Estrogen receptors ERα and ERβ are members of the family of nuclear hormone receptors and act as ligand-inducible transcriptional factors, which regulate the expression of target genes on binding to cognate response elements. We report here the characterization of steroid receptor coactivator-3 (SRC-3), a cofactor of nuclear receptor transcription that is a member of a family of steroid receptor coactivators that includes SRC-1 and transcription intermediate factor-2. SRC-3 enhanced ERα and progesterone receptor-stimulated gene transcription in a ligand-dependent manner, but stimulation of ERβ-mediated transcription was not observed. Protein–protein interaction assays, including real-time interaction analyses with BIAcore, demonstrated that the affinity of the ERα interaction with SRC-3 was much higher than that observed for the ERβ interaction with SRC-3. Mutational analysis suggests a potential interplay between the transactivation function-1 and -2 domains of ERα and SRC-3. Furthermore, an intrinsic transactivation function was observed in the C-terminal half of SRC-3. Finally, SRC-3 was differentially expressed in various tissues and, among several tumor cells examined, was most abundant in the nuclear fraction of MCF-7 breast cancer cells. Therefore, SRC-3, a third member of a family of steroid receptor coactivators, has a distinct tissue distribution and intriguing selectivity between ERα and ERβ.

Estradiol (E2)1 exerts numerous biological effects in different tissues through an interaction with the estrogen receptor (ER), a member of the steroid/nuclear hormone receptor superfamily (1, 2). Amino acid sequence analyses, transient transfection studies, and mutational dissections of ER indicate that ER can be subdivided into several functional domains (3). The N-terminal A/B domain contains a transactivation function, referred to as TAF-1. The DNA binding domain, the C region, contains two zinc fingers and is responsible for DNA recognition. The ligand binding domain (LBD) and a second transactivation function, referred to as TAF-2, is located at the C-terminal of ER. On binding to hormone, the receptor undergoes an activation and transformation step. The activated ER interacts with specific estrogen response elements that are located in the promoter region of estrogen-regulated genes and influences the rate of gene transcription. Over the past decade, numerous studies have provided a basic understanding of both the effects of ligand (agonist and antagonist) on the ER and the relationship between the structure and function of the ER (4). Nevertheles, little is known regarding the mechanisms involved in the gene-specific and tissue-selective effects mediated by either estrogens or antiestrogens. Furthermore, the molecular mechanisms by which ligand-activated ER influences the basal transcriptional machinery and regulates target gene transcription are mostly unknown.

Recently, a new estrogen receptor, named ERβ, has been isolated from rat prostate and human testis (5, 6). The DNA binding domain of ERβ is 90% identical to that of ERα. However, the overall homology between the LBD of ERα and ERβ is <60%. Like ERα, ERβ can stimulate transcription from an estrogen response element in a ligand-dependent manner. Currently, the biological significance of the existence of two ER subtypes is not clear. However, the potential functional differences and differential localization between ERα and ERβ (7) may contribute to the selective actions of E2 in different target tissues.

The mechanisms by which nuclear hormone receptors (NRs) regulate target gene transcription is currently under intensive investigation. The ligand-activated NRs may promote formation of the preinitiation complex of the basal transcriptional apparatus and facilitate transcription by RNA polymerase II. These effects may be transmitted in part by direct interactions between NRs and basal transcriptional factors (8–12). In addition, NR-induced transcription of different target genes may be transmitted through indirect interactions, mediated by intermediary transcriptional coactivators. Recently, a number of NR-associated proteins that interact with steroid and thyroid receptors have been reported (for review, see Refs. 13, 14). Among these NR-associated proteins, several corepressors have been described that inhibit basal level transcription by interacting with unliganded thyroid hormone receptor and retinoic acid receptor (15–17), and ligand causes the dissociation of these corepressors from the thyroid hormone receptor and retinoic acid receptor. Furthermore, proteins that interact with NRs in a ligand-dependent manner and augment transcription have also been identified (12). To date, steroid receptor coactivator-1 (SRC-1) (18), transcription intermediate factor-2 (TIF-2) (19), androgen receptor-associated protein 70 (ARA70) (20), cAMP response element-binding protein (CBP) (21) and TAFI135 (22) have been shown to function as NR transcriptional coactivators, and NR-stimulated gene transcription was markedly enhanced by coexpression of these coactivators with

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1 Estradiol (E2) exerts numerous biological effects in different tissues through an interaction with the estrogen receptor (ER), a member of the steroid/nuclear hormone receptor superfamily.

