Phosphorylation of Steroid Receptor Coactivator-1

IDENTIFICATION OF THE PHOSPHORYLATION SITES AND PHOSPHORYLATION THROUGH THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY*

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Steroid receptor coactivator-1 (SRC-1) is a member of a coactivator family that enhance the activation of the steroid/nuclear receptor superfamily of ligand-stimulated transcription factors. To study the regulation of SRC-1 by signaling pathways in the cell, the major phosphorylation sites of SRC-1 were identified in COS-1 cells using a combination of in vivo labeling with [32P]H3PO₄, modified manual Edman degradation, phosphoamino acid analysis, endoproteinase digestion, and mutagenesis of the SRC-1 phosphorylation sites. Seven phosphorylation sites were identified in SRC-1: serine 372, serine 395, serine 517, serine 569, serine 1033, threonine 1179, and serine 1185. All the sites contained consensus sequences for the serine/threonine-proline-directed family of protein kinases, and two sites (serine 395 and threonine 1179) contained a perfect consensus sequence for the mitogen-activated protein kinase family (Erk-1 and Erk-2). Furthermore, Erk-2 phosphorylated threonine 1179 and serine 1185 (and to a lesser extent, serine 395) in vitro, suggesting the importance of this pathway for SRC-1 regulation. Treatment of cells expressing SRC-1 with epidermal growth factor enhanced the ligand-dependent, progesterone receptor-mediated activation of a target reporter gene. These results identify phosphorylation as a regulatory modification of SRC-1 and provide a basis upon which to identify signaling pathways that regulate SRC-1 function and, consequently, modify steroid/nuclear receptor action.

Coactivators are a class of proteins that interact with sequence-specific transcription factors to enhance their effect on gene transcription. Steroid receptor coactivator 1 (SRC-1) (1) (also referred to as NCoA-1 (2)) was the first member identified in a family of coactivators that modulate the activity of the steroid/nuclear receptor superfamily of ligand-stimulated transcriptional activators. Our laboratory proposed a two-step model for steroid receptor induction of gene expression in which liganded receptor recruits coactivator proteins that can 1) open up local chromatin structure through its intrinsic histone acetyltransferase (HAT) activity, and 2) contribute to the stabilization of the preinitiation complex at the promoter (3).

Subsequent to the identification of SRC-1, two other members of the SRC gene family were cloned: TIF-2 (4) (also known as GRIP1 (5) and NCoA-2 (6)) and RAC3 (7) (also known as ACTR (8), AIB1 (9), P/CIP (6), and TRAM (10)). SRC-1 is ubiquitously expressed in mammalian tissues and has been shown to stimulate the expression of steroid receptor-dependent target genes (1, 2) by interaction with receptor and basal transcription factors through multiple domains (11). SRC-1 has been shown to functionally interact with another coactivator, cAMP-response element-binding protein-binding protein (CBP) to synergistically enhance both estrogen receptor- and progesterone receptor-mediated gene activation (2, 12). SRC-1 has an intrinsic HAT activity and also recruits other strong HATs, such as P300/CBP-associated factor (PCAF) (13), giving SRC-1 a role in chromatin remodeling.

Recent evidence has suggested that certain functions of coactivators and corepressors (a class of proteins that interact with sequence-specific transcription factors to repress their effect on gene transcription) can be modulated by a number of different signal transduction pathways. Interaction of the corepressor N-CoR with the estrogen receptor is decreased by treatment with forskolin and epidermal growth factor (14), and interaction between the progesterone receptor and two corepressors, N-CoR and SMRT, is decreased by treatment with 8-bromo cAMP (15). Tyrosine kinase signaling pathways were similarly shown to disrupt interactions of the corepressor SMRT with the thyroid hormone receptor, v-Erb A, and retinoic acid receptors. Numerous studies have implicated different cell signaling pathways in the regulation of CBP function, including cyclic AMP and protein kinase A (16–20), the MAP kinase pathway (17, 19), nuclear calcium (18), and the cell cycle-dependent kinases (22).

Whereas most studies have examined the role of CBP as a signal-regulated coactivator for several different classes of transcription factors, including members of the steroid receptor superfamily, no studies have focused on SRC-1 as a potential regulatory target. Given the importance that SRC-1 has for steroid-mediated responses in mammals (23, 24), a study of the regulation of SRC-1 is important for predicting steroid-dependent responses in various tissues. To predict which kinases may be important in regulating SRC-1 activity, the major phosphorylation sites in SRC-1 expressed in COS-1 cells were identified. Furthermore, the effect of activation of MAP kinase on SRC-1-mediated progesterone receptor activation and the role of progesterone treatment in modulating SRC-1 phosphorylation were examined. This information will allow a determina-

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The abbreviations used are: SRC-1, steroid receptor coactivator-1; HAT, histone acetyltransferase; CBP, cAMP-response element-binding protein-binding protein; PR, progesterone receptor; P/CAB, P300/CBP-associated factor; MED, modified manual Edman degradation; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s); HPLC, high performance liquid chromatography; MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; Erk, extracellular signal-regulated kinase; CAT, chloramphenicol acetyltransferase; hpr, human progesterone receptor.
tion of those signaling pathways that regulate SRC-1 function through alterations in SRC-1 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture reagents and LipofectAMINE were purchased from Life Technologies, Inc. Thin layer cellulose plates were obtained from EM Science (Gibbstown, NJ). Carrier-free [32P]Pi, PO₄, and γ-[32P]ATP, were purchased from NEN Life Science Products. pSVL plasmid and protein A-Sepharose were purchased from Amer sham Pharmacia Biotech. The oligonucleotides used in the plasmid construction and sequencing were synthesized by GenoSys (The Woodlands, TX). Sequencing grade endonucleases Asp-N, Glu-C, Arg-C, and Lys-C were obtained from Roche Molecular Biochemicals. Triethanolamine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 8-methoxypsoralen, sequencing grade trifluoroacetic acid, poly-L-lysine, phosphoserine, phosphothreonine, phosphotyrosine, protease inhibitors, and monothioglycerol were purchased from Sigma. Xylene was purchased from Fisher Scientific (Pittsburgh, PA). Ninhydrin was obtained from Pierce. Tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ). Phenyloisothiocyanate and HPLC reagents were obtained from J. T. Baker Chemical Corp. (Phillipsburg, NJ). Anti-SRC-1 IgG (clone 1135) was prepared as described previously (13). Rabbit anti-mouse IgG (H+L) antibody was obtained from Zymed Laboratories Inc. (San Francisco, CA). Sequelon-AA disks were obtained from Millipore Corp. (Milford, MA). Epidermal growth factor (EGF) was purchased from Collaborative Biomedical Products. Erk-2 was a giftkind of Melanie Cobb.

