Striatin-3γ inhibits estrogen receptor activity by recruiting a protein phosphatase

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Abstract

A splicing variant of rat striatin-3 (rSTRN3γ) was found to associate with estrogen receptor-α (ERα) in a ligand-dependent manner. In two-hybrid and pull-down analyses, estradiol induced an interaction between rSTRN3γ and ERα. STRN3γ protein was found in nuclear extracts from rat uterus and human cell lines. Overexpression of rSTRN3γ induced a decrease in ERα transcriptional activity but had no effect on ERβ activity. Immunoprecipitation analyses showed that rSTRN3γ interacts with both the ERα and the catalytic subunit of protein phosphatase 2A (PP2A(C)). The transrepressor action of rSTRN3γ was overcome by okadaic acid, an inhibitor of PP2A(C), and by cotransfection of PP2A(C) siRNA. rSTRN3γ caused dephosphorylation of ERα at serine 118 and this was abrogated by okadaic acid. ERα lacking phosphorylation sites at either serine 118 or 167 was insensitive to the corepressor action of rSTRN3γ. These observations suggest that an rSTRN3γ-PP2A(C) complex is recruited to agonist-activated ERα, thereby leading to its dephosphorylation and inhibiting transcription.

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Introduction

Estrogen receptors (ERα and ERβ) are the ligand-activated, phosphorylated transcription factors (McKenna & O’Malley 2002, Edwards 2005). Like other nuclear receptors, ERα exerts its transactivational function through interaction with coregulatory proteins, coactivators and corepressors. Recent advances with cDNA microarrays have allowed an appreciation of the magnitude of the genomic response to 17β-estradiol (E2). In the estrogen-responsive breast cancer cells, MCF-7, 438 out of the 12 000 genes examined were regulated by E2; 70% of these 438 genes were down-regulated (Frasor et al. 2003). About one-third of the genes that were up-regulated by E2 responded transiently, i.e., their mRNA levels increased dramatically within the first few hours following the addition of E2 but then decreased to near pre-stimulation levels over the course of the next few hours, even though E2 remained in the culture medium (Frasor et al. 2003). This type of transient transactivational response was observed previously for the early response genes, c-fos, c-myc, and c-jun (Loose-Mitchell et al. 1988, Weisz & Bresciani 1988, Bigsby & Li 1994). On the other hand, the E2-induced increase in mRNA levels for pS2 (Brown et al. 1984, Cavailles, et al. 1989, Metivier et al. 2003), cathepsin D (Cavailles et al. 1989), and growth factors, amphiregulin and SDF-1 (Frasor et al. 2003), occurs early after stimulation and is maintained through at least 24 h. It is not known how some genes are down-regulated by estrogen while others are up-regulated. Furthermore, the mechanisms responsible for quickly shutting down E2-induced transactivation of some genes, but not others, are unknown.

Transactivational effects of ERα are regulated via proteins that become associated with the receptor. Upon activation with ligand and/or phosphorylation via growth factor signaling pathways, ERα binds to estrogen response elements or to other transcription factors, thereby tethering it to the promoter region in target DNA; simultaneously, there is a conformational change in ERα, permitting it to interact with a wide array of nuclear receptor coregulatory proteins (McKenna & O’Malley 2002, Smith & O’Malley 2004, Edwards 2005). Coactivator proteins have intrinsic enzymatic activity or they recruit other proteins with enzymatic action that modifies histones, thereby changing the chromatin structure and allowing the formation of a complex of proteins, which directly or indirectly interacts with the pre-initiation complex (McKenna & O’Malley 2002, White et al. 2004). One of the major coactivator proteins, steroid receptor coactivator-3 (SRC-3), is active only if it is in its fully phosphorylated state (Wu et al. 2004). Corepressor proteins, such as silencing mediator of the retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR), negatively regulate ERα activity.
when an antagonist occupies the receptor’s ligand pocket; SMRT and NCoR bring proteins with histone deacetylase (HDAC) activity into the complex, thereby shutting down transactivation (Smith & O’Malley 2004, White et al. 2004). Negative regulation in the presence of agonist is achieved through recruitment of corepressors, LCoR, RIP140, and HDAC proteins (Metivier et al. 2003, White et al. 2004). In addition, ERz protein levels are down-regulated by E2-induced interactions between the receptor and the members of the proteosomal pathway (Fan et al. 2002, 2003). Elegant experiments using immunoprecipitation (IP) and chromatin immunoprecipitation techniques have shown that the recruitment of regulatory proteins occurs in an ordered and cyclical fashion (Shang, et al. 2000, Metivier et al. 2003).

