AD_____

Award Number: DAMD17-98-1-8195

TITLE: Chromatin HMG-I(Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Viroj Boonyaratankornkit, Ph.D.
Dean P. Edwards, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado
Health Science Center
Denver, Colorado 80262

REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Annual Summary (1 APR 98 - 31 MAR 01)

4. TITLE AND SUBTITLE
Chromatin HMG-I(Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-98-1-8195

6. AUTHOR(S)
Viroj Boonyaratankornkit, Ph.D.
Dean P. Edwards, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Science Center
Denver, Colorado 80262

E-Mail: viroj.boon@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Steroid hormones have rapid non-genomic effects on cell signaling pathways, but the receptor mechanisms responsible for this effect are not understood. We have identified a specific polyproline helix motif in the amino-terminal domain of conventional progesterone receptor (PR) that mediates direct progestin-dependent interaction of PR with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases. Through this interaction, PR is a potent activator of Src kinases working by an SH3 domain displacement mechanism. By mutagenesis we also show that rapid progestin-induced activation of Src and down-stream MAP kinase in mammalian cells is dependent on PR-SH3 domain interaction, but not on the transcriptional activity of PR. Evidence for biological significance of this non-genomic PR signaling pathway through regulatory SH3 domains was shown with respect to an influence on progestin-induced growth arrest of breast epithelial cells and induction of *Xenopus* oocyte maturation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, c-Src, Progesterone Receptor, Estrogen Receptor, SH3	29
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Summary of 2000-2001 Research

As indicated in my annual summary of 1999-2000 research, the original research title "Chromatin HMG-I(Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells" failed to demonstrate a functional biological significance of HMG-I(Y) on estrogen receptor (ER) dependent gene transcription in whole cells. I had proposed new statement of work, which has recently been forwarded to my Grant Officer's Representative, Dr. Kandasamy. The result and training gained from these studies is presented below.

Molecular Mechanism of Non-genomic Actions of Estrogen and Progesterone in Breast Cancer Cells

Introduction

As members of the superfamily of nuclear receptors, steroid hormone receptors are well established as ligand-dependent transcriptional activators (Mangelsdorf et al., 1995). However, there is increasing evidence that not all the biological effects of steroid hormones are mediated by receptor binding to DNA and direct modulation of target gene expression. Examples have been reported of rapid effects of all classes of steroid hormones on cell membrane/cytoplasmic signal transduction pathways that do not require changes in gene transcription or protein synthesis (Revelli et al., 1998; Wehling, 1997; Watson and Gametchu, 1999). Estrogen rapidly stimulates the activity of G protein coupled effectors such as phospholipase C and adenylate cyclase resulting in increased production respectively of inositol lipid and cAMP second messengers (Aronica et al.; Razandi et al., 1999). Estrogens have also been reported to rapidly increase intracellular Ca^{++} and to activate growth factor related mitogen activated protein (MAP) kinase signaling pathways in different cell types (Migliaccio et al., 1996, 1998; Improta-Brears et al., 1999; Kahlert et al., 2000). Estrogen has long been known to have rapid effects on vasodilation of blood vessels; an action now believed to involve calcium and PI(3) kinase/Akt mediated stimulation of endothelial nitric oxide synthase activity (Kim et al., 1999; Chen et al., 1999; Simoncini et al., 2000).

Rapid non-transcriptional actions of progesterone include maturation of amphibian oocytes (Ferrell, 1999), stimulation of the acrosomal reaction in sperm (Revelli et al., 1998), modulation of neurotransmitter receptor activity and neuronal excitability (McEwen, 1991) and stimulation of the mitogenic Src/$p21^{ras}$/MAP kinase signaling pathway in breast cancer cells (Migliaccio et al., 1998). In *Xenopus laevis* oocytes, progesterone stimulation brings about cdc2/cyclin B activation and resumption of the meiotic cell cycle. This effect is linked to a rapid decrease in adenylate cyclase activity and

intracellular cAMP followed by activation of the c-mos/MEK1/p42 MAP kinase cascade and the kinases/phosphatases that act immediately upstream of cdc2/ cyclin B. The effect of progesterone on the acrosomal reaction is associated with a rapid influx of Ca^{++} and Cl^{-} ions in parallel with hydrolysis of phospholipids and induction of protein tyrosine phosphorylation.

The receptors and molecular mechanisms responsible for mediating rapid non-genomic effects of estrogen and progesterone on cytoplasmic/membrane signaling pathways are not known. Studies have suggested the existence of separate membrane steroid receptors unrelated to conventional intracellular receptors (Revelli et al., 1998; Wehling, 1997; Watson and Gametchu, 1999; Ferrell, 1999; Filardo et al., 2000). However, cloning, characterization and confirmation of *bone-fide* novel membrane steroid receptors has not been accomplished. Other studies have indicated that a subpopulation of the conventional steroid receptor associates with cell membrane or cytoplasmic components and is responsible for many of the rapid effects of steroids (Aronica et al., 1994; Razandi et al., 1999; Migliaccio et al., 1996, 1998; Improta-Bears et al., 1999; Kim et al., 1999; Chen et al., 1999; Simoncini et al., 2000). The recent cloning of an amphibian homolog of mammalian PR (termed X-PR) revealed that X-PR is involved in progesterone-induced *Xenopus* oocyte maturation. Injection of X-PR antisense oligonucleotides into oocytes inhibited this non-genomic progesterone response which could be restored by overexpression of X-PR or human PR. Additionally, overexpression of X-PR increased sensitivity to progesterone and accelerated the rate of progesterone-induced meiosis (Tian et al., 2000; Bayaa et al., 2000).

How ER or PR can associate with and activate cytoplasmic/membrane signaling molecules pathways is not known. Membrane localization of ER has been detected biochemically by cell fractionation and by immunocytochemistry (Razandi et al., 1999; Norfleet et al., 1999; Kim et al., 1999). A physical association of ER with insulin-like growth factor receptor (IGF-R), c-Src, p85 subunit of PI3/kinase and caveolin-1 (Kahlert et al., 2000; Simoncini et al., 2000; Migliaccio et al., 1998 and Schlegal et al., 1999) has been reported. The only report of PR association with a membrane/cytoplasmic signaling molecule is with c-Src and this was suggested to be indirect through association with ER that binds directly to Src (Migliaccio et al., 1998). The identification of specific motifs in ER and PR capable of mediating interaction with cytoplasmic/membrane signaling molecules and how these interactions can activate signaling pathways are important unknowns.

In this report, we have identified a polyproline helix motif in the amino terminal domain of conventional PR that is both necessary and sufficient for mediating direct hormone-dependent binding of

PR to SH3 domains of various cytoplasmic signaling molecules and we show how this interaction can activate Src tyrosine kinases. We also show that the previously described ability of progestins in mammalian cells to stimulate a rapid activation of the c-Src/p21ras/MAP kinase signaling pathway (Migliaccio et al., 1998) is dependent on the ability of PR to interact with SH3 domains. Finally, we provide evidence that this non-genomic PR signaling domain pathway influences two known biological actions of progesterone, growth arrest of normal breast epithelial cells and induction of *Xenopus* oocyte maturation.

Experimental Procedures

Yeast two-hybrid screen. A yeast two hybrid screening was performed using the Match-Maker Yeast System 2 (Clontech). Human PR-A cDNA was fused to the GAL4 DNA binding domain in the yeast expression vector pAS2. The yeast strain CG1945, containing integrated HIS3 and β-galactosidase reporter genes under the control of GAL4 upstream activator sequences was transformed with GALDBD/PRA and a U2-OS osteosarcoma cDNA expression library fused to GAL4 activation domain (AD) in vector pACT2 vector. Approximately $3x10^6$ independent clones were screened for R5020 dependent growth and induction of ß-galactosidase activity. His^+ and ß-gal^+ plasmids were recovered from yeast, amplified in *Escherichia coli* DH5α cells and sequenced.

GST pull-down assays. Bacterial strains expressing SH3 or other domains of various signaling molecules (CAP, cortactin, Grb-2, Hck, Fyn, p85, NCK, CAS and c-Src) as GST fusion were constructed, expressed and bound to glutathione Sepharose 4B as described ((Ribon et al., 1998b; Burton et al., 1997; Melvin and Edwards, 2000). GST-Src-pp60-SH3 was provided by CA Cartwright (Stanford University). PR and other nuclear receptors were then incubated with the resins for 1 hr at 4 C, washed with TEDGN100 (10 mM Tris, pH8.0; 1 mM EDTA; 1mM DTT; 10% Glycerol, and 100 mM NaCl) and bound proteins were detected by Western blot with antibodies specific for PR (1294), 6X histidine-tags (1162/F6) or GST (794/H2) (Clemm et al., 2000; Boonyaratanakornkit et al. 1998). Detection was by enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ).

***In vitro* site-directed mutagenesis.** Point mutations in human PR cDNA (P422A, P423A, P426A) were generated *in vitro* using *Pfu*-Turbo DNA polymerase (Stratagene) in the presence of sense and antisense strand annealing oligonucleotides with the indicated mutated sequences as described by the manufacturer. Mutations were confirmed by DNA sequencing.

Expression and purification of nuclear receptors in the baculovirus system. Polyhistidine tagged PR and other nuclear receptors were expressed from baculovirus vectors in Sf9 insect cells in the presence of their cognate hormones and were purified by nickel affinity resins as previously described (Boonyaratanakornkit et al., 1998).

