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REGULATION OF PROGESTERONE RECEPTOR ACTIVITY BY CYCLIN DEPENDENT KINASES 1 AND 2 OCCURS IN PART BY PHOSPHORYLATION OF THE SRC-1 CARBOXYL-TERMINUS

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Abstract

We described previously a novel role for cyclin A2/Cdk2 as a progesterone receptor (PR) coactivator. In reporter gene assays, cyclin A2 overexpression enhanced PR activity while inhibition of Cdk2 activity using the chemical inhibitor roscovitine or Cdk2 siRNA strongly inhibited PR activity. We demonstrate here that both Cdk1 and Cdk2 contribute to maximal induction of endogenous progestin responsive genes in T47D breast cancer cells. Our earlier studies suggested that the mechanism by which cyclin A2/Cdk2 enhances PR activity is via phosphorylation of steroid receptor coactivator-1 (SRC-1), which increases PR-SRC-1 interactions. To assess the importance of SRC-1 phosphorylation in the regulation of PR activity, SRC-1 was phosphorylated by cyclin A2/Cdk2 in vitro and seventeen phosphorylation sites were identified using biochemical techniques. We show that one of these sites, T1426 (adjacent to the C-terminal LXXLL nuclear receptor interaction motif), is an in vivo target of Cdks in mammalian cells and an in vitro target of Cdk1 and Cdk2. Phosphorylation of T1426 also contributes to SRC-1 coactivation potential, as mutation of the threonine target site to alanine results in reduced stimulation of PR activity by SRC-1. Together, these results suggest a role for Cdk1 and Cdk2 in the regulation of endogenous PR activity in part through phosphorylation of SRC-1.

Keywords

Cdk1; Cdk2; SRC-1; progesterone receptor; phosphorylation

1. INTRODUCTION

The ovarian hormone progesterone regulates female reproductive processes through the progesterone receptor (PR), which is expressed as two isoforms, PR-B and PR-A (Kastner et al., 1990). Coregulators are essential for optimal function of PR and other transcription factors; the best characterized steroid receptor coregulators are the three members of the steroid receptor coactivator (SRC) family, SRC-1, SRC-2/GRIP1/TIF2 and SRC-3/AIB1/RAC3/TRAM-1/ACTR/pCIP (McKenna et al., 1999). Typically, the ligand-bound receptor binds to DNA in the regulatory regions of target genes where it interacts with coactivators

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and directs assembly of a multi-component transcription complex to facilitate gene transcription.

Our laboratory has shown that the cell cycle regulator cyclin A2/cyclin dependent kinase 2 (Cdk2) is a PR coactivator, and that Cdk2 kinase activity is required for optimal PR function (Narayanan et al., 2005a). Coactivation of PR by cyclin A2/Cdk2 is independent of its ability to phosphorylate PR, as a PR-A with all previously identified Cdk2 target sites mutated to alanine retains coactivation by cyclin A2 in transient transfection assays (Narayanan et al., 2005a). Coactivation of PR by cyclin A2/Cdk2 instead likely involves phosphorylation of SRC-1, which enhances the interaction between SRC-1 and PR (Narayanan et al., 2005a).

Posttranslational modifications such as phosphorylation can lead to remarkable complexity in the regulation of protein function depending on the pattern of sites targeted in response to different stimuli. Phosphorylation, and de-phosphorylation, can modify interactions, localization, activity, structure and stability of numerous nuclear receptors and coactivators (Han et al., 2009; Li and Shang, 2007; Munz et al., 2010; Weigel and Moore, 2007). We identified previously seven phosphorylation sites in SRC-1, of which two (T1179 and S1185) are in vitro targets of the mitogen activated protein kinase (MAPK) pathway (Rowan et al., 2000b) and required for optimal coactivation of chicken PR-A and human androgen receptor (AR) (Rowan et al., 2000a; Ueda et al., 2002). SRC-1 is also phosphorylated at S103, S565, S566, S569 and S582 following DNA damage; most likely by ATM (ataxia telangiectasia mutated) or ATR (ataxia telangiectasia and Rad3 related) pathways (Matsuoka et al., 2007; Stokes et al., 2007). Other kinases that may phosphorylate SRC family members include protein kinase A (Rowan et al., 2000a), Abl (Oh et al., 2008), glycogen synthase kinase-3 (Wu et al., 2007), I kappa B kinase (Park et al., 2005; Wu et al., 2002), casein kinase 1 δ (Giamas et al., 2009), atypical protein kinase C (Yi et al., 2008) and Cdk2 (Narayanan et al., 2005a). Although SRC family members exhibit similarity in sequence and domain structure it is likely that differential phosphorylation patterns induced by different kinases may confer the tissue, cell and promoter specific actions of SRCs described in studies of knockout mice and cell lines (Karmakar et al., 2009; Shang and Brown, 2002; Xu and Li, 2003).

Our previous studies on PR regulation by cyclin A2/Cdk2 were, like many others investigating coactivator function, largely performed with transient transfection assays. This study aimed to investigate the role of Cdk2, as well as Cdk1, in regulating endogenous PR activity and, to further evaluate the mechanism by which this occurs, identify the sites in SRC-1 that are phosphorylated by these kinases. The results further support a role for cell cycle proteins in the regulation of gene expression by PR via phosphorylation of SRC-1.

2. MATERIALS AND METHODS

2.1 Cell culture

General reagents were purchased from Invitrogen (Carlsbad, CA). All cell lines were obtained from the American Type Culture Collection (Manassas, VA). T47D cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 5 μ g/ml insulin at 37°C with 5% CO₂. HeLa and COS-1 cells were maintained in DME medium containing 10% FBS with penicillin and streptomycin at 37°C with 5% CO₂. Roscovitine and NU6102 were obtained from Calbiochem (San Diego, CA). R5020 and R1881 were from Perkin Elmer (Waltham, MA). 1,25(OH)₂D₃ (1 α ,25-dihydroxyvitamin D₃) was from Solvay Pharmaceuticals (Weesp, The Netherlands).