In a recent report, striatin (STRN) was described as an ERz-interacting protein (Lu et al. 2004). STRN is a member of a family of multimodal proteins that include striatin-3 (STRN3) and striatin-4 (STRN4). Striatins have several putative functional domains, including caveolin- and calmodulin-binding domains and protein-interacting motifs (Muro et al. 1995, Castets et al. 1996, Castets et al. 2000, Moreno et al. 2000). Shang et al. (2000) and Lu et al. (2003) found that the ERz–STRN interaction plays a role in the non-genomic effects of estrogen in endothelial cells (Lu et al. 2004). Herein, we describe the agonist-induced interaction between the ERz and an isoform of STRN3, rSTRN3γ, isolated from the rat uterus. Evidence is presented indicating that rSTRN3γ represses ERz transactivational activity through a novel mechanism involving a protein phosphatase (PP2A).

Materials and methods

Chemicals and cells

Treatment chemicals, E2, 4-hydroxytamoxifen (Tam), and okadaic acid (OA), were purchased from Sigma Corp. Human breast cancer cell lines, MDA-MB-231 and MCF-7; the human cervical carcinoma cell line, HeLa; the human ovarian cancer cell line, BG-1; and immortalized monkey kidney cells, Cos-1, were purchased from ATCC (Manassas, VA, USA). The cell lines were maintained in DMEM supplemented with 10% FBS. Experimental stimulation with hormone was performed in media free of phenol red and supplemented with 3% charcoal-stripped serum (HyClone Laboratories Inc., Logan, UT, USA).

Plasmids

rSTRN3γ cDNA was cloned into the expression vectors, pcDNA3 and pcDNA3-His/myc tag, purchased from Invitrogen. The estrogen-responsive luciferase reporter gene, 2XERE-pS2-luc, was reported earlier (Long et al. 2001); briefly, it was generated by ligating two consensus ERE sites into the minimal promoter region of the pS2 gene and ligating this into the pGL3 luciferase (firefly) reporter plasmid (Promega, Madison, WI, USA). The ERz expression vector (HEGO) was obtained from Dr P Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Phosphomutants of ERz were the generous gift of Dr Simala Ali (Imperial College, London) and are described earlier (Ali et al. 1993, Campbell et al. 2001). Control reporter plasmids, pCMV-β-galactosidase (pCMV-β-gal) and pRL-tk-renilla-luciferase (pRL-TK), were purchased from Promega.

Yeast two-hybrid reporter assays

The yeast two-hybrid screening was performed as described previously (Fan et al. 2002). Briefly, a triple selection system was used. The yeast strain J69-4A was cotransformed with the GAL4 DNA-binding domain plasmid containing the ERz hybrid, pBD-GAL4-ERzAF2 (amino acid residues 290–600 of rat ERz) and the rat uterine cDNA library cloned into the GAL4 activation domain plasmid, pAD-Gal4. Yeast transformants were plated onto synthetic minimal medium agar lacking leucine, tryptophan, histidine, and adenine for 6 days at 30 °C. ERz-interacting clones were identified by their ability to grow in the selective plates and to activate LacZ reporter gene as indicated by blue colonies when X-Gal (Boehringer Mannheim, Indianapolis, IN, USA) was added to the culture. To further investigate the ligand-dependent and -independent interactions between rSTRN3γ with the AF2 domain of ER, we used the yeast two-hybrid system in solution culture, also as described previously (Fan et al. 2002). Yeast transformed with pBD-GAL4-ERzAF2 and pAD-GAL4+rSTRN3γ was grown in liquid culture containing 10⁻⁸ M E₂, 10⁻⁶ M Tam, or vehicle. The β-gal expression levels were determined using a chemiluminescent reporter assay (PE Applied Biosystems, Foster City, CA, USA).

GST pull-down assay

Glutathione S-transferase (GST) fusion pull-down experiments were performed as described previously (Fan et al. 2002). 35S-labeled full-length ERz or -β was incubated with GST–rSTRN3γ bound to glutathione–Sepharose beads in the absence or presence of 10⁻⁷ M E₂. After washing four times, specific interacting protein was eluted and analyzed by SDS–PAGE and autoradiography.