Activation of down-regulated Hck by progesterone receptor. Hck was produced from baculovirus vectors in Sf9 insect cells and purified in down-regulated form as previously described (Moarefi et al., 1997). Kinase activity was measure by a spectrophotometric assay that couples the production of ADP to the oxidation of NADH (Barker et al., 1995). Enzyme reactions (100 µl) contained 600 µM peptide substrate (Porter et al., 2000), 10 nM Hck and 500 µM ATP in buffer containing 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.28 mM NADH, 89 units/ml pyruvate kinase and 124 units/ml lactate dehydrogenase. The reduction in absorbance of NADH at 30°C was measured continuously at 340 nM in a VersaMax plate reader (Molecular Devices). The fold activation of Hck by ER and PR and apparent activation constants, K_{act}, were determined as previously described (Moarefi et al., 1997; Porter et al., 2000).

Cell cultures and transfections. T47D breast cancer cells and Cos-7 monkey cells were grown as previously described (Boonyaratanakornkit et al., 1998). Cos-7 cells were plated in 100mm dishes (1.7×10^6 cell) and 24 hr later were transiently transfected with plasmids indicated in figures by an attenuated adenovirus-mediated procedure described previously (Boonyaratanakornkit et al., 1998). Cells were then grown for 24 hr on phenol-red free DMEM with 5% fetal bovine serum (FBS) treated with dextran coated charcoal (DCC), followed by growth for another 48 hr with 0.2% DCC-FBS prior to hormone treatment. Immortalized normal MCF-12A human breast epithelial cells were maintained in DMEM/F-12 supplemented with cholera toxin (100ng/ml), insulin (10µg/ml), hydrocortisone (0.5µg/ml), EGF (20ng/ml) and 5% horse serum as described (Paine et al., 1992). MCF-7 breast cancer cells were stably transfected by a calcium phosphate method with human PR, or PR-B_{mPro}, inserted into XhoI, EcoRI sites of pZeoSV2(-) and pZeoSV-Lac-Z (Invitrogen, Carlsbad, CA). Stable transfected colonies were selected in 750 ug/ml of Zeocin. For experiments, subconfluent cells were maintained for 3 days in phenol red-free MEM with 5% DCC-FBS, followed by another 48 hr prior to hormone addition in the same medium supplemented with 1% DCC-FBS.

Coimmunoprecipitation assays. T47D cell lysates (1mg total protein) were prepared as previously described (Boonyaratanakornkit et al., 1998) except the lysis buffer contained the addition of 1% NP-40. Cell lysates were incubated with 1µg of anti-Src antibody (327 MAb) (gift from Dr. Joan

Brugge, Harvard Medical School, MA) for 90 min at 4° C, followed by absorption for 1 hr at 4°C with protein A Sepharose that was precoated with rabbit anti-mouse secondary IgG. After washing beads with lysis buffer, proteins were eluted and analyzed by Western blot with PR specific monoclonal antibody 1294.

c-Src kinase catalytic assay. Cell lysates were immunoprecipitated with the c-Src monoclonal antibody, 327, as described above. Protein A Sepharose beads containing bound Src were washed three times with lysis buffer and once with Src kinase reaction buffer (10 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, and 1 mM sodium orthovanadate). Kinase assays were performed by incubating the washed protein-A Sepharose beads with ^{32}p-γAPT and 5ug of acid modified enolase as the peptide substrate (Hunter et al., 1998). Incorporation of ^{32}P into enolase was detected by SDS-PAGE and autoradiography and quantified by Phosphorimager (Molecular Devices) or densitometry.

***Xenopus* oocytes germinal vesicle breakdown.** Stage VI *Xenopus* oocytes were isolated and microinjected as described previously (Schwab et al., 1999). mRNA encoding PR-B, PR-B_{mPro}, or β-galactosidase (control) was transcribed using a T7 mMessage mMachine kit (Ambion), concentrated to 1.5mg/ml, and injected in 50nl volumes. After 24 hr of incubation at 19°, oocytes were treated with progesterone (10μg/ml) and induction of maturation was assessed by white spot formation, indicative of germinal vesicle breakdown (GVBD).

Results

Identification of a polyproline motif in progesterone receptor that mediates direct binding to SH3 domains of signaling molecules.

Human PR is expressed as two proteins from a single gene, PR-B and the N-terminal truncated PR-A that lacks the first 164 amino acids (Kastner et al., 1990). The two PR proteins have identical sequence in the C-terminal ligand binding domain (LBD), the central DNA binding domain (DBD) and about 2/3 of the N–terminal domain (Figure 1A). PR-B is generally a stronger transactivator than PR-A and in certain contexts, PR-A has no transcriptional activity and can act as a transrepressor of other steroid receptors (Giangrande and McDonnell, 1999). Using PR-A as the bait with a human U2-OS osteosarcoma expression cDNA library, a yeast two-hybrid screen designed to identify novel amino-terminal interacting proteins resulted in isolation of a clone with 72% amino acid sequence identity to the carboxyl terminal Src homology 3 (SH3) domain of the mouse c-Cbl-associated protein (CAP) (Ribon et al., 1998b). CAP contains three adjacent SH3 domains in the C-terminus and the cDNA obtained from our screening corresponds to the C-terminal most SH3 domain, termed SH3-C. As shown

by yeast two-hybrid assay, a weak interaction above background between PR-A (fused to GAL4 DBD) and the CAP-SH3 domain (fused to GAL4 activation domain-AD) was observed in the absence of hormone that was stimulated another 8-10 fold by the synthetic progestin R5020 (Figure 1B). The LBD of PR failed to interact significantly with the CAP-SH3 domain, indicating that sequences within the DBD or the N-terminal domain of PR are required for this interaction (Figure 1B).

To determine whether PR interaction with the SH3 domain of CAP is due to a direct association between the two proteins, and whether this extends to SH3 domains of other signaling molecules, GST pull-down experiments were performed. Free GST, or GST fused to SH3 domains of CAP and various other signaling molecules were immobilized to glutathione Sepharose beads and incubated with baculovirus expressed PR. Bound receptors were eluted and detected by Western blot. As shown in Figure 1C, PR-B (bound to R5020) prepared as a whole cell extracts from Sf9 insect cells, did not bind to free GST but did bind efficiently to the SH3-C domain of CAP and to SH3 domains of Hck, Grb-2 and c-Src (Figure 1C upper panel). PR interacted minimally with Fyn and Crk and failed to interact at all with Cas, Nck and the other two SH3 domains of CAP. Highly purified PR also bound efficiently to SH3 domains of CAP (not shown) and c-Src (Figure 1D), and PR-A gave similar binding results as PR-B (Figure 3A). Thus, PR selectively interacts directly with a subset of SH3 domains, including those from the c-Src tyrosine kinase family members, Hck and c-Src itself.

The region of PR required for interaction with SH3 domains was broadly mapped, by GST-pull down assays with various baculovirus expressed domains of PR, to a region within the N-terminus PR (aa 165 - 552) that is common to both the A and B forms of the receptor (not shown). SH3 domains recognize short contiguous proline-rich motifs that adopt an extended left-handed polyproline (PPII) helix. Class I SH3 ligands have the sequence RXLPPZP(Z=L or R and X= any aa), while class II ligands contain the sequence XPPLPXR. The difference in position (N or C-terminal) of the conserved arginine residue results in the two ligands binding with the peptide backbone in the opposite orientation (Yu et al., 1994). Human PR contains a type II sequence in the amino terminus between amino acids 421-428 (PPPPLPPR). To determine whether this sequence is important for PR-SH3 domain interaction, three key prolines were converted to alanines (P422A, P423A, and P426A) by site directed mutagenesis and the resultant mutant receptor (PR-B_{mPro}), was expressed in the baculovirus system (bound to R5020) and analyzed for binding with these same SH3 domain constructs. These mutations abolished binding, indicating that the PPII motif is essential for PR-SH3 domain interaction (Figure 1C, bottom panel). The failure of PR_{mPro} to bind to SH3 domains is not due to a general effect on stability or

overall folding of the receptor. Intact PR_{mPro} is expressed in Sf9 cells at the same level as PR-B and it binds hormone and DNA normally (not shown). An 18 amino acid peptide containing the PR proline-rich motif (aa 421-428) and several flanking sequences on either side, (DFPLGPPPPLPPRATPSR) was synthesized along with a peptide in which the three key prolines were changed to alanines (DFPLGAAPLAPRATPSR). The wild-type peptide effectively inhibited PR association with Src in a dose dependent manner, while the mutant peptide had no effect (Figure 1D). These results taken together with the failure of PR-B_{mPro} to bind SH3 domains (Figure 1C), strongly supports the conclusion that the PPII motif is both necessary and sufficient for PR interaction with SH3 domains.

PR-SH3 domain interaction is hormone-dependent *in vitro* and in cells.

