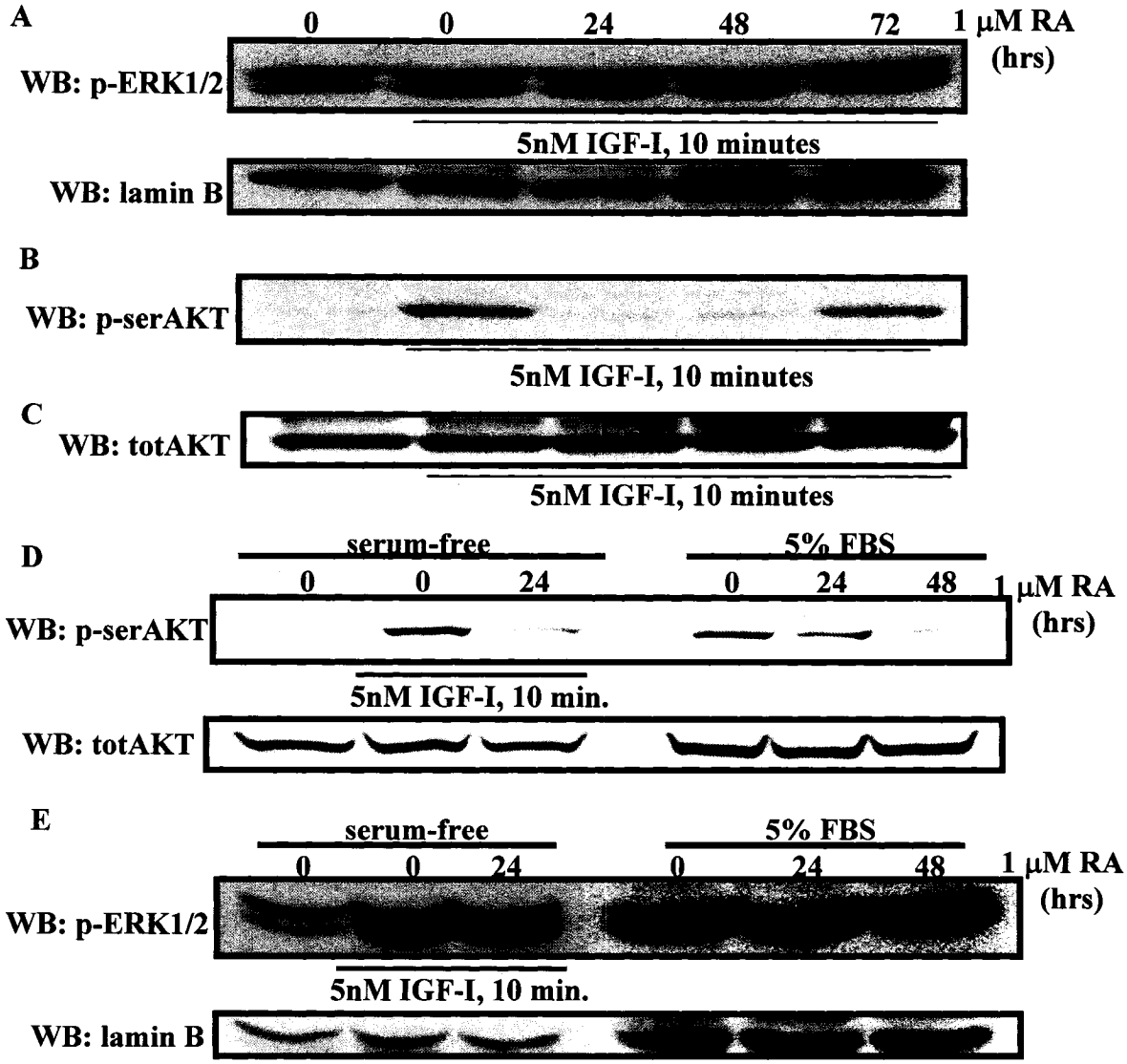
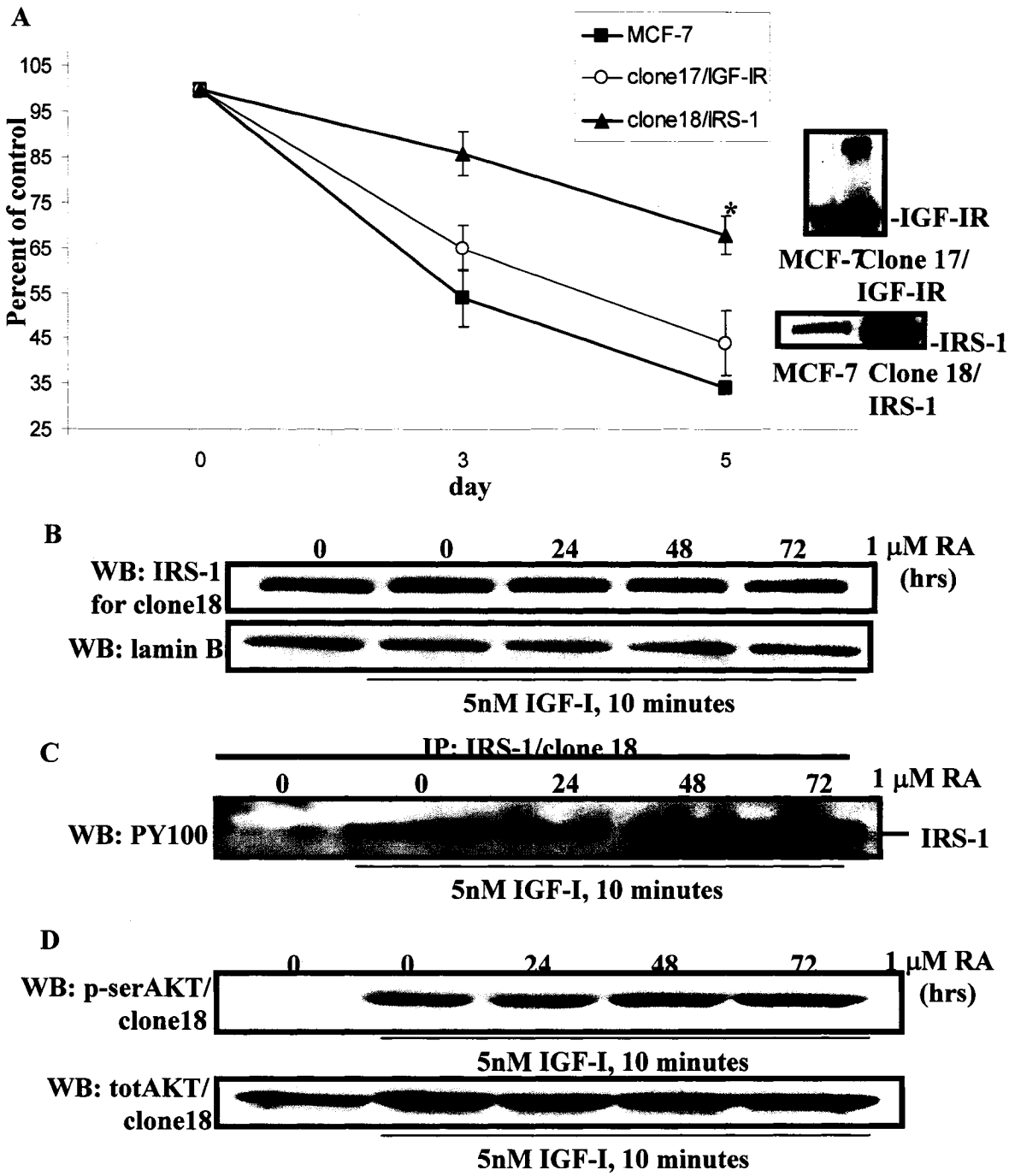


Figure 2.4 - RA regulates the phosphorylation of AKT but not of ERK1/2.

MCF-7 cells were treated as in Figure 1 (B). Western blot was used to determine the levels of: (A) phosphorylated p44/42 MAP kinase (on Thr202/Tyr204). Lamin B was used to show equal loading of lanes. (B) phosphorylated AKT (on Ser-473) and (C) AKT to show equal levels of total AKT. In (D) and (E), MCF-7 cells were cultured in serum free media and stimulated with 5 nM IGF-I for 10 minutes (left panels) or cultured in the presence of 5% FBS (right panels). (D) phosphorylated AKT (on Ser-473) (upper panel) and total AKT (lower panel). (E) phosphorylated p44/42 MAP kinase (on Thr202/Tyr204) (upper panel) and lamin B (lower panel). These data represent three independent experiments.

Figure 2.5 - RA inhibits the growth of MCF-7 cells over-expressing specific components of the IGF-I signal transduction pathway.

(A) Parental (vector alone), IGF-IR-transfected (clone 17/IGF-IR), and IRS-1-transfected (clone 18/IRS-1) MCF-7 cells were plated at 1×10^4 cells/well in 24-well plates in DMEM/F12 medium + 5% FBS. All cell lines were incubated with 1 μM RA for five days. Cells were counted using a hemacytometer on day three and five. Each data point represents an average of triplicate wells +/- standard deviation. (* There was a significant difference between clone 18 and the MCF-7 parental $p < 0.005$, while there was no significant difference between clone 17 and MCF-7). Insets: Western blots confirming an over-expression of IGF-IR in clone 17, and an over-expression of IRS-1 in clone 18. For (B)-(D): MCF-7 cells over-expressing IRS-1 (clone 18) were pre-treated with 1 μM RA for 24, 48 and 72 hours in SFM. Prior to protein extraction, cells were stimulated with 5 nM IGF-I for 10 minutes. (B) Western blot was used to determine the levels of IRS-1 in clone 18. (C) Phosphorylated IRS-1 in clone 18 was detected by immunoprecipitating cell lysates with an anti-IRS-1 pAb and immunoblotting with PY100. (D) Western blot was used to determine the levels of phosphorylated AKT (on Ser-473) (upper panel) and total AKT (lower panel). Data depicted are representative of at least three independent experiments.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MCF-7
clone17/IGF-IR
clone18/IRS-1
Percent of control
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-IRS-1
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clone18
5nM IGF-I, 10 minutes

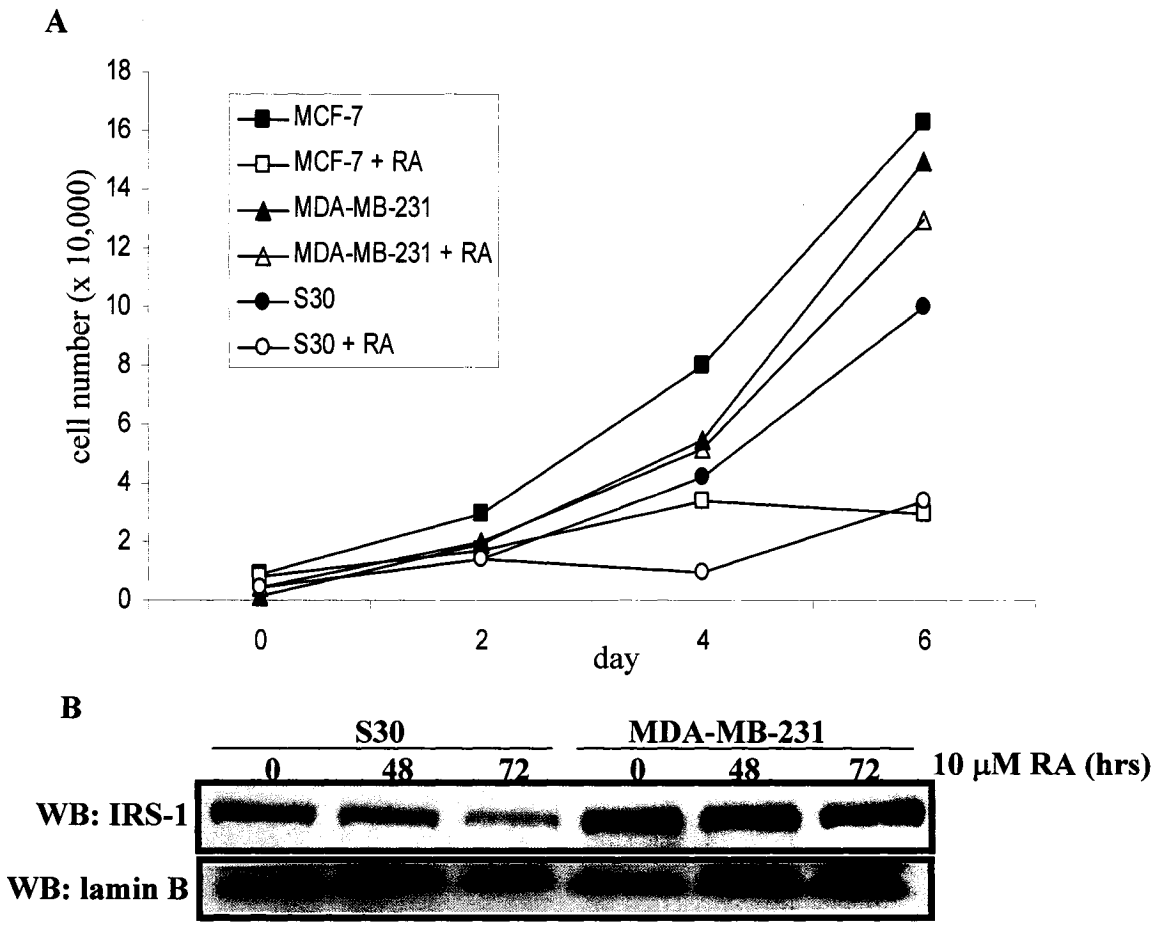

Figure 2.6 – RA-mediated growth inhibition involves IRS-1 regulation.

(A) MCF-7 cells, MDA-MB-231, and S30 were plated at $1'10^4$ cells/well in 24-well plates in the presence of 5% FBS (MCF-7) or 5% charcoal stripped serum (MDA-MB-231 and S30). MCF-7 cells were incubated with 1 μM RA for six days. MDA-MB-231 and S30 were incubated with 10 μM RA for six days. Cells were counted in triplicate using a hemacytometer on days two, four, and six. There was a significant difference between S30 and MDA-MB-231 ($p < 0.005$). (B) Western blot was used to determine the levels of IRS-1 in S30 and MDA-MB-231 cells treated with 10 μM RA for 48 and 72 hours. Lamin B was used to show equal loading of all lanes.