2 The abbreviations used are: E2, estradiol; ER, estrogen receptor; hER, human ER; TAF, transactivation function; LBD, ligand binding domain; NR, nuclear hormone receptor; SRC, steroid receptor coactivator; TIF, transcription intermediate factor; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; PR, progesterone receptor.
NRs. SRC-1, TIF-2, and a more recently described coactivator (ACTR (23), RAC3 (24), AIB1 (25), TRAM-1 (26)) share significant homologies and constitute a family of coactivators. Members of this family have been shown to augment ERα-stimulated gene transcription and, to date, only SRC-1 has been shown to modulate ERβ-mediated gene transcription (27).

To further understand the molecular mechanisms of gene transcription mediated by ERα, a human cDNA library was screened using the yeast two-hybrid assay to identify potential coactivators. One potential coactivator was discovered, which we referred to as SRC-3, that was homologous to SRC-1 and TIF-2, and we now know that it is identical to ACTR/RAC3/AIB1/TRAM-1. Interestingly, SRC-3 enhanced ERα- and progesterone receptor-stimulated gene transcription, but stimulation of ERβ-mediated transcription was not observed. Protein-protein interaction assays, including real-time interaction analyses with BLAcore, demonstrated that the affinity of the ERs interaction with SRC-3 was much higher than that observed for the ERβ interaction with SRC-3. The distinct tissue distribution and intriguing selectivity between ERα and ERβ make SRC-3 a unique member of a family of steroid receptor coactivators.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids—**All recombinant DNA and plasmid constructions were performed according to standard procedures, and DNA sequences of plasmid constructs were verified by standard DNA sequencing (28). Plasmids were transfected into human cell lines using DEAE-dextran (29) or lipofectamine (30). The expression vector pcDNA3 was obtained from the supplier.

**Yeast Two-hybrid Screen—**The MATCHMAKER two-hybrid system (CLONTECH) was used according to the manufacturer's instructions (31). Briefly, yeast CG1945, colonies were selected on SD/-His/-Trp/-Leu plates. For mammalian SRC-1 and SRC-2, the surface of a CM 5 sensor chip (certified) was first modified with biotinylated antibody. The immunoreactive SRC-3 was visualized using the ECL antibody. The membrane was washed in 0.1% SSC, 0.1% SDS at 65 °C for 30 min and exposed to X-OMAT AR film (Eastman Kodak Co.). Electric autoradiograph film was subjected to autoradiography, and bound proteins were eluted with 50–500 mM NaCl gradient of the same buffer. Fractions containing receptor, eluting between 350 and 450 mM, were pooled and applied onto a Fast Desalting column equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 50 mM imidazole. Receiver was eluted with two column volumes of a linear gradient of imidazole 50–500 mM in the same buffer. ERβ-containing fractions were pooled and loaded on a Superdex 200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 0.005% Nonidet P-40. Fractions containing ERβ were pooled and loaded onto a HITrap 5′ affinity column equilibrated with 20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 50 mM imidazole. Receptor-containing fractions were pooled and loaded on a Fast Desalting column equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 0.005% Nonidet P-40. The resulting fractions were analyzed by SDS-PAGE and Western blot analysis, and binding of [3H]estradiol and the purity of the receptor was ~80%.

