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Cloning and Characterization of *Xenopus laevis* Insulin Receptor Substrate (xIRS-u) and Progesterone Receptor (xPR)

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
Ottawa, Ontario, Canada
December 2001

Abstract

Xenopus laevis oocytes are physiologically arrested at G_2 of meiosis I. Resumption of meiosis, or oocyte maturation, is triggered in vivo by progesterone, and in vitro by many hormones, such as insulin. Downstream of IGF-1 receptor activation is the activity of docking proteins such as xIRS-l and xIRS-u. Sequence analysis suggested that xIRS-u was a novel member of the IRS family rather than a *Xenopus* homolog of an existing member. The cloned xIRS-u cDNA contains an amino-terminal pleckstrin homology (PH) domain and phosphotyrosine-binding (PTB) domain. The carboxy terminus of xIRS-u contains several potential Src homology 2 (SH2)-binding sites, five such sites are potential binding sites for phosphatidylinositol 3-kinase (YM/LXM). It also contains a putative binding site for Grb2 (YINID). The injection of xIRS-u mRNA accelerated insulin-induced MAP kinase activation and oocyte maturation. An amino-terminal deletion of the PH domain reduced the ability of xIRS-u to potentiate insulin signaling. In contrast to the full-length protein, injection of deletion mutants encoding either the PH and PTB domains or the PH domain alone of xIRS-u, blocked insulin signaling in *Xenopus* oocytes. Finally, the injection of a deletion mutant that had a truncated PH domain and an intact PTB domain of xIRS-u had no effect on insulin signaling. This is the first report that the PH domain of an IRS protein can function in a dominant negative manner to inhibit insulin signaling.

Progesterone-induced *Xenopus* oocyte maturation is mediated via an extranuclear receptor and is independent of gene transcription. The identity of this extranuclear oocyte progesterone receptor (PR), however, has remained a longstanding problem. In an attempt to identify the oocyte PR, screening of a *Xenopus* oocyte cDNA library using the

hormone binding domain (HBD) of human PR as a probe, lead to the isolation of the amphibian homologue of human PR. The cloned *Xenopus* progesterone receptor (xPR) had an open reading frame of 583 amino acids. It functioned in heterologous cells as a progesterone-regulated transcription activator. However, endogenous xPR was excluded from the oocyte nucleus and instead appeared to be a cytosolic protein not associated with any membrane structures. Injection of xPR mRNA into *Xenopus* oocytes accelerated the progesterone-induced oocyte maturation and reduced the required concentrations of progesterone. In enucleated oocytes, xPR accelerated the progesterone-induced mitogen-activated protein kinase activation. These data suggest that xPR is the long sought after *Xenopus* oocyte receptor responsible for progesterone-induced oocyte maturation.

To my mother and father
who have given me unconditional love and endless support

ACKNOWLEDGEMENTS

I would like to thank Dr. Johne Liu for his guidance throughout the pursuit of my M.Sc. My gratitude goes to him for motivating me to attain high standards. Thank you for teaching me the meaning of hard work.

Dr. Nicholas Ohan for being there for me and providing great help in and outside of the lab. For reviewing my thesis, and providing constructive criticisms throughout my M.Sc. For all the deep thought discussions we had in the small frog room. His sweet wife Dorothy for her endless hospitality.

Sahar Farah for her help in the lab, on my thesis, and for being such a great friend.

Cathy Cummings for her excellent technical assistance.

Dr. G. Prefontaine for all his help and assistance.

Dr. Yinglun Sheng, Ron Booth, Dr. Sasaki, Dr. Yehenew Agazie, Dr. Qing Wei Chu, Nicholas Lemee, Sherine Rahal, Dr. Nadra Ginting, Geoff Morris, Helena Hamdan, Debra Weiss and all members of my lab for the pleasant memories, frequent help and kind companionship.

I would like to thank the rest of the LOEB Health Research Institute for making my stay a very enjoyable one.

My brothers Ahmad and Abdallah for keeping me company while writing my thesis; my brothers Hassan and Ibrahim for the good times we had.

My family members in Canada (Adnan and Zahira), United Arab Emirates, Jordan and Lebanon for their support and their favourite questions "Are you writing? When will you finish?"

The Shahin family all over the globe for making me feel like one of the family.

My best friends Botros, Fawaz, and Zaki for the endless nights they spent in the lab with me. The Anani brothers, Najjar family, Mohico and Hanico, Ziad Wehbe , Dakwar family and Moatasim (my hubbly partner) for having the ability to distract me from work.

Finally, I would like to thank two special people in my life, Seren and Razan Shahin. Seren (my thesis writing partner) for being a person I look up to and admire for her lively spirit, perseverance and will to excel in life.

Razan, my wife, a person that words would do no justice in describing. For being there for me every step of the way, from my B.Sc. in McGill to the final days of my M.Sc. in Ottawa. A rare individual with endless beauty, she is a ray of light in my life, and the reason for my success.

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List of Abbreviations

A.A.	amino acid
AC	adenylate cyclase
AcD	actinomycin D
AD	activating domain
BD	binding domain
cAMP	cyclic AMP
CAT	chloramphenicol acetyl transferase
chPR	chicken progesterone receptor
DBD	DNA binding domain
ER	estrogen receptor
GPCR	G-protein coupled receptor
GSP	gene specific primer
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HBD	hormone binding domain
IGF-1	insulin like growth factor-1
IR	insulin receptor
IRS-1	insulin receptor substrate-1
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MPF	maturation-promoting factor
NLS	nuclear localization signal

PH	pleckstrin homology
PI 3-kinase	phosphatidylinositol 3-OH kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMSG	pregnant mare serum gonadotropin
PR	progesterone receptor
PTB	phosphotyrosine binding
PTX	Bordetella pertussis toxin
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcriptase – polymerase chain reaction
SAIN	Shc and IRS-1 NPXY binding
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Sos	son-of-sevenless
xIGF-1	*Xenopus* Insulin-Like Growth Factor-1
xIGF-1R	*Xenopus* Insulin-Like Growth Factor-1 Receptor
xIRS-1	*Xenopus* IRS-1
xIRS-u	*Xenopus* IRS-u (u for unique, no known mammalian homologue)
xPR	*Xenopus* progesterone receptor

CHAPTER ONE

GENERAL INTRODUCTION

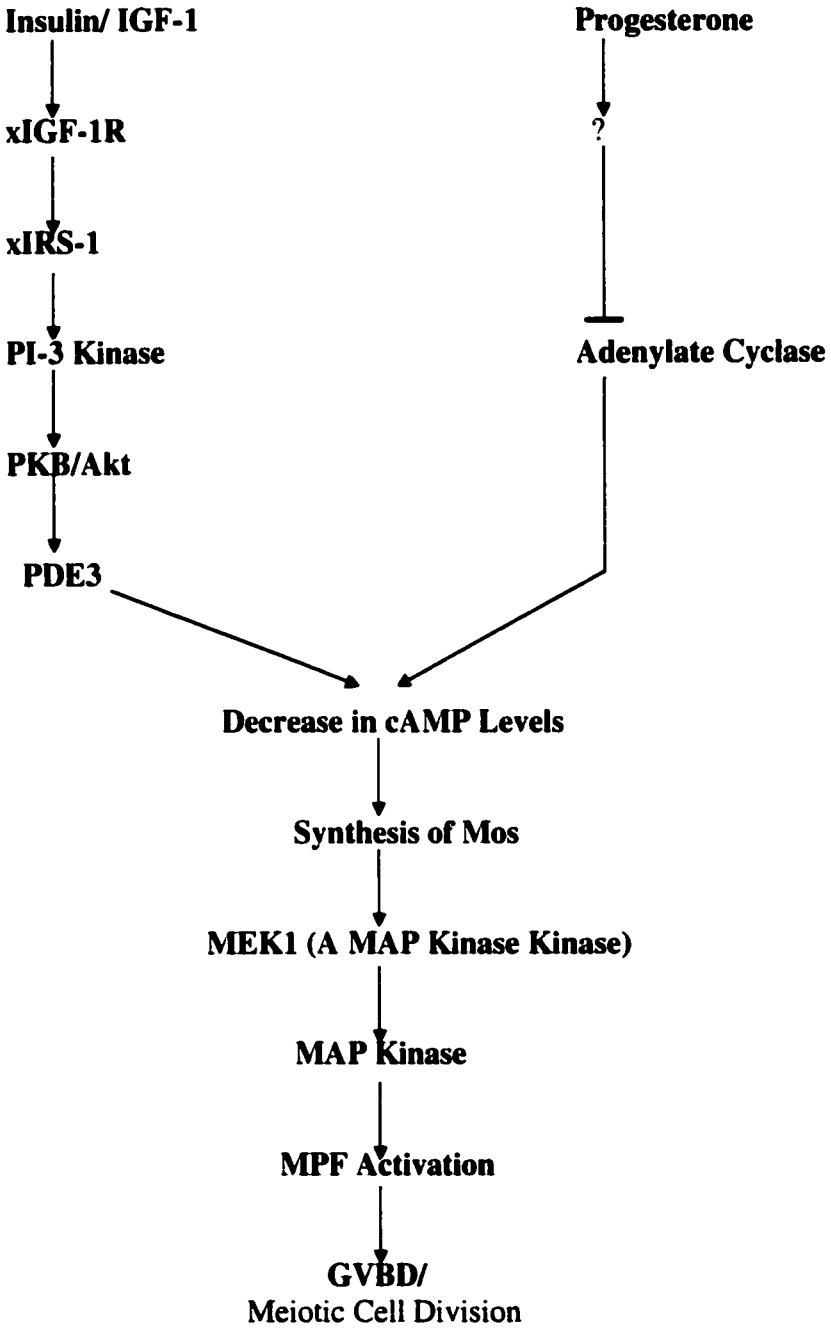
This thesis is comprised of two chapters examining different components of the insulin and progesterone signaling pathways using *Xenopus laevis* oocytes maturation as a model system. A schematic representation of the insulin and progesterone signaling pathways is presented in Figure 1. Both pathways converge at the reduction of cAMP and ultimately result in germinal vesicle breakdown (GVBD), which is readily observed in *Xenopus* oocytes as a white spot. The introduction will start with a general overview of the insulin signaling pathway. This is followed by an overview of the classical progesterone receptor, as well as the progesterone signaling pathway. It will also include a review of the history of progesterone signaling in *Xenopus*. The data presented in these two chapters has been published, Ohan et al. 1998 and Bayaa et al. 2000, respectively.

INSULIN SIGNALING PATHWAY

Insulin Hormone

The discovery of the insulin hormone by Banting and Best in 1922 lead to the uncovering of a very important regulator of metabolic physiology (Myers and White 1996). The most prominent role of insulin is the control of blood glucose levels in mammals (White and Kahn 1994). The action of insulin is very diverse and dependant on the tissue or cell. Such actions range from stimulation of Na^+/K^+-ATPase in muscle, to regulation of protein synthesis, gene expression and apoptosis (Myers and White 1996). Another important function of insulin is its ability to stimulate cell growth and differentiation (Rechler and Nissley 1985).

Figure 1
Insulin/Progesterone Pathways
A brief schematic representation of insulin and progesterone signaling pathways that are involved in the induction of meiotic cell division in *Xenopus laevis*. Adapted from Liu et al. 1995; Myers and White 1996; Gebauer and Richter 1997; Andersen et al. 1998 and Maller 2001.


Insulin/ IGF-1
xIGF-1R
xIRS-1
PI-3 Kinase
PKB/Akt
PDE3
Progesterone
?
Adenylate Cyclase
Decrease in cAMP Levels
Synthesis of Mos
MEK1 (A MAP Kinase Kinase)
MAP Kinase
MPF Activation
GVBD/
Meiotic Cell Division

Insulin and Insulin-like Growth Factor-1 (IGF-1) Receptors

At non-physiologically high concentrations, insulin is capable of binding and activating IGF-1 receptor (IGF-1R). Binding and activation of the IGF-1R when insulin is present in excess, is due to the similarity in structure of the IGF-1R and insulin receptor (IR). IGF-1 receptor is present in *Xenopus* oocytes (Hainaut et al. 1991; Zhu et al. 1998), while the presence of IR could not be confirmed in oocytes (Hainaut et al. 1991). The IGF-1 and insulin receptors have similar heterotetrameric structures. These structures consist of disulphide-linked α–and β-subunits, which represent the extracellular ligand-binding site and an intrinsic intracellular tyrosine kinase site, respectively (Yarden & Ullrich 1988). The same gene encodes both subunits of the receptor (IGF-1R or IR). The assembly of the receptor at the cell surface is achieved after the post-translational proteolytic cleavage of the receptor precursor (Rechler and Nissley 1990). Insulin-induced and insulin-regulated tyrosine autophosphorylation of the IGF-1R within the kinase domains stimulates kinase activity towards endogenous substrates, such as IRS (White et al. 1988).

Insulin Signal Transduction

The binding of insulin to the IR activates its tyrosine kinase and initially causes autophosphorylation of several tyrosine residues on the intracellular β-subunit (Karlsson et al. 1979; White and Kahn 1989). The activated IR acts on intracellular proteins, most notably IRS-1 (Myers and White 1995) and Shc (Pellici et al. 1992), to propagate a host of secondary messages. The insulin signaling system and IRS proteins have been

extensively studied and reviewed (White 1997; Yenush and White 1997; White and Kahn 1994; Myers and White 1996).

IRS Family and SH2 Containing Proteins

The first member of the insulin receptor substrate family, IRS-1, was identified as a 180 kDa phosphotyrosine-containing protein in insulin-stimulated cells (White 1985). In essence, the IRS family members act like docking proteins, which have no catalytic activity but serve to link the receptor to downstream signaling proteins. Unlike adapter proteins, which link two proteins together, as with Grb2 linking IRS-1 with Sos, a docking protein has the ability to recruit several signaling components. The first major IR substrate identified was insulin receptor substrate-1 (IRS-1) which is phosphorylated on tyrosine residues by the ligand activated IR and the IGF-1R (Sun et al. 1991). IRS is an example of a docking protein, since its phosphotyrosines serve as docking sites for a number of signaling molecules. Since then, a number of IRS and IGF-1R substrates have been identified, including IRS-2, 3, and 4 (Fig.2), as well as Shc.

The aforementioned conserved phosphotyrosine sites can bind Src homology 2 (SH2) domain containing proteins such as PI 3-kinase (Backer et al. 1992), Nck (Lee et al. 1993), among others. PI 3-kinase, a lipid kinase, consists of two subunits, the p85 regulatory subunit and p110 catalytic subunit. The IRS-1 phosphotyrosines $pTyr^{608}$ and $pTyr^{628}$ in the pYMXM motif are binding sites for the two SH2 domains of p85 (Sun et al. 1993). PI 3-kinase's main products, 3-phosphoinositids (Carpenter et al. 1990), act as second messengers that are capable of inducing multiple downstream signals, such as protein kinase B (PKB) activation.

Another SH2 containing, small cytoplasmic protein that binds IRS-1 is Grb2. Grb2 also contains two Src homology domains (SH3) (Mayer et al. 1988) that can bind a guanine nucleotide exchange factor for $p21^{ras}$, Sos. The binding of IRS-1 $pTyr^{895}$ to the SH2 domain of Grb2, helps to illustrate how the Grb2-Sos complex protein might serve as an adapter molecule linking phosphorylated IRS-1 to the Ras pathway (Baltensperger et al. 1993)

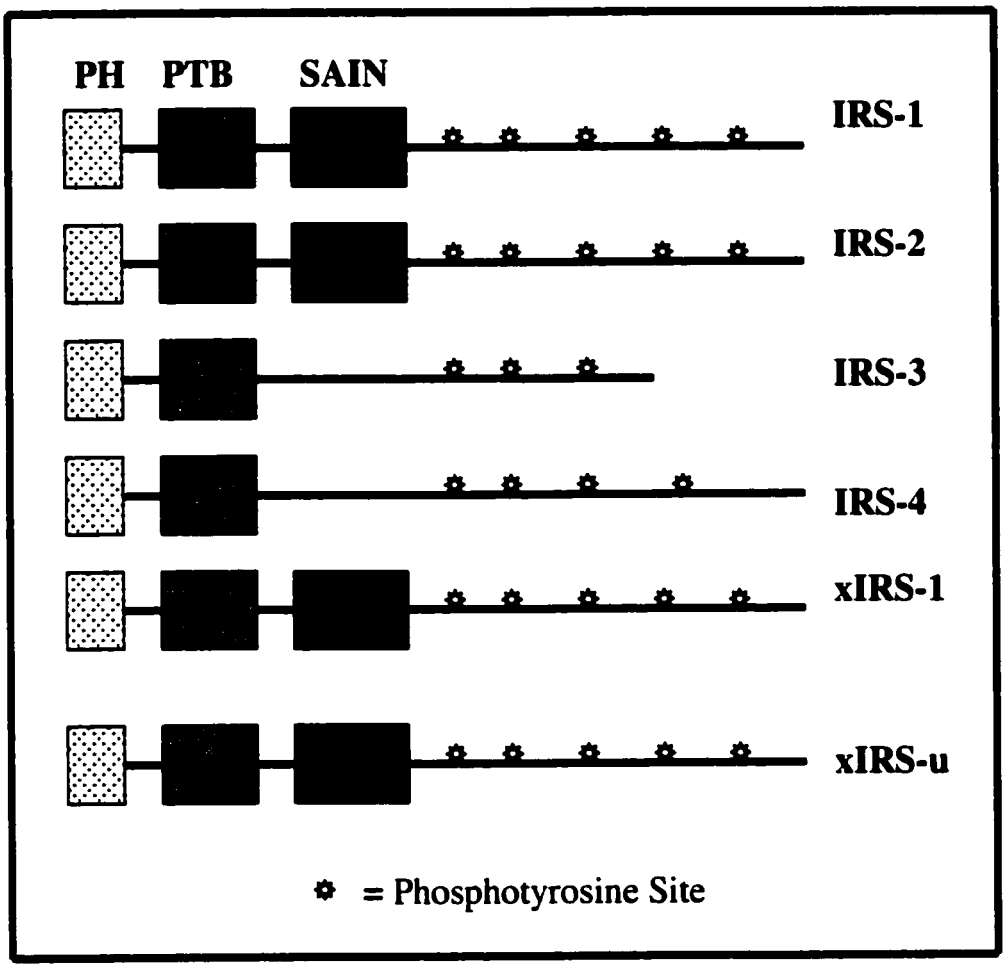
Conserved Protein Domains in the IRS Family

Tyrosine phosphorylation sites are among several conserved protein/lipid binding motifs within the IRS family members. Almost all of the IRS family members contain a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain. Several insulin receptor substrates contain the Shc and IRS-1 NPEY binding (SAIN) domain (Pawson 1995). A brief schematic representation of the different IRS family proteins and their domains is provided (Fig. 2).

PH Domain

The PH domain was first detected as an internal repeat in pleckstrin, a 47 kDa protein substrate of protein kinase C (PKC) in platelets (Mayer et al. 1993; Haslam et al. 1993). Now more than 90 different PH-containing proteins involved in cytoskeletal functions or cellular signaling have been identified (Gibson et al. 1994). The structure of the PH domain seems to be composed of two anti-parallel β–sheets that are roughly perpendicular, followed by carboxy-terminal α–helix (Riddihough 1994). The PH

Figure 2
Structure of IRS Proteins
Schematic representation of mammalian and Xenopus IRS proteins. The amino terminus contains the PH, PTB and SAIN domains (not in IRS-3 and IRS-4). The C-terminus contains the SH2 binding phosphotyrosine residues.


PH
PTB
SAIN
IRS-1
IRS-2
IRS-3
IRS-4
xIRS-1
xIRS-u
= Phosphotyrosine Site

domains of different proteins have two main functions; membrane binding and protein localization (Shaw 1996).