Transient transfection reporter assays

HeLa, MDA-MB-231, and BG-1 cells were maintained in DMEM with 5% FBS. Two days before transfection, the
cells were seeded onto 12-well dishes (10^5 cells/well) in phenol red-free DMEM containing 3% charcoal-stripped charcoal-stripped serum. The cells were transfected with equal amounts of total plasmid DNA (adjusted by adding corresponding empty vectors) using Tfx-20 reagent (Promega) according to the manufacturer’s guidelines. After 1 h, transfection medium was replaced with phenol red-free medium containing 3% charcoal-stripped charcoal-stripped serum and appropriate treatments (vehicle, hormone, or okadaic acid plus hormone). Cell lysates were prepared 20 h after treatment using reporter lysis buffer (Promega). All cells were cotransfected with a non-inducible reporter, either pCMV-β-gal or pRL-TK. Luciferase activities (firefly and renilla) were determined using the Promega Luciferase Assay System; β-galactosidase was assayed with a luminescence reagent kit (Tropix, Foster City, CA, USA). The level of firefly luciferase (2XERE-luc) was expressed as relative light units, normalizing against β-gal or renilla luciferase activity to correct for transfection efficiency. All experiments were performed in quadruplicate and repeated at least two times.

PP2A(C) siRNA

HeLa cells were seeded onto a 24-well dish. Using the same reagents as described above for reporter assays, the cells were transfected with ERz, 2XERE-luc, pCMV-β-gal, and STRN3γ or its empty vector, and with siRNA for PP2A(C) (Dharmacon, Lafayette, CO, USA; 100 pmol/well) or with a non-target siRNA supplied by the manufacturer. The following day the cells were treated with vehicle or 10^{-8} M E_2 and then lysed 18 h later. Luciferase activity in the lysate was normalized against β-galactosidase activity. In separate culture dishes, HeLa cells were similarly transfected with or without the addition of PP2A(C) siRNA and lysed 24 h later. Immunoblot analysis of PP2A(C) and STRN3γ in the lysate was performed and the density of the PP2A(C) bands was measured.

Western blot and immunoprecipitation

Whole cell lysates, cytoplasmic extracts, and nuclear extracts were prepared using the reagents and the procedures provided in a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Briefly, cells were seeded onto 100 mm dishes in complete culture media (with phenol red) containing 10% FBS and grown to approximately 75% confluence. The cells were washed with PBS and collected by scraping in a buffer containing protease and phosphatase inhibitors. The cells were either lysed in complete lysis buffer to produce whole cell lysate or treated sequentially with kit components according to the manufacturer’s instructions to produce cytoplasmic and nuclear extracts. Endometrial scrapings from ovary-intact or ovarioctomized rats (done using an IACUC-approved protocol) were lysed in the buffer containing protease inhibitors (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, PMSF).

Proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunobotted. Antibodies used included: anti-phospho-S118-ERz (Cell Signaling, Beverly, MA, USA), anti-ERz (HC20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SG2NA (S-68, Upstate Biotechnologies, Lake Placid, NY, USA), and anti-PP2A(C) (C-20, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) were applied and immunobotted proteins were visualized using Lumiglo reagent (Cell Signaling).

Cos-1 cells were cotransfected with HEGO and a vector that expresses His-tagged rSTRN3γ. The following day the cells were used in an immunoprecipitation assay to determine whether rSTRN3γ interacts with PP2A and ERz. For immunoprecipitation, 10 μl anti-His-tag polyclonal antibody (Cell Signaling) were added to 200 μl cell lysate and incubated overnight at 4 °C. A slurry of Sepharose protein A plus protein G was added to 200 μl cell lysate and incubated overnight at 4 °C. A slurry of Sepharose protein A plus protein G was added, and the mixture was shaken for 4 h at 4 °C. After washing four times with cell lysis buffer, the proteins were extracted from the beads in SDS–sample buffer and applied to 10% SDS–PAGE. Immunoblot analysis was performed as described above.

Statistical analyses

Assays were done in quadruplicate. All error bars represent standard deviation from the mean. ANOVA was applied to data, followed by Bonferroni’s tests to determine differences between individual means.