The PR-SH3 domain interaction results above were obtained with baculovirus expressed PR bound to R5020. Although liganded PR is functional and stable, unliganded baculovirus expressed PR has little hormone or DNA binding activity, indicating that it is not correctly folded (Christensen et al., 1991). Thus baculovirus expressed PR is not a suitable source for determining the influence of hormone on PR-SH3 domain interaction *in vitro*. To circumvent this problem we expressed PR-B and PR-B_{mPro} from recombinant adenovirus vectors in MCF-12A immortalized normal human breast epithelial cells. Expression was controlled at a low M.O.I. to give PR levels comparable to that of endogenous PR in breast cancer cells (not shown). As detected by GST-pull down assay, PR-B extracted from MCF-12A cells in the absence of ligand interacted weakly with the SH3 domain of c-Src, while this interaction was greatly increased by treatment of cells with R5020. PR-B_{mPro} expressed in MCF-12A cells exhibited little to no binding with Src in the presence or absence of R5020 (Figure 2A).

A hormone-dependent association of PR with c-Src in T47D breast cancer cells, which express both proteins endogenously, was also observed. Progestin treatment of cells for 5 min stimulated an association of PR (both A and B forms) with c-Src as detected by coimmunoprecipitation assay (Figure 2B). From these results, we conclude that PR-SH3 domain interaction is hormone-dependent *in vitro* and *in vivo*.

Other steroid/thyroid hormone receptors do not interact with SH3 domains.

Within the nuclear hormone receptor superfamily, only PR contains an obvious polyproline SH3 recognition sequence. Nonetheless, the ability of other nuclear receptors to bind to SH3 domains *in vitro* was tested directly. Human ER, glucocorticoid receptor (GR), androgen receptor (AR) and thyroid hormone receptor-β (TRβ) were expressed in the baculovirus system in the presence of their cognate

hormonal ligands and were analyzed for binding to the SH3 domains of CAP or c-Src by GST pull-down assay. As directly compared to efficient binding with human PR-A (bound to R5020), no interaction was observed between the SH3 domains of CAP (Figure 3A) or Src (not shown) with any of the other nuclear hormone receptors. Thus, SH3 domain interaction appears to be a unique property of PR, at least among the steroid/thyroid class of the nuclear receptors tested.

Previous studies from Auricchio and colleagues have suggested that PR does not associate directly with c-Src but interacts through ER which in turn binds to the SH2 domain of Src (Migliaccio et al., 1998, 2000). To reconcile the apparent discrepancy between our data and these, we have further analyzed interactions of ER and PR with Src *in vitro* and the consequence of these interactions on kinase catalytic activity. Members of the Src-family of tyrosine kinase possess an N-terminal unique domain (U) followed by SH3 and SH2 domains, a linker region, the catalytic domain and a short carboxyl terminal tail (Thomas and Brugge, 1999; Brown and Cooper, 1996). Highly purified baculovirus expressed human ERα (bound to estradiol) interacted directly with the SH2 domain of c-Src, but showed no interaction with SH3 or the unique domain (U). ER also bound with a construct containing all three regulatory domains (U-SH3-SH2), indicating that the SH2 domain is the primary ERα binding site (Figure 3B). PR-B interaction with Src occurred primarily through the SH3 domain; interactions were not observed with SH2 or U domains (Figure 3B). The two receptors added together formed a ternary complex with Src but only with a construct containing all the regulatory domains (U-SH3-SH2), not with SH3 or SH2 alone (Figure 3B). Furthermore, the binding of one receptor did not appear to significantly influence the binding of the other (Figure 3B). These results suggest that ER and PR can interact Src simultaneously through independent binding respectively to SH2 and SH3 domains.

PR Activation of tyrosine kinase activity by SH3 domain displacement.

In addition to mediating protein-protein interaction with targets, the SH2 and SH3 domains of Src tyrosine kinases are involved in autoinhibition through intramolecular associations. The SH2 domain interacts with a conserved tyrosine (527) in the C-terminal tail that is phosphorylated by c-Src kinase (CSK). The SH3 domain interacts with a polyproline-like helix in the linker region between the SH2 and catalytic domains (Xu et al., 1999; Schindler et al., 1999). These intramolecular interactions maintain the enzyme in a closed inactive conformation. Conversion to the open catalytically active conformation can be achieved by dephosphorylation of tyrosine 527, or by displacement of SH2 or SH3

domain interactions with high affinity external SH2 and SH3 ligands (Thomas and Brugge, 1997; Brown and Cooper, 1996; Moarefi et al., 1997).

Because c-Src and Hck have a similar closed-conformation structure as determined by X-ray crystallography (Schindler et al., 1999; Xu et al., 1999), we used down-regulated Hck as a general model of c-Src family tyrosine kinases to investigate the mechanism of activation by PR. Hck was co-expressed with CSK in Sf9 cells to stoichiometrically phosphorylate tyrosine 527 and produce the enzymatically down-regulated closed conformation (Porter et al., 2000). (The following experiments were done in collaboration with Dr. W. Todd Miller at Department of Physiology and Biophysics at State University of New York at Stony Brok, Stony Brook, New York.) When varying concentrations of baculovirus expressed PR-B (bound to R5020) purified to near homogeneity were incubated with down-regulated Hck, a PR concentration-dependent stimulation (3-4 fold) of kinase activity was observed (Figure 4A). Similar results were obtained with purified PR-A, whereas PRB_{mPro} had no effect on Hck catalytic activity (Figure 4A). The apparent k_{act} for PR-B and PR-A, which is the concentration of receptor which gives half-maximal activation of Hck, was 23± 18nM. These results indicate that PR is a potent activator of c-Src tyrosine kinases through displacement of intramolecular SH3 domain interactions.

In contrast to the ability of PR to activate down-regulated Hck, highly purified ER α (bound to estradiol) at the same effective concentration as PR (1.5μM), failed to activate tyrosine kinase catalytic activity (Figure 4B). When the two receptors were added together, ER did not potentiate Hck activation by PR, but at the highest concentration, slightly attenuated induction by PR (Figure 4B). These results taken together with binding properties of ER and PR to Src, indicate that PR *in vitro* is perfectly capable of binding to and activating Src kinases independent of ER.

PR-SH3 domain interaction is required for rapid progesterone-induced activation of c-Src and MAP kinase in mammalian cells.

To determine whether PR-SH3 domain interaction has a role in rapid progestin stimulation of Src kinase activity in mammalian cells, we turned to the use of PR negative cells and ectopic expression of wild PR and PR_{mPro}. Three cell systems were used: transient co-transfection of Cos-7 cells with PR and c-Src; transient expression of PR from recombinant adenovirus vectors in MCF-12A cells, and stable transfection of MCF-7 breast cancer cells with PRs. Although MCF-7 cells express PR, it is estrogen

regulated thus enabling the elimination of endogenous PR by growing cells under estrogen depleted conditions.

We first evaluated an earlier report that progestin-induced Src activation in transfected Cos-7 cells was dependent on ER (Migliaccio et al., 1998), finding that the effect of ER was to suppress elevated basal Src activity in transiently transfected cells. ER and PR negative Cos-7 cells were transiently co-transfected with PR and c-Src, with and without ER. c-Src activity in response to short term treatment with R5020 was assayed by an immunoprecipitation kinase procedure using ^{32}P incorporation into enolase as a substrate. As shown in Figure 5A, ER lowered the basal activity of Src and thus enabled stimulation by progestin (Figure 5A). We consistently observed a higher basal Src activity in cells expressing PR alone, which approached that of the progestin stimulated state in cells cotransfected with ER and PR. Thus in Cos-7 experiments, cells were routinely cotransfected with ER (in the absence of estrogen) to lower basal Src activity.

Rapid progestin induction of c-Src activity in Cos-7 cells was dependent on PR-B. No effect was observed in mock transfected cells that lack PR (not shown). In PR-B expressing Cos-7 cells, progestin stimulation of c-Src activity was maximal between 2 and 5 min (ave 2.7 fold induction ± .057), and returned to baseline by 10 to 15 min. R5020 failed to stimulate c-Src activity in cells transfected with PR-B_{mPro} or with PR truncation constructs lacking either the C-terminal LBD (BNDBD) or the N-terminal domain (dhLBD) (Figure 5B). In cells expressing PR-B, the progestin antagonist RU486 behaved as a pure antagonist. RU486 itself failed to stimulate c-Src activity but effectively inhibited induction by the agonist R5020 (Figure 5C). Other classes of steroid hormones had no effect on c-Src activity (Figure 5C), and response to progestins was dose dependent over physiological (0.5 to 10nM) concentrations (not shown).

The inability of progestins to stimulate rapid activation of Src in Cos-7 cells expressing PR-B_{mPro} does not appear to be due to an instability of the protein or to a significant alteration in conformation. PR-B_{mPro} expressed in Cos-7, or MCF-12A cells bound hormone and transactivated reporter gene constructs in a manner that was indistinguishable from that of wild type PR-B (data not shown). Thus, the SH3 domain interaction motif does not appear to influence or be required for transcriptional activity of PR. Conversely PR containing a point mutation in the second zinc finger of the DNA binding domain (C587A) that cripples the ability of PR to function as a transcription factor, was fully capable of mediating progestin-induced Src activity (Figure 5D). These results with transfected Cos-7 cells show that rapid progestin stimulation of c-Src kinases in cells is dependent on the ability of the conventional

PR to interact with SH3 domains and that this non-genomic pathway is separable from the transcriptional activity of PR.