Drawing comparisons to SH domains, it was thought that the PH domain mediated protein-protein interactions. Studies suggest that the PH domain can associate with cell membranes by binding to lipid molecules (Harlan et al. 1994). This helps explain the interaction of the cytoplasmic IRS-1 with the transmembrane IGF-1R. The PH domain is believed to be responsible for the interaction between IRS-1 and IR (Yenush et al. 1996). The PH domain has also been implicated in the Ras-MAPK signaling pathways, through its interaction with $G_{\beta\gamma}$ (Inglese et al. 1995). This interaction with the $G_{\beta\gamma}$ is achieved via the PH domain's α–helix (Touhara et al. 1994).

Although PH domains have a similar function and are conserved between different proteins, studies have shown that heterologous PH domains do not couple IRS-1 to the IR (Burks et al. 1997). This suggests that PH domains can be protein specific and play a vital role in the specific function of each protein.

PTB Domain

In cells stimulated with several different growth factors, the coimmunoprecipitation of a 145 kDa phosphotyrosine-containing protein with Shc was observed (Kavanaugh and Williams 1994). Sequence analysis of Shc lead to the discovery of the PTB domain that bound to the 145 kDa phosphotyrosine-containing protein. Although the PTB domain binds phosphotyrosines, it is structurally different from the SH2 domain (Bork and Margolis 1995). The PTB domain specifically recognizes NPXpY motif (N=Asn, P=Pro,X=any a.a., pY=phospho-Tyr) . Nonetheless,

the ability of the PTB domain to bind phosphotyrosines suggests that it plays a similar role as the SH2 domain (van der Geer & Pawson 1995). A review by Lemmon et al. (1996) suggests that the PTB domain represents a class of PH domains, that serve to bring Shc and IRS-1 close to the membrane surface.

Oocyte Maturation as a Model for Insulin Signaling

Xenopus oocytes have been used extensively as a model system to reconstitute part of the mammalian insulin signaling pathway, but the endogenous mechanism of insulin signaling in *Xenopus* has not been fully elucidated. The oocyte, which is not a somatic cell, is a useful system to study insulin signaling due to its size and ease of manipulations, such as microinjection of mRNA. Postvitellogenic, or Stage VI, *Xenopus* oocytes are physiologically arrested at the G_2/M transition of the first meiotic prophase. These oocytes must complete meiosis I and proceed to the second meiotic metaphase before fertilization is possible. Oocyte maturation, which involves the resumption of meiosis and the breakdown of the germinal vesicle (GV), is believed to be triggered *in vivo* by progesterone (Schuetz and Glad, 1985), but several other substances have been used to induce maturation *in vitro*.

Two known maturation inducers are insulin and IGF-1. Previous studies have shown that insulin/IGF-1-induced oocyte maturation is mediated by endogenous IGF-1 receptor (Maller & Koontz 1981). Insulin or progesterone signal transduction can be studied via oocyte maturation. Oocyte maturation is phenotypically monitored by germinal vesicle break down (GVBD) resulting in a characteristic white spot in the pigmented animal hemisphere. MAP kinase activation causes MPF activation that

eventually leads to GVBD (Fig. 1). The phosphorylation of MAP kinase acts as a biochemical marker for oocyte maturation, as the phosphorylation of MAP kinase can be visualized by anti-phospho-MAP kinase antibodies and also by a characteristic SDS-PAGE mobility shift induced by its phosphorylation. Vitellogenic (stage IV) oocytes cannot undergo maturation nor MAP kinase activation after stimulation with hormones (El-Etr et al. 1979; Masui and Clarke 1979; Maller and Koontz 1981).

Insulin/IGF-1 Pathway

Unlike other tyrosine kinase receptors such as epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR), IR and IGF-1R do not necessarily bind signaling molecules directly. These receptors predominantly use docking proteins, such as IRS-1, to transmit the signals downstream. Downstream signals include Ras, MAP kinase, and PI 3-kinase activation.

The Role of Ras, MAP Kinase and PI 3-Kinase

Shc, another IR and IGF-1R substrate, serves to activate the Ras/mitogen-activated protein kinase (MAP kinase) pathway. Shc binds the tyrosine-phosphorylated IR and IGF-1R with its SH2 domain and is itself tyrosine phosphorylated (Dey et al. 1996). The phosphorylated tyrosines on Shc act as docking sites for the SH2 domain of a cytoplasmic protein, growth factor receptor-binding protein (Grb2) (Giorgetti et al. 1994). Grb2 is constitutively bound to son of sevenless (Sos) through its two SH3 domains (Baltensperger et al. 1993; Skolnik et al. 1993). Upon ligand binding to the receptor, Shc is recruited to the phosphorylated receptor. The subsequent

phosphorylation of Shc recruits the Grb2/Sos complex to the receptor/Shc complex. The recruitment of Grb2/Sos to the membrane catalyses the exchange of GTP for GDP on the small membrane-bound G-protein Ras. GTP bound Ras, at this point activates the serine/threonine kinase Raf, which phosphorylates MEK. MEK then phosphorylates MAP kinase on threonine and tyrosine residues activating its kinase activity. MAP kinase then phosphorylates numerous cytoplasmic and nuclear proteins, on serine and threonine residues, involved in cell cycle progression, transcription, translation and differentiation (Guan 1994).

The over expression of xIRS-1 (Liu et al. 1995) in oocytes potentiates insulin induced GVBD and MAP kinase activation, but does not spontaneously induce GVBD. The presence of xIRS-1 in stage VI oocytes suggests that it plays a role in insulin signaling. In contrast, others have shown that Ras is important for oocyte maturation (Shibuya et al. 1992), and therefore *Xenopus* insulin signaling may involve Shc rather than IRS. In mammalian cells Shc seems to be associated with Ras activation more than IRS, but IRS can activate Ras (Chuang et al. 1994). The evidence that suggests that Ras is involved in insulin signaling in oocytes is the fact that injection of activated Ras (v-ras) spontaneously activates MAP kinase and induces GVBD in stage VI oocytes (Birchmeier et al. 1985; Allende et al. 1988). In contrast, the injection of v-ras causes the activation of MAP kinase but not GVBD in stage IV oocytes (Fabian et al. 1993; Muslin et al. 1993). Also, it has been shown that injection of dominant negative Ras inhibits insulin induced GVBD (Chuang et al. 1994). The fact that injection of activated Ras protein or mRNA induces GVBD is not under dispute, but until activation of endogenous Ras by insulin is shown, these results must be taken with caution. The need for PI 3-kinase in inducing

GVBD has also been demonstrated in our laboratory (Liu et al. 1995). There have been some reports of PI 3-kinase activating Ras (Hu et al. 1995; Potempa and Ridley 1998) which would be consistent with both pathways being necessary for signaling, but no reports have shown a link between Ras and PI 3-kinase in oocytes. Considering the data that two pathways, PI 3-kinase and Ras/MAP kinase, seem to be involved in insulin induced *Xenopus* oocyte maturation, more research is necessary to understand the signaling molecules that are involved in insulin stimulated oocyte maturation.

The Involvement of SH2/SH3 Domain-Containing Proteins

The SH2/SH3 adapters NcK, Grb2 and Crk promote the assembly of signaling complexes by binding to tyrosine phosphorylated proteins using their SH2 domains and binding to proline-rich sequences on effector molecules using their SH3 domains (Gupta and Mayer 1998). Several studies have been conducted to clarify the role of Grb2 and its SH2 and SH3 domains in both the mammalian and *Xenopus* systems. One study done by Chuang et al. in 1994, involved co-injecting glutathione S-transferase (GST)-SH2 domain fusion proteins and ras into stage VI oocytes. They were able to observe that the different insulin actions are mediated by IRS-1 through two independent converging pathways that involve Grb2 and PI 3-kinase. The microinjection of SH2/GST-fusion proteins of either p85 (subunit of PI-3K) or GRB2 inhibited IRS-1 dependent activation of MAP kinase and oocyte maturation. They state that GRB2 is a molecule believed to link IRS-1 to the Ras pathway. Recall that in stage IV oocytes, the MAP kinase activation cascade downstream of ras is intact (Chesnel et al. 1997). Insulin-induced autophosphorylation of xIGF-1R is detected in stage IV oocytes (Bayaa and Liu, unpublished data). In further

experiments, insulin-induced MAP kinase activation could not be achieved in xIRS-1 injected stage IV oocytes (Bayaa and Liu, unpublished data).

In a study by Simon and Schreiber (1995), they investigated the interaction between Grb2 and hSos peptides. They were able to conclude that this interaction is predominantly via the amino-terminal SH3 domain, although the carboxy-terminal SH3 domain played a role in stabilizing the Grb2-hSos complex. Finally, a very significant study was carried out by Aroca et al. (1996) on SH2 domains of several proteins including p85 and Grb2 and their functional interactions with the insulin/Ras signaling pathways in *Xenopus.* They found that the injection of the unique SH2 domain of Grb2 into full grown, stage VI xenopus oocytes caused slight inhibition of insulin induced GVBD. The Grb2 SH2 domain also failed to co-operate with normal ras protein to induce GVBD. In their study, they demonstrated that the purified, isolated SH2 domains retain structural and functional specificity and that *Xenopus* oocytes constitute a useful biological system to analyse their functional roles.

PROGESTERONE SIGNALING PATHWAY

The Steroid Hormone Progesterone

Progesterone, a progestin, is the precursor to many important steroid hormones such as estrogen and testosterone. It is made from the sterol pregnenolone, which is in turn made from cholesterol, which is made from acetate, a product of the breakdown of sugar and fat in the body. Corticosteroids, which are essential for stress response, sugar and electrolyte balance and blood pressure, are also derived from progesterone (Graham and Clarke 1997).

Physiological Action of Progesterone

In mammals, progesterone plays a crucial role in the regulation of female reproductive function. It plays various tissue-specific roles (Graham and Clarke 1997). These roles range from the release of mature oocytes in the ovary, mediation of sexual responsiveness in the brain, modulation of bone mass and proliferation regulation of endothelial cells (Vásquez et al. 1999). The progesterone receptor (PR) mediates the effects of progesterone, and is induced by estrogen in most tissues. Regulation of gene expression in target tissues in response to progesterone has been observed in cases such as progesterone-induced transcription activation by the chicken progesterone receptor (cPR) (Tsai and O'Malley 1994). The action of progesterone is reviewed extensively by Graham and Clarke (1997).

Steroid Receptor Superfamily

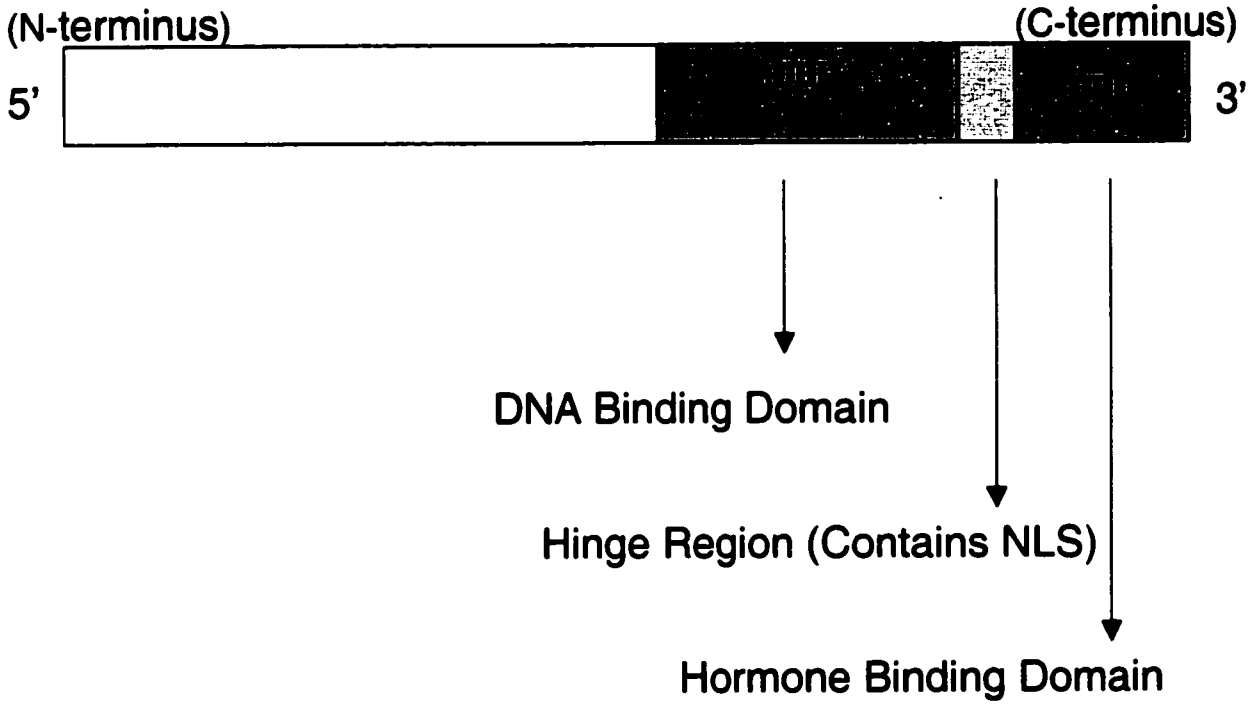
The largest known family of transcription factors in eukaryotes is represented by the steroid receptor superfamily (Tsai and O'Malley 1994). All members of the family

have a highly conserved structure and include similar domains (Fig. 3). The amino-terminal is usually variable in sequence and in length. It contains a transactivation function (AF), which activates target genes by interacting with coactivators. The amino-terminal is important for determining target gene specificity for receptor isoforms (Tora et al. 1988; Bocquel et al. 1989). Following the amino terminal there is a region containing two type II zinc fingers (Cys-X_2-Cys-X_{1-3}-Cys-X_2-Cys, also known as the Cys_2/Cys_2 finger), which is responsible for DNA binding (DBD) and aids in dimerization of steroid receptors. This is followed by a variable hinge region, which contains a nuclear localization signal (NLS). The variable hinge region aids in dimerization (Tetel et al. 1997) and allows conformation alterations of the receptor to alter function. Finally, the carboxy-terminal contains the hormone binding domain (HBD). The HBD is functionally complex and in addition to ligand binding, it is important for heat shock protein association, dimerization, nuclear localization, transactivation, intermolecular silencing and intramolecular repression (Tsai and O'Malley 1994; Xu et al. 1996). Steroid receptors have either a neutral or inhibiting activity in the absence of ligand. In general, ligand binding allows the receptor to bind DNA and activate target gene expression (Carson-Jurica et al., 1990).

The Progesterone Receptor

In the early 1970's, the first progesterone-binding, estrogen-inducible receptor proteins were characterized from mammalian uterus and chick oviduct (Milgrom and Baulieu 1970; Milgrom et al. 1970; Sherman et al. 1970; Leavitt and Blaha 1972). There are two forms of the human PR, hPR_A and hPR_B. A single gene under the control of

Figure 3
Structure of Steroid Receptor
Schematic representation of the classical steroid receptor. The amino terminal contains the most variable region among steroid receptors. The carboxy terminal contains the DBD, hinge region, and HBD, in that order, and is highly conserved.


(N-terminus)
(C-terminus)
5'
3'
DNA Binding Domain
Hinge Region (Contains NLS)
Hormone Binding Domain

distinct promoters encodes these two proteins, which are 81 kDa and 115 kDa, respectively (Kastner et al. 1990). PR belongs to the steroid receptor superfamily and is a ligand-activated nuclear transcription factor. There is strong evidence that hPR_A and hPR_B function in different manners. Their function is promoter- and cell-specific (Meyer et al. 1992; Vegeto et al. 1993). Studies report that hPR_A can act as a transcriptional repressor, while hPR_B is a transcriptional activator of progesterone-responsive genes (Vegeto et al. 1993). A recent study mapped and characterized the functional domains responsible for the different activities of the two hPR isoforms (Giangrande et al. 1997). The repressor activity of hPR_A was mapped to the first 140 a.a. of the protein.

Overview of Progesterone-Induced *Xenopus* Oocyte Maturation

In sexually mature vertebrate females, gonadotropin secretion by the pituitary triggers oocyte maturation and accompanying ovulation (Masui et al. 1979). The primary sites of gonadotropin action are the somatic cells in the ovary, rather than the oocytes themselves. In most amphibian species, including *Xenopus laevis*, the pre-ovulatory gonadotropin surge causes the production of the steroid hormone progesterone by follicular cells. Fully grown *Xenopus* oocytes are arrested at prophase (G2/M) of meiosis I. Progesterone is responsible for the initiation of oocyte maturation and resumption of the meiotic cell cycles (Masui et al. 1979). In turn, the oocyte becomes a fertilizable egg. The molecular mechanism by which progesterone induces oocyte maturation has been studied extensively (Sadler and Maller 1981; Sadler and Maller 1984; Smith et al. 1991; Maller 1998).

The initial detectable biochemical change that arises minutes following progesterone stimulation is a reduction of cAMP in the oocyte cytoplasm. This reduction in cAMP is a result of both the inhibition of adenylate cyclase (AC) and stimulation of cAMP specific phosphodiesterases by progesterone (Smith and Manganiello 1989). In contrast, progesterone-induced oocyte maturation is blocked by the activation of AC (elevation of intracellular cAMP levels). It has been shown that cAMP-dependent protein kinase A (PKA) is an inhibitor of progesterone-induced oocyte maturation (Maller et al. 1977). In that study, over-expression of the catalytic subunit of PKA inhibited progesterone-induced oocyte maturation, while inhibition of endogenous PKA by the injection of its regulatory subunit induced hormone-independent GVBD.

Hours after the initial progesterone stimulation, a germ cell specific serine/ threonine kinase MOS is synthesized and subsequently activated (Sagata et al. 1988). Two more serine/threonine kinases are subsequently activated by post-translational modifications, MAPK and MPF (p34cdc kinase/cyclin B). This process of maturation takes between 4-6 h, with MAPK phosphorylation taking place before any phenotypic changes occur. MPF is responsible for the nuclear division (Sagata 1997; Gebauer and Richter 1997).

Role of Kinase Eg2

Based on the notion that the initial signal transduced by progesterone is very rapid, thus most likely involving protein phosphorylation, a group devised a screening strategy to identify proteins involved in progesterone signaling (Andresson and Ruderman 1998). The screening strategy involved the in vitro translation and incubation

of small pools of cDNA with extracts from either progesterone-stimulated/unstimulated oocytes. The proteins were then analyzed by SDS-PAGE to look for mobility shifts due to protein phosphorylation. This led to the identification of a serine/threonine kinase, Eg2. Progesterone-induced phosphorylation of Eg2 was noted. Eg2 overexpression accelerates progesterone-induced MOS synthesis and MAPK activation. Eg2 overexpression also seems to decrease the progesterone concentration that is needed to induce maturation. This discovery proposes the identification of a protein that might be involved directly downstream of a putative *Xenopus* oocyte PR.

Progesterone Signaling in Stage IV Oocytes

As mentioned previously, stage IV oocytes are unable to undergo progesterone-induced maturation (Sadler et al. 1983). There are many proposed reasons explaining this. For example, it has been demonstrated that stage IV oocytes lack the high affinity binding sites for progesterone found in stage VI oocytes (Liu et al. 1993). Earlier studies have shown that oncogenic ras protein is able to induce hormone-independent MOS synthesis (Chesnel et al. 1997), MAPK activation, and oocyte maturation (Birchmeier et al., 1985) in stage VI oocytes. In stage IV oocytes, although the oncogenic ras protein has been observed to cause hormone-independent MOS sythesis and MAPK activation, it is unable to cause oocyte maturation (Chesnel et al. 1997). This suggests a defect downstream of MAPK in the oocyte maturation system of stage IV oocytes.

