Ligand-independent activation of oestrogen receptor α by caveolin-1

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Expression of caveolin-1 in the human mammary adenocarcinoma cell line MCF-7 causes ligand-independent concentration of oestrogen receptor α (ERα) in the nucleus, and potentiates ligand-independent and ligand-dependent transcription from an oestrogen response element-driven reporter gene. Furthermore, caveolin-1 co-immunoprecipitates with ERα [Schlegel, Wang, Katzenellenbogen, Pestell and Lisanti (1999) J. Biol. Chem. **274**, 33551–33556]. In the present study we show that caveolin-1 binds directly to ERα. This interaction is mediated by residues 82–101 of caveolin-1 (i.e. the caveolin scaffolding domain) and residues 1–282 of ERα. The caveolin-binding domain of ERα includes the ligand-independent transactivation domain, activation function (AF)-1, but lacks the hormone-binding domain and the ligand-gated transactivation domain, AF-2. In co-transfection studies, caveolin-1 potentiates the transcriptional activation of ERα(1–282), a truncation mutant that has intact AF-1 and DNA-binding domains. Since AF-1 activity is regulated largely by phosphorylation we determined that co-expression with caveolin-1 increased the basal phosphorylation of ERα(1–282), but blocked the epidermal growth factor-dependent increase in phosphorylation. Indeed, caveolin-1 interacted with and potentiated the transactivation of an ERα mutant that cannot be phosphorylated by extracellular signal-regulated kinase (ERK)1/2 [ERα($Ser^{118} \rightarrow Ala$)]. Thus caveolin-1 is a novel ERα regulator that drives ERK1/2-independent phosphorylation and activation of AF-1.

Key words: caveolae, receptor activation, sex hormone receptors, signal transduction.

INTRODUCTION

Nuclear receptors are regulated by many molecules. In addition to their cognate hormone ligands, these transcription factors bind to cytosolic chaperone proteins [1,2] and nuclear co-regulators [3–5]. The integration of these multiple interactions manifests in the co-ordinate expression of genes that