PREFACE to CHAPTER 3

IRS-1 is the main adaptor molecule phosphorylated by IGF-I in ER+ breast cancer cells and several studies have highlighted the importance of IRS-1 in breast cancer pathogenesis. This suggests that we may develop molecular strategies targeting IRS-1 by understanding the mechanisms controlling its expression and turnover. The past two decades have focused on examining transcriptional mechanisms linked to the effects of RA, however, it is becoming evident that RA exerts many of its effects also via posttranslational modification of proteins. Since in Chapter 2 I found that IRS-1 levels were regulated at the level of protein but not mRNA, and given the importance of IRS-1 in breast cancer this provided me with the rationale in Chapter 3 to investigate the molecular mechanism by which RA regulates IRS-1 levels.

Elucidate the mechanism by which RA regulates IRS-1 protein levels.

1.1 Determine if IRS-1 is degraded by the ubiquitin-proteasome pathway.

1.2 Define the RA activated signaling pathway responsible for the serine phosphorylation of IRS-1.

CHAPTER 3

Retinoic acid mediates degradation of IRS-1 by the ubiquitin-proteasome pathway, via a PKC-dependant mechanism.

This paper was accepted to *Oncogene*, August 2004.

3.1 ABSTRACT

Insulin receptor substrate-1 (IRS-1) mediates signaling from the insulin-like growth factor type-I receptor. We found that all-trans retinoic acid (RA) decreases IRS-1 protein levels in MCF-7, T47-D, and ZR75.1 breast cancer cells, which are growth arrested by RA, but not in the RA-resistant MDA-MB-231 and MDA-MB-468 cells. Based on prior reports of ubiquitin-mediated degradation of IRS-1, we investigated the ubiquitination of IRS-1 in RA-treated breast cancer cells. Two proteasome inhibitors, MG-132 and lactacystin, blocked the RA-mediated degradation of IRS-1, and RA increased ubiquitination of IRS-1 in the RA-sensitive breast cancer cells. In addition, we found that RA increases serine phosphorylation of IRS-1. To elucidate the signaling pathway responsible for this phosphorylation event, pharmacologic inhibitors were used. Two PKC inhibitors, but not a MAPK inhibitor, blocked the RA-induced degradation and serine phosphorylation of IRS-1. We demonstrate that RA activates PKC-δ in the sensitive, but not in the resistant cells, with a time course that is consistent with the RA-induced decrease of IRS-1. We also show that: 1) RA-activated PKC-δ phosphorylates IRS-1 *in vitro*, 2) PKC-δ and IRS-1 interact in RA-treated cells, and 3) mutation of three PKC-δ serine sites in IRS-1 to alanines results in no RA-induced *in vitro* phosphorylation of IRS-1. Together, these results indicate that RA regulates IRS-1 levels by the ubiquitin-proteasome pathway, involving a PKC-sensitive mechanism.

3.2 INTRODUCTION

The family of insulin receptor substrate (IRS) proteins (IRS 1-4) function as the central substrates in the insulin and insulin-like growth factor type-I (IGF-I) receptor signal transduction pathways [55, 323]. Specifically, IRS-1 functions as a critical scaffolding protein between the activated IGF-IR and various downstream signaling pathways, including the PI3-kinase/AKT and MAPK pathways [324, 325]. Among its many functions, this protein has been shown to regulate cell proliferation and survival [61]. In hematopoietic cells, IRS-1 is an essential regulator of proliferation in response to insulin [62], and in NIH 3T3 cells, cellular transformation can be directly induced by overexpressing IRS-1 [326]. Several studies have highlighted the importance of IRS-1 in breast cancer pathogenesis: IRS-1 overexpression in breast cancer cells causes a loss of estrogen-dependant growth [125], high levels of IRS-1 in human breast tumors correlate with increased disease recurrence [6, 94], constitutive IRS-1 signaling exists in breast tumors [127], and expression of dominant negative or antisense IRS-1 vectors in breast cancer cells decreases their transformation potential [127, 133]. This suggests that we may develop molecular strategies targeting IRS-1 by understanding the mechanisms controlling its expression and turnover.

Previous studies have suggested that the expression of IRS-1 can be regulated at the level of transcription and proteolysis. 17-beta-estradiol (E2) increases IRS-1 levels by increasing mRNA and protein levels [93-95], while antiestrogens decrease IRS-1 mRNA and/or protein levels [97, 98]. Glucocorticoids also reportedly decrease the levels of IRS-1 mRNA and protein [327, 328]. Using various cellular systems, including MCF-7 breast cancer cells, it has been shown that prolonged treatment with IGF-I and high

concentrations of insulin can induce degradation of IRS-1 and IRS-2 via the ubiquitin proteasome pathway [90-92, 329].

Retinoids, including all-trans-retinoic acid (RA), have been shown to induce G1 arrest and differentiation in several cancer cell types by regulating various cellular factors. We previously showed that RA-mediated growth arrest of MCF-7 cells involves a decrease in IRS-1 protein levels [240], and others have associated G1 arrest of bronchial epithelial cells with an RA-mediated decrease of cyclin D1 protein levels [266, 267] and RA-mediated differentiation of F9 embryonal carcinoma cells with a decrease in the protein levels of the transcriptional co-activator, p300 [330, 331]. Although the past two decades have focused on examining transcriptional mechanisms linked to the pleiotropic effects of RA, the importance of posttranslational modification of various proteins has been recently highlighted. RA has been shown to induce ubiquitination of a number of proteins, including cyclin D1 [232], p300 [330], and Skp2 [234], and IRS-1 is known to be conjugated by ubiquitin [90]. However, the ubiquitination of IRS-1 by RA has not been reported. Therefore, we tested the hypothesis that RA-mediated growth inhibition of breast cancer cells is associated with proteolytic degradation of IRS-1 by the ubiquitin-proteasome pathway.

3.3 MATERIALS AND METHODS

Reagents

All-trans-retinoic acid (RA), 9-cis-retinoic acid (9cRA), TTNPB, N-(4-hydroxyphenyl)retinamide (4-HPR), and 17-beta-estradiol (E2) were purchased from Sigma. Bexarotene was a generous gift of Ligand Pharmaceuticals, Inc. (San Diego,

CA). Recombinant human IGF-I was purchased from PeproTech (Princeton NJ). MG-132 (MG), 1,25-Dihydroxyvitamin D3, 15-deoxy-delta 12,14-prostaglandin J2, Rottlerin and GF109203X were purchased from Calbiochem. Protein G-agarose, and Nonidet-P40 (NP-40) were purchased from Sigma (Oakville, Canada). Enhanced chemiluminescence (ECL) detection system and Protein A-sepharose were purchased from Amersham Biosciences. The following antibodies (Abs) were used for immunoprecipitations: anti-C-terminal IRS-1 pAb (Upstate Biotechnology), anti-FLAG pAb (Sigma), anti-PKC-delta (δ) pAb (Santa Cruz Biotechnology). Serine phosphorylation was detected with an anti-phospho-serine pAb (Zymed). The following antibodies were used for western blotting: anti-IRS-1 pAb, anti-ubiquitin mAb (Santa Cruz Biotechnology), anti-HA-Peroxidase mAb (clone 12CA5, Roche), anti-phospho-IRS-1 (Ser^{307}) pAb (Upstate Biotechnology), anti-GRB2 mAb (Transduction laboratories), anti-c-Jun pAB (Santa Cruz), anti-phospho-PKC-δ (Thr^{505}) pAb (Cell Signaling), anti-PKC-δ pAb (Santa Cruz), and anti-GST mAb (Santa Cruz). Equal loading in western blotting experiments was assessed using either an anti-lamin B pAb (Santa Cruz) or anti-β-actin mAb (Sigma).

Cell maintenance and total cell lysate preparation

MCF-7 cells, ZR75.1 cells, and MDA-MB-468 cells were maintained in phenol red containing α-MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). T47-D cells and MDA-MB-231 cells were maintained in phenol red containing DMEM/F-12 (Life Technologies, Inc) supplemented with 10% FBS. When experiments were performed under serum-free conditions, cells at 70% confluence were washed twice with phosphate-buffered saline and changed to phenol red-free α-MEM supplemented with BSA and holo-transferrin (serum free media - SFM). When

experiments were performed in the presence of serum, cells were treated in the same media in which they were routinely maintained. For total cell lysate preparation, all cell lines were washed twice with cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.05% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Subsequent to incubation of lysate on ice for 30 minutes, the extracts were centrifuged at 13,000 *g* at 4° C for 30 min to remove insoluble material. After centrifugation, the protein content was measured by the Bradford assay using Bio-Rad reagents and BSA as standard.

Subcellular Fractionation

Following the treatment periods in DMSO or RA, MCF-7 cells were washed in PBS and incubated on ice for 10 minutes in Buffer 1 (10 mM Tris-Cl pH 7.8, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 300 mM sucrose, 0.5 mM DTT, plus protease and phosphatase inhibitors). 5 µl of 10% NP-40 was added to each sample and vortexed to lyse cells. The samples were centrifuged for 1 min at ~1000 rpm, and the supernatant collected (cytosolic fraction). The pellet was washed 3 times with washing buffer (50 mM NaCl, 10 mM Hepes, pH8, 25% glycerol, 0.1 mM EDTA, 100 mM DTT, plus protease and phosphatase inhibitors), and resuspended in Buffer 2 (20 mM Tris-Cl pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, plus protease and phosphatase inhibitors). Tubes were incubated on ice for 30 minutes followed by centrifugation for 15 minutes at 13,000 *g*; the supernatant represented the nuclear fraction. 20 µg of cytosolic and nuclear proteins were then subjected to SDS-PAGE and transferred to nitrocellulose membrane filters. The membranes were processed

for Western blotting of IRS-1, GRB-2 (for cytoplasmic purity), c-Jun (for nuclear purity), and actin (loading control).

Immunoprecipitation and Western Blotting

For western blotting, cell lysates were boiled for 3 minutes in 2X SDS sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% β-mercapto-ethanol, Bromophenol Blue) and resolved in 8-10% SDS-polyacrylamide gels (SDS-PAGE). For immunoprecipitation, 500 μg – 1mg of pre-cleared cell lysates were incubated with the indicated antibody overnight at 4 °C, followed by the addition of protein G-agarose overnight to collect immune complexes. The immunoprecipitates were washed in RIPA buffer, resuspended in SDS sample buffer, and boiled for five minutes. The solubilized proteins were resolved by SDS-PAGE. Proteins on the gel were transferred to nitrocellulose membrane (Bio-Rad) and detected by western blotting with the indicated antibody using ECL. Some membranes were stripped to prepare them for a second round of probing.

Ubiquitination of IRS-1

The ubiquitination of IRS-1 was examined by transfecting (using FuGENE, Roche) breast cancer cells with a FLAG-tagged human IRS-1 cDNA in an expression construct provided by Dr. Chris Sell, and a hemagglutinin (HA)-tagged ubiquitin cDNA in an expression construct provided by Dr. Dirk Bohmann [332]. 24 hours following transfection, cultures were washed twice with PBS and incubated in media containing 10 μM RA for 24 hours in the presence or absence of 10 μM MG132 for the last 12 hours of RA treatment. FLAG-tagged IRS-1 protein was immunoprecipitated using an anti-FLAG antibody (Sigma) and after separation of the immunoprecipitated proteins by SDS-

PAGE, the ubiquitinated IRS-1 was detected using an anti-HA-Peroxidase mAb (clone 12CA5, Roche).

Northern Blotting

Total cellular RNA was isolated using guanidinium thiocyanate extraction as previously described [333]. For Northern blotting, 20 μg of RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta probe (BioRad, Mississauga, Ontario, Canada) transfer membranes. cDNA probes were labelled by random priming (Amersham Biosciences). Hybridization and autoradiography was performed as previously described [334]. The full-length cDNA encoding IRS-1 was the generous gift of Dr. Khan (Joslin Diabetes Center).

RT-PCR

Total cellular RNA was isolated using guanidinium thiocyanate extraction as previously described [333]. The methods described by Morelli *et al.* were used for IRS-1 RT-PCR [335]. In brief, RNA (1 μg) was reverse transcribed (Superscript First Strand synthesis system - Gibco) and then amplified by PCR to obtain products corresponding to cDNA fragments of IRS-1. The following primers were used: IRS-1 upstream primer 5'-TCCACTGTGACACCAGAATAAT-3' and IRS-1 downstream primer 5'-CGCCAACATTGTTCATTCCAA-3'. The following PCR conditions were used for IRS-1: 1 min at 94°C, 1 min at 50°C, 2 min at 72°C. The amplification products obtained in 30 cycles were analyzed in a 1% agarose gel.

Pulse Analysis

To determine the synthesis rate of IRS-1, we followed the methods described by Zhang *et al.* with some modifications [92]. 24 hours after plating, MCF-7 cells were

treated with DMSO vehicle or 10 μM RA for 24 hours. Prior to labeling, the media was replaced with methionine-free RPMI media (Wisent Inc.) for 1 hour. During the last 12 hours of RA treatment, the cells were labeled with 100 μCi of [^{35}S]-methionine (Pro-mix, Amersham Biosciences) and cells were collected after 0, 4, 8, and 12-hour time intervals. Total cellular proteins were isolated and IRS-1 immunoprecipitated (as described above). The IRS-1 immunoprecipitates were subjected to SDS-PAGE, and following transfer to nitrocellulose filters, labeled IRS-1 was visualized by autoradiography.

Pulse-Chase Analysis

To determine the degradation rate of IRS-1, we followed the methods described by Zhang *et al.* with some modifications [92]. 24 hours after plating, MCF-7 cells were treated with either DMSO vehicle or 10 μM RA for 24 hours. Prior to labeling, the media was replaced with methionine-free RPMI media (Wisent Inc.) for 1 hour. The cells were then pulsed for 4 hours with 100 μCi of [^{35}S]-methionine (Pro-mix, Amersham Biosciences). Following the pulse, the cells were chased for 0, 4, 8, and 12 hours in 10% FBS containing media. After these chase times, total cellular proteins were isolated and processed as described above for the Pulse analysis experiments.

Immunofluorescence

One day after plating on coverslips, MCF-7 cells were treated with DMSO vehicle or 10 μM RA for 48 hours. For the last 12 hours of RA treatment, 10 μM MG-132 was added. Following the treatment period, the cells were fixed in 3% formaldehyde in PBS for 30 minutes. Next, the cells were permeablized in PBS containing 0.2% Triton X-100 for 5 minutes. The cells were then incubated for 3 hours with a rabbit anti-IRS-1 antibody (2 μg/ml), washed in PBS, and incubated for 1 hour with a rhodamine-conjugated donkey

anti-rabbit IgG secondary antibody. Following additional washes in PBS, the coverslips were incubated with 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes and mounted on glass slides in anti-fade medium. The images were then collected using a flourescence microscope.

