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Signalling Pathways Controlling the Initiation of

*Xenopus* Oocyte Maturation

by

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A thesis submitted to
the Faculty of Graduate and Postdoctoral Studies
in partial fulfilment of
the requirements for the degree of

Doctorate of Philosophy, in Biochemistry

Department of Biochemistry, Microbiology and Immunology
University of Ottawa
Ottawa, Canada
September 2002

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Abstract

*Xenopus laevis* oocytes are physiologically arrested at the first meiotic prophase. Re-initiation of meiosis, or oocyte maturation, is triggered *in vivo* by progesterone, but can also be triggered by insulin and insulin-like growth factor-1 (IGF-1) *in vitro*. The mechanism by which these two very different hormones regulate the same physiological process is poorly understood.

Chapter 2 describes my research that contributed to the characterization of the progesterone receptor responsible for inducing oocyte maturation. We demonstrated that the *Xenopus* progesterone receptor (xPR) is a dual functional protein. When expressed in the heterologous COS-7 cells, xPR is imported into the nucleus and functions as a progesterone-regulated transcription factor. In contrast, the endogenous xPR in *Xenopus* oocytes is restricted in the cytoplasm and appears to mediate cytoplasmic signalling.

Chapter 3 describes a functional link between the IGF-1 receptor and G-protein signalling in the control of oocyte maturation. The *Xenopus* homologue of GIPC, a PDZ-domain-containing protein, was identified as a binding partner for the cytoplasmic domain of the IGF-1 receptor. GIPC is known to interact with the C-terminus of a Gai-specific GAP, RGS-GAIP. Expression of two dominant negative forms of xGIPC blocked insulin-induced MAPK activation and oocyte maturation, while full-length xGIPC synergized with human RGS-GAIP to enhance insulin signalling. This is the first demonstration that the GIPC/RGS-GAIP complex acts positively in IGF-1 receptor signalling.
Acknowledgements

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<tbody>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mM</td>
<td>nanomolar</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
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<tr>
<td>μL</td>
<td>microlitre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>U</td>
<td>international units</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurrie</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AD</td>
<td>activation domain</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>AF-1, -2</td>
<td>activation function-1, -2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine-3',5'-monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>chloroamphenicol acetyl transferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CG</td>
<td>chorionic gonadotropin</td>
</tr>
<tr>
<td>CPEB</td>
<td>cytoplasmic polyadenylation element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>E2</td>
<td>17-β estradiol</td>
</tr>
<tr>
<td>EC50</td>
<td>50% effective concentration</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAIP</td>
<td>Gqi interacting protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GIPC</td>
<td>GAIP interacting protein, C-terminus</td>
</tr>
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<td>G-protein coupled receptor</td>
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<tr>
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<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>GV</td>
<td>germinal vesicle</td>
</tr>
<tr>
<td>GVBD</td>
<td>GV break down</td>
</tr>
<tr>
<td>HBD</td>
<td>hormone binding domain</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>LH</td>
<td>luteninizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MPF</td>
<td>m-phase (maturation) promoting factor</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD95/DLG/ZO-1 interaction domain</td>
</tr>
<tr>
<td>PI-3 K</td>
<td>phosphotyidyinositol-3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PRE</td>
<td>progesterone response element</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signalling</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology-2 domain</td>
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Chapter 1

General Introduction
GENERAL INTRODUCTION

Oocytes from the African clawed frog, *Xenopus laevis*, have been used as a model system for diverse studies including signal transduction (Maller, 1998; Ferrell, 1999). Large numbers of oocytes can be readily obtained using a simple surgical incision. The oocytes are quite large (ca. 1.3 mm in diameter, with a volume of about 1 μL) allowing them to be easily manipulated under a dissecting microscope. Their size also facilitates techniques such as microinjection of DNA, RNA and proteins using a simple microinjection apparatus.

Like the oocytes in many other vertebrate species, *Xenopus* oocytes are arrested at two points during meiosis (Figure 1.1) (Masui, 1985). The first arrest occurs at the first meiotic prophase (prophase I), during which the oocyte accumulates the components necessary for early embryogenesis. During this time, the oocytes substantially increase in size and pigmentation, and the changes in these characteristics have been used to categorize the different stages of growth (Stages I to VI) (Figure 1.1) (Dumont, 1972). The nucleus, referred to as the germinal vesicle (GV), occupies roughly half the volume of the animal hemisphere by stage VI, at which point the oocyte is considered fully-grown. The fully-grown oocyte is then capable of re-entering meiosis and undergoing oocyte maturation (Sadler and Maller, 1983).

The steroid hormone progesterone triggers *Xenopus* oocyte maturation (Masui and Markert, 1971). Upon stimulation by progesterone secreted from the surrounding follicle cells, the oocyte re-enters meiosis, the GV breaks down (GVBD), chromosomes condense, spindle formation occurs and the first polar body is extruded (Yamashita,
1998). At this point meiosis is arrested for a second time (metaphase II) where the now mature egg awaits fertilization (Figure 1.1).

Much research has focused on the cascade of events in which progesterone stimulation leads to the production of a mature egg (Maller, 1998; Yamashita, 1998). This has resulted in the elucidation of signalling pathways involving many known and novel proteins including adenyl cyclase (AC), mitogen activated protein kinase (MAPK), and maturation promoting factor (MPF). An overview of the current understanding of the pathway leading to oocyte maturation is presented in Figure 1.2.

As this thesis details work which aided in clarifying early signalling events in both the progesterone and insulin/insulin-like growth factor-1 (IGF-1) pathways, a review of the relevant literature is divided into two sections. The first section describes the currently understood signalling pathway by which progesterone stimulates oocyte maturation, while the second section describes the early signalling events triggered by insulin/IGF-1.
Figure 1.1
Schematic representation of oocyte maturation and early embryogenesis in *Xenopus laevis*. Modified from Ferrell (1999). During oogenesis, the oocytes are arrested in a G2-like interphase while they accumulate the components necessary for early embryogenesis. The oocytes are staged according to their size and colouration. Once stage VI is reached the oocytes are competent to undergo maturation in response to follicular progesterone and arrest for a second time at metaphase of meiosis II. The mature oocytes, now referred to as eggs, are able to be fertilized and undergo embryogenesis. The red and green lines refer to the activities of MAPK, and MPF respectively during late embryogenesis, oocyte maturation and early embryogenesis. The times indicated refer to both MAPK and MPF timing and cell cycle state (yellow, blue and green bar)
Figure 1.2
Schematic representation of the known steps involved in activation of MPF (cyclin B/Cdc2), release from prophase I arrest and arrest in metaphase II. Upon progesterone stimulation of stage VI oocytes, adenylyl cyclase (AC) activity is reduced. This reduction, through an unknown mechanism, activates the translation of Mos protein. Mos then activates the MAPK pathway, which through Rsk and Myt1, activates MPF. Other proteins proposed to be involved in activation of MPF include Plx1 and Ringo. Once MPF is active, it activates the anaphase promoting complex (APC) which down regulates MPF by proteolysis. MAPK remains active while the APC is active and aids in de novo synthesis of cyclin B and inhibition of S-phase during oocyte maturation. MAPK also aids in the maintenance of metaphase II arrest.
Progesterone Mediated Oocyte Maturation

Reproductive function of progesterone in mammals

The main function of progesterone in the mammalian reproductive system is the maintenance of pregnancy (Lodish et al., 1997). In brief, there exists a feedback loop between the ovary and the hypothalamus/anterior pituitary glands of the brain. The release of follicle-stimulating hormone (FSH) by the anterior pituitary gland stimulates the growth of the follicle. The follicular cells release estrogens that stimulate growth of the uterine walls in preparation for implantation. Estrogen feeds back to the hypothalamus and the anterior pituitary to decrease the amount of FSH. As the follicle matures, luteinizing hormone (LH), another pituitary hormone, is released. Maturation is completed in the presence of LH, and the ovum is released into the oviduct. At this point, the follicle is transformed into an endocrine organ called the corpus luteum, which secretes progesterone and causes additional growth of the endometrium. When the ovum is not fertilized, the corpus luteum degenerates and the decreased production of estrogen and progesterone causes the uterine wall to break down. If fertilization and implantation occur, progesterone stimulates the placental tissues to produce chorionic gonadotropin (CG). CG then prevents the degradation of the corpus luteum and maintains progesterone production. The production of progesterone throughout pregnancy is mandatory for maintenance of a proper blood supply for the developing embryo.
Classical genomic action of progesterone

Progesterone exerts its actions through its classical nuclear receptor (PR), which is a progesterone activated transcription factor. The PR receptor is a member of a large family of steroid-activatable nuclear transcription factor receptors that bind to steroid response elements upstream of genes, and induce their transcription. The classical human PR is transcribed from two alternative start codons within the same gene producing two isoforms, an A-isof orm and a B-isof orm. Transcription of the A-isof orm starts at codon 165 of the human B-isof orm, producing a truncated form of the receptor. The PR consists of three main domains (Figure 1.3), a large N-terminal domain which contains a transcriptional activation domain (AF-1), followed by the DNA binding domain (DBD), a hinge region connecting the DBD and the C-terminal hormone binding domain (HBD). Within the HBD there is a second transcriptional activation domain (AF-2), and overlapping the end of the DBD and the hinge region is the nuclear localization sequence (NLS).

A complex process of receptor maturation occurs before the receptor has the ability to interact with its ligand and activate transcription. The receptor must associate with a number of accessory proteins to obtain the mature transcriptionally active stage. The assembly of a mature (ligand binding competent) PR appears to occur in a number of stages, and involves numerous accessory proteins including Hsp70, Hsp90, immunophilins FKBP51 and FKBP52, and possibly the phosphatase PP5 (Dittmar et al., 1998; Johnson et al., 1996; Prapapanich et al., 1998; Smith, 2000). Although the mature conformation appears to be the most stable state, the receptor complex is constantly in a cycle between all states. As with other steroid hormone receptors, in the absence of
Figure 1.3
Schematic representation of the human progesterone receptor. Diagram denotes A and B isoform translation start positions. Numbers indicate amino acid positions. DBD, DNA binding domain; HBD, hormone binding domain; AF, activation function; NLS, nuclear localization sequence.
hormone, the receptor’s transcriptional ability is silenced. The association of the chaperone proteins inhibits the ability of the receptor to dimerize, and masks its DNA binding domain and potential co-regulatory domains (Smith, 2000).

The binding of progesterone to the receptor complex does not cause dissociation of the chaperones, rather, it inhibits the re-initiation of the assembly cycle (Smith, 1993). Once the receptor is activated by binding progesterone, it dimerizes with a second PR, which then binds to the DNA at specific progesterone response elements (PREs) (Guiochon-Mantel et al., 1989). The binding of ligand to the PR inhibits the re-association of its chaperone proteins, which unmask transcriptional activation domains. This allows the AF domains of PR to interact with proteins involved in activating transcription, including the SRC (steroid receptor co-activator) proteins (Onate et al., 1995; Hong et al., 1997; Voegel et al., 1996). In this way, the receptor is thought to activate transcription through initiation or stabilization of the pre-initiation complex in the promoter region of the genes, which activates transcription (Tsai and O'Malley, 1994).

While there does not seem to be a difference in the proteins associated with the two PR isoforms, there are functional differences (Smith, 2000). The absolute levels of PR and the stoichiometric ratio of PR-A to PR-B differ in tissues depending on their developmental and hormonal state. Which genes are activated and to what extent by progesterone appears to be modulated by this ratio. The additional N-terminal 165 amino acid region, present only in the PR-B isoform, has been shown to contain a third transactivation domain (AF-3) that plays an essential role in activating specific target genes (Giangrande and McDonnell, 1999).
When PR-A and PR-B are expressed separately in various cell types, they each display different transactivation characteristics (Tora et al., 1988; Conneely and Lydon, 2000). Specifically, PR-B functions as a strong activator of transcription, even in cells in which PR-A does not support transcription. In addition, when both PR-A and PR-B are co-expressed, PR-A has the ability to act as a dominant repressor of PR-B activity in some cell types (Vegeto et al., 1993). These findings demonstrate the ability of PR-A to modulate the activity of progesterone action in cells depending on both the expression of PR-A and PR-B.

The repressor capability of PR-A is also evident with other steroid receptors such as the estrogen receptor, the glucocorticoid and mineralocorticoid receptors (McDonnell et al., 1994; Wen et al., 1994). PR-A and PR-B each respond to progesterone antagonists differently, both PR-A and PR-B’s transcriptional ability are inhibited, while PR-B can be converted to a strong activator of transcription by modulating specific phosphorylation pathways (Sartorius et al., 1993; Sartorius et al., 1994; Musgrove et al., 1993). Taking the above into account it suggests that the two PR isoforms seem to have distinct yet connected functions within cells.

**Subcellular localization of progesterone receptor**

The nuclear envelope is composed of double membranes contiguous with the endoplasmic reticulum (Macara, 2001). This membrane structure is dotted with transport channels, the nuclear pore complexes (NPC), which control the movement of proteins and substances into and out of the nucleus. The NPC allow proteins smaller than ca. 50 kDa to passively diffuse into and out of the nucleus (Komeili and O’Shea, 2000),
however this does not account for the presence of larger molecules in the nucleus. Specific sequences, the nuclear localization sequences (NLS), direct larger proteins into the nucleus. The NLS sequences were first identified in the simian virus 40 large-T antigen and nucleoplasmin proteins, which had the sequences, PKKRRK, and KRPAATKKAGQAKKKKLD, respectively. These sequences became the prototypes of the mono- and bipartite NLS sequences, respectively (Kalderon et al., 1984; Robbins et al., 1991). The NLS sequence works by interacting with specific importin proteins, such as importin-α, which in turn binds to a second nuclear import protein importin-β (Radu et al., 1995). The latter protein docks with and promotes translocation of the protein complex through the NPC (Macara, 2001). Once in the nucleus, the translocated protein is dissociated from the importin-α/β complex by the action of GTP-Ran, a small G-protein involved in nuclear import and export (Azuma and Dasso, 2000). High levels of GTP-Ran in the nucleus, and GDP-Ran in the cytoplasm are maintained by selective localization of Ran guanine nucleotide exchange factors and Ran GTPase-activating proteins to the nucleus and cytoplasm, respectively (Azuma and Dasso, 2000).

Not all nuclear proteins contain a classical NLS. The hnRNPA1 protein, which efficiently shuttles between the nucleus and cytoplasm, does not contain a classical NLS. Instead, a glycine and asparagine rich region interacts with a protein, transportin. Transportin has homology to importin-β and serves to transport hnRNPA1 into the nucleus in a similar manner to the importin-α/β complex (Pollard et al., 1996).

In addition to nuclear import, proteins can also be actively exported out of the nucleus. The first protein to be identified having a role in nuclear export was Crm1 (Fukuda et al., 1997). It recognises a short leucine rich or hydrophobic motif present in
numerous proteins, including the protein kinase inhibitor PKI (Macara, 2001). The prototypical nuclear export signal recognized by Crm1 has the sequence LxxxLxxLxL, however other hydrophobic residues can be substituted for several of the Leu residues (Macara, 2001). As with nuclear import, Crm1 binds to the NES, as well as GTP-Ran, exporting its cargo through the NPC into the cytoplasm (Komeili and O’Shea, 2000). Once in the cytoplasm, GTP-Ran is hydrolysed to GDP-Ran causing the dissociation of the transport complex (Azuma and Dasso, 2000).

The activity of either the NLS or the NES can be modulated through a number of mechanisms including phosphorylation. An example of this mechanism is the translocation of the transcription factor NF-AT. NF-AT is a transcriptional activator whose localization is regulated by intracellular calcium levels (Matsuda et al., 1998). Under resting conditions NF-AT is phosphorylated and localized to the cytoplasm. When intracellular calcium levels increase, NF-AT is de-phosphorylated and migrates to the nucleus. The phosphorylation sites are near the NLS and prevent nuclear localization, presumably by blocking interaction with importin-α (Hogan and Rao, 1999). Another nucleocytoplasmic control mechanism is through masking of either NLS or NES sequences. Export of NF-AT from the nucleus requires Crm1, as it is sensitive to a Crm1-specific inhibitor leptomycin B (a NES-mediated export inhibitor; Zhu and McKeon, 1999). Zhu and McKeon (1999) showed that the NES overlapped with the calcineurin (a phosphatase which interacts with and dephosphorylates NF-AT) binding site, and that constitutively active calcineurin blocked Crm1-mediated export of NF-AT, suggesting that Crm1 was not able to access the NES when calcineurin was active.
The human PR contains two functional NLS sequences (Guiochon-Mantel et al., 1989). One located in the hinge region between the C-terminal hormone binding domain and the N-terminal DNA-binding domain and has the sequence $^{637}\text{RKFKKFNK}^{644}$ (Guiochon-Mantel et al., 1989). This NLS functions independent of ligand binding and therefore is known as a constitutive NLS. In contrast, a more N-terminal NLS contained within the DNA binding domain (amino acids 593 to 638) only functions in the liganded receptor and therefore is termed ligand-dependent NLS (Guiochon-Mantel, et al., 1992). The PR-A isoform is completely localized in the nucleus, while human PR-B is distributed between the cytoplasm and the nucleus (Guiochon-Mantel et al., 1989) and upon stimulation with progesterone, human PR-B translocates to the nucleus (Guiochon-Mantel et al., 1989). The mechanism that distributes PR-B between the cytoplasm and nucleus is unknown, but may involve an unidentified NES present in the N-terminal 164 amino acids or through a conformational difference between PR-A and PR-B which masks the constitutive NLS until hormone is bound (Baumann et al., 1999).