Results

rSTRN3γ associates with ERz

Using the carboxy portion of ERz as bait in a yeast two-hybrid system, we identified a rat protein that associates with ERz in a ligand-dependent manner. Sequence analysis of the cDNA retrieved from the yeast clone revealed that this protein was a member of the striatin family of proteins that include striatin (STRN, calmodulin-binding protein), striatin-3 (STRN3, SG2NA, calmodulin-binding protein-3), and striatin-4 (STRN4, zinedin, calmodulin-binding protein-4) (Castets et al. 1996, 2000, Bartoli, et al. 1998). The particular form of striatin that we found is a hitherto unreported splice
variant of striatin-3 that we refer to as rSTRN3γ (GenBank DQ473607). The rat STRN3 gene (GenBank gene ID 114520) is composed of 22 putative exons, out of which 18 code for amino acid sequence. The structure of the 18 coding exons for rat, mouse (GenBank gene ID 94186), and human STRN3 (GenBank gene ID 29966) are similar, and, as shown in Fig. 1, they produce nearly identical proteins. Two splicing isoforms of the human gene have been identified, hSTRN3α and -β (as designated in Swiss-Prot, ID Q13033; http://us.expasy.org/uniprot/Q13033); the α-isofrom (713 aa) is produced when exons 8 and 9 are omitted from the transcript; the full-length protein (797 aa) is referred to as the β-isofrom.

The rat γ-isofrom that we have identified also omits the two middle exons, identified as exons 12 and 13 in the rat genomic sequence. In addition, the rSTRN3γ transcript represents a read-through of the intron that follows exon 15, adding three amino acid codons and a STOP codon to the in-frame sequence. Furthermore, the in-frame STOP codon is conserved between the rat and human exon/intron sequences, suggesting that the truncated version may exist and has a functional role in humans as well. In the cloned cDNA for rSTRN3γ, the sequence that follows this STOP codon is identical to the ensuing intron, exons 16 and 17, plus approximately 1 kb of intronic sequence that follows exon 17. Several putative functional domains have been

Figure 1 Alignment of published mouse (m), rat (r), and human (h) STRN3 sequences and the deduced amino acid sequence for rSTRN3γ. Functional domains identified in the literature reports are indicated as follows: caveolin binding, underline; calmodulin binding, double underline; transactivation domain, shaded; transrepression domain, boxed WD40 repeat, bold.

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identified in STRN3 proteins, including caveolin- and calmodulin-binding domains, transactivation and trans-repression domains, and six WD40 repeat domains believed to function in protein–protein interactions. Thus, the protein sequence of rSTRN3γ is similar to hSTRN3α but is truncated in the carboxy end, having only one out of the six WD40 repeat motifs found in other striatin proteins.

Two-hybrid analysis shows that interaction between ERα and rSTRN3γ is dependent upon agonist stimulation; protein–protein interaction was induced by estradiol but not by the receptor antagonist, 4-hydroxytamoxifen (Fig. 2). In an in vitro, GST pull-down assay, estradiol induced the interaction between rSTRN3γ and ERα, but not with ERβ (Fig. 3).

The truncated nature of rSTRN3γ was confirmed through immunoblot analysis of the protein produced by expression vectors transfected into mammalian cells. The full-length cDNA was cloned into an expression vector and when transfected into Cos-1 cells (Fig. 4) or HeLa cells (not shown), it produces a protein of approximately 50 kDa that is detectable with an antibody, S-68, directed against a peptide common to the N-terminal amino acid sequence of striatin and STRN3 in both humans and rats. The S-68 antibody also detects endogenous STRN3α (94 kDa), -β (102 kDa), and -γ in the lysates of cell lines and homogenates from rat uterus (Fig. 4). Cos-1 cells expressed STRN3α but not STRN3β. Proteins from human cell lines were derived from either whole cell lysates or extracts made from the cytosolic or nuclear fractions. Human cell lines expressed the three STRN3 isoforms variably. HeLa and MCF-7 cells expressed all three proteins; STRN3β was found in both cytoplasmic and nuclear extracts while STRN3α and -γ were found only in the nuclear extracts. BG-1 cells did not express STRN3α and only a very small amount of STRN3γ could be detected in the nuclear extract. The rat uterus of ovari-intact animals expressed the α, β, and γ STRN3 isoforms, and after ovariectomy they also expressed an additional antibody-interacting protein of approximately 62 kDa; this latter observation was made in three separate sets of uterine homogenates. Whether the 62 kDa band represents yet another isoform of STRN3 remains to be determined. In the original report on the characteristics of cloned SG2NA (STRN3β), Muro et al. (1995) found that the expressed cDNA produced three bands; it was suggested that the two lower bands that were in the range of 60–66 kDa were the result of proteolytic degradation. Perhaps the intermediate band found in homogenates of ovariectomized rat uterus is also a product of proteolysis.

rSTRN3γ represses activity of ERα

In mammalian cells, including human breast cancer cells, MDA-MB-231; human cervical cancer cells, HeLa (Fig. 5); or monkey kidney cells, Cos-1 (not shown) rSTRN3γ blocks ERα transcriptional activity in a dose-dependent manner without altering basal levels of the
reporter gene. While rSTRN3γ is effective against ERα, it has no effect on transactivation by ERβ (Fig. 5b). The dose of STRN3γ required for inhibition of E2-induced ERα activity is not affected by cotransfection of SRC-3 (Fig. 5c), indicating that the inhibitory effect is not a matter of competition for coactivator binding to receptor.