2.2 Small interfering RNA (siRNA) transfection

T47D cells in medium with 10% FBS and 5 µg/ml insulin (200,000 cells/well in 12 well plates) were transiently transfected with siRNA using Lipofectamine reagent (Invitrogen) for five hours in serum free medium before addition of RPMI containing 10% FBS. Cdk2 (Catalog number AM51333, ID 42820, Ambion, Austin, TX), Cdk1 (Catalog numbers J-003224-13 and J-003224-16, Dharmacon, Lafayette, CO) and control non-targeting (D-001206-13, Dharmacon) siRNAs were used. Hormone or vehicle (0.1% ethanol) was added 48 hours after transfection for another four hours.

2.3 Real-time reverse transcription (RT)-PCR

RNA was isolated using TRIzol Reagent (Invitrogen) and reverse transcribed using random primers and SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Master Mix or SYBR Green PCR Master Mix using standard conditions on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers and probes (indicated in Supplemental Table S1) were purchased from Sigma-Genosys (The Woodlands, TX). Control 18S rRNA primer-probe mix was purchased from Applied Biosystems. The relative mRNA level of each target gene was determined using a standard curve and normalized to the level of 18S rRNA.

2.4 Western blotting

For siRNA experiments, cells were lysed in reporter lysis buffer (Promega, Madison, WI) containing 0.4 M NaCl and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml each of leupeptin, antipain, aprotinin, benzamidin HCl, chymostatin and pepstatin) using four freeze-thaw cycles. For SRC-1 Western blotting in parallel to luciferase assays, cells were lysed in cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.0]), 0.5% NP-40, protease inhibitors as above). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. Primary antibodies used were: PR 1294 (provided by Dr. Dean Edwards, Baylor College of Medicine, described in (Press et al., 2002)), actin (Chemicon, Temecula, CA), Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA), Cdk1 (Cell Signaling Technology, Danvers, MA), GST (GE Healthcare, Buckinghamshire, England), SRC-1 (BD Biosciences, San Jose, CA), FLAG (Sigma, St Louis, MO) and beta-tubulin (Upstate, Billerica, MA). For detection of phosphorylated T1426, a rabbit polyclonal antibody raised against the peptide CPTSGPQ(pT)PQAQQ was generated and epitope purified by Bethyl Laboratories (Montgomery, TX). Proteins were detected using ECL (Enhanced Chemiluminescence) Detection Reagents (GE Healthcare). Band intensity was measured using ImageJ software version 1.40g (<http://rsb.info.nih.gov/ij/>).

2.5 Purification of SRC-1-FLAG

Baculoviruses encoding full length human SRC-1 (FLAG tagged on the carboxyl terminus) and histidine-tagged human PR-B were co-expressed in Sf9 cells as previously described (Thackray and Nordeen, 2002). SRC-1-FLAG was isolated by lysis and purification using anti-FLAG M2 affinity gel (Sigma) as previously described (Thackray and Nordeen, 2002); however, we eluted SRC-1-FLAG in fractions.

2.6 Identification of phosphorylated sites

Purified full length SRC-1-FLAG, glutathione-S-transferase (GST)-SRC-1 fragments or GST alone (Sigma) were phosphorylated in vitro with GST-cyclin A/Cdk2 (2.8 µg, purified as previously described, (Harper et al., 1993)) in Cdk2 reaction buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl₂) with [γ -³²P]ATP (MP Biomedicals, Solon, OH) at a specific activity of 30,000–40,000 dpm/pmol. The reactions were incubated at 30°C for 1 hour, terminated by

addition of Laemmli sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.004% bromophenol blue, 10% β -mercaptoethanol) and separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography and bands were excised from the gel. Gel slices were incubated with sequencing grade modified trypsin (Promega) to elute and digest proteins and phosphopeptides were separated on a C-18 reversed-phase high performance liquid chromatography (HPLC) column as previously described (Knotts et al., 2001). Phosphopeptide peaks were detected with a Flow Scintillation Analyzer (Packard Instrument Company, Meriden, CT) and subjected to manual Edman degradation (MED), phosphoamino acid analysis (PAA) and secondary endoprotease digestions with GluC, AspN or LysC (Roche, Indianapolis, IN) to determine phosphorylation sites based on the known protein sequence of SRC-1 (**NP_003734.3**). Detailed procedures have been described previously (Knotts et al., 2001).

2.7 Generation of GST-SRC-1 fragments

Eight fragments of SRC-1 (F1–F8) were amplified by PCR from the full length wild type (wt) pCR3.1-SRC-1 construct using Platinum Pfx polymerase (Invitrogen) and primers indicated in Supplemental Table S2, with incorporation of BamHI and NotI restriction sites at the 5' and 3' ends, respectively. PCR products were ligated in frame into pGEX-4T-1 (GE Healthcare) and clones were verified by sequencing. Constructs were transformed into *E. coli* BL21 (DE3) cells (Stratagene, La Jolla, CA) and fusion protein expression was induced with 1 mM IPTG. Proteins were extracted with B-PER (Pierce, Rockford, IL), purified with GST sepharose 4B (GE Healthcare) and eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl [pH 8.0] as previously described (Harper et al., 1993).

2.8 Plasmid constructs and site directed mutagenesis

The pCR3.1-PR-A, pCR3.1-PR-B, pCR3.1-AR and pCR3.1-SRC-1 expression constructs and the glucocorticoid responsive element (GRE) GRE₂-*E1b*-luc and vitamin D responsive element (VDRE) VDRE-tk-luc luciferase reporter constructs have been described previously (Agoulis et al., 2003; Jenster et al., 1997; Narayanan et al., 2005a; Nawaz et al., 1999; Zou et al., 1997). pSG5-KM3F2-SRC-1-FLAG was provided by Dr. Dennis Dowhan (Baylor College of Medicine). An alanine mutation at amino acid T1426 was introduced into pCR3.1-SRC-1 and pSG5-KM3F2-SRC-1-FLAG by site directed mutagenesis using the primers 5'-CAGGACCACAGGCACCCAGGCCAGC-3' and 5'-GCTGGGCCTGGGGTGCCTGTGGTCCTG-3'. The mutant constructs were verified by sequencing.