The cytoplasmic or nuclear localization of PR however, is not static; the receptors constantly undergo nucleocytoplasmic shuttling. The mechanism involved in this shuttling does not appear to be mediated by traditional NES sequences, and may involve the NLS. Tyagi et al. (1998) showed that rabbit PR was exported from the nucleus under conditions in which NES mediated export was blocked by leptomycin B and GTP-γS (an inhibitor of GTP hydrolysis), suggesting NES mediated nuclear export may not be important in PR shuttling.
Non-genomic actions of steroid receptors

Although the major action of progesterone and other steroid hormones is to activate transcription, other more rapid non-genomic effects of steroid hormones have been reported, but only recently have some been shown to be mediated by classical receptors.

The classical estrogen receptor (ERα) has been found to act in a non-genomic fashion in cancer cells. That is, it rapidly activates MAPK after stimulation with estrogen, without the need for transcription. Auricchio and co-workers (Migliaccio, et al., 1996; Migliaccio, et al., 1998; Migliaccio, et al., 2000) have shown there is an interaction between the ER and Src kinase, which is mediated through the SH2 domain (a phosphotyrosine binding domain) of Src and the HBD of ER (Migliaccio, et al., 1996; Migliaccio, et al., 1998; Migliaccio, et al., 2000). Mutation of ER Tyr537 to Phe, blocks this association, and inhibits the activation of Src by ER (Migliaccio, et al., 2000). MAPK activation occurs through Src phosphorylation of Shc and Ras-GAP p190, which in turn leads to activation of Ras, and ultimately MAPK (Castoria et al., 1999).

Progesterone also functions in a non-genomic manner in some cell types. Progesterone acts as a stimulus of sperm which affects several functions, including motility, capacitation, and the acrosome reaction (Baldi et al., 1999). The effects of progesterone are mediated through an increase in intracellular calcium, as well as activation of phospholipases, kinases, and efflux of chloride ion (Baldi et al., 1999). The efflux of chloride, which is essential for the acrosome reaction, is attributed to progesterone activation of a membrane located neuronal GABA(A)-like receptor (Calogero et al., 2000). The mechanism that mediates the other effects of progesterone in
sperm has not been identified and may act through the classical PR or as yet unidentified membrane receptors.

As with estrogen, the classical PR may activate the MAPK pathway in tumour cells. In T47D cells and in COS-7 cells over-expressing progesterone and estrogen receptors, progesterone stimulation is able to activate MAPK in a non-genomic manner (Migliaccio et al., 1998). This stimulatory action of progesterone was found to act through the estrogen receptor, since stimulation of cells with an anti-estrogen or ablation of the estrogen receptor, blocks MAPK activation by progesterone (Migliaccio et al., 1998). PR has been found to bind the estrogen receptor through its N-terminal 168 amino acids and this association stimulates estrogen receptor to activate the Src tyrosine kinase. Src then activates the Ras/MAPK pathway (Migliaccio et al., 1998). In this way, the classical PR, through association with the classical estrogen receptor, stimulates the MAPK pathway without the need for transcription.

**Progesterone stimulation of oocyte maturation**

The steroid hormone progesterone is the natural hormone that releases the prophase I arrest and induces oocyte maturation in *Xenopus*. The identification of progesterone as the natural inducer of GVBD answers one question yet raises a number of others. The first detectable change in oocytes stimulated by progesterone appears to be a decrease in the intracellular concentration of cAMP (Cicirelli and Smith, 1985; Maller, 1982). The main question is how progesterone causes a decrease in the intracellular cAMP concentration to induce GVBD. In most other cells, progesterone acts as a transcriptional activator. However, in *Xenopus* oocytes, transcription is not necessary for oocyte
maturation (Masui and Markert, 1971). The action of progesterone in *Xenopus* oocytes is of a non-classical nature; that is, transcription is not activated, and somehow progesterone decreases the concentration of cAMP, activates transcription of Mos mRNA and activates MAPK and MPF, ultimately causing GVBD and oocyte maturation.

*Non-genomic action of progesterone in *Xenopus* oocytes*

Oocyte maturation in *Xenopus* is initiated by release of pituitary gonadotropin, which acts on the follicle cells surrounding the oocyte. The follicle cells then release progesterone, which directly stimulates oocyte maturation (Masui and Markert, 1971). Since the identification of progesterone as the inducer of oocyte maturation, researchers have been searching for the receptor through which progesterone acts. Early in the study of progesterone signalling in oocytes, Masui and Markert (1971) were able to demonstrate that the nucleus was unnecessary for MPF activation. They were able to remove the nuclei of oocytes, and subsequent stimulation with progesterone still resulted in MPF activation. Masui and Markert (1971) and Smith and Ecker (1971) then injected progesterone into oocytes and showed that this did not induce oocyte maturation, which suggests that progesterone needs to be delivered to the outside of the oocyte to activate the progesterone receptor, implying a membrane localized receptor. Oocytes also underwent maturation in response to progesterone coupled to a polymer (Godeau *et al.*, 1978). The polymer-linked progesterone was not expected to diffuse through the oocyte membrane and therefore would only activate a receptor present on the membrane. Again this suggested a membrane bound progesterone receptor.
The lipophilic nature of progesterone causes non-specific association with hydrophobic proteins and membrane structures and hence, presents problems in identifying a progesterone receptor associated with the membrane. Binding studies to identify the receptor in isolated membranes and melanosomes revealed a number of candidate proteins (Belle, et al., 1977; Liu and Patino, 1993), but after further characterization, they were discarded as non-specific binding proteins (Maller, 1998). To circumvent the non-specific binding, photoaffinity labelling with R5020 identified a 110 kDa protein in the oocyte membrane, but after molecular characterization, it was found not to be a receptor (Maller, 1998).

*Cyclic-AMP and protein kinase A’s role in oocyte maturation*

The proposed interaction of progesterone with a membrane receptor initially led investigators to test the hypothesis that cAMP was involved as a second messenger in oocyte maturation. However, a statistically significant change in cAMP was difficult to observe due to the inherent variability of cAMP levels in oocytes (Smith and Ecker, 1971; Brachet et al., 1974). This lack of statistical significance caused investigators to question the involvement of cAMP. However, O'Connor and Smith (1976) found theophylline (a phosphodiesterase inhibitor, which maintains cAMP high) inhibited oocyte maturation, suggesting a reduction in cAMP was necessary for oocyte maturation to proceed. These results were mirrored by treatment of oocytes with forskolin (which activates adenyl cyclase and maintains high cAMP) (Schorderet-Slatkine and Baulieu, 1982), and IBMX (3-isobutyl-1-methylxanthine; a phosphodiesterase inhibitor), which further reinforced the potential necessity for reducing cAMP (Maller and Krebs, 1980).
Consistent with the above results, within 15 minutes of the addition of progesterone, a drop in cAMP of about 20% can be measured (Cicirelli and Smith, 1985; Sadler and Maller, 1981; Sadler and Maller, 1983). This drop is relative to the total amount of cellular cAMP, although the local effective drop may be much more significant. The mechanism in which progesterone causes the drop in cAMP is thought to occur through inhibition of oocyte adenylyl cyclase.

Progesterone was found to reduce either basal or cholera toxin (increases adenylyl cyclase activity) treated oocytes adenylyl cyclase (AC) activity by approximately 50% (Finidori-Lepicard et al., 1981). Finidori-Lepicard et al. (1981) also showed that in manually isolated membranes, progesterone reduced the activity of AC by a similar amount. These results suggest a component present in the oocyte membrane interacts with progesterone and inhibits the activity of AC or that progesterone directly inhibits AC activity. This reduced level of cAMP was thought to reduce the activity of one of the main effectors of cAMP, the cAMP dependant protein kinase A (PKA).

PKA is a tetrameric protein kinase consisting of two catalytic (C) subunits and two regulatory (R) subunits. PKA is held in an inactive state by interacting with the R subunits, upon binding of cAMP to the R subunits, they dissociate from the C subunits, activating PKA’s kinase activity. In this way, the activity of PKA is directly proportional to the local concentration of cAMP. Injection of either the R subunit of PKA or the PKA protein inhibitor I, induced hormone independent oocyte maturation, while injection of the C subunit blocked progesterone stimulated maturation (Huchon et al., 1981; Maller and Krebs, 1977). This suggests that a reduction in the activity of PKA is necessary for oocyte maturation.
Although the cAMP levels appear to return to normal within approximately 20 minutes of progesterone stimulation (Maller et al., 1979), the activity of PKA must be regulated until the time of MPF activation. Any increase in cAMP concentration or elevation of PKA activity inhibits the activation of MPF caused by various inducers of GVBD, even those thought to be effectors of PKA (Matten et al., 1994), including Cdc25 (Rime et al., 1994). This suggests that PKA exerts a number of negative controls on MPF activation, and that in turn, MPF could exert a negative feedback control on the cAMP level (Karaiskou et al., 2001). Although the effectors of PKA involved in oocyte maturation have not been determined, a reduction in PKA activity stimulates the next step in oocyte maturation, the translation of the Mos mRNA, and activation of MAPK. The above data demonstrate the importance of cAMP in controlling oocyte maturation, but suggest that its involvement is more complex than a simple transient decrease.

**Involvement of the Mos/MAPK pathway in oocyte maturation**

The Erk1/Erk2 MAPK pathway is a ubiquitous signalling pathway that regulates growth and differentiation in numerous cell types (Liebmann, 2001). Erk1 (p44 MAPK) and Erk2 (p42 MAPK) are Erk family MAPK kinases, which are most notably activated by cell surface growth factor tyrosine kinase receptors (Liebmann, 2001), although other mechanisms are known to activate the Erk family kinases (Liebmann, 2001). *Xenopus* oocytes only contain a single Erk kinase, the p42 MAPK, which will be referred to as MAPK throughout this thesis (Gotoh et al., 1991). MAPK is the final member of a conserved kinase cascade, which consists of a MAPK kinase kinase (MAPKKK; which is Mos in the case of *Xenopus* oocytes), a MAPK kinase (MAPKK), MEK1, and finally
MAPK (Erk1/2) (Figure 1.2). The MAPK pathway is universally activated during oocyte maturation among vertebrates (Nebreda and Ferby, 2000), although the timing and requirement for activation of MAPK in oocyte maturation between various species can differ substantially (Nebreda and Ferby, 2000).

Mos Translation

Approximately 2 to 4 hours after Xenopus oocytes are stimulated with progesterone, MAPK is activated. This is in contrast to other cell types where MAPK is activated within seconds of the addition of external stimuli. Xenopus oocytes require the de novo synthesis of Mos from a pool of stored maternal mRNA before the MAPK pathway is activated. Two mechanisms maintain low levels of Mos protein in oocytes: First, the Mos mRNA is "masked" by specific proteins, and therefore poorly translated, and second, any translated Mos is rapidly degraded or remains inactive (Karaiskou et al., 2001a).

Oocytes store a large number of maternal mRNAs, including Mos mRNA, which are selectively activated during oocyte maturation and early development. The translational control mechanism appears to be through cytoplasmic polyadenylation. Messenger RNAs with a short poly(A)-tail (ca. 20-30 nt) are poorly translated, while ones with longer tails (100+ nt) are translated well. The Mos mRNA and other masked mRNAs have relatively short poly(A)-tails (ca. 20 nucleotides in length), and elongation of this tail is necessary for translation of the mRNA (Barkoff et al., 1998; Hake and Richter, 1997). The mechanism that maintains the poly(A)-tail short involves a "masking" complex. The Mos mRNA has been found to be complexed with a number of proteins,
including the cytoplasmic polyadenylation binding protein (CPEB), Maskin, and eIF-4E, which hold the mRNA in a hairpin structure that blocks polyadenylation (Stebbins-Boaz et al., 1999). Phosphorylation of CPEB by Eg2 dissociates the CPEB/Maskin/eIF-4E complex and allows poly(A) polymerase access to the mRNA, resulting in the elongation of its poly(A)-tail and increasing translation (Mendez et al., 2000a; Mendez et al., 2000b). Once Mos protein translation increases, it phosphorylates MEK1 (a MAPKK) (Resing, et al., 1995), which then activates MAPK. The increase in translation is enough to activate the MAPK pathway at a low level. Through a positive feedback mechanism, MAPK phosphorylates Mos on Ser3, which inhibits its degradation (Matten et al., 1996). In this way, MAPK activity increases and maintains Mos activity. Without the phosphorylation of Ser3, Mos is rapidly degraded through ubiquitin directed proteolysis (Nishizawa et al., 1993).

**MEK1**

The MEK kinases are dual-specificity kinases that activate Erk1 and Erk2 MAPK by tyrosine and threonine phosphorylation in their activation loop. In oocytes, the MAPKK MEK1 phosphorylates Thr183 and Tyr185 of MAPK (Kosako et al., 1993). The requirement for activation of MEK1 in oocyte maturation has been demonstrated through microinjection of anti-MEK1 antibodies, which have been found to block progesterone induced GVBD (Kosako et al., 1994), and the use of the MEK1 inhibitor, PD98059, which also inhibits GVBD (Cross and Smythe, 1998). Microinjection of constitutively active MEK1 has been found to induce oocyte maturation, even in the presence of the
translation inhibitor cycloheximide, demonstrating its position below Mos and upstream of MAPK in the signalling pathway (Gotoh and Nishida, 1995)

MAPK

When MEK1 activates MAPK, it appears that the total pool of MAPK is activated at essentially the same time due to a positive feedback mechanism involving Mos (Ferrell and Machleder, 1998). Therefore, MAPK is in one of two possible states, completely inactive (unphosphorylated) or completely active (phosphorylated). This presents the ability to biochemically monitor whether the oocyte maturation pathway has been activated (phosphorylated MAPK, activated pathway, unphosphorylated MAPK, inactive pathway). MAPK activation occurs about 2 to 5 hours after stimulation, at the time of MPF activation and just preceding GVBD. Recently, it has been suggested there is a brief burst of MAPK activity shortly after progesterone stimulation (Fisher et al., 1999a; Fisher et al., 2000a). This might serve to activate a small amount of MPF, which would account for the translation of Mos and MAPK/MPF full activation through a feedback mechanism. Whether or not there is a MAPK burst at the time of progesterone stimulation, the point at which sustained MAPK activation occurs is approximately the same time as Mos translation and MPF activation (Ferrell, 1999).

The activation of MAPK has been assumed to be a necessary step in oocyte maturation (Ferrell, 1999). Inactivation of MEK1, either by anti-MEK antibodies or by the inhibitor PD98059, blocks progesterone induced GVBD (Cross and Smythe, 1998). Similarly, injection of the MAPK phosphatase CL100 blocks progesterone induced GVBD (Gotoh et al., 1995). However, there have been reports where U0126 (a MEK1
inhibitor), geldanamycin (an Hsp90 inhibitor), or Pyst1 (a MAPK phosphatase) blocked MAPK activation and oocytes still underwent GVBD (Fisher et al., 1999a; Gross et al., 2000). Although oocytes treated with U0126 (an inhibitor of MEK) are able to activate MPF and undergo GVBD, they do not form metaphase I spindles, re-accumulate cyclin B or hyperphosphorylate the Cdc27 component of the anaphase-promoting complex (Gross, et al., 2000). Instead, the oocytes enter S-phase with subsequent DNA replication. This suggests that although there appears to be a MAPK independent mechanism for activating MPF, MAPK activity is necessary for suppression of S-phase and entry into meiosis II.

**Maturation promoting factor in the control of meiosis**

Masui and Markert (1971) first detected the presence of MPF activity in oocytes when they microinjected immature oocytes with cytoplasmic extract obtained from progesterone-treated mature eggs. The injected oocytes underwent maturation suggesting that the cytoplasmic extracts of the mature eggs contained a maturation promoting factor (MPF). Furthermore, even if the egg extract was diluted, or if protein synthesis inhibitors were added, the injected, immature oocytes still underwent maturation and had full MPF activation. Thus, MPF had the ability to autoamplify its activity. They surmised the autocatalytic nature of MPF was due to a pre-existing component in the oocyte, which is in some way modified and activated. The mechanism of the autoamplification (described in more detail later) is still under investigation (Karaiskou et al., 2001a).

MPF has been found to consist of a complex of the serine/threonine kinase Cdc2 and the regulatory cyclin, cyclin B (Lohka et al., 1988). The Cdc kinases, also referred to as
cyclin dependent kinases (Cdks), regulate the progression of cell division. The Cdks interact with cyclins, a family of non-catalytic proteins, in order to ultimately regulate entry and exit from the cell cycle (Morgan, 1995). MPF not only regulates oocyte maturation, but also universally regulates the G2/M transition of the eukaryotic cell cycle (Wuarin and Nurse, 1996; Yamashita, 1998; Masui, 1992).

**Regulation of maturation promoting factor activity in *Xenopus* oocytes**

In *Xenopus* oocytes, the MPF complex is pre-formed and held in an inactive form referred to as pre-MPF (Karaiskou *et al.*, 2001a). The pre-MPF complex is maintained in an inactive state by inhibitory phosphorylation of the Cdc2 component on residues Thr14 and Tyr15 (Yamashita, 1998). Thr161 phosphorylation is required for activity, and appears to be constitutively phosphorylated by cyclin-dependent kinase activating kinase (CAK) (Yamashita, 1998). It is the dephosphorylation of residues Thr14 and Tyr15 that activates MPF (Yamashita, 1998). The opposing activities of the Wee1 and Myt1 kinases (Morgan, 1995) and the Cdc25 phosphatase (Gautier *et al.*, 1991; Sclafani, 1996), respectively, control the phosphorylation state of Thr14 and Tyr15.