**rSTRN3γ acts through protein phosphatase PP2A**

The corepressor action of rSTRN3γ may be related to protein dephosphorylation. Others had reported that the STRN3 behaves as a B-subunit of the PP2A (Moreno et al. 2000). PP2A acts on phosphorylated proteins only as a trimeric complex including the catalytic subunit (PP2A(C)), a stabilizing subunit (PP2A(A)), and the substrate-recognizing subunit (PP2A(B)) (Depaoli-Roach et al. 1994, Csortos et al. 1996, Janssens & Goris 2001). We tested whether the corepressor action of rSTRN3γ was related to its function as a PP2A(B) subunit. Accordingly, the cells were pretreated with 10⁻⁷ M okadaic acid, the inhibitor of PP2A, prior to stimulation with estrogen in the presence or absence of the vector expressing rSTRN3γ; pretreatment allows okadaic acid to enter the cells in sufficient quantity to reduce PP2A(C) activity by 50% at the time of adding E2 (Favre, et al. 1997). The ERα-positive human ovarian cancer cell line, BG-1 was used in this analysis because, unlike the breast cancer cell lines, it was found to be tolerant of okadaic acid treatment. Okadaic acid slightly enhanced ERα action and it blocked the repressive effect of rSTRN3γ (Fig. 6a).

Since okadaic acid can inhibit other protein phosphatases, we used an siRNA technique to determine the effect of specifically knocking down PP2A(C) (Fig. 6b-d). HeLa cells were transfected with expression vectors for ERα and rSTRN3γ and the estrogen-responsive reporter, with or without the addition of siRNA for PP2A(C). The cells were left untreated for 24 h to allow clearance of endogenous PP2A(C) and then they were treated with vehicle or E2 for 18 h. E2-induced enhancement of luciferase expression was inhibited by rSTRN3γ and this effect was blocked by the addition of PP2A(C) siRNA. The addition of non-target siRNA had no effect on the repressive effect of rSTRN3γ. In separate cultures, HeLa cells were similarly transfected with or without the addition of PP2A(C) siRNA and lysed 24 h later. Immunoblot analysis of total cellular lysate showed that siRNA treatment reduced PP2A(C) protein levels by an average of 52%. Since transfection efficiency in HeLa cells is never better than approximately 50% under the conditions employed in the siRNA experiment (data not shown), it is likely that there is very little, if any, PP2A(C) protein in the transfected cells.

The above results suggest that rSTRN3γ, acting as a B-subunit of PP2A, directs dephosphorylation of ERα or the proteins associated with ERα at the promoter, thereby turning off the transactivation of the estrogen-responsive gene. Using immunoprecipitation of the His-tagged rSTRN3γ, we demonstrated that rSTRN3γ, ERα, and PP2A(C) form a complex in the cell and estrogen activation of ERα enhances the formation of this complex (Fig. 7). If the corepressor action of rSTRN3γ derives from its ability to bring PP2A(C) into the complex of proteins formed through interactions with ERα at the promoter, then we would expect it to cause dephosphorylation of ERα and/or those proteins in the complex. To test this we examined the phosphorylation state of ERα, with and without the addition of rSTRN3γ (Fig. 8). In the absence of rSTRN3γ, estrogen induced a dramatic increase in the amount of ERα that was phosphorylated at serine 118; the addition of rSTRN3γ abrogated this effect. Furthermore, the effect of rSTRN3γ on ERα phosphorylation was blocked by okadaic acid. Thus, the presence of rSTRN3γ caused an okadaic acid-dependent dephosphorylation of ERα in at least one known phosphorylation site and this action correlates to inhibition of ERα transactivational activity. Using ERα phosphorylation mutants, we found that one other phosphorylation site, serine 167 is also critical to the inhibitory action of rSTRN3γ. When either serine 118 or serine 167 of ERα was mutated to alanine, the inhibitory action of rSTRN3γ was abrogated (Fig. 9). The suppressor action of rSTRN3γ was unaffected by

![Figure 4](https://www.endocrinology-journals.org/2533/article-pdf/20/3/199/10643073/2533.pdf)
mutation of serines to alanines at positions 102, 104, and 106 (Fig. 9). These observations suggest that phosphorylated serines at 118 and 167 are required for the regulatory effects of rSTRN3γ.