In vitro kinase assay using histone H1

Cells were treated with RA or Rottlerin for the indicated times and then lysed in phosphorylation lysis buffer A (20 mM Tris-Cl pH7.5, 150 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1% NP-40, 1 mM sodium orthovanadate, 10 mM NaF, 2 mM sodium pyrophosphate, 1 mM PMSF). Cell lysates were immunoprecipitated with anti-PKC-δ antibody, followed by the addition of protein A-sepharose overnight to collect immune complexes. Immunoprecipitates were washed twice with lysis buffer A and twice with wash buffer B (25 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$). Protein A-sepharose beads were resuspended in 30 µl of kinase buffer (25 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 20 µg of phosphatidylserine, and 20 µM ATP) containing 5 µg of histone H1 (Sigma Type III) as an exogenous substrate, and [γ-^{32}P]ATP. The reaction was incubated for 30 minutes at room temperature and terminated by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE, and phosphorylated histone H1 was detected by autoradiography.

In vitro kinase assay using GST constructs

Cells and cell extracts were treated as described above (*In vitro* kinase assay using histone H1), however, in the kinase reaction, GST-IRS-1$^{288\text{-}678WT}$, GST-IRS-1$^{288\text{-}678MUT}$, or GST alone was used as an exogenous substrate. The GST constructs were a generous gift from Dr. R. Roth, and have been previously described in [81]. GST-IRS-1$^{288\text{-}678WT}$ or

GST were then purified from the reaction mix with GST-Sepharose beads, resolved by SDS-PAGE, and phosphorylated GST-IRS-$1^{288\text{-}678WT}$ was detected by autoradiography. Decayed membranes were then incubated with an anti-GST antibody to ensure equal pull-down of GST.

3.4 RESULTS

Regulation of IRS-1 protein levels in MCF-7 cells and other retinoid-responsive breast cancer cell lines.

Consistent with our previous findings, exposure of MCF-7 cells to 1 μM RA for 72 hours in serum free media (SFM) results in a dose-dependant decrease in IRS-1 protein levels (Figure 3.1a) [240]. Previous studies have shown that antiestrogens and glucocorticoids alter IRS-1 levels [97, 98, 327, 328], thus we investigated whether other nuclear receptor-selective ligands would induce a similar decrease in IRS-1 levels. As expected, treatment of MCF-7 cells with Tamoxifen and ICI 182780 results in decreased IRS-1 levels (Figure 3.1c). We also observed a decrease with RAR-α (TTNPB), RXR-selective ligands (LGD1305 and Bexarotene), and N-(4-hydroxyphenyl)retinamide (4-HPR) (Figure 3.1b), while no significant effect was observed after treatment with the vitamin D3 selective ligand (1,25-Dihydroxyvitamin D3) or the PPAR-selective ligand (15-deoxy-delta 12,14-prostaglandin J2) for 3 days (Figure 3.1c).

Although the mechanism of RA-mediated growth inhibition is unclear, it has generally been found that RA inhibits the growth of breast cancer cells that are ER-positive (MCF-7, T47-D, ZR75.1), while having minimal effects on breast cancer cells that are ER-negative (MDA-MB-231, MDA-MB-468) [160, 182]. We hypothesized that regulation of IRS-1 levels by RA would correlate with RA-mediated growth inhibition of

breast cancer cells. We treated RA-sensitive and RA-resistant breast cancer cell lines with 1 μM RA for 24, 48 and 72 hours in SFM. In support of our hypothesis, the RA-sensitive cells responded to 1 μM RA treatment with a similar decrease in IRS-1 protein levels as MCF-7 cells, while the two RA-resistant cell lines did not (Figure 3.1d).

RA induces a posttranslational modification of IRS-1 in MCF-7 cells.

Previous studies have suggested that IRS-1 can be regulated at the transcriptional level [94, 95, 336]. MCF-7 cells were treated with 10 μM RA for 24, 48 and 72 hours, total RNA was extracted and IRS-1 mRNA expression was examined by Northern blot, wherein the 8.5 kb transcript is shown (Figure 3.2a, top panel). Using RT-PCR, we observed no change in IRS-1 mRNA expression at any of the time points examined in the presence of 1 or 10 μM RA, however, ICI 182780 was shown to decrease IRS-1 mRNA levels, consistent with previously published reports [98] (Figure 3.2a, bottom panel).

Recently, IRS-1 has been shown to translocate to the nuclei in various cellular backgrounds stimulated with insulin or IGF-I [337-339]. Since the regulation of IRS-1 nuclear/cytoplasmic trafficking is not a well understood process, we examined whether the RA-mediated decrease in IRS-1 protein levels might involve the regulation of IRS-1 localization. We prepared cytosolic and nuclear extracts from MCF-7 cells grown in serum-free medium with or without 1 μM RA for 72 hours. Under these conditions, there did not appear to be any redistribution of IRS-1 into the nucleus (Figure 3.2b).

Based on these results, we hypothesized post-transcriptional effect of RA on the synthesis or degradation rate of IRS-1. We first examined whether the decline in IRS-1 protein in MCF-7 cells treated with RA results from a decrease in the rate of IRS-1 synthesis. Using pulse-labeling with [^{35}S]-methionine, we measured the synthesis rate of

IRS-1 in MCF-7 cells pre-treated with RA. The rate of IRS-1 synthesis was similar in cells treated with DMSO vehicle or RA, suggesting that this is not the mechanism by which RA alters IRS-1 protein levels (Figure 3.2c). We then measured the rate of degradation of IRS-1 in the presence of DMSO or RA by pulse-chase analysis and found that RA-treated MCF-7 cells showed an accelerated rate of IRS-1 degradation compared to those cells treated with DMSO alone (Figure 3.2d). This suggests that RA activates a proteolytic pathway in MCF-7 cells that may be responsible for the degradation of IRS-1.

Effects of proteasome and protease inhibitors on degradation of IRS-1 by RA in breast cancer cells.

Previous studies have reported that the levels of IRS-1 can be regulated by IGF-I and insulin at the protein level via the ubiquitin-proteasome pathway [90-92, 319]. We therefore tested whether the effects of RA on IRS-1 were mediated via a similar mechanism. The proteasome inhibitors lactacystin and MG-132 are reagents that inhibit the activity of the 26S proteasome, causing the accumulation of ubiquitinated proteins otherwise degraded by the ubiquitin-proteasome pathway. As shown in Figure 3.3a the decrease in IRS-1 levels in MCF-7 cells treated with 10 μM RA for 48 hours was rescued by the addition of lactacystin to the culture medium for the last 12 hours of RA treatment. Similarly, MG-132 reversed the RA-mediated decline in IRS-1 levels (Figure 3.3b). Cyclin D1 is a protein known to be regulated by RA-mediated ubiquitination and subsequent proteolysis [232, 234, 267]. In Figure 3.3b, we show a similar rescue of cyclin D1 levels by MG-132 in RA-treated MCF-7 cells.

We next assessed whether IRS-1 levels could be restored by the addition of proteasome inhibitors in other RA-sensitive breast cancer cells. In the retinoid-

responsive breast cancer cell lines T47-D and ZR75.1, we added MG-132 for the last 12 hours of a 48-hour 10 μM RA treatment and observed a restoration of IRS-1 levels (Figure 3.3c). We further demonstrate the effect of RA on IRS-1 by immunoflourescence in T47-D cells (Figure 3.3d). Consistent with our western blot data, treatment with RA for 48 hours decreased IRS-1 protein staining, and treatment with MG-132 for the last 12 hours of RA treatment blocked this effect. It has been reported that IRS-2 is regulated at the post-translational level via increased protein-ubiquitination [335, 340], however we failed to observe any regulation in the protein expression of IRS-2 in any of the breast cancer cell lines treated with RA (data not shown). Although MG-132 is an inhibitor of the proteasome, it also inhibits calpains, calcium-activated cysteine proteases. Furthermore, some studies have shown that IRS-1 degradation can be mediated via calpains [341], so we assessed whether RA induces a decline in IRS-1 levels via a mechanism involving calpains. In our system, the addition of the cell-permeable calpain inhibitor, calpeptin, could not restore IRS-1 levels (data not shown). This suggests that RA does not decrease IRS-1 levels via activation of a calpain-dependent pathway, but rather it may be increasing proteasomal activity.

RA Enhances Ubiquitination of IRS-1 in breast cancer cells.

Proteasomal degradation of proteins involves the prior conjugation of ubiquitin to the targeted protein. We examined whether ubiquitin-IRS-1 complexes could be formed in breast cancer cells treated with RA. IRS-1 immunoprecipitation and ubiquitin western blotting revealed that the level of ubiquitin-IRS-1 conjugates is augmented in MCF-7 cells treated with RA and MG-132 compared to either agent alone (Figure 3.4a). This result was supported by co-transfection experiments using expression vectors for flag-

IRS-1 and HA-Ub in MCF-7 cells. The cells were transfected with both vectors and treated with either 10 μM RA or 10 nM IGF-I for 24 hours, with or without the addition of MG-132 for the last 12 hours of RA treatment. To show that IRS-1-ubiquitin conjugates bound more effectively in the presence of RA, these cells were lysed and the lysate immunoprecipitated with anti-flag antibodies and immunoblotted with anti-HA antibodies (Figure 3.4b). RA and MG-132 induced a marked increase in the association of flag-IRS-1 and Ha-Ub compared to MG-132 alone (Figure 3.4b, compare lanes 3 and 5). Furthermore, consistent with prior reports, IGF-I and MG-132 also augmented the level of detectable IRS-1-Ub conjugates in MCF-7 cells compared to MG-132 alone (Figure 3.4b, compare lanes 3 and 6) [90]. In two other RA-sensitive breast cancer cell lines, we found a similar dramatic increase in IRS-1-ubiquitin conjugates upon treatment with RA and MG-132 (Figure 3.4c). Taken together, these results show that RA enhances the ubiquitination of IRS-1.

Inhibiting the PKC pathway blocks the RA-mediated phosphorylation and degradation of IRS-1.

Recent studies have shown that serine/threonine phosphorylation of IRS-1 signals its degradation, suggesting a requirement for the activation of specific kinases to regulate the levels of IRS-1. In support of this hypothesis, we find that RA-treated MCF-7 cells grown in 10 % FBS have a greater and more rapid decrease in IRS-1 protein levels than when grown in serum free media (Figure 3.5a). Consistent with the activation of a serine/threonine kinase in breast cancer cells treated with RA, we observe an increase in the total serine phosphorylation of IRS-1 (Figure 3.5b). Based on these data, we investigated the role of specific signaling pathways in mediating the RA-induced decline

in IRS-1 protein levels. Using a chemical inhibitor approach, the breast cancer cell lines, MCF-7, T47-D, and ZR75.1, were pre-treated for 60 minutes with inhibitors of the MAPK (PD98059) and PKC (Rottlerin and GF109203X) signaling cascades and then treated for an additional 48 hours with RA in the presence of the inhibitors. The decrease in IRS-1 protein levels could be inhibited in all of the breast cancer cells by treatment with Rottlerin and GF109203X, but not with PD98059 (Figure 3.5c). Consistent with this result, we also observed a block in the RA-mediated increase in serine phosphorylation of IRS-1 when the breast cancer cells were co-treated with Rottlerin (Figure 3.5d). These results suggest that a PKC-sensitive pathway is involved in the RA-mediated decline in IRS-1 levels.

In vitro phosphorylation of IRS-1 by RA-activated PKC-δ in retinoid-sensitive breast cancer cells.

The PKC family of serine/threonine kinases are involved in signaling pathways controlling cell growth, transformation, and differentiation [342]. There are at least 10 PKC isoforms, however, Kambhampati *et al.* recently identified PKC-δ as a selective target of RA [343]. We thus asked whether RA would activate PKC-δ in the breast cancer cell lines used in this study. We found that RA potently induces the phosphorylation of the threonine 505 residue in PKC-δ (Figure 3.6a). To directly measure if this increase in phosphorylation corresponded to increased activation of PKC-δ, we performed an *in vitro* kinase assays using histone H1 as a substrate. Figure 3.6b shows a clear increase in kinase activity with a time course of activation that is consistent with that observed for the RA-mediated decline in IRS-1 protein levels (compare, Figure 3.6b with Figure 3.5b). There was no change in PKC-δ activity in the RA-resistant breast

cancer cell line MDA-MB-231, suggesting a tight correlation between activation of PKC-δ by RA and regulation of IRS-1 levels (Figure 3.6c). We confirm that treatment of the cells with 0.5 μM Rottlerin abrogates the RA-induced activity of PKC-δ (Figure 3.6d, lower panel), consistent with the ability of this inhibitor to rescue the decrease in IRS-1 levels in the RA-sensitive breast cancer cell lines. In addition, we show in Figure 3.6e that 0.5 μM Rottlerin also blocks basal PKC-δ activity.

Recently Greene *et al.* [81] identified 18 PKC-δ serine/threonine phosphorylation sites on IRS-1. When three of these sites, serine 307, serine 323, and serine 574, were mutated to alanines, the phosphorylation of IRS-1 in response to activated PKC-δ was significantly decreased. To examine whether activation of PKC-δ by RA increases phosphorylation of IRS-1 on these sites, we performed an *in vitro* kinase assay using the wild-type human IRS-1–GST-fusion protein (GST-IRS-$1^{288\text{-}678WT}$) or the GST-IRS-$1^{288\text{-}678}$ with serine 307, serine 323, and serine 574 mutated to alanines (GST-IRS-$1^{288\text{-}678MUT}$) as a substrate. Our results show that PKC-δ immunoprecipitated from RA-treated breast cancer cells can phosphorylate the GST-IRS-$1^{288\text{-}678WT}$ construct, and that this phosphorylation event is abrogated by co-incubation with the PKC-δ inhibitor Rottlerin (Figure 3.7b, left panel). In addition, when the three putative PKC-δ serine sites are mutated to alanines no RA-induced phosphorylation of the GST-IRS-$1^{288\text{-}678MUT}$ construct is observed (Figure 3.7b, right panel). The possibility that this phosphorylation may result from an interaction between IRS-1 and PKC-δ in the presence of RA is supported by our finding that IRS-1 co-immunoprecipitates with PKC-δ in ZR75.1 cells (and T47-D, data not shown) treated with 1 μM RA (Figure 3.7c).

3.5 DISCUSSION

Although immediate/early retinoid signaling events are initiated via transcriptional activation of retinoid receptors, later retinoid signaling events can occur through the posttranslational regulation of proteins. Considerable attention has been given in recent years to identifying retinoid-regulated proteins to gain insight into the mechanisms involved in their growth inhibitory and differentiating effects [344]. There is indeed a growing list of proteins known to be regulated by RA via mechanisms involving the ubiquitin-proteasome pathway: cyclin D1 [232], PML/RAR alpha [320], RAR alpha and RAR gamma [238], CDK-4 [237], p300 [330], and Skp2 [234]. Our findings support the addition of IRS-1 to this list. Using breast cancer cell lines as a model, we are the first to show that RA regulates IRS-1 protein levels through a posttranslational mechanism involving the ubiquitin-proteasome pathway. Moreover, we find that PKC inhibitors rescue the RA-mediated loss in IRS-1 protein, and that RA-mediated activation of PKC-δ phosphorylates IRS-1 *in vitro*.