Activation of Cdc25 appears to be controlled through hyperphosphorylation by Plx1 and its subcellular localization (Qian *et al.*, 2001). Plx1 is a member of the newly discovered polo-like kinase (plk) family that is involved in the regulation of the cell cycle (Glover *et al.*, 1998). Plx1 is activated by an upstream kinase, Plkk1, suggesting a kinase cascade may be involved in activation of Cdc25 (Karaiskou *et al.*, 1999). The nuclear localization of Cdc25 is also required for entry into meiosis (Kumagai *et al.*, 1998), however premature entry of Cdc25 into the nucleus bypasses cell cycle checkpoints and
initiates mitosis (Peng et al., 1997). The regulation of the localization of Cdc25 appears to be accomplished through interaction with the 14-3-3 proteins. Phosphorylation of Ser$^{216}$ of Cdc25 creates a binding site for 14-3-3, which retains Cdc25 in the cytoplasm (Kumagai et al., 1998), thus blocking the progression of the cell cycle until the S-phase is complete. Cyclin B/Cdc2 has also been shown to phosphorylate and activate Cdc25, forming a positive feedback loop. Through this feedback loop, once MPF is activated it can maintain its activity level, which presumably accounts for the autoamplification of MPF activity, however it does not account for its initial activation, since some other mechanism is needed to activate Cdc25 (Izumi and Maller, 1993).

The Wee1 gene was originally identified in *Schizosaccharomyces pombe* as a negative regulator of mitosis (Nurse and Thuriaux, 1980). When the *Xenopus* homologue of Wee1 was cloned, it only had the ability to phosphorylate Cdc2 on one of the two sites required for inactivation, Tyr$^{15}$ (Mueller et al., 1995a). The fact that MPF in oocytes is phosphorylated on both Tyr$^{15}$ and Thr$^{14}$ suggests that another kinase is present that will phosphorylate Thr$^{14}$ (Lundgren et al., 1991). The kinase responsible for this Thr$^{14}$ phosphorylation in *Xenopus* has been identified as Myt1, a Wee1 homologue (Mueller et al., 1995b). Thus, two possible kinases have the ability to inactivate MPF. However, only Myt1 has been identified in immature *Xenopus* oocytes, and is therefore assumed to be the only kinase of relevance in oocytes (Murakami and Vande Woude, 1998).

As with Cdc25 regulation, Myt1 is activated by phosphorylation (Mueller et al., 1995a; 1995b; Palmer et al., 1998). The activation of Myt1 appears to be by a MAPK effector, p90$^{rsk}$ kinase (Rsk) (Palmer et al., 1998). This demonstrates a link between the
activation of MAPK and the subsequent activation of MPF, which may participate in the initial activation and maintenance of MPF activity.

*Xenopus* oocytes contain inactive pre-MPF as well as an excess of free Cdc2 (Nebreda *et al.*, 1995). Injection of a kinase inactive Cdc2 into oocytes blocks GVBD. This suggests that this free Cdc2 is necessary for activation of MPF, since the pre-existing Cdc2/cyclin B complex can be activated directly by Cdc25 (Nebreda *et al.*, 1995). The most likely candidate for association and activation of free Cdc2 kinase would be a cyclin, yet synthesis of the known cyclins are not necessary for activation of GVBD (Nebreda and Ferby, 2000). Recently, during screening to identify proteins that activate GVBD in *Xenopus* oocytes without progesterone stimulation, a novel protein, Ringo, which has no similarity to cyclins, was identified (Ferby *et al.*, 1999). Over-expression of Ringo activates GVBD without the need for progesterone stimulation, even in the presence of protein synthesis inhibitors (Ferby *et al.*, 1999). Moreover, removal of Ringo mRNA by antisense RNA, blocks progesterone induced activation of MPF (Ferby *et al.*, 1999). The mechanism of Ringo's activation of Cdc2 seems to involve binding to and activating Cdc2 directly, without the need for Thr^{160} phosphorylation (Karaiskou *et al.*, 2001b). Therefore, Ringo may represent a novel type of cyclin that must be synthesized upon progesterone stimulation to activate MPF. Ringo's synthesis may also explain the requirement for activation of free Cdc2 in oocyte maturation.
Insulin Mediated Oocyte Maturation

Insulin-like growth factor-1 signalling

Insulin-like growth factor-1 (IGF-1) has numerous functions within an organism including growth, inhibition of apoptosis, and differentiation. In *Xenopus laevis* oocytes, insulin and IGF-1 have the ability to stimulate oocyte maturation through the IGF-1 receptor. The IGF-1 receptor consists of two extracellular ligand-binding α-subunits, and two transmembrane β-subunits that contain a tyrosine kinase domain (Figure 1.4).

Upon activation, the IGF-1 receptor autophosphorylates on tyrosines, recruiting a number of signalling molecules including insulin receptor substrate (IRS) proteins, Shc and many other substrates (Cheatham and Kahn, 1995). The diverse set of substrates phosphorylated by the IGF-1 receptor activates numerous signalling pathways within the cell including the Ras/MAPK and the phosphotidylinositol 3-kinase (PI 3-K) pathways. These same pathways are activated by many other growth factors, yet the cellular function of these other growth factors produces distinct effects on the cell. The mechanism by which IGF-1 and other growth factors produce distinct effects has not been clarified. Clearly, there are other signals originating from the IGF-1 receptor that have yet to be identified to produce its specific cellular effects. One of these might be heterotrimeric G-proteins. Indeed the IGF-1 receptor has also been shown to activate a heterotrimeric G-protein signalling pathway (Luttrell *et al.*, 1995).
Figure 1.4

Representation of the *Xenopus* insulin-like growth factor-1 receptor including tyrosine phosphorylation sites. The external $\alpha$-subunit is involved in ligand binding and is disulfide bonded to the transmembrane $\beta$-subunit. The $\beta$-subunit contains a kinase domain, which is activated upon ligand binding, as well as a number of tyrosine residues. The tyrosines can be trans-phosphorylated creating binding sites for numerous signalling proteins.
**Heterotrimeric G-protein signalling**

Heterotrimeric G-proteins are composed of three different protein subunits, Ga, Gβ, and Gγ, which are commonly associated with G-protein coupled receptors (GPCR, also known as seven transmembrane receptors). While in association with the GPCR, the G-protein heterotrimer is in an inactive state. GDP bound Ga is considered inactive, while GTP-Ga is considered active. Activation of the GPCR stimulates the exchange of GDP for GTP, activating the trimeric complex, which dissociates into two separate signalling components, the Ga-GTP subunit and the βγ subunit. G-proteins are commonly classified into classes according to their Ga subunit: Gas (Gas and Ga\textsubscript{OLF}), Gai (Gai1, Gai2, Gai3, GaoA, GaoB, Gat1, Gat2, Gag, and Gaz), Gaq (Gaq, Ga11, Ga14, Ga15, and Ga16), and Ga12 (Ga12, and Ga13). Gαs class subunits function to activate AC, while the Ga1 type inhibits a subset of ACs (Simonds, 1999). In this way, Gas and Gai control the level of cAMP present in the cell. The Gai subgroup can also activate a variety of phospholipases and phosphodiesterases, and promote the opening of several ion channels (Gutkind, 2000). Gaq are involved in activating phosphotyridinositol-specific phospholipases such as phospholipases C-β (PLC-β) (Rhee and Bae, 1997), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5-triphosphate (IP\textsubscript{3}) and dicylglycerol (DAG). IP\textsubscript{3} and DAG in turn lead to an increase in the intracellular calcium concentration and the activation of a number of protein kinases (Berridge, 1993). One function of Ga12 may be to provide a link between G-protein coupled receptors (GPCR) and the small G-protein Rho (Hart et al., 1998).
Regulation of G-protein signalling by RGS proteins

Hydrolysis of the bound GTP to GDP inactivates the Gα subunit and promotes re-association of the βγ subunits with Gα. The regulators of G-protein signalling (RGS) potentiate this GTP hydrolysis. The RGS proteins are a very diverse group of proteins all of which share a common domain referred to as the RGS domain. The RGS domain consists of approximately 130 amino acids arranged to produce a 4-helix bundle, which directly interacts with Gα subunits and stimulates their GTP hydrolytic activity (Popov et al., 1997). RGS proteins have been categorized into four subgroups, A, B, C, and D, based on their sequence similarity outside of the RGS domain (Druey, 2001). Although the RGS proteins do selectively interact and catalyse hydrolysis of certain Gα subgroups, the functional domains outside the RGS domain most likely determine the location and specificity of RGS proteins.

GAIP is a member of the RGS subgroup A, and is capable of activating the GTPase activity (acting as a GTPase activating protein (GAP)) of the Gαi and Gαq subunits. GAIP was first identified in a yeast 2-hybrid screen using the C-terminus of Gαi3 as a bait (De Vries et al., 1995). It contains a central RGS domain flanked by a C-terminal PDZ binding site and C-terminal cystine string (De Vries et al., 1995). The GAP activity of GAIP has been determined to be selective for Gαi and Gαq type G-proteins (Berman et al., 1996; Hepler et al., 1997). The C-terminal cystine string is palmitoylated, which localises GAIP to clathrin-coated vesicles (Fisher et al., 2000b). Ogier-Denis et al., (2000) found that the GAP activity of GAIP was modified by phosphorylation. They have shown that phosphorylation by MAPK increased GAIP's GAP activity toward Gαi3 in HT-29 cells, suggesting a possible feedback mechanism for the activation of GAIP.
Although the exact function of GAIP in vivo is unknown, it does inhibit Gαi signalling and may be involved in vesicle transport and fusion (Fisher et al., 1999b; Fisher et al., 2000b).

**G-protein βγ complex in intracellular signalling**

The Gβγ subunits, of which there are 12 Gγ subunits and 6 Gβ subunits, also have numerous functions in signalling including, negative regulation of GPCRs, and activation of phosphatidylinositol 3-kinases, phospholipases, ACs and receptor kinases (Vanderbeld and Kelly, 2000). The βγ-dimer does not dissociate under physiological conditions and can therefore be considered as a single signalling unit (Schwindinger and Robinshaw, 2001). The members of the Gβ family are all highly homologous while the Gγ subunits are slightly more diverse in sequence (Schwindinger and Robinshaw, 2001). Thus the pairing of specific β-subunits with specific γ-subunits activates distinct downstream effectors (Schwindinger and Robinshaw, 2001).

The Gβγ complex has been shown to affect the activity of certain AC types. In the presence of activated Gαs, Type II and IV AC are activated by βγ, while type I is inhibited (Simonds, 1999). Some forms of PI-3K are regulated by βγ-dimers. Class IB PI 3-K isoforms, which consist of a p110γ catalytic subunit and a p101 regulatory subunit, are activated by βγ-dimers (Stephens et al., 1997). The mechanism involves direct binding of βγ-dimers to the p110γ/p101 heterodimer (Leopoldt et al., 1998, Krugmann et al., 1999). Gβγ-dimers have also been shown to activate the MAPK pathway. Although there does not seem to be a direct interaction between MAPK and βγ-dimers, they do stimulate a number of pathways that activate MAPK. The activation of
the small G-protein Ras seems to be necessary for activation of MAPK by βγ-dimers, since expression of dominant negative N-17Ras blocks βγ mediated MAPK activation (Koch et al., 1994). Gβγ-dimers may serve to initiate a Ras activation complex. Binding of β-arrestin to a phosphorylated GPCR serves to down regulate signalling and initiate receptor internalization. However, β-arrestin may also function as an adapter protein for the binding of Src and activation of the MAPK pathway (Luttrell et al., 1999). In this scenario, βγ induces the translocation of G-protein coupled receptor kinase (GRK) to the activated receptor, which initiates the complex formation.
Chapter 2

The Classical Progesterone Receptor Mediates *Xenopus* Oocyte Maturation Through a Non-genomic Mechanism.
**ABSTRACT**

*Xenopus laevis* oocytes are physiologically arrested at G2 of meiosis I. Progesterone triggers the resumption of meiosis through an extranuclear receptor, which is independent of gene transcription. However, the identity of this extranuclear oocyte progesterone receptor (PR) has remained a longstanding question. The amphibian homologue of human PR previously identified from a *Xenopus* oocyte cDNA library was characterized. The cloned *Xenopus* progesterone receptor (xPR) exhibited all sequence characteristics of a classical mammalian progesterone receptor. Indeed, when expressed in COS-7 cells, xPR is imported into the nuclei. In the presence of progesterone, but not several other steroid hormones, xPR was capable of activating transcription of a reporter gene driven by a progesterone response element. Furthermore, the transcriptional activity of xPR was inhibited by the antiprogestin RU486. However, endogenous xPR was exclusively localized to the oocyte's cytoplasm. Similarly, the majority of xPR derived from mRNA injection also resided in the oocytes cytoplasm. The cytoplasmic localization of xPR suggested that it functioned to promote signal transduction. Indeed, injection of xPR accelerated progesterone induced oocyte maturation. It also accelerated progesterone induced MAPK activation in enucleated oocytes. Taken together, these results demonstrate that xPR signals both through classical transcriptional activation in COS-7 cells and in a novel cytoplasmic cascade in *Xenopus* oocytes demonstrating its dual functional ability.
INTRODUCTION

Ovarian progesterone is the natural trigger of amphibian oocyte maturation, commonly assessed by GVBD (Masui, 1967). Masui and Markert (1971) demonstrated that progesterone-induced activation of maturation promoting factor (MPF or Cdc2/cyclin B) could occur in enucleated oocytes, therefore establishing the cytoplasmic nature of the putative progesterone receptor. Furthermore, Masui and Markert (1971) and Smith and Ecker (1971) found that injection of progesterone into the oocyte cytoplasm, unlike progesterone added to the incubation medium, did not cause GVBD, suggesting that the oocyte progesterone receptor may be a transmembrane protein. The notion of an oocyte surface receptor was further supported by others who demonstrated that extracellular application of cell-impermeable, polymer-linked progesterone was able to induce GVBD (Ishikawa et al., 1977; Godeau et al., 1978). Furthermore, Sadler and Maller (1981) demonstrated that in partially isolated plasma membrane (via peeling), progesterone was able to inhibit oocyte adenylate cyclase, implying association of the putative PR with the plasma membrane. Inhibition of adenyl cyclase is thought to be a key event in progesterone-induced GVBD (Maller and Krebs, 1980). However, many attempts to identify a plasma membrane-bound receptor by classical ligand binding and cross-linking have failed to yield physiologically relevant progesterone-binding proteins in Xenopus oocytes (Belle, et al., 1977; Liu and Patino, 1993; Sadler and Maller, 1998).

Although progesterone action in amphibian oocytes is mechanistically distinct from that in somatic cells in which progesterone regulates transcription via its nuclear progesterone receptor (PR), no direct evidence is available to rule out the possibility that
PR may function in a novel, cytoplasmic fashion. In fact, the amphibian homologue of nuclear PR has not been identified or cloned. Indeed, nuclear PR is known to shuttle between the nucleus and the cytoplasm (Tyagi et al., 1998; Hager et al., 2000) and yet its possible cytoplasmic function has not been delineated. Furthermore, Sadler et al. (1985) reported that the anti-progestin RU486 could induce oocyte GVBD. RU486 and progesterone interact with the hormone-binding domain (HBD) of PR (Edwards et al., 2000), suggesting that the oocyte progesterone receptor at least contains a classical progesterone HBD.

In this study, the putative oocyte progesterone receptor cDNA was isolated via molecular cloning of *Xenopus* genes that contained classical progesterone HBD utilizing the HBD of human PR as a probe (completed by M. Bayaa). The receptor appeared to be the bona fide *Xenopus* homologue of progesterone receptor (xPR). In further studies utilizing both frog oocytes and the heterologous COS-7 cells, xPR was demonstrated to function as a dual functional protein. It was imported into the nucleus and functioned as a progesterone-regulated transcriptional activator in heterologous COS-7 cells (described in this thesis). In contrast, the endogenous xPR was restricted in the oocyte cytoplasm and appeared to mediate intracellular signal transduction leading to MAP kinase activation (M. Bayaa, MSc thesis).
MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma-Aldrich Co. unless otherwise stated.

Animal and oocyte manipulation

All procedures involving live oocytes were carried out in a room maintained between 18-20 °C. Sexually mature, oocyte positive Xenopus laevis were purchased from NASCO and maintained according to local animal care guidelines. The frogs were injected with pregnant mare serum gonadotropin (Sigma, 50 IU/frog) 3-10 days before operations. A fragment of ovary was removed surgically under hypothermia. For all experiments, unless otherwise stated, stage VI oocytes were manually defolliculated in culture medium OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.8). For other experiments, ovary sections were treated with collagenase solution (0.2 % collagenase, 0.1 M NaH₂PO₄, 0.1 % BSA, 0.1% trypsin inhibitor) for 3 h at room temperature with shaking, and the various stages of oocytes used were then individually selected according to Smith et al. (1991).