2.9 Reporter gene assays

HeLa cells in medium with 10% charcoal stripped FBS and penicillin-streptomycin (100,000 cells/well in 12 well plates) were transiently transfected using lysine-coupled inactivated adenovirus as previously described (Nazareth and Weigel, 1996). To measure receptor activity cells were transfected with pCR3.1-PR-A (0.1 ng/well), pCR3.1-PR-B (0.5 ng/well) or pCR3.1-AR (0.5 ng/well) with, pCR3.1 or pCR3.1-SRC-1 (250 ng/well) and GRE₂-*E1b*-luc (250 ng/well) or VDRE-tk-luc (125 ng/well). To evaluate SRC-1 protein levels, HeLa cells (1,000,000 cells/10 cm dish) were transiently transfected in parallel as above with ten volumes of the same transfection mix used for 12 well plates. Hormone or vehicle was added 24 hours after transfection. Eighteen hours later, cells for luciferase assay were lysed with reporter lysis buffer. Luciferase activity, measured using Luciferase Assay Reagent (Promega) and a LuminoSkan Ascent luminometer (Thermo Scientific, Waltham, MA), was normalized to protein concentration, measured using Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Cells for parallel Western blot analysis were harvested at the same time and processed as described above.

2.10 Immunoprecipitation

COS-1 cells in medium with 10% FBS (2,000,000 cells/15 cm dish) were transiently transfected with pSG5-KM3F2-SRC-1-FLAG (5 µg/dish) and pCR3.1-PR-B (500 ng/dish) using adenovirus as described above then treated with hormone and/or inhibitors. Cells were resuspended in immunoprecipitation (IP) lysis buffer (20 mM Tris [pH 7.5], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5% glycerol, 200 µM sodium vanadate, 10 mM sodium molybdate, 20 mM NaF, protease inhibitors as above) and lysed by four freeze-thaw cycles. Lysates were diluted 1:1 in IP lysis buffer without NaCl, clarified by ultracentrifugation and added to EZview Red ANTI-FLAG M2 Affinity Gel (Sigma). Following overnight incubation, beads were washed and proteins were eluted with Laemmli sample buffer then analyzed by Western blotting.

2.11 λ-phosphatase and kinase treatments

For λ-phosphatase experiments, an aliquot of ANTI-FLAG M2 beads bound to SRC-1-FLAG was incubated with 400 U λ-phosphatase and 2 mM MnCl₂ (New England Biolabs, Ipswich, MA) at 30°C for one hour. For radioinert phosphorylation experiments, COS-1 cells in medium with 10% charcoal stripped FBS (2,000,000 cells/15 cm dish) were transfected with SRC-1-FLAG (5 µg/dish) using adenovirus. Lysates were prepared and immunoprecipitated as described above in the absence of phosphatase inhibitors. Beads were incubated with 50 mM ATP in the presence or absence of cyclin A2/Cdk1 (Cell Signaling) or purified GST-cyclin A2/Cdk2 at 30°C for one hour. SRC-1 was eluted as described above and analyzed by Western blotting.

3. RESULTS

3.1 Endogenous PR activity is regulated by Cdk1 and Cdk2

We have previously shown that the Cdk2 inhibitor roscovitine potently reduces PR activity (Narayanan et al., 2005a). However, roscovitine can inhibit numerous kinases in addition to Cdk2 (Bach et al., 2005). To further define the role of Cdk2 in regulating endogenous PR activity, we treated T47D breast cancer cells with NU6102, which has a much higher potency and specificity against Cdk1 and Cdk2 than other kinases (Davies et al., 2002) and measured the effect on expression of SGK1 (serum and glucocorticoid regulated kinase-1), a well characterized progesterin responsive gene (Boonyaratanakornkit et al., 2007). NU6102 reduced the hormone-induced SGK1 mRNA levels by approximately 50% compared to control, indicating that Cdk2 (and/or Cdk1) activity is required for maximum endogenous PR activity (Fig. 1A). In comparison, roscovitine almost completely inhibited SGK1 expression (Fig. 1A), suggesting that other kinases in addition to Cdk1/2 contribute to PR function. Neither inhibitor reduced PR protein levels, and we occasionally observed a slight increase in PR (Fig. 1A).

We then investigated the specific role of Cdk2 in T47D cells by depleting its expression using siRNA. We have shown previously that siRNA depletion of Cdk2 reduces progesterin induction of a transiently transfected reporter gene (Narayanan et al., 2005a). We confirmed this effect using a different Cdk2 siRNA by showing that Cdk2 is important for maximal expression of SGK1 and cyclin D1 (Fig. 1B), another known endogenous PR target gene (Boonyaratanakornkit et al., 2007). The siRNA effectively depleted Cdk2 mRNA and protein but did not reduce PR protein (Fig. 1B). As Cdk2 also phosphorylates PR and SRC-1 (Knotts et al., 2001; Narayanan et al., 2005a; Zhang et al., 1997) and is recruited to a progesterin responsive promoter (unpublished observations), these results support an important role for Cdk2 in regulating endogenous PR activity.

Recent studies have indicated that Cdk1 can interact with other cyclins including cyclin A2 to regulate interphase and mitosis in the absence of other interphase Cdks (Hochegger et al., 2008; Santamaria et al., 2007). Because NU6102 inhibits Cdk1 and Cdk2, we tested whether Cdk1 depletion can also affect PR activity in T47D cells. Two independent siRNAs against Cdk1 (#13 and #16) each significantly reduced the hormone dependent mRNA levels of SGK1 and cyclin D1 (Fig. 1C), suggesting an important role for Cdk1 in regulating endogenous PR activity. Cdk1 mRNA and protein levels were also reduced by both siRNAs while PR protein was slightly increased by both Cdk1 siRNAs (Fig. 1C).