**Human Lung A549 Cells—**Human lung A549 cells were routinely maintained in minimal essential medium containing 5% FBS. Cells were seeded into six-well plates (Falcon), and plasmids were transiently introduced using calcium phosphate coprecipitation method (Promega). Transfections were done in the presence or absence of 10% fetal bovine serum (10 mM) using lipofectamine (25 μg) as an intracellular marker plasmid pEGFP-C1 (2 μg). Cells were harvested 48 h after transfection, cell pellets were washed with PBS, and bound proteins were assayed for β-galactosidase activity using filter assays. Plasmids of these positive clones were cloned into yeast. Full-length cDNA was obtained by screening a human lymphoma cDNA library (CLONTECH).**Northern Blot—**Poly(A)+ mRNAs (5 μg) of various human tissues (CLONTECH) were separated in a formaldehyde gel and transferred to a Hybond-N membrane (Amersham Corp.). Hybridization was carried out for 4 h in 50% formamide, 5 × saline/sodium phosphate/EDTA, 5 × Denhardt's solution, 1% glycine, and 100 μg/ml denatured salmon sperm DNA at 42 °C. Hybridization was conducted overnight under the same conditions with 2 × 10⁶ cpm/ml denatured probe (0.8-kbp fragment of SRC-3). The membrane was washed in 0.1 × SSC, 0.1% SDS at 65 °C for 30 min and exposed to X-Omat XAR film (Eastman Kodak Co.) overnight at ~80 °C with intensifying screens.

**Nuclear Extract Preparation and Western Blot—**Human cancer cells lines used in this study, including MCF-7 human breast cancer cells, A549 human lung cancer cells, HeLa human cervical carcinoma cells, and Ishikawa endometrial adenocarcinoma cells, were grown in 150-mm cell culture plates with DMEM, 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml). When cells reached confluence, nuclear extract and cytosolic proteins were prepared as described previously (29). For Western blots, 100 μg of nuclear and cytosolic proteins were separated on 10% SDS-polyacrylamide gels. After electrophoretic transfer to nitrocellulose membranes, Ponceau S staining was performed to ensure equal loading of each sample. SRC-3 protein was detected using an rabbit antipeptide antibody specific to an internal 15-amino acid sequence (912–926, CQTPSSGWDGPLPNSK) that shows minimal sequence similarity to both SRC-1 and TIF-2. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive SRC-3 was visualized using the ECL detection system (Amersham) following the procedures recommended by the supplier.

**Expression and Purification of hERβ—**The full-length cDNA encoding hERβ was cloned into a pET-28 (Novogen) expression vector using HindIII and XhoI restriction sites. This plasmid was transformed into Escherichia coli BL21(DE3)pLyS7. The transfected were grown to 0.6 OD units at 37 °C. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside, cells were grown for 3 h and harvested by centrifugation (10,000g, 10 min). The cell pellet was resuspended in four volumes of lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 2 mM dithiothreitol, 1 mM phenylmethylsulfonl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.1 mg/ml soyosyme) and sonicated three times for 30 s on ice. After a 10-min incubation on ice, sodium deoxycholate was added to 0.05%. The mixture was stirred at 4 °C for 15 min, and crude cell extract was obtained by centrifugation at 50,000 × g. Receptor-containing supernatant was loaded on a HiTrap 5′ affinity column equilibrated with 20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 50 mM imidazole. Receptor was eluted with two column volumes of a linear gradient of imidazole 50–500 mM in the same buffer. ERβ-containing fractions were pooled and loaded on a Superdex 200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 0.005% Nonidet P-40. Fractions containing ERβ were pooled and loaded onto a HITrap 5′ affinity column equilibrated with 20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 50 mM imidazole.

**Experimental Procedures—**The yeast MATCHMAKER two-hybrid system (CLONTECH) was used according to the manufacturer's instructions (31). Briefly, yeast CG1945 cells along with a human liver cDNA library constructed in the pM vector (ERβ) were transfected into yeast strain CG1945, colonies were selected on SD/-His/-Trp/-Leu plates. For mammalian two-hybrid assays, the LBDs of hERα and hERβ were ligated into the pM vector (ERα/pM and ERβ/pM), and clone 31 was ligated into the pVP16 vector (31/VP16). The reporter plasmid expressing the chloramphenicol acetyltransferase (CAT) gene was under control of the GAL4 response element (pGS5CAT). Human lung A549 cells were routinely maintained in minimal essential medium containing 5% FBS. Cells were seeded into six-well plates (Falcon), and plasmids were transiently introduced using calcium phosphate coprecipitation method (Promega). Transfections were done in the presence or absence of 17β-estradiol (10 mM) using lipofectamine (0.25 μg) as an intracellular marker plasmid pEGFP-C1 (2 μg), ERα/pM (0.8 μg), 31/VP16 (0.8 μg), and pGEM-4Z as a carrier DNA (Promega). In preliminary experiments, increasing amounts of pcDNA vector containing ERα provided increasing estrogen-induced luciferase activity without changing the levels of the β-galactosidase activity in the co-transfection, thus demonstrating that the amount of pcDNA vector did not significantly alter the expression of the control plasmid. After 48 h, cells were harvested, and extracts were assayed for CAT activity.