Messenger RNA was transcribed (mMessage mMachine, Ambion) from purified linearized plasmid DNA. Typically, oocytes were injected with 20 nL of mRNA (1 μg/μL) and incubated in OR2 medium for 24-36 h (unless otherwise stated) before being subjected to hormonal treatment.
**COS-7 cell transfection and CAT assay**

For immunohistochemistry, COS-7 cells were seeded on cover slips in DMEM containing 10% stripped FCS (containing no steroid hormones; HyClone, USA) and at approximately 70% confluence were either mock-transfected (no DNA), transfected with 0.2 µg of pCS2+MT-xPR plasmid (xPR) or pCS2+MT-xIRS-u plasmid (as a control) using 2 µL of Lipofectamine reagent (GibcoBRL, USA). The pCS2+MT-xPR plasmid was constricted by inserting amino acids 1-583 in-frame with the Myc tag of the pCS2+MT vector (Turner and Weintraub, 1994), resulting in a plasmid expressing five copies of the 13 amino acid Myc tag fused to xPR. The pCS2+MT-xIRS-u plasmid was constructed by inserting amino acids 1-1003 of xIRS-u (Ohan *et al.*, 1998) in frame with the Myc tag of pCS2+MT resulting in a plasmid expressing five copies of the Myc tag fused to xIRS-u. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 60 min. at room temperature. The cover slips were then washed 3 times with PBS, for a minimum of 5 min. each wash. The cells were permeabilized in 0.1% Triton-X-100, 0.1% sodium citrate for 2 min. on ice. To block non-specific binding, the cover slips were incubated for 20 min. in blocking buffer (PBS containing 1% BSA (United States Biochemical, USA), 2% normal goat serum (GibcoBRL, USA)) at room temperature. The cover slips were then placed in blocking buffer with anti-Myc antibodies (9E10 ascites (Farah *et al.*, 1998; 1:500) overnight at 4°C and then washed 5 times with PBS. After washing, the cover slips were again blocked with blocking buffer for 20 min. at room temperature and then incubated in anti-mouse fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch Inc.,
USA; 1:100 in blocking buffer) for 2 hours at room temperature. After washing 5 times in PBS, the cover slips were mounted on slides using Cytoseal 60 (Stephens Scientific, USA). Results were visualized using a BioRad model # MRC 1024 confocal microscope equipped with an excitatory laser of 488 nm.

In some experiments for transcriptional activation, COS-7 cells were seeded in 23 mm dishes that were co-transfected with mouse mammary tumour virus (MMTV)-chloramphenicol acetyltransferase (CAT) reporter plasmid (0.25 ug/dish) and one of the various test constructs (vector pCS2+MT, xPR, or xPR-ER). In other experiments, to equalize the amount of plasmid transfected per 23 mm dish, COS-7 cells in 10 cm dishes were transfected with MMTV-CAT reporter plasmid (1.5 μg/10 cm plate), together with vector pCS2+MT, or Myc-xPR (1.5 μg/10 cm plate, unless otherwise stated) and then equally divided between 23 mm dishes for hormonal treatment. For experiments using 1/5th the amount of xPR plasmid DNA, 0.3 μg/10 cm plate was used while maintaining the original amount of reporter plasmid. The cells were transfected overnight in OPTI-MEM media (GibcoBRL). The next morning the cells were lifted and re-plated equally between 23 mm dishes containing charcoal/dextran treated serum (serum lacking steroid hormones; HyClone, USA) and allowed to recover overnight. Forty-eight hours after transfection, the cells were either left unstimulated, or incubated for 18 h with various concentrations of hormones: progesterone, R5020 (synthetic progestin; Steraloids Inc., USA), 17-β estradiol (E2; Steraloids Inc., USA), or dexamethasone (Steraloids Inc., USA). After hormonal treatment, the cells were washed once with PBS and scraped into a 1.5 mL centrifuge tube. The cells were collected by centrifugation at 1500 rcf for 1 min. and resuspended in 200 μL of FT buffer (0.25 M sucrose, 10 mM Tris pH 7.4, 10
mM EDTA). The resuspended cells were subjected to 3 freeze/thaw cycles using liquid N₂ and a 37 °C water bath. One hundred and ninety microlitres of the lysate was transferred to a fresh tube and stored at -80 °C until used. One hundred microlitres of the lysate was mixed with 2 μL of ¹⁴C-chloramphenicol (0.05 μCi; NEN Dupont, USA), 5 μL butyryl Co-A (5 mg/mL in 0.25 M Tris, pH 7.8) and 93 μL of 0.25 M Tris, pH 7.8 and incubated for 2 h at 37 °C. The reaction was stopped and extracted with the addition of 750 μL of ethyl acetate. The samples were vortexed and briefly centrifuged to separate the phases. The upper phase (ethyl acetate) was transferred to a fresh tube and dried under vacuum. The pellet was resuspended in 10 μL of ethyl acetate, spotted onto a TLC plate (Silica Gel 60; EM Science, Germany), and resolved in a chloroform:methanol (95:5) mixture. Quantification of the CAT assay was performed using a PhosphorImager (Bio-Rad, model# GS 525). The activation of each sample was calculated by selecting equal sized areas around the uppermost spot in each lane, and using the BioRad Molecular Analyst analysis program, the volume within each area was determined. The percent activation was calculated relative to the highest concentration used in the experiment.

**Immunoblotting**

For immunoblotting, oocytes were lysed in PBS lysis buffer (Farah et al., 1998) (10 mM phosphate buffer, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 10 μg/mL Leupeptin, 100 μM sodium orthovanadate), and immediately centrifuged at 4 °C to clear the lysate. The supernatant was then mixed with 2X Laemmli sample buffer and separated using SDS-PAGE. The proteins were
transferred to nitrocellulose membranes (Schleicher & Schuell, Germany), which were probed using anti-\textit{Xenopus} MAPK antibodies (a gift from J.A. Cooper, Fred Hutchinson Cancer Research Centre, Seattle). For immunoblotting of COS-7 cell, an aliquot of the lysate used in the CAT assay was mixed with an equal volume of 2X Laemmli sample buffer and separated using SDS-PAGE. The proteins were transferred to nitrocellulose membranes and probed using anti-Myc antibodies (4E10, M. Tyres)
RESULTS

The identification of xPR as dual functional protein was jointly carried out by a fellow student, Mr. Mustafa Bayaa, and myself. Mr. Bayaa has presented his portion of the data in his MSc thesis. The data presented in this chapter were entirely mine. In order to keep the presentation more relevant, however, I will refer to Mr. Bayaa’s work in our joint paper (Bayaa et al., 2000).

xPR functions as a progesterone-regulated transcriptional activator in COS-7 cells

As the cloned xPR resembled a classical nuclear progesterone receptor, we were puzzled by its localization to the cytoplasm in frog oocytes (Bayaa et al., 2000). Since xPR contained a perfect nuclear localization signal (286RKFKKFGGR) (Guiochon-Mantel, et al., 1992) we wished to determine if xPR was capable of translocating to the cell nucleus. COS-7 cells were chosen partly because they contained no endogenous progesterone receptor (Savouret et al., 1991) which otherwise might interfere with our functional assays (see later). COS-7 cells were transfected with Myc-xPR and cultured in medium containing steroid-free fetal bovine serum. Using anti-Myc antibodies, Myc-xPR was detected mainly in the nucleus, although faint cytoplasmic staining was also detectable (Figure 2.1A). To control for the possibility that the Myc tag may play a role in the nuclear localization of xPR in COS-7 cells, a cytoplasmic Myc-tagged protein, Myc-xIRS-u (Ohan et al., 1998), was used in immunohistochemistry experiments. In
**Figure 2.1**
xPR localizes to the nucleus of COS-7 cells. (A) COS-7 cells were transfected with Myc-xPR or mock (no DNA) transfected. Forth eight hours after transfection the cells were fixed and stained with anti-myc antibodies. Shown are typical confocal images of mock-transfected (Left) or Myc-xPR transfected COS-7 cells (Right). The mock transfected cells showed no fluorescence, while the nuclei of Myc-xPR transfected cells fluoresced. Some cytoplasmic fluorescence was seen in heavily overexpressing cells. (B) COS-7 cells were transfected with Myc-IRS-u plasmid DNA. Forth eight hours after transfection the cells were fixed and stained with anti-myc antibodies. Intense fluorescence was seen in the cytoplasmic region of the cells. The cells shown are representative examples of 2 independent experiments.
Figure 2.2
Stimulation of Myc-xPR transfected cells with progesterone does not alter its localization. COS-7 cells were transfected with Myc-xPR or mock transfected. Forty eight hours after transfection the cells were fixed and stained with anti-myc antibodies. Shown are typical confocal images of mock transfected (left) and Myc-xPR transfected (right) COS-7 cells stimulated with 1 μM progesterone for 18 hours. Fluorescent signals originated from the nuclei of the transfected cells.
Mock

Myc-xPR + 1 μ M Prog.
contrast to Myc-xPR which was primarily localized in the nucleus, Myc-xIRS-u was predominantly in the cytoplasm (Figure 2.1B), suggesting that the Myc tag did not play a role in the nuclear localization of xPR. As might be expected, the addition of 1 μM progesterone to the medium 18 hours before fixation did not cause appreciable changes in xPR localization (Figure 2.2). These results suggested that xPR contained a ligand-independent nuclear localization signal.

To determine whether xPR functioned as a progesterone-regulated transcriptional activator, a reporter construct that contained a chloramphenicol acetyl transferase (CAT) gene downstream of a mouse mammary tumour virus (MMTV) promoter was employed (Prefontaine et al., 1999). The MMTV promoter contained 4 copies of the progesterone-response element (PRE). COS-7 cells were co-transfected with Myc-xPR and MMTV-CAT. Stimulation of co-transfected COS-7 cells with 1 μM R5020 (a synthetic progestin) for 18 hours caused a substantial increase in CAT activity (Figure 2.3A, comparing lane 2 to lane 3). The lower band corresponds to the un-acetylated form of chloramphenicol, while the middle and upper bands correspond to double and triple acetylated forms of chloramphenicol respectively. An increase in the acetylated forms of chloramphenicol reflects an increased amount of cat gene transcription induced by the MMTV promoter, which contains progesterone response elements (PRE).

Oocytes not only undergo maturation in response to progesterone, but also respond to other steroids including testosterone and androstenedione but do not respond to estrogen. In order to determine the specificity of xPR, several other steroid hormones
Figure 2.3
Transcriptional activation induced by xPR. COS-7 cells were transfected with mouse mammary tumour virus-chloramphenicol acetyl transferase reporter plasmid, together with either pCS2+Myc vector, Myc-xPR or Myc-xPR-ER in 23 mm dishes. Forty-eight hours after transfection, the cells were stimulated with either 1 μM R5020, 17-β estradiol (E2), or dexamethasone (Dex) for 18 hours. Cell lysates were prepared as in Methods and subjected to CAT assays. (B) Western blot of COS-7 cell lysates showing expression of the Myc-xPR and Myc-xPR-ER constructs. Shown is a representative example of three independent experiments.
Figure 2.4
xPR mediated transcription is not induced by testosterone. COS-7 cells transfected with mouse mammary tumour virus-chloramphenicol acetyl transferase reporter plasmid in 10 cm dishes, together with either pCS2+Myc vector or Myc-xPR and divided between 23 mm dishes for hormonal treatment. Forty-eight hours after transfection the cells were stimulated with 1 μM R5020 or 1 μM testosterone for 18 hours. Cell lysates were prepared as in Methods and subjected to CAT assays. Shown is an example of two independent experiments.
xPR
- + - - -
- - - + +

Myc

R5020
Testosterone
were tested for their ability to modulate xPR mediated transcription in COS-7 cells. Stimulation of co-transfected COS-7 cells with 1 μM estradiol or dexamethasone (a glucocorticoid receptor agonist) (Figure 2.3A, lanes 4 and 5, respectively), as well as stimulation with 1 μM testosterone (Figure 2.4) did not result in any significant increase in CAT activity. Expression of the xPR and xPR-ER constructs are shown in Figure 2.3B.

To further demonstrate the ligand specificity of xPR, the ligand-binding domain of xPR was replaced with that of human ER (Green et al., 1986), generating a chimeric receptor xPR-ER. As shown in Figure 2.3, whereas xPR responded to R5020, but not to estradiol, xPR-ER, in contrast, responded to estradiol but not to R5020 (Figure 2.3A, comparing lanes 7 and 8). Our original hope was that injection of this estradiol-responsive xPR-ER into frog oocytes might render oocytes responsive to estradiol in GVBD induction. As oocytes normally do not respond to estradiol, a successful “rescue” would help establish the functionality of xPR in frog oocyte maturation. However, repeated injection of xPR-ER had failed to cause any changes in estradiol responsiveness (Bayaa et al., 2000).

**Similar concentrations of progesterone are required to activate transcription and stimulate GVBD**

To estimate the binding affinity of xPR for R5020 or for progesterone, COS-7 cell experiments were performed using 0.1 to 1000 nM R5020 or progesterone. Figures 2.5 shows that 50% activation of CAT activity was achieved at approximately 10 nM of R5020, whereas maximum activation required 100 nM. Using linear regression analysis
of 4 independent experiments (Figure 2.6) the 50% activation was calculated to be 16.96 ± 3.85 nM. Similar results were obtained when COS-7 cells were transfected with 1/5 the amount of xPR DNA with the exception that the absolute amounts of CAT activity were correspondingly less (Figure 2.5, 250 ng/23 mm dish xPR vs. 50 ng/23 mm dish xPR). Similarly, the EC₅₀ for progesterone in CAT activation was 20.22 ± 8.92 nM as determined from 3 independent experiments (Figure 2.7) using linear regression analysis over the linear activation range (10 nM to 100 nM). An example of a representative autoradiograph is presented in Figure 2.8. Interestingly, the EC₅₀ for progesterone in the induction of oocyte maturation was between 10 and 20 nM (Figure 2.9). As determined previously in the lab (Ohan et al., 1999), the percent GVBD of any group of oocytes correspond with the ratio of active MAPK (upper band) vs. inactivate MAPK (lower band) (Figure 2.9). These results suggest that the same receptor (xPR) was responsible for both transcription in COS-7 cells and signal transduction in frog oocytes. Significantly, these values were also in close agreement with the intra-ovarian progesterone concentration (estimated at 10 nM) (Smith, 1989).

**xPR responds to the anti-progestin RU486**

The progesterone antagonist RU486 is capable of inducing oocyte GVBD (Sadler et al., 1985), presumably by binding the putative oocyte progesterone receptor. RU486 binds human PR but not chicken PR and this difference has been attributed to a single amino acid (Gly⁷²² in human PR and Cys⁵⁷⁵ in the corresponding position of chicken PR) within the HBD (Benhamou et al., 1992). Inspecting the xPR sequence revealed the
Figure 2.5
R5020 induces xPR mediated transcription. COS-7 cells transfected in 10 cm dishes with either 1.5 μg of Myc-xPR (equivalent of 250 ng per 23 mm dish) or 0.3μg of Myc-xPR (equivalent of 50 ng per 23 mm dish) and divided between 23 mm dishes for hormonal treatment. Forty-eight hours after transfection the cells were simulated with increasing concentrations of R5020 as indicated. The percent of chloramphenicol acetylation relative to Myc-xPR transfected cells stimulated with 1 μM R5020 is indicated above each lane. Shown is a representative example of three independent experiments.
Figure 2.6
Graphical representation of four independent CAT assay experiments. Four independent CAT assays were performed by transfecting COS-7 cells in 10 cm plates with 1.5 μg of Myc-xPR. After transfection the cells were equally divided between 23 mm dishes for R5020 treatment. The percent activation was calculated relative to the 100 nm R5020 treated sample.
Figure 2.7
Grasphical representation of three independent CAT assays for progesterone treatment. Three independent CAT assays were performed by transfecting COS-7 cells in 10 cm plates with 1.5 μg of Myc-xPR. After transfection the cells were equally divided between 23 mm dishes for progestosterone treatment. The percent activation was calculated relative to the 100 nm progesterone treated sample.
Figure 2.8
Progesterone induces xPR mediated transcription. COS-7 cells were transfected in 10 cm dishes with Myc-xPR plasmid DNA and transferred to 23 mm dishes for hormonal treatment. Forty-eight hours after transfection the cells were simulated with increasing concentrations of progesterone as indicated. The percent of chloramphenicol acetylation relative to Myc-xPR transfected cells stimulated with 1 μM progesterone is shown above each lane. Shown is a representative of three independent experiments.
Figure 2.9
Progesterone induces oocyte maturation with an EC$_{50}$ between 10 and 20 nM. Freshly isolated oocytes were manually defolliculated and groups of 20-25 oocytes were treated overnight with the increasing concentrations of progesterone as indicated below each lane. After scoring each sample for GVBD (expressed as % of total treated oocytes), extracts were prepared and analysed by MAPK immunoblotting. The lower band corresponds to the non-phosphorylated, inactive form of MAPK, while the upper band corresponds to the active phosphorylated form of MAPK.
presence of a cysteine (Cys$^{372}$) at the corresponding position (Benhamou et al., 1992), indicating similarity to chicken PR. To examine whether xPR was sensitive to RU486, xPR-transfected COS-7 cells were treated with R5020, RU486 or both. Indeed, RU486, at concentrations ranging from 10-50 μM, inhibited hormone-induced transcription by xPR (Figure 2.10) or human PR-A (Figure 2.11). RU486 (10-50 μM) alone did not alter basal transcription in COS-7 cells or COS-7 cells transfected with xPR cDNA (not shown). Significantly, the same concentrations of RU486 were also effective in inducing oocyte GVBD and MAP kinase activation (Figure 2.12). Again, these data are consistent with the notion that xPR was responsible for the effect of RU486 in COS-7 cells and frog oocytes.
Figure 2.10
RU486 inhibits transcription activated by xPR. COS-7 cells were transfected in 10 cm dishes with mouse mammary tumour virus-CAT reporter plasmid along with Myc-xPR and then divided between 23 mm dishes for hormonal treatment. Forty-eight hours after transfection the cells were either left unstimulated (lane 1), stimulated with 1μM R5020 (lane 2) or co-stimulated with 1μM R5020 and increasing concentrations of RU486 for 18 hours. For samples containing both R5020 and RU486, both compounds were added simultaneously. Lysates were prepared as in Methods and assayed for CAT activity. Indicated is the percent acetylation induced by R5020 in the presence of RU486 relative to cells transfected with Myc-xPR and stimulated with 1μM R5020 only. Shown are two independent experiments.
% of activity at 1 μM R5020

Lane
- + + + + + - + + + + R5020 (1μM)
0 0 1 10 20 50 0 1 10 20 50 0 RU486 (μM)
**Figure 2.11**

RU486 inhibits transcription activated by human PR-A. Human PR-A was used to transfect 10 cm dishes that were equally divided between 23 mm dishes for hormonal treatment. COS-7 cells were treated with 1 μM R5020 (lane 1) or 1 μM R5020 together with the indicated concentrations of RU486 (lanes 2-5). For samples containing both R5020 and RU486, both compounds were added simultaneously and incubated for 18 hours. Lysates were prepared as in Methods and assayed for CAT activity. Indicated is the percent acetylation induced by R5020 in the presence of RU486 relative to cells transfected with PR-A and stimulated with only 1 μM R5020. Shown is an example of three independent experiments.
% of activity at 1 μM R5020.