**Discussion**

In this report, we describe an agonist-dependent association of ERα and rSTRN3γ, a protein that recruits the protein phosphatase PP2A, thereby leading to receptor dephosphorylation and reduced transcriptional activity. Nuclear receptor activity depends not only on ligand binding but also on phosphorylation events that occur through intracellular signaling pathways. The mitogen-activated protein kinases (MAPK), ERK-1, and ERK-2 phosphorylate ERα at serine 118 and receptor coactivator proteins at several sites, thereby enhancing transactivational function (Smith 1998, Font de Mora & Brown 2000, Kato 2001, Levin 2003). TFIIH/CDK7 also phosphorylates ERα at serine 118 (Chen et al. 2000, 2002). RSKI and -2 kinases activated by MAPK, and AKT phosphorylate ERα at serine 167 (Frodin & Gammeltoft 1999, Martin et al. 2000, Clark et al. 2001, Likhite et al. 2006). Recent evidence indicates that all of the six identified phosphorylation sites on the coactivator protein, SRC-3, are required for its transactivational function (Wu et al. 2004). Crosstalk between typical growth factor pathways and ERα are highly dependent upon phosphorylation events and the pathways involved are known to impinge on cellular proliferation, apoptosis, and differentiation (Kato 2001, Migliaccio et al. 2002, Levin 2003). It is therefore likely that PPs play a significant role in regulation of the individual pathways and in the crosstalk between them. Our observations suggest that rSTRN3γ modulates ERα activity by acting as a B-subunit of PP2A, thereby dephosphorylating key amino acid residues in the receptor protein. Perhaps proteins that associate with ERα, such as SRC-3, are also dephosphorylated through this mechanism.

Others have shown that phosphorylation of S118 and S167 of ERα regulates DNA-binding affinity, coactivator-binding affinity, and ligand-specific effects (Likhite et al. 2006). We showed that ERα phosphorylation at serine

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**Figure 5** rSTRN3γ represses ERα transactivation of an ERE-reporter. (a) MDA-MB-231 cells were transfected with expression vectors for ERα, 2XERE-luciferase, and the control reporter, CMV-β-gal, without or with increasing amounts (ng/well) of the vector for rSTRN3γ. The cells were treated with vehicle or 10^-8 M estradiol (E2) for 20 h. The amount of luciferase and β-gal activities in cellular lysates were determined in a luminometer and expressed as relative light units (RLU). (b) HeLa cells were transfected with either the ERα or -β expression vector and the reporter genes plus varying amounts (ng/well) of rSTRN3γ.

(c) HeLa cells were transfected with ERα and reporter vectors and SRC3 and/or rSTRN3γ as indicated. Values represent means ± s.d., n=4. *P<0.01 versus E2 treated without rSTRN3γ.
118 is decreased in an okadaic acid-dependent manner by experimental expression of rSTRN3γ. Using ERα mutants, we found that both S118 and serine S167 are required for the inhibitory effects of rSTRN3γ. It is interesting that the activity of the ERαS167A mutant was not reduced by STRN3γ because we might expect that the S118 residue within that receptor protein would be dephosphorylated by the addition of rSTRN3γ to the cells, and thus the receptor would effectively lack phosphorylation at both sites. This observation suggests that both S118 and S167 are required for the formation of protein complex of ERα, STRN3γ, and PP2A(C), a possibility that will require further testing.

PP2A plays a role in the regulation of multiple cellular signaling pathways, including regulation of steroid receptor activity (Sola et al. 1991, Borras et al. 1994, Galigniana et al. 1999, Bhattacharjee et al. 2001), and aberrant expression of the enzyme may be involved in cancer etiology (Csoros et al. 1996, Smith 1998, Schonthal 2001, Sontag 2001). PP2A and another protein phosphate PP5, have been previously implicated in regulation of ER action. It has been noted that PP2A activity is higher and PKC activity is lower in ERα-positive breast cancer cell lines compared with