3.2 Identification of Cyclin A2/Cdk2 phosphorylation targets in full length SRC-1

We have shown previously that cyclin A2/Cdk2 phosphorylates SRC-1 in vitro which increases PR-SRC-1 interactions and enhances PR activity (Narayanan et al., 2005a). To further characterize this mechanism we looked for Cdk2 phosphorylation sites in SRC-1. The amount and integrity of FLAG-tagged full length human SRC-1 purified from Sf9 cells was confirmed by SDS-PAGE, silver staining (Fig. 2A) and Western blotting with FLAG and SRC-1 antibodies (Fig. 2B). SRC-1-FLAG was then phosphorylated in vitro by cyclin A2/Cdk2 in the presence of [γ -³²P]ATP (Fig. 2C, left panel). A 35-fold increase in SRC-1 phosphorylation was observed compared to control (Fig. 2C, right panel).

Phosphopeptides were released from the gel slice containing full length SRC-1, separated by HPLC and peaks were selected for site identification (fraction numbers indicated in Fig. 2D). Manual Edman degradation (MED) determined the position of phosphorylated residue(s) relative to the N-terminus while phosphoamino acid analysis (PAA) determined the identity (i.e. serine or threonine) of the phosphorylated residue(s). Supplemental Fig. S1A shows the sequence of predicted SRC-1 tryptic phosphopeptides and MED release cycle numbers for each candidate site. In fraction 8, a cycle 7 release (supplemental Fig. S1B) suggests S569 or T979. However PAA indicates serine only in this fraction (supplemental Fig. S1C), confirming S569 as a cyclin A2/Cdk2 target. In fraction 16, a cycle 4 release suggests S1033. However, based on our previous studies, we know that the tryptic peptide containing this site elutes later from the column (Rowan et al., 2000b). We did observe a cycle 4 release in fraction 21 suggesting that this fraction contains S1033. The cycle 4 release in fraction 16 likely corresponds to S22. Although S22 should release at cycle 2, trypsin is less efficient in cleaving adjacent lysines/arginines and thus partially digested peptides are often detected. A release at cycle 4 is predicted if trypsin cleaves between K18 and R19. Fraction 19 shows a cycle 10 release, confirming S395 as a site. Fractions containing other phosphopeptide peaks were also analyzed in the same manner, however the data obtained was insufficiently clear to confirm the phosphorylated sites (data not shown). Of the four candidate cyclin A2/Cdk2 phosphorylation sites identified in full length SRC-1, three (S395, S569, S1033) were previously identified as in vivo phosphorylation sites (Rowan et al., 2000b) and one (S22) is a novel site (Fig. 2D). However, full length SRC-1 has 29 SP and TP candidate motifs and the large number of tryptic peptides generated, and the length and charge of many of them, make a thorough analysis of all the sites difficult. Because of this, an alternative strategy using SRC-1 fragments was designed to facilitate identification of other sites.

3.3 In vitro phosphorylation of SRC-1 fragments enables identification of additional Cdk2 target sites

Eight fragments of SRC-1 (F1–F8) were designed to collectively encompass all 29 potential SP and TP motifs while bringing each motif closer to the N-terminus of tryptic peptides to facilitate site identification by MED (Fig. 3A). Silver stain and Western blot analysis of *E. coli* BL21 cells expressing SRC-1 fragments indicated that multiple GST fusion proteins were produced; however, proteins of the predicted molecular weight (Supplemental Table

S2) for each fragment were present (data not shown). All eight fragments were phosphorylated by GST-cyclin A2/Cdk2 although no phosphorylation of purified GST alone was detected (Fig. 3B). Many phosphorylated bands were observed in each reaction although bands of the predicted molecular weight were present, and these were selected for site identification.

Results are summarized in Table 1 while data are presented in supplemental Figs. 2–9 and the supplemental results section. In total, 17 candidate cyclin A2/Cdk2 phosphorylation sites were identified in the eight SRC-1 fragments, nine were excluded as sites under these conditions and three remain unknown. Target sites were located throughout the protein (Fig. 3C). Five (S395, S569, S1033, T1179, S1185) of the seven previously identified S/TP *in vivo* sites were identified as Cdk2 targets, while S372 and S517 were not observed to be phosphorylated by Cdk2, suggesting that kinase pathways can differentially phosphorylate SRC-1. NetPhosK 1.0 (Blom et al., 2004) predicts phosphorylation of two common sites by Cdk1 and ten by Cdk5 and Scansite 2.0 (Obenauer et al., 2003) predicts 14 common sites by Cdk1 and 15 by Cdk5 (Fig. 3C). Of the 17 Cdk2 candidate phosphorylation sites identified in *Homo sapiens* SRC-1, 16, 16 and nine are conserved as S/TP motifs in *Mus musculus*, *Bos taurus* and *Xenopus tropicalis*, respectively (Fig. 3C). Of the published phosphorylation sites in SRC-2 and SRC-3, only two are homologous to SRC-1 candidate sites (Fig. 3C). SRC-1 S22 is homologous to SRC-3 T24 (Wu et al., 2004) and SRC-1 S488 is homologous to SRC-2 S493 (Olsen et al., 2006), although this lack of conserved sites likely is due to the low sequence similarity between the human SRC paralogs. Only three sites are found within a full consensus Cdk target motif (S/TPXR/K) while the majority of sites were found within the minimal Cdk consensus motif (S/TP) (Fig. 3C).

3.4 Mutation of T1426 reduces SRC-1 coactivation potential

The carboxyl-terminus of SRC-1 contains a unique LXXLL motif that is not conserved in other SRC family members but which interacts strongly with numerous nuclear receptors, particularly PR (Ding et al., 1998; Onate et al., 1998), and is required for maximal coactivation of PR by SRC-1 in a chromatin transcription assay (Liu et al., 2001). One of the cyclin A2/Cdk2 candidate phosphorylation sites, T1426, lies adjacent to this LXXLL motif (Fig. 4A). Because we have previously shown that the interaction between PR and SRC-1 is increased following phosphorylation of SRC-1 by Cdk2 (Narayanan et al., 2005a), it is possible that this site contributes to the dynamics of PR-SRC-1 interactions. The potential importance of T1426 is further highlighted by its evolutionary conservation and prediction as a Cdk target by NetPhosK and Scansite (Fig. 3C). To determine whether T1426 phosphorylation regulates SRC-1 activity, a non-phosphorylatable alanine mutation was introduced at this site in the full length SRC-1 and the effect on PR coactivation in transiently transfected HeLa cells was assessed. Under the conditions used, PR-A coactivation by SRC-1 was partially reduced by the T1426A mutation relative to wild type (wt) while PR-B coactivation was almost completely abolished (Fig. 4B and 4C left panels). Western blotting of extracts prepared in parallel indicated that SRC-1-T1426A protein levels were not reduced compared to wt SRC-1 (Fig. 4B and 4C right panels). Similarly, the T1426A mutation significantly compromised coactivation of AR (Fig. 4D) and vitamin D receptor (VDR) (Fig. 4E) by SRC-1 compared to wild type. These results suggest that phosphorylation at T1426 is important for the coactivation potential of SRC-1.