**Plasmid Construction**—Full-length SRC-1 (amino acids 1–1441) was transferred from the pCRS-1-hSRC-1A (3) into the XhoI site (5') of the Smal site (3') of the pSVL plasmid. A double-stranded oligonucleotide (sense, 5'-TGCAGGGCCCATGAGAGTCATCCATCTCATCATCATCATCATCATCATCATCATATGAGATGTATC-3'); antisense, 5'-CGCGGGTTGATCTTCTCATCAGAGATGATGATGATGATGAGAACCTCCTGTTGCCCC-3') was inserted into the SRC-1 sequence, which added 6 histidine residues to the expressed protein. An N-terminal deletion mutant of SRC-1 (amino acids 498–1414) was created by transferring XcmI/SwaI digested pSVL (3') and Smal (3') sites of the pSVL plasmid with a double-stranded oligonucleotide (sense, 5'-CCGCGGCGGCACTGAGAGTTCATCCATCTCATCATCATCATCATCATCATCATCATCATCATCATATGAGATGTATC-3'); antisense, 5'-CTGGTTGAATGATGATGATGATGAGAACCTCCTGTTGCCCC-3') inserted 5' to the SRC1 sequence, which added 6 histidine residues to the expressed protein. An N-terminal deletion mutant of SRC-1 (amino acids 498–1414) was created by digesting the first deletion mutant with BglII (within the His6 oligonucleotide) and XhoI (within the SRC-1 coding sequence) and religation along with plasmid. Mutagenesis was performed using the Stratagene Quick Change kit. SRC-1 sequence and mutations were confirmed by DNA sequencing.

**Transfection of SRC-1 in COS-1 Cells and Metabolic Labeling**—SRC-1 expression in COS-1 cells was obtained using a nonrecombinant adenovirus transfer procedure as described previously (26). Briefly, polyclonal antisera to the adenovirus was prepared and then mixed with plasmid DNA. The plasmid DNA binds to the adenovirus particle through the polylsine chains. The adenovirus-plasmid particles were then added to cells, and the plasmid DNA was carried into the cells along with the adenovirus. COS-1 cells were plated in 150-mm dishes (2 × 10⁶ cells/dish) in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) that had been treated with dextran-coated charcoal and cultured at 37 °C with 5% CO₂. 24 h after plating, the medium was removed and replaced with DMEM without FBS to prepare the cells for the adenovirus infection. SRC-1 expression plasmid (0.1 μg/dish) was mixed with adenovirus (virus to cell ratio, 400:1) and the resulting mixture was added to the dishes. Two hours postinfection, DMEM with FBS was added to the dishes to give a final concentration of 2.5% FBS. After 24 h, the medium was removed and replaced with phosphate-free DMEM for 1 h at 37 °C. Subsequently, the phosphate-free medium was removed and replaced with phosphate-free DMEM with 1% dialyzed, stripped FBS, 2–4 μCi of [32P]Pi (0.13–0.26 μCi/μl) was added to each dish, and cells were cultured for 12–14 h at 37 °C prior to harvest.

**Preparation of SRC-1 for Purification**—Medium containing [32P]Pi was removed from the dishes, and the cells were centrifuged at 500 × g for 10 min at 4 °C. All subsequent procedures were carried out at 4 °C. Cell pellets were resuspended in 2.5 ml of a denaturing homogenization buffer (10 mM Tris, pH 8, 50 mM potassium phosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium chloride, 5 mM α-monoothioglycerol, 8 mM urea, protease inhibitor mix). The resulting mixture was passed through an 18 gauge needle three times. Following centrifugation at 100,000 × g for 30 min at 4 °C, 47.5 ml of phosphate-buffered saline containing 0.2% bovine serum albumin was added to the supernatant to reduce the urea concentration to 0.4 M and prepare the immunoprecipitation reaction for use.

**Preparation of SRC-1 Immunoprecipitation Columns and Purification of SRC-1**—0.2 ml of soluble protein A-Sepharose beads were incubated with 25 μg of rabbit anti-mouse IgG (H+L) for 1 h at room temperature followed by incubation with 0.25 ml of clone 1135 anti-SRC-1 antibody tissue culture supernatant overnight at 4 °C. The beads were transferred to a 3-ml column, and the column was washed extensively with phosphate-buffered saline (PBS). SRC-1 was washed by passing the entire supernatant (50 ml) over the immunoprecipitation column over a 2–3-h period. The column was washed with 200 ml of wash buffer (10 mM Tris, pH 8, 400 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Purified SRC-1 was eluted from the column with 1 M acetic acid, and the eluted fractions were placed in a SpeedVac Concentrator® (Savant, Farmingdale, NY) overnight to evaporate the acetic acid. Purified SRC-1 was then electroeluted on a 6.5% SDS-PAGE gel, and the wet gel was exposed to X-AR film (Eastman Kodak) for 1–2 h. The phosphorylated SRC-1 band was cut out of the gel, and the gel slice was incubated with 50% methanol for 1 h followed by incubation with HPLC grade water for 30 min. The gel slice was ready for endoprotease digestion.

**Endoprotease Digestion and HPLC Analysis of SRC-1 Phosphopeptides**—For trypsin digestion, the gel slice was incubated in 0.5 ml of 50 mM Tris (pH 8) containing 10 μl of 1 M monothioglycerol was added to the gel slice every 4 h at 37 °C for a total of 12 h. For Arg-C digestion, the gel slice was incubated in 0.5 ml of 100 mM Tris, 10 mM CaCl₂, pH 7.6, and 1.25 μg of Arg-C (0.1 μg/ml in 50 mM Tris, 10 mM CaCl₂, 5 mM EDTA, pH 8) was added to the gel slice four times over a 24-h period at 37 °C. For Lys-C digestion, the gel slice was incubated in 0.5 ml of 25 mM Tris, 1 mM EDTA, pH 8.5, and 1.25 μg of Lys-C (0.1 μg/ml in buffer supplied by the manufacturer) was added to the gel slice four times over a 24-h period at 37 °C. The released phosphopeptides were separated by reversed-phase HPLC as described previously (26). Briefly, phosphopeptides were loaded onto a C-18 reversed-phase HPLC column, and the phosphopeptides were eluted from the column using a 0–45% gradient of acetonitrile containing 0.1% trifluoroacetic acid over a period of 90 min. Phosphopeptide peaks were detected with a Packard model 4910 FLS detector (radioactive flow detector).