**Treatment and Data Analysis—**Each binding cycle was performed with a constant flow (10 ml/min) of buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5. Samples of ER were
injected across the surface via a sample loop. Once the injection plug had passed the surface, the formed complexes were washed with the buffer for an additional 500–1000 s. All experiments were performed at 25 °C. Data were collected at 1 Hz and analyzed on a Compaq PC using the Clamp 3 program obtained from Dr. David Mishka (University of Utah). This program uses a “global fitting” analysis method for the determination of rate binding constants for macromolecular interactions. The best description for experimental data was obtained with a model describing monomolecular interactions with surface heterogeneity.

Transcriptional Coactivator Activity (Luciferase) Assays—CHO cells were maintained in DMEM/F-12 tissue culture medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). To measure the coactivator activity of SRC-3, CHO cells (1.5 × 10^5 cells/well) were seeded in six-well plates in phenol red-free DMEM/F-12 supplemented with 5% charcoal- and dextran-treated FBS. Using the calcium phosphate/DNA precipitation method, cells were transfected with 0.05 µg of receptor expression vector, 2 µg of reporter plasmid, and 0–3 µg of SRC-3 expression vectors. Rous sarcoma virus-β-galactosidase served as an internal control, and pGEM4Z plasmid was used as a carrier DNA. After 4 h, cells were treated with 10% glycerol and incubated in the presence or absence of 10 nM E2 and/or 10 nM antiestrogen ICI 182,780 in phenol-red free medium for 20 h, unless otherwise stated. Luciferase assays were performed according to the supplier's
RESULTS

Identification of SRC-3 cDNA—The yeast two-hybrid assay using the LBD of human ERα was used to identify a clone, clone 31, that interacted only in the presence of ligand. A full-length cDNA was isolated, and the sequence revealed a long open reading frame of 1423 amino acids with a predicted molecular mass of ~155 kDa. This protein, referred to as SRC-3, had significant homology (30–40%) to SRC-1 and TIF-2 (18, 19) and has recently been described by others under various names (ACTR/RAC3/AIB1/TRAM-1; Refs. 23–26).

Tissue Distribution of SRC-3—A Northern analysis using 5 μg of poly(A)⁺ mRNA from various human tissues was performed to determine the size of the message and the tissue distribution of SRC-3. A major mRNA transcript with an approximate size of 8.5–9.0 kb was detected (Fig. 1A). The transcript was abundant in uterus, mammary gland, pituitary, testis, heart, and skeletal muscle, it was relatively low in bone marrow, and it was barely detectable in liver, lung, brain, kidney, stomach, and adrenal gland (Fig. 1A; data not shown) indicating that SRC-3 is differentially expressed among tissues. Also, a minor species (~5.5 kb) was found in testis and skeletal muscle, suggesting a potential alternative splicing variant of SRC-3. Finally, the transcript of SRC-3 was expressed abundantly in Burkitt’s lymphoma cells, colorectal SW480 cells (Fig. 1B), and MCF-7 breast cancer cells (data not shown).

Antipeptide antibodies were generated and used to examine the expression of SRC-3 protein. A single band with a molecular weight of ~160 kDa was detected predominantly in the nuclear fraction of MCF-7 breast cancer cells, suggesting that SRC-3 is present in the nucleus (Fig. 1C). Furthermore, Western blot analysis of nuclear extracts prepared from several human cancer cell lines clearly showed that SRC-3 was highly expressed and most abundant in human breast cancer MCF-7 cells (Fig. 1D).