The proteasomal degradation of proteins requires prior binding of ubiquitin to the target protein via three successive reactions. First, the ubiquitin-activating enzyme, E1, activates ubiquitin in an ATP-dependant reaction. Secondly, the ubiquitin molecule is transferred from E1 to the ubiquitin conjugating enzyme, E2. Thirdly, ubiquitin is transferred from E2 to the substrate-specific ubiquitin ligase, E3. In this paper we show an increased level of IRS-1-ubiquitin conjugates in the presence of RA in MCF-7 cells when proteasomal activity is blocked using MG-132, and confirm this result with co-transfection experiments using flag-IRS-1 and HA-Ub constructs in the three RA-sensitive breast cancer cell lines. We observed that the RA-mediated increase in the level

of IRS-1-Ub conjugates in MCF-7 cells (Figure 3.4b) is comparable to that previously reported in IGF-I-induced proteasomal degradation of IRS-1 [90]. Interestingly, we did not observe a decrease in the levels of IRS-2 protein when any breast cancer cell line was treated with RA, suggesting that a specific motif in IRS-1 is responsible for the selective degradation of this protein by RA. It is believed that substrate-specificity of the ubiquitin-proteasome system is due to the specific E3, however, the E3 responsible for the degradation of IRS-1 remains unknown. One can speculate that this E3 may be an SCF ligase complex, since all identified SCF complexes target phosphorylated substrates, and prior studies have shown that IRS-1 needs to be phosphorylated on serine residues prior to its recognition by the ubiquitin machinery [89, 345, 346].

Our observation that RA decreases the levels of IRS-1 more rapidly in the presence of serum than in serum free media (Figure 3.6a) suggested that an activated signaling pathway was involved in the degradation of IRS-1. Consistent with the idea that phosphorylated-IRS-1 is targeted for ubiquitination, we show that RA induces total serine phosphorylation of IRS-1. We used a chemical inhibitor approach to begin to clarify the signals generated that target IRS-1 for ubiquitination. We found that PKC inhibitors can rescue the RA-mediated loss in IRS-1 protein in the three RA-sensitive breast cancer cell lines examined. The activation of a number of PKC isoforms, including PKC-δ [81], has been linked to serine phosphorylation of IRS-1, and interestingly, Greene *et al.* mentioned an observed decrease in IRS-1 levels when CHO cells were transfected with a constitutively active PKC-δ construct, suggesting the involvement of this kinase in the regulation of IRS-1 protein levels. Our data supports such a role of PKC-δ: 1) RA stimulates PKC-δ activity (Figure 3.6b), 2) RA-activated

PKC-δ can phosphorylate IRS-1 *in vitro*, and this is blocked by the PKC-δ inhibitor Rottlerin (Figure 3.7b, left panel) or by mutation of three critical PKC-δ serine sites in IRS-1 (Figure 3.7b, right panel), and 3) PKC-δ and IRS-1 interact in the presence of RA (Figure 3.7c). Consistent with the inability of RA to regulate IRS-1 levels in the RA-resistant MDA-MB-231 breast cancer cell line, we also failed to observe any regulation of PKC-δ in these cells. Although activating PKC has not been previously reported to be required for the ubiquitination of IRS-1, the phosphorylation of p53 by PKC was shown to regulate p53 degradation by stimulating its ubiquitination [347]. Interestingly, three of the serine sites (serines 307, 323, and 574) phosphorylated by PKC-δ lie within the C-terminal domain of IRS-1 that contains potential PEST sequences, as identified by a PEST-FIND program (http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/) (Figure 3.7a). PEST sequences are rich in proline (P), glutamate (E), serine (S) and threonine (T) residues and frequently serve as signals for proteolytic degradation [348]. It is tempting to speculate that PKC-δ-mediated phosphorylation of these three serine sites may induce a conformational change in the C-terminal domain of IRS-1 that enables unmasking of these PEST domains and subsequent recognition by some component of the ubiquitin machinery. We thus propose a model of RA-mediated IRS-1 degradation whereby RA first activates PKC-δ, which in turn phosphorylates IRS-1 on serine residues, allowing for its subsequent recognition by the ubiquitin machinery.

Breast cancer cells exhibit elevated levels of known ubiquitinated proteins, such as cyclin D1 [349], cyclin E [350], and IRS-1 [6]. Although the ubiquitin ligase, or E3, for cyclin D1 and IRS-1 remain unknown, future identification of these ligases, may lead to the development of compounds that specifically activate E3, thus inhibiting the

accumulation of cyclin D1 and IRS-1 in breast tumors where these proteins are stabilized. A possible novel chemotherapeutic approach for the treatment of breast tumors may be to combine RA with activation of specific E3s to target oncogenic proteins.

3.6 ACKNOWLEDGEMENTS

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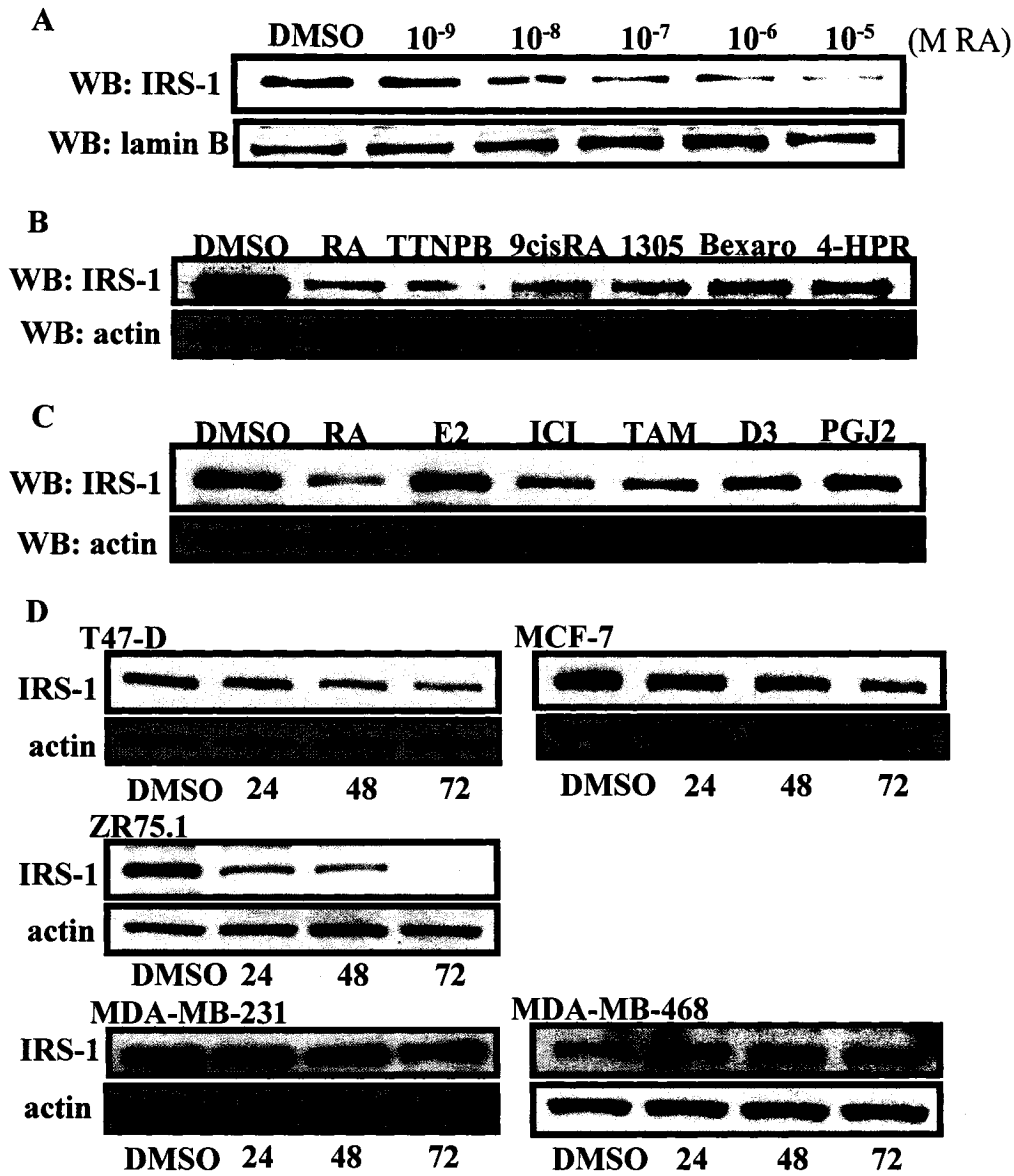
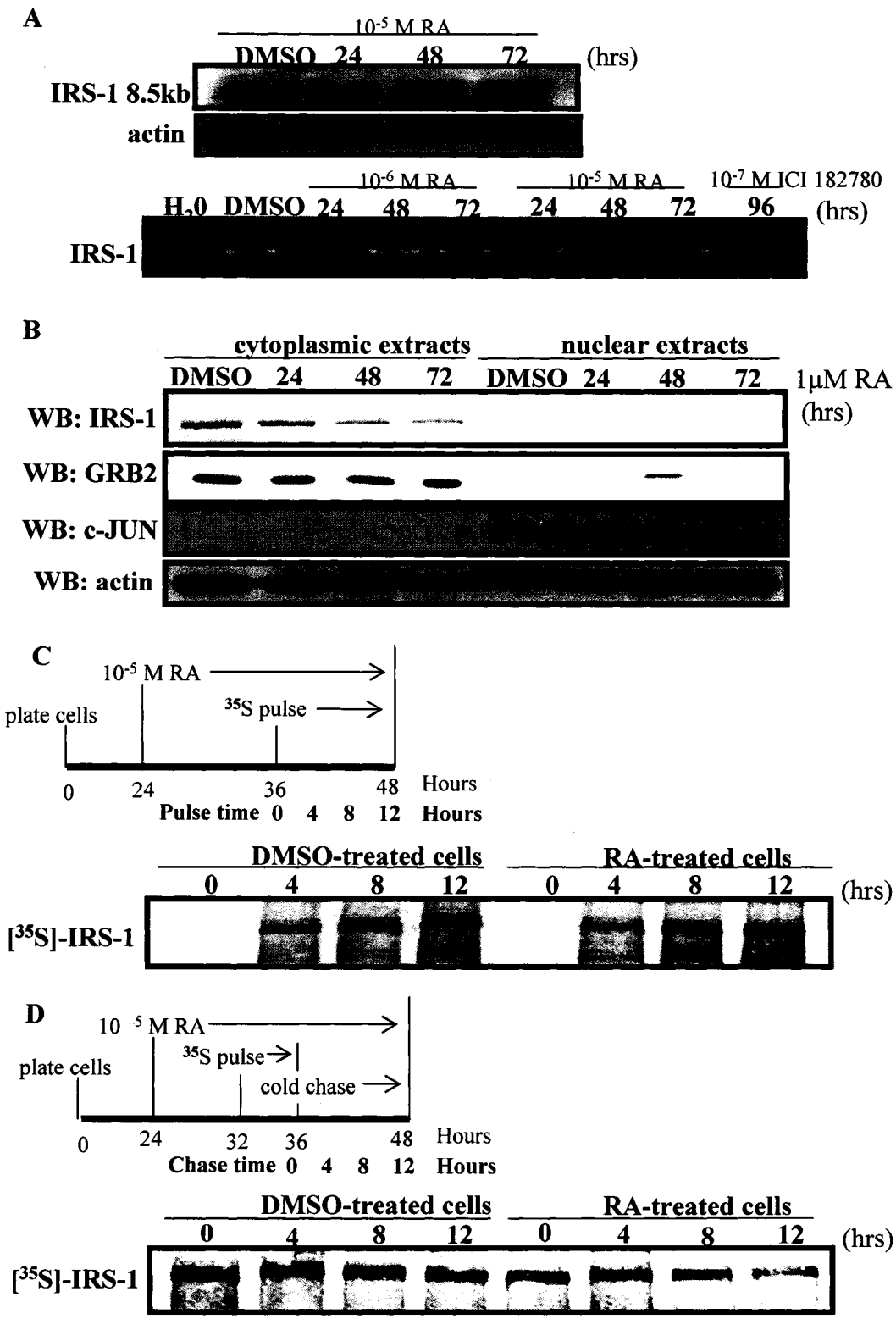


Figure 3.1 - IRS-1 protein regulation in breast cancer cell lines.

Western blotting (WB) was used to determine the expression level of IRS-1 in (A)-(D). (A) MCF-7 cells were treated for 72 hours in serum free media (SFM) with RA. Lamin B was used as a loading control. (B) MCF-7 cells were treated with: TTNPB, 9cisRA, LGD1305 (1305), Bexarotene (Bexaro), N-(4-hydroxyphenyl)retinamide (4-HPR) for 72 hours in SFM. (C) MCF-7 cells were treated with: 17-beta-estradiol (E2), Tamoxifen (Tam), ICI 182780 (ICI), 1,25-Dihydroxyvitamin D3 (D3), and 15-deoxy-delta 12,14-prostaglandin J2 (PGJ2) for 72 hours in SFM. β-actin (actin) was used as a loading control. (D) T47-D, MCF-7, ZR75.1, MDA-MB-231, MDA-MB-468 breast cancer cells were treated with 1μM RA for 24, 48 and 72 hours in SFM.

Figure 3.2 - RA induces a posttranslational modification of IRS-1 in MCF-7 cells.

(A) MCF-7 cells were treated with 10^{-5} M RA in SFM for 24, 48 or 72 hours and the expression of IRS-1 mRNA was evaluated by Northern blot (top panel). Actin was used to ensure equal loading. RT-PCR was used to detect IRS-1 levels in MCF-7 cells treated with 10^{-6} M or 10^{-5} M RA, or 10^{-7} M ICI 182780 (bottom panel). All data are representative of four independent experiments. (B) Subcellular fractionation was used to examine the expression of IRS-1 in cytoplasmic and nuclear protein lysates obtained from MCF-7 cells treated with RA for 24, 48, and 72 hours. To control for the purity of the fractions, the levels of a nuclear protein (c-Jun), and a cytoplasmic protein (Grb2) were assessed by stripping and reprobing membranes. (C) The synthesis rate of IRS-1 in MCF-7 cells treated for 24 hours in the presence of DMSO or 10^{-5} M RA was determined by pulse labeling with [^{35}S]-methionine. IRS-1 abundance was analyzed at 0, 4, 8, and 12 hour time intervals after the pulse. (D) The degradation rate of IRS-1 in MCF-7 cells treated for 24 hours in the presence of DMSO or 10^{-5} M RA was determined by pulse-chase analysis with [^{35}S]-methionine. IRS-1 abundance was analyzed at 0, 4, 8, and 12 hour time intervals after the chase.


