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

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Abstract

A splicing variant of rat striatin-3 (rSTRN3 γ) was found to associate with estrogen receptor- α (ER α) in a ligand-dependent manner. In two-hybrid and pull-down analyses, estradiol induced an interaction between rSTRN3 γ and ER α . STRN3 γ protein was found in nuclear extracts from rat uterus and human cell lines. Overexpression of rSTRN3 γ induced a decrease in ER α transcriptional activity but had no effect on ER β activity. Immunoprecipitation analyses showed that rSTRN3 γ interacts with both the ER α and the catalytic subunit of protein phosphatase 2A (PP2A(C)). The transrepressor action of rSTRN3 γ was overcome by okadaic acid, an inhibitor of PP2A(C), and by cotransfection of PP2A(C) siRNA. rSTRN3 γ caused dephosphorylation of ER α at serine 118 and this was abrogated by okadaic acid. ER α lacking phosphorylation sites at either serine 118 or 167 was insensitive to the corepressor action of rSTRN3 γ . These observations suggest that an rSTRN3 γ -PP2A(C) complex is recruited to agonist-activated ER α , thereby leading to its dephosphorylation and inhibiting transcription.

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Introduction

Estrogen receptors (ER α and ER β) are the ligand-activated, phosphorylated transcription factors (McKenna & O'Malley 2002, Edwards 2005). Like other nuclear receptors, ER α 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 17 β -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 ER α are regulated via proteins that become associated with the receptor. Upon activation with ligand and/or phosphorylation via growth factor signaling pathways, ER α 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 ER α , 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 ER α 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, ER α 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 ER α -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, such as 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 ER α -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 ER α and an isoform of STRN3, rSTRN3 γ , isolated from the rat uterus. Evidence is presented indicating that rSTRN3 γ represses ER α 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

rSTRN3 γ 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 ER α expression vector (HEGO) was obtained from Dr P Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). Phospho-mutants of ER α 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 ER α hybrid, pBD-GAL4-ER α AF2 (amino acid residues 290–600 of rat ER α) 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. ER α -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 rSTRN3 γ 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-ER α AF2 and pAD-GAL4-rSTRN3 γ 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 ER α or - β was incubated with GST-rSTRN3 γ bound to glutathione-Sepharose beads in the absence or presence of 10⁻⁸ 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% dextran-coated 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% 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 ER α , 2XERE-luc, pCMV- β -gal, and STRN3 γ 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 STRN3 γ 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 ovariectomized 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 $_3$ VO $_4$, 1 μ g/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, PMSF).

Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted. Antibodies used included: anti-phospho-S118-ER α (Cell Signaling, Beverly, MA, USA), anti-ER α (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 immunoblotted proteins were visualized using Lumiglo reagent (Cell Signaling).

Cos-1 cells were cotransfected with HEGO and a vector that expresses His-tagged rSTRN3 γ . The following day the cells were used in an immunoprecipitation assay to determine whether rSTRN3 γ interacts with PP2A and ER α . For immunoprecipitation, 10 μ l anti-His-tag polyclonal antibody (Cell Signaling) were added to 200 μ l 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

rSTRN3 γ associates with ER α

Using the carboxy portion of ER α as bait in a yeast two-hybrid system, we identified a rat protein that associates with ER α 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 rSTRN3 γ (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, hSTRN3 α and - β (as designated in Swiss-Prot, ID Q13033; <http://us.expasy.org/uniprot/Q13033>); the α -isoform (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 -isoform.