Protein-Protein Interaction Between SRC-3 and ERα and ERβ—In yeast, clone 31, containing the last 803 amino acids of SRC-3, interacted with the LBD (TAF-2) but not the N terminus (TAF-1) of hERα (Fig. 2A). Furthermore, this interaction was dependent on the presence of ligand (Fig. 2A). To test whether this interaction between the hERα and clone 31 occurs in mammalian cells, a mammalian two-hybrid protein-protein interaction assay was conducted. In the absence of clone 31 (31/VP16), E₂ (10 nM) stimulated CAT activity ~8-fold when cells were transfected with the LBD of ERα (ERα/PM), consistent with the presence of a known transcription activation function (TAF-2) in this region (Fig. 2B). However, in the presence of clone 31, E₂-stimulated CAT activity was increased by an additional 10–15-fold. Furthermore, the interaction between clone 31 and hERα was dependent on the presence of E₂, and antiestrogen ICI 182,780 inhibited this interaction. Recently, a second estrogen receptor, ERβ, has been cloned, and the homology between the LBD of the ERα and ERβ is ~55%. Therefore, it was of interest to determine whether clone 31 can also interact with the LBD of ERβ. Like ERα, CAT activity was stimulated by E₂ when cells were transfected with the LBD of ERβ (ERβ/pM), suggesting the presence of a transcription activation function within the LBD of ERβ. Nevertheless, in the presence of clone 31, E₂-stimulated CAT activity was only marginally enhanced (Fig. 2C). These results indicate that the affinity of the interaction of clone 31 with ERα is higher than that with ERβ.

To evaluate further the interaction between clone 31 and ERα, surface plasmon resonance analyses, which effectively monitor interactions between macromolecules in real time, were conducted. One of the interacting components, clone 31, was immobilized on the surface of a sensor chip, whereas the other, either ERα or ERβ, was injected by constant flow. A surface with 347 resonance units of the immobilized clone 31 was tittered with increasing amounts of purified ERα or ERβ and saturable interactions were detected for both proteins (data not shown). Two overlaid injections of equal concentrations of each receptor in the presence of 1 μM E₂ are presented (Fig. 2D). “On” and “off” rates as well as the equilibrium dissociation constant of the protein-protein interactions were obtained using global fitting analysis. The affinity of clone 31 interaction with ERα is significantly higher than the affinity of interaction with ERβ. The apparent Kd was 1.02 nM for the interaction between SRC-3 and ERα and 768 nM for the interaction with ERβ. Furthermore, the effect of ligand on the interaction of clone 31 with ERα was also determined. ERα was incubated overnight with E₂ (1 μM) or ICI 182,780 (1 μM) before it was injected over the surface with immobilized clone 31. Overlaid injections of ERα liganded with either E₂ or ICI 182,780 and unliganded ERα are presented (Fig. 2E). Analysis indicated that E₂ promotes and ICI 182,780 inhibits clone 31 interaction with ERα.

SRC-3 Functions as a Transcriptional Coactivator and Selectively Augments Transcriptional Activity—The transcriptional activity of ERα in the presence or absence of SRC-3 was assessed to determine whether SRC-3 is indeed a transcriptional coactivator that enhances E₂-stimulated gene transcription. In the absence of SRC-3, E₂-stimulated ERα-mediated reporter gene transcription ~4–5-fold over that of the control (Fig. 3A and B). However, the E₂-stimulated transcriptional activity was enhanced up to 22-fold in the presence of increasing amounts of SRC-3, and antiestrogen ICI 182,780 completely blocked this transcriptional activation. Furthermore, in the absence of E₂, SRC-3 did not affect basal level transcription of ERα under the assay conditions used (data not shown).

The protein-protein interaction assay described above demonstrated a preferential interaction of clone 31 with the LBD of ERα over ERβ (Fig. 2C). Therefore, we investigated whether ERβ-stimulated reporter gene transcription could also be augmented by SRC-3. As in the case of hERα, E₂ stimulated ERβ-mediated reporter gene transcription (Fig. 3C). However, this ERβ-stimulated transcriptional activity was not augmented with increasing amounts of SRC-3 (Fig. 3C). In some experiments, a slight augmentation was observed, but never >2-fold. A radioligand binding assay indicated that similar...
A Coactivator with Nuclear Hormone Receptor Selectivity

**Fig. 2**

Panel A: Images of petri dishes labeled with 'Clone 31 + TAF1', 'Clone 31 + TAF2 + E2', and 'Clone 31 + TAF2 + E2'.