A
10-5 M RA
DMSO 24 48 72 (hrs)
IRS-1 8.5kb
actin
10-6 M RA
10-5 M RA
10-7 M ICI 182780
H2O DMSO 24 48 72 24 48 72 96 (hrs)
IRS-1
B
cytoplasmic extracts
nuclear extracts
DMSO 24 48 72 DMSO 24 48 72 1μM RA
(hrs)
WB: IRS-1
WB: GRB2
WB: c-JUN
WB: actin
C
10-5 M RA
plate cells
35S pulse
0 24 36 48 Hours
Pulse time 0 4 8 12 Hours
DMSO-treated cells
RA-treated cells
0 4 8 12 0 4 8 12 (hrs)
[35S]-IRS-1
D
10-5 M RA
plate cells
35S pulse
cold chase
0 24 32 36 48 Hours
Chase time 0 4 8 12 Hours
DMSO-treated cells
RA-treated cells
0 4 8 12 0 4 8 12 (hrs)
[35S]-IRS-1

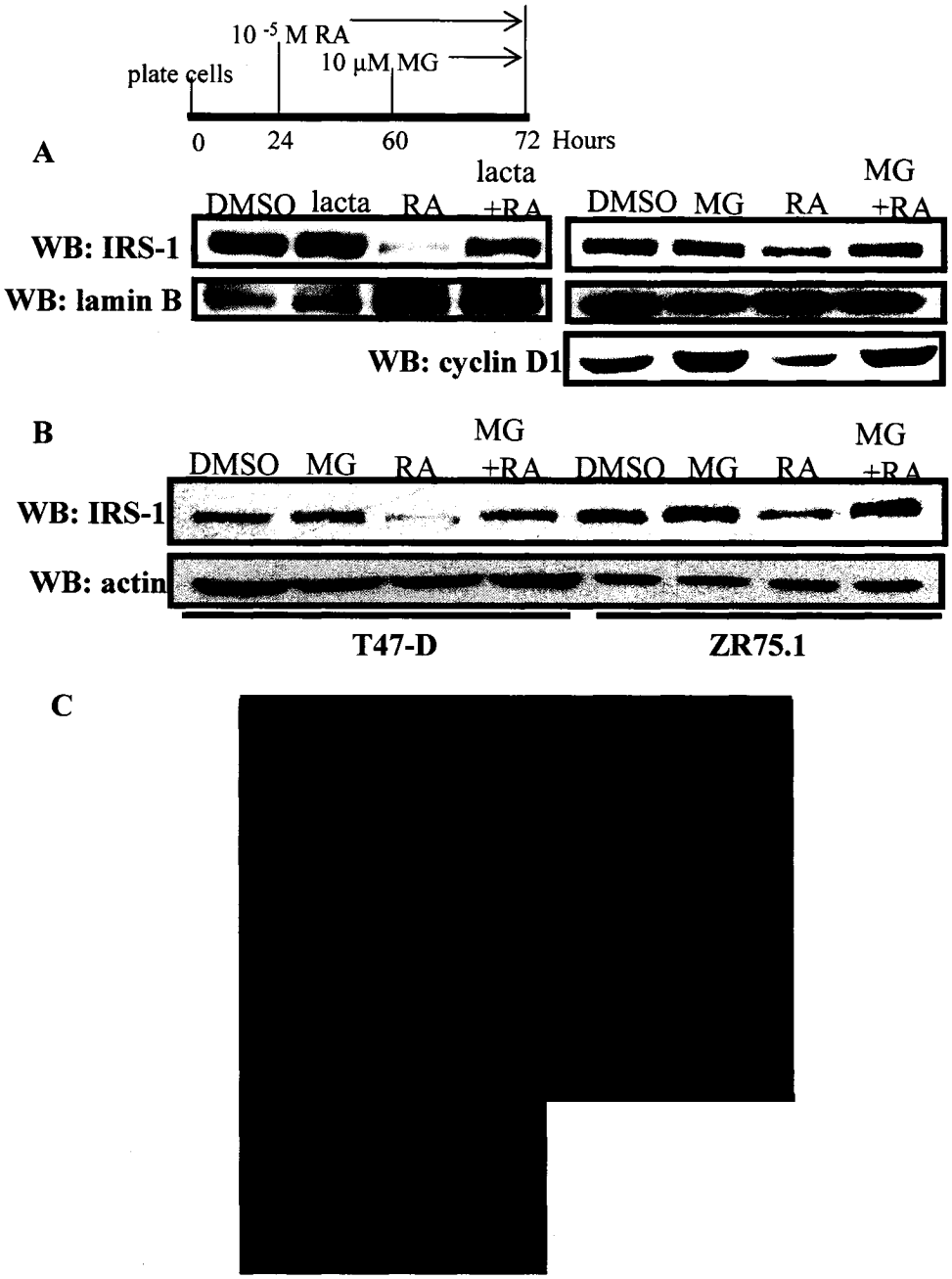


Figure 3.3 - Proteasome inhibitors block the RA-mediated decrease in IRS-1 protein.

WB was used to determine the expression level of IRS-1 in (A) and (B). (A) MCF-7 cells were treated with DMSO or 10^{-5} M RA for 48 hours in SFM. 10^{-5} M Lactacystin (lacta) or MG-132 (MG) was added for the last 12 hours of RA treatment. Cyclin D1 levels were detected by WB. (C) T47-D and ZR75.1 cells were treated as in (A). (C) T47-D cells were treated as in (A). Immunoflourescence was used to detect IRS-1 proteins (red stain) in cytoplasm of T47-D cells. DAPI staining revealed the nucleus (blue stain).

Figure 3.4 - RA Enhances Ubiquitination of IRS-1 in breast cancer cells.

(A) MCF-7 cells were treated with DMSO or 10^{-5} M RA for 48 hours in SFM. 10^{-5} M MG-132 was added for the last 12 hours of RA treatment. IRS-1 was immunoprecipitated (IP) from protein lysates, and the level of ubiquitination was evaluated by WB with an anti-ubiquitin (Ub) antibody. The same membrane was stripped and reprobed for IRS-1. (B) MCF-7 cells were transiently transfected with HA-Ub and flag-IRS-1 constructs. Cells were then treated in SFM for 24 hours with 10nM IGF-I or 10^{-5} M RA. 10-5 M MG-132 was added for the last 12 hours of RA or IGF-I treatment. FLAG was immunoprecipitated (IP) from protein lysates, and the level of flag-IRS-1/HA-Ub interaction was evaluated by WB with an anti-HA (HA) antibody. The same membrane was stripped and reprobed for IRS-1. (C) T47-D and ZR75.1 cells were transfected and treated as described in (B).

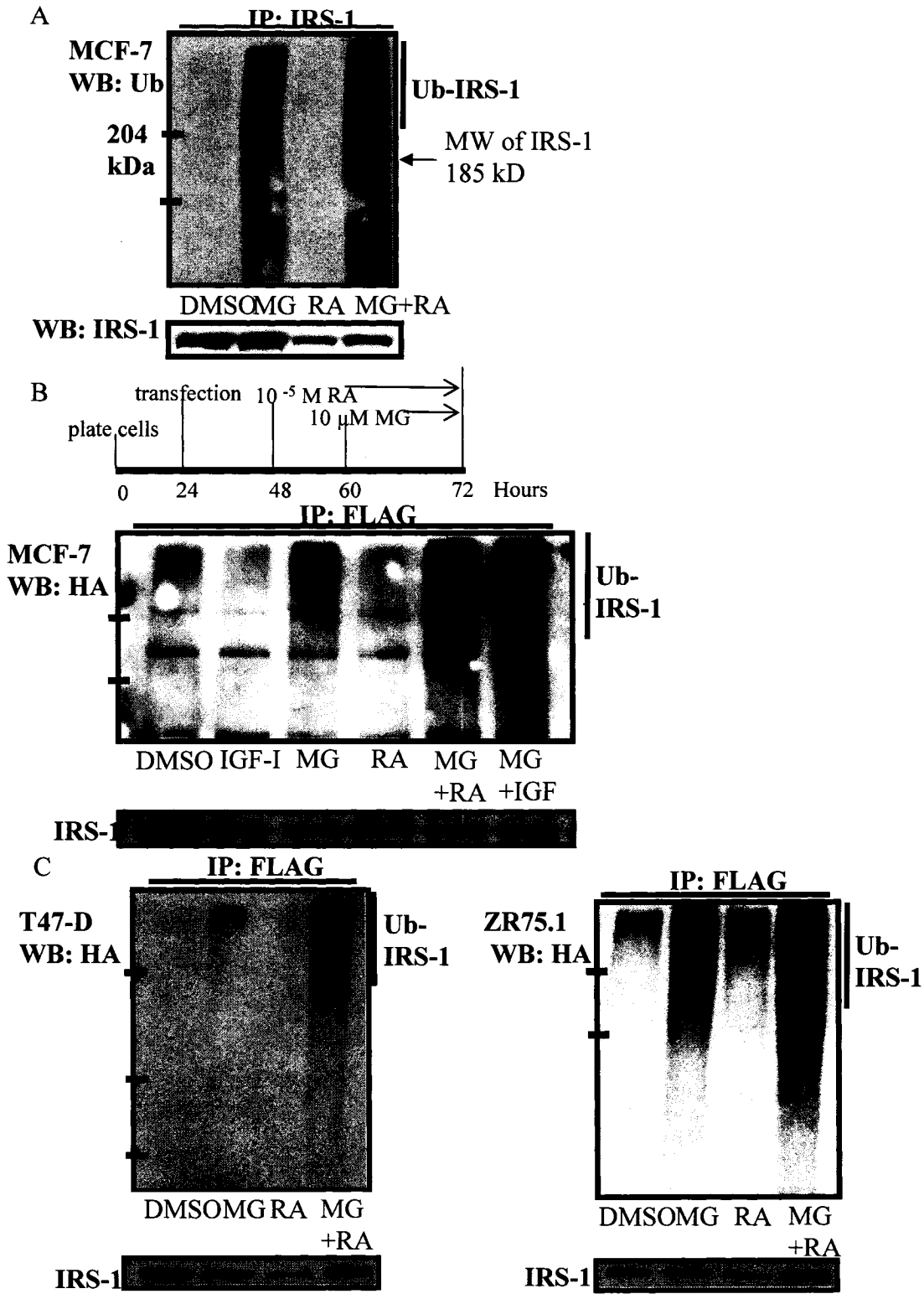

A
IP: IRS-1
MCF-7
WB: Ub
Ub-IRS-1
204
kDa
MW of IRS-1
185 kD
DMSO MG RA MG+RA
WB: IRS-1
B
transfection
10 -5 M RA
10 μM MG
plate cells
0 24 48 60 72 Hours
IP: FLAG
MCF-7
WB: HA
Ub-
IRS-1
DMSO IGF-I MG RA MG +RA MG +IGF
IRS-1
C
IP: FLAG
T47-D
WB: HA
Ub-
IRS-1
DMSO MG RA MG +RA
IRS-1
IP: FLAG
ZR75.1
WB: HA
Ub-
IRS-1
DMSO MG RA MG +RA
IRS-1

Figure 3.5 - PKC inhibitors block the RA-mediated phosphorylation and degradation of IRS-1.

WB was used to determine the expression level of IRS-1 in (A)-(C). (A) MCF-7 cells were treated with 1 μM RA for 24, 48 and 72 hours in SFM or 10% fetal bovine serum (FBS). (B) Top panel: MCF-7 cells were treated with 1 μM RA for the times indicated in 10% FBS. Lower panel: The total serine phosphorylation status of IRS-1 was determined by immunoprecipitating (IP) IRS-1 from MCF-7 protein lysates and immunoblotting with an anti-phosphoserine (p-serine) antibody. (C) MCF-7 and T47-D cells were pre-treated for 60 minutes with inhibitors of the MAPK (25 μM PD98059) and PKC (0.5 μM Rottlerin and 1 μM GF109203X) signaling cascades and then treated for an additional 48 hours with 1 μM RA in the presence of the inhibitors. (D) ZR75.1 cells were treated as in (C), and the serine phosphorylation status of IRS-1 was determined as in (B).