R5020 (1μM)

RU486 (μM)
Figure 2.12
RU486 stimulated GVBD and MAPK activation in oocytes. Freshly isolated oocytes were manually defolliculated and groups of 20-25 oocytes were treated overnight with increasing concentrations of RU486. After scoring each sample for GVBD (expressed as % of total treated oocytes), extracts were prepared and analysed by MAPK immunoblotting. The upper band corresponds to the phosphorylated active form of MAPK, while the lower band corresponds to the unphosphorylated inactive form of MAPK.
DISCUSSION

Subsequent to our publication (Bayaa et al., 2000), Tian et al. (2000) reported the cloning of XPR-1 (GenBank #AF279335). With the exception that XPR-1 contained an additional 149 amino acids at the N-terminus, it was 92% identical to xPR. Over-expression of XPR-1, similar to xPR over-expression (Bayaa et al., 2000), increased the sensitivity of oocytes to progesterone and accelerated progesterone induced-GVBD (Tian et al., 2000). Furthermore, Tian et al (2000) was able to show that injection of XPR-1-specific antisense oligonucleotides blocked progesterone-induced GVBD, and that subsequent injection of XPR-1 mRNA rescued progesterone responsiveness. Antisense oligonucleotides directed against the N-terminus of xPR were employed similarly in our lab and unfortunately they did not cause reduction of xPR proteins (M. Bayaa and J. Liu, unpublished results). The reason for the apparent discrepancy between the above result and that from Tian et al. (2000) is unknown. It is possible that the difference in the targeted regions accounted for the different outcome of these experiments. Our work (Bayaa et al., 2000), together with that of Tian et al. (2000) demonstrated that the classical progesterone receptor (xPR) is responsible for the non-genomic action of progesterone in the induction of frog oocyte maturation.

Previous publications, using intracellular delivery of progesterone and polymer-linked progesterone, suggested that the oocyte progesterone receptor is associated with the plasma membrane. However, xPR does not appear to be associated with the plasma membrane (Bayaa et al., 2000). The possibility that xPR is associated with a membrane
structure that co-isolates with the cytoplasm cannot be ruled out, however the early data which suggested the oocyte PR is membrane associated can be re-examined.

Masui and Markert (1971), and Smith and Ecker (1971) reported that intracellular delivery of progesterone did not stimulate oocyte maturation, while Tso *et al.* (1982) reported that oocyte maturation was stimulated by intracellular injection of progesterone. Our lab injected free progesterone and found that the injection of 5 nL of 1 mM progesterone (in ethanol) caused 100% of injected oocytes to undergo maturation (Bayaa *et al.*, 2000). This result agrees with Tso *et al.* (1982) that intracellular delivery of progesterone does induce maturation, suggesting that the earlier reports (Masui and Markert, 1971; Smith and Ecker 1971) likely suffered from technical problems involving progesterone solubility, since progesterone diffuses through the membrane and will activate either a membrane bound or intracellular receptor.

The use of polymer- or BSA-linked progesterone to induce oocyte maturation by Godeau *et al.* (1978) and Ishikawa *et al.* (1977) suggested a plasma membrane location for the oocyte PR, since the attached polymer should block the progesterone from entering the oocyte and activating an intracellular receptor. Our lab has tested BSA-progesterone (Steraloids, USA) and found it equally potent in inducing maturation whether added externally (to the culture medium) or injected into the oocyte (Bayaa *et al.*, 2000). One interpretation of this data is that a substantial amount of free progesterone is present in the BSA-progesterone sample. Recently, a report by Stevis *et al.* (1999) indicated that a commercial preparation of BSA-E2 (Sigma, USA) contained significant levels of free hormone, and when the free hormone was removed by filtration the BSA-E2 lost its biological function.
What are the molecular determinant(s) that render xPR its dual functionality (as a transcription regulator in COS-7 cells and a signal transducer in frog oocytes)? The simplest explanation appears to be its differential subcellular localization. Therefore, in COS-7 cells, and presumably in somatic cells of *Xenopus laevis* as well, xPR is imported into the nucleus and is fully functional as a progesterone-regulated transcriptional activator. In contrast, in frog oocytes, it is restricted in the cytoplasm, even in the presence of bound ligand (progesterone). Support of this simple model (subcellular localization determining mode of action) came from both our group and that of Dr. Ruderman’s. Tian *et al.* (2000) show that frog oocytes failed to exhibit any transcriptional activity in the presence of progesterone and a reporter construct (luciferase downstream from a progesterone response element or PRE-luc), even though they do contain endogenous xPR (Bayaa *et al*., 2000). This is consistent with our data, which demonstrates that the endogenous xPR is restricted in the oocyte cytoplasm even in the presence of progesterone (Bayaa *et al*., 2000). However, over-expression of XPR-1 in oocytes resulted in significant levels of luciferase activity in the presence of progesterone (Tian *et al*., 2000). Bayaa *et al.* (2000) demonstrated that over-expression of xPR does indeed lead to nuclear localization of a portion of the expressed xPR. However, Tian *et al.* (2000) were unable to determine the localization of XPR-1 due to the lack of specific antibodies, and therefore could not determine the localization of the endogenous protein, while over-expressed XPR-1 was present in nuclear, cytoplasmic and cortical fractions of the oocytes. These data suggest that endogenous xPR is retained in the oocyte cytoplasm via a saturable mechanism and that over-expression leads to over-saturation of this mechanism and therefore a proportion was “free” to translocate into the nucleus.
How does xPR regulate cytoplasmic signalling and how does this differ from its activity in regulating gene transcription? Upon ligand binding, progesterone receptor is thought to modulate transcription by binding to a specific sequence, the PRE, within the promoter region of target genes and, simultaneously, interacting with other proteins (co-activators and/or components of basal transcription machinery) (Rowan and O’Malley, 2000). The regions of PR thought to be responsible for these protein-protein interactions are the AF-1 and AF-2 domains, the latter being within the hormone binding domain (Tsai and O’Malley, 1994). RU486 is known to interact with PR through its hormone binding domain and hence antagonise the ability of progesterone to activate transcription (el-Ashry et al., 1989; Meyer et al., 1990). As shown here, RU486 also antagonises the ability of xPR to activate transcription, despite the presence of Cys\textsuperscript{372} in xPR, a position corresponding to Gly\textsuperscript{722} in human PR. Clearly, the previous assignment of Gly\textsuperscript{722} to be the only residue important for RU486 binding (Benhamou et al., 1992) requires further clarification. Interestingly though, RU486 functions as an agonist for xPR in its ability to induce frog oocyte maturation (Figure 2.9). The opposite effects of RU486 in COS-7 cells (transcription) and in frog oocytes (signal transduction) suggest that the conformation assumed by xPR, upon binding to RU486, is not compatible with its transcription activity but it is compatible with its ability to signal in the cytoplasm. We hope to utilize RU486 in our search for cytoplasmic “effectors” of xPR. One possible technique for detecting RU486 specific interactions is the yeast 2-hybrid assay. Conducting screens using xPR in the presence of RU486 may identify novel interaction partners involved in cytoplasmic signalling.
Finally, our work also clearly hints at the importance of the HBD of xPR in cytoplasmic signalling, in addition to its ability to bind progesterone. We demonstrate that xPR-ER, in which the xPR HBD was replaced with the HBD of human estrogen receptor (ER), was able to function as an estrogen-regulated transcription activator in COS-7 cells. However, this receptor failed to “signal” in the cytoplasm of frog oocytes (Bayaa et al., 2000). This is reminiscent of steroid receptors’ (PR included) function in transcriptional regulation in which the HBD also possesses AF-2 activity in addition to binding hormones (Tsai and O’Malley, 1994). Interestingly, a recent study by Kousteni et al. (2001) shows the importance of the HBD of estrogen receptor and of androgen receptor in the cytoplasmic activation of MAPK and inhibition of apoptosis. They found that truncation mutants of the respective receptors are capable of mediating estrogen or androgen activities in inhibiting apoptosis in osteoblasts and osteocyes, as long as the mutant receptors contain a functional HBD and are being restricted in the cytoplasm (with their nuclear localization signals deleted).
Chapter 3

GIPC Participates in G-protein Signalling Downstream of the IGF-1 Receptor
ABSTRACT

Several recent studies have demonstrated that insulin-like growth factor (IGF) 1-induced mitogen activated protein kinase (MAP kinase) activation is abolished by pertussis toxin, suggesting that trimeric G proteins of Gi class are novel cellular targets of the IGF-1 signalling pathway. We report here that the intracellular domain of the Xenopus IGF-1 receptor is capable of binding to the Xenopus homolog of mammalian GIPC, a PDZ domain-containing protein previously identified as a binding partner of Gi-specific GAP (RGS-GAIP). Binding of xGIPC to xIGF-1 receptor is independent of the receptor's kinase activity and appears to require the PDZ domain of xGIPC. Injection of two C-terminal truncation mutants that retained the PDZ domain blocked IGF-1-induced Xenopus MAP kinase activation and oocyte maturation. While full-length xGIPC injection did not significantly alter insulin response, it greatly enhanced human RGS-GAIP in stimulating insulin response in frog oocytes. This represents the first demonstration that GIPC/RGS-GAIP complex acts positively in IGF-1 receptor signal transduction.
INTRODUCTION

Insulin-like growth factor 1 (IGF-1) exerts its biological roles by activating the intrinsic protein tyrosine kinase activity of the IGF-1 receptor. The activated IGF-1 receptor autophosphorylates its cytoplasmic domain and phosphorylates insulin receptor substrate 1 (IRS-1) and many other protein substrates. Phosphorylation of these protein substrates lead to changes in multiple intracellular signalling pathways including the Ras-Raf- MAP kinase pathway and the phosphatidylinositol 3 kinase/Akt pathway (Cheatham and Kahn, 1995). However, these same signalling pathways are also similarly regulated by insulin/insulin receptor and other growth factors such as epidermal growth factor and platelet-derived growth factor. Clearly, simple tissue- and time-specific expressions of the receptors and/or components of these signalling pathways does not fully explain the distinct biological activities of these various growth factors.

Several recent studies have demonstrated that IGF-1 receptors activate heterotrimeric G proteins in some cell types. Luttrell et al., (1995) first demonstrated that IGF-1-induced MAP kinase activation in Rat-1 fibroblasts was inhibited by either treating cells with pertussis toxin (PTX) or transfecting them with a Gβγ scavenger (β-ARK-CT), suggesting the involvement of a PTX-sensitive G protein (of the Gi class). This study further suggests that the released Gβγ subunits, rather than the activated, GTP-bound Gα subunit, is responsible for IGF-1-induced MAP kinase activation. Similar results have since been obtained using human intestinal smooth muscle cells (Kuemmerle and Murthy, 2001), 3T3-L1 mouse pre-adipose cells (Dalle, et al., 2001) and in rat cerebellar granule neurons (Hallak et al., 2000). These latter studies further demonstrated that the
IGF-1 receptor forms a complex with a G protein α subunit of the Gi class with its associated Gβ subunit (and presumably Gγ subunit) (Dalle, et al., 2001; Hallak et al., 2000). Kueemmerle and Murthy (2001) were able to demonstrate that IGF-1 selectively activated Gai2 but not Gai1, Gai3 or Gαq in the same cells. As heterotrimeric G proteins are known to be associated with, and activated by, heptahelical G-protein coupled receptors, it remains unclear how IGF-1/IGF-1 receptors activate the Gi proteins.

Our lab has been studying insulin mediated oocyte maturation using the yeast 2-hybrid assay. The IGF-1R (Zhu et al., 1998) and xIRS-13,500 (Liu et al., 1995) were used as baits in the 2-hybrid assay in an attempt to isolate potential downstream signalling proteins. A number of clones were identified with xIRS-13,500 (the N-terminal region of xIRS-1 containing the PH, PTB and SAIN domains) as the bait, including Maskin (Stebbins-Boaz et al., 1999) and a novel protein with unknown function. Using the cytoplasmic portion of the xIGF-1 receptor the *Xenopus* homologue of GIPC as an xIGF-1 receptor-binding protein was identified. Evidence supporting a functional role for xGIPC in the regulation of G protein signalling downstream of the xIGF-1 receptor in the induction of oocyte maturation is presented.
MATERIALS AND METHODS

All reagents were supplied by Sigma-Aldrich Co. unless otherwise stated.

Animal and oocyte manipulation

Refer to: Chapter 2 Materials and Methods

Cloning and cDNA manipulation

The nucleotide sequence encoding the cytoplasmic domain (amino acids 958-1358) of the *Xenopus* IGF-1R (Zhu et al., 1998) was PCR-amplified using the following primers: forward primer 5' TATG AAT TCT AAG AAG AGA AAC AGC AAC C; reverse primer 5' TAT GAA TTC ACT GAT ACA GCG GG 3'. The amplified cDNA was digested with *EcoRI*, treated with the Klenow fragment and ligated into the pAS2 (CLONTECH, USA) vector that had been digested with *BamHI* and treated with the Klenow fragment. The resulting clone, designated pAS-xIGF-1Rcyto, expressed a fusion protein between the GAL4 DNA-binding domain and the xIGF-1R cytoplasmic domain. The kinase deficient mutant of xIGF-1R (xIGF-1RKAcyto) in pAS2 was constructed by the two-step PCR procedure (Vallette et al., 1989) using the same forward and reverse primers (above), in combination with the following internal primers changing the catalytically essential Lys(K)-1029 to Ala (A): forward primer=5'-A GTT GCC ATA GCG ACG GTC AAC G; reverse primer=5'-C GTT GAC CGT CGC TAT GGC AAC TTT C). To create xIGF-1Rcyto and xIGF-1RKAcyto for transfection or mRNA
synthesis, the same PCR products were digested with EcoRI and ligated into pCS2+Myc previously treated with EcoRI.

Upon sequence analysis, YA 5-2, the clone identified in the yeast 2-hybrid screen, was found to have its open reading frame or ORF (xGIPC) inserted in a reverse orientation relative to the GAL4 Activation Domain (AD). To clone xGIPC in-frame with the GAL4AD, a 5' PCR primer was designed to amplify the coding region (5'-TAT GAA TTC ATG CCT CTG GGA TTG CGC GTA AAG). This primer, along with a pGAD10 (two-hybrid vector) specific primer, was used to amplify the ORF. The PCR product was digested with EcoRI and ligated into the pGAD10 vector previously digested with EcoRI. To create an xGIPC plasmid for transfection and mRNA synthesis, the same PCR product was digested with EcoRI and treated with Klenow before ligation into pCS2+HA previously digested with XbaI and treated with Klenow.

pCS2+HA was modified from pCS2+ (Turner and Weintraub, 1994) as follows. Two complementary oligonucleotides were made to code for the HA epitope (YPYDVPDYA) with a translation initiation codon and cohesive BamHI ends. The sequences of the two oligos were: forward=5'GAT CCA CCA TGT ACC CAT ACG ATG TTC CAG ATT ACG CTT CCA TG; reverse=5'GAT CCA TGG AAG CGT AAT CTG GAA CAT CGT ATG GGT ACA TGG TG. The oligos were annealed to each other which generated the cohesive ends needed for direct ligation into the pCS2+ vector. The annealed oligos were then ligated with the pCS2+ vector previously digested with BamHI.