To determine which PR is capable of mediating rapid progestin induction of c-Src kinase activity independent of ER, MCF-12A cells lacking both ER and PR were transduced to express PR-B or PR-B_{mPro} from adenovirus vectors, without the introduction of ER. Viral transduction conditions were used to express PRs at levels comparable to endogenous receptors in breast cancer cells (not shown). As shown in Figure 5E, the progestin R5020 stimulated a rapid activation of c-Src in cells transduced with wild type PR, but not in cells that express PR_{mPro}.

To determine the consequence of progestin activation of c-Src, we analyzed the effect of progestins on the down-stream effector MAP kinase (p42/p44) in stably transfected MCF-7 cells. Cells were grown on estrogen depleted medium for five days to eliminate expression of endogenous PR. Progestin treatment of MCF-7 cells expressing wild type PR-B gave a rapid induction of c-Src kinase activity at 2 min (not shown) and an activation of MAPK at 5 min that was about 20-25% of the magnitude stimulated by EGF (Figure 5F). In contrast, the progestin R5020 had no effect on Src (not shown) or MAPK activity in cells stably transfected with PR_{mPro} or with a control Lac Z vector (Figure 5F). Thus the SH3 domain interaction motif in PR is also required for rapid progestin activation of MAP kinase in mammalian cells.

PR-SH3 domain interaction influences two biological responses to progesterone: growth arrest of normal breast epithelial cells and induction of *Xenopus* oocyte maturation.

Progesterone can either stimulate or inhibit proliferation of epithelial cells, and which effect it has is dependent on cell type, duration of response and interaction with other hormones or growth factors. The predominant effect of progestins on breast cancer cells and normal mammary epithelium appears to be growth inhibition (Sutherland et al., 1998; and Lange et al., 1999). MCF-12A cells were transduced with adenovirus vectors to express PR-B, PR-B_{mPro}, or green fluorescent protein (GFP) as a control, and were analyzed for growth response to progestin. In the absence of progestins, PR-B, but not PR_{mPro}, expressing cells exhibited a partial inhibition of proliferation as compared to GFP controls. However, continuous treatment of PR-B expressing cells with R5020 resulted in significant growth inhibition and that resulted in complete arrest by day 5. Progestin-dependent growth inhibition was delayed in cells expressing PR_{mPro}, resulting in a significantly greater number of cells at the time of growth arrest which occurred later at day 7 (Figure 6A).

Overexpression of the amphibian homolog (X-PR) of mammalian PR was recently shown to increase the sensitivity and accelerate the rate of progesterone induced *Xenopus* oocyte maturation (Tian et al., 2000; Bayaa et al., 2000). (The following experiments were done in collaboration with Dr. James L. Maller at Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado). We similarly observed that expression of human PR-B by RNA injection into *Xenopus* oocytes, resulted in acceleration of the rate of progesterone induced maturation as measured by the percentage of cells undergoing germinal vesicle membrane break down (GVDB) as a function of time after progesterone administration (Figure 6B). In contrast, the rate of progesterone induced GVDB was unaffected in oocytes injected with PR-B_{mPro}, and was the same as control oocytes injected with β-galactosidase RNA (Figure 6B). The main effect of PR-B on this non-genomic progesterone response appeared to be kinetic; shortening the time that it takes to achieve maximal GVDB by about 30 min. At the longest time point analyzed, all experimental groups reached approximately the same maximum % of oocytes entering into meiosis (Figure 6B). These results confirm that overexpression of conventional PR accelerates the rate of progesterone induced oocyte maturation and further shows that this effect requires the ability of PR to interact with SH3 domains.

Discussion

The results in this report define for the first time a motif in a steroid receptor that mediates direct hormone-dependent interaction with a signaling molecule and provides a mechanism for how this interaction can activate signaling pathways. We show that the N–terminus of PR contains a previously unrecognized polyproline class II SH3 ligand sequence that is both necessary and sufficient for mediating direct interaction of PR with SH3 domains of c-Src tyrosine kinases and other selected signaling molecules. We also show that a consequence of this interaction is an activation of Src-kinases *in vitro* and *in vivo* by PR through an SH3 domain displacement mechanism. In support of this mode of Src activation point mutations in the PR proline-rich motif that disrupts interaction with SH3 domains *in vitro*, also abolished rapid progesterone stimulation of c-Src kinase activity in cells and the ability of PR to activate down-regulated Hck *in vitro* (Figures 1C, 4A). Additionally, PR activated a mutant form of down-regulated HcK in which the low affinity C-terminal tail SH2 tyrosine phosphorylation site YQQQ was replaced with a high affinity consensus site YEEI (data not shown). The substituted high affinity site does not permit displacement by exogenous SH2 peptide, leaving SH3 displacement as the only activation mechanism (Porter et al., 2000). Binding affinities for SH3 domains are typically modest with dissociations constants in the low μM range (Brown and Cooper, 1996; Thomas and Brugge, 1997). PR

was estimated to have an apparent Kd of 0.1μM (not shown). Other activators of Src kinases including focal adhesion kinase (FAK), the HIV protein Nef, Cas, synapsin I, sin, and $p68^{sam}$ have reported K_{acts} for down-regulated Hck or Src in the 100-200nM range (Thomas and Brugge, 1997; Moarefi et al., 1997; M. LaFevre-Bernt and W. Todd Miller, unpublished). An apparent k_{act} of 28nM for PR activation of down-regulated Hck (Figure 4A) is comparable to if not better than other activators.

Our results suggest that binding to SH3 domains is a unique property of PR within the steroid thyroid class of nuclear receptors. Several other receptors tested failed to interact directly *in vitro* and analysis of the protein sequence data base revealed consensus PPII SH3 ligand sequences only in PR. Motifs that conform to class II SH3 ligands are also present in the N-terminal domains of other species of PRs including mouse, rat, rabbit and *Xenopus*. This conservation across species further suggests that PR-SH3 domain interaction is physiologically important. Migliaccio et al. (2000) reported a physical hormone-dependent association of human AR with the SH3 domain of Src. The reason for the apparent discrepancy with out results is not known. AR used in pull-down assays was in crude cell lysates, so it is not known whether physical association was direct or indirect. A proline rich motif in the N-terminus of AR proposed to be the SH3 domain interaction site was not directly tested and does not conform to a consensus SH3 ligand.

PR interaction with SH3 domains was observed to be hormone–dependent *in vitro* (Figure 2A). Also, physical association of PR with Src *in vivo* (within intact cells) was rapidly and transiently induced by hormone (Figure 2B) and PR-SH3 domain interaction in the yeast two hybrid system was highly dependent on hormone (Figure 1B). We have also assessed the interaction of the N-terminal domain alone to see if it can constitutively interact with and activate Src kinases in the absence of hormone. When expressed in cells, the N-domain had no effect on Src activity (Figure 5B). The expressed purified N-domain *in vitro* is able to bind to the SH3 domain of Src and activate down-regulated Hck, but does so much less efficiently than full-length PR bound to hormone (not shown). A synthetic peptide containing the minimal polyproline sequence of PR was also capable of binding (as detected through competition- Figure 1D) to Src and activating down-regulated Hck, but required a 500-fold high molar concentration than PR (not shown). These results taken together suggest that ligand induces a conformational change in the N-terminus of PR that exposes the SH3 interaction motif and that the polyproline motif within the context of PR is a much better SH3 ligand than the minimal motif. To what extent hormone *in vivo* is also required to colocalize PR and Src in the same cell compartment is not known. Based on immunohistochemistry, PR is predominantly a nuclear protein in most cells and

tissues examined. However, PR exhibits a rapid shuttling between the nucleus and cytoplasm by active nuclear import and export mechanisms and in some cells a sizable fraction of PR-B has been detected in the cytoplasm of living cells with GFP-tagged PR (Tyagi et al, 1998; Lim et al. 1999). The dynamic localization of PR in the cells implies that it may be capable of encountering signaling molecules in the cytoplasm and of having extranuclear functions. By use of fluorescent tagged proteins, it will be important in future studies to determine whether and where PR and Src (or other SH3 domain containing proteins) interact in the cell in response to hormone treatment.

Mutations in the proline rich motif that disrupt PR interaction with SH3 domains *in vitro* also abolished rapid stimulation by progestins of c-Src and MAP kinase enzyme activities in mammalian cells. These results suggest that a functional PR- SH3 domain interaction is required for the progestin induced activation of the entire Src/ras/MAPK pathway reported by Migliaccio et al. (1998). Importantly, mutations that abolished PR interaction with SH3 domains had no influence on the ability of PR to function as a hormone–dependent transcription activator (not shown). Conversely a DNA binding deficient mutant PR was perfectly capable of mediating rapid effects of progesterone on activation of c-Src (Figure 5D). These receptor mutagenesis experiments define separable and independent functions for PR as a direct modulator of signaling pathways and as a transcription factor.