The rat γ -isoform that we have identified also omits the two middle exons, identified as exons 12 and 13 in the rat genomic sequence. In addition, the rSTRN3 γ 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 rSTRN3 γ , 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

mSTRN3	MDELAGGGGGGMAAPRPQGGPGLNLSLPPGANGAPGGGGPPAAEAGPPAGPELSRPQQYTI PGILHYIQHEWARFE	80
rSTRN3	T.....	79
hSTRN3 β	Q.....G.S.G.A.....S.G.A.....N.....	80
hSTRN3 α	Q.....G.S.G.A.....S.G.A.....N.....	80
rSTRN3 γ	V.....T.....	79
mSTRN3	MERAHWEVERAELQARIAFLQGERKQENLKKDLVRRIMKLEYALKQERAKYHKLKYGTENQGDLMKPTFESEETKDVE	160
rSTRN3	A.....	159
hSTRN3 β	T.....	160
hSTRN3 α	T.....	160
rSTRN3 γ	A.....	159
mSTRN3	APPAPQNSQLTWKQGRQLLRQYLQEVGYTDTILDVRSQRVRSLLGLSNSEPNGSVEAKNLEQILNGGESPKQKGQEIKR	240
rSTRN3	I.....	238
hSTRN3 β	..T.....T.....S	240
hSTRN3 α	..T.....T.....S	240
rSTRN3 γ	I.....	238
mSTRN3	PGDVLETFNPLENADDSDE--EENDMIEGIPEGKDKLRIHKHKIGNEGLAADLTDDPDTEALKEFDLFTAEDGEGAGEA	319
rSTRN3	S.....	317
hSTRN3 β	S.....D.....H.MN.....	320
hSTRN3 α	S.....D.....H.MN.....	320
rSTRN3 γ	S.....	317
mSTRN3	RSSGDGTWEDKDDLSPTEAVVDVQGLISKLEQYKQKRGKGVKRVNRTNLCMDITDLGDDLPHPISGIINQSRSSAS	399
rSTRN3	M.....A.....	397
hSTRN3 β	A.....K.....A.....	400
hSTRN3 α		369
rSTRN3 γ		367
mSTRN3	TRMADHEGARAEAEAPITFPSSGGGKSFIMGSDVLLSVLGLGDLADLTVTNDADYSYDLPANKDAFRKTWNPKVTLRSHF	479
rSTRN3	S.....L.....	477
hSTRN3 β	..T.....	480
hSTRN3 α		396
rSTRN3 γ	L.....	394
mSTRN3	DGVRALAFHPVEPVLVTASEDHTLKLWNLQKTVPAKKSASLDVEPIYTFRAHIGPVLSLAISSNGEQCFSGGIDATIQWM	559
rSTRN3		557
hSTRN3 β		560
hSTRN3 α		476
rSTRN3 γ	(QVfstop).....	434
mSTRN3	NMPSPNVDPYDTYESNVLAGTLVAHTDAVNGLAYSGIKNQLLSCSADGTIRLWNPQEKLPVCVCTYNGDKEHGIPTSVDFI	639
rSTRN3	H.....I.....	637
hSTRN3 β	S.....P.....I.....K.....	640
hSTRN3 α	S.....P.....I.....K.....	556
mSTRN3	GCDPAHMTVSFNTGSAVIYDLETSQSLVMLSSQVDSGLQSSNHINRVVSHPTLPVTITAHEDRHKKFFDNKTGKMIHSMV	719
rSTRN3	I.....	717
hSTRN3 β	I.....	720
hSTRN3 α	I.....	636
mSTRN3	AHLDAVTSLAVDPNGIYLMSSGSHDCSIRLWNLDKSTQVCEITAHRRKLDSEIYDVAFHPSPKAYIASAGADALAKVVF	796
rSTRN3		794
hSTRN3 β		797
hSTRN3 α		713

Figure 1 Alignment of published mouse (m), rat (r), and human (h) STRN3 sequences and the deduced amino acid sequence for rSTRN3 γ . 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.

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 rSTRN3 γ is similar to hSTRN3 α 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 ER α and rSTRN3 γ 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 rSTRN3 γ and ER α , but not with ER β (Fig. 3).