Structure of the Putative *Xenopus* Oocyte PR

The nature and identity of the receptor responsible for transducing the progesterone signal remains elusive. It is known that RU486, a synthetic progestin antagonist, binds to the HBD of PR and functions as a progesterone antagonist or agonist in somatic cells, depending on the receptor conformation (Horwitz et al. 1995). RU486 and progesterone interact with distinct, but overlapping regions within the HBD of PR (Vegeta et al. 1992). In *Xenopus* oocytes, RU486 could induce oocyte GVBD (Sadler and Maller 1985). This demonstrated that the putative oocyte PR contains at least a classical HBD.

Evidence of a Cell Surface Receptor

Early evidence supporting the theory of the putative *Xenopus* progesterone receptor being a cell surface receptor that is distinct from the classical PR has been extensive. Progesterone-induced MPF activation occurrs in enucleated oocytes, or in the presence of actinomycin D or ethidium bromide, both of which are transcription inhibitors (Baulieu et al. 1978). A study demonstrated that the addition of progesterone to the oocyte medium was able to induce GVBD, while its delivery to the cytoplasm by injection did not induce GVBD (Smith and Ecker 1971; Baulieu et al. 1978; Masui et al. 1979). Furthermore, progesterone analogs chemically coupled to polymers, which are not internalized by endocytosis, were capable of inducing GVBD (Godeau et al. 1978). A relatively recent study (Liu et al. 1993) reported high affinity binding sites for progesterone or meiosis-competent progesterone analogs in oocyte membrane preparations ($K_d=10^{-9}M$), and this is consistent with the theory of a cell surface receptor.

A contradictory piece of evidence was reported when injection of progesterone dissolved in paraffin oil (Tso et al. 1982) induced GVBD, unlike the previously used injection of progesterone dissolved in aqueous solution (Smith and Ecker 1971; Baulieu et al. 1978). In the latter study, the authors suggested that the aqueous-based progesterone leaked out too rapidly from the oocyte cytoplasm thus making it unable to trigger an intracellular response, while the paraffin oil-based progesterone is retained longer in the cytoplasm.

Similarity to Classical Cell Surface Receptors

The activation of mammalian sperm is an example of a non-genomic action of progesterone (Baldi et al. 1999). The initial binding of the sperm to the egg zona causes the sperm exocytosis (acrosome reaction). In vitro, the acrosome reaction can be initiated by incubation with Ca^{++} ionophore or with progesterone. It has been suggested that the putative sperm surface PR is a GABA receptor-like chloride channel (Roldan et al. 1994; Meizel 1997). The *Xenopus laevis* sperm receptor was found to be a homolog of the mammalian sperm receptor ZP2 (Tian et al. 1999). Studies carried out in our laboratory and in collaboration with that of J.M. Baltz eliminate the possibility that the putative oocyte PR is a GABA receptor-like chloride channel (J.M. Baltz and X.J. Liu, unpublished). In these studies, chloride channel blockers (DIDS, picrotoxin) were unable to inhibit progesterone-induced oocyte maturation. Moreover, image analysis of single oocytes injected with MQAE, a chloride-sensitive dye, failed to detect any progesterone-induced alteration of chloride permeability of the plasma membrane.

A study reported that progesterone binds oxytocin receptors in the uterus and therby competitively inhibits oxytocin binding (Grazzini et al. 1998). *Xenopus* oocytes do

not appear to express endogenous oxytocin receptor (Morley et al. 1988) and therefore it is unlikely to be involved in oocyte maturation. From the data available, the involvement of a GABA like receptor or oxytocin receptor, both of which are transmembrane receptors, appears unlikely.

The Estrogen Receptor (ER)

Besides its genomic function (transcriptional), estrogen is also known to exert nongenomic action, mainly Ca^{++} release and/or MAP kinase activation. In the same manner that early studies suggested that the putative *Xenopus* PR was a cell surface receptor, the existence of an estrogen cell surface receptor was also speculated. This notion was based on studies that employed BSA-E2 or anti-estrogen receptor antibodies (Watson and Gametchu 1999). Recent studies (Migliaccio et al. 1998; Castoria et al. 1999; Razandi et al. 1999) have demonstrated that the classical, intracellular estrogen receptor is also responsible for the non-genomic action of estrogen. These studies involved reconstitution of the non-genomic action of estrogen in heterologous cells. Interestingly, the non-genomic action of progesterone receptor in COS cells transfected with exogenous genes apparently requires both the classical PR and ER, in the form of a heterologous receptor dimer (Castoria et al. 1999). Estrogen is inactive in oocytes (Smith 1989). It is improbable that the progesterone action in oocytes requires an ER.

Cross-Talk Between PR and ER

Several studies concerning the cross-talk between ER and PR have been conducted recently, demonstrating the non-genomic nature of their action (Migliaccio et al. 1998; Castoria et al. 1999). The Src/$p21^{ras}$/Erk pathway is activated by estrogen in

human breast cancer cells. This activation is due to an interaction of ER with c-Src (Migliaccio et al. 1998). The study demonstrated that in Cos-7 cells, progestin-induced MAPK pathway activation is dependent on co-transfection of PR_B and ER. To prove that the action of PR_B was independent of its ability to regulate trascription, they co-transfected a transcriptionally inactive PR_B, which also activated the signaling pathway. PR_B associated via the N-terminal 168 a.a. with ER. The study proposed that the action of ER and agonist-activated PR_B activated the Src/$p21^{ras}$/Erk pathway. The transcription-independent activity of oestradiol and progestin triggered DNA synthesis and cell cycle progression (Castoria et al. 1999).

CHAPTER TWO

XENOPUS INSULIN RECEPTOR SUBSTRATE U (XIRS-U)

Introduction: Molecular Cloning of a Novel xIRS

Work described here has been published (Ohan et al. 1998). The initial cloning of xIRS-u was carried out in Dr. Liu's laboratory before my arrival. My contributions to this project include constructing all of the expression plasmids and the majority of the microinjection experiments. Dr. Ohan, a post-doctoral fellow in Dr. Liu's laboratory, taught me most of the techniques and provided guidance and help throughout this project, including performing some of the injection experiments.

MATERIALS AND METHODS

Construction of Expression Plasmids

An NcoI-EcoRI fragment of the xIRS-u cDNA insert was ligated into pCS2+MT (Fig. 4, Turner et al. 1994), which was also treated with NcoI and EcoRI. This resulted in an expression plasmid that encoded the PH-PTB domain of xIRS-u (nucleotides 1-299), and was preceeded by 5 copies of the 13-amino acid Myc tag. To clone the full length xIRS-u in pCS2+MT, the EcoRI-EcoRI fragment (nucleotides 1029 to the 3'end of the cDNA) was ligated to an EcoRI digested pCS2+MT/PH-PTB vector. Deletion of the amino terminal 118 amino acids from the PH-PTB and the full-length xIRS-u generated ΔPH-PTB and xIRS-u ΔPH respectively. This was accomplished by replacing the NcoI-EcoRI fragment with a PCR-amplified fragment encoding amino acids 119-299. The primer used was xIRS-u ΔPH NcoI (5'-TAT CCA TGG CCGC TGT ACC TGT GTT TC-3') . The PH domain construct, xIRS-u PH, was constructed by using the Myc tagged PH-PTB construct and deleting a fragment from a unique SacI site in the cDNA to an XbaI site in the vector, resulting in expression of the first 170 amino acids, which aligned with the PH domain.

mRNA Synthesis

All plasmids were linearized with a NotI restriction enzyme, which cut after the SV40 poly-A sequence in the vector. In vitro transcription was carried out using SP6 RNA polymerase (Ambion, Austin, TX). The synthesized mRNA was dissolved in water to a concentration of 1mg/ml, as estimated by comparison to RNA standards (GIBCO-

BRL, Gaithersburg, MD) of known concentration separated by formaldehyde-agarose gel electrophoresis.

Oocyte Manipulation

Manipulation of live oocytes was carried out in a room maintained at 18°C. Adult female *Xenopus laevis* were injected with pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co., St Louis, MO; 50 IU/frog) 3-10 days before oocyte isolation. Preparation of the frog for surgical removal of the ovaries required the induction of hypothermia. This was achieved by placing the frog in ice water for 15 minutes, followed by an additional 10 minutes on crushed ice. The surgery was performed under sterile conditions. A 3 to 5 cm incision was made through both the skin and muscle layers in the frog's abdomen area. The ovary was pulled out and an appropriately sized piece was cut out and immediately placed in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8). Stage VI oocytes were manually defolliculated according to Smith (Smith, 1989), from surgically removed fragments of ovary. Oocyte manipulations, including mRNA microinjection, oocyte incubation and hormonal stimulation was carried out in regular OR2, unless otherwise specified. For experiments involving mRNA injection, 10 ng (in 10 nL) of mRNA or 10 nL of water were injected per oocyte, unless otherwise mentioned. The injected oocytes were incubated in OR2 medium for a minimum of 6 hrs before the addition of hormones or a second microinjection.

Insulin stimulation (final concentration, 5 μM) was carried out in OR2 lacking K^+ ions for maximal response of the oocytes to insulin (Cicirelli et al. 1990; Tonks et al.

1990). Absence of K^+ ions did not induce or inhibit oocyte maturation, and had no effect on oocyte viability (Cummings et al. 1996). Progesterone stimulation (20 μM) was carried out in unmodified OR2. In vitro GVBD was assayed after incubation of the oocytes overnight with insulin or progesterone. Oocytes that underwent GVBD had the distinct appearance of a white spot at the center of the animal hemisphere. In some instances, GVBD was confirmed by bisecting trichloroacetic acid (5%) fixed oocytes. The presence (GVBD negative) or absence (GVBD positive) of a germinal vesicle could be directly observed in this way.

Protein Isolation and Western Blotting

For protein isolation, the desired number of oocytes were forced through a pipette tip and lysed in PBS lysis buffer (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 μg/ml each of leupeptin and aprotinin, 1mM phenylmethylsulfonate, and 1mM sodium orthovanadate; 10 μl lysis buffer per oocyte). To isolate the protein fraction from the homogenate, the lysate was centrifuged at maximum speed in an Eppendorf centrifuge for 15 minutes at 4°C. The middle clear fraction was collected, mixed with equal amount of 2X reduced SDS sample buffer, and analyzed by SDS-PAGE on a 15% polyacrylamide gel. The yolk proteins, present in the top yellow layer, and the lower dense fraction were not collected. Depending on the protein assay being performed the amount of protein loaded on the gel varied as indicated. This was followed by immunoblotting with anti-xMAP kinase antiserum and anti-Myc antibody (9E10), both used at 1:1000 dilutions. The immunoblots were then developed using an ECL kit (Amersham, Arlington Heights, IL).

RESULTS

Sequence Analysis of xIRS-u

Analysis of the nucleotide sequence of the xIRS-u cDNA revealed two possible translation initiation sites (ATG codons that are preceded by a consensus translation initiation). The first ATG was chosen as the putative translation initiation site to avoid possible truncation of xIRS-u at the amino terminus. The cDNA also contained a unique NcoI site at the first ATG. This allowed for the easy generation of a Myc-tagged full length xIRS-u construct. The xIRS-u cDNA contained an open reading frame of 1003 amino acids with an estimated molecular mass of 110 kDa.

From pair-wise amino acid sequence comparisons (Table 1) it was observed that xIRS-u has similar overall sequence homology to all the members of the mammalian IRS family (33-45% identical in predicted amino acid sequence). On the other hand, the previously cloned xIRS-1 (Liu et al. 1995) is particularly similar to rat IRS-1 (67%) and much less similar to the other mammalian IRS members (31-46%). Comparing individual domains revealed similar patterns of sequence homology (data not shown, Ohan et al. 1998). Unlike the PH and PTB domains that are readily identifiable in all IRS members (Fig. 2), the SAIN domain has only been identified in IRS-1 (Gustafson et al. 1995), xIRS-u (Liu et al. 1995) and IRS-2 (He et al. 1996). IRS-3 (Lavan et al. 1997a) and IRS-4 (Lavan et al. 1997b) lack an apparent SAIN domain. From the extensive pair-wise comparisons it appears that xIRS-u is a new member of the IRS family, rather than an amphibian homolog of an existing IRS member. On that basis the nomenclature xIRS-u

Table 1

A Novel IRS Protein, xIRS-u

Pair-wise amino acid sequence comparisons (BESTFIT, Genetic Computer Group, Madison, WI) of xIRS-u and xIRS-1 to the mammalian IRS family members. The table shows the percent identity between each pair.

	xIRS-1	rIRS-1
xIRS-u	100	47
xIRS-1	47	100
rIRS-1	37	67
mIRS-2	45	46
rIRS-3	35	31
hIRS-4	33	33

(unique or undetermined designation) was chosen since no mammalian homolog has been identified.

Sequence analysis of xIRS-u also revealed multiple (5) tyrosine residues in the context of the YM/LXM motif (Fig. 2). Phosphorylation of such motifs creates binding sites for the SH2 domains of PI 3-kinase (Songyang et al. 1993). Another interesting site was a putative phosphorylation site (Y789 INID) that is a putative binding site for the GRB2 SH2 domain (Myers et al. 1994).

Expressing Full-Length and Deletion Mutants of xIRS-u

The expression vector pCS2+MT (Turner & Weintraub, 1994) was used for the functional characterization of xIRS-u. xIRS-u, and its mutants, were inserted in the NcoI site of pCS2+MT (Fig. 4). This resulted in a plasmid encoding five copies of the Myc tag followed by the desired xIRS-u coding sequence. The SV40 polyA sequence present in pCS2+MT at the end of the cDNA insert likely increases the stability of the mRNA in oocytes.

In this study, 5 different constructs were utilized (Fig. 5). xIRS-u represents the full- length protein initiating at amino acid 1. xIRS-u PH-PTB contains the amino acids 1-299. xIRS-u ΔPH was constructed by deletion of the first 118 amino acids. Previously, such a deletion compromised the function of the PH domain of IRS-1, and ultimately the function of IRS-1 (Voliovitch et al. 1995). ΔPH-PTB was constructed by deleting amino acids 1-118 from the xIRS-u PH-PTB construct. Finally, xIRS-u PH (amino acids 1-170) was derived from the PH-PTB construct by an internal deletion of amino acids 171-299.

Figure 4
xIRS-u in pCS2+MT vector
CloneMap™ generated diagram showing pCS2+MT/xIRS-u. Fusing the xIRS-u coding sequence to the five copies of Myc-tag present in pCS2+MT made it possible to analyze expressed proteins by immunoblotting with an anti-Myc antibody as described in Materials and Methods. Also shown are several key restriction enzyme sites in the cDNA, the SP6 promoter used for *in-vitro* transcription, as well as the SV40 polyadenylation site.

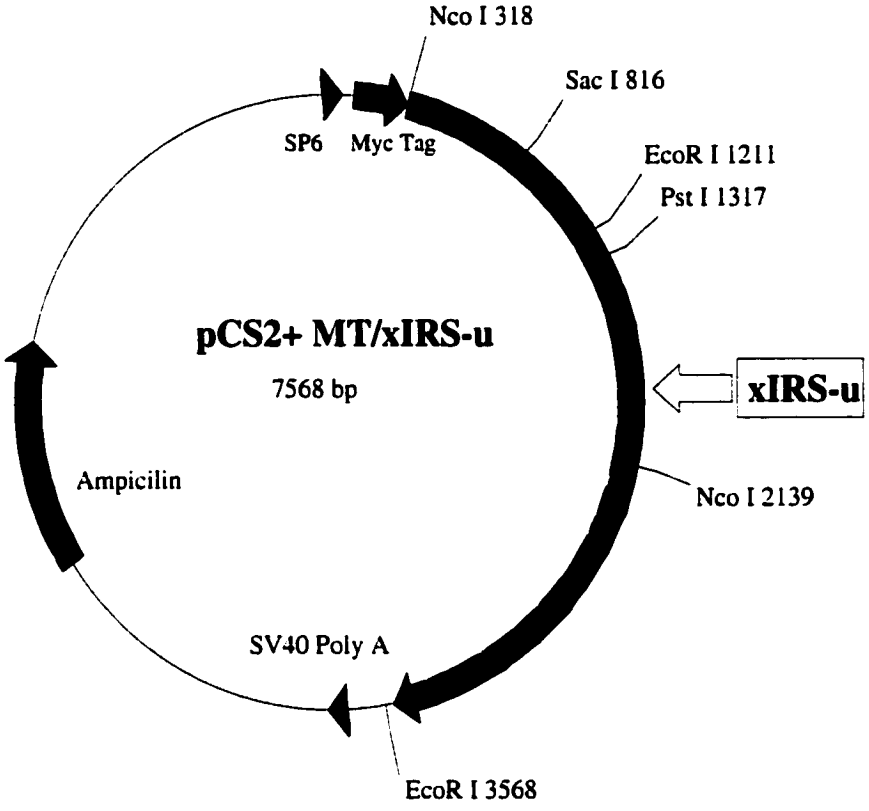
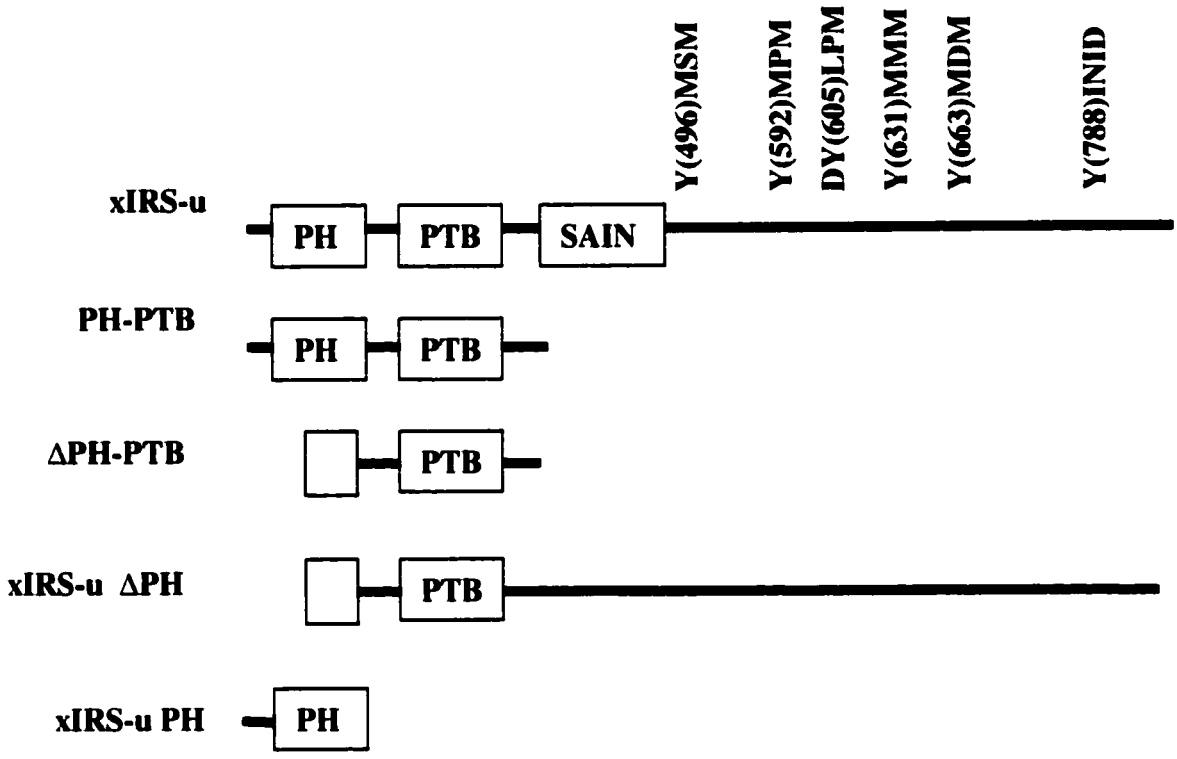

Nco I 318
Sac I 816
SP6
Myc Tag
EcoR I 1211
Pst I 1317
pCS2+ MT/xIRS-u
7568 bp
xIRS-u
Ampicilin
Nco I 2139
SV40 Poly A
EcoR I 3568

Figure 5
Schematic Representation of xIRS-u and the Different Truncation Mutants
All the constructs represented were cloned in the pCS2+MT vector and thus are fused to five copies of the Myc tag. Putative SH2 binding sites are indicated.