Our results support the conclusion that progestin activation of Src in cells is through a direct PR-Src interaction, not through cross-talk with ER as previously reported (Migliaccio et al,. 1998, 2000). A dependency on ER for progestin activation of Src was observed only in transiently transfected Cos-7 cells and the effect of ER was to lower an elevated basal activity of Src (Figure 5A). Transient transfections tend to overexpress proteins in a small fraction of cells, suggesting that overexpressed PR constitutively activates Src in Cos-7 cells. In MCF-12A cells that do not overexpress PR, basal Src activity was lower and rapid progestin activation of Src was readily detected in the absence of ER (Figure 5 E). How ER can suppress Src activity is not clear. Although ER binds to the SH2 domain of Src (Migliaccio et al., 2000 and Figure 3B) it did not activate down-regulated Hck tyrosine kinase, implying that ER did not compete with the phosphotyrosine site in the C-terminal tail to effectively displace the SH2 domain. Instead, ER partially attenuated PR-induced activation (Figure 4B). Because ER and PR can interact simultaneously with Src through SH3 and SH2 domains respectively (Figure 3B), raises the possibility that the two receptors have the potential to influence each others ability to affect Src activity. Precisely how ER and PR cooperate to modulate Src activity is a complicated problem that remains to be determined.

To begin to explore the physiological significance of non-genomic PR signaling through SH3 domain interactions, we analyzed the influence of mutations in the proline rich–motif of PR on two different biological responses to progesterone; growth arrest of normal breast epithelial cells and induction of Xenopus oocyte maturation. In both systems, loss of the ability of PR to interact with SH3 domains altered the rate of response to progesterone. Progestin–induced arrest of MCF12-A cells was delayed while the ability of overexpressed PR to accelerate the rate of progestin-induced oocyte maturation was lost (Figure 6). As determined by flow cytometry, progestin-dependent growth arrest of MCF-12A was due to a dramatic and rapid accumulation of cells in G1 of the cell cycle without significant apoptosis during the time course of the hormone treatment. Whether altered progesterone responses in the presence of PR_{mPro} are due to a lack of PR activation of Src or other SH3 domain containing signaling molecules is not known. In the Xenopus oocyte system, the effect is presumably through Src, since overexpression of constitutively active v-Src was shown previously to accelerate the rate of progesterone–induced oocyte maturation (Spivack et al., 1984). In addition to Src, PR interacts efficiently with SH3 domains of other signaling molecules (Figure 2A). Thus other potential cellular targets may be activated through PR interaction with SH3 domains. The CAP protein initially identified in our yeast two-hybrid screen is a potentially interesting candidate because it has a role in the focal adhesion signaling pathway (Ribon et al., 1998a) and progesterone induces cell spreading and focal adhesion of breast cancer cells through stimulation of tyrosine phosphorylation of FAK and paxillin (Lin et al, 2000). CAP also targets Cbl to specialized compartments of the plasma membrane implicated in localization of signaling pathways termed caveolae, suggesting the possibility that CAP may assist PR in associating with signaling molecules at the cell membrane (Baumann et al., 2000).

Progesterone is an important hormone in regulating developmental cycles in the normal mammary gland. It has both proliferative and differentiative effects at different stages of development and the requirement of progesterone for morphogenesis of lobulo-alveolar glands during pregnancy has been well established (Lydon et al, 1995). Because progesterone can switch from proliferative to differentiative effects at stages of mammary gland development, we propose that a physiological role of non-genomic signaling of PR through Src, or other SH3 domain signaling molecules, may be to regulate the timing of this developmental switch by inducing inhibition of proliferation. By directly modulating the activity of signaling molecules, PR can immediately (or within minutes) activate or shut off a signaling pathway, while regulating the levels of the signaling molecules in a pathway by transcription takes hours or days and may be required for sustained effects. In the Xenopus oocyte system all the effects of progesterone are non-genomic on cytoplasmic signaling pathways. Thus the altered kinetics of

progesterone-induced oocyte meiosis observed with PR_{mPro}, suggests that PR modulation of Src, or other signaling molecules is not essential for the response to progesterone, but may also be involved in regulating the timing of the response. The c-Src tyrosine kinases are involved in regulating a variety of cellular processes including cell proliferation, cell cycle progression, cell adhesion and migration, differentiation, survival and angiogenesis (Thomas and Brugge, 1997; Brown and Cooper, 1996). This wide range of activities is the consequence of c-Src association with a variety of cell surface receptors and occupying a central position in the signal transduction pathways linked to these receptors. Src tyrosine kinase have also been implicated in human cancers, including colon and breast cancer, through overexpression and constitutive activation, but not through mutations. Src is normally repressed and transiently activated by extracellular signals. Thus, constitutive activation of c-Src by PR may have a role in tumorigenesis of the mammary gland and other reproductive tissues.

We propose that PR is a dual function protein capable of directly modulating cell signaling pathways in the cytoplasm through hormone-dependent interaction with regulatory SH3 domains of signaling molecule and of functioning in the nucleus in its well established role as a hormone-dependent transcription factor (Figure 7). Our identification of mutations in PR that are capable of separating these functions will provide the opportunity in the future to analyze the physiological roles of these two distinct PR signaling pathways through gene replacement in mice of wild type PR with mutant PRs.

Key Research Accomplishments

- Human PR through its polyproline helix motif (aa 421-428) interacts with SH3 domains of several signaling molecules, including c-Src tysosine kinases. This interaction does not extend to other members of nuclear receptor superfamily tested (GR, AR, ER and TR)
- Through this polyproline motif and SH3 interaction, PR is a potent activator of Src and Hck. working by an SH3 displacement mechansim.
- Progesterone treatment causes a rapid and transient interaction between PR and c-Src and rapid activation of c-Src kinase and down-stream MAP kinase in breast cancer cells. This rapid effect of progesterone is dependent on PR-SH3 interaction.
- PR and ER can interact with Src simultaneously. PR interacts through SH3 domain. ER interacts with SH2 domain of c-Src but failed to activate down-regulated Src tyrosine kinase, Hck, *in vitro*.
- This non-genomic action of PR through SH3 interaction affects progestin-induced growth arrest of breast epithelial cells and induction of *Xenopus* oocyte maturation

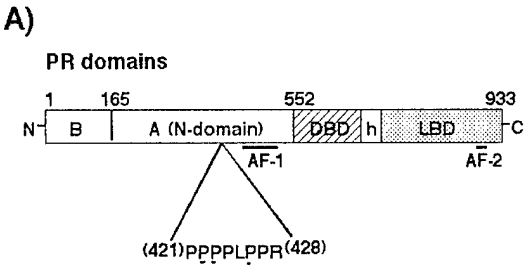
Reportable Outcomes

Abstracts for each presentation is included in Appendices

- Oral presentation at the Endocrine Society's 82nd Annual Meeting, June 21-24, 2000, Toronto, Canada, Abstract # 1971
- The 2000 Burroughs Wellcome Fund Travel Grant Award for the presentation at 82nd Annual Meeting of the Endocrine Society.
- Oral presentation at the 11th International Congress of Endocrinology (ICE2000), Sydney, Australia, November 2, 2000 Abstract OR326
- Manucript Title " Progesterone receptor contains a proline-rich sequence in the amino terminus that directly interacts with SH3 domains and activates Src family tyrosine kinases." *(Submitted to Molecular Cell).*

Conclusion

We had unexpectedly discovered a novel direct interaction between polyproline helix motif in the N-terminus of PR and SH3 domain of several cytoplasmic signaling molecules including Src tyrosine kinases. This interaction mediates rapid, non-genomic progestin-mediated activation of Src and its downsteam target MAP-kinase in breast cancer and normal breast epithelial cells. This interaction is unique property of PR. No interaction was observed with other nuclear hormone receptor tested Mutation of this polyproline motif resulted in delayed progestin-induced growth arrest of breast epithelial cells and delayed progestin induced *Xenopus* oocyte maturation. The new findings in this report may help us to understand the molecular basis of the rapid non-genomic action of progesterone in breast cancer cells.



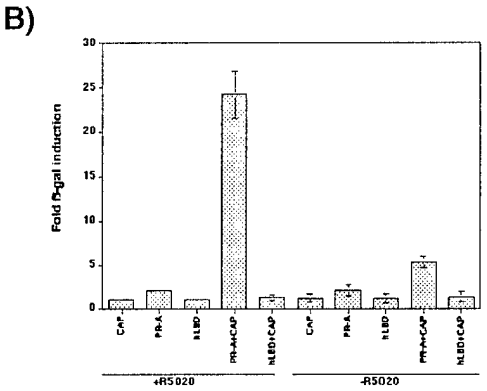


Figure 1. PR interaction with SH3 domains mediated by a polyproline motif. A) Schematic of human PR and the polyproline motif between amino acids 421 to 428. The proline residues underlined were converted to alanines to create mutant PR($_{mPro}$). LBD, ligand binding domain; DBD, DNA binding domain; h, hinge region; AF-1, transcriptional activation domain –1; AF-2 transcriptional activation domain -2. **B)** Hormone-dependent PR interaction with the SH3 domain of CAP *in vivo* by yeast two hybrid assay. PR-A or the ligand binding domain of PR (LBD) (aa 633-933) were fused to GAL4DBD and expressed in yeast (CG1945) as single hybrids, or with CAP-SH3 fused to the GAL4 activation domain (AD). CG1945 contains an integrated β-galactosidase reporter harboring upstream GAL4 activator sequences. The data are represented as average fold inductions (-/+ SEM) of enzyme activity (normalized to OD600) mediated by the presence of two hybrids over the one-hybrid controls.