**Purification of SRC-1 Phosphopeptides by Alkaline Polyacrylamide Gels or Two-dimensional Phosphopeptide Analysis—HPLC fractions were evaporated using a SpeedVac, and phosphopeptides were visualized by autoradiography following electrophoresis on a 25% alkaline polyacrylamide gel (27). Individual phosphopeptides were eluted from the dried gel as described previously (26). Two-dimensional analysis of SRC-1 phosphopeptides was carried out using the IETLE-7000 electrofocusing gel system from CBB Scientific and was visualized previously (28). Briefly, SRC-1 phosphopeptides were spotted onto 20 × 20 cm thin layer cellulose plates and separated in the first dimension by electrophoresis at 1000 V for 35 min in pH 1.9 buffer (2.2% v/v formic acid, 7.8% v/v acetic acid). The cellulose plates were then placed in a chromatography tank containing phosphochromatography buffer (38% (v/v) n-butanol, 25% (v/v) pyridine, 7.5% (v/v) acetic acid) for 6–8 h to separate the phosphopeptides in the second dimension. The dried cellulose plate was then exposed to X-AR film. Individual phosphopeptides were isolated by scraping and collecting the cellulose from 50% formic acid.

**Modified Manual Edman Degradation and Secondary Endoproteinase Digestion**—Phosphopeptides purified by alkaline polyacrylamide gel electrophoresis and two-dimensional analysis were subjected to modified manual Edman degradation as described previously to identify the phosphorylation sites (26). Briefly, phosphopeptides were covalently coupled to Sequelon-AA disks and subjected to consecutive cycles of the Edman degradation. After each cycle, the disc was treated with trifluoroacetic acid to cleave and release the N-terminal amino acid, and the released ³²P was determined by Cerenkov counting. Trypsin digestion was performed on the phosphopeptides with phosphopeptides with ³²P that were then subjected to digestion with Asp-N and Glu-C as described previously to further identify the phosphorylation sites (26). Briefly, tryptic phosphopeptides were treated with either 0.1 μg of Asp-N in 50 mM sodium phosphate buffer, pH 8, and incubated for 4 h at 37 °C or with 0.5 μg of Glu-C in 25 mM ammonium bicarbonate buffer for 8 h at 37 °C. Untreated phosphopeptides were incubated in either Asp-N or Glu-C buffer in the absence of endoprotease for 8 h at 37 °C for comparison. The doubly
digested peptides were then electrophoresed on a 25% alkaline polyacrylamide gel and visualized by autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described previously (28).

Immunoblotting Analysis—Purified SRC-1 was electrophoresed on a 6.5% SDS-PAGE gel and transferred to a nitrocellulose membrane for Western blotting. SRC-1 was detected using anti-SRC-1 antibody (clone 1135) followed by chemiluminescent detection using ECL reagent (Amersham Pharmacia Biotech).

In Vitro Phosphorylation of SRC-1—SRC-1 was subcloned into a baculovirus transfer plasmid (Invitrogen) followed by infection of SF9 cells with the recombinant virus as described previously (29). The SF9 cell pellets were extracted in Buffer A (10 mM Tris, pH 8, 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM monothioglycerol, protease inhibitor mix (Sigma)) by 10 strokes with a Dounce homogenizer on ice and purified by immunoprecipitation. The SRC-1-bound protein A-Sepharose beads were incubated with kinase reaction buffer (final concentration, 20 mM HEPES, pH 7.6, 1 mM MgCl₂, 2 mM EDTA, 2 mM EGTA, 0.1 mM sodium vanadate) and 1–3 µL of Erk-2 with a final specific activity of [γ-32P]ATP of 33,000 dpm/pmol ATP, in a final reaction volume of 40 µL. The reaction was incubated for 30 min at 30 °C and terminated by addition of 4 × Laemmli sample buffer followed by electrophoresis on a 6.5% SDS-PAGE gel. The phosphorylated SRC-1 band was visualized by autoradiography and cut out of the gel, and the gel slice was subjected to trypsin digestion. The released phosphotryptic peptides of SRC-1 were separated by two-dimensional electrophoresis on cellulose plates as described under “Experimental Procedures,” and the plates were exposed to X-AR film for autoradiography.

Expression, an adenosine-mediated DNA transfer technique was used to express SRC-1 in COS-1 cells. This method resulted in high efficiency DNA transfer and, consequently, a higher percentage of transfected cells than could be achieved with a conventional transient transfection procedure (25). To assess the effect of progesterone treatment on SRC-1 phosphorylation, SRC-1, human progesterone receptor B and the GRE_E1bCAT reporter were coexpressed in COS-1 cells, and the cells were labeled in vivo with [32P]H₃PO₄ in the presence or absence of 10⁻⁸ M progesterone for 16 h. Treatment with progesterone did not significantly alter the overall level of phosphorylation (Fig. 1A). Phosphorimage analysis of the SRC-1-32P signal revealed that there was a slight decrease in the overall level of SRC-1 phosphorylation in response to progesterone treatment (23% ± 2% S.E. for three experiments) when normalized to the amount of SRC-1 protein present by densitometric scans of the ECL Western blot. Two-dimensional phosphopeptide maps show no consistent differences in the intensity of the SRC-1 tryptic phosphopeptides in the presence and absence of progesterone treatment (Fig. 1B). These results are in marked contrast to steroid-mediated hyperphosphorylation of the progesterone receptor (30, 31) and to ligand-dependent hyperphosphorylation of the nuclear receptor-associated protein TIF1-α (32).

SRC-1 Is Phosphorylated on Multiple Sites in COS-1 Cells—SRC-1 was highly phosphorylated on multiple sites, as indicated by the numerous tryptic phosphopeptide peaks detected by HPLC analysis (Fig. 2A) and the large number of phosphopeptides bands detected on alkaline polyacrylamide gels (Fig. 2B). The major phosphopeptide bands were numbered (1–6) as a reference for the other figures. Sequence information could not be obtained for low stoichiometry phosphorylation sites and for several of the phosphopeptides that were detected at later retention times (62–75 min; Fig. 2B). It is possible that several of these phosphopeptides were partial tryptic digestion products of phosphopeptides 1–6.