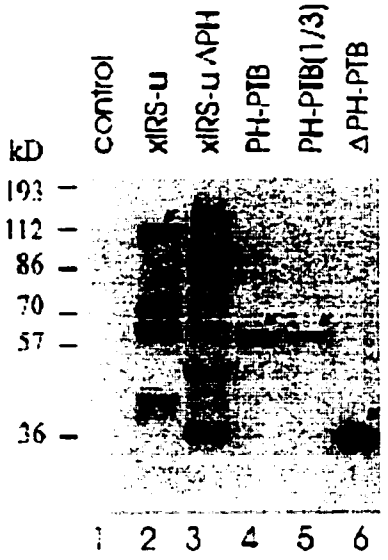

Y(496)MSM
Y(592)MPM
DY(605)LPM
Y(631)MMM
Y(663)MDM
Y(788)INID
xIRS-u
PH
PTB
SAIN
PH-PTB
PH
PTB
ΔPH-PTB
PTB
xIRS-u ΔPH
PTB
xIRS-u PH
PH

To examine the expression of the different constructs, oocytes were injected with the various xIRS-u in vitro transcribed mRNAs and incubated overnight. The oocytes were lysed and analyzed by immunoblotting to assess the accumulation of the Myc-tagged proteins (Fig. 6). The predicted molecular mass of the Myc-tagged xIRS-u protein is approximately 117 kDa (110 kDa plus 7 kDa from 5 copies of the 13 amino acid Myc tag). Immunoblotting with anti-Myc antibodies revealed that xIRS-u mRNA injection resulted in the production of a 120 kDa protein, within the predicted range. xIRS-u ΔPH resulted in an 86 kDa protein, consistent with the deletion of the amino terminal 118 amino acids. xIRS-u PH-PTB produced a protein slightly larger than the expected 40 kDa, at about 57 kDa. The size of ΔPH-PTB was as predicted, taking into consideration the 118 a.a. deletion. Finally, injection of the xIRS-u PH domain mRNA resulted in the expression of a protein close to the predicted size (data not shown; Ohan et al. 1998).

xIRS-u Potentiates Insulin Signaling

Injection of *Xenopus* oocytes with xIRS-u mRNA accelerated their response to insulin stimulation and potentiated the effect of insulin, as determined by GVBD assay (data not shown). As an alternative method of quantifying insulin signaling in *Xenopus* oocytes, anti-xMAP kinase immunoblotting was used to determine the ratio of activated MAP kinase compared to total MAP kinase. Activation of xMAP kinase and MPF are recognized biochemical events upstream of GVBD during hormone-induced oocyte maturation (Smith, 1989; Matten et al. 1994). Utilizing immunoblotting, the ratio of active xMAP kinase over total xMAP kinase is indicative of GVBD in a group of oocytes (Cummings et al. 1996; Posada et al. 1993).

Figure 6
***In-vivo* Expression and Immunoblot Analysis of xIRS-u and Truncation Mutants**
Groups of at least 20 oocytes were injected with water (control) or mRNA as described in Materials and Methods. After overnight incubation, the oocytes were lysed. Immunoblotting using an anti-Myc antibody showed the expression of the various proteins. Sizes are indicated in kilodaltons according to the prestained protein markers.


kD
193
112
86
70
57
36
control
xIRS-u
xIRS-u ΔPH
PH-PTB
PH-PTB(1/3)
ΔPH-PTB
1 2 3 4 5 6

xMAP kinase activation was not observed in xIRS-u injected oocytes in the absence of insulin stimulation (Fig. 7, 0 insulin). Seven hours after insulin stimulation, acceleration of xMAP kinase activation was detected in xIRS-u injected oocytes compared to control oocytes, in which no xMAP kinase activation was seen. This acceleration of xMAP kinase activation was also evident at 10.5 hours. Longer incubation periods, such as 15 h, showed equivalent xMAP kinase activation levels (maximal activation). The injection of xIRS-u mRNA had no effect on progesterone induced MAPK activation or GVBD (data not shown). This data clearly indicated that over expression of xIRS-u accelerated insulin signaling in *Xenopus* oocytes.

Importance of the PH Domain in Potentiation of Insulin Signaling

Earlier studies have shown that the PH domain of IRS-1 increases the efficiency of IRS-1 as a substrate for the ligand-activated insulin receptor, thus causing it to be a better mediator of insulin signaling (Myers et al. 1995; Yenush et al. 1996; Voliovitch et al. 1995). In order to study the effect of the PH domain of xIRS-u on the potentiation of insulin signaling, the ability of xIRS-u and xIRS-u ΔPH domain to potentiate insulin-induced xMAP kinase activation were compared (Fig. 8). Ten hours after insulin stimulation, it was clear that both constructs were successful in accelerating insulin-induced xMAP kinase activation (Fig. 8, lanes 5 and 6) as compared to control oocytes (lane 4). On the other hand, at 5 h after insulin stimulation, xIRS-u ΔPH was less effective in accelerating xMAP kinase activation (comparing lanes 2 and 3). The injection of xIRS-u or xIRS-u ΔPH mRNA had no effect on progesterone induced MAPK

Figure 7
xIRS-u Accelerates Insulin-Induced MAP Kinase Activation
Control and xIRS-u injected oocytes (at least 150/group) were incubated overnight in OR2. An initial sample of oocytes was lysed prior to hormonal stimulation, the remaining oocytes were stimulated with insulin and samples (30 oocytes) were lysed after different time points for a MAP kinase immunoblotting assay.

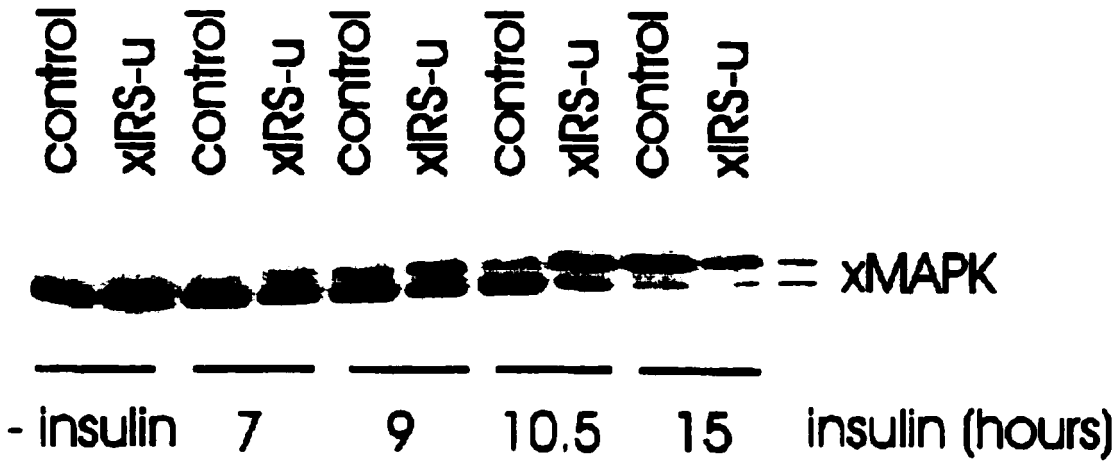
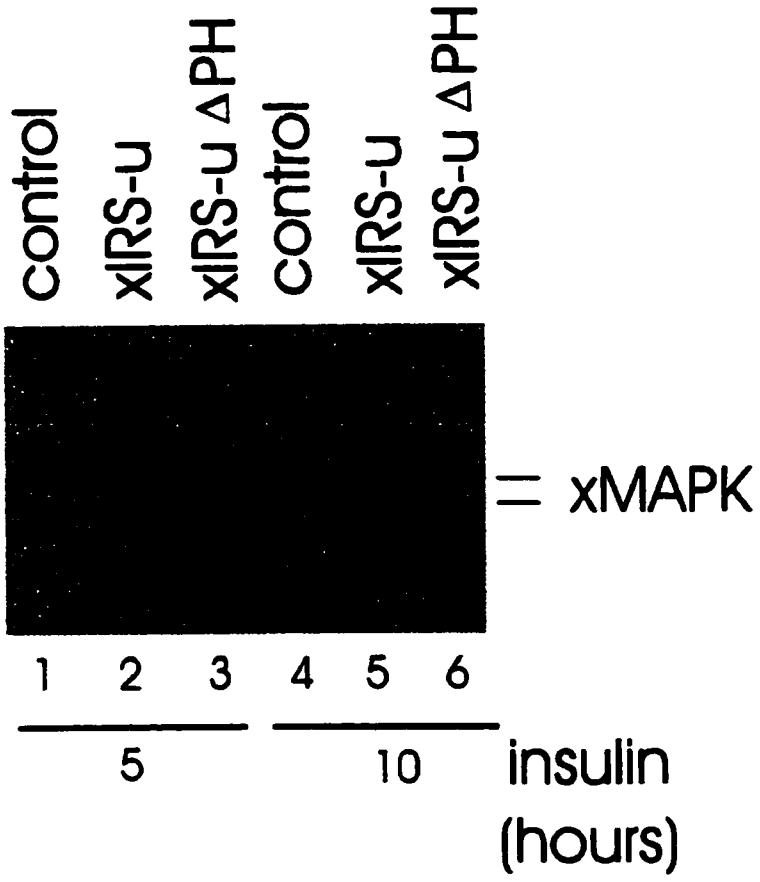

control
xIRS-u
control
xIRS-u
control
xIRS-u
control
xIRS-u
control
xIRS-u
= xMAPK
- insulin
7
9
10.5
15
insulin (hours)

Figure 8

xIRS-u ΔPH is Less Efficient in Enhancing Insulin Signaling than xIRS-u

Oocytes (80) injected with water (control), xIRS-u and xIRS-u ΔPH, were incubated overnight in OR2. Samples (40 oocytes) were taken at 5 h and 10 h after insulin stimulation, and the lysates were used in a MAP kinase immunoblotting assay.


control
xIRS-u
xIRS-u ΔPH
control
xIRS-u
xIRS-u ΔPH
= xMAPK
1 2 3 4 5 6
5
10
insulin
(hours)

activation and GVBD (data not shown). The results suggested that the PH domain contributed to the ability of xIRS-u to potentiate insulin signaling.

Inhibition of Insulin Signaling by xIRS-u PH-PTB Domain

A previous study (Tanaka & Wands, 1996) demonstrated that overexpression of the amino-terminal region of IRS-1 (PH and PTB domains) inhibits insulin signaling. The study suggested that the amino-terminal region functions as a dominant negative inhibitor of endogenous IRS-1. Their study did not clarify if either or both (PH and PTB domains) are required for this inhibition. Based on these studies, the ability of xIRS-u PH-PTB (a.a. 1-299) to inhibit insulin signaling in oocytes was studied (Fig. 9). At 15 h after insulin stimulation, control oocytes had completely activated xMAP kinase (lane 1) as expected. Meanwhile there was an obvious inhibition of xMAP kinase activation (lane 3) of xIRS-u PH-PTB mRNA injected oocytes (lane 3). Even when the amount of mRNA was reduced to one third, significant inhibition was still observed (compare lanes 4 and 1), with a corresponding reduction in protein expression (Fig. 6, lanes 4 and 5). On the other hand, xIRS-u ΔPH-PTB (a.a. 119-299) containing an intact PTB domain, but lacking a functional PH domain, did not inhibit insulin-induced xMAP kinase activation (Fig. 9, lane 5).

To confirm that the observed inhibitory effect of xIRS-u PH-PTB was specific to insulin signaling, we observed its effect on progesterone-induced xMAP kinase activation (Fig. 10). As anticipated, inhibition of insulin-induced xMAP kinase activation was observed in xIRS-u PH-PTB injected oocytes (compare lanes 3 and 4). xIRS-u PH-PTB

Figure 9

xIRS-u PH-PTB Inhibits Insulin-Induced MAP Kinase Activation

Oocytes (30) injected with water (control), xIRS-u, xIRS-u PH-PTB (10 ng and 3.3 ng) and xIRS-u ΔPH-PTB were incubated overnight in OR2. Insulin was added, and the oocytes incubated for 15 h (overnight) before being lysed for MAP kinase immunoblotting assay.

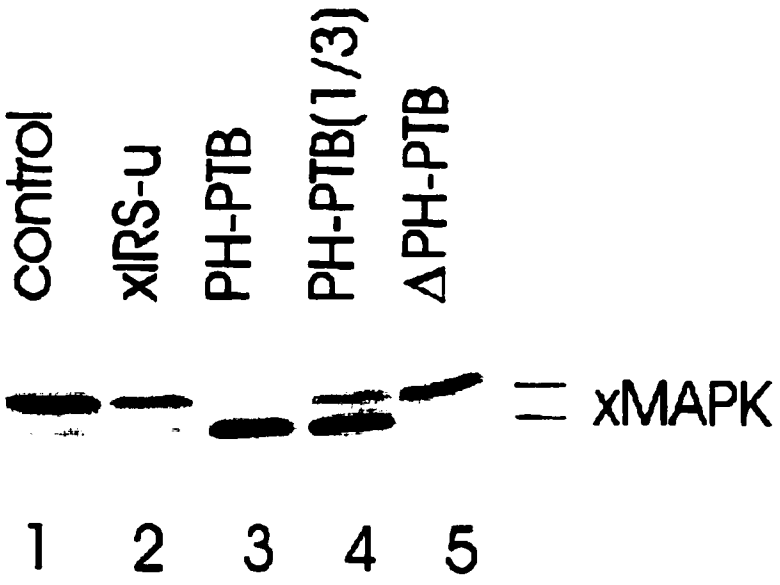
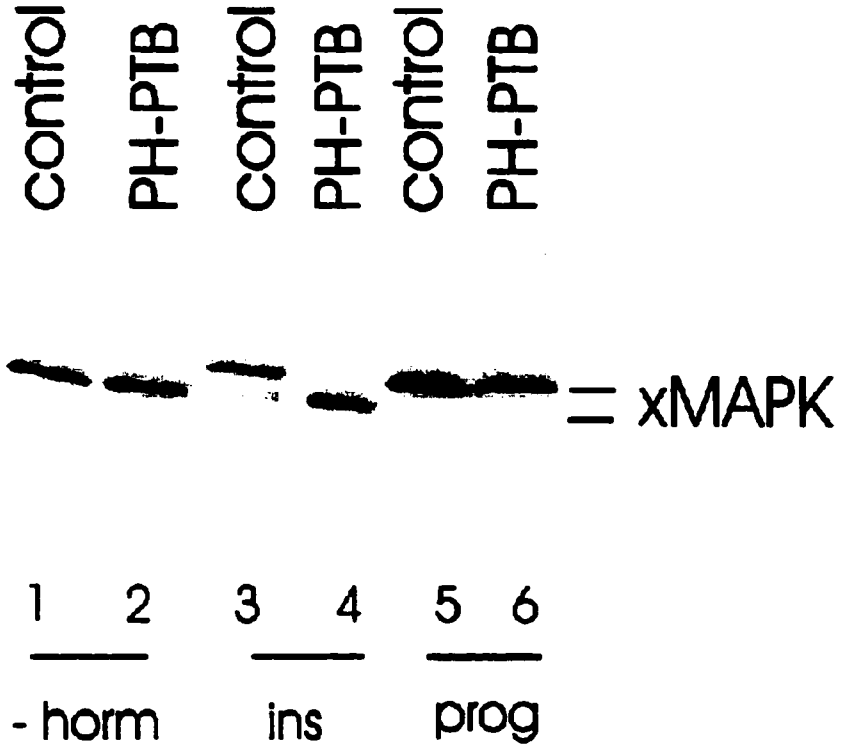

control
xIRS-u
PH-PTB
PH-PTB(1/3)
ΔPH-PTB
xMAPK
1
2
3
4
5

Figure 10
xIRS-u PH-PTB Specifically Inhibits Insulin-Induced MAP Kinase Activation
Oocytes were injected with water (control) or xIRS-u PH-PTB mRNA, and incubated overnight in OR2. The oocytes were divided into groups and were incubated overnight in OR2 lacking hormone (control), or with insulin or progesterone. Samples were lysed and the lysates were subjected to MAP kinase immunoblotting assay.

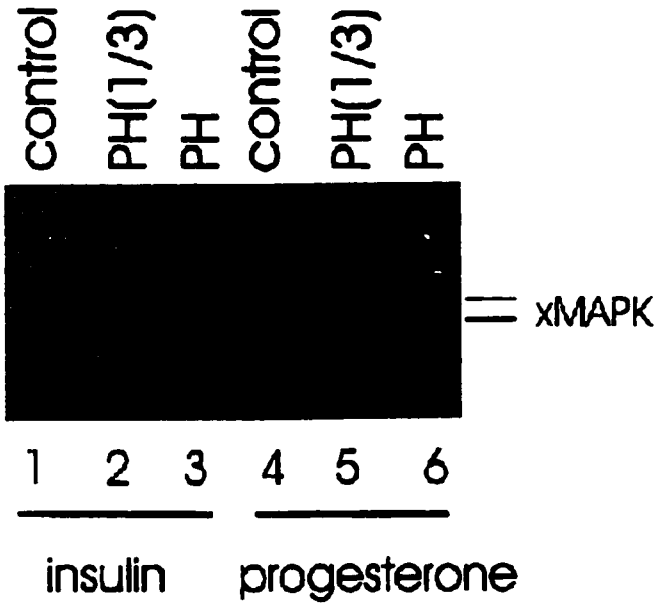

control
PH-PTB
control
PH-PTB
control
PH-PTB
= xMAPK
1 2 3 4 5 6
- horm
ins
prog

mRNA injection had no effect on progesterone-induced xMAP kinase activation (compare lanes 5 and 6) or GVBD (data not shown).

PH Domain of xIRS-u is Sufficient for Inhibition of Insulin Signaling

To determine if the xIRS-u PH domain could inhibit insulin signaling in *Xenopus* oocytes independently, oocytes were injected with either 3.3 ng (1/3X) or 10 ng (1X) of xIRS-u PH mRNA. Injection of the PH domain significantly inhibited insulin-induced xMAP kinase activation when compared to control injected oocytes (Fig. 11, lanes 1-3). A similar pattern of GVBD inhibition was noted (data not shown, Ohan et al. 1998). This inhibition of xMAP kinase activation and GVBD by the PH domain was specific to insulin signaling, as it did not affect progesterone-induced GVBD (data not shown, Ohan et al. 1998) or xMAP kinase activation (Fig. 11, lanes 4-6).

Figure 11
xIRS-u PH Inhibits Insulin-Induced MAP Kinase Activation
Oocytes (30) injected with water (control) and xIRS-u PH (10 ng and 3.3 ng) were incubated overnight in OR2. Insulin was added, and the oocytes incubated for 15 h (overnight) before being lysed for MAP kinase Western blotting assay.


control
PH(1/3)
PH
control
PH(1/3)
PH
xMAPK
1 2 3 4 5 6
insulin
progesterone

DISCUSSION

A Novel Insulin Receptor Substrate, xIRS-u

Xenopus laevis has a pseudo tetraploid genetic makeup (Graf et al. 1991), and many genes are represented in nonallelic duplicates. Genes that are represented by non-allelic duplicates include the insulin receptor gene (Scavo et al. 1991) and the proinsulin gene (Shuldiner et al. 1989). Usually the duplicate loci of the same gene are more than 90% identical in the coding regions (Graf et al. 1991). However, the sequence identity between xIRS-u and the previously cloned xIRS-1 is relatively low (47%). This indicates that xIRS-u and xIRS-1 are distinct genes rather than nonallelic copies of the same gene.