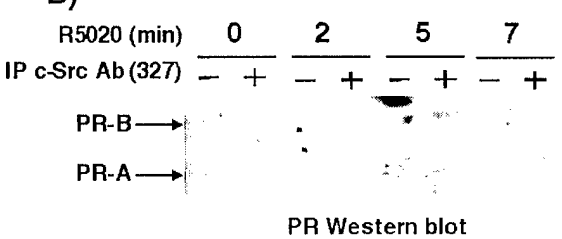
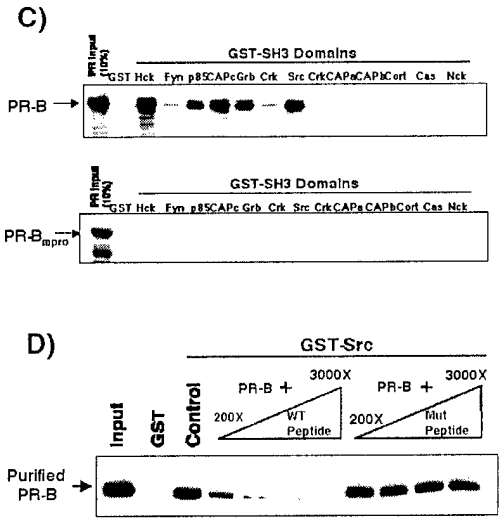
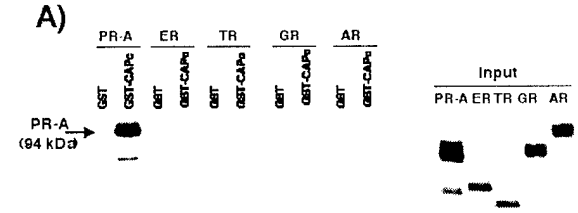


Figure 2. PR-SH3 interaction is hormone-dependent *in vitro* and within cells. A). PR-B or PR-B$_{mPro}$ were expressed for 48 hr from adenovirus vectors at an M.O.I. of 50 in MCF-12A human breast epithelial cells. Cells were then treated for 1 hr at 37°C with R5020 (10nM) prior to harvest. Interaction of receptors in cell lysates with the SH3 domain of Src by GST-pull down assay was done as above (Figure 1 C&D). Equal levels of PR-B and PR-B$_{mPro}$ were expressed in MCF-12A cells as detected by Western blot (not shown). **B)** T47D breast cancer cells were treated for 0, 2, and 5 min with R5020 (10nM). Cell lysates were prepared and immunoprecipitated with c-Src antibody (327) or an unrelated control antibody (-). Immunoprecipitates were analyzed for PR by Western blot with the PR specific antibody 1294.



C) Direct *in vitro* interaction of PR with SH3 domains of signaling molecules is dependent on the polyproline motif. PR-B or PR$_{mPro}$ bound to the synthetic progestin R5020 was expressed in the baculovirus system. Crude extracts from Sf9 cells were incubated with free GST or the indicated GST SH3 domains immobilized to glutathione Sepharose beads. PR bound to the beads was eluted and analyzed by Western blotting with the PR specific monoclonal antibody 1294. The input lanes are 10% of Sf9 cell extracts. As determined by Western blot with a GST specific antibody (794/H12), free GST and each GST-fusion protein were equally loaded (not shown). **D)** A synthetic peptide containing the PR proline-rich motif inhibits PR-SH3 interaction. PR-B binding to GST-Src (U-SH3-SH2 domains) by pull-down assay was performed as in C above except in the presence and absence of varying concentrations of a synthetic peptide (200-3,000 fold molar excess over PR-B) containing either the wild type PR proline-rich motif (WT) or a mutant motif (Mut). PR-B was purified by nickel affinity resins.



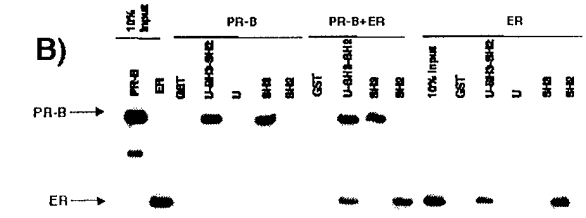


Figure 3. Other nuclear receptors do not bind SH3 domains directly. A) Human PR-A, ER, TRβ, GR and AR were expressed in the baculovirus system with N-terminal polyhistidine tags and incubated with their cognate hormone ligands in Sf9 cells. Crude extracts of Sf9 cells were incubated with GST or GST-CAP SH3 immobilized to glutathione Sepharose beads. Bound receptors were analyzed by Western blot with antibody specific for the 6x polyhistidine tag (upper panel). The right panel is 10% of the input for each receptor. **B)** PR and ER bind Src simultaneously through SH3 and SH2 domains respectively. Baculovirus expressed PR-B and ERα bound to their respective hormonal ligands (R5020 and estradiol), were purified by nickel affinity resins. Binding of each receptor separately, or as an equal molar mixture, with the indicated GST-domain constructs of c-Src was performed by pull-down assay. Bound receptors were detected by Western blot with 6x polyhistidine antibody.

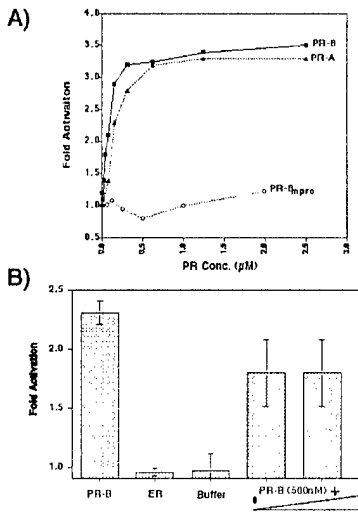


Figure 4. Down-regulated HcK is activated by PR *in vitro* through SH3 domain displacement. A) Down-regulated Hck was incubated with varying concentrations (6 nM to 2.5uM) of purified PR-A, PR-B or mutant PR-B $_{mPro}$ each bound to the synthetic progestin (R5020). Receptors were expressed in baculovirus Sf9 insect cells with 6x histidine tags and purified by nickel affinity resins. Hck enzymatic activity was measured by a spectrophotometric method for detection of phosphate incorporation into a synthetic peptide substrate. The values represent fold-induction of Hck enzyme activity by PR over a control with PR purification buffer. **B)** Down-regulated Hck was incubated with a single concentration of purified PR-B (1.4μm), ERα (1.5μm), the ER purification buffer, or a mixture of PR-B (500nm) and ERα (0, 200 and 500nm). Hck catalytic activity was measured as in A above.

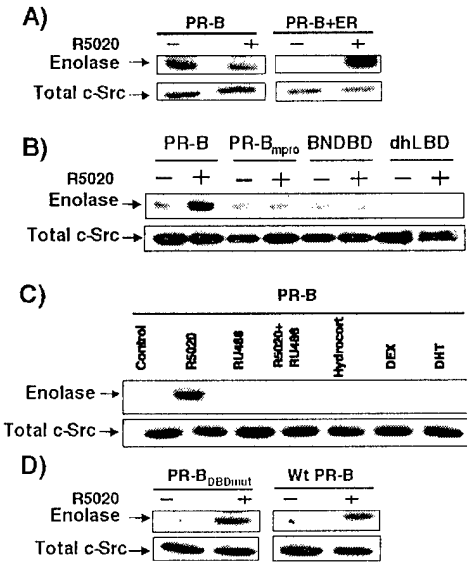
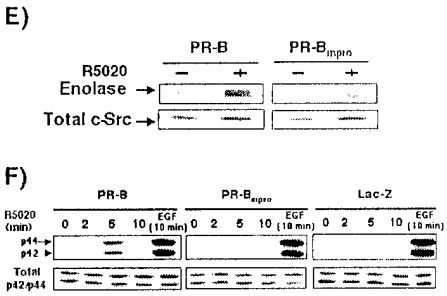


Figure 5. Rapid progestin stimulation of c-Src and MAP kinase activity in mammalian cells is dependent on PR-SH3 domain interactions. A-D) Cos-7 cells co-transfected with expression vectors for c-Src, and the indicated PR or ER constructs, (0.1ug, 2ug and 0.25ug respectively), were treated for 0 or 2 min with R5020 or the other hormone or ligands indicated (all at 10nM). Immunoprecipitated c-Src was assayed for kinase activity by 32P-incorporation into the substrate enolase (upper panels). Total c-Src protein in cell lysates was detected by Western blot with the 327 antibody (lower panels). The receptor constructs were all inserted into a CMV based mammalian expression vector pcDNAI (InVitrogen) and includes PR-B, ERα, PR-B $_{mPro}$, PR truncation mutants lacking either the ligand binding domain (BNDBD) or the N-terminal domain (dhLBD), and PR-B with a point mutation in the DNA binding domain C537A (PR-B DBDmut).



E) MCF-12A breast epithelial cells were grown for 24 hr to 70-80% confluence in 100mm dishes and were then infected with PR or GFP adenovirus at an M.O.I of 10.0. At 48 hr after infection, cells were treated for 0 or 2 min with R5020 (10nM) and c-Src activity was measured as in A-D above. **F)** MCF-7 breast cancer cells stably transfected with PR-B, PR-B $_{mPro}$ or Lac Z were grown for 5 days on estrogen-depleted medium and were then treated for 0 or 2 min with R5020 (10nM). Activated MAP kinase was detected by Western blot with a phosphospecific p42/p44 antibody and total MAPK protein with a pan p42/p44 antibody (Cell Signaling Technology).