3.5 T1426 is an *in vivo* target of Cdk1/2 in mammalian cells

To further investigate the role of T1426 phosphorylation in the regulation of SRC-1 activity, a specific antibody against phosphorylated T1426 was generated. The specificity of this antibody was tested in COS-1 cells transiently transfected with wt SRC-1-FLAG. COS-1 cells were chosen for high protein yield and FLAG-tagged SRC-1 was used for enhanced

immunoprecipitation efficiency and elimination of background from endogenous SRC-1. The phospho-T1426 antibody detected a protein of the expected size for wt SRC-1-FLAG (Fig. 5A, lane 1). Immunoreactivity was blocked when the phospho-T1426 antibody was pre-incubated with the corresponding phosphorylated peptide (Fig. 5A, lane 3) but not with the non-phosphorylated peptide (Fig. 5A, lane 4) or a non-specific phosphorylated peptide (Fig. 5A, lane 5). Furthermore, immunoreactivity was abolished by incubation of immunoprecipitated SRC-1-FLAG with λ -phosphatase (Fig. 5A, lane 2), indicating that the antibody is specific for phosphorylated SRC-1. Specificity was further confirmed by the lack of immunoreactivity with an SRC-1 T1426A mutant lacking the target phosphorylation site (Fig. 5B). These results confirm that the antibody specifically recognizes phosphorylated T1426-SRC-1 and that this site is an authentic, novel *in vivo* phosphorylation target.

To determine whether T1426 phosphorylation is regulated by Cdks *in vivo*, COS-1 cells expressing wt SRC-1-FLAG were treated with roscovitine. Immunoreactivity with the T1426 phospho-antibody was reduced in roscovitine treated cells compared to controls (Fig. 5C left panel). Surprisingly, total immunoprecipitated SRC-1 was also reduced by roscovitine. The relative decrease of phosphorylated and total SRC-1 was therefore analyzed by imaging densitometry. Following correction for total SRC-1 levels, a 78% decrease in T1426 phosphorylation was observed in roscovitine treated cells in the representative experiment shown (Fig. 5C left panel) while over three independent experiments the average decrease was 73% (Fig. 5C center panel). To gain insight into why immunoprecipitated total SRC-1 was reduced by roscovitine, the whole cell extracts used for immunoprecipitations were also analyzed by Western blotting. Roscovitine reduced total SRC-1 levels while equal protein loading was confirmed by tubulin (Fig. 5C right panel), suggesting that SRC-1 protein steady state levels are also regulated by Cdks. NU6102 treatment also reduced T1426 phosphorylation (Fig. 5D left panel), although less than roscovitine (43% when averaged over three independent experiments, Fig. 5D right panel). This indicates that Cdk1 and/or Cdk2 can target T1426 *in vivo*. NU6102 did not decrease total SRC-1 protein levels, suggesting that Cdk1 and Cdk2 do not contribute to mechanisms regulating SRC-1 protein stability and that other targeted kinases are likely responsible for the effect of roscovitine on total SRC-1 levels. To clarify whether both Cdk1 and Cdk2 can target SRC-1 T1426, full length SRC-1-FLAG immunoprecipitated from COS-1 cells was phosphorylated *in vitro* with cyclin A2/Cdk1 or cyclin A2/Cdk2. Both kinases phosphorylated T1426 although Cdk2 appeared more efficient than Cdk1 (Fig. 5E). Together, these results suggest that both Cdk1 and Cdk2 regulate SRC-1 coactivation potential via phosphorylation of T1426.

4. DISCUSSION

We have confirmed a role for Cdk2 in regulating PR activity by demonstrating that it is required for maximum progestin induction of two endogenous PR target genes in T47D cells and have shown for the first time that Cdk1 is also required for optimal PR activity. Our original study reported that cyclin A2/Cdk2 coactivates PR; cyclin A interacts directly with PR and the associated Cdk2 kinase activity can phosphorylate the coactivator SRC-1 (Narayanan et al., 2005a). Cdk1 also interacts with cyclin A2 (Pagano et al., 1992) and contemporary models of the cell cycle describe both complexes controlling S phase (Hochegger et al., 2008). Regulation of PR activity by Cdk1 and Cdk2 is therefore consistent with our previous work showing that PR activity is cell cycle dependent, with the highest activity in S phase (Narayanan et al., 2005b). The major partner for Cdk1 is cyclin B and this complex regulates mitosis. While our *in vitro* studies showed that cyclin A2/Cdk1 phosphorylates SRC-1 at T1426, the role of cyclin B in regulating SRC-1 phosphorylation and PR activity is yet to be determined. However, PR activity and SRC-1 recruitment to a progestin responsive promoter are lower in the G2/M phase compared to S phase (Narayanan et al., 2005b); therefore cyclin B/Cdk1 may not be a strong regulator of PR.