Subclones of xGIPC for mapping the binding region of xGIPC and for injection into oocytes were similarly generated by PCR using primers containing an EcoRI site.
xGIPC<sub>N-term</sub> was generated using the forward primer used to generate xGIPC in-frame in the pGAD10 vector in combination with a gene specific reverse primer (reverse=5’ TAT GAA TTC TGA TTT TAA AAC TTC CAC TTC C) producing a protein truncated at amino acid 139. xGIPC<sub>P</sub>DZ was generated using two gene specific primers generating a clone encompassing amino acids 114 to 232 (forward=5’ TAT GAA TTC GGT GGA CAG ATT GGG CTT G, and reverse=5’ TAT GAA TTC TGT TTT ACC ACC TCT TG). The xGIPC<sub>N+P</sub>DZ construct was created using the forward primer from the N-terminal construct in combination with the reverse primer used to make the PDZ construct producing a protein truncated at amino acid 232. The xGIPC<sub>C-term</sub> construct was generated using one gene specific forward primer (forward=5’ TAT GAA TTC CCA TTT ACG TTA AAG TTG ATA G) in combination with the pGAD10 specific primer as above producing a protein encompassing amino acids 206 to 320. The PCR fragments were ligated into either the pGAD10 vector or pCS2+HA vector. The resulting clones generated were fusion proteins with the GAL4AD or an HA tag, respectively. For the sake of brevity, the amino acids comprising the various constructs are indicated in the figures. To reconstitute full-length xGIPC, we PCR-amplified xGIPC<sub>1-320</sub> with a reversed primer encoding 11 extra amino acids (GAIGDAKQGRF) derived from the sequence of the Xenopus EST clone (Blackshear et al., 2001).

Human GAIP coding sequence was PCR-amplified from a human brain cDNA library (a gift of J. K. Ngsee) using the following primers: forward primer=5’TAT GAA TTC A AT GCC CAC CCC GCA TGA GG; reversed primer=5’TAT GAA TTC TGT GCT GCT GGG GGC GGC C. The amplified DNA was digested with EcoRI and inserted at the EcoRI site of pCS2+ or pCS2+MT (Turner and Weintraub, 1994),
resulting in the expression of an untagged or Myc-tagged version of human GAIP. The identity of the sequence was confirmed by DNA sequence analysis.

**Yeast 2-hybrid analysis**

These procedures are essentially the same as those described in the Yeast Protocol Handbook provided by CLONTECH. *Saccharomyces cerevisiae* strain Y190 were grown overnight with shaking at 30 ºC in YPAD (1% bacto-yeast extract, 2% Bactopeptone, 2% dextrose, 0.003% adenine sulphate). Five mL of the overnight culture was then added to 100 mL of fresh YPAD and grown for a further 4 h (or until O.D.₆₀₀ between 0.6 and 0.9 was achieved) with shaking at 30 ºC. The 100 mL culture was then centrifuged at 3000 rcf for 7 min. at 4 ºC. The cell pellet was then washed once with sterile distilled water and re-centrifuged. The water was removed, and on top of the pellet in this order was added: 4.8 mL of 50% polyethyleneglycol (3350), 720 µL 1.0 M lithium acetate, 500 µL fish sperm DNA (sheared, 2 mg/mL), 50 µL (0.5 mg/mL) bait plasmid (pAS-xIGF-1Rcyto), 50 µL (0.5 mg/mL) of a *Xenopus laevis* oocyte cDNA library constructed in the pGAD10 vector (constructed using oligonucleotide(dT) and random priming generating 2.5 x 10⁶ independent clones; CLONTECH, USA). The mixture was vortexed for 1 minute until the pellet was fully resuspended. The transformation mixture was incubated at 30 ºC for 30 min. and then heat-shocked at 42 ºC for 30 min., with gentle shaking. After heat-shocking, the transformation mix was centrifuged for 7 min. at 3000 rcf at 4 ºC. The pellet was gently resuspended in 15 mL of sterile water and 500 µL was spread on 150 mm synthetic dropout medium lacking amino acids His, Leu, Trp, containing 3-amino-1,2,4-triazole [50mM] plates and
incubated at 30 °C, until colonies reached 1-2 mm. β-Galactosidase assays for the
detection of potential positive clones were performed according to the protocols supplied
by CLONTECH. The colonies were lifted from the plates using filterpapers (#413, VWR
Scientific Products, USA) which were then immersed in liquid nitrogen and placed in a
minimal volume of Z buffer/X-gal solution (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM
KCl, 1mM MgSO₄, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal;
United States Biochemical, USA) at 30 °C until the potential positive colonies turned
blue. A colony was considered positive if a blue colour developed within 18 hours.
Potential positive clones were grown in liquid synthetic dropout medium/-Leu and the
pGAD10 library plasmid isolated using the fast plasmid rescue (Dr. B. Wedland). The
liquid cultures were centrifuged at 3000 rcf for 7 min. and the pellet resuspended in 200
μL of rescue buffer (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 10% SDS).
Glass beads (0.45 mm) equal to the amount of the cell pellet were added and the mixture
vortexed for 1 min. The resulting solution was phenol:chloroform:isoamyl alcohol
(25:24:1) extracted and the aqueous phase transferred to a fresh tube containing 25 μL of 3
M sodium acetate, pH 5.2 and 750 μL 95% ethanol. The plasmid DNA was collected by
centrifugation at 18,000 rcf for 15 min. and resuspended in 10 μL distilled water. One
μL of the plasmid DNA was used to transform Escherichia coli strain MH6 by
electroporation. The transformants were plated on M9 medium (11 mM Na₂HPO₄, 22
mM KH₂PO₄, 8.5 mM NaCl, 19 mM NH₄Cl, 1 mM MgSO₄, 100 μM CaCl₂, 2 mg/mL
thiamine, 20 mg/L uracil, 100 μg/mL ampicillin) and incubated at 37 °C for 20h. Five
isolated MH6 colonies were chosen for plasmid prep. The isolated plasmid was used in
cotransformation assays with the original bait, plated on SD/-Leu/-Trp plates. Colonies
were tested for filter-based β-galactosidase activity. Yeast protein extraction for immunoblotting was carried out using the urea/SDS method supplied by CLONTECH.

**Polyclonal antibodies against xGIPC**

An internal HindIII fragment of xGIPC, encoding amino acids 216-305, was excised and inserted into pGEX-KT (Guan and Dixon, 1991). GST fusion protein were induced and purified by binding to glutathione-agarose beads. Immunization of rabbits was carried out according to Harlow and Lane (Harlow and Lane, 1988). Immune sera were used without further processing.

**Co-immunoprecipitation experiments**

COS-7 cells were maintained in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco-Life Technologies, Inc., USA), 50 U/mL penicillin-streptomycin and 2ml/L Fungizone Gibco-Life Technologies, Inc., USA) at 37 °C in a humidified atmosphere containing 5% CO₂.

Transient transfection of COS-7 cells was accomplished using LipofectAMINE (Gibco-Life Technologies, Inc., USA). Briefly, cells in a 6-well dish at 70% confluence were washed once with PBS and incubated with 1 mL of OPTI-MEM (Gibco-Life Technologies, Inc., USA) media containing 5 μL of LipofectAMINE reagent (Gibco-Life Technologies, Inc., USA), and 0.25 μg of each plasmid for 18 h. Following the transfection, the media was changed to DMEM-10% FCS with antibiotics. The cells were allowed to grow for a further 24-30 h and lysed for co-immunoprecipitation or kinase assays.
Transiently transfected COS-7 cells were washed with PBS and then lysed with 200 μL of ice-cold PBS-Lysis buffer (Farah et al., 1998) (10 mM phosphate buffer, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 10 μg/mL Leupeptin, 100 μM sodium orthovanadate). The lysate was cleared by a 10 min centrifugation at 15,000 rcf. One hundred and fifty microlitres of the cleared lysate was added to 450 μL of PBS-Lysis buffer containing 10 μL of anti-Myc or anti-HA sepharose beads (Sheng et al., 2001). Lysates were incubated at 4 °C with rocking for 2 h. Immunocomplexes were washed 5 times with PBS-Lysis buffer, after washing, 20 μL of 2X Laemli sample buffer was added and the samples separated using SDS-PAGE. The proteins were transferred to a nitrocellulose membrane for western blotting.

Co-immunoprecipitation experiments using frog oocyte extracts were performed essentially the same way. Oocytes were injected with the various mRNA and incubated overnight. Extracts were prepared in PBS lysis buffer (10 μL per oocyte) and subjected to immunoprecipitation (200-400 μL extract with 5 μL of either pre-immune serum or anti-xGIPC immune serum).

**Protein kinase assays**

Protein kinase assays were performed on immunoprecipitated xIGF-1Rcyto or xIGF-1RKAcyto. Briefly, immunoprecipitation procedures were carried out as above with the exception that the last wash step was done with kinase buffer (50 mM Hepes, pH 6.9, 1 mM EDTA, 0.1% Triton X-100). After the final wash, 23 μL of kinase buffer was added and the reaction was started with the addition of 1 μCi [\(^{32}\)P]γ-ATP (10 mCi/mL > 3000 Ci/mmol; Amersham, USA), 25 μM ATP and 10 MgCl₂ and 2 mM MnCl₂. Kinase
reactions were carried out at room temperature for 30 min. and were stopped with the addition of 2X Lamelli sample buffer.

MPF extracts were prepared and assayed according to Nebreda and Hunt (1993). Briefly, oocytes were crushed in extraction buffer (20 mM Hepes pH7.3, 80 mM glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1.5 µL of 10 µM ATP, 0.15 mM NaF, 10 µg/mL leupeptin, 200 µM PMSF, 25 µg/mL benzamadine; 20 µL/oocyte). Following centrifugation, 8 µL of lysates were used in a kinase reaction (total volume of 12 µL) with the addition of 2 µg of histone H1, 5 µCi [³²P]γ-ATP (10 mCi/mL > 3000 Ci/mmol; Amersham, USA) and 33 µM ATP. Kinase reactions were carried out at room temperature for 20 min. before the addition of 12 µL of 2X Lamelli sample buffer. Proteins were separated on a 10% SDS-PAGE, dried and visualized by autoradiography.
RESULTS

Yeast 2-hybrid screening using xIRS-1 as bait

My main thesis project was to utilize yeast 2-hybrid screening to identify proteins involved in insulin-induced oocyte maturation. The first bait used was the N-terminus of *Xenopus* insulin receptor substrate-1 (xIRS-1\(_{13,500}\)), previously generated by S. Farah (Farah *et al.*, 1998). Following screening 1.34 X 10\(^6\) colonies, a total of 124 potential positive clones were obtained. Of these, 6 were determined to be true positive as indicated by their ability to induce β-galactosidase activity upon re-transformation of the yeast with the bait, but not with a control plasmid. Four of these corresponded to the same gene, tentatively termed D-19, of unknown function (based on database searches which revealed numerous EST clones, the closest match being accession number AW646322, in *Xenopus* and other species but no defined functions) (Figure 3.1). The missing 5' end of the coding sequence was amplified by anchored PCR using pGAD10 specific primer. To examine the possible involvement of D-19 in insulin signalling, we generated a Myc-D-19 construct. Messenger RNA was synthesized and injected into oocytes. However, no specific interaction between Myc-D19 and xIRS-1 (Liu *et al.*, 1995) was detected (data not shown). Furthermore, over-expression of Myc-D-19 had no effect on insulin-induced GVBD (data not shown). Therefore we did not continue pursuing this project.
Figure 3.1
Sequence and hypothetical translation of the novel gene termed D-19. The underlined sequence was identified by 5' anchored PCR and was not present in the original yeast 2-hybrid clone. Numbers indicate amino acid position.
tagtccacagaagggcacagatgaggaaggttaccagacgcgtgtgagactacaggtgattctg
MKRVTSAVKTTVIL
aatatagagattatgaggagtgttatgtcctcaagacgagatagtgatatccatacagag
NIEIIMRSVMSKSGEVVFHTE
atgaccacagaatataatagggccagaggtgagccctctacagagccgacaacttgagcagcacagag
MTQNIIDGEVSSLRLADNLSTE
aaagcaagatggctacagagaaagaagccatactacccagcctgctcgctcagagacgccgt
KAKMATGKRHTTSSLSPETACA
acccgcaagagagacaccctttcttttggcgagctctctctctgctaatccgacagatatttc
HRKEETLPLLSSLSDLRIL
acacatagtagatgacgctccagttgtagacgcagaaaggtactacaccagacagagggaa
TSHRSGGGVSSRSYSRPDRGKK
ctctaccctctccccacacacagaggaagagagacggtacagctccccctccagca
SYPSHQQHRSSKERSSTTHPPA
gtatccccgcaagttgtctgcaattccttacctacaaagggccagatagatgaaaagtv
VSPITSSAAAPPTKGAEALS
rgccagacttgtctgcaccaatatttattggagctgtttgaattggttgtggctgagaagaatg
CRLSDPIFMEAACKWVAPR
agggagttcagacgaatgcaattgcagatgtacagagacagcatgtggtgatctctctct
EKVTECKLQDGTTEEHAGSSS
kacgtgcagttcagagatagttcagtggcaaatgtttctgggtattcttagatgc
ALHGDQIAELEANVSGNSEF
ctatgatgcaaatcccacatccagcgggccctgaagcaacttggagacgaaagaagacacacaagaatc
HDANPHTSRSRAIAAKTKEI
gaagaggttatagcgagactgtgagagatgtgctagtttagagatgtaataatagat
EVEYRQDCETFGMVVKMLID
aggaccccgcaatgtgaanaaaacattcagtttgcgttgagcagacatttaagcagagtc
KDPALQIFQFALRQNLSEI
gggaaagatgtcattgaagacgttaaatgtattatatcagactatagtggtgctgcacaccag
GERCIESLKMITYDAAHQ
gaatggataggtaggtgacctacagagacacatatttttcagatttaattaagattttcct
EElV-
The two remaining positive clones contained an open reading of 729 nucleotides encoding the C-terminal 243 amino acids of the protein maskin (Stebbins-Boaz et al., 1999). Maskin is involved in oocyte maturation by maintaining the Mos mRNA in an un-translatable “masked” conformation. Progesterone stimulation causes the activation of Eg2 (Andresson and Ruderman, 1998), which in turn phosphorylates CPEB (Mendez et al., 2000a) and dissociates CPEB from Maskin, hence “unmasking” the mRNA and allowing translation (Mendez et al., 2000b). Insulin induced oocyte maturation, like progesterone, requires the translation of Mos mRNA. The investigation of the interaction between xIRS-1 and maskin may provide additional information about the link between activation of the xIGF-1 receptor and translation of Mos, however, due to time constraints this project was not pursued.

**xGIPC interacts with the cytoplasmic domain of xIGF-1 receptor in yeast**

The cytoplasmic domain of xIGF-1 receptor (Vallette et al., 1989) was inserted into the bait vector, pAS2. The resultant plasmid (pAS-xIGF-1Rcyto) encodes an HA epitope tag followed by the DNA-binding domain of GAL4 and then by the xIGF-1 receptor cytoplasmic domain. A similar bait (pAS-xIGF-1RKAcyto) with a single point mutation changing the catalytically essential lysine-1029 (K) to alanine (A) was also constructed. Transforming yeast with each plasmid resulted in expression of the fusion protein with the anticipated size, as indicated by both anti-xIGF-1 receptor blots and anti-HA blots (Figure 3.2). As expected, pAS-xIGF-1Rcyto was phosphorylated on tyrosine due to autophosphorylation whereas phosphorylation of pAS-xIGF-1RKAcyto was undetectable.
Using pAS-xIGF-1Rcyto as bait, an oocyte cDNA library constructed in the pGAD10 vector (encoding the GAL4 activating domain or GAL4AD) (by CLONTECH) was screened. After screening a total of $1.1 \times 10^6$ colonies, 74 potential positive clones were identified. Of these potential positives, 3 true positive clones resulted. All three were identical in sequence and cloning site, suggesting they originated from a single cDNA-vector ligation event. These clones, collectively entitled YA5-2, contained an open reading frame (ORF) homologous to a mammalian gene, GIPC, previously identified as a binding partner for a Gi-specific GTPase activating protein (RGS-GAIP or GAIP) (De Vries et al., 1998). The literature suggested that GIPC might provide a functional link between IGF-1 receptor and Gi protein signalling. However, the ORF of 320 amino acids, which included a candidate translation start site but lacked a translation termination codon, was inserted in pGAD10 with a reverse orientation. Therefore xGIPC could not have been made as part of a GAL4AD fusion protein. This clone was therefore initially discarded as "false positive" until a search of the literature provided the information that the ADH1 termination sequences actually contained a functional promoter sequences capable of initiating transcription opposite to the ADH promoter in pGAD10 (Chien et al., 1991). Consequently, xGIPC could have been expressed in yeast from the promoter contained within the ADH1 termination sequences as a non-fusion protein.