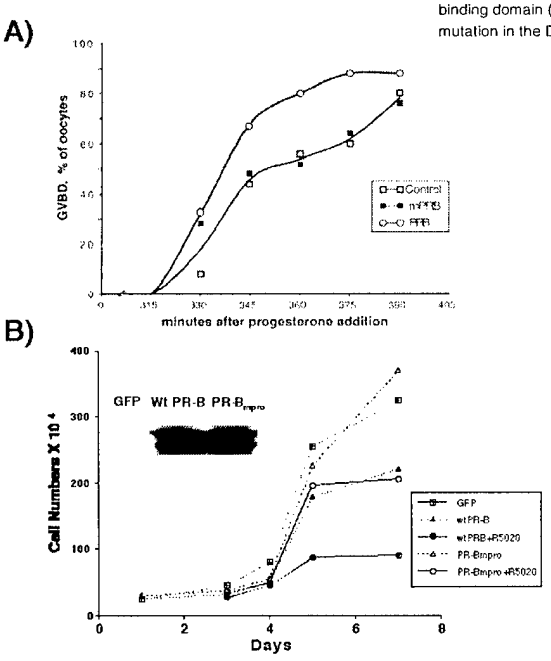


Figure 6. The role of PR-SH3 domain interaction in biological responses to progesterone. A) Progesterone induced *Xenopus* oocyte maturation. Oocytes were injected with mRNA (1.5mg/ml) encoding either β-gal (control), PR-B, or PR-B $_{mPro}$ as indicated, and incubated for 24 hr to allow accumulation of recombinant protein. Then oocytes were treated with progesterone and the kinetics of GVBD determined as described in Materials and Methods. **B)** Growth arrest of immortalized normal human breast epithelial cells. MCF-12A cells (plated at 200,000/well in 6 well dishes) grown in complete medium were infected at 24 hr after plating with adenovirus vectors for PR-B, PR-B $_{mPro}$ or GFP at M.O.I.s of 10.0. At 24 hr after infection to allow PR to accumulate, cells were treated with and without R5020 (10nM) and were harvested and counted at the times indicated. The data points are average values from quadruplicate culture wells and this is representative of four independent experiments. Wild type and mutant PR were expressed at equal levels out to 7 days in culture, while no PR was detected in GFP control cells (Western blot inset).

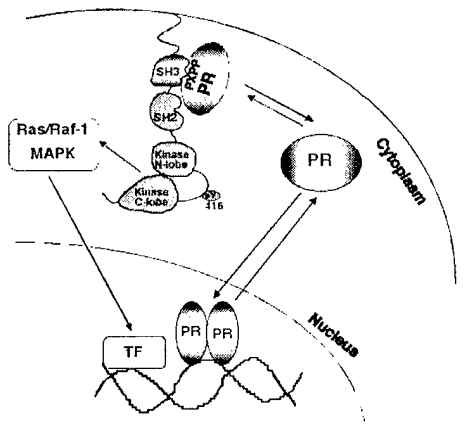


Figure 7. Model of PR function as a dual function protein. PR modulates cell signaling pathways in the cytoplasm through hormone-dependent interaction with regulatory SH3 domains and in the nucleus as a hormone-dependent transcription factor.

References

Aronica, S.M., Kraus, W.L., and Katzenellenbogen, B.S. (1994). Estrogen action via the cAMP signaling pathway: Stimulation of adenylate cyclase and cAMP-regulated gene transcription. Cell Biol. 91, 8517-8521.

Barker, S.C., Kassel, D.B., Weigel, D., Huang, X., Luther, M.A., and Knight, W.B. (1995). Characterization of pp60c-src tyrosine kinase activities using a continuous assay:autoactivation of the enzyme is an intermolecular autophosphorylation process. Biochemistry 34, 14843-14843.

Bayaa, M., Booth, R.A., Sheng, Y., and Liu, X.J. (2000). The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proc. Natl. Acad. Sci. USA 97, 12607-12612.

Baumann, C.A., Ribon, V., Kanzaki, M., Thurmond, D.C., Mora, S., Shigematsu, S., Bickel, P.E., Pessin, J.E., and Saltiel, A.R. (2000). CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 407:202-207.

Boonyaratanakornkit, V., Melvin, V., Prendergast, P., Altman, M., Ronfani, L., Bianchi, M.E., Taraseviciene, L., Nordeen, S.K., Allergretto, E.A., and Edwards, D.P. (1998). High-mobility group chromatic protein 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding *in vitro* and transcriptional activity in mammalian cells. Mol. Cell Biol. 18, 4471-4487.

Brown, M.T., and Cooper, J.A. (1996). Regulation, substrates and functions of SRC (Review). Biochimica et Biophysica Acta. 1287, 121-149.

Burton, E., Hunter, S., Wu, S., and Anderson, S. (1997). Binding of Src-like kinases to the beta subunit of the interleukin-3 receptor. J. Biol. Chem. 272, 16189-16195.

Chen, Z., Yuhanna, I.S., Galcheva-Gargova, Z., Karas, R.H., Mendelsohn, M.E., and Shaul, P.W. (1999). Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J. Clin. Invest. 103, 401-406.

Clemm, D.L., Sherman, L., Boonyaratanakornkit, V., Schrader, W.T., Weigel, N.L., and Edwards, D.P. (2000). Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol. Endocrinol. 14,52-65.

Christensen, K., Estes, P.A., DeMarzo, A.M., Oñate, S.A., Beck, C.A., Nordeen, S.K., and Edwards, D.P. (1991). Characterization and functional properties of A and B forms of human progesterone receptor synthesized in insect cells in a baculovirus system. Mol. Endocrinol. 5, 1755-1770.

Ferrell, J.E. (1999). Xenopus oocyte maturation: New lessons from a good egg. Bio Essays 21, 833-842.

Filardo, E.J., Quinn, J.A., Bland, K.I., and Frackelton, Jr. A.R. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GRP30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Mol. Endo. 14, 1649-1660.

Giangrande, P.H., and McDonnell, D.P. (1999). The A and B isoforms of the human progesterone receptor: Two functionally different transcription factors encoded by a single gene. Recent Progress in Hormone Research 54, 291-300.

Hunter, S., Burton, E., Wu, S., and Anderson, S. (1998). Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphotidylinositol 3-kinase. J. Biol. Chem. 274, 2097-2106.

Improta-Brears, T., Whorton, A.R., Codazzi, F., York, J.D., Meyer, T., and McDonnell, D.P. (1999). Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc. Natl. Acad. Sci. USA 96, 4686-4691.

Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyers, R., and Grohé, C. (2000). Estrogen receptor α rapidly activates the IGF-1 receptor pathway. J. Biol. Chem. 275, 18447-18453.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-related promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. 9:1603-1614.

Kim, H.P., Lee, J.L., Jeong, J.K., Bae, S.W., Lee, H.K., and Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor α localized in caveolae. Biochem. Biophy. Res. Comm. 263, 257-262.

Lange, C.A., Richer, J.K., and Horwitz, K.B. (1999). Hypothesis: Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. Mol. Endocrinol. 13, 829-836.

Lim, C.S., Baumann, C.T., Htun, H., Xian, W., Irie, M., Smith, C.L., and Hager, G.L. (1999). Differential localization and activity of the A- and B-form of the human progesterone receptor using green fluorescent protein chimeras. Mol. Endocrinol. 13, 366-375.

Lin, V.C.-L., Ng, E.H., Aw, S.E., Tan, M.G.-K., Ng, E.H.-L., and Bay, B.H. (2000). Progesterone induces focal adhesion in breast cancer cells MDA-MB-231 transfected with progesterone receptor complementary DNA. Mol. Endocrinol. 14, 348-358.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, Jr. C.A., Shyamala, G., Conneely, O.M., O'Malley, B.W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 9, 2266-2278.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995). The nuclear receptor superfamily: The second decade. Cell 83, 835-839.

McEwen, B.S. (1991). Non-genomic and genomic effects of steroids on neural activity. TIPS Reviews 12, 141-147.

Melvin, V., and Edwards D.P. (in press). Expression and purification of recombinant progesterone receptor in baculovirus and bacterial systems. In Methods in Molecular Biology, J.M. Walker, B. Liberman eds. (Humana Press, Inc.).

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola E., and Auricchio, F. (1996). Tyrosine kinase/p21^{ras} /MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. 15, 1292-1300.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21^{ras}/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J. 17, 2008-2018.

Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M.V., Ametrano, D., Zannini, M.S., Abbondanza, C., and Auricchio, F. (2000). Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation. EMBO J. 19, 5406-5417.

Moarefi, I., Lafevre-Bernt, M., Sicheri, F., Huse, M., Lee, C-H., Kuriyan, J., and Miller, W.T. (1997). Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature 385, 650-653.

Norfleet, A.M., Thomas, M.L., Gametchu, B., and Watson, C.S. (1999). Estrogen receptor-α detected on the plasma membrane of aldehyde-fixed GH_3/B6/F10 rat pituitary tumor cells by enzyme-linked immunocytochemistry. Endocrinology 140, 3805-3814.

Paine, T.M., Soule, H.D., Pauley, R.J., and Dawson P.J. (1992). Characterization of epithelial phenotypes in mortal and immortal human breast cells. Intl J. Cancer 50, 463-473.