Panel B: Bar graph showing CAT activity with fold induction. The y-axis represents CAT activity, and the bars indicate the effects of various conditions.

Panel C: Graph showing CAT activity with fold induction. The x-axis represents time in seconds, and the y-axis represents CAT activity.

Panel D: Graph showing RU over time for various conditions.

Panel E: Graph showing RU over time for different ligands and conditions.

**Legend:**
- E2 (10 nM)
- ERα/pM
- 3VP16
- ICI (1 μM)

**Time (sec)**
amounts of ERα and ERβ were expressed in the assay, a result consistent with the similar amounts of transcriptional activity observed in the absence of SRC-3 (data not shown). This preferential stimulation of ERα activity by SRC-3 is consistent with our previous finding that clone 31, a C-terminal derivative of SRC-3, interacted preferentially with the LBD of the ERα.

To further characterize this potential receptor selectivity of SRC-3, the activity of progesterone receptor (PR)-mediated gene transcription was analyzed. Progesterone stimulated PR-mediated gene transcription ~20-fold over the basal level transcription (Fig. 3D). As in the case of ERα, the transcriptional activity of PR was gradually enhanced by adding increasing amounts of SRC-3. However, this transcriptional enhancement of PR was attenuated when larger amounts of SRC-3 were expressed, and this squelching was not observed with ERα using similar amounts of SRC-3 (Fig. 3, B and D).

To determine whether intact TAF domains of ERα are involved in the coactivation of ERα-dependent transcription by SRC-3, expression vectors of wild-type ERα, ERα-TAF-1, ERα-TAF-2, and ERα-null (Fig. 4A) were introduced individually into CHO cells with SRC-3 in the presence and absence of E2. Compared with the wild-type ERα, the E2-stimulated reporter gene transcriptional activities of these mutant receptors were relatively weak (Fig. 4B). The activity of ERα-TAF-2, a TAF-1 deletion, and ERα(Term)-TAF-2, containing three amino acid substitutions within the LBD of ERα, was augmented by SRC-3 ~8- and 5-fold, respectively, suggesting that SRC-3 can, to varying degrees, augment transcription in the absence of either TAF-1 or TAF-2 in this cell system. However, SRC-3 was not able to augment transcription of the ERα-null construct, suggesting that one intact TAF domain of ERα was required for SRC-3 coactivator activity.

**SRC-3 Contains an Autonomous Transcriptional Activation Function**—To determine whether SRC-3 contains an autonomous transcriptional activation function, both full-length SRC-3 and clone 31 were fused to the DNA binding domain of GAL (1–147) and transfected into the CHO cells with a GAL4 reporter plasmid. The reporter activity was stimulated by both SRC-3 (SRC-3/pM) and clone 31 (31/pM) (Fig. 5). Interestingly, a greater stimulation was consistently observed with clone 31 when the same amounts of plasmids were used. Regardless, this study clearly suggests that a potential transcriptional activation function is present within SRC-3 and the location of this activity is within the C-terminal half of the protein.
Several models have been proposed to understand the potential mechanism(s) by which nuclear receptors stimulate gene transcription. Earlier models implicated a direct binding of NRs to proteins in the preinitiation complex such as TFIIIB, TBP, and TAFs (8–12, 31, 32). However, this simplified mechanism in which NRs directly interact with the basal transcriptional apparatus does not accommodate the known complexities involved in either ligand specificity or promoter and tissue selectivity. Transcriptional interference and squelching experiments (33–35) suggest additional proteins, referred to as co-activators and co-repressors, function as bridging molecules between the NRs and the basal transcriptional apparatus and regulate the expression of different target genes. Consistent with this model, several candidate proteins that interact directly with NRs have recently been identified (for review, see Ref. 12). Among these proteins, SRC-1 (18), TIF-2 (19), androgen receptor-associated protein 70 (ARA70) (20), and cAMP response element-binding protein (CBP)p300 (21) have been shown to enhance ligand-stimulated gene transcription by a number of NRs, suggesting that these proteins function as coactivators.