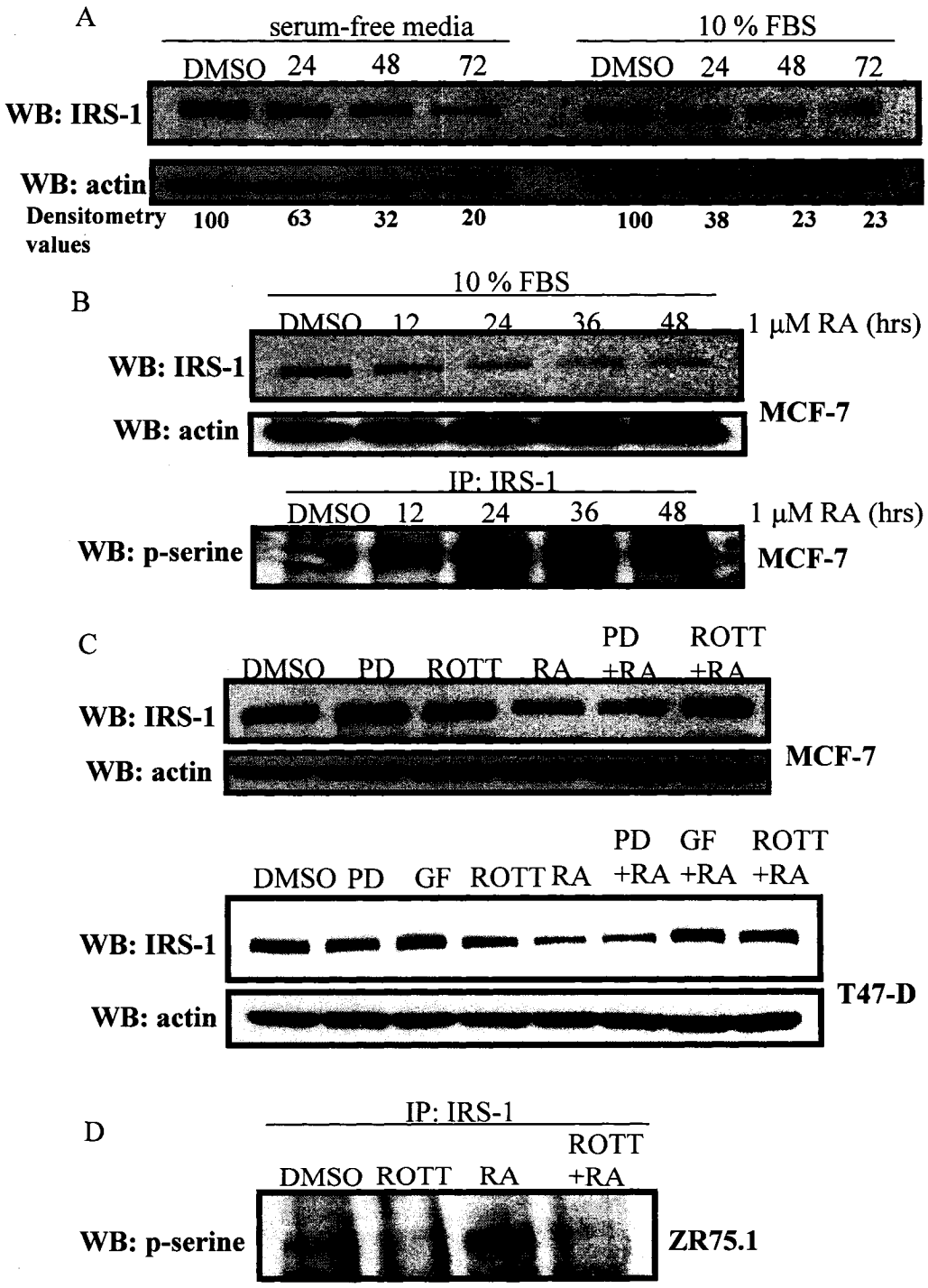
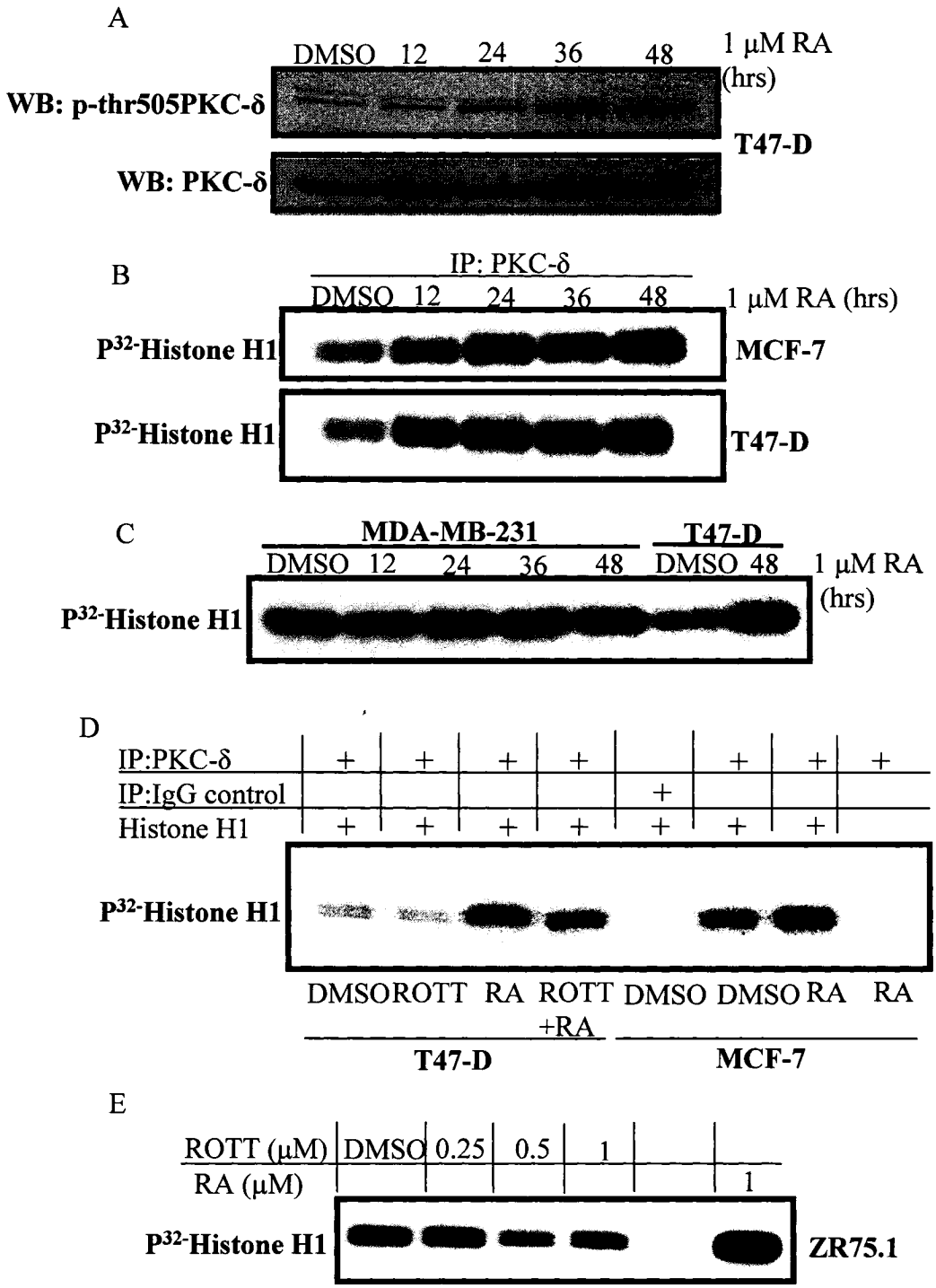

A
serum-free media
10 % FBS
DMSO 24 48 72
DMSO 24 48 72
WB: IRS-1
WB: actin
Densitometry values
100 63 32 20
100 38 23 23
B
10 % FBS
DMSO 12 24 36 48
1 μM RA (hrs)
WB: IRS-1
WB: actin
MCF-7
IP: IRS-1
DMSO 12 24 36 48
1 μM RA (hrs)
WB: p-serine
MCF-7
C
DMSO PD ROTT RA PD +RA ROTT +RA
WB: IRS-1
WB: actin
MCF-7
DMSO PD GF ROTT RA PD +RA GF +RA ROTT +RA
WB: IRS-1
WB: actin
T47-D
D
IP: IRS-1
DMSO ROTT RA ROTT +RA
WB: p-serine
ZR75.1

Figure 3.6 – RA activates PKC-δ in retinoid-sensitive breast cancer cells.

(A) T47-D cells were treated with 1μM RA for 12, 24, 36, and 48 hours in 10% FBS. Western blot was used to determine the phosphorylation status of anti-PKC-δ at threonine 505 (p-thr505-PKC-δ). This membrane was then stripped and reprobed with an antibody against total PKC-δ. (B) MCF-7 and T47-D cells were treated with 1 μM RA for the time indicated. Lysates were immunoprecipitated (IP) with an anti-PKCδ antibody, and immunoprecipitates were subjected to an *in vitro* kinase assay using histone H1 as an exogenous substrate. Phosphorylated histone H1 was detected by autoradiography. (C) The *in vitro* kinase assay using MDA-MB-231 cell extracts was performed as in (B). (D) T47-D cells were pre-treated for 60 minutes with 0.5 μM Rottlerin and then treated for an additional 36 hours with 1 μM RA in the presence of the inhibitor. The *in vitro* kinase assay was performed as in (B). (E) ZR75.1 cells were treated with 0.25 μM, 0.5 μM, or 1 μM Rottlerin for 36 hours, and the *in vitro* kinase assay was performed as in (B).


A
DMSO 12 24 36 48
1 μM RA (hrs)
WB: p-thr505PKC-δ
T47-D
WB: PKC-δ
B
IP: PKC-δ
DMSO 12 24 36 48
1 μM RA (hrs)
P32-Histone H1
MCF-7
P32-Histone H1
T47-D
C
MDA-MB-231
T47-D
DMSO 12 24 36 48 DMSO 48
1 μM RA (hrs)
P32-Histone H1
D
IP:PKC-δ + + + + + + + +
IP:IgG control +
Histone H1 + + + + + + +
P32-Histone H1
DMSO ROTT RA ROTT +RA DMSO DMSO RA RA
T47-D
MCF-7
E
ROTT (μM) DMSO 0.25 0.5 1
RA (μM) 1
P32-Histone H1
ZR75.1

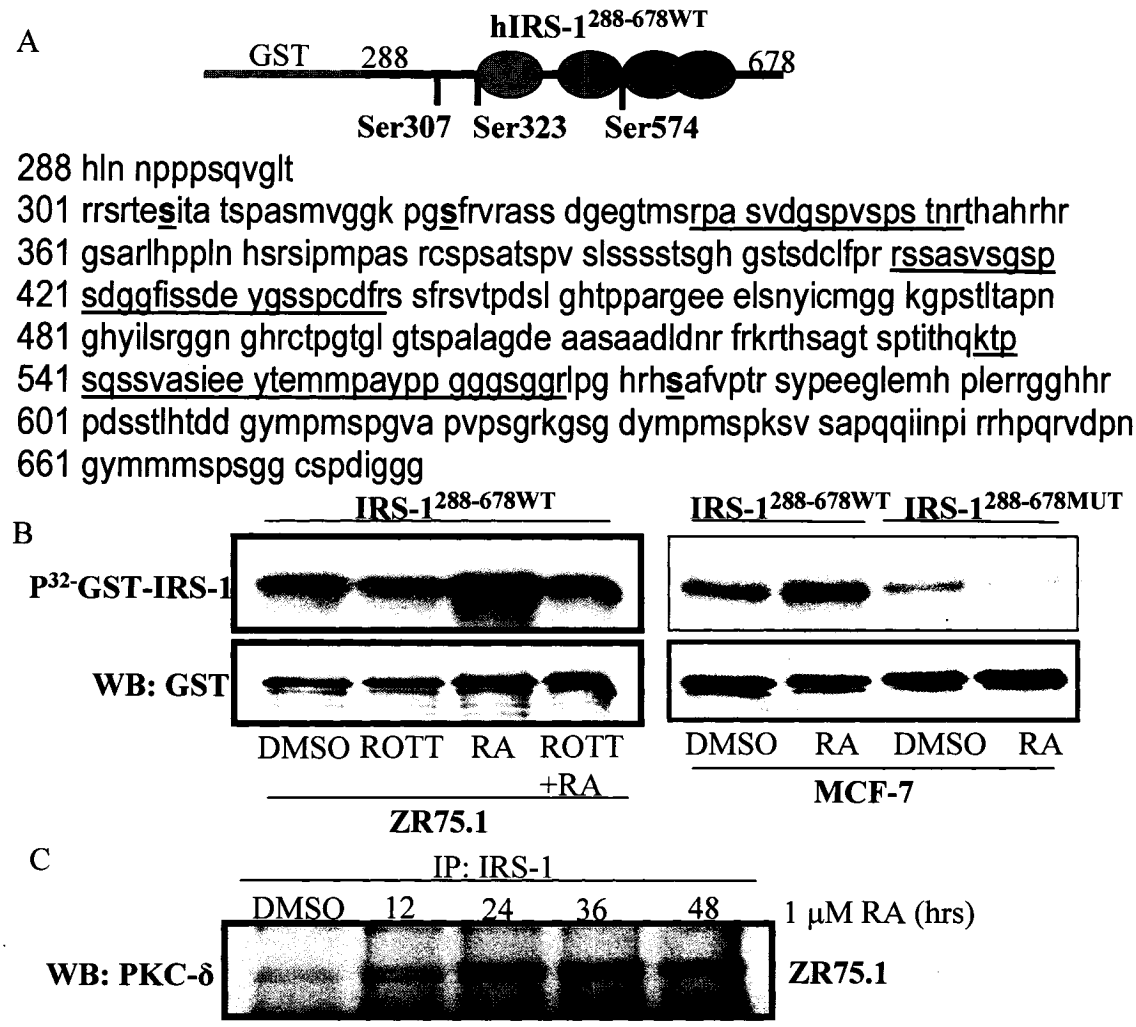


Figure 3.7 - *In vitro* phosphorylation of IRS-1 by RA-activated PKC-δ.

(A) Schematic: the human IRS-1 protein used in the GST-IRS-$1^{288\text{-}678WT}$ construct. Larger font represents the three serine sites (307, 323, and 574) phosphorylated by activated PKC-δ. Underlined in the IRS-1 amino acid sequence (288 to 678) are putative PEST domains. Serines 307, 323, and 574 are in bold/underlined. (B) Cells were pre-treated for 60 minutes with 0.5 μM Rottlerin and then co-treated for an additional 36 hours with 1 μM RA. Lysates were immunoprecipitated (IP) with an anti-PKC-δ antibody, and immunoprecipitates were subjected to an *in vitro* kinase assay using a GST-IRS-$1^{288\text{-}678WT}$ or GST-IRS-$1^{288\text{-}678MUT}$ (with serines 307, 323, and 574 mutated to alanines) as a substrate. Phosphorylated GST-IRS-$1^{288\text{-}678}$ was detected by autoradiography. (C) ZR75.1 cells were treated with RA, and cell lysate was IP with an anti-IRS-1 antibody and immunoblotted with an anti-PKC-δ antibody.

CHAPTER 4

GENERAL DISCUSSION

DISCUSSION

RTKs and their ligands are important for normal and malignant epithelial cell growth. Deregulation of various components of RTK signal transduction cascades are frequent events leading to carcinogenesis, making RTKs and their downstream signaling events attractive therapeutic targets. This thesis project began with the observation, from our lab and others, that RA is able to antagonize the growth stimulatory effects of growth factors, including IGF-I [245, 247, 248, 260]. In this study, I examined the possibility that RA inhibits breast cancer cell proliferation by impairing the mitogenic actions mediated by the activation of the IGF-IR signaling pathway. Although the inhibition of EGF-stimulated growth by RA can be understood to result from the observed downregulation of EGFR levels and activation [351-353], conflicting results exist as to the RA-mediated regulation of the IGF-IR [181, 248]. As such, it was my objective to determine whether the inhibition of IGFs mitogenic effects on MCF-7 cells was simply due to the regulation of the IGF-IR or due to the regulation of some other aspect of IGF-IR signaling. In understanding where retinoids act upon the IGF-IR signal transduction cascade, it may be possible to develop innovative therapeutic and preventive approaches combining retinoids with inhibitors of IGF-IR signaling.