The conclusion that xIRS-u is a novel member of the IRS family, rather than a *Xenopus* homolog of an existing mammalian IRS gene, was mainly based on pairwise comparisons of the deduced amino acid sequence of xIRS-u with those of other known members (Table 1). Based on these comparisons, xIRS-u has similar sequence homology to all mammalian IRS family members (33-45%). This contrasts with xIRS-1, which was particularly similar to mammalian IRS-1 (67% identical) and less similar to the remaining mammalian IRS family members (31-46%). These comparisons lead to the conclusion that a novel member of the IRS family, with no existing mammalian homolog, was identified. The total number of IRS genes in *Xenopus* (as well as in mammals) is still undetermined, but this data points to the presence of several IRS genes in the Xenopus genome.

Characterization of xIRS-u : Functional Importance of its PH Domain

Sequence analysis of xIRS-u indicated the presence of PH, PTB and SAIN domains, as well as several potential tyrosine phosphorylation sites that conform to the binding consensus sequence for known SH2 domain-containing proteins. The data clearly demonstrated the ability of xIRS-u in potentiating insulin signaling (Fig. 7). xIRS-u lacking the PH domain was far less effective at potentiating insulin induced MAPK activation and GVBD (Fig. 8). Earlier studies demonstrated the importance of the PH domain, as its deletion from IRS-1 or IRS-2 diminishes tyrosine phosphorylation of the respective IRS proteins by ligand-activated insulin receptors (Myers et al. 1995, Voliovitch et al. 1995, Yenush et al. 1996). This reduces the ability of the IRS proteins to mediate insulin-induced mitogenesis (Yenush et al. 1996). In the same studies, IRS tyrosine phosphorylation or insulin-induced mitogenesis were not as affected by the deletion of the PTB domain (Yenush et al. 1996). These studies clearly demonstrated the more essential role played by the PH domain, as compared to the PTB domain, in IRS-1 and IRS-2 phosphorylation by the insulin receptor. The presence of the insulin receptor substrate, Gab1, that contains a PH domain while lacking a PTB domain (Holgado-Madruga et al. 1996), supports the importance of the PH domain.

The data presented in this thesis further supports this notion. In addition to the less efficient potentiation of insulin signaling of xIRS-u lacking the PH domain, it was demonstrated that the PH domain, and not the PTB domain, could function in a dominant-negative manner to specifically inhibit insulin-induced MAPK activation and oocyte maturation. At the same time, there are conflicting reports (Sharma et al. 1997) that either the PTB or SAIN domain of IRS-1 can function in a dominant negative way to

inhibit insulin-induced MAP kinase activation in 3T3 L1 adipocytes. This study and subsequent results are the first to demonstrate the actions of the PH domain of an IRS protein alone in inhibiting insulin signaling through dominant negative activity. A potential mechanism of inhibition could involve the binding and saturation of IRS binding proteins or lipids. In this mechanism, the overexpression of the xIRS-u PH domain binds to downstream signaling proteins and renders them inactive because they are not necessary in the vicinity of an active receptor, that is needed to propagate the signal. Further studies are needed to test this theory.

In summary, a novel member of the IRS family, xIRS-u, was identified and characterized. Furthermore, the functional importance of its PH domain was demonstrated. The results suggest an interesting interaction between the PH domain of IRS proteins with other components of the insulin-signaling pathway. Studies revealing the mammalian homolog of xIRS-u and the mechanism of PH domain action will prove to be interesting.

CHAPTER THREE

XENOPUS PROGESTERONE RECEPTOR (xPR)

Introduction: The Identification of xPR

Work described here has been published (Bayaa et al. 2000). I was responsible for all of the cloning and generation of the expression plasmids. I also performed the majority of the microinjection experiments. Ronald A. Booth, a fellow student in Dr Liu's laboratory, was responsible for characterizing xPR in cos-7 cells (published in Bayaa et al. 2000, but not included in this thesis).

MATERIALS AND METHODS

Genomic Library Screening

Screening of a *Xenopus* genomic library (in λ phage, a gift from M. King) with PCR-amplified hPR hormone binding domain HBD (sequence encoding hPR amino acids 686-933 (Misrahi et al., 1987)) as a probe was carried out under reduced stringency conditions (Liu et al., 1995). The probe was radioactively (^{32}P) labeled using random priming (Boehringer Manheim, Germany). A total of 600,000 genomic clones, average insert size of 15-20 kb, were screened by filter hybridization. This covered the *Xenopus* genome (3 X 10^9 bp) around 3-4 times. The prehybridization buffer consisted of 50% formamide, 5X Denhardt's solution, 5X SSPE (1X SSPE is 0.18 M NaCL, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 8.0]), 0.1% SDS and 0.1 mg/ml heat denatured salmon sperm DNA. The hybridization buffer was the same as the prehybridization buffer but with 1X Denhardt's solution (instead of 5X), 90 mg/ml dextran sulfate and the desired radioactive probe. Both prehybridization and hybridization were performed at 42°C for 2 hours. The filters were then washed repeatedly with large volumes of increasingly dilute SSC (1X SSC is 150 mM NaCl plus 15 mM sodum citrate) containing 0.1% SDS at increasing temperatures, until the background level became stable. Finally, the membranes were exposed to Kodak (XAR-5) film. Positive clones were then confirmed and purified by secondary and tertiary screening using the same hPR HBD probe and hybridization conditions. Five positive clones were isolated from the genomic library screening.

Isolation of Bacteriophage DNA

To further characterize the positive clones obtained from library screens, the bacteriophage DNA was isolated. An isolated GEM11 phage plaque was picked with the

small end of a sterile pasture pipette and resuspended in 1 ml of SM buffer (90 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl (pH 7.5), and 0.01% gelatin solution; Sambrook et al. 1989) and a drop of chloroform. This mixture was incubated overnight on a nutator at 4°C. In a 50 ml sterile Falcon tube, 200 µl of the phage suspension was allowed to adsorb to 0.6 ml of LE392 liquid bacterial culture [grown overnight in liquid broth (LB – components per liter: 10 g tryptone, 5 g yeast extract, and 10 g NaCl) and 0.2% maltose] for 10 minutes at 37°C. Ten milliliters of LB containing 0.5% maltose and 10 mM $MgCl_2$ were added to the phage/bacterium mixture and the tubes were shaken overnight at 37°C. Cell lysis occurred after 6-9 hours. The bacterial debris was removed by centrifugation twice at 5,000 rpm, saving the supernatant after both spins. The phage particles were pelleted by high speed centrifugation (30,000 rpm for 30 minutes) and resuspended in 200 µl SM. One hundred microliters of freshly prepared proteinase K (Promega, 2 mg/ml in SM) were added, and incubated at 37°C for 2 hours. The samples were then extracted twice with phenol and once with chloroform. After ethanol precipitation, the DNA was pelleted, washed with 70% ethanol, dried and dissolved in ddH_2O or TE (10 mM Tris-HCl pH 7.4 and 1 mM EDTA pH 8.0).

Southern Blot Analysis

Southern blot analysis was performed to confirm and further characterize isolated bacteriophage DNA, as well as characterizing xPR in the *Xenopus* genome. DNA was purified from bacteriophage isolated after a tertiary screen. The DNA was digested with EcoRI restriction enzyme to excise the insert. The DNA fragments were separated by agarose gel electrophoresis and transferred by capillary blotting to Hybond-N

nitrocellulose membranes (Amersham) following standard procedures (Sambrook et al. 1989). The DNA was cross-linked to the membranes by exposing it to UV light using a GS Gene linker UV chamber (Biorad). DNA probe labeling, prehybridization, hybridization, and membrane washing were performed in the same manner as with the library screening. The membranes were exposed to Kodak (XAR-5) film.

Sequence Analysis

DNA sequence analysis on positive clones was carried out using the *LICOR* automated sequencer. Infrared fluorescent dye IRD4 labeled primers were used in sequithermal cycle sequencing (*LICOR*). The resultant labelled PCR products were resolved on a Long Range 4% polyacrylamide gel and sequenced using the *LICOR* automated sequencer. In some instances, manual sequencing was also performed following the protocol provided in the USB sequencing kit.

cDNA library Screening

Characterization of a potential genomic clone by Southern blotting following an EcoRI digestion yielded exons E1 and E2 that were located in separate EcoRI fragments within the clone. The two exons were PCR amplified separately and combined equally for probe synthesis in a subsequent oocyte cDNA library screen (Rebagliati et al., 1985). The cDNA library screening followed the same methods as with the genomic library screen, and resulted in the isolation of cDNA clone-6.

5' Rapid Amplification of cDNA Ends (RACE)

To isolate the remainder of the putative xPR cDNA, 5' RACE was carried out using a kit from Life Technologies according to manufacturer's instructions. Briefly, the first strand cDNA was synthesized using gene specific primer 1 (GSP1, Table 2). The cDNA was then purified using a GLASSMAX DNA isolation spin cartridge (Life Technologies, Inc.). A TdT-tailing reaction followed, in which terminal deoxynucleotidyl transferase (TdT) adds dCTP to the 5' end of the purified cDNA. A nested GSP2 and Abridged Anchor Primer (AAP), were used to PCR amplify the desired gene. Often a single PCR will not generate enough specific product to be visualized, or may sometimes generate numerous nonspecific products. To eliminate these problems, a nested amplification reaction using a GSP3 and Abridged Universal Amplification Primer (AUAP) was performed. The 5'RACE yielded RACE clone 6.

Cloning and Subcloning of xPR

cDNA clone 6 and RACE clone 6 were then ligated together via an overlapping BglIII restriction site and subcloned into the pCS2+MT (Turner et al., 1994) expression vector. The final plasmid construct encoded 5 copies of the Myc tag followed by xPR (a.a. 1-583), and was titled 'pCS2+MT-xPR'. For some experiments, the xPR gene was characterized by using hybrid receptors, in which the HBD was swapped. To generate the xPR-ER hybrid receptor, the xPR plasmid was digested with BglII (following codon ATC for I^{339}) and XbaI (3' non-translating region) to remove xPR amino acids 340-583 (xPR HBD is L^{336} to K^{583}). Human ER HBD (amino acids 282 to 595 (Green et al. 1986) were PCR amplified with an EcoRI adapter at each end. To create pCS2+MT-xPR-ER,

Table 2
Primers used for sequencing, RT-PCR, RACE and other purposes in the xPR chapter of the thesis

PRIMER	SEQUENCE (5'→3')	PURPOSE
λgt10For	AGC AAG TTC AGC CTG GTT AAG T	PCR of λgt10 phage DNA
λgt10Rev	CTT ATG AGT ATT TCT TCC AGG G	PCR of λgt10 phage DNA
EF1α 3'	CAG ATT GGT GCT GGA TAT GC	RT Control
EF1α 5'	ACT GCC TTG ATG ACT CCT AG	RT Control
hPR-HBD 5'	CAC CAC TGA TCA ACC TG	PCR HBD for library screening.
xPR6-for1	ACC ATT GTG TGA TAA TGT GC	Sequencing primer xPR 6KB RI insert that contains E2
xPR6RT5'	TAT GGA TCC TAC ATA AGA GAG CTG GCC	RT primer for tissue expression
xPR6exon3'	CAT GCA TGG AGT CCA TGA G	For PCR of E2 from 3' of xPR
xPR6-1/T3-1	AAC TAG CAA AGA ACC AAC G	Sequencing of xPR6-1
xPR6-1/T7-1	GGG CAA ACA CCT ACC CAC TCC	Sequencing of xPR6-1
xPR6-1/T3-2	TGG GAT CAC CCA CTG TGG	Sequencing of xPR6-1
xPR/HBD/ RACE-1	CAC AAA CGA GTT GTC GTT CAC	RACE from the N-terminus of HBD of xPR6
xPR/HBD/ RACE-2	CTG ATT GAG ACT ACT CAA CAA TG	Nested primer for cloning 5' end of xPR
xPR/HBD/ RACE-3	GTC TCG GGC TGT GTG GTA TC	5' RACE
xPRace4	GCC CTC GCT GCT AGG TAT GC	5' RACE
xPRace5	GGG TTC AAT GGC AGG ATG GG	5' RACE
xPRace6	ATT GTC CAG GTT CAG GTC TGG	5' RACE
xPR/DBD	CCT GCT TGG CAA CAC TTT CT	Sequencing of clone RACE-6

all ends were blunted before ligation. As a result of these manipulations, a single amino acid (Asn) was introduced between xPR I^{339} and hER S^{282}.

Anti-xPR Antibodies

A 645 bp fragment of xPR was isolated by digestion with NcoI and BglII (encoding for amino acids 1-215), followed by Klenow treatment, to fill in the 3' and 5' overhangs and create a blunt-ended fragment. The isolated fragment was inserted into pGEX-KT (Guan and Dixon 1991) previously digested with SmaI restriction enzyme. The pGEX-KT vector was used to allow the fusion of the xPR fragment to the C-terminus of glutathione S-transferase (GST). Induction of the GST fusion protein in bacteria by isopropyl thio-β-D-galactoside (IPTG) was followed by affinity purification according to Frangioni and Neel's (1993) method. The addition of SDS (1% final concentration) in the elution buffer was necessary due to the inability of high concentrations of free glutathione to elute the GST fusion protein from the glutathione beads. Before immunizing the rabbits, the excess SDS was removed by dialysis under electric current. For the first injection, the GST purified protein was mixed with complete Freund's adjuvant, and for the subsequent injections, incomplete adjuvant was used. The rabbit was boosted 4 and 6 weeks after the initial injection. Two weeks later, the rabbits were bled. The blood was incubated at 37°C for 1 hr, and then overnight at 4°C. After a low speed centrifugation (4,000 rpm), the serum was recovered and used directly. To purify xPR-specific IgG, the immune serum was first fractionated by ammonium sulfate (50% saturation) to precipitate total IgG. To deplete GST-specific IgG, the IgG fraction was incubated with purified GST that was previously coupled to sepharose beads. The

supernatant from the GST beads was then incubated with purified GST-xPR (a.a.1-215), previously coupled to sepharose beads. The beads were then washed extensively to remove all the unspecific IgG. The antibodies were then eluted by acidic pH buffer (0.2 M sodium acetate, pH 2.3) and dialyzed thoroughly against phosphate buffer saline. For immunoblotting either antiserum (1:500 dilution) or affinity purified IgG (2 μg/mL) was used as indicated.

Oocyte Manipulation

The same procedures were followed as in Part I, with the following differences. Oocytes injected with mRNA were incubated for 24-36 hrs prior to the addition of hormones. This longer incubation period was necessary to ensure a high level of protein expression and proper post-translational processing of the expressed recombinant protein. Insulin stimulation was carried out in regular OR2 rather than potassium-free OR2.

For the experiment dealing with endogenous xPR detection in oocytes from different stages, the oocytes were isolated using the collagenase method. The collagenase method was used because it was too difficult to isolate the smaller (stage I-III) oocytes by manual defolliculation. This was achieved by treating pieces of isolated ovary tissue for 3 hours with collagenase solution (0.2%, type I, in 0.1 M sodium phosphate buffer, pH 7.4, 0.1% bovine serum albumin, 0.1% soybean trypsin inhibitor, all from Sigma) to release the oocytes. The oocytes were then individually selected and grouped into the various stages as detailed in Smith et al. 1991. Removal of the vitelline membrane was attained by rinsing the collagenase-treated oocytes in regular OR2 before being placed in hypertonic OR2 (2X OR2). After a 5-10 minute incubation in the 2X OR2 the oocyte

cytoplasm shrank, resulting in the separation of the plasma membrane and the overlaying vitelline membrane. The vitelline membrane was then removed with forceps and discarded (Methfessel et al. 1986). The devitellinated oocytes were transferred to an eppendorf tube and lysed with PBS lysis buffer as described previously.

RNA Isolation

Isolation of RNA was carried out using the RNAZOL^{TM} B method (TEL-TEST). Briefly, oocytes or tissue samples were homogenized with RNAZOL^{TM} B (2 mL per 100 mg) in a glass-Teflon homogenizer. Two hundred microliters of chloroform was mixed in with the homogenate. After a 5 minute incubation on ice, the suspension was centrifuged (12,000 rpm, 4°C, 15 minutes). Two phases resulted, a phenol-chloroform phase and an upper aqueous phase. The aqueous phase was ethanol precipitated and washed with 75% ethanol, and the RNA precipitate was resuspended in 0.1% DEPC-treated water.

RT-PCR

cDNA synthesis was carried out on total RNA (1 μg). In the reverse transcriptase reaction, random hexamers (20 μM) and Superscript II RT enzyme (Gibco BRL) were used. The PCR was carried out for 30 cycles, and in some instances [^{32}P]-α-dCTP was used as a tracer. Conditions of the PCR reactions were as follows, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 2U Taq polymerase, and 5 μl cDNA. The thermal profile of the PCR reaction started with an initial 2 minute denaturation at 94°C, then 30 cycles as follows 30 seconds at 94°C, 30 seconds at a temperature depending on the T_m of the primers, and 30 seconds at 72°C. The xPR primers were

specifically designed to eliminate the possibility of amplifying sequence derived from contaminating genomic DNA by flanking two different exons (Fig. 11). Therefore, the amplified product should span both exons (E1 and E2). The EF1α primers (Table 2) were used for control purposes for the RT-PCR.

Subcellular Fractionation

Biochemical isolation of oocyte membranes was attained by lysing, typically 30 oocytes, in ice-cold hypotonic homogenization buffer (10 μL/oocyte, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.9, 0.5 mM PMSF, 10 μg/mL of leupeptin). Two rounds of low-speed centrifugation (900g for 5 minutes) clarified the homogenate (discarding the pellet after every round). This was followed by a centrifugation at 100,000g for 60 minutes, resulting in total oocyte membrane (pellet) and cytosol (supernatant) fractions. Any samples that were meant for anti-β-integrin blotting had to be dissolved in 2X non-reduced SDS sample buffer (lacks β–mercaptoethanol), since the antibodies did not recognize reduced proteins (Gawantka et al., 1992).

Oocyte germinal vesicles were isolated for immunoblotting according to Heine et al. 1993. First, the oocytes' membranes were pierced with forceps in the middle of the animal hemisphere. The GVs were then squeezed out of the oocytes in Joe Gall's 5:1 plus Mg^{++} (83 mM KCl, 17 mM NaCl, 1 mM $Mg^{++}Cl_2$, 10 mM Tris, pH 7.2). Isolated GVs were transferred to an Eppendorf tube with 1 ml of 100 mM Na-acetate, pH 5.2, 5 mM EDTA and kept on ice. The acetate buffer allowed the precipitation of the nuclear contents. After brief centrifugation to collect the nuclei, the supernatant was discarded. The nuclei were dissolved in SDS sample buffer for PAGE.