The truncated nature of rSTRN3 γ 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

Figure 2 Association of rSTRN3 γ with ER α in a yeast two-hybrid assay. The cDNA for the carboxy half of ER α was ligated to the Gal4 DNA-binding domain and used as 'bait'. The rSTRN3 γ cDNA was ligated to the Gal4 transcriptional domain and used as target. Yeast was transformed with the two expression vectors and treated with estradiol (E₂) or 4-hydroxytamoxifen (Tam). Ligand-induced β -gal expression indicates a protein–protein interaction. **P* < 0.001 versus vehicle control.

Figure 3 GST pull-down analysis of ER and rSTRN3 γ interactions. The cDNA for rSTRN3 γ was cloned into a vector to yield the GST–rSTRN3 γ hybrid protein that was isolated on glutathione-Sepharose beads. Full-length ER α and - β proteins were produced by *in vitro* translation in the presence of ³⁵S-methionine. The radiolabeled receptor proteins were incubated with the GST–rSTRN3 γ on the Sepharose beads in the presence or absence of estradiol (E₂). After centrifugation and washing, the protein on the beads was solubilized in SDS buffer and electrophoresed. The autoradiogram of the electrophoresis gel is shown. Samples of the radiolabeled receptors were included (input) for reference.

the N-terminal amino acid sequence of striatin and STRN3 in both humans and rats. The S-68 antibody also detects endogenous STRN3 α (94 kDa), - β (102 kDa), and - γ in the lysates of cell lines and homogenates from rat uterus (Fig. 4). Cos-1 cells expressed STRN3 α but not STRN3 β . 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; STRN3 β was found in both cytoplasmic and nuclear extracts while STRN3 α and - γ were found only in the nuclear extracts. BG-1 cells did not express STRN3 α and only a very small amount of STRN3 γ could be detected in the nuclear extract. The rat uterus of ovary-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 (STRN3 β), 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.

rSTRN3 γ represses activity of ER α

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) rSTRN3 γ blocks ER α transcriptional activity in a dose-dependent manner without altering basal levels of the

Figure 4 STRN3 isoforms expressed in rat uterus and human cell lines. Proteins from tissue homogenates of rat uterus or lysates of cell lines were separated by SDS-PAGE and analyzed by immunoblot using the S-68 SG2NA antibody. Cos-1 cells (Cos) were transfected with empty vector (–) or the expression vector for STRN3 γ (+). Proteins from human cell lines were derived from whole cell lysates (W), cytoplasmic extracts (C), or nuclear extracts (n). Uteri were derived from either ovary-intact (int) or ovariectomized (ovx) rats and endometrial scrapings were homogenized in lysis buffer.

reporter gene. While rSTRN3 γ is effective against ER α , it has no effect on transactivation by ER β (Fig. 5b). The dose of STRN3 γ required for inhibition of E₂-induced ER α 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.

rSTRN3 γ acts through protein phosphatase PP2A

The corepressor action of rSTRN3 γ 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, Csontos *et al.* 1996, Janssens & Goris 2001). We tested whether the corepressor action of rSTRN3 γ 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 rSTRN3 γ ; 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 ER α -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 ER α action and it blocked the repressive effect of rSTRN3 γ (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 ER α and rSTRN3 γ 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 rSTRN3 γ 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 rSTRN3 γ . 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 rSTRN3 γ , acting as a B-subunit of PP2A, directs dephosphorylation of ER α or the proteins associated with ER α at the promoter, thereby turning off the transactivation of the estrogen-responsive gene. Using immunoprecipitation of the His-tagged rSTRN3 γ , we demonstrated that rSTRN3 γ , ER α , and PP2A(C) form a complex in the cell and estrogen activation of ER α enhances the formation of this complex (Fig. 7). If the corepressor action of rSTRN3 γ derives from its ability to bring PP2A(C) into the complex of proteins formed through interactions with ER α at the promoter, then we would expect it to cause dephosphorylation of ER α and/or those proteins in the complex. To test this we examined the phosphorylation state of ER α , with and without the addition of rSTRN3 γ (Fig. 8). In the absence of rSTRN3 γ , estrogen induced a dramatic increase in the amount of ER α that was phosphorylated at serine 118; the addition of rSTRN3 γ abrogated this effect. Furthermore, the effect of rSTRN3 γ on ER α phosphorylation was blocked by okadaic acid. Thus, the presence of rSTRN3 γ caused an okadaic acid-dependent dephosphorylation of ER α in at least one known phosphorylation site and this action correlates to inhibition of ER α transactivational activity. Using ER α phosphorylation mutants, we found that one other phosphorylation site, serine 167 is also critical to the inhibitory action of rSTRN3 γ . When either serine 118 or serine 167 of ER α was mutated to alanine, the inhibitory action of rSTRN3 γ was abrogated (Fig. 9). The suppressor action of rSTRN3 γ was unaffected by