Porter, M., Schindler, T., Kuriyan, J., and Miller, W.T. (2000). Reciprocal regulation of Hck activity by phosphorylation of Tyr^{527} and Tyr^{416}. J Biol. Chem. 275, 2721-2726.

Razandi, M., Pedram, A., Greene, G.L., and Levin, E.R. (1999). Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol. Endocrinol. 13, 307-319.

Revelli, A., Massobrio, M., and Tesarik, J. (1998). Nongenomic actions of steroid hormones in reproductive tissues. Endo. Rev. 19, 3-17.

Ribon, V., Herrera, R., Kay, B.K., and Saltiel, A.R. (1998a). A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. J. Biol. Chem. 273, 4073-4080.

Ribon, V., Printen, J.A., Hoffman, N.G., Kay, B.K. and Saltiel, A.R. (1998b). A novel, multifunctional c-Cbl binding protein in insulin receptor signaling in 3%3-L1 adipocytes. Mol. Cell. Biol. 18, 872-879.

Schindler, T., Sicheri F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999). Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. Mol Cell. 3, 639-648.

Schlegel, A., Wang, C., Katzenellenbogen, B.S., Pestell, R.G., and Lisanti, M.P. (1999). Caveolin-1 potentiates estrogen receptor α (ERα) signaling. J. Biol. Chem 274, 33551-33556.

Schwab, M.S., Kim, S.H., Terado, N., Edfjäll, C., Kozma, S.C., Thomas, G., and Maller, J.L. (1999). $P70^{S6K}$ controls selective mRNA translation during oocyte maturation and early embryogenesis in *Xenopus* laevis. Mol. Cell. Biol. 19, 2485-2494.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407, 538-541.

Spivac, J.G., Erikson, R.L., and Maller, J.L. (1984). Microinjection of $PP60^{v\text{-}src}$ into *Xenopus* oocytes increases phosphorylation of ribosomal protein S6 and accelerates the rate of progesterone-induced meiotic maturation. Mol. Cell. Biol 4, 1631-1634.

Sutherland, R.L., Prall, O.W.J., Watts, C.K.W., Musgrove, E.A. (1998). Estrogen and progestin regulation of cell cycle progression. J. Mammary Gland Biol. Neoplasia 3, 63-72.

Thomas, S.N., and Brugge, J.S. (1997). Cellular functions regulated by SRC family kinases. Ann. Rev. Cell Dev. Biol. 13, 513-609.

Tian, J., Kim, S., Heilig, E., and Ruderman, J.V. (2000). Identification of XPR-1, a progesterone receptor required for Xenopus oocyte activation. Proc. Natl. Acad. Sci. USA 97, 14358-14363.

Tyagi, R.K., Amazit, L., Lescop, P., Milgrom, E., and Guiochon-Mantel, A. (1998). Mechanism of progesterone receptor export from nuclei: Role of nuclear localization signal, nuclear export signal, and ran guanosine triphosphate. Mol. Endocrinol. 12, 1684-1695.

Watson, C.S., and Gametchu, B. (1999). Membrane-initiated steroid actions and the proteins that mediate then. Proc. Soc. Exp. Biol. Med. 9-19. Review.

Wehling, M. (1997) Specific, non-genomic actions of steroid hormones. Annu. Rev. Physiol. 59, 365-393.

Xu, W., Doshi, A., Lei, M., Eck, M.J., and Harrison, S.C. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol. Cell. 3, 629-638.

Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W., and Schreiber, S.L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76, 933-945.

Appendices

Abstracts presented at The Endocrine Society's 82nd Annual Meeting, June 21-24, 2000, Toronto, Canada. Abstract #1971

Progesterone receptor contains proline-rich sequences that directly interact with SH3 domains of Src-tyrosine kinase family members. V. Boonyaratanakornkit[1], M.P. Scott[2], S.M. Anderson[1], W.T. Miller[2], and D.P. Edwards[1], Pathology Dept and Mol. Bio. Prog , U of Colorado HSC, Denver, CO 80262[1]. Dept of Physiology and Biophysics, SUNY, Stony Brook, NY 11794.

As sequence specific transcriptional activators, progesterone (PR) and estrogen receptor (ER) mediate many of the biological actions of progesterone and estrogen through direct activation of target genes. There is increasing evidence that PR and ER can also mediate non-genomic effects through interaction with and activation of the Src/Ras/Erk signaling pathway. Using full length PR (A-form) as the target in a yeast two hybrid screen, the SH3 domain of c-Cbl-associated protein (CAP) was identified to interact with the N-terminal domain of PR in a hormone dependent manner *in vivo*. By GST-pull down experiments, PR was found to interact directly *in vitro* with the SH3 domain of CAP as well as selected SH3 domains of other signaling molecules including the Src-tyrosine kinase family members: c-Src and Hck. The N-domain of PR contains a proline-rich sequence (aa 421-428) that conforms to a consensus class II polyproline helix (PPII) ligand for SH3 domains and was shown by mutagenesis to be required for PR interaction with SH3 domains. Other nuclear receptors lack the PPII sequence and those tested (ER, GR, AR and TR) failed to interact with SH3 domains *in vitro*. PR interaction with full length Src and Hck involved additional hormone-dependent determinants in the ligand binding domain (LBD) of PR and unknown kinase determinants outside of the SH3 domain. The interaction between PR and Src *in vitro* was enhanced by addition of ER and a tertiary complex between PR-Src and ER was also detected. The interaction between ER and Src was mapped to the SH2 domain. Using coimmunoprecipation, a transient hormone-dependent interaction between PR and Src was detected in mammalian cells that was optimal at 5 min of treatment. The Src-family kinases are autoinhibited by intramolecular associations between the SH2 domain and a C-terminal tyrosine phosphorylation site and between the SH3 domain and a polyproline like helix in the linker connecting the catalytic domain keeping the kinase in an inactive conformation. Disruption of these interactions by dephosphorylation and/or competition by external ligands converts the kinase to an active conformation. *In vitro*, PR was a potent activator of tyrosine phosphorylated down regulated Hck via displacement of the intramolecular association of SH3 domain with the catalytic domain. These results suggest a novel function role for PR as an activator of Src-kinase signaling pathways through direct interaction with the SH3 domain and a

possible role of ER which enhances Src-PR interaction. Together, these findings may begin to unravel the molecular mechanism for non-genomic actions of estrogen and progesterone.

Abstracts presented at the 11th International Congress of Endocrinology (ICE2000), Sydney, Australia, November 2, 2000 Abstract OR326

A PROLINE-RICH SEQUENCE IN PROGESTERONE RECEPTOR DIRECTLY INTERACTS WITH AND ACTIVATES SRC TYROSINE KINASES VIA SH3 DOMAIN DISPLACEMENT.

V. Boonyaratanakornkit*[1], M.P. Scott[2], S.M. Anderson[1] , W.T. Miller[2], and D.P. Edwards[1], Pathology, U of Colorado HSC, Denver, CO 80262, USA[1]. Physiol. & Biophys, SUNY, Stony Brook, NY 11794, USA[2].

There is increasing evidence that the proliferative effects of estrogen (E) and progesterone (PG) can be mediated through rapid estrogen (ER) and progesterone receptor (PR) interaction with the Src/Ras/Erk signalling pathway. Using a yeast two-hybrid screen, we have identified that PR interacts with SH3 domains of selected signalling molecules including, c-Src and Hck. PR-SH3 domain interaction is direct as determined by GST-pull down assays with purified proteins. The site of interaction was mapped to a proline rich sequence (aa 421-428) in the N-terminus of PR that conforms to a consensus class II polyproline helix (PPII) ligand for SH3 domains. Point mutations in the PPII (PR_{mpro}) abolished the interactions. Other nuclear receptors lack the PPII and those tested (ER, GR, AR, TR) failed to interact with SH3 domains *in vitro*. The PR-Src interaction *in vitro* was enhanced by addition of ER and a PR-Src-ER tertiary complex was detected. ER interacted only with the SH2 domain of Src. Using coimmunoprecipation, a transient hormone-dependent PR-Src interaction was detected in breast cancer cells (T47D). c-Src kinase activity was also transiently increased between 2-5 min of treatment. Cotransfection experiments in COS-7 cells showed that wild type PR mediated a rapid PG activation of Src kinase activity, whereas PR_{mpro} did not. The Src-family kinases are autoinhibited by intramolecular associations between the SH2 domain and a C-terminal tyrosine phosphorylation site and the SH3 domain and a PPII-like helix in the linker region, keeping the kinase in an inactive conformation. Disruption of these interactions by competition with external ligands converts the kinase to an active conformation. *In vitro*, PR was a potent activator of down-regulated Hck via displacement of SH3 with the catalytic domain, whereas PR_{mpro} had no effect. These results suggest a novel role for PR as an activator of Src-kinase signalling pathways through direct interaction with the SH3 domain and a possible role of ER to enhance PR-Src interaction. These findings may begin to unravel the mechanism for non-genomic actions of ER and PR on cell proliferation.

THE ENDOCRINE SOCIETY

Burroughs Wellcome Fund
Travel Grant Award

is presented to

Viroj Boonyaratanakornkit, Ph.D.

for exceptional research presented at the
82nd Annual Meeting of The Endocrine Society

Toronto, Canada
June 20, 2000

J Larry Jameson

President