In order to determine the number of phosphopeptides that were present in the N-terminal region of SRC-1, two N-terminal deletions of SRC-1 were prepared (aa 409–1441 and 783–1441). These deletion constructs, as well as full-length SRC-1 (aa 1–1441), were expressed in COS-1 cells and labeled in vivo with [32P]H₃PO₄, and the tryptic phosphopeptides were separated by two-dimensional phosphopeptide mapping (Fig. 2C). To identify the spots on the two-dimensional phosphopeptide map that corresponded to phosphopeptides 1–6 from Fig. 2B,
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Fig. 2. Comparison of HPLC and two-dimensional phosphopeptide maps of full-length SRC-1 (aa 1-1441) and two N-terminally deleted forms of SRC-1 (aa 409-1441 and 783-1441). A, 5 x 10⁶ COS-1 cells were plated on 150-mm dishes (2 x 10⁶ cells/dish), and cells were infected with the expression vector for full-length SRC-1 (0.1 µg/dish) followed by incubation of cells with [³²P]H₃PO₄ (2 mCi/dish). SRC-1 was immunopurified and then electrophoresed on a 6.5% SDS-PAGE gel. The SRC-1 band was cut out of the gel, and the gel slice was subjected to trypsin digestion. The released SRC-1 phosphotryptic peptides were separated by C-18 reversed-phase HPLC. B, HPLC fractions containing full-length SRC-1 phosphopeptides were electrophoresed on a 25% alkaline polyacrylamide gel. The gel was dried and then exposed to X-AR film for autoradiography (72 h) as described under “Experimental Procedures.” Numbers 1–6 were assigned to the phosphopeptide bands. C, for each form of SRC-1, 1.4 x 10⁷ COS-1 cells were plated on 150-mm dishes (2 x 10⁶ cells/dish), and cells were infected with the expression vectors (0.1 µg/dish) for either full-length SRC-1, a deletion mutant of SRC-1 (aa 409–1441), or another deletion mutant of SRC-1 (aa 783–1441). Following incubation of cells with [³²P]H₃PO₄ (2 mCi/dish), both the full-length and N-terminally deleted SRC-1 proteins were immunopurified, and tryptic phosphopeptides were prepared as described in A. The SRC-1 phosphotryptic peptides from each protein were separated by two-dimensional phosphopeptide mapping as described under “Experimental Procedures,” and the plates were exposed to X-AR film for autoradiography. Origin indicates the site at which the tryptic phosphopeptides were spotted onto the cellulose plates. Numbers 1–6 were assigned to the phosphopeptide bands. These are the same phosphopeptides as those shown in B.

Individual phosphopeptides were scraped from the two-dimensional phosphopeptide map and electrophoresed on an alkaline peptide gel side by side with phosphopeptides 1–6 isolated by HPLC (data not shown). The majority of the tryptic phosphopeptides detected in full-length SRC-1 were also detected in N-terminally deleted SRC-1 (aa 409–1441). Only phosphopeptides 2 and 4 were not detected in N-terminally deleted SRC-1, indicating that these sites were located in the first 408 amino acids of SRC-1 (Fig. 2C). Phosphopeptides 3 and 6 were also detected in SRC-1 aa 783–1441. Although in SRC-1 aa 783–1441 there was a heavy phosphopeptide(s) signal in the region in which phosphopeptide 1 migrated, further experimentation revealed that phosphopeptide 1 was not present in SRC-1 aa 783–1441 (see Table I). This heavy signal may be related to aberrant phosphorylation as a result of deleting the N-terminal half of SRC-1.

Identification of the Major Phosphorylation Sites in SRC-1—Several mapping tools were used to identify the major phosphorylation sites in SRC-1. Following in vivo labeling of SRC-1 in COS-1 cells with [³²P]H₃PO₄, SRC-1 was purified and digested with trypsin, and the phosphopeptides were separated by HPLC, two-dimensional phosphopeptide maps, and alkaline polyacrylamide gel electrophoresis. Subsequent experiments were performed on individual phosphopeptides that were isolated from HPLC fractions, alkaline polyacrylamide gels, or cellulose plates (two-dimensional phosphopeptide maps).

To determine the position of the phosphoamino acid relative to the N-terminal of the tryptic phosphopeptide, each phosphopeptide was subjected to modified manual Edman degradation (MED) (Fig. 3A and Table I). Data for MED and secondary proteinase digestion of phosphopeptide 6 are shown in Fig. 3A; the results for the remaining phosphopeptides are listed in Table I. MED of phosphopeptide 6 indicated that the phosphorylated amino acid was present 21 amino acid residues from the N-terminal of the phosphopeptide (Fig. 3A, left side). Phosphoamino acid analysis indicated that phosphopeptides 1–5 contained only phosphoserine and that phosphopeptide 6 contained both phosphoserine and phosphothreonine (Table I). Phosphotyrosine was not detected in any of the individual phosphopeptides examined.

Phosphopeptides 1–6 were subjected to secondary proteinase digestion with Asp-N and Glu-C (Fig. 3A and Table I). Asp-N digests peptides on the N-terminal side of aspartate residues, and Glu-C digests peptides on the C-terminal of glutamate residues. Phosphopeptide 6 was digested with Glu-C but not Asp-N, as shown by the altered migration of the phosphopeptide after incubation with Glu-C (Fig. 3A, right side). This indicates that phosphopeptide 6 contains a glutamate residue but not an aspartate. Although it appears that phosphopeptide 6 may be slightly reduced in the Asp-N lane, this is most likely due to loading differences between the – and Asp-N lanes, as it was not reproducible. The same secondary proteinase digestions were performed for phosphopeptides 1–5, and the data are summarized in Table I.