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 rSTRN3 γ .

Discussion

In this report, we describe an agonist-dependent association of ER α and rSTRN3 γ , 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 ER α 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). TFIIF/CDK7 also phosphorylates ER α at serine 118 (Chen *et al.* 2000, 2002). RSK1 and -2 kinases activated by MAPK, and AKT phosphorylate ER α at serine 167 (Frodin & Gammelfo 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 ER α 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 rSTRN3 γ modulates ER α activity by acting as a B-subunit of PP2A, thereby dephosphorylating key amino acid residues in the receptor protein. Perhaps proteins that associate with ER α , such as SRC-3, are also dephosphorylated through this mechanism.

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

Figure 5 rSTRN3 γ represses ER α transactivation of an ERE-reporter. (a) MDA-MB-231 cells were transfected with expression vectors for ER α , 2XERE-luciferase, and the control reporter, CMV- β -gal, without or with increasing amounts (ng/well) of the vector for rSTRN3 γ . 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 ER α or - β expression vector and the reporter genes plus varying amounts (ng/well) of rSTRN3 γ . (c) HeLa cells were transfected with ER α and reporter vectors and SRC3 and/or rSTRN3 γ as indicated. Values represent means $\pm$ S.D., n=4. *P<0.01 versus E₂ treated without rSTRN3 γ .

Figure 6 rSTRN3 γ repression of ER α 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 rSTRN3 γ . 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 ER α , ERE-luc, pCMV- β -gal, and rSTRN3 γ 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 STRN3 γ 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 $\pm$ s.d., $n=4$; means with different superscripts differ from each other ($P<0.05$).

118 is decreased in an okadaic acid-dependent manner by experimental expression of rSTRN3 γ . Using ER α mutants, we found that both S118 and serine S167 are required for the inhibitory effects of rSTRN3 γ . It is interesting that the activity of the ER α S167A mutant was not reduced by STRN3 γ because we might expect that the S118 residue within that receptor protein would be dephosphorylated by the addition of rSTRN3 γ 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 ER α , STRN3 γ , 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, Borrás *et al.* 1994, Galigniana *et al.* 1999, Bhattacharjee *et al.* 2001), and aberrant expression of the enzyme may be involved in cancer etiology (Csontos *et al.* 1996, Smith 1998, Schonthal 2001, Sontag 2001). PP2A and another

protein phosphatase 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 ER α -positive breast cancer cell lines compared with

Figure 7 rSTRN3 γ interacts with the catalytic subunit of PP2A in cells. Cos-1 cells were transfected with His-rSTRN3 γ and ER α 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 (α -His). The precipitate was probed in a western blot with an antibody against PP2A(C) and ER α . A sample of total lysate (T) was included on the western blot for comparison with the immunoprecipitated material.

Figure 8 rSTRN3 γ decreases phosphorylation of ER α . Cells were transfected with ER α alone (lanes 1–3) or with ER α plus rSTRN3 γ (lanes 4 and 5). The cells were treated with E₂ and/or okadaic acid as indicated. Separate blots were probed for S118 phosphorylated ER α (ER α -P), total ER α , and rSTRN3 γ .