RESULTS

Genomic and partial cDNA cloning of xPR

The most likely domain to be conserved between human PR and the elusive xPR is the hormone binding domain (HBD) that binds progesterone. Using the human PR HBD to screen a *Xenopus* genomic library (a generous gift from M. King), two putative exons highly similar to the C-terminus of hPR (Fig. 12) were identified. A total of 600,000 genomic clones were screened, covering the *Xenopus* genome 3 to 4 times. Then hybridization screening of an oocyte cDNA library (Rebagliati et al. 1985) was carried out, using the putative exons E1 and E2 as probes, as well as performing 5'RACE on oocyte RNA in order to clone the putative full-length xPR cDNA. The cloned cDNA contained an open reading frame of 583 a.a. (Fig. 13), and it contained a putative translation start codon (ACCATGG). A nuclear localization signal was detected (^{286}RKFKKFGR). The cloned cDNA contained a putative HBD and a DNA binding domain (DBD) that are 86% and 92% identical in amino acid sequence, respectively, to their counterparts in hPR. No significant homology was detected between the N-terminal region of hPR and the cloned cDNA (a.a. 1-200). There was significant homology found with chPR, 65/200 a.a. sequence identity (Fig. 14). Based on these findings, the cloned cDNA likely represents the amphibian homologue of mammalian and avian PR and was termed xPR.

xPR in Stage I-VI Oocytes

To determine whether xPR was expressed in *Xenopus* oocytes, RT-PCR analysis on oocyte RNA was performed. As shown in Figure 15, oocytes of all developmental

Figure 12
Schematic Representation of xPR
Schematic comparison of xPR (accession=AY007198) and hPR (accession=M151716). Both the cDNA and 5'RACE clones that were isolated are illustrated. The figure also shows the characterized genomic clone with the internal EcoRI site, noting that the introns (line) and the two isolated exons (E1 and E2) are not drawn in proportion in the approximately 9 Kb genomic clone.

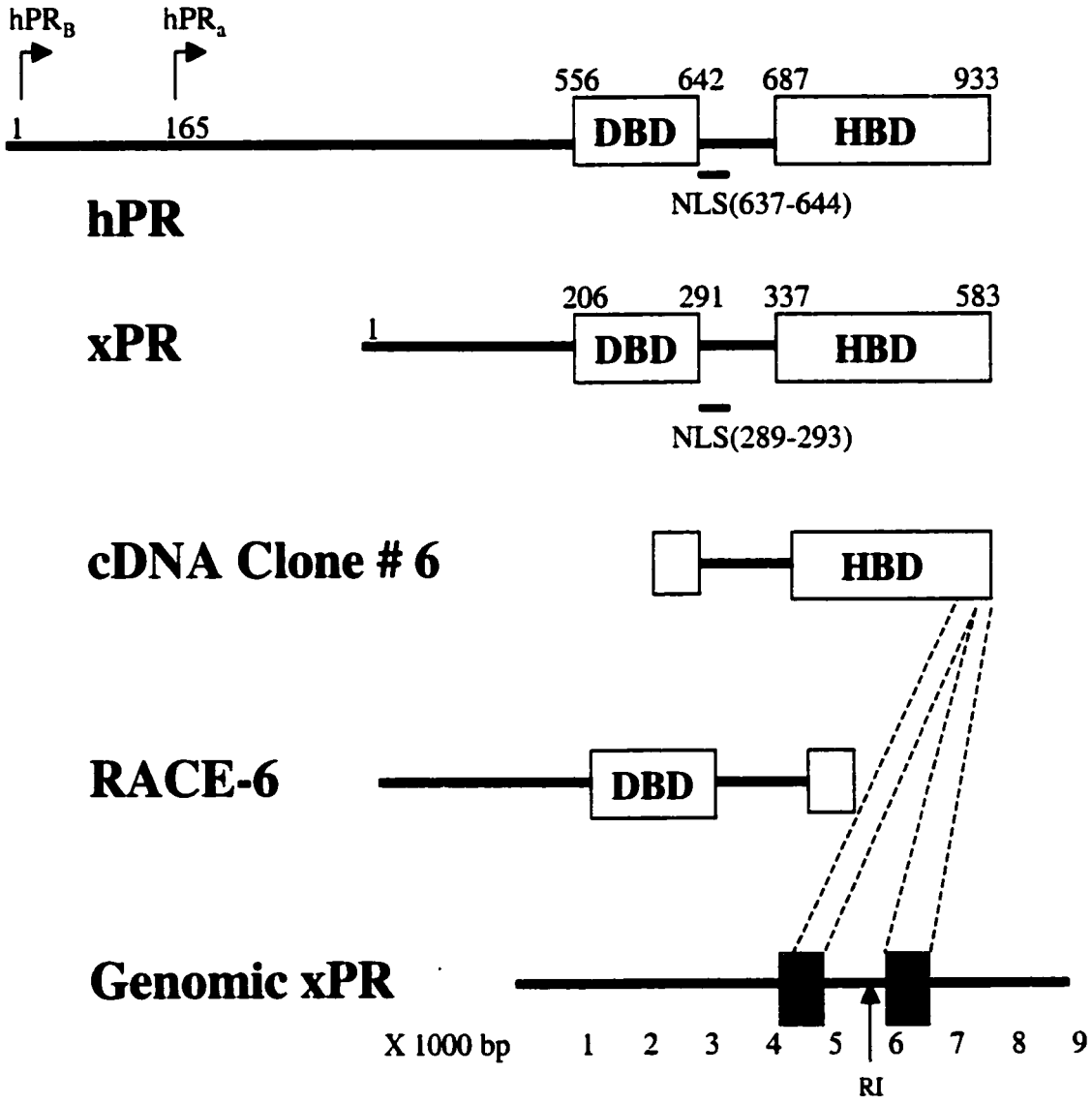

hPR$_B$
hPR$_a$
1
165
556
642
687
933
DBD
HBD
NLS(637-644)
hPR
xPR
1
206
291
337
583
DBD
HBD
NLS(289-293)
cDNA Clone # 6
HBD
RACE-6
DBD
Genomic xPR
X 1000 bp
1
2
3
4
5
6
7
8
9
RI

Figure 13
Nucleotide and Predicted Amino Acid Sequence of xPR
The bold face regions, from the amino terminus, are the DNA and Hormone binding domains respectively. The NLS is underlined in wave. E1(underlined) and E2(double underlined) are indicated. Numbers in superscript indicate amino acid positions.

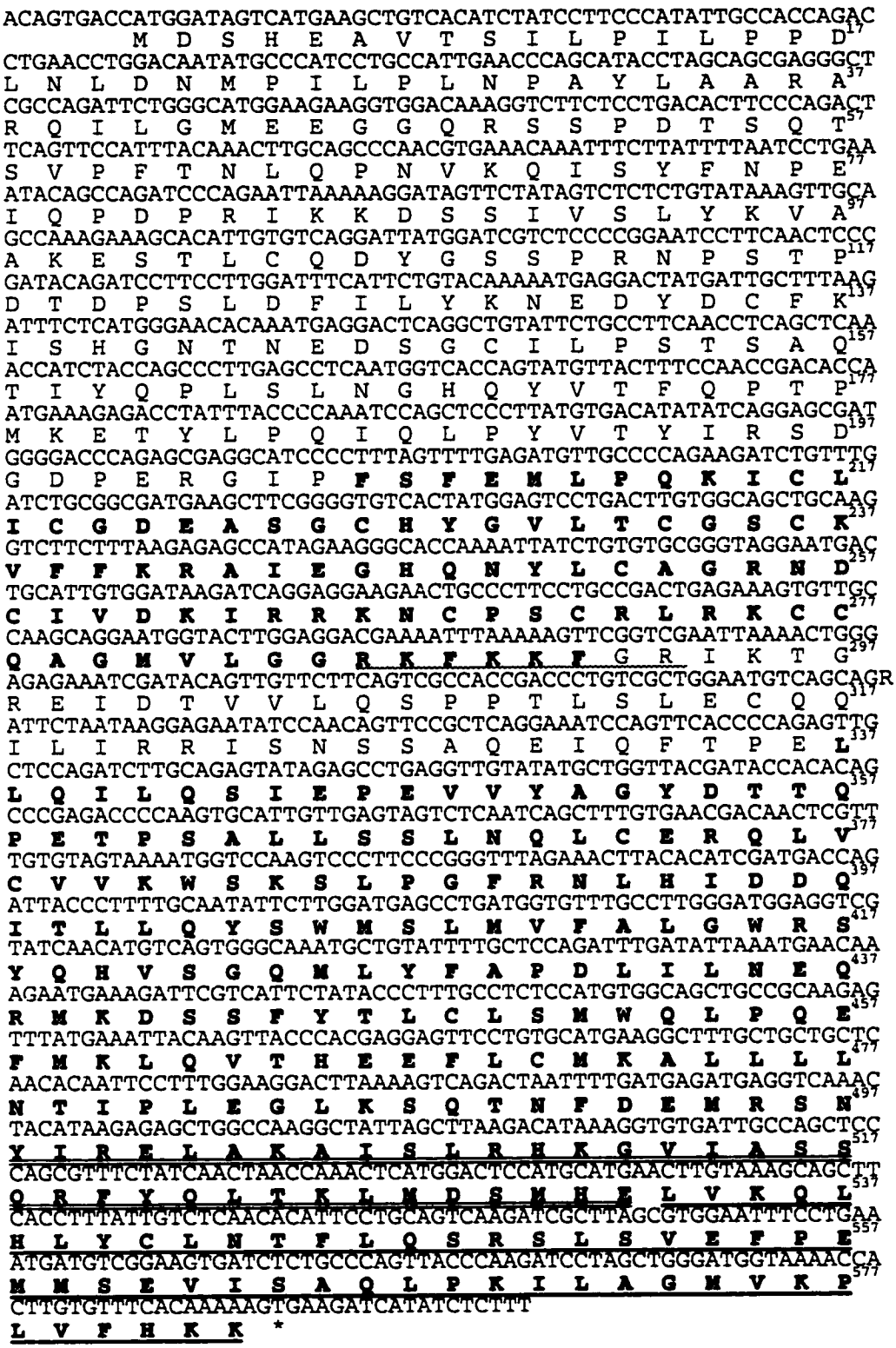

ACAGTGACCATGGATAGTCATGAAGCTGTCACATCTATCCTTCCCATATTGCCACCAGAC
M D S H E A V T S I L P I L P P D 17
CTGAACCTGGACAATATGCCCATCCTGCCATTGAACCCAGCATACCTAGCAGCGAGGGCT
L N L D N M P I L P L N P A Y L A A R A 37
CGCCAGATTCTGGGCATGGAAGAAGGTGGACAAAGGTCTTCTCCTGACACTTCCCAGACT
R Q I L G M E E G G Q R S S P D T S Q T 57
TCAGTTCCATTTACAAACTTGCAGCCCAACGTGAAACAAATTTCTTATTTTAATCCTGAA
S V P F T N L Q P N V K Q I S Y F N P E 77
ATACAGCCAGATCCCAGAATTAAAAAGGATAGTTCTATAGTCTCTCTGTATAAAGTTGCA
I Q P D P R I K K D S S I V S L Y K V A 97
GCCAAAGAAAGCACATTGTGTCAGGATTATGGATCGTCTCCCCGGAATCCTTCAACTCCC
A K E S T L C Q D Y G S S P R N P S T P 117
GATACAGATCCTTCCTTGGATTTCATTCTGTACAAAAATGAGGACTATGATTGCTTTAAG
D T D P S L D F I L Y K N E D Y D C F K 137
ATTTCTCATGGGAACACAAATGAGGACTCAGGCTGTATTCTGCCTTCAACCTCAGCTCAA
I S H G N T N E D S G C I L P S T S A Q 157
ACCATCTACCAGCCCTTGAGCCTCAATGGTCACCAGTATGTTACTTTCCAACCGACACCA
T I Y Q P L S L N G H Q Y V T F Q P T P 177
ATGAAAGAGACCTATTTACCCCAAATCCAGCTCCCTTATGTGACATATATCAGGAGCGAT
M K E T Y L P Q I Q L P Y V T Y I R S D 197
GGGGACCCAGAGCGAGGCATCCCCTTTAGTTTTGAGATGTTGCCCCAGAAGATCTGTTTG
G D P E R G I P F S F E M L P Q K I C L 217
ATCTGCGGCGATGAAGCTTCGGGGTGTCACTATGGAGTCCTGACTTGTGGCAGCTGCAAG
I C G D E A S G C H Y G V L T C G S C K 237
GTCTTCTTTAAGAGAGCCATAGAAGGGCACCAAAATTATCTGTGTGCGGGTAGGAATGAC
V F F K R A I E G H Q N Y L C A G R N D 257
TGCATTGTGGATAAGATCAGGAGGAAGAACTGCCCTTCCTGCCGACTGAGAAAGTGTTGC
C I V D K I R R R K N C P S C R L R K C C 277
CAAGCAGGAATGGTACTTGGAGGACGAAAATTTAAAAAGTTCGGTCGAATTAAAACTGGG
Q A G M V L G G R K F K K F G R I K T G 297
AGAGAAATCGATACAGTTGTTCTTCAGTCGCCACCGACCCTGTCGCTGGAATGTCAGCAGR
R E I D T V V L Q S P P T L S L E C Q Q 317
ATTCTAATAAGGAGAATATCCAACAGTTCCGCTCAGGAAATCCAGTTCACCCCAGAGTTG
I L I R R I S N S S A Q E I Q F T P E L 337
CTCCAGATCTTGCAGAGTATAGAGCCTGAGGTTGTATATGCTGGTTACGATACCACACAG
L Q I L Q S I E P E V V Y A G Y D T T Q 357
CCCGAGACCCCAAGTGCATTGTTGAGTAGTCTCAATCAGCTTTGTGAACGACAACTCGTT
P E T P S A L L S S L N Q L C E R Q L V 377
TGTGTAGTAAAATGGTCCAAGTCCCTTCCCGGGTTTAGAAACTTACACATCGATGACCAG
C V V K W S K S L P G F R N L H I D D Q 397
ATTACCCTTTTGCAATATTCTTGGATGAGCCTGATGGTGTTTGCCTTGGGATGGAGGTCG
I T L L Q Y S W M S L M V F A L G W R S 417
TATCAACATGTCAGTGGGCAAATGCTGTATTTTGCTCCAGATTTGATATTAAATGAACAA
Y Q H V S G Q M L Y F A P D L I L N E Q 437
AGAATGAAAGATTCGTCATTCTATACCCTTTGCCTCTCCATGTGGCAGCTGCCGCAAGAG
R M K D S S F Y T L C L S M W Q L P Q E 457
TTTATGAAATTACAAGTTACCCACGAGGAGTTCCTGTGCATGAAGGCTTTGCTGCTGCTC
F M K L Q V T H E E F L C M K A L L L L 477
AACACAATTCCTTTGGAAGGACTTAAAAGTCAGACTAATTTTGATGAGATGAGGTCAAAC
N T I P L E G L K S Q T N F D E M R S N 497
TACATAAGAGAGCTGGCCAAGGCTATTAGCTTAAGACATAAAGGTGTGATTGCCAGCTCC
Y I R E L A K A I S L R H K G V I A S S 517
CAGCGTTTCTATCAACTAACCAAACTCATGGACTCCATGCATGAACTTGTAAAGCAGCTT
Q R F Y Q L T K L M D S M H E L V K Q L 537
CACCTTTATTGTCTCAACACATTCCTGCAGTCAAGATCGCTTAGCGTGGAATTTCCTGAA
H L Y C L N T F L Q S R S L S V E F P E 557
ATGATGTCGGAAGTGATCTCTGCCCAGTTACCCAAGATCCTAGCTGGGATGGTAAAACCA
M M S E V I S A Q L P K I L A G M V K P 577
CTTGTGTTTCACAAAAAGTGAAGATCATATCTCTTT
L V F H K K *

Figure 14
Amino Acid Sequence Comparison of xPR and chPR
Amino acid sequence alignment between the N-terminus of xPR and chPR (chicken, accession=M37518). Black and shaded blocks indicate sequence identity and amino acid similarity respectively. Numbers within the parenthesis indicate the amino acid position in its respective protein.

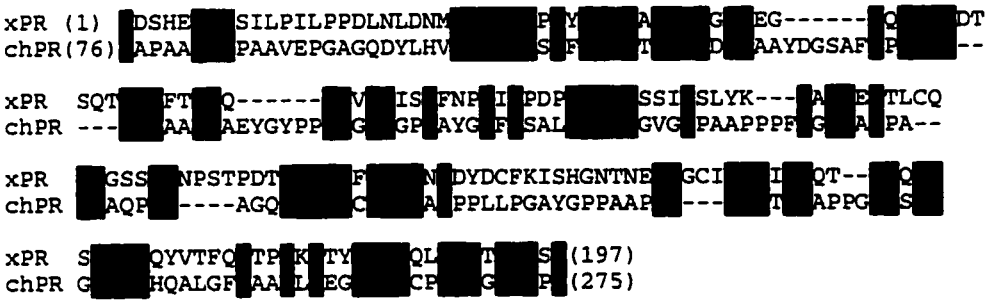
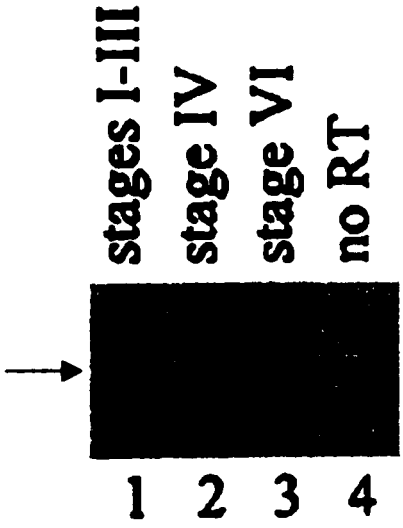


Figure 15
RT-PCR Analysis of Various Oocyte Stages
Total RNA (1 μg each) from oocytes of the different stages (stages I-III mixed with unknown ratio) was reverse-transcribed. This was followed by PCR amplification using xPR primers encompassing exons E1 and E2. Lane 4 is a negative control in which PCR was performed directly on input stage VI oocyte RNA without performing the reverse transcription reaction. Arrows indicate the specifically amplified product.


stages I-III
stage IV
stage VI
no RT
1 2 3 4

stages contained xPR mRNA. The maturation-incapable oocytes (stage I-IV), and the maturation-capable stage V and VI oocytes, all contain xPR mRNA.

Polyclonal anti-xPR antibodies detected a prominent band (designated as xPR) of approximately 78 kDa. The protein detected was slightly larger than the predicted size of 66 kDa. Results indicated the presence of xPR protein at similar levels in both small (stage IV) and large (stage V and VI) oocytes (Fig. 16). To rule out the possibility that the xPR protein was from contaminating attached follicle cells rather than being in the oocytes, the vitelline membrane was removed (Methfessel et al. 1986). Similar amounts of xPR were detected before and after the vitelline membrane was removed (Fig. 16, lanes 3 and 4, respectively).

Extranuclear Localization of xPR : xPR Appears to be a Cytosolic Protein

The localization of endogenous xPR was further characterized by employing enucleation experiments (Smith et al. 1991; Heine et al. 1993). Extracts from enucleated stage VI oocytes or the GV were analyzed by immunoblotting with anti-xPR and antibodies against other marker proteins. The results clearly showed that xPR was retained in the enucleated oocytes but was absent in the GV (Fig. 17, lanes 5 and 2 respectively). Meanwhile nucleolin, an intranuclear protein (Heine et al. 1993) was detected in the GV but was undetectable in protein extracted from the enucleated oocytes. Incubation of the oocytes with progesterone for 15 and 45 minutes did not cause translocation of xPR to the nucleus (Fig. 17, lanes 3 and 4). To further narrow down where xPR is located within the oocyte, subcellular fractionation experiments were

Figure 16
Immunoblot Analysis of Endogenous xPR in Various Oocyte Stages
Extracts from stage IV, V or VI oocytes (lanes 1 to 3 respectively), were isolated following collagenase treatment of ovarian tissues, or extracts from collagenase-treated and devitellinated oocytes (lane 4), were immunoblotted with anti-xPR or anti-xMAP kinase antiserum. An equal amount of protein was loaded on each lane.