The cloned xGIPC was sequenced from both directions and the sequences assembled and compared with GIPC from several other species (Figure 3.3). From these comparisons, it seemed that YA 5-2 only contained a partial xGIPC coding sequence, lacking the extreme C-terminus of perhaps as few as 11 amino acids. Repeated attempts
Figure 3.2
xIGF-1Rcyto is active in yeast. Extracts from control (untransformed) yeast or yeast transformed with pAS-xIGF-1Rcyto or with pAS-xIGF-1RKAcyto were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Expression of the pAS-xIGF-1Rcyto and pAS-xIGF-1RKAcyto fusion proteins is shown by anti-xIGF-1R and anti-HA antibodies respectively. Arrows indicate GAL4 fusion proteins containing xIGF-1Rcyto or xIGF-1RKAcyto. To monitor the phosphorylation state of the fusion proteins, anti-phosphotyrosine antibodies were used. anti-pY=anti phosphotyrosine.
Figure 3.3
Sequence comparison between mouse, rat, human and *Xenopus* GIPC. GIPC amino acid sequences from the various species were aligned using the web-available CLUSTAL W Multiple Sequence Alignment Program. The stars (*) indicate matches whereas the colon (:) and period (.) indicate strong and weaker conservative changes respectively. The PDZ domain is underlined. Numbers indicate positions of the amino acids in xGIPC. The C-terminal 11 amino acids (bold-face) were derived from a *Xenopus* EST clone (AW646188).
Mouse_GIPC
  MPLGLGRRKAPPLVENEEAEPREPPQGLLPPQGGGAGQSMGLPPFPASLPRPLVF
Rat_GIPC
  MPLGLGRRKAPPLVENEEAEPREPPQGLLPPQGGGAGQSMGLPPFPASLPRPLVF
Human_GIPC
  MPLGLGRRKAPPLVENEEAEPREPPQGLLPPQGGGAGQSMGLPPFPASLPRPLVF
Xenopus_GIPC
  MPLGLGRRKAPPLVENEEAEPREPPQGLLPPQGGGAGQSMGLPPFPASLPRPLVF

Mouse_GIPC
  HTQLAHGSPTRIGFTNVEVYKIAAEAFRLPAAEMVFTLNLHVKVRDMLKLLGQIGLE
Rat_GIPC
  HTQLAHGSPTRIGFTNVEVYKIAAEAFRLPAAEMVFTLNLHVKVRDMLKLLGQIGLE
Human_GIPC
  HTQLAHGSPTRIGFTNVEVYKIAAEAFRLPAAEMVFTLNLHVKVRDMLKLLGQIGLE
Xenopus_GIPC
  HTQLAHGSPTRIGFTNVEVYKIAAEAFRLPAAEMVFTLNLHVKVRDMLKLLGQIGLE

Mouse_GIPC
  DFIDAHVKGQREVEFVFSKEdALGLTIDNAGAYAFIKRIKEXGISVIDHILISVGDMIAE
Rat_GIPC
  DFIDAHVKGQREVEFVFSKEdALGLTIDNAGAYAFIKRIKEXGISVIDHILISVGDMIAE
Human_GIPC
  DFIDAHVKGQREVEFVFSKEdALGLTIDNAGAYAFIKRIKEXGISVIDHILISVGDMIAE
Xenopus_GIPC
  DFIDAHVKGQREVEFVFSKEdALGLTIDNAGAYAFIKRIKEXGISVIDHILISVGDMIAE

Mouse_GIPC
  INGQSLGCRHYEVARLKLKPRCTFTLLKTLEPRKAFDMISQRSAGQVHPSGPQGLTGR
Rat_GIPC
  INGQSLGCRHYEVARLKLKPRCTFTLLKTLEPRKAFDMISQRSAGQVHPSGPQGLTGR
Human_GIPC
  INGQSLGCRHYEVARLKLKPRCTFTLLKTLEPRKAFDMISQRSAGQVHPSGPQGLTGR
Xenopus_GIPC
  INGQSLGCRHYEVARLKLKPRCTFTLLKTLEPRKAFDMISQRSAGQVHPSGPQGLTGR

Mouse_GIPC
  GTLRLRSRPATVDELPSAFEKAIKEDDLLLESYMGRDELAATVESLGVKDKRNPDEL
Rat_GIPC
  GTLRLRSRPATVDELPSAFEKAIKEDDLLLESYMGRDELAATVESLGVKDKRNPDEL
Human_GIPC
  GTLRLRSRPATVDELPSAFEKAIKEDDLLLESYMGRDELAATVESLGVKDKRNPDEL
Xenopus_GIPC
  GTLRLRSRPATVDELPSAFEKAIKEDDLLLESYMGRDELAATVESLGVKDKRNPDEL

Mouse_GIPC
  AEALDEKLGDPFEDFVFDVWGAIGDAKVGRY-
Rat_GIPC
  AEALDEKLGDPFEDFVFDVWGAIGDAKVGRY-
Human_GIPC
  AEALDEKLGDPFEDFVFDVWGAIGDAKVGRY-
Xenopus_GIPC
  AEALDEKLGDPFEDFVFDVWGAIGDAKVGRY-(331)

* **** ****************************
Figure 3.4
Interaction of xGIPC with xIGF-1Rcyto in the yeast 2-hybrid assay. (A) Schematic representations of the various xGIPC clones described in this report, GAL4AD, GAL4 activation domain. Numbers indicate positions of amino acids in xGIPC. Arrows indicate direction of the xGIPC ORF. 5'UTR=5' untranslated region. The EST clone-derived C-terminal 11 amino acids are shown in single amino acid codes. (B) Summary of yeast two-hybrid assays using the colony-lift method. A “+” indicates that the colonies turned dark blue following overnight incubation with the substrate. A “-“ indicates that colonies remained pink/white at least 24 hours after the addition of substrate.
to identify the C-terminus using a xGIPC specific primer and an anchor primer specific for the pGAD10 yeast 2-hybrid vector containing the cDNA library, as well as other oocyte cDNA libraries (C. Ma unpublished results; Bayaa et al., 2000) did not generate further 3' sequence. Freshly isolated oocyte RNA was also used in 3' RACE (rapid amplification of cDNA ends) with no avail.

Further yeast two-hybrid assays were carried out to confirm that xGIPC was indeed capable of interacting with xIGF-1Rcyto, and attempted to define the region of xGIPC responsible for the interaction. xGIPC$_{1-320}$, the PDZ domain, or the arbitrarily defined N- or C-terminus (Figure 3.4A) were PCR-amplified and inserted into pGAD10. Cotransformation of each plasmid with xIGF-1Rcyto, xIGF-1RKAcyto or a control plasmid xIRS-1 (3-500) (Farah et al., 1998) was carried out. The GAL4AD-xGIPC$_{1-320}$, like YA 5-2, was shown to interact strongly with xIGF-1Rcyto. Both YA 5-2 and GAL4AD-xGIPC$_{1-320}$ also interacted strongly with the kinase deficient mutant, xIGF-1RKAcyto. Neither was able to interact with xIRS-1$_{3-500}$ (Farah et al., 1998), cloned in the same vector or the vector alone (not shown). A deletion mutant (xGIPC$_{N-PDZ}$) missing the C-terminus behaved very similarly to YA 5-2 or xGIPC$_{1-320}$ (Figure 3.4B). However, none of three regions of xGIPC (the N-terminus, the PDZ domain or the C-terminus), when expressed separately with GAL4AD, were able to interact with xIGF-1Rcyto or xIGF-1RKAcyto (Figure 3.4B).

**xGIPC co-immunoprecipitates with xIGF-1 receptor**

Co-immunoprecipitation of endogenous xIGF-1 receptor and xGIPC were attempted in *Xenopus* oocytes, however the anti-xIGF-1 receptor antibody did not
Figure 3.5
Binding of xIGF-1Rcyto to xGIPC1-320 in COS-7 cells. (A) Extracts from COS-7 cells, mock transfected or transfected with either Myc-xIGF-1Rcyto or Myc-xIGF-1RKAcyto. After 48 hours the cells were lysed and immunoprecipitated with anti-Myc sepharose beads. The immunoprecipitates were subjected to in vitro kinase assays, separated by SDS-PAGE and visualized by autoradiography (upper panel). The intense band in the Myc-xIGF-1Rcyto lane indicates autophosphorylation of the xIGF-1Rcyto, while the lack of a band in the Myc-xIGF-1RKAcyto indicates the lack of kinase activity of the receptor. Total cell extracts were also analysed by immunoblotting using anti-Myc antibodies in the lower panel, demonstrating expression of both the kinase active and kinase dead receptor forms. (B) Total cell extracts (lanes 1-4) of COS-7 cells transfected with HA-xGIPC, Myc-xIGF-1Rcyto or Myc-xIGF-1RKAcyto, or anti-HA immunoprecipitates (lanes 5-8) from the variously transfected COS-7 cells were analysed by immunoblotting using anti-Myc (upper panel) or anti-HA (lower panel) antibodies. The presence of Myc-xIGF-1Rcyto and Myc-xIGF-1RKAcyto in the HA immunoprecipitates demonstrates the association of xGIPC with both the kinase active and kinase dead forms of the xIGF-1Rcyto.
A

Anti-myc I.P.

<table>
<thead>
<tr>
<th>Myc-xIGF-1R&lt;sub&gt;cyto&lt;/sub&gt;</th>
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<tr>
<td>Myc-xIGF-1RKA&lt;sub&gt;cyto&lt;/sub&gt;</td>
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Kinase Assay

|xIGF-1R<sub>cyto</sub>|

Anti-Myc blot

|xIGF-1R<sub>cyto</sub>|

Total Lysate

B

<table>
<thead>
<tr>
<th>Total Lysate</th>
<th>HA I.P.</th>
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<tr>
<td>- - + +</td>
<td>- - + + HA-xGIPC</td>
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<td>- + + -</td>
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Anti-Myc blot

Anti-HA blot

1 2 3 4 5 6 7 8

105
immunoprecipitate the xIGF-1 receptor, and did not recognize a specific band after anti-xGIPC immunoprecipitation. The lack of immunoprecipitation is likely due to the inability of the anti-xIGF-1 receptor antibodies to recognize non-denatured xIGF-1 receptor. The reason for the lack of a specific band after anti-xGIPC immunoprecipitation is unknown. Therefore, binding experiments in transiently transfected COS-7 cells were used to demonstrate an interaction between xIGF-1 receptor and xGIPC. To facilitate these experiments, xIGF-1Rcyto or xIGF-1RKAcyto were subcloned into pCS2+Myc (Blackshear et al., 2001) and xGIPC, and its truncation mutants (Figure 3.4A) into pCS2+HA (HA=hemeaglutinin epitope tag). Transient transfection of Myc-xIGF-1Rcyto, or Myc-xIGF-1RKAcyto, resulted in similar levels of the two proteins, recognized by anti-Myc (Figure 3.5A, lower panel). Immunoprecipitation followed by in vitro kinase assay confirmed that Myc-xIGF-1RKAcyto was indeed deficient in catalytic activity (Figure 3.5A). Co-transfection of each plasmid with HA-xGIPC followed by co-immunoprecipitation experiments clearly indicated that xGIPC was able to bind both the kinase active and kinase deficient cytoplasmic domain of the xIGF-1 receptor (Figure 3.5B, lanes 7 and 8). Lanes 5 and 6 represent HA immunoprecipitation from extracts derived from cells expressing only the Myc-tagged proteins, indicating that the co-immunoprecipitation observed in lanes 7 and 8 were not due to the over-expressed Myc-fusion proteins being "pulled down" by anti-HA antibodies nonspecifically.
Over-expression of xGIPC C-terminal truncations inhibit oocyte maturation

Does over-expression of xGIPC affect IGF-1 receptor signalling in oocytes? To answer this question, 15 nL (approximately 1 μg/μL) of mRNA encoding GFP (Ohan et al., 2000) (used as a control), HA-xGIPC_{1-320} or HA-xGIPC_{PDZ} were injected into freshly defolliculated immature oocytes. Following overnight incubation, to allow translation and accumulation of the corresponding proteins, oocytes were incubated with insulin (200 nM or 500 nM). It has previously been shown that these concentrations of insulin activate the endogenous xIGF-1 receptor (Cummings et al., 1996; Zhu et al., 1998), and ultimately cause oocyte maturation, indicated by, among other criteria, xMAP kinase activation and GVBD. Figure 3.6A shows that injection of the PDZ domain mRNA had no significant effect (compared to similar amount of GFP mRNA or water) on insulin-induced GVBD. In contrast, injection of xGIPC_{1-320} mRNA severely reduced the ability of insulin to induce GVBD. Figure 3.6B shows a typical experiment when we analysed xMAP kinase phosphorylation (indicative of activation (Ohan et al., 1999; Posada et al., 1993)) following scoring oocytes for GVBD (Figure 3.6A). Again, xGIPC_{1-320}, but not the PDZ domain in isolation, significantly reduced the ability of insulin to activate xMAP kinase phosphorylation (activation). Immunoblotting using anti-HA antibodies demonstrated that both xGIPC_{1-320} and HA-xGIPC_{PDZ} were expressed and accumulated in oocytes (Figure 3.6A, inset). Similarly, injection of mRNA encoding either the N-terminus or the C-terminus of xGIPC did not significantly change insulin-induced xMAP kinase phosphorylation (Figure 3.7A) or GVBD (Figure 3.7B).
Figure 3.6
xGIPC \textsubscript{1-320} blocks insulin-induced oocyte maturation. (A) Oocytes were injected with mRNA for GFP (as a control), xGIPC \textsubscript{1-320} or xGIPC \textsubscript{PDZ}. After 8 h of incubation the oocytes were stimulated with 0.2 μM insulin. Oocytes were scored for GVBD following an overnight incubation with insulin. Shown are the percent of oocytes which underwent GVBD with the total number of oocytes indicated above each bar. Inset shows an anti-HA immunoblot indicating protein expression in mRNA-injected oocytes. (B) Oocytes were treated similarly as in A with the exception that two different concentrations of insulin were used. Following an overnight incubation with insulin, extracts were prepared and subjected to immunoblotting with anti-xMAP kinase antibodies. The upper band and the lower band represent phosphorylated (or active) and unphosphorylated (or inactive) *Xenopus* MAP kinase.
Figure 3.7
The N-terminus and C-terminus of xGIPC do not affect insulin signalling. (A) Groups of 25-30 oocytes were injected with water, or mRNA encoding the xGIPC<sub>N-term</sub> or the xGIPC<sub>C-term</sub>. After 8 h of incubation, the oocytes were then stimulated with 0.2 μM insulin and incubated overnight. Shown are the percent of oocytes that underwent GVBD after overnight stimulation. (B) After scoring GVBD the groups of oocytes were lysed and analysed by immunoblotting with anti-xMAP kinase antibodies.
Figure 3.8
xGIPC\textsubscript{N-PDZ} blocked insulin-induced oocyte maturation (A) Oocytes were injected with water (control) or mRNA for xGIPC\textsubscript{N-PDZ} or full-length xGIPC. After 8 h of incubation the oocytes were stimulated with 0.2 μM insulin. Oocytes were scored for GVBD following an overnight incubation with insulin. Shown are the means of 2 experiments with the total number of oocytes indicated above each bar. Inset shows an anti-HA immunoblot indicating expression of the corresponding protein in mRNA-injected oocytes. (B) Oocytes injected with water or mRNA were incubated with the indicated concentrations of insulin for 9 hours. MPF extracts were prepared and subjected to in vitro kinase assays using histone H1 as a substrate. (C) The same extracts from B were immunoblotted with anti-xMAP kinase antibodies.
A recently deposited *Xenopus* EST clone (AW646188) (Blackshear *et al*., 2001) apparently contains the missing 3' end of our xGIPC sequence. This clone contains 240 base pairs of coding sequence that is identical to the corresponding region of xGIPC (with the exception, of course, of the 11 extra codons that are missing from our clone). Full-length xGIPC was therefore generated by PCR amplification of xGIPC\(_{1-320}\) with a 3' primer that contained 11 extra codons (GAIGDAKQGRF). Surprisingly, the full-length xGIPC, unlike xGIPC\(_{1-320}\), did not inhibit insulin-induced oocyte maturation, assayed by three different criteria: GVBD (Figure 3.8A), MPF activation (Figure 3.8B) and xMAP kinase phosphorylation (Figure 3.8C). In contrast a C-terminal deletion mutant (xGIPC\(_N\)\(_{PDZ}\)), which was capable of binding xIGF-1 receptor cytoplasmic domain in the yeast assays (Figure 3.4B), significantly reduced insulin-induced oocyte maturation. Immunoblotting indicated that xGIPC and xGIPC\(_N\)\(_{PDZ}\) were expressed at similar levels in the respectively injected oocytes (Figure 3.8A, inset). The inhibitory effect of xGIPC\(_N\)\(_{PDZ}\) was reminiscent of that of xGIPC\(_{1-320}\) (Figures 3.6A and B).