ER α -negative lines (Gopalakrishna *et al.* 1999). Estradiol-induced down-regulation of ER α 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 ER α mRNA and reduced receptor protein levels (Keen *et al.* 2005). Experimental overexpression of PP5 reduced transcriptional activities of both ER α and β , and the reduced activity of ER α was correlated

Figure 9 Effect of rSTRN3 γ on transactivation by ER α phosphomutants. Cos-1 cells were transfected with 2XERE-luciferase and control reporter and ER α mutants (ER-mut) in which the indicated serine residues were replaced with alanines, and either 0 or 400 ng rSTRN3 γ . After 24 h the cells were treated with 10⁻⁸ M E₂ or vehicle and cell lysates were prepared 18 h later. rSTRN3 γ caused a significant (**P* < 0.05) decrease in E₂-induced transcription only in the cells transfected with the ER α (S102, 104, 106A) mutant.

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 corepressor 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 (Csontos *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 ER α associates with STRN3 γ and this association is enhanced by estrogen. Furthermore, our immunoprecipitation data indicate that the formation of STRN3 γ -PP2A(C) complex is enhanced by estrogen-activated ER α . It may be that the interaction between ER α and STRN3 γ alters the conformation of STRN3 γ so that it has a higher affinity for PP2A(C); this would increase the specificity of the interaction.

Lu *et al.* (2003) reported that PP2A(C) exerts its dephosphorylating effect by binding directly to ER α , 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 STRN3 γ does not bind to ER β in a ligand-stimulated fashion and does not affect ER β -induced transcription suggests that its B-subunit activity is restricted to ER α .

The present observations suggest a role for a striatin protein in the mechanism through which PP2A is recruited to a nuclear receptor. rSTRN3 γ 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

GI nos 10985167, 5853476, 5178755, 4898431). There are at least three members of the striatin gene family, and in the human they are each located on separate chromosomes as follows: STRN on chromosome 2 (gene ID: 6801); STRN3 on chromosome 14 (gene ID: 29966); and STRN4 on chromosome 19 (gene ID: 97387). Homologs for all three types of striatin have been found in the mouse, rat, and many lower species. Sequence analyses show that all of these proteins contain a putative nuclear localization sequence (Muro *et al.* 1995) but 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 rSTRN3 γ 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 ER α binding and repression.

In a recent report, Lu *et al.* (2004) described an interaction between ER α and STRN. Unlike the interaction between rSTRN3 γ and ER α , 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 ER α . It was demonstrated that the ER α –STRN interaction plays a role in the non-genomic effects of estrogen in endothelial cells; furthermore, the evidence suggests that STRN localizes ER α to the cytoplasm. Experimental disruption of the STRN–ER α interaction blocked estradiol induction of MAPK phosphorylation. It was pointed out that disruption of STRN–ER α did not block transactivation of an estrogen-responsive reporter gene; however, interference with this protein–protein interaction actually enhanced the transactivational effect of ER α . This observation suggests that STRN may, like rSTRN3 γ , act as a repressor protein, dampening the transactivational effects of estrogen.

In summary, rSTRN3 γ is a novel isoform of the STRN3 gene product that behaves as a B-subunit of PP2A and also interacts with ER α in an agonist-dependent manner. Thus, the repressor activity of rSTRN3 γ derives from its capacity to target PP2A(C) to ER α and perhaps to other proteins associated with the receptor. The intramolecular domains within rSTRN3 γ that are responsible for these activities are unknown. In addition, evidence presented by others (Lu *et al.* 2004) suggests that STRN may also exhibit ER α corepressor activity. Whether STRN, STRN3 α/β , or STRN4 all

represent B-subunits of PP2A and/or nuclear receptor-associated proteins remains to be determined.

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