Results from MED and secondary proteinase digestion allowed the unambiguous identification of phosphopeptides 5 and 6 as serine 517 and threonine 1179, respectively. The regions of SRC-1 encompassing these sites (see Fig. 2C) contained unique tryptic peptides with properties of a serine in position 15 with neither aspartate nor glutamate (for serine 517) and a threonine in position 21 with a glutamate but not an aspartate (for threonine 1179). For phosphopeptides 1–4, fur-
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Identification of SRC-1 phosphorylation sites

The combined data for each phosphopeptide were sufficient to identify the phosphorylation sites. ND, not determined.

| Phosphopeptide | Phosphoamino acid | MED release | Asp-N/Glu-C | Arg-C/Lys-C | SRC-1 409–1441 | SRC-1 783–1441 | Identity |
|----------------|-------------------|-------------|-------------|-------------|----------------|----------------|----------|
| 1              | Serine            | 7           | Arg-C       | +           | ND             | Ser-569        |          |
| 2              | Serine            | 10          | Arg-C       | −           | −              | Ser-395        |          |
| 3              | Serine            | 4           | Arg-C       | +           | +              | Ser-1033       |          |
| 4              | Serine            | 6           | Asp-N       | Arg-C       | +              | −              | Ser-372   |
| 5              | Serine            | 15          | Glu-C       | ND          | +              | Ser-517        |          |
| 6              | Serine/threonine  | 21          |            |             | +              | +              | Thr-1179/Ser-1185|

**Fig. 3. Identification of SRC-1 phosphorylation sites.** 6 × 10⁷ COS-1 cells were plated on 150-mm dishes (2 × 10⁶ cells/dish), and cells were infected with the expression vector for full-length SRC-1 (0.1 µg/dish) followed by incubation of cells with [³²P]H₃PO₄ (2 mCi/dish). SRC-1 was purified and extensively digested with either trypsin, Arg-C, or Lys-C, and the phosphopeptides were separated by either HPLC and 25% alkaline polyacrylamide gels or two-dimensional phosphopeptide mapping as described in Fig. 2. Following autoradiography of the alkaline gels or cellulose plates, individual phosphopeptides were purified from the alkaline gels or from the cellulose plates and subjected to modified manual Edman degradation and phosphoamino acid analysis. Alternately, tryptic phosphopeptides were eluted from the HPLC and subjected to secondary proteinase digestion with Asp-N and Glu-C or comparative phosphopeptide mapping of SRC-1 digested with trypsin, Arg-C, or Lys-C as described under “Experimental Procedures.” A modified manual Edman degradation (left panel) and secondary proteinase digestion with Asp-N and Glu-C (right panel) of tryptic phosphopeptide 6. Phosphopeptide 6 purified from alkaline polyacrylamide gels was subjected to MED as described under “Experimental Procedures.” HPLC fractions containing phosphopeptide 6 were used for secondary proteinase digestion with Asp-N and Glu-C, and secondary digests were electrophoresed on 25% alkaline polyacrylamide gels followed by autoradiography to visualize the phosphopeptides. B, comparison of the phosphopeptide maps of SRC-1 digested with trypsin, Arg-C, or Lys-C. For each proteinase digestion, 1 × 10⁷ COS-1 cells were plated on 150-mm dishes (2 × 10⁶ cells/dish) and cells were infected with the expression vector for full-length SRC-1 (0.1 µg/dish) followed by incubation of cells with [³²P]H₃PO₄ (2 mCi/dish). SRC-1 was purified as described in Fig. 2 above and then digested with either trypsin, Arg-C, or Lys-C prior to HPLC analysis. Corresponding HPLC fractions from trypsin-, Arg-C-, or Lys-C-digested SRC-1 were then electrophoresed on a 25% alkaline polyacrylamide gel, and the dried gel was subjected to autoradiography. C, comparison of the phosphopeptide maps of SRC-1 mutated at aa 1033 (serine to alanine) with SRC-1 mutated at aa 1118 (serine to alanine). The expression vector for SRC-1 was mutated to alanine at each residue 1033 or residue 1118, and each mutant vector (0.1 m/hr) was used to infect 1.8 × 10⁷ COS-1 cells plated on 150-mm dishes (2 × 10⁶ cells/dish). Cells were incubated with [³²P]H₃PO₄ (2 mCi/dish) for 15 h, and subsequently, SRC-1 was purified, extensively digested with trypsin, and subjected to HPLC analysis as described in Fig. 2. HPLC fractions were electrophoresed on 25% alkaline polyacrylamide gels. The phosphopeptides eluted at a later retention time than that shown in Fig. 2B because a different HPLC system was used for this experiment.

FIG. 2. Identification of SRC-1 phosphorylation sites. 6 × 10⁷ COS-1 cells were plated on 150-mm dishes (2 × 10⁶ cells/dish), and cells were infected with the expression vector for full-length SRC-1 (0.1 µg/dish) followed by incubation of cells with [³²P]H₃PO₄ (2 mCi/dish). SRC-1 was purified and extensively digested with either trypsin, Arg-C, or Lys-C, and the phosphopeptides were separated by either HPLC and 25% alkaline polyacrylamide gels or two-dimensional phosphopeptide mapping as described in Fig. 2. Following autoradiography of the alkaline gels or cellulose plates, individual phosphopeptides were purified from the alkaline gels or from the cellulose plates and subjected to modified manual Edman degradation and phosphoamino acid analysis. Alternately, tryptic phosphopeptides were eluted from the HPLC and subjected to secondary proteinase digestion with Asp-N and Glu-C or comparative phosphopeptide mapping of SRC-1 digested with trypsin, Arg-C, or Lys-C as described under “Experimental Procedures.” A, modified manual Edman degradation (left panel) and secondary proteinase digestion with Asp-N and Glu-C (right panel) of tryptic phosphopeptide 6. Phosphopeptide 6 purified from alkaline polyacrylamide gels was subjected to MED as described under “Experimental Procedures.” HPLC fractions containing phosphopeptide 6 were used for secondary proteinase digestion with Asp-N and Glu-C, and secondary digests were electrophoresed on 25% alkaline polyacrylamide gels followed by autoradiography to visualize the phosphopeptides. B, comparison of the phosphopeptide maps of SRC-1 digested with trypsin, Arg-C, or Lys-C. For each proteinase digestion, 1 × 10⁷ COS-1 cells were plated on 150-mm dishes (2 × 10⁶ cells/dish) and cells were infected with the expression vector for full-length SRC-1 (0.1 µg/dish) followed by incubation of cells with [³²P]H₃PO₄ (2 mCi/dish). SRC-1 was purified as described in Fig. 2 above and then digested with either trypsin, Arg-C, or Lys-C prior to HPLC analysis. Corresponding HPLC fractions from trypsin-, Arg-C-, or Lys-C-digested SRC-1 were then electrophoresed on a 25% alkaline polyacrylamide gel, and the dried gel was subjected to autoradiography. C, comparison of the phosphopeptide maps of SRC-1 mutated at aa 1033 (serine to alanine) with SRC-1 mutated at aa 1118 (serine to alanine). The expression vector for SRC-1 was mutated to alanine at each residue 1033 or residue 1118, and each mutant vector (0.1 µg/dish) was used to infect 1.8 × 10⁷ COS-1 cells plated on 150-mm dishes (2 × 10⁶ cells/dish). Cells were incubated with [³²P]H₃PO₄ (2 mCi/dish) for 15 h, and subsequently, SRC-1 was purified, extensively digested with trypsin, and subjected to HPLC analysis as described in Fig. 2. HPLC fractions were electrophoresed on 25% alkaline polyacrylamide gels. The phosphopeptides eluted at a later retention time than that shown in Fig. 2B because a different HPLC system was used for this experiment.
digestion of phosphopeptide 4 with Glu-C because glutamate residues at the end of a peptide are rarely cleaved by Glu-C (33).