My first aim was to determine if RA regulates the levels or activation state of the IGF-IR. I showed that RA does not regulate the protein levels or the IGF-I-stimulated tyrosine phosphorylation of the IGF-IR, leaving the regulation of downstream signaling as a possible mechanism to explain the RA-mediated inhibition of IGF-I stimulated growth. As IRS-1 is the predominant signaling intermediate activated by IGF-I in MCF-7 cells [70], I first assessed if the levels of this protein were altered in response to RA

treatment. I was able to show that IRS-1 protein levels were greatly decreased upon RA treatment. Furthermore, the reduction in IRS-1 levels seemed to be selective, since the levels of two other IGF-IR downstream substrates, IRS-2 and SHC remained unchanged. I also showed that the tyrosine phosphorylation of IRS-1 in response to IGF-I stimulation was hampered by RA treatment. Interestingly, the inhibition of IRS-1 tyrosine phosphorylation occurred at a time point earlier than the decrease in IRS-1 protein levels, suggesting that the decrease in IRS-1 activation was not a direct consequence of decreased protein levels. Although I did not elucidate the mechanism for the reduction of IRS-1 phosphorylation, I can hypothesize, based on the findings in Chapter 3 of this dissertation, that this decrease in IRS-1 tyrosine phosphorylation may result from the increased serine phosphorylation of IRS-1 also observed in response to RA treatment. This hypothesis is supported by several studies reporting that serine/threonine phosphorylation of IRS-1 can prevent further tyrosine phosphorylation [354-356]. However, since it is also recognized that phosphatases such as PTP1B and SHP-2 can regulate IRS-1 phosphorylation, a role of these protein tyrosine phosphatases cannot be excluded as a possible mechanism for the observed tyrosine dephosphorylation of IRS-1 in RA-treated breast cancer cells.

Tyrosine phosphorylated IRS-1 can recruit SH2-containing proteins such as the p85 subunit of PI 3-kinase or the Grb2 adapter protein, to activate PI 3-kinase or MAPK, respectively. Therefore, I next assessed if a specific IGF-I stimulated pathway was altered as a result of reduced IRS-1 activity in RA treated MCF-7 cells. Interestingly, I found that the IGF-I-stimulated interaction between IRS-1 and p85 was significantly reduced, and this correlated with a reduction in AKT activity. These data are in

agreement with numerous studies showing that the PI 3-kinase/AKT pathway has a critical role in RA signaling and that constitutive activation of this pathway is associated with RA resistance [264, 270, 318, 357]. My results also complement those of at least one other study showing that increased IGF-I production is associated with increased tyrosine phosphorylation of p85 and constitutive activation of the PI 3-kinase/AKT pathway in RA resistant leukemic cells [272]. In contrast, my results also indicated that the RA-dependent reduction in IRS-1 levels and activity is associated with selective changes in IGF-I signaling, since the SHC/GRb2 interaction and MAPK activity remained unaffected. This result indicates that perhaps the SHC branch of signaling is activated in MCF-7 cells by a RTK other than the IGF-IR, since it was observed in Chapter 2 that the level of SHC tyrosine phosphorylation, SHC/Grb2 interaction, and MAPK activation was not greatly increased by IGF-I stimulation. This is not unreasonable to postulate, since SHC is frequently constitutively phosphorylated in tumors and forms tight interactions with a number of RTKs including, the EGFR [358], nerve growth factor [359], and platelet-derived growth factor receptors [358], all of which can lead to MAPK activation.

IRS-1 was observed to be regulated by RA only in breast cancer cells responsive to the growth inhibitory effects of RA, providing preliminary evidence of the significance of IRS-1 regulation in mediating the growth inhibitory effects of RA. I thus hypothesized that overexpressing this protein in an RA-sensitive cell line such as MCF-7 would render these cells resistant to RA. I was able to show that overexpression of the IGF-IR in MCF-7 cells (MCF-7/IGF-IR) had little effect on their sensitivity to RA-mediated growth inhibition, while IRS-1 overexpression (MCF-7/IRS-1) resulted in a significant decrease

in sensitivity to RA. In the MCF-7/IRS-1 cells, RA was unable to significantly alter the levels of IRS-1, and AKT activity remained unaffected, suggesting that regulation of this pathway is important for RA-mediated growth inhibition. Prior studies using inhibitors of PI 3-kinase or AKT suggest these compounds might be effective in rescuing the RA resistance observed in the MCF-7/IRS-1 cells.

Recent studies reflect the importance of IRS-1 in breast carcinogenesis and the potential for this intracellular substrate as a target for prevention and treatment of this disease. High levels of IRS-1 in human breast tumors have been correlated with increased disease recurrence [6, 94], and subsequent studies have shown that constitutive IRS-1 signaling exists in breast tumors [127]. An improved understanding of the mechanisms regulating IRS-1 expression may help develop molecular strategies targeting IRS-1. I reasoned that it would be of significance to the action of retinoids in breast cancer to examine the molecular mechanism by which RA regulated IRS-1 levels. This was especially important since, IRS-1 protein levels were reduced only in the RA sensitive breast cancer cell lines, MCF-7, T47-D, and ZR75.1, but not in the RA resistant breast cancer cell lines, MDA-MB-231 and MDA-MB-468, and the decline in protein was not due to an effect of RA on IRS-1 mRNA status. Since pulse-chase experiments revealed that the degradation rate of IRS-1 was accelerated in MCF-7 cells treated with RA, I formulated the hypothesis that RA was enhancing the ubiquitination of IRS-1. To test this hypothesis, I first used a chemical inhibitor approach and found that two proteasome inhibitors, lactacystin and MG 132 were able to rescue the RA-mediated decrease in IRS-1 levels. Using numerous other approaches such as

immunoflourescence, co-immunoprecipitation, and transfection, I was able to validate these results.

Often proteins, such as IRS-1, are recognized by the ubiquitin-proteasome machinery by first becoming phosphorylated [360]. Recent reports demonstrate that the phosphorylation of serine residues in IRS-1 regulates its subsequent ubiquitination and degradation [89, 346, 361]. In breast cancer cells, RA treatment induces a robust increase in serine phosphorylation of IRS-1 that corresponds to the observed decline in IRS-1 protein level. To determine which kinase was responsible for this phosphorylation event, I again used a chemical inhibitor approach and found that the RA-mediated decrease in IRS-1 levels and increase in serine phosphorylation could be counteracted by addition of a PKC-δ inhibitor. I also showed that in the RA-sensitive, but not in the RA-resistant breast cancer cell lines that RA potently activated PKC-δ activity. Importantly, RA treatment of breast cancer cells resulted in the association between PKC-δ and IRS-1. In addition, RA-activated PKC-δ could phosphorylate IRS-1 *in vitro*; however, when three known PKC-δ serine sites in IRS-1 (serines 307, 323, and 574) were mutated to alanines this phosphorylation was lost. Upon sequence analysis of the region of the IRS-1 protein containing these three putative PKC-δ serine sites, I found the presence of PEST domains which can potentially serve as a recognition signal for degradation [348]. One could postulate that phosphorylation of these PKC-δ serine sites induces a conformational change in the C-terminal domain of IRS-1 that results in the unmasking of these PEST domains. In the *in vitro* kinase assay used to show PKC-δ-induced phosphorylation of IRS-1, I used a GST-IRS-1 construct containing only 390 amino acids of the 1243 amino acids in the full length protein. It is well known that IRS-1 contains well over 30 serine

phosphorylation sites, and thus I can not say if other serine residues need to be phosphorylated in response to RA treatment for the protein to become ubiquitinated. Unfortunately, given the technical problems of performing mass spectrometry on a protein such as IRS-1, large and heavily phosphorylated, I could not exploit this technology to attempt the mapping of all serine sites phosphorylated in response to RA treatment.

The association between the ubiquitin-proteasome pathway and RA-mediated regulation of IRS-1 expression is reminiscent of the well documented decrease in cyclin D1 levels by RA [267]. In fact, although an increasing number of proteins are known to be regulated by RA at the level of ubiquitination, the underlying mechanism for this effect of RA remains unclear. Though not discussed in the manuscripts included in Chapters 2 and 3 of this dissertation, I felt it would be an important initial step to search for components of the ubiquitin-proteasome pathway that are regulated by RA in breast cancer cells. UBE1L is considered to be a putative tumor suppressor gene, and though it is expressed in normal lung tissue, its expression is decreased or lost in the majority of lung carcinomas [362]. Interestingly, the status of UBE1L expression in normal breast epithelia and breast tumor tissue remains unknown. Characterization of the gene revealed its homology to the gene coding for E1, thus it was named UBE1L [363]. UBE1L activates the conjugation of the ubiquitin-like protein ISG15 to target proteins [364]. Since ISG15 substrates can be simultaneously modified by ubiquitin and ISG15 [365], one could hypothesize that some of the substrates already thought to be ubiquitinated by RA are also ISG15ylated via a UBE1L-dependant mechanism. Consistent with this idea, the ubiquitin-dependant degradation of the APL fusion

oncoprotein PML/RARα by RA has been shown to depend on the RA-mediated induction of UBE1L [241]. Late in my doctoral degree I began to test the hypothesis that RA may mediate proteolytic regulation of proteins important for tumor cell growth, such as IRS-1, by induction of UBE1L expression in breast cancer cells. Since this was not the main focus of my thesis project, I will only briefly summarize my findings to date and present these in appendix A. Indeed, UBE1L mRNA and protein expression are induced in RA sensitive breast cancer cell lines but not in the resistant cell lines (Figure A.1). Forcing the stable expression of UBE1L in T47-D cells induces G1 arrest, which is also observed with RA treatment of T47-D cells (Figure A.2). Unfortunately, I did not observe any regulation of IRS-1 levels in the UBE1L stable clones produced. This indicates that the regulation of IRS-1 ubiquitination does not depend on RA's ability to regulate the UBE1L/ISG15 pathway, but may be depend on RA-mediated regulation of a component of the classical ubiquitin-proteasome pathway. Consistent with this idea, I have found that RA increases the levels of the ubiquitin conjugating enzyme UbcH8 in breast cancer cells (Figure A.3). This is of particular interest, since UbcH8 both induces ubiquitin conjugation and is also the major E2 for ISG15 conjugation, indicating that the ISG15 conjugation pathway can indeed overlap or converge with ubiquitin conjugation pathways [366, 367]. Although an in depth analysis of the role of UBE1L/UBCH8/ISG15/ubiquitin pathway in breast cancer was well beyond the scope of this thesis project, it would certainly be of general interest to pursue these initial findings in hopes of understanding the more general mechanism by which RA regulates the posttranslational modification of proteins.

Combining Retinoids with Inhibitors of Signal Transduction

For rational development of novel combination therapies with retinoids, it is important to clarify how retinoids exert their effects on breast cancer cells. My data support the novel idea that RA abrogates breast cancer cell proliferation by inhibiting the action of the IRS-1 signaling intermediate used by the IGF-IR to propagate its mitogenic signal downstream. The data I presented in this dissertation suggest the possibility of examining the use of small molecule IGF-IR inhibitors in combination with RA, to determine if there is a synergistic inhibition of the activated signaling cascades frequently observed in transformed cells. Given the availability of recent promising results with these small molecule inhibitors *in vitro* and *in vivo* [147, 148], it seems quite possible that this combination would yield significant therapeutic effects, especially if tolerable doses of retinoids could be administered. On the other hand, my data also suggest that immediate substrates of the RTKs, rather than the RTKs themselves, might also serve as effective targets for anti-tumor combination therapies with retinoids. This is an important point since breast tumors often exhibit alterations in a number of different signal transduction pathways whose activity ultimately results in the activation of genes involved in tumor growth, such as the cyclin D1 gene which is induced by activation of the IGF-IR and EGFR signal transduction pathways. For example, if IRS-1 is overexpressed in a breast tumor that also overexpresses the Her2/neu receptor, an inhibitor of the IGF-IR may not be effective at synergizing with retinoids for combating tumor growth. In this case it might be more reasonable to combine an agent that decreases IRS-1 level and function, such as a retinoid/rexinoid, with an agent blocking the Her2/neu receptor, such as Herceptin. This combination strategy should receive more

attention since Herceptin has been shown to overcome RA resistance in breast cancer cells [264]. Knowledge of the genetic profile of the breast tumor would greatly increase our ability to design target-specific chemotherapy. More generally then, the data presented in this thesis has contributed to a better understanding of the intracellular events that occur in breast cancer cells treated with RA and form a conceptual rationale for the use of anti-signal transduction strategies and retinoids/rexinoids in combination therapy for inhibiting breast cancer cell growth.

When considering the future of retinoids in cancer treatment and prevention it is also important not to forget about the synthetic compounds 4-HPR and bexarotene that will surely open new avenues for their use in combination with other drugs. Since IRS-1 levels were reduced by 4-HPR and bexarotene treatment of MCF-7 cells, perhaps combining either of these agents with specific inhibitors of over-expressed/constitutively active RTKs would be more effective at blocking growth factor-stimulated signaling.