Figure 6 rSTRN3γ repression of ERα transactivation is dependent on PP2A activity. (a) Okadaic acid: BG-1 were cotransfected with cDNA for 2XERE-luciferase and control reporter (pCMV-β-gal) with or without 200 ng cDNA for rSTRN3γ. Cells were pretreated for half an hour with PBS vehicle or 100 nM okadaic acid (OA) and then with DMSO or 10^-11 M E2 for 8 h. Luciferase activity in cell lysates is expressed as relative light units (RLU). (b–d) PP2A(C) siRNA: HeLa cells were seeded onto a 24-well dish and transfected with ERα, ERE-luc, pCMV-β-gal, and rSTRN3γ or its empty vector, with (b) PP2A(C) siRNA or (c) with non-target siRNA. The next day the cells were treated with vehicle or 10^-8 M E2 and then lysed 18 h later. Luciferase activity in cell lysates is expressed as relative light units (RLU). In separate culture dishes, HeLa cells were similarly transfected with or without the addition of PP2A(C) siRNA and lysed 24 h later. Immunoblot analysis of PP2A(C) and STRN3γ in the lysate was performed (c); the density of the PP2A(C) bands was measured (arbitrary units below bands). Values for luciferase activity (a–c) represent means ± S.D., n=4; means with different superscripts differ from each other (P<0.05).

Figure 7 rSTRN3γ interacts with the catalytic subunit of PP2A in cells. Cos-1 cells were transfected with His-rSTRN3γ and ERα expression vectors. The following day the cells were left untreated (control) or treated with 10^-8 M E2 1 h prior to harvest. Lysate was subjected to immunoprecipitation (IP) with an antibody against polyhistidine (x-His). The precipitate was probed in a western blot with an antibody against PP2A(C) and ERα. A sample of total lysate (T) was included on the western blot for comparison with the immunoprecipitated material.
ERα-negative lines (Gopalakrishna et al. 1999). Estradiol-induced down-regulation of ERα protein was inhibited by 100 nM okadaic acid, suggesting that a dephosphorylation event via either PP2A or PP1A was required (Borras et al. 1994). On the other hand, experimental reduction of PP2A activity, through either expression of siRNA specific to the catalytic subunit of PP2A or by the addition of okadaic acid, decreased the stability of ERα mRNA and reduced receptor protein levels (Keen et al. 2005). Experimental overexpression of PP5 reduced transcriptional activities of both ERα and -β, and the reduced activity of ERα was correlated with a decrease in the phosphorylation state of the serine 118 residue (Ikeda et al. 2004).

Protein kinases and phosphatases have been shown to regulate other nuclear receptors and their associated proteins as well. Inhibition of protein phosphatases with okadaic acid or stimulation of PKA enhances transactivational activity of androgen receptor (AR) and progesterone receptor (PR; Beck et al. 1992, Ikonen et al. 1994). PR transcriptional activity is cell cycle dependent, probably due to phosphorylation/dephosphorylation events (Narayanan et al. 2005). Phosphorylation of specific residues regulates nuclear translocation of PR (Qiu et al. 2003). Dissociation from heat shock protein-90 (HSP90) and subsequent nuclear translocation of GR is dependent upon a dephosphorylation event induced by PP2A, PP1A, or PP5 (DeFranco et al. 1991, Galigniana et al. 1999, Zuo et al. 1999, Dean et al. 2001, Ismaili & Garabedian 2004). Likewise, the constitutive AR (CAR) ligand, phenobarbital, induces recruitment of PP2A to the CAR–HSP90 complex and the subsequent okadaic acid-sensitive nuclear translocation of the receptor (Yoshinari et al. 2003). On the other hand, nuclear localization of the two co-repressor proteins, N-CoR and SMRT, is dependent upon PP1 activity (Hermanson et al. 2002, McKenzie et al. 2005).

Specificity of PP2A(C) action derives from association with a regulatory B-subunit that controls its intracellular localization and/or substrate recognition (Csordos et al. 1996, Janssens & Goris 2001, Sontag 2001). STRN3 behaves as a B-subunit of PP2A, forming a complex with the regulatory subunit, PP2A(A) and the catalytic subunit, PP2A(C) (Moreno et al. 2000). We found that ERα associates with STRN3γ and this association is enhanced by estrogen. Furthermore, our immunoprecipitation data indicate that the formation of STRN3γ·PP2A(C) complex is enhanced by estrogen-activated ERα. It may be that the interaction between ERα and STRN3γ alters the conformation of STRN3γ so that it has a higher affinity for PP2A(C); this would increase the specificity of the interaction.