**xGIPC and hGAIP over-expression synergise to potentiate oocyte maturation**

To further investigate the mechanism of xGIPC in insulin signalling, the possible functional interaction between xGIPC and GAIP in *Xenopus* oocytes was explored. Human GAIP (hGAIP) cDNA was PCR-amplified and expressed with or without an N-terminal Myc-tag. The preliminary injection experiments indicated that hGAIP significantly accelerated insulin-induced GVBD (not shown). Therefore, a concentration of insulin (50 nM), which was not sufficient to induce oocyte maturation in most batches of *Xenopus* oocytes, was used. Under these conditions, injection of hGAIP mRNA
**Figure 3.9**
xGIPC synergized with hGAIP in stimulating insulin response in frog. (A) Oocytes were injected with water (control) or the indicated mRNA. After 8 h of incubation the oocytes were stimulated with 0.05 μM insulin. Oocytes were scored for GVBD following an overnight incubation with insulin. Shown are means of three independent experiments with the total number of oocytes indicated above each bar. (B) Following GVBD scoring, MPF extracts were prepared and subjected to in vitro kinase assays using histone H1 as a substrate (upper panel) or immunoblotted with anti-xMAP kinas antibodies (lower panel). Shown is a representative of three independent experiments. (C) Oocytes were first injected with mRNA for Myc-hGAIP followed by injection with mRNA for the indicated xGIPC forms. Following an overnight incubation, extracts were prepared and immunoprecipitated (IP) with either pre-immune serum or anti-xGIPC serum. The IPs were immunoblotted with anti-Myc antibodies (upper panel). The blots were re-probed with anti-HA antibodies (lower panel) following stripping of anti-Myc-antibodies Shown is a representative of two independent experiments.
(either untagged or Myc-tagged) resulted in oocyte maturation in a significant percentage of oocytes (Figures 3.9A and B). Although xGIPC alone had little effect, it greatly enhanced the ability of hGAIP in stimulating insulin-induced oocyte maturation (Figures 3.9A and B). To confirm that xGIPC bound hGAIP in *Xenopus* oocytes, co-immunoprecipitation experiments were performed. Oocytes were injected with Myc-hGAIP, alone or together with HA-xGIPC, HA-xGIPC1-320 or HA-xGIPC-N-PDZ. Immunoprecipitation with antibodies against xGIPC pulled down both endogenous xGIPC (not shown) and all three forms of mRNA-derived xGIPC (Figure 3.9C, lower panel). Co-immunoprecipitation of Myc-hGAIP with the endogenous xGIPC was evident (Figure 3.9C, lane 5, compared to lane 1, which represents pre-immune control). The amounts of Myc-hGAIP co-precipitated in xGIPC IPs increased significantly in all three groups of oocytes which had been injected with the various xGIPC mRNA (Figure 3.9C, lanes 6-8, as compared to lane 5). These results clearly indicated the ability of all three forms of xGIPC to bind Myc-hGAIP. As would be expected, non-specific binding of Myc-GAIP in pre-immune IPs (Figure 3.9C, lanes 1-4) did not change regardless whether oocytes had received xGIPC mRNA injection.
DISCUSSION

The original xGIPC\textsubscript{1-320} clone (YA 5-2) isolated in the yeast two-hybrid screen was inserted in the pGAD10 vector with a reverse orientation relative to that of the GAL4AD. This unusual result meant that xGIPC\textsubscript{1-320} was not made as a GAL4AD fusion protein or driven by the ADH promoter. Instead, it was transcribed from an intrinsic promoter lying within the ADH1 termination sequences (Chien et al., 1991) and translated as a non-fusion protein. Under this circumstance, xGIPC\textsubscript{1-320} should bind the bait (GAL4DBD-xIGF-1Rcyto) but should also function as a "transcription activator" capable of replacing the GAL4AD in activating the Gal1-driven reporters in the two-hybrid assay. This is not surprising, given that more than 1\% of random bacterial genomic fragments demonstrate "transcription activator" function when fused in frame with the yeast GAL4 DNA binding domain (Ma and Ptashne, 1987). The common feature of these "transcriptional activators" is a relative abundance of acidic amino acids (Ma and Ptashne, 1987). Indeed, xGIPC\textsubscript{1-320} is an acidic protein (overall pI of 6.16) with a particularly acidic C-terminus (pI of 4.71 over the last 114 amino acids). A second possibility was for xGIPC\textsubscript{1-320} to simultaneously bind the bait and a GAL4AD fusion protein derived from the anti-sense direction of xGIPC\textsubscript{1-320}.

A recent study (Lou et al., 2001) indicates that the PDZ domain of mammalian GIPC is sufficient to mediate interaction to TrkA (receptor for nerve growth factor). Lou et al. (2001) have also defined the juxtamembrane domain of TrkA as the likely docking site for the GIPC PDZ domain. Limited, but noticeable, sequence homology was found between the juxtamembrane domains of TrkA and xIGF-1 receptor (not shown). We
have not yet determined whether this region of the xIGF-1 receptor is similarly required for interacting with xGIPC. Our data also indicate that both xGIPC<sub>1-320</sub> and xGIPC<sub>N-PDZ</sub> are capable of binding to xIGF-1 receptor, consistent with the notion that the PDZ domain is also involved in binding the intracellular domain of xIGF-1 receptor. However, the PDZ domain alone did not suffice to bind xIGF-1 receptor, either in the yeast two-hybrid assays (Figure 3.4B) or in COS-7 cells (not shown). The fact that the PDZ domain alone did not bind the xIGF-1R may suggest that although the PDZ domain is necessary for the interaction, sequence N-terminal to the PDZ domain are also necessary for a stable interaction. Further studies will be required to clarify the nature of this apparent difference, possibly by narrowing the area that is involved in the binding using further deletion mutants.

Clearly the most interesting implication lies in the possible link between IGF-1 receptor signalling and G protein functions during oocyte maturation. High levels of cAMP are thought to be responsible for maintaining oocyte meiotic arrest (Smith, 1989). Progesterone, the natural maturation-inducing ovarian hormone, binds to the cytoplasmic PR (xPR) (Chapter 2; Bayaa et al., 2000; Tian et al., 2001) and triggers a reduction of cAMP by inhibiting oocyte AC (Sadler and Maller, 1981; Finidori-Lepicard et al., 1981). Insulin and IGF-1 can also induce maturation in vitro (El-Etr et al., 1979), by activating the endogenous IGF-1 receptor (Zhu et al., 1998). Forskolin, a potent AC activator, similarly blocks both progesterone-induced and insulin-induced oocyte maturation (Schorderet-Slatkine and Baulieu, 1982; Schorderet-Slatkine and Schorderet, 1984), suggesting that a reduction of cAMP is a necessary event in both cases. It has been reported that insulin is able to stimulate oocyte cAMP phosphodiesterase activity (Sadler
and Maller, 1987). A recent study has provided a possible link between the insulin/IGF-1 receptor and the activation of oocyte phosphodiesterase. Andersen et al. (1998) reported that injection of PKB/Akt results in activation of cAMP phosphodiesterase (PDE3) and induction of oocyte maturation. PKB/Akt is a serine/threonine kinase activated downstream of phosphatidylinositol 3-kinase (PI3-kinase). Our lab (Liu et al., 1995) and others (Deuter-Reinhard et al., 1997) have previously shown that insulin/IGF-1 receptor activates PI 3-kinase in Xenopus oocytes. Therefore, one signalling pathway may consist of IGF-1 receptor-activated PI 3-kinase-PKB/Akt, which, directly or indirectly activates oocyte PDE3 (Anderson et al., 1998) and ultimately results in the reduction of oocyte cAMP.

The model put forward is, following ligand binding; xIGF-1 receptor activates an oocyte Gi, in addition to the PI 3-kinase/Akt/PDE3 pathway. The co-operation of the two signalling pathways ensures the required cAMP reduction by inhibiting oocyte AC (via Gαi-GTP) and activates oocyte PDE3 (via PKB/Akt). The function of xGIPC is likely to promote GAIP-mediated Gαi-GTP hydrolysis that timely terminates the G protein signal and recycles the resulting Gαi-GDP for another round of G protein activation. It is interesting to note that several groups have recently demonstrated that the IGF-1 receptor forms a complex with trimeric Gαi proteins (Dalle et al., 2001; Hallak et al., 2000). Whether the interaction between IGF-1 receptor and Gαi is direct or mediated by other proteins, it is conceivable that xGIPC could function to link GAIP to the receptor complex. A receptor complex containing both the signal initiator (IGF-1 receptor) and signal terminator (GAIP) may be a unique feature to trimeric G protein signalling downstream of receptor protein tyrosine kinases (such as IGF-1 receptor and TrkA). The
two C-terminally truncated xGIPC mutants, xGIPC_{N-PDZ} or xGIPC_{1-320}, may act in a
dominant-negative fashion over the endogenous xGIPC. The fact that both behaved
similarly to full-length xGIPC in binding xIGF-1R (Figure 3.4B) and hGAIP (Figure
3.9C) would seem to rule out the simple explanation that the mutants fail to link GAIP to
the IGF-1 receptor. However, the reasons for the apparently dominant-negative effect of
these mutants therefore remain unknown. One possible explanation is that xGIPC needs
to interact with another protein and that this interaction is mediated by the C-terminus of
xGIPC.

How might the complex of xGIPC and RGS-GAIP stimulate IGF-1 receptor
signalling in frog oocytes? Two non-mutually exclusive possibilities are considered here.
First, over-expression of xGIPC and hGAIP may increase the IGF-1 receptor-associated
GTPase activity of hGAIP towards endogenous Gi-GTP, hence accelerate recycling of
Gi-GDP for further rounds of activation by the IGF-1 receptor. This clearly would have
the net effect of increased Gi activity, resulting in greater inhibition of oocyte AC and
reduction of cAMP. Second, the complex of xGIPC and hGAIP may function
downstream of xIGF-1 receptor, independently of Gi, to activate a maturation
promoting signalling pathway.
Chapter 4

Concluding Remarks
CONCLUDING REMARKS

The prophase I arrest in Xenopus oocytes is maintained by high levels of cAMP, and a reduction in the level of cAMP must occur before meiosis can re-initiate. Stimulation of oocytes with progesterone causes a transient reduction in cAMP concentration through inhibition of AC (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981). This reduction of cAMP is thought to cause a decrease in the activity of the cAMP-dependant protein kinase PKA. The importance of inhibiting PKA in the resumption of meiosis has been demonstrated by the fact that inhibiting PKA activity spontaneously induced oocyte maturation, while injection of active PKA blocked hormone induced maturation (Maller and Krebs, 1977; Speaker and Butcher, 1977; Sadler and Maller, 1981; Sadler and Maller, 1983).

The concentration of cAMP within a cell results from a balance between its formation, catalysed by AC, and its destruction, by PDEs. The control of AC is mediated by Gas and Gai G-proteins that activate and inhibit AC respectively. Gallo et al. (1995) showed that injection of a neutralizing antibody against Gas induced hormone independent oocyte maturation, suggesting the requirement of Gas in maintaining oocyte arrest. Recently, our lab showed the importance of G-βγ subunits in maintaining oocyte meiosis arrest. Two βγ scavengers, βARK-C (C-terminal Gβγ binding domain of bovine β adrenergic receptor kinase) and Gat (α subunit of bovine transducin), which inhibit βγ signalling were shown to induce hormone independent oocyte maturation (Sheng et al., 2001). Conversely, over-expression of xGβ1 together with bovine Gγ2 elevated
intracellular cAMP, and blocked progesterone induced GVBD. These results suggest that both \( \alpha \) and \( G\beta\gamma \) are involved in maintaining the cAMP concentration. Interestingly, AC type II and IV are thought to be activated by \( \beta\gamma \) only in the presence of activated \( \alpha \) (Federman et al., 1992; Tang and Gilman 1991), suggesting an AC of type II or IV is active in prophase arrested oocytes. In order for oocyte maturation to proceed, the activity of AC must be inhibited. A number of AC types are inhibited by \( G\alpha i \) proteins, yet progesterone induced oocyte maturation is not blocked by pertussis toxin (specific inhibitor of \( G\alpha i \) G-proteins) (Onate et al., 1984; Sadler et al., 1994), suggesting AC inhibition by progesterone does not involve activation of a \( G\alpha i \) protein.

One possible mechanism for maintaining AC activity in oocytes may consist of an endogenous GPCR that activates a G-protein, likely a \( G\alpha \) family member, and releases \( G\alpha \)-GTP and \( G\beta\gamma \) which co-operatively activate an oocyte AC of type II or IV (Figure 4.1). In this model, the GPCR is constitutively active, possibly through an autocrine loop where its ligand is secreted by the oocyte or in a ligand independent manner. Progesterone action in oocytes may result in the inhibition of this GPCR, therefore promoting the re-association of \( G\alpha \) and \( G\beta\gamma \) subunits. The re-association of the subunits removes the AC activation signal and the concentration of cAMP falls, initiating oocyte maturation.

Similar to progesterone-induced maturation, stimulation of oocytes with insulin also causes a reduction of cAMP, although likely through activation of a phosphodiesterase (PDE) that hydrolyses cAMP (Sadler, 1991a; Sadler, 1991b; Andersen et al., 1998). One way in which insulin can regulate PDE activity is through the PI 3-K pathway. Oocytes activate PI 3-K in response to insulin stimulation, and the use of a specific
Figure 4.1
Proposed mechanism of regulation of cAMP by progesterone and insulin. High levels of cAMP are responsible for maintaining G2 arrest. An endogenous GPCR* (the receptor and its cognate ligand secreted by the oocytes) activates a G protein, releasing GTP-Gq6 and a βγ complex, which together activate an oocyte adenyly cyclase. Progesterone, through xPR, releases prophase arrest by inhibiting GPCR* signalling. IGF-1 receptor activates two parallel signalling pathways. It activates xIRS-1 and phosphotidylinositol 3-kinase (PI 3-K). PI 3-K, via the generation of PI[3,4,5]P3, activates protein kinase B (PKB)/Akt, which in turn activates phosphodiesterase 3 (PDE3) and thus reduces cAMP. This chapter describes our work which identifies a novel signalling pathway in which IGF-1 receptor, through direct interaction with xGIPC and GAI, activates Goα and hence inhibits adenylyl cyclase. The co-operation of the two IGF-1 receptor activated signalling pathways serves to decrease cAMP.
inhibitor of PI 3-K blocks insulin-induced oocyte maturation (Liu et al., 1995). Recently, Andersen et al (1998) suggested a mechanism of how PI 3-K activation aids in the induction of oocyte maturation. PI 3-K activates PKB/Akt, a serine threonine kinase, which has been shown to increase the activity of a phosphodiesterase, PDE3 (Andersen et al., 1998). Since the reduction of cAMP is necessary for oocyte maturation to occur, the activation of a phosphodiesterase by insulin may provide the link from the IGF-1 receptor to reduction of cAMP.

In chapter 3 of my thesis, I describe my work that has revealed another possible mechanism by which insulin can regulate cAMP. Previously it was shown that in various cell types there is an association of the IGF-1 receptor with heterotrimeric G-proteins of the Gi type (Luttrell et al., 1995; Kuemmerle and Murthy, 2001), suggesting a Gi signal originates for the IGF-1 receptor. The identification of GIPC as an IGF-1 receptor binding protein may provide a functional link between the IGF-1 receptor and GaI. The mechanism by which GIPC modulates IGF-1 signalling is thought to involve the regulation of G-proteins associated with the IGF-1 receptor. GIPC's function presumably involves the complexing of GAIP, a regulator of G-protein signalling (RGS), with the IGF-1 receptor, since GIPC has previously been shown to interact with GAIP (De Vries et al., 1998). GAIP, a member of the RGS family of proteins which GAP proteins of Ga subunit, specifically activates the GTPase activity of Gi type Ga subunits (De Vries et al., 1995; Druey, 2001). The association of GIPC with both the IGF-1 receptor and GAIP presumably forms a negative regulatory complex at the receptor. However, the over-expression of xGIPC and hGAIP in oocytes potentiated insulin signalling in inducing oocyte maturation (Figure 3.8). How is it possible that over-expression of presumably
negative regulators of G-protein signalling act in a positive manner? Two mechanisms are possible. 1). Upon receptor activation, the increased amount of GIPC and GAIP cause the activated Gai-GTP to hydrolyse its bound GTP to GDP, which readies the Gai subunit for another round of activation by the receptor. This increased cycling of the G-protein may potentiate the Gai signal from the IGF-1 receptor and further decrease the activity of AC (Figure 4.1). 2). The GIPC/GAIP complex may function as an IGF-1 receptor effector and activate downstream components in the oocyte maturation pathway (Figure 4.1).

In summary, stimulation of oocyte IGF-1 receptor may activate two parallel signalling pathways: one leads to activation of PI 3-K/Akt/PDE3 and hydrolysis of cAMP and the other leads to activation of Gai and inhibition of AC. The coordination of these two pathways ensures the reduction of cAMP and resumption of oocyte meiosis.
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Curriculum Vitae

Education

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Master of Science in Biology and Biotechnology
Rapid enzyme linked immunosorbent assays (ELISA) for the detection of Escherichia coli O157:H7 (the cause of Hamburger Disease) in foods and clinical samples through the detection of surface lipopolysaccharide (LPS) antigens and elaborated verocytotoxins were developed.

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An honours research project was completed which entailed the characterization of the mutational specificity of 1-nitroso-6-nitropyrene in the lac I gene of Escherichia coli deficient in nucleotide excision repair.

Scientific Publications

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Karen P. Phillips, Mary Ann Petrunewich, Jennifer L. Collins, Ronald A. Booth, X. Johné Liu, and Jay M. Baltz
Inhibition of MEK parthenogenetically activates mouse eggs and yields the same phenotypes as mos+ parthenogenotes. Dev. Biol. 2002 247: 210-223

Yinglun Sheng, Mario Tiberi, Ronald A. Booth, Chunqi Ma, and X. Johné Liu.
Mustafa Bayaa*, Ronald A. Booth*, Yinglun Sheng, and X. Johné Liu
* These two authors contributed equally to this work.

Nicholas Ohan, David Sabourin, Ronald A. Booth and X. Johné Liu

Nicholas Ohan, Yahenew Agazie, Cathy Cummings, Ronald A. Booth, Mustafa Bayaa, and X. Johné Liu.

Cellular determinants of the mutational specificity of 1-nitroso-6-nitropyrene and 1-nitroso-8-nitropyrene in the lacI gene of Escherichia coli. Accepted by: Mutation Research.

Objective analysis of medium-induced fragility of Schwanniomyces occidentalis. Submitted for publication in: Lab Manual on Non-conventional Yeasts Genetics, Biochemistry, Molecular Biology, and Biotechnology.


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