This study suggests that one of the mechanisms by which Cdk1/2 can regulate PR activity is via phosphorylation of SRC-1 T1426, a site targeted by both kinases in vitro and in vivo and required for the maximum coactivation potential of SRC-1. Global phosphorylation of SRC-1 by Cdk2 increases its affinity for PR (Narayanan et al., 2005a). Because of its close proximity to an LXXLL motif that is required for interaction with PR (Ding et al., 1998), it was possible that mutation of the phosphorylation site would reduce the coactivation potential of SRC-1 by altering its interaction with PR. However, results from mammalian two-hybrid (supplemental Fig. S10) and co-immunoprecipitation (data not shown) assays comparing wild type and phospho-mutant SRC-1 suggest that elimination of T1426 phosphorylation alone is insufficient to inhibit PR-SRC-1 interactions. This is further supported by the reduced coactivation observed for the T1426A mutant SRC-1 on AR compared to wild type; unlike other steroid hormone receptors, AR does not interact with SRC-1 via LXXLL motifs but instead interacts predominantly with the glutamine rich region of SRC-1 (Bevan et al., 1999; Ma et al., 1999). Therefore, loss of SRC-1 phosphorylation at T1426 would not be expected to change interaction with AR and must cause an alternative defect in SRC-1 function. Moreover, T1426 is unique to the SRC-1a form of SRC-1; a somewhat shorter form with a unique carboxyl terminus (SRC-1e) also functions as a PR coactivator (Takeshita et al., 1998) and must, therefore, interact with PR. Previous studies have shown that phosphorylation can affect coactivator interaction with other components of the transcription complex, subcellular localization, stability and conformation (Han et al., 2009). Mechanism(s) responsible for the functional deficiency associated with loss of T1426 phosphorylation are currently being investigated in our laboratory.

If loss of phosphorylation at T1426 does not diminish SRC-1 interaction with PR, one or more of the other phosphorylation sites identified must be responsible for this effect. It may not be possible to predict which sites are involved based on their location within SRC-1 since many phosphorylation sites in SRC-3 have been shown to affect mechanisms associated with distant functional domains (Oh et al., 2008; Wu et al., 2004). Preliminary studies indicated that other phosphorylation sites we identified are also required for maximal coactivation of PR-B by SRC-1 (data not shown); therefore one or more of these may regulate interactions with PR. Although no sites were identified in the study, Munz et al. recently found that phosphorylation of a roscovitine sensitive site inhibited the interaction between STAT6 and SRC-1 (Munz et al., 2010). Because they had previously localized the minimum interaction region in SRC-1 to amino acids 260–370 (Litterst and Pfizner, 2001) we would speculate, based on our findings, that phosphorylation of S325 may be responsible for inhibition of the STAT6-SRC-1 interaction. Interestingly, they also demonstrated that inhibition of cyclin dependent kinases by roscovitine reduced overall SRC-1 phosphorylation in vivo and that total SRC-1 phosphorylation was greatest in S phase where Cdk2 was most active (Munz et al., 2010).

Seventeen sites in SRC-1 out of a possible 29 S/TP Cdk target motifs were identified as in vitro Cdk2 targets. While not all of these sites were detected in the context of the full length protein, and some of them may not be authentic in vivo targets, it highlights the complexity of regulation of the SRC family of coactivators by phosphorylation. Some of the Cdk2 target sites have been reported to be phosphorylated by other kinases (Matsuoka et al., 2007; Rowan et al., 2000b; Stokes et al., 2007), while others may be unique. Our finding that phosphorylation of T1426 is partially inhibited by roscovitine and NU6102 suggests that it may be a target of other kinases as well although we cannot completely exclude the possibility that the remaining phosphorylation is due to incomplete inhibition of target kinases. This is the first study to identify Cdk target sites on an SRC family member so it is not yet possible to compare the numbers of target sites found within this family. Only eleven, four and fourteen total sites have previously been published for SRC-1, 2 and 3

respectively, these being targets of a variety of kinases. As mass spectrometry techniques improve, it is likely that additional *in vivo* sites will be identified and that some will be phosphorylated only in response to specific stimuli.

We have shown that Cdk1 and Cdk2 contribute to optimal expression of two endogenous PR target genes, SGK1 and cyclin D1, which are regulated by different mechanisms of PR action. SGK1 is a classical PR regulated gene, with response elements in the promoter region that directly recruit PR following progestin treatment (Boonyaratanakornkit et al., 2007; Boonyaratanakornkit et al., 2008). In contrast, cyclin D1 is strongly regulated by the rapid signaling actions of PR (Boonyaratanakornkit et al., 2007), with some regulation by genomic PR actions through the cyclin D1 promoter (Quiles et al., 2009). Cyclin D1 is well known as a target of a variety of transcription factors including estrogen receptor (Eeckhoute et al., 2006) and NF-kappa-B (Witzel et al., 2010) and cyclin dependent kinases may affect these pathways as well. However, in our experiments (Fig. 1), the basal expression of cyclin D1 was essentially unchanged by Cdk1 or Cdk2 depletion whereas R5020 dependent induction was inhibited. This suggests that a component of PR signaling was targeted.

As Cdk1 and Cdk2 are required for optimal R5020-dependent induction of cyclin D1, this raises the question of whether these kinases also regulate the rapid extra-nuclear actions of PR or whether they contribute to the genomic component of cyclin D1 regulation. Cyclin/Cdk1 and Cdk2 complexes shuttle between the nucleus and cytoplasm (Jackman et al., 2002), suggesting that they can potentially influence PR action in either compartment. The current study together with our previous work (Narayanan et al., 2005a) support a mechanism involving assembly of a transcription complex including PR, cyclin A2/Cdk1/2 and SRC-1 at the promoters of direct PR target genes such as SGK1, followed by phosphorylation of SRC-1 by Cdk1/2 at numerous sites including T1426 and increased target gene expression. Results observed with cyclin D1 suggest an alternative mechanism where Cdk1/2 can influence PR mediated activation of rapid signaling pathways which may or may not depend on SRC-1 phosphorylation. Investigation of these multidimensional actions of Cdk1 and 2 will provide important insights into how PR functions integrate to control gene expression and cell proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

ANOVA	analysis of variance
AR	androgen receptor
Cdk	cyclin dependent kinase
ECL	enhanced chemiluminescence
ER	estrogen receptor
FBS	fetal bovine serum
GRE	glucocorticoid responsive element
GST	glutathione S-transferase
HPLC	high performance liquid chromatography
IP	immunoprecipitation

luc	luciferase
MAPK	mitogen activated protein kinase
MED	manual Edman degradation
NT	non-targeting
PAA	phosphoamino acid analysis
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
RLU	relative light units
RT-PCR	reverse transcription-PCR
SGK1	serum and glucocorticoid regulated kinase-1
siRNA	small interfering RNA
SRC-1	steroid receptor coactivator-1
VDR	vitamin D receptor
VDRE	vitamin D responsive element
wt	wild type

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Figure 1.