Concluding Remarks

The results presented in this dissertation addressed the topic I initially set out to investigate: Molecular interactions between insulin-like growth factor signal transduction and retinoids in breast cancer cells. I was the first to report that RA mediates its growth inhibitory effects by regulating IRS-1 protein levels in breast cancer cells. I was also the first to show that the functional consequence of decreased IRS-1 levels in RA treated breast cancer cells is the selective regulation of the PI 3-kinase/AKT pathway, thus elucidating a novel cross-talk between retinoid and IGF signaling. I went on to demonstrate that the regulation of IRS-1 by RA was at the level of its increased ubiquitination and degradation. The enhanced ubiquitination of IRS-1 by RA was found

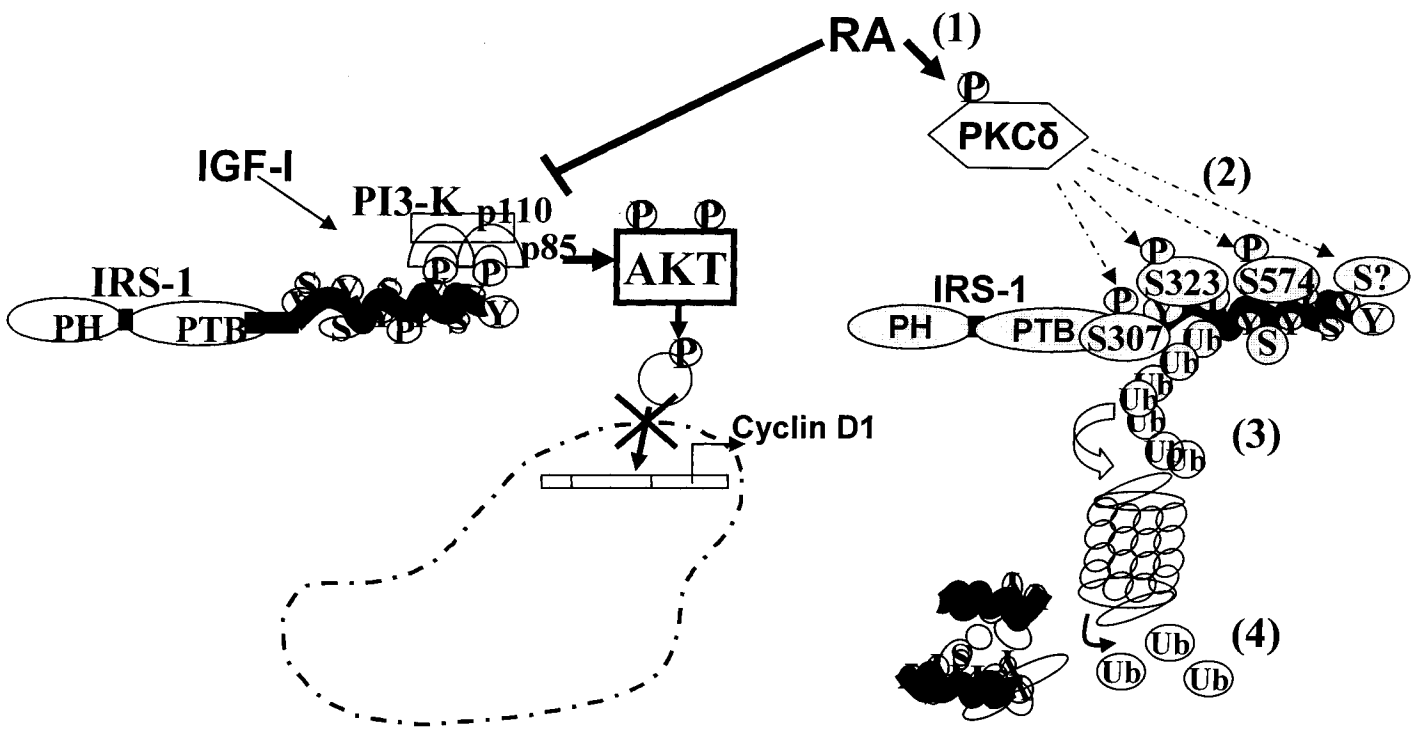


Figure 4.1 - RA regulates IRS-1 protein levels and interferes with its downstream signaling in breast cancer cells.

In the presence of RA, IGF-I-stimulated IRS-1/p85 interaction is abrogated and AKT activity is decreased. This may result in a reduction in transcription of growth promoting genes, such as cyclin D1.

RA reduces IRS-1 protein levels by increasing its proteasomal degradation. This is believed to occur in the following manner: (1) RA activates PKC-δ, (2) activated PKC-δ interacts with, and phosphorylates IRS-1 on serine residues, (3) IRS-1 is recognized by the ubiquitin-proteasome machinery, and (4) IRS-1 is degraded, and ubiquitin is recycled.

to involve a mechanism requiring the activation of PKC-δ, that in turn phosphorylates IRS-1 on serine sites which presumably serve as a signal to target IRS-1 for ubiquitination and degradation. Together, the conclusions derived from this research project have contributed to an enhanced understanding of the effect of retinoids in human breast cancer cells (Figure 4.1).

Contributions to Original Knowledge

The work presented in this doctoral thesis has provided several original contributions to the existing body of scientific knowledge to understanding the mechanism of retinoid action in breast cancer.

In Chapter 2, I show that RA-mediated regulation of breast cancer cell growth is mediated through interference with the IGF signal transduction pathway. This study has demonstrated that the intracellular substrate IRS-1, frequently overexpressed in breast cancer, is a novel target of RA in breast cancer cells. RA decreases IRS-1 levels and inhibits IGF-I-stimulated tyrosine phosphorylation of IRS-1. The regulation of IRS-1 was selective since RA did not affect IRS-2 or SHC levels. I further demonstrated that the functional consequence of regulating IRS-1 was the selective downregulation of the IGF-I-stimulated PI 3-kinase/AKT pathway, since IGF-I-stimulated erk1/2 activity remained unchanged. I also confirmed the importance of RA-mediated regulation of IRS-1 by showing that RA resistance is observed in MCF-7 cells overexpressing IRS-1 but not MCF-7 cells overexpressing IGF-IR.

In Chapter 3, I elucidated the mechanism by which RA regulates IRS-1 levels. I show that the mRNA levels remain unchanged and that the degradation rate of IRS-1 in RA-treated breast cancer cells is accelerated. Using various techniques, I demonstrated that RA enhances the ubiquitination of IRS-1 and that this is responsible for its degradation, since inhibitors of the proteasome are able to rescue the decline in IRS-1 levels. I further showed that RA reduces IRS-1 levels in a PKC-dependant manner, since inhibitors of this pathway blocked the RA-mediated decline in IRS-1 levels. In support of this, I show that RA activates PKC-δ in RA sensitive but not in RA resistant breast

cancer cells. In addition, I demonstrate that PKC-δ interacts with IRS-1 in RA treated cells and that IRS-1 can be phosphorylated *in vitro* by RA-activated PKC-δ.

Together, the conclusions derived from this research project have contributed to an enhanced understanding of the mechanism of action of retinoids in human breast cancer cells.

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Appendix A

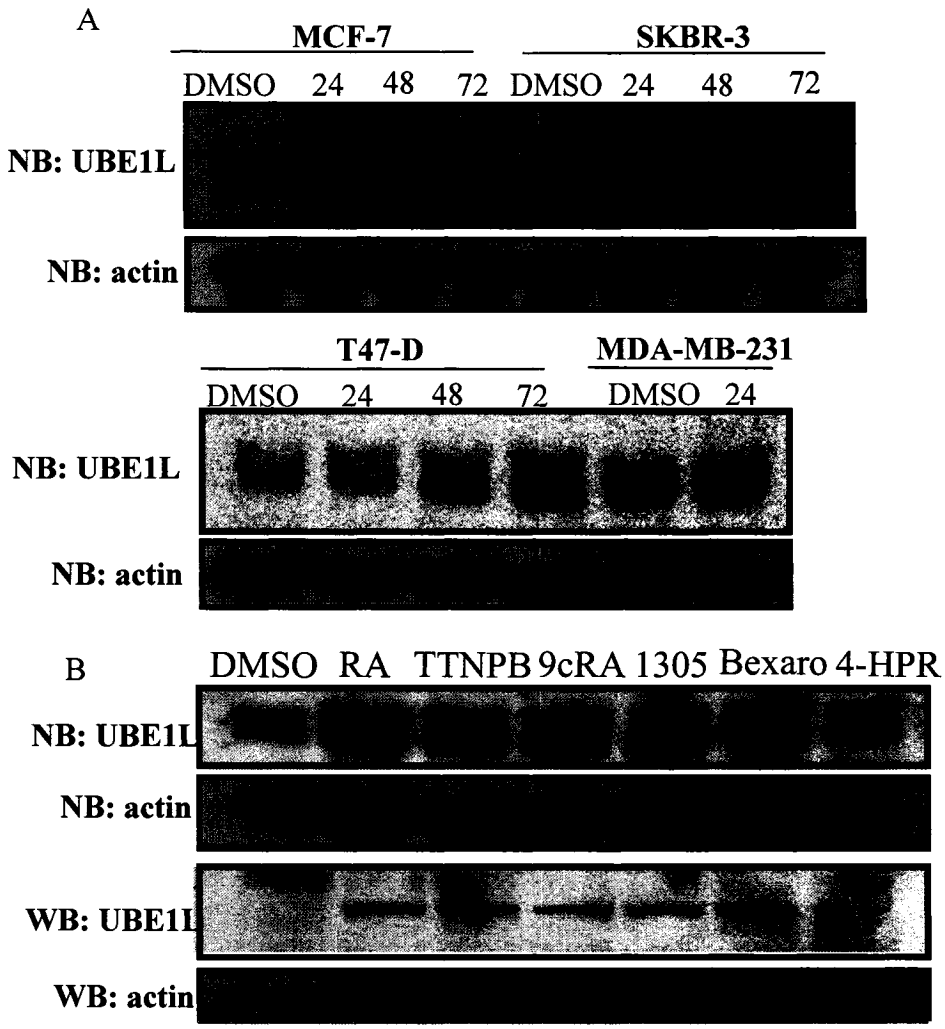

Figure A.1 - RA increases UBE1L levels in breast cancer cells.

(A) The breast cancer cell lines indicated were treated with 1 μM RA for 24, 48 and 72 hours. Northern blot (NB) was used to determine the expression level of UBE1L. Actin was used as a loading control. (B) T47-D cells were treated with: RA, TTNPB, 9cisRA, LGD1305 (1305), Bexarotene (Bexaro), N-(4-hydroxyphenyl)retinamide (4-HPR) for 72 hours. NB and Western blot (WB) were used to detect UBE1L.

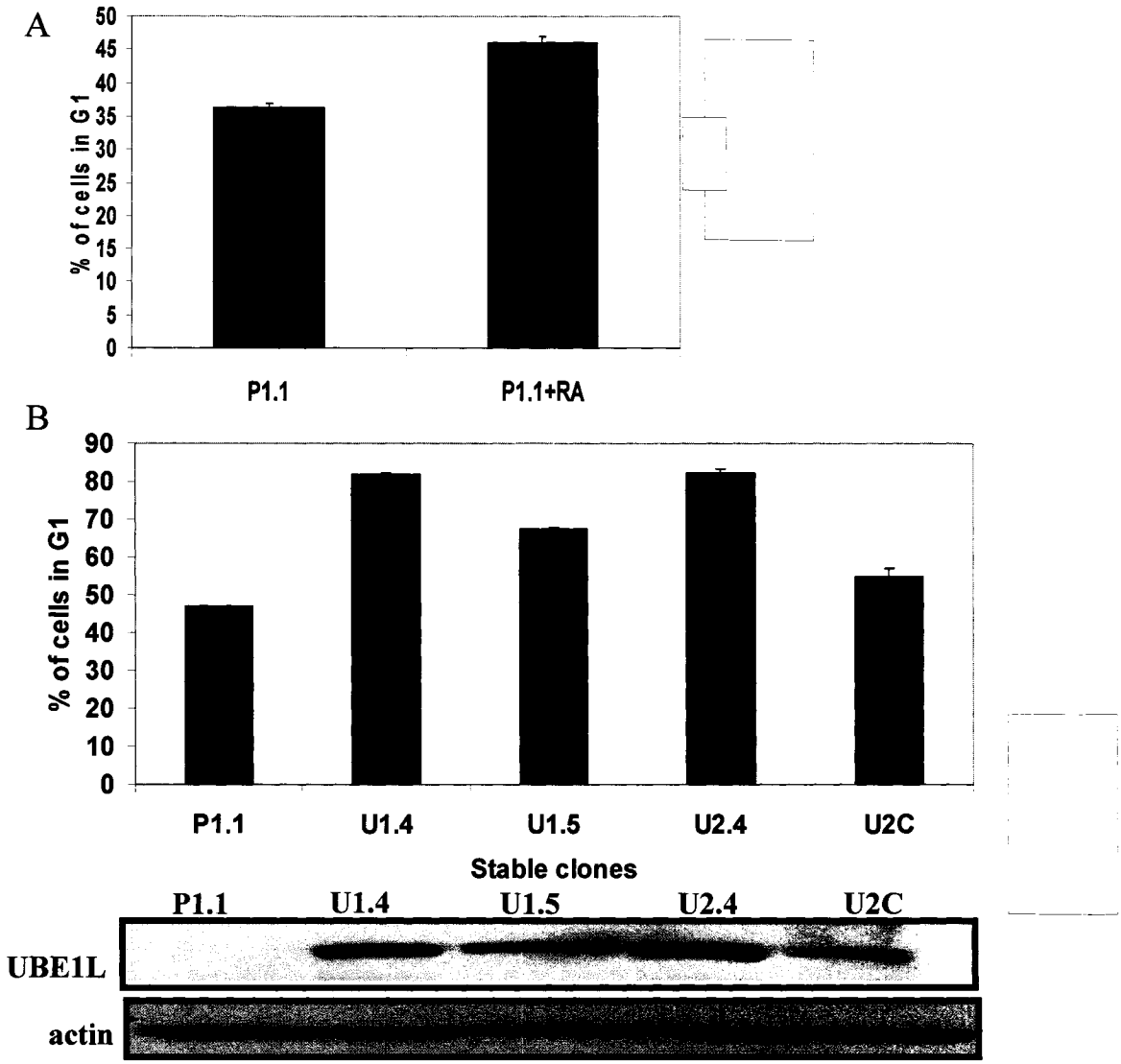

Figure A.2 –T47-D cells stably overexpressing UBE1L accumulate in the G1phase of the cell cycle.

(A) P1.1 cells are T47-D cells stably expressing the empty vector pcDNA3. These cells were treated with 1 μM RA for 48 hours, harvested, and stained with propidium iodide (PI). The distribution of P1.1 cells in the cell cycle determined by flow cytometry. (B) U1.4, U1.5, U2.4, U2C are T47-D cells stably expressing UBE1L. The cells were stained with PI and the distribution of cells in the cell cycle determined by flow cytometry. WB shows the level of UBE1L expression in each stable clone.

Appendix A

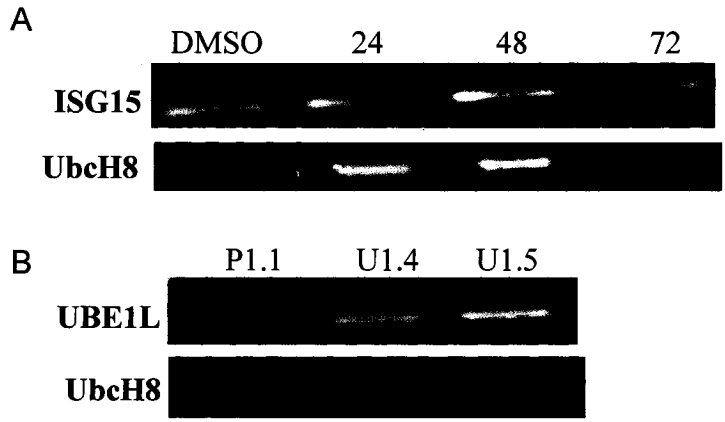

Figure A.3 - RA increases UbcH8 levels inMCF-7 cells.

(A) MCF-7 cells were treated with 1 μM RA for 24, 48 and 72 hours. RT-PCR was used to determine the expression level of ISG15 and UbcH8. (B) T47-D cells stably expressing UBE1L (U1.4 and U1.5) or stably expressing the empty vector control (P1.1) were used to detect the expression of UBE1L and UbcH8 by RT-PCR.

Research Compliance Certificate