We report here a steroid receptor coactivator, SRC-3, that selectively augments ERα- over ERβ-mediated reporter gene transcription. In addition, transcriptional activity of PR is also enhanced with coexpression of SRC-3.

The overall homologies and local alignment similarities among SRC-3, SRC-1, and TIF-2 clearly define a family of nuclear receptor coactivators that have (i) an N-terminal region containing a bHLH-PAS domain, (ii) an autonomous transcription activation function, and (iii) consensus core motifs (LXXLL) within the central region of the coactivators required for nuclear receptor interaction. These consensus core motifs (LXXLL), also referred to as leucine charged domains, were recently identified (36, 37) as sequences involved in the ligand-dependent interaction between the nuclear hormone receptors and several transcriptional coactivators. A total of five LXXLL motifs are present in SRC-3, and three are located between residues 621 and 742. Interestingly, the relative positions of the three motifs in the central region of SRC-3 are conserved in SRC-1, TIF-2, and p/CIP and are present in the region of these coactivators that interacts with nuclear hormone receptors (36, 37). Also, results from the protein-protein interaction assays (Fig. 2, A and B) suggest that the LXXLL motif at residues 113–117 within the bHLH-PAS domain, a region not present in

**Fig. 4.** SRC-3 enhances the transcriptional activity of ERα-TAF-1 and ERα-TAF-2 but not ERα-null. A, Schematic organization of wild-type ERα (ERα-wt) and mutant ERs ERα-TAF-1, ERα-TAF-2, and ERα-null, used in this experiment. B, ERα-wt and mutant ERα (0.05 μg) with the reporter (2 μg) were introduced into CHO cells with or without SRC-3 (3 μg). After 24 h of treatment, luciferase activity was measured. The data shown indicate the mean ± S.E. of triplicates.
the clone 31, is dispensable for a strong interaction between SRC-3 and ERα. Similarly, the LXXLL motifs at residues 45–53 and 111–118 of SRC-1a lack strong nuclear receptor binding activity (36).

Compared with SRC-1 (18) and TIF-2 (19), the expression pattern of SRC-3 is more restricted (Fig. 1, A and B), suggesting that differential expression of these coactivators among tissues may be involved in the regulation of tissue-selective gene expression. Significantly, SRC-3 is highly expressed in a breast cancer cell, MCF-7, compared with some tumor cell lines of non-breast origin (Fig. 1D). Furthermore, SRC-3 was observed in the nuclear fraction of MCF-7 cells (Fig. 1C). Recently, the gene encoding SRC-3, referred as AIB1, was identified as a segment of chromosome 20 that is amplified in some primary breast tumors, and the expression of AIB1 mRNA was increased in more than half of these tumors (25). As suggested by Anzick et al. (25), SRC-3/AIB1 may contribute to the development of cancers in tissues in which it is expressed, particularly steroid-dependent cancers (25).

Recently, a second ER, referred to as ERβ, has been identified and shown to activate transcription of an estrogen response element-containing reporter gene construct in the presence of E2 (5, 6). However, the amino acid homologies within the TAF-1 (16%) and TAF-2 (56%) regions of ERα and ERβ suggest that the regulation of ERα and ERβ responsive genes may use different molecular mechanisms, including the use of distinct coactivators. Consistent with this hypothesis, clone 31 was shown to preferentially interact with ERα over ERβ (Fig. 3, C and D), and the transcriptional activity of ERα but not ERβ was augmented by SRC-3 (Fig. 4, B and C). Nevertheless, this transcriptional enhancement is not exclusive to ERα, because SRC-3 also enhanced PR-stimulated transcriptional activity. Interestingly, smaller amounts of SRC-3 were required to observe the transcriptional augmentation of PR than ERα (Fig. 4, B and D). This suggests that varying ratios of SRC-3 and a particular NR are required to regulate the expression of different target genes. Selective enhancement of subsets of nuclear hormone receptor-mediated gene transcription has also been observed with TIF-2 (19), androgen receptor-associated protein 70 (ARA70) (20), and TAF₁₁₁₃₅ (22). Therefore, as the number of putative coactivators and/or corepressors grows and as functional activities are identified (e.g., histone acetyltransferase activity), the interplay among the NRs, coactivators and/or corepressors, integrators, and the basal transcriptional apparatus will facilitate, at least in part, an understanding of tissue- and promoter-specific gene expression mediated by ligand-activated NRs.