The preceding data eliminated all but two candidate peptides for the phosphorylation site for phosphopeptide 3: serine 1033 and serine 1118. Both peptides fit all the criteria established in the experiments performed in Fig. 3. To determine which candidate peptide contained the phosphorylation site for phosphopeptide 3, serine 1033 or serine 1118 was mutated to alanine. Both mutant constructs were expressed in COS-1 cells, labeled in vivo with $[^{32}P]Pi$, and the tryptic HPLC phosphopeptide map of each was compared. Mutation of serine 1033 to alanine resulted in loss of phosphopeptide 3 (Fig. 3C, top), whereas mutation of serine 1118 to alanine had no effect on phosphopeptide 3 (Fig. 3C, bottom). This identified phosphopeptide 3 as serine 1033.

MAP Kinase (Erk-2) Phosphorylates SRC-1 in Vitro—Consensus phosphorylation sites for MAP kinase (PXX(S/T)P) (34, 35) were identified at serine 395 and threonine 1179 (Table II). To determine whether Erk-2 could phosphorylate SRC-1 at these sites, in vitro phosphorylation of immunopurified SRC-1 was performed using Erk-2. Erk-2 phosphorylated SRC-1 in vitro at several sites as shown by two-dimensional phosphopeptide mapping of the SRC-1 tryptic phosphopeptides (Fig. 4, A and B). One phosphopeptide (numbered 6 in Fig. 4A) co-migrated with the threonine 1179 phosphopeptide identified in COS-1 cells. Further analysis of this Erk-2-phosphopeptide revealed that it exhibited the same characteristics as the threonine 1179 phosphopeptide identified in COS-1 cells. The peptide eluted from the HPLC at the same retention time, showed the same relative migration on alkaline polyacrylamide gels (phosphopeptide 6 in Fig. 4B) and gave the same secondary proteinase digestion pattern with Asp-N and Glu-C (compare Fig. 4C with Fig. 3A, right side). Furthermore, phosphoamino acid analysis of in vitro phosphopeptide 6 detected both phosphoserine and phosphothreonine identical to that found for the threonine 1179 phosphopeptide identified in COS-1 cells (Fig. 4D and Table I).

Phosphopeptide 6, identified in COS-1 cells and phosphorylated by Erk-2 in vitro, contained a phosphoserine residue as well as a phosphothreonine residue, indicating that there are at least two phosphorylation sites in this peptide. Phosphopeptide 6 contains four serine residues, but only one serine (serine 1185) would be predicted to be a substrate for Erk-2 in vitro. Serine 1185 is very close to a perfect Erk-2 consensus phosphorylation site (there are three instead of two amino acids separating the N-terminal proline and serine 1185). The other three serines in phosphopeptide 6 do not have the mandatory proline residue immediately to the C-terminal that is requisite for Erk-2 phosphorylation, nor do these sites resemble consensus sequences for any known kinases. Phosphoamino acid analysis of the threonine 1179 phosphopeptide identified in COS-1 cells also detected phosphoserine; however, MED of this phosphopeptide only showed a $^{32}P$ release at cycle 21 (three experiments). It is possible that the hydrophobic nature of this peptide or the numerous proline residues in the peptide may inhibit the MED reaction. Because serine 1185 is the only serine residue in phosphopeptide 6 that has the Ser-Pro motif for proline directed kinases and is almost a perfect MAP kinase consensus site, it is highly probable that this residue is the serine that is phosphorylated in COS-1 cells.

It is clear that Erk-2 phosphorylated SRC-1 on several other phosphopeptides, including phosphopeptides 1, 2, 3, and possibly 5, as well as several unidentified phosphopeptides (Fig. 4B). Although only phosphopeptide 2 contains a perfect consensus sequence for Erk-2, phosphopeptides 1, 2, 3, and 5 all contain the Ser-Pro kinase motif that is required for Erk-2 phosphorylation. These data suggest that the MAP kinase family of kinases may play an important role in the phosphorylation of SRC-1.

**Treatment of Cells with EGF Enhances SRC-1 Coactivation of Progesterone Receptor**—To determine whether activation of the MAP kinase pathway has an effect on SRC-1 coactivation, COS-1 cells expressing SRC-1, hPR$_{alpha}$, and a reporter gene were treated with progesterone or progesterone + EGF. Coexpression of SRC-1 increased steroid-dependent progesterone receptor activation approximately 2–3-fold (Fig. 4E, black bars). Co-treatment with EGF enhanced progesterone-dependent activation in the absence and presence of cotransfected SRC-1, respectively (Fig. 4E, compare gray bars to black bars). The effect of EGF was greater when a subsaturating level of progesterone ($5 \times 10^{-10}$ M) was used, resulting in an approximately 2-fold increase in progesterone receptor activation. These data are consistent with SRC-1 and/or progesterone receptor as a target for the MAP kinase pathway. EGF treatment also enhanced progesterone receptor activation in the absence of coexpressed SRC-1. It is possible that lower levels of endogenous SRC-1 detected in COS-1 cells compared with transfected cells is a target of the MAP kinase pathway.

**DISCUSSION**

The SRC-1 family of coactivators for the nuclear receptor superfamily are large modular proteins that contain multiple protein interaction domains (with nuclear receptors, HATS, CBF, and basal transcription machinery) and an intrinsic HAT activity. The coactivator function of SRC-1 requires the combined action of each of these domains. It is now established that protein-protein interactions, as well as the HAT activity of coactivator proteins, can be controlled through signal transduction pathways that presumably modulate phosphorylation of the coactivators. At present, there has not been a detailed analysis of the major phosphorylation sites in coactivators or corepressors. To begin to identify signaling pathways that may be important in modulating SRC-1 function, we have identified the major phosphorylation sites in SRC-1 and examined the role of progesterone and the MAP kinase pathway in modulating SRC-1 phosphorylation and progesterone receptor activation. We found that SRC-1 is multiply phosphorylated in COS-1 cells, suggesting a role for phosphorylation in SRC-1 regulation.