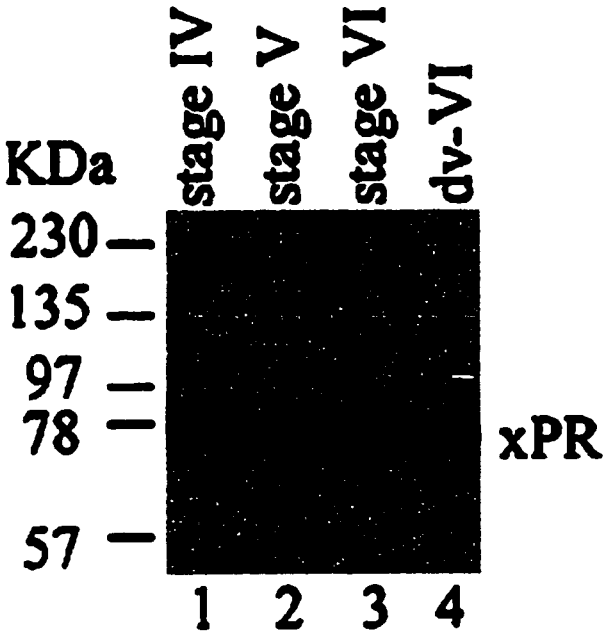
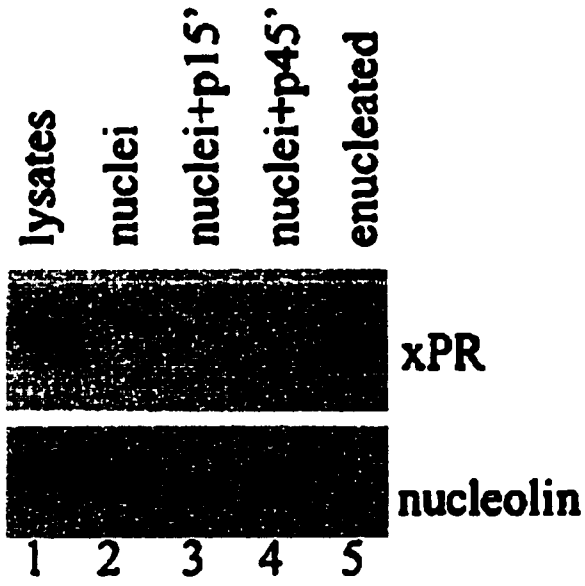

KDa
stage IV
stage V
stage VI
dv-VI
230
135
97
78
57
xPR
1
2
3
4

Figure 17
Localization of Endogenous xPR Outside the Nucleus
Extracts from intact oocytes (lane 1, equivalent of ½ oocyte), isolated nuclei (lane 2, 1 germinal vesicle), nuclei isolated from oocytes following 15 or 45 minute incubation with 0.5 μM progesterone (lanes 3 and 4 respectively, 1 GV each), and extracts from enucleated oocytes (lane 5, equivalent of ½ oocyte), were immunoblotted with anti-xPR or anti-*Xenopus* nucleolin (R2D2).


lysates
nuclei
nuclei+p15'
nuclei+p45'
enucleated
xPR
nucleolin
1 2 3 4 5

performed. This indicated that xPR was not associated with any membrane structures but instead was located in the cytoplasm (Fig. 18).

Enhancement of Progesterone Signaling by xPR via a Non-genomic Mechanism

Several approaches were used to determine whether xPR is indeed functional in mediating oocyte maturation.

Acceleration of Progesterone-Induced Signal

Oocytes injected with xPR or, for control, xPR-ER (hybrid receptor with the ER-HBD) mRNA, or an equal amount of water, were incubated with progesterone and observed for GVBD. Results indicated acceleration of progesterone-induced GVBD in oocytes injected with xPR mRNA when compared to oocytes injected with xPR-ER mRNA or with water (Fig. 19). Oocytes from different animals tend to respond differently and have varying GVBD response times. Acceleration time (time for 50% GVBD in control oocytes minus that in xPR-injected oocytes), is a more reliable measure of statistical significance among independent experiments. In four independent experiments, an acceleration time of 1-3.5 hours was observed with a p-value of 0.002 by the Student t-Test. Comparing both control groups, water injected and xPR-ER mRNA injected oocytes, no evident difference in response time was found (Fig. 19). For further confirmation, when metabolically more stable promegestone (synthetic progestin R5020 at 0.5 μM) or RU486 (50 μM) were used instead of progesterone, injection of xPR mRNA accelerated oocyte GVBD (data not shown).

Figure 18
Localization of Endogenous xPR in the Cytosol
Extracts from intact oocytes (lane 1), 100,000g pellet (lane 2) or the supernatant (lane 3) were blotted with anti-xPR or anti-β-integrin. The equivalent of 1 oocyte was loaded in each lane.

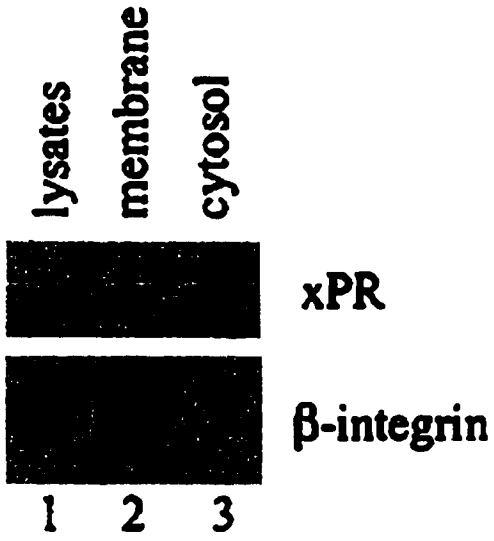
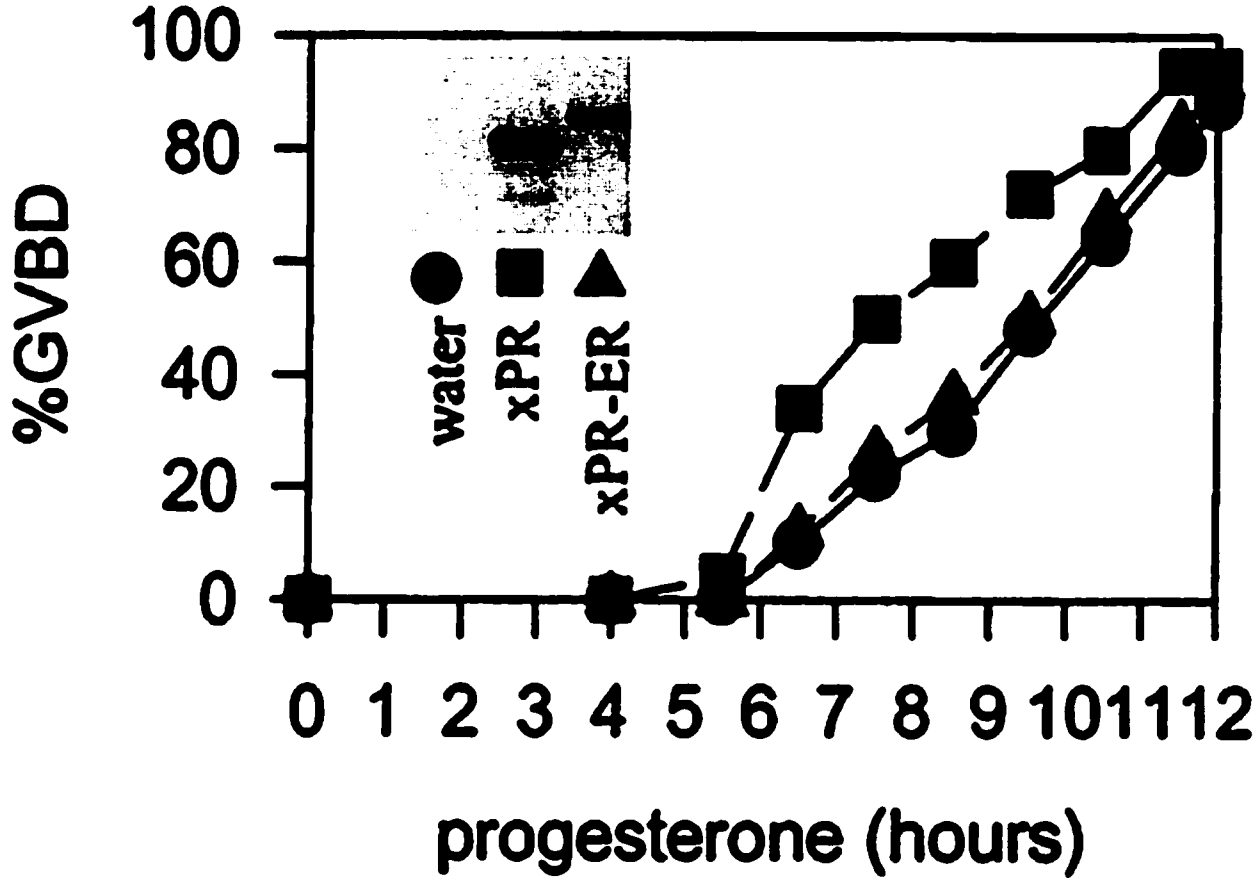

lysates
membrane
cytosol
xPR
β-integrin
1
2
3

Figure 19
xPR Potentiates Progesterone-Induced GVBD
Oocytes were injected with water, xPR or xPR-ER mRNA and were incubated for 24 hours before 0.5 μM progesterone stimulation. GVBD was scored at several time points (as indicated) after progesterone stimulation. GVBD was expressed as % of total treated oocytes. A typical sample consisted of at least 60 oocytes. Inset shows protein expression following mRNA injection, as determined by immunoblotting with anti-Myc ascites.


100
80
60
40
20
0
%GVBD
water
xPR
xPR-ER
0 1 2 3 4 5 6 7 8 9 10 11 12
progesterone (hours)

Progesterone response was further characterized in xPR mRNA injected oocytes by analysis of progesterone-induced synthesis of MOS, a germ-cell specific protein kinase (Benhamou et al. 1992). Detection of MOS synthesis was assayed by immunoblotting with anti-MOS antibodies. Progesterone-induced MOS synthesis was significantly accelerated in xPR injected oocytes when compared to water injected oocytes (Fig. 20). MOS functions as a MEK kinase in *Xenopus* oocytes (Fig. 1), which eventually leads to xMAP kinase activation (Posada and Cooper 1992; Posada et al. 1993). Anti-xMAP kinase immunoblotting was used to assess the phosphorylation (and hence activation) of xMAP kinase, which leads to a slight upward shift in migration (Posada and Cooper 1992; Ohan et al. 1999). As expected, xPR mRNA injection accelerated xMAP kinase activation (Fig. 21) and GVBD (data not shown).

Sensitization of Oocytes to Progesterone Concentrations

An increase of xPR in oocytes due to mRNA injection might also reduce the concentrations of progesterone required to attain a response. Water or mRNA injected oocytes were incubated for 24 to 36 hours before the addition of progesterone. In xPR mRNA injected oocytes, xMAP kinase activation (Fig. 22) and GVBD (data not shown) were significantly enhanced as compared to H_2O injected controls when sub-optimal concentrations of progesterone (0.05 or 0.1 μM) were used.

Non-genomic Action of xPR

To determine if the extranuclear xPR was responsible for the acceleration of the progesterone response an enucleation approach was utilized. Masui and Markert

Figure 20
xPR Accelerates MOS Synthesis in Progesterone-Induced Oocytes
Oocytes were injected with water (control) or xPR (≥200 per group) and incubated for 24 hours prior to progesterone (0.5 μM) addition. 20-25 oocytes were randomly withdrawn and lysed immediately at several time points after hormonal stimulation. All samples were then immunoblotted with anti-MOS.

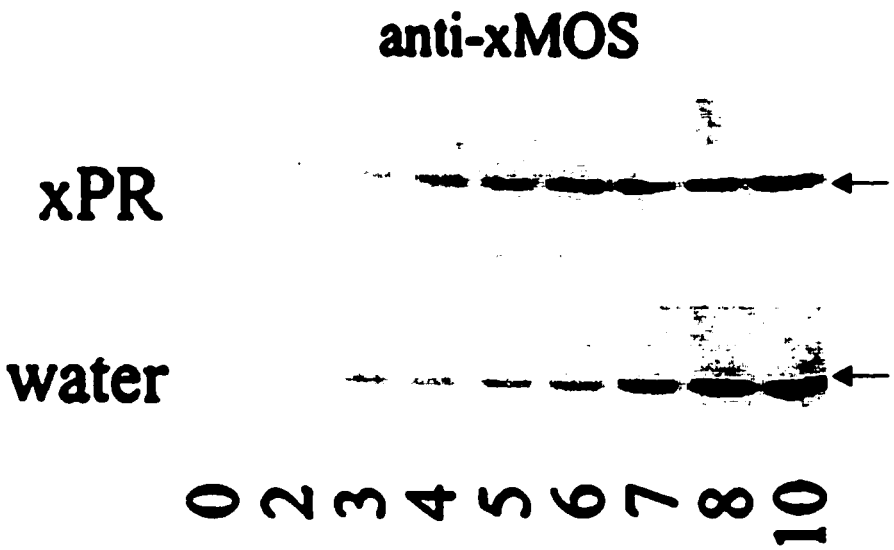

anti-xMOS
xPR
water
0 2 3 4 5 6 7 8 10
Hours

Figure 21
xPR Potentiates Progesterone-Induced MAP Kinase Activation
Oocytes were injected with water (control) or xPR (≥200 per group) and incubated for 24 hours prior to progesterone (0.5 μM) addition. 20-25 oocytes were randomly withdrawn and lysed immediately at several time points after hormonal stimulation. All samples were then immunoblotted with anti-xMAP kinase.

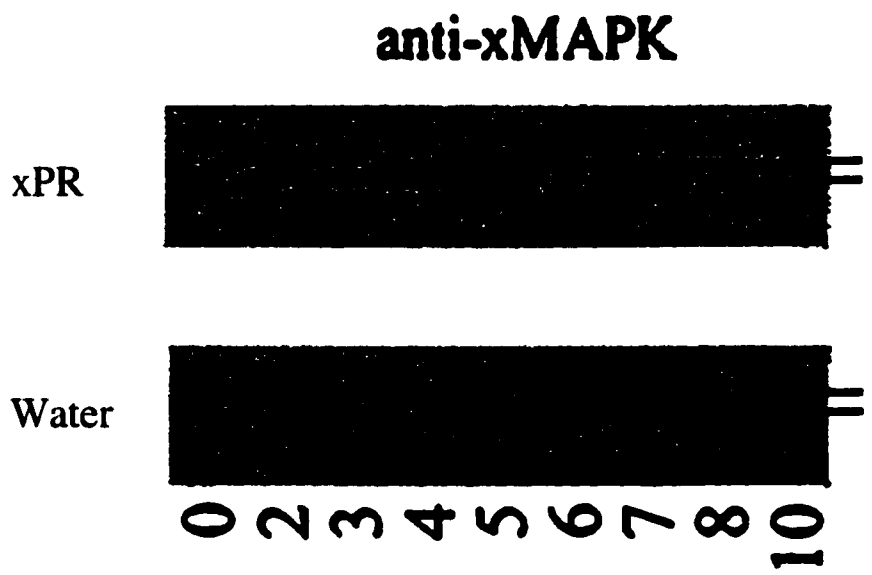
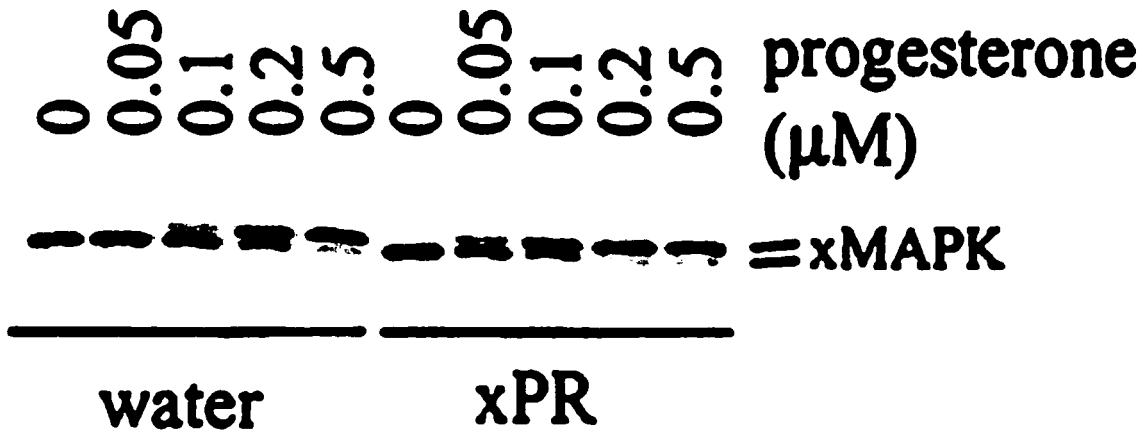

anti-xMAPK
xPR
Water
0 2 3 4 5 6 7 8 10
Hours

Figure 22
xPR Sensitizes Oocytes to Lower Concentrations of Progesterone
Oocytes injected with water (control) or xPR, were incubated for 36 hours prior to the addition of the indicated concentrations of the progesterone. The oocytes were incubated overnight in the presence of the hormone, each group (15 oocytes) was then lysed and the lysates were subjected to MAP kinase immunoblotting assay.


0
0.05
0.1
0.2
0.5
0
0.05
0.1
0.2
0.5
progesterone (μM)
=xMAPK
water
xPR

employed this enucleation approach in their study (Masui & Markert 1971) that led to the discovery of cdc2/cyclin B and the establishment of the cytoplasmic nature of the putative oocyte progesterone receptor. This approach involved injecting the oocytes with xPR mRNA or water, incubating them overnight to allow for protein expression. They were then enucleated and stimulated with progesterone. xMAP kinase activation was accelerated in xPR mRNA injected, enucleated oocytes compared to water injected, enucleated oocytes (Fig. 23).

To address the possibility that the over-expressed xPR might have caused a genomic (transcription) effect prior to enucleation, the general transcriptional inhibitor Actinomycin D (AcD) was used. Incubation of oocytes in AcD containing media did not affect the ability of xPR to accelerate progesterone induced GVBD (Fig. 24). The concentration of AcD used has been established according to earlier studies (Schorderet-Slatkine 1972). As a control, it was shown that the concentration of AcD used was sufficient to abolish transcriptional activity of xPR in COS cells (data not shown). These results demonstrated that the cloned xPR functions in a non-genomic fashion in *Xenopus* oocytes.

Figure 23

xPR Potentiates Progesterone-Induced MAP Kinase Activation in Enucleated Oocytes. Oocytes injected with water and xPR (100 oocytes per group) were incubated for 24 hours and then individually enucleated. The enucleated oocytes were pooled, and then divided into 6 groups of 15 oocytes each. Progesterone was added to 0.5 μM. The enucleated oocyte samples were lysed at the indicated times after progesterone stimulation, and the lysates were immunoblotted with anti-xMAP kinase.