PR activity in T47D breast cancer cells is regulated by Cdk1 and Cdk2. (A) T47D cells growing in RPMI with 10% FBS were pre-treated with vehicle (DMSO), 10 μ M NU6102 (NU) or 30 μ M roscovitine (Ros) for one hour before addition of vehicle (ethanol) or 10 nM R5020 for another four hours. Relative mRNA levels for SGK1 were determined using real-time RT-PCR and normalized to 18S rRNA levels. PR and actin protein levels were determined by Western blot of cell extracts prepared in parallel. * $p < 0.05$ Two-way analysis of variance (ANOVA) with Tukey's post-hoc test, DMSO vs. inhibitor. (B) T47D cells were transiently transfected with 25 pmol/well control non-targeting siRNA (NT) or siRNA against Cdk2. After 48 hours, cells were treated with 10 nM R5020 (or ethanol vehicle) for another 4 hours. mRNA levels for SGK1, cyclin D1 and Cdk2 were measured by real-time RT-PCR and normalized to 18S rRNA levels. Cdk2, PR and actin protein levels in parallel wells were analyzed by Western blot analysis. (C) T47D cells were transiently transfected with 50 pmol/well control non-targeting siRNA (NT) or two independent siRNAs against Cdk1 (si 13 and si 16). After 48 hours, cells were treated with 10 nM R5020 (or ethanol vehicle) for another 4 hours. mRNA levels for SGK1, cyclin D1 and Cdk1 were measured by real-time RT-PCR and normalized to 18S rRNA levels. Cdk1, PR and actin protein levels were analyzed by Western blot analysis. * $p < 0.05$ Two-way ANOVA, NT vs. Cdk siRNA. All bars represent the average $\pm$ standard error of the mean of triplicate samples.

Figure 2.

Cyclin A2/Cdk2 phosphorylates full length SRC-1 in vitro. (A) Silver stain analysis of full length SRC-1-FLAG (fractions 4–10) purified from Sf9 insect cells. (B) Western blot analysis of purified full length SRC-1-FLAG with anti-FLAG (left panel) and anti-SRC-1 (right panel) antibodies. Note that there are some proteolytic fragments of SRC-1, presumably due to degradation after column elution. (C) SRC-1-FLAG (1 µg) was incubated with [γ - 32 P] ATP in the presence or absence of purified GST-cyclin A2/Cdk2 and the phosphorylated product was visualized using SDS-PAGE followed by autoradiography (left panel). The band corresponding to full length SRC-1 was excised from the gel and subjected to Cerenkov counting to determine the extent of phosphorylation (right panel). (D) Tryptic phosphopeptides from the excised band were separated by HPLC; peaks indicate elution of phosphorylated peptides. Arrows below the trace indicate fractions analyzed by manual Edman degradation and/or phosphoamino acid analysis that led to identification of sites. Serine or threonine residues identified by these techniques as being phosphorylated are shown with the corresponding elution peaks for their phosphopeptides. Asterisks indicate sites previously identified as in vivo sites.

Figure 3.

Phosphorylation of GST-SRC-1 fragments enables identification of additional cyclin A2/Cdk2 target sites. (A) Schematic diagram depicting full length wild type SRC-1 (wt) and the eight fragments (F1–F8) generated by cloning into the pGEX-4T-1 vector. The N-terminal GST tag is indicated adjacent to each SRC-1 fragment. Positions of S/T-P motifs are indicated by triangles and labeled with their amino acid numbers. Functional domains of SRC-1 are indicated (bHLH = basic helix-loop-helix, PAS = Per/Arnt/Sim homology domain, NRID = nuclear receptor interaction domain, AD = activation domain) while positions of LXXLL nuclear receptor interaction motifs are shown as white bars. (B) Purified GST-SRC-1 fragments or GST alone (1 µg) were incubated with [γ - 32 P] ATP in the presence or absence of purified GST-cyclin A2/Cdk2 followed by SDS-PAGE. Phosphorylated bands were visualized by autoradiography. Asterisks indicate phosphorylated SRC-1 fragments of the predicted molecular weight (sizes of molecular weight markers are indicated to the right of each panel). The arrow indicates auto-phosphorylated GST-cyclin A2 in each lane containing kinase. (C) Schematic diagram indicating the 17 candidate cyclin A2/Cdk2 target sites within the known functional domains of Homo sapiens SRC-1. Black circles indicate whether the sites shown above were previously identified as in vivo targets, predicted to be phosphorylated by Cdk2 according to NetPhosK 1.0 or ScanSite 2.0 software, conserved in the Mus musculus, Bos taurus or Xenopus tropicalis orthologs of SRC-1 or contained within a full (S/TPXR/K) or minimal (S/TP) Cdk2 consensus site. Gray circles indicate partial conservation, i.e. exact amino acid not conserved but site is still a proline directed serine or threonine residue at that position able to be targeted by Cdk2.

Figure 4. Mutation of the T1426 phosphorylation site reduces the coactivation potential of SRC-1. (A) Sequence of the SRC-1 protein surrounding the T1426 residue indicating its proximity to the C-terminus and LXXLL motif. (B–E) HeLa cells were transiently transfected with empty pCR3.1 vector, wild type (wt) or T1426A mutant SRC-1 expression constructs, the GRE₂-*Elb*-luc (B, C, D) or VDRE-tk-luc (E) reporter constructs and PR-A (B), PR-B (C) or AR (D) expression constructs. HeLa cells express endogenous vitamin D receptor (VDR) and therefore were not transfected with a VDR expression construct. After 24 hours cells were treated with vehicle or 10 nM R5020 (B, C), 3 nM R1881 (D) or 100 nM 1,25(OH)₂D₃ (1,25 D) (E) and harvested 18 hours later for luciferase activity or Western blot analysis.