Liu et al. (2003) reported that PP2A(C) exerts its dephosphorylating effect by binding directly to ERα, but this is unlikely to occur without the benefit of B- and A-subunits; since PP2A subunits are ubiquitous throughout the plant and animal kingdoms, the cell lysates used in their experimental systems could have provided the required A- and B-subunits. The observations that STRN3γ does not bind to ERβ in a ligand-stimulated fashion and does not affect ERβ-induced transcription suggests that its B-subunit activity is restricted to ERα.

The present observations suggest a role for a striatin protein in the mechanism through which PP2A is recruited to a nuclear receptor. rSTRN3γ belongs to the striatin family of multimodal proteins. Striatins have been identified in a variety of tissues (Landberg & Tan 1994, Muro et al. 1995, Castets et al. 1996; see also GenBank:

![Figure 8](Image 84x141 to 269x364)

**Figure 8** rSTRN3γ decreases phosphorylation of ERα. Cells were transfected with ERα alone (lanes 1–3) or with ERα plus rSTRN3γ (lanes 4 and 5). The cells were treated with E2 and/or okadaic acid (lanes 4 and 5). The cells were treated with E2 alone (lanes 1–3) or with ERα plus rSTRN3γ. After 24 h the cells were treated with 10⁻⁸ M E2 or vehicle and cell lysates were prepared 18 h later. rSTRN3γ caused a significant (*P<0.05) decrease in E2-induced transactivation by ERα.

![Figure 9](Image 90x585 to 227x640)

**Figure 9** Effect of rSTRN3γ on transactivation by ERα phosphomutants. Cos-1 cells were transfected with 2XERE-luciferase and control reporter and ERα mutants (ER-mut) in which the indicated serine residues were replaced with alanines, and either 0 or 400 ng rSTRN3γ. After 24 h the cells were treated with 10⁻⁸ M E2 or vehicle and cell lysates were prepared 18 h later. rSTRN3γ caused a significant (*P<0.05) decrease in E2-induced transcription only in the cells transfected with the ERα (S102, 104, 106A) mutant.
In addition, evidence presented by others (Lu et al. 2000) that are responsible for these activities are unknown. In some cells, particularly neuronal tissues, they are located entirely in the cytoplasm (Castets et al. 2000, Moreno et al. 2001). Striatin proteins are characterized by several WD40 repeat motifs in their carboxy terminal region, and caveolin- and calmodulin-binding sites in their amino terminal region (Castets et al. 2000, Moreno et al. 2000). Transactivation and transrepression domains have been identified in STRN3/SG2NA (Zhu et al. 2001). Sequence analysis of rSTRN3γ indicates that it is an isoform of rat STRN3 that arises from a splicing variation, producing a truncated version of the protein. The carboxy terminal truncation yields a protein containing only one out of the six WD40 repeats, the putative transactivation domain and a portion of the transrepression domain. Further investigation is required to determine what portions of the protein are required for ERα binding and repression. In a recent report, Lu et al. (2004) described an interaction between ERα and STRN. Unlike the interaction between rSTRN3γ and ERα, which we describe as occurring through the carboxy half of the receptor protein, the STRN-interacting domain was localized to the N-terminal portion of ERα. It was demonstrated that the ERα-STRN interaction plays a role in the non-genomic effects of estrogen in endothelial cells; furthermore, the evidence suggests that STRN localizes ERα to the cytoplasm. Experimental disruption of the STRN-ERα interaction blocked estradiol induction of MAPK phosphorylation. It was pointed out that disruption of STRN-ERα did not block transactivation of an estrogen-responsive reporter gene; however, interference with this protein–protein interaction actually enhanced the transactivational effect of ERα. This observation suggests that STRN may, like rSTRN3γ, act as a repressor protein, dampening the transactivational effects of estrogen.

In summary, rSTRN3γ is a novel isoform of the STRN3 gene product that behaves as a B-subunit of PP2A and also interacts with ERα in an agonist-dependent manner. Thus, the repressor activity of rSTRN3γ derives from its capacity to target PP2A(C) to ERα and perhaps to other proteins associated with the receptor. The intramolecular domains within rSTRN3γ that are responsible for these activities are unknown. In addition, evidence presented by others (Lu et al. 2004) suggests that STRN may also exhibit ERα corepressor activity. Whether STRN, STRN3α/β, or STRN4 all represent B-subunits of PP2A and/or nuclear receptor-associated proteins remains to be determined.

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