One of the expected properties for NR coactivators is that transcriptional enhancement would be dependent upon the presence of an intact NR TAF domain. Previously, it has been shown that the substitution of three amino acids in the ligand-binding domain disrupts TAF-2 activity but does not disrupt receptor dimerization or ligand binding affinity (38). However, it was also shown that the complex estrogen-responsive complement 3 promoter can be induced by this mutant construct in the presence of estrogen, suggesting that this activity is mediated by TAF-1 (39). Recently, Smith et al. (40) reported that SRC-1 efficiently enhances E₂-stimulated complement 3 promoter transcriptional activity of this TAF-2-defective ER mutant, indicating that an intact TAF-2 domain is not required for SRC-1 coactivator activity in this cell and promoter context. The activity of the SRC-1 on the TAF-1 mutant was not analyzed in this system. We have demonstrated that SRC-3 enhances transcriptional activity of both the TAF-1-deleted ERα (ERα-TAF-2) and the TAF-2-defective ERα (ERα-TAF-1) to varying degrees (Fig. 4B). The TAF-1 deletion mutant had a minor impact on the ability of SRC-3 to enhance transcription, as might be anticipated, because SRC-3 did not interact with the TAF-1 region (Fig. 2A). Furthermore, SRC-3 was able to augment the transcription of the TAF-2-defective mutant ~5 fold (Fig. 4B), suggesting that SRC-3 does indeed augment transcription through TAF-1 despite a lack of direct interac-
tion. This activity of SRC-3 may use an adaptor protein that mediates the interaction of SRC-3 and the TAF-1 domain. Alternatively, an interaction with the TAF-1 domain could require the presence of the TAF-2 domain. These observations are consistent with those recently reported for two other coactivators. Norris et al. (41) also reported that GRIP-1 can enhance the transcriptional activity of the defective TAF-2 ERα mutant and therefore conclude that TAF-2 is likely not to be the sole determinant in the interaction of GRIP-1 with ERα (41). Furthermore, SRC-1 has been proposed to mediate the transcriptional synergy that exists between TAF-1 and TAF-2 of ERα (42). Therefore, three different coactivators have now been demonstrated to play an intriguing role in TAF-1-mediated transcription.

Finally, a transcriptional coactivator is thought to enhance gene transcription by bridging transcription factors with the components of the basal transcriptional machinery. Therefore, a transcriptional coactivator would be expected to have a transcriptional activation function that activates target gene expression through either the disruption of the nucleosome structure or by modulating the preinitiation complex (43–45), resulting in an increased rate of transcription. Like SRC-1, TIF-2, and cAMP response element-binding protein (CBP), both clone 31 and SRC-3 activated heterologous gene transcription through either the disruption of the nucleosome structure or by modulating the preinitiation complex (43–45), resulting in an increased rate of transcription. Like SRC-1, a transcriptional coactivator would be expected to have a transcriptional activation function (Fig. 5). These results also suggest that the bHLH-PAS domain at the N terminus of SRC-3 is not essential for either receptor interaction or transcriptional activation.

In summary, this study reports the cloning and characterization of SRC-3, a transcriptional coactivator involved in ERα- and PR-stimulated gene transcription. Most importantly, SRC-3 selectively activates the transcriptional activity of ERα over ERβ. Both the unique tissue distribution and the ability to selectively augment transcriptional activity suggest that SRC-3 may play an important role in steroid hormone-mediated gene expression. The precise role(s) of SRC-3 in steroid hormone actions, particularly tissue- and promoter-specific regulated gene expression. The precise role(s) of SRC-3 in steroid hormone actions, particularly tissue- and promoter-specific regulated gene expression. The precise role(s) of SRC-3 in steroid hormone actions, particularly tissue- and promoter-specific regulated gene expression.