Luciferase activity is expressed as relative light units (RLU) normalized to total protein concentration. SRC-1 and actin protein levels in HeLa cells transfected in parallel are shown for PR experiments (B and C right panels). Images are divided at the dotted lines to omit unrelated lanes but all samples are from the same gel. Band intensity was measured by densitometry and relative SRC-1 protein levels, corrected for actin levels, are indicated below each image (with wild type SRC-1 set to a value of 1). All bars represent the average $\pm$ standard error of the mean of triplicate samples. * $p < 0.05$ Two-way ANOVA with Tukey's post-hoc test, wt vs. T1426A.

Figure 5.

A phosphorylation specific antibody confirms T1426 as an authentic *in vivo* target of Cdk1 and Cdk2 in mammalian cells. (A) COS-1 cells were transiently transfected with PR-B and wild type (wt) SRC-1-FLAG constructs followed by overnight treatment with 10 nM R5020. Whole cell lysates were immuno-purified using FLAG affinity beads. Following the wash steps, an aliquot of beads (1/5 volume) was removed and incubated with λ -phosphatase (PPase) for one hour at 30°C prior to elution. Eluates were separated using SDS-PAGE and analyzed by Western blotting using the T1426 phospho-specific antibody (upper panel). Peptide competition experiments were performed by preincubating the phospho-antibody overnight in the presence or absence of a 200-fold molar excess of competing phosphorylated or non-phosphorylated peptide as indicated prior to addition to Western blot membranes (upper panel). Following detection of phosphorylated SRC-1, membranes were re-probed with an antibody for FLAG, detecting total SRC-1 (lower panel). (B) COS-1 cells were transiently transfected with PR-B and wt or T1426A mutant SRC-1-FLAG construct and treated with 10 nM R5020 overnight then cell lysates were immuno-purified using FLAG affinity beads. Eluates were separated using SDS-PAGE and analyzed by Western blotting using the T1426 phospho-specific antibody (upper panel) or an antibody recognizing total SRC-1 (lower panel). (C) COS-1 cells were transiently transfected with PR-B and wt SRC-1-FLAG constructs. Cells were pre-treated with vehicle or 30 μ M roscovitine for 1 hour followed by treatment with 10 nM R5020 for 5 hours prior to preparation of whole cell extracts. Following immunoprecipitation (IP) using FLAG affinity

beads, Western blot analysis was performed for phosphorylated (upper panel) and total (lower panel) SRC-1. Roscovitine-induced changes in phosphorylation were quantified by imaging densitometry; the numbers below the image indicate relative intensity of phosphorylated SRC-1 corrected for the intensity of total SRC-1 for the representative experiment shown. The graph represents average quantitation $\pm$ standard error of the mean over three independent experiments, * $p = 0.017$ independent samples t-test, DMSO vs. Roscovitine. Whole cell extracts (WCE) from the same experiment were analyzed by SDS-PAGE and Western blotting for total SRC-1 and tubulin. (D) COS-1 cells were transiently transfected with PR-B and wt SRC-1-FLAG constructs. Cells were pre-treated with vehicle or 10 μ M NU6102 for 1 hour followed by treatment with 10 nM R5020 for 5 hours prior to preparation of whole cell extracts. Following IP using FLAG affinity beads, Western blot analysis was performed for phosphorylated (upper panel) and total (lower panel) SRC-1. NU6102-induced changes in phosphorylation were quantified by imaging densitometry as described for part C. The reduction in SRC-1-T1426 phosphorylation was almost statistically significant, $p = 0.054$ independent samples t-test, DMSO vs. NU6102. (E) COS-1 cells were transiently transfected with the wt SRC-1-FLAG construct. SRC-1 was immunoprecipitated using FLAG affinity beads and incubated with cyclin A2/Cdk1 (0.6 μ g) or cyclin A2/Cdk2 (0.023 or 0.23 μ g) for 60 minutes at 30°C prior to elution. Eluates were separated using SDS-PAGE and analyzed by Western blotting with the phospho-T1426 (upper panel) and total (lower panel) SRC-1 antibodies.

Summary of PAA, MED and secondary digest data used to identify candidate cyclin A2/Cdk2 target sites in GST-SRC-1 fragments F1 through F8.

Table 1

Fraction	PAA data	MED release cycle (s)	Secondary digest data	Sites confirmed	Sites excluded	Sites unverified
GST-SRC-1-F1 (1 potential site, supplemental Fig. S2)						
14	ND	3, 4	ND	S22		
GST-SRC-1-F2 (4 potential sites, supplemental Fig. S3)						
11	ND	5	ND	S325		
18	ND	10	ND	S395		
35	ND	none	ND		S351, S372	
GST-SRC-1-F3 (8 potential sites, supplemental Fig. S4)						
10	ND	7		S569		
31	ND	none for trypsin digest 11 for trypsin + GluC digest	digests with GluC, not AspN or LysC	S488	S444, S698 S558 S517, S763	S474
GST-SRC-1-F4 (3 potential sites, supplemental Fig. S5)						
25	ND	7	ND	T979		
38	ND	8	ND	T789	T795	
GST-SRC-1-F5 (6 potential sites, supplemental Fig. S6)						
20, 21	S, T	4, 13	ND	T1026, S1033		
23	T	13	ND	T1026		
27	S	8, 17	ND	S997, S1006	S999	
31	S, T	8, 17	ND	S997, S1006		T1099
GST-SRC-1-F6 (3 potential sites, supplemental Fig. S7)						
26	ND	13	ND	S1185		
27	ND	7	ND	T1179		
GST-SRC-1-F6 + GluC (3 potential sites, supplemental Fig. S7)						

Fraction	PAA data	MED release cycle (s)	Secondary digest data	Sites confirmed	Sites excluded	Sites unverified
18	ND	8	ND	S1250		
GST-SRC-1-F7 (3 potential sites, supplemental Fig. S8)						
19	S	16	ND	S1279		
20	S	16	ND	S1279		
22	S, T	8, 16	ND	T1271, S1279		
26	ND	none	ND			T1305
GST-SRC-1-F8 (1 potential site, supplemental Fig. S9)						
10	T	ND	ND	T1426		

PAA = phosphoamino acid analysis, MED = manual Edman degradation, ND = not determined