### Table II

| Phosphorylation site | Phosphopeptide sequence | Consensus sequence |
|----------------------|-------------------------|--------------------|
| Ser-569              | QMSSQN S FSRS           | Proline-directed kinase |
| Ser-395              | VNPSVSNPSI S PAHVVAR    | MAP kinase |
| Ser-1033             | GAF S PGMMGMP          | Proline-directed kinase |
| Ser-372              | EHSGL S PQDDTNSGMSIPIR  | Proline-directed kinase |
| Ser-517              | MPNSFPPNITLS S PVGMTSSACNNNR | Proline-directed kinase |
| Thr-1179/Ser-1185    | LNQGAPQFFYPFPNGTFPAST S PF | MAP kinase |
|                      | SFLANENFEASLANK        |                    |

*Notes: The preceding data eliminated all but two candidate peptides for the phosphorylation site for phosphopeptide 3: serine 1033 and serine 1118. Both peptides fit all the criteria established in the experiments performed in Fig. 3. To determine which candidate peptide contained the phosphorylation site for phosphopeptide 3, serine 1033 or serine 1118 of SRC-1 was mutated to alanine. Both mutant constructs were expressed in COS-1 cells, labeled in vivo with $[^{32}P]Pi$, and the tryptic HPLC phosphopeptide map of each was compared. Mutation of serine 1033 to alanine resulted in loss of phosphopeptide 3 (Fig. 3C, top), whereas mutation of serine 1118 to alanine had no effect on phosphopeptide 3 (Fig. 3C, bottom). This identified phosphopeptide 3 as serine 1033.*
Phosphorylation of SRC-1

Fig. 4. Comparison of SRC-1 phosphorylated in COS-1 cells and SRC-1 phosphorylated in vitro with Erk-2 and effect of EGF on the activation of hPRb in COS-1 cells. Baculoviral-expressed SRC-1 was immunoprecipitated under native conditions, and SRC-1 bound to the protein A beads was incubated with purified Erk-2 and [γ-^32P]ATP as described under “Experimental Procedures.” SDS-PAGE sample buffer was added, and the reaction was heated to 100 °C for 5 min to stop the reaction. Following electrophoresis on a 6.5% SDS-PAGE gel, the SRC-1 band was cut out, and the gel slice was subjected to trypsin digestion. The released phosphotryptic peptides of SRC-1 were separated by two-dimensional phosphopeptide mapping (A) and HPLC followed by 25% alkaline polyacrylamide gel electrophoresis (B). This also differs from the nuclear receptor-regulated phosphorylation, which is markedly enhanced in response to steroid treatment (30, 31). This also differs from the nuclear receptor-regulated phosphorylation, which is markedly enhanced in response to steroid treatment (30, 31). This also differs from the nuclear receptor-regulated phosphorylation, which is markedly enhanced in response to steroid treatment (30, 31).

Fig. 5. SRC-1 phosphorylation sites. Domain structure of SRC-1 showing the location of the identified phosphorylation sites. bHLH, basic helix-loop-helix motif; PAS, Per-Arnt-Sim domain; S/T, serine-threonine-rich region; AD, putative activation domain; Q, glutamine-rich region; NR, nuclear receptor interaction domain; CBP, CBP-interaction domain; P/CAF, P300/CBP-associated factor interaction domain; HAT, histone acetyltransferase domain. Black, vertical bars indicate LXXLL motifs.

Coexpression of SRC-1 and progesterone receptor in COS-1 cells and treatment with 10^-7 M progesterone had little, if any, effect on the overall level or site-specific phosphorylation of SRC-1. This is in contrast to progesterone receptor phosphorylation, which is markedly enhanced in response to steroid treatment (30, 31). This also differs from the nuclear receptor-associated protein TIF1-α, which binds to retinoid X receptor-α and estrogen receptor-α and becomes hyperphosphorylated in a ligand-dependent manner (32). Because hormone treatment induces an interaction between progesterone receptor and SRC-1 (1), it is possible that specific changes in progesterone receptor phosphorylation following steroid treatment may regulate binding to SRC-1. It has recently been shown that two phosphorylation sites in the AF-1 region of estrogen receptor-β regulate the binding of SRC-1 to the receptor (36) and a single phosphorylation site in the AF-1 region of the orphan receptor steroidogenic factor 1 regulates binding of the SRC-1 family member GRIP1 to the receptor (37). It remains to be determined whether specific phosphorylation sites in SRC-1 regulate the interaction with progesterone receptor or other proteins in the nuclear receptor coregulator complex (38).

The major phosphorylation sites in SRC-1 are clustered in two groups (Fig. 5). The first group of sites (between residues 372–569) lies within or adjacent to a region of SRC-1 that is rich in serine and threonine residues. Although serine 569 lies adjacent to a region that interacts with nuclear receptors, the other three phosphorylation sites in this group do not lie within identified functional domains of SRC-1. Serine 569 is located within 64 amino acids of the first LXXLL motif of SRC-1 (6, 39). It is possible that changes in the phosphorylation of this site as well as other phosphorylation sites in this region may affect the conformation of SRC-1 in a way that alters the accessibility of the LXXLL motif for nuclear receptor interaction. The second group of phosphorylation sites in the C-terminal (serine 1033, threonine 1179, and serine 1185) lie within a region of SRC-1 containing several functional domains. These sites are adjacent to a major progesterone receptor interaction domain (1) and lie within the region of SRC-1 that interacts with the histone acetyltransferase, P/CAF (13). Threonine 1179 and serine 1185 also lie within the region of SRC-1 that was recently shown to possess HAT activity (13). This raises the possibility that alteration in phosphorylation at these sites could affect multiple functional interactions between SRC-1 and other proteins as well as affect the intrinsic HAT activity of SRC-1.
There were several phosphopeptides eluting at later retention times from the HPLC that could not be identified. Late eluting phosphopeptides are generally large phosphopeptides that are very hydrophobic and are therefore not amenable to modified manual Edman degradation. It is likely that some or all of these phosphopeptides were partial tryptic digestion products of phosphopeptides 1–6. We found that the degree of phosphorylation of these late eluting phosphopeptides was markedly reduced when the phosphopeptide map of a mutant form of SRC-1 (Thr-1179 → Ala) was expressed and compared with the phosphopeptide map of WT SRC-1 (data not shown). This suggests that some of these later eluting phosphopeptides could be partial tryptic digestion products of phosphopeptide 6.

All of the identified phosphorylation sites contained consensus sequences for the serine/threonine-proline-directed family of protein kinases. Two sites (serine 395 and threonine 1179) contained a perfect consensus sequence for the MAP kinase family members (Erk-1 and Erk-2). Erk-2 phosphorylated threonine 1179 as well as serine 1185 (an adjacent site containing an imperfect Erk-1/2 motif) in vitro. Serine 372 is contained within a consensus sequence for casein kinase II, as well as the sites in SRC-1 at serine 395, threonine 1179, and serine 1185 that interact with P/CAF and the region of SRC-1 that possess HAT activity. A detailed analysis of the role of individual phosphorylation sites in regulating SRC-1 function is currently under way.

Only two of the identified SRC-1 phosphorylation sites are conserved in other members of the SRC-1 gene family. Serine 569 is conserved in SRC-2 (also known as TIF-2 and GRIP-1), and serine 517 is conserved in both SRC-2 and SRC-3 (also known as RAC-3 and AIB-1). These conserved phosphorylation sites in the SRC-1 family may play a role in modulating nuclear receptor action in tissues and cells in which multiple coactivators are present. Furthermore, a change in SRC-1 phosphorylation has the potential to simultaneously regulate the action of several different nuclear receptors present in the same target cell or tissue. Phosphorylation of the SRC-1 family of coactivators represents another regulatory step for nuclear receptor activation.

REFERENCES

1. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) Science 270, 1354–1357
2. Kamei, Y., Xu, L., Heinzl, T., Torchia, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 403–414
3. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7879–7884
4. Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) EMBO J. 15, 3667–3677
5. Hong, H., Kohl, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4948–4952
6. Torchia, J., Rose, D. W., Inostroza, J., Kamey, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684
7. Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479–8484
8. Chen, H., Lin, R. J., Shultz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 586–590
9. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, Kallogeri, O. P., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965–968
10. Takeshita, A., Yen, P. M., Misihi, S., Cardona, G. R., Liu, Y., and Chin, W. W. (1996) Endocrinology 137, 3594–3597
11. Onate, S. A., Boonyaratankonkulli, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O'Malley, B. W. (1998) J. Biol. Chem. 273, 12010–12018
12. Smith, C. L., Onate, S. A., Tsai, M. J., and O'Malley, B. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8884–8888
13. Spencer, T. E., Jenster, G., Burcin, M. M., Alits, C. D., Zhou, J. X., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Nature 389, 194–198
14. Lavinsky, R. M., Jepsen, K., Heinzl, T., Torchia, J., Mullen, T. M., Schiﬀ, R. Delrio, A. L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S. G., Osborne, C. K., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2929–2925
15. Wagner, B. L., Norris, J. D., Knotts, T. A., Weigel, N. L., and Mendenhall, D. P. (1989) Mol. Cell. Biol. 18, 1369–1374
16. Xu, L., Lavinsky, R. M., Davis, S. B., Flynn, S. E., McNerney, E. M., Mullen, T. M., Heinzl, T., Szeto, D., Korzus, E., Kurokawa, R., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998) Nature 395, 301–306
17. Lecq, Y. Z., Chrvia, J. C., and Latchman, D. S. (1998) J. Biol. Chem. 273, 32400–32407
18. Chawla, S., Hardingham, G. E., Quinn, D. R., and Bading, H. (1998) Science 281, 1505–1509
19. Zanger, R., Cohen, L. E., Hashimoto, K., Radovic, S., and Wondisford, F. E. (1999) Mol. Endocrinol. 13, 288–295
20. Jainkeith, R., and Nordheim, A. (1996) Oncogene 12, 1961–1969
21. Jainkeith, R., and Nordheim, A. (1996) Biochem. Biophys. Res. Commun. 228, 831–837
22. Ait, S. A., Ramirez, S., Barre, F. X., Dkhissi, F., Maghni-Jaulin, L., Giraud, J. A., Robin, P., Knobehler, M., Pritchard, L. L., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998) Nature 396, 184–186
23. Xu, J., Qiu, Y., Demayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998) Science 279, 1922–1925
24. Weiss, R. E., Xu, J., Ning, G., Kohlen, J., O'Malley, B. W., and Refetoff, S. (1998) EMBO J. 17, 1900–1904
25. Allgood, V. E., Zhang, Y., O'Malley, B. W., and Weigel, N. L. (1997) Biochem. J. 324, 224–232
26. Zhang, Y., Beck, C. A., Peletti, A., Edwards, D. P., and Weigel, N. L. (1994) J. Biol. Chem. 269, 31034–31040
27. West, M. H. P., Wu, R. S., and Banerji, G. W. (1984) Electrophoresis 5, 133–138
28. van der Geer, P., and Hunter, T. (1984) Electrophoresis 5, 544–554
29. Boonyaratankonkulli, V., Melvin, V., Prendergast, G., Altman, Rnolfi, L., Bianchi, M. E., Tarasvichev, I., Neoroen, S. K., Allegretto, E. A., and Edwards, D. P. (1998) Mol. Cell. Biol. 18, 4471–4477
30. Denner, L. A., Weigel, N. L., Schraden, W. T., and O'Malley, B. W. (1998) Endocrinology 125, 3051–3058
31. Beck, C. A., Weigel, N. L., and Edwards, D. P. (1992) Mol. Endocrinol. 6, 607–620
32. Fraser, R. A., Heard, D. J., Adam, S., Lavigne, A. C., LeDeurain, B., Tora, L., Losson, B., Rochetteeyley, C., and Chambon, P. (1998) J. Biol. Chem. 273, 16199–16204
33. Austen, B. M., and Smith, E. L. (1976) Biochem. Biophys. Res. Commun. 72, 411–417
34. Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991) J. Biol. Chem. 266, 4482

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35. Alvarez, E., Northwood, I. C., Gonzales, F. A., Latour, D. A., Seth, A., Abate, C., Curran, T., and Davis, R. J. (1991) *J. Biol. Chem.* 266, 15277–15285
36. Tremblay, A., Tremblay, G. B., Labrie, F., and Giguere, V. (1999) *Mol. Cell* 3, 513–519
37. Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel, N. L., and Ingraham, H. A. (1999) *Mol. Cell* 3, 521–526
38. McKenna, N. J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11697–11702
39. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* 387, 733–736
40. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) *EMBO J.* 15, 2174–2183
41. Kato, S., Endoh, H., Masuhire, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) *Science* 270, 1491–1494