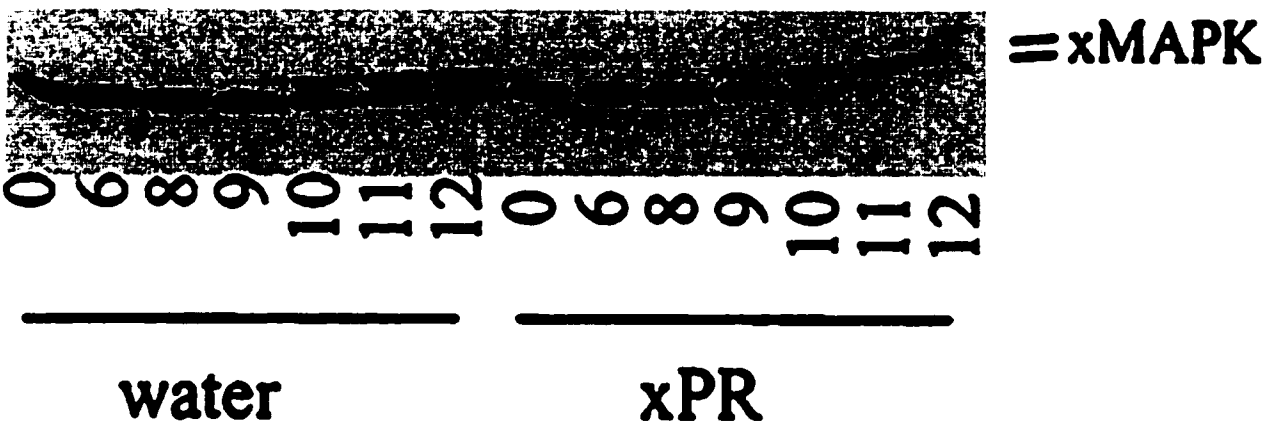
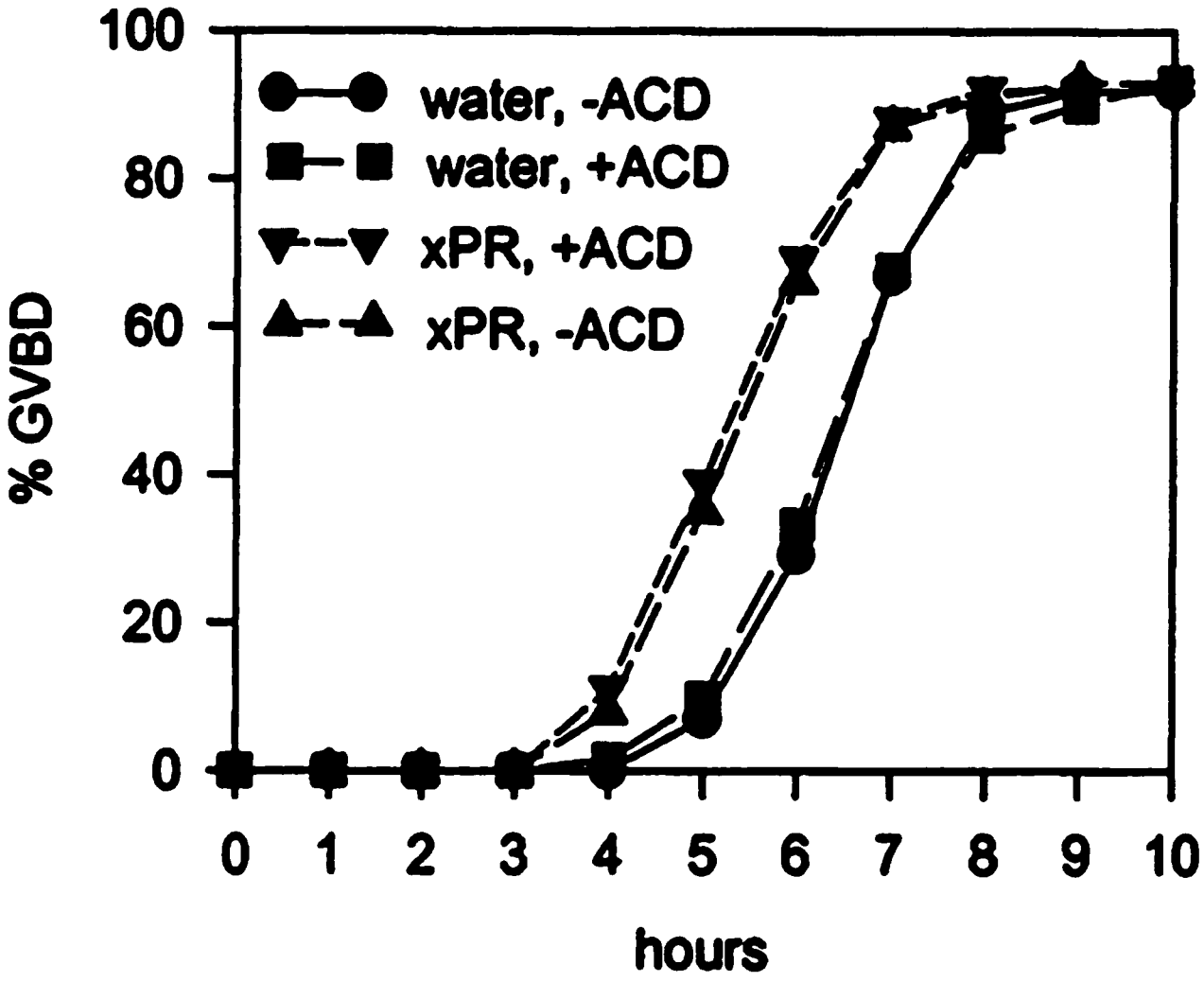

=xMAPK
0 6 8 9 10 11 12
0 6 8 9 10 11 12
water
xPR

Figure 24

xPR Potentiates Progesterone-Induced GVBD in the Presence of Actinomycin D (AcD)

Oocytes injected with water or xPR mRNA, were split into two groups each of 60 oocytes. The two groups were incubated for 24 hours, one in OR2 and the other in OR2 containing 5 μg/mL of AcD. Progesterone was added to 0.5 μM to all four groups and GVBD was scored at the indicated time points after hormonal stimulation. GVBD was expressed as % of total treated oocytes.


100
80
60
40
20
0
% GVBD
water, -ACD
water, +ACD
xPR, +ACD
xPR, -ACD
0 1 2 3 4 5 6 7 8 9 10
hours

DISCUSSION

In this study, the first amphibian PR cDNA was cloned from a *Xenopus* oocyte cDNA library, and termed xPR. xPR appears to be the long sought receptor responsible for progesterone-induced *Xenopus* oocyte maturation. This is an example of a progesterone induced physiological non-genomic action of a steriod receptor, xPR. The characterization of xPR was carried out using two principal systems, *Xenopus* oocytes (described in this thesis) and COS cells (Work done by Ronald A. Booth).

Sequence Analysis

The cloned xPR had all the indications of being a classical progesterone receptor. A nuclear localization signal was detected (^{286}RKFKKFGR). In the C-terminal, the cloned cDNA contained a putative HBD and a DBD that are 86% and 92% identical in amino acid sequence, respectively, to their counterparts in hPR. There was significant homology found between the N-terminal region of cPR and the cloned cDNA (a.a. 1-200), 65/200 a.a. sequence identity. Based on the presented sequence data, the cloned cDNA represents the amphibian homologue of the classical progesterone receptor.

To further support this conclusion, another group cloned a longer cDNA, termed xPR-1, that they believe is the full length *Xenopus* progesterone receptor (Tian et al. 2000). This xPR-1 is 145 a.a. longer than xPR (all at the 5' end), but beyond that, xPR and xPR-1 are identical. A Southern blot analysis of *Xenopus* genomic DNA using the two isolated exons (E1 and E2) indicated that xPR was coded by a single gene (Bayaa et

al. 2000). The reason that xPR is 145 a.a. shorter is either due to the limitations of the 5'RACE technique and the quality of RNA being used, or it could be a shorter isoform.

Hormonal Response of xPR

One set of experiments revolved around the receptiveness of oocytes to different hormones and a parallel set of experiments carried out by Ronald A. Booth examined the nature of hormonal activity of xPR in COS cells (Bayaa et al. 2000). The effective concentration of progesterone, or promegestone (R5020, synthetic progestin), needed to induce xPR-dependent transcription in COS cells was about 10 nM and maximum activation required 100 nM (Bayaa et al. 2000). These concentrations were similar to the hormonal concentrations required to induce GVBD or MAP kinase activation in freshly isolated oocytes (Bayaa et al. 2000).

xPR transfected COS cells exhibited steroid receptor mediated transcriptional control at effective concentrations of antiprogestin RU486 that resembled those concentrations required to induce GVBD and MAP kinase activation in oocytes (Bayaa et al. 2000). Dexamethasone (a glucocorticoid agonist) induced oocyte GVBD was observed at concentrations (10-100 μM, Data not shown) that are known to activate progesterone receptor non-specifically (Tsai et al. 1994).

Progesterone signaling and xPR over-expression

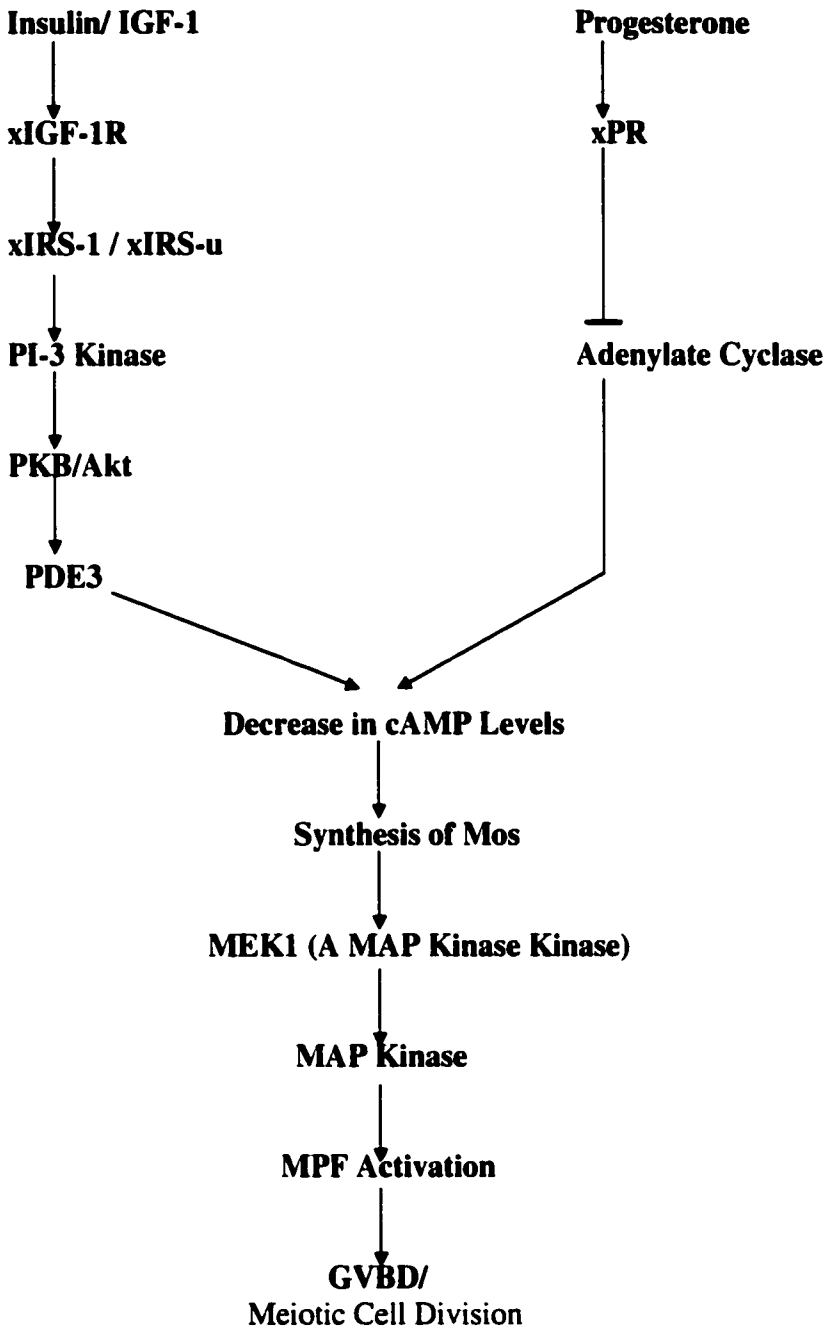
The proposed involvement of xPR in the progesterone signaling pathway can be seen in figure 25. Injection of xPR mRNA enhanced progesterone response (MOS synthesis, MAP kinase activation and GVBD) as compared to control mRNA or water

injected oocytes. The acceleration of progesterone-induced MAP kinase activation and GVBD via xPR mRNA injection can be simply explained by the notion that less time would be needed to accumulate the threshold level of activated receptor signaling components. Furthermore, the over-expression of xPR should reduce the concentrations of progesterone required to achieve the threshold level of activation, as shown in Fig. 21. These notions were also supported by data from COS cell studies (Bayaa et al. 2000). In COS cells, higher level of xPR generated higher level of CAT transcription under the same hormonal concentrations. This indicated that a reduced concentration of hormone will be required to reach the same threshold level of activation when the receptor is over-expressed in oocytes (through mRNA injection). Hormonal stimulation of oocytes (with progesterone) did not cause an increase in the level of endogenous xPR (data not shown).

Non-genomic action of xPR

Of great importance in cloning the *Xenopus* progesterone receptor is the non-genomic nature of its activity that was demonstrated almost three decades ago (Masui and Markert 1971). Masui and Markert first demonstrated that progesterone-induced MPF activation could occur in enucleated oocytes. Acceleration of progesterone induced MAP kinase activation was observed in xPR injected, enucleated ooctyes. Furthermore, xPR was also able to accelerate progesterone response in the presence of actinomycin D in the oocyte medium, supporting the non-genomic effect of the cloned xPR.

Figure 25
Proposed Insulin/Progesterone Pathways
A brief schematic representation of insulin and progesterone signaling pathways that are involved in the induction of meiotic cell division in *Xenopus laevis*, with the proposed involvement of xIRS-u and xPR. Adapted from Liu et al. 1995; Myers and White 1996; Gebauer and Richter 1997; Andersen et al. 1998 and Maller 2001.


Insulin/ IGF-1
xIGF-1R
xIRS-1 / xIRS-u
PI-3 Kinase
PKB/Akt
PDE3
Progesterone
xPR
Adenylate Cyclase
Decrease in cAMP Levels
Synthesis of Mos
MEK1 (A MAP Kinase Kinase)
MAP Kinase
MPF Activation
GVBD/
Meiotic Cell Division

Cytoplasmic xPR Localization

Extranuclear localization of xPR in the oocytes was shown. The nuclear exclusion of xPR is consistent with its non-genomic activity. The reason for the nuclear exclusion of xPR is still under investigation, although xPR contains a seemingly functional NLS as demonstrated by COS cell transfection studies (Bayaa et al. 2000). The exact localization of xPR still remains in question. Upon homogenization of the oocytes, xPR was separated from the membrane. The lack of a signal peptide or a transmembrane domain (Fig. 13), coupled with its fractionation in the high-speed supernatant (Fig. 18), reduces the likelihood that xPR is accessible from the cell surface.

The data is in contrast with many previous studies that suggest that the responsible receptor is a transmembrane, cell surface receptor. The majority of these studies have employed binding assays involving radiolabeled progesterone (or synthetic progestin such as R5020) and oocyte membrane preparations. This was addressed in a review by J. Maller (Maller 1998), pertaining to non-specific binding of lipophilic steroids to membrane components (proteins or lipids). This probably accounts for the majority of these presented binding activities. In another pertinent experiment, the injection of progesterone into oocytes did not cause GVBD (Masui et al. 1971; Smith et al. 1971), suggesting that progesterone had to be presented outside of the cells. This was disputed (Tso et al. 1982) when progesterone injected in an oil-based medium induced GVBD. They claimed the use of an oil-based medium limited the leaking of progesterone to the outside of the cell, and allowed for sufficient time for the action of progesterone to take place within the oocyte. In other experiments (Ishikawa et al. 1977; Godeau et al. 1978) it was demonstrated that when oocytes were incubated in cell impermeable,

polymer-linked (or BSA-linked) progesterone analog was capable of inducing GVBD, supporting a commonly believed theory of a transmembrane cell surface receptor. Take note that a small amount of uptake of the macromolecules and/or a minor contamination of uncoupled analog may have accounted for the observed effects. Godeau et al. (1978) reported an estimated contamination of 0.15% when they utilized 30 μM of polymer-linked progesterone analog. This contamination alone would yield 45 nM of free progesterone analog, a concentration which we have shown to be sufficient to cause GVBD (Bayaa et al. 2000).

HBD Swapping

xPR-ER was a construct of interest, in which PR HBD was swapped with the ER HBD. The theory was that the injection of xPR-ER mRNA might cause the oocytes to undergo GVBD when stimulated with estradiol (E2). Although there was proper expression of the fusion protein (Fig. 19, inset), the oocytes did not respond to E2 stimulation. COS cells that were transfected with xPR-ER, responded weakly to E2 treatment (Bayaa et al. 2000). The results point to the importance of the xPR HBD in signaling, in addition to its role in hormone binding. This is supported by the presence of one of the transcriptional activation domains (AF2) in the hPR HBD (Danielian et al. 1992).

Inhibition of Endogenous xPR Function

Different approaches to inhibit endogenous xPR function to demonstrate the necessity of xPR for progesterone-induced GVBD were employed unsuccessfully. One

such approach was the injection of affinity-purified anti-xPR antibodies. This failed to inhibit progesterone-induced GVBD. This result was perhaps not surprising since the antibody was raised against SDS-denatured protein and the antibodies did not immunoprecipitate xPR (data not shown).

Another approach involved the injection of several xPR-specific, phosphorothioate-containing antisense oligos, to reduce levels of endogenous xPR. When the oligo-injected oocytes were incubated for as long as one week, no reduction of endogenous xPR was detected. The oocytes lost their viability beyond a week, and therefore, inhibition of progesterone-induced GVBD was not achievable using this method.

On the other hand, Tian et al. (2000) reported that injection of xPR-1 antisense oligos inhibited progesterone-induced oocyte maturation. Three antisense oligos were designed around a.a. 87, 381 and 459 (the latter two sites are present in xPR). They were then able to rescue these oocytes by injection of xPR-1 or hPR_B. The level of endogenous xPR protein could not be assayed due to the lack of an antibody. This further supports the identity and function of the cloned xPR.

With these two papers, it is believed that one of the longest cell biological puzzles is finally resolved (Maller 2001).

General Discussion

Xenopus laevis oocyte maturation, or resumption of meiosis, is triggered in vivo by progesterone. It was recognized long ago that insulin/IGF-1 can also induce oocyte maturation, establishing it as an in vitro model to study insulin signaling. The ease of manipulation of oocytes, as well as the physical and biochemical markers that are available to follow the progression of both pathways, make *Xenopus* oocytes an excellent biological model. In the preceding two chapters two novel genes were cloned and characterized, xIRS-u and xPR. xIRS-u is an insulin receptor substrate that acts downstream of the xIGF-1 receptor. xPR is the long sought progesterone receptor responsible for mediating progesterone-induced in vivo oocyte maturaion. The characterization of these two new genes will aid in the better understanding of the insulin and progesterone signaling pathways in *Xenopus* oocytes, and their proposed implication is shown in Fig. 25.

In addition to the subcloning of xIRS-u, the potentiation of insulin induced oocyte maturation due to the over-expression of xIRS-u was demonstrated. Furthermore, I demonstrated the importance of the PH domain of xIRS-u in this potentiation, as well as the ability of the PH domain alone to act as a specific inhibitor of insulin signaling. This was the first study showing the ability of a PH domain of an IRS to act in this manner.

In the second part of this thesis, I have detailed my work in the cloning of xPR and the functional evidence that eventually lead to our conclusion that xPR is the oocyte progesterone receptor that had eluded the scientific community for almost thirty years. The elusive progesterone receptor (Maller 2001) has at long last been identified (Sadler

2001). This work not only resolved an old scientific puzzle, it also represents a milestone in the area of nongenomic (transcription independent) action of steroid hormones.

Three years of research in Dr. Liu's Laboratory has added an extensive, diversified and comprehensive list of molecular biology techniques to my repertoire. This work has also offered me an opportunity to learn how to efficiently comb the scientific literature to present and defend one's scientific ideas, as well as experience the excitement of scientific discoveries. In short, I shal say, all the long hours spent in the lab have paid off handsomely!

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1. Mustafa Bayaa, Ronald Booth, Yinglun Sheng, and X.J. Liu. The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proc Natl Acad Sci U S A Nov 7;97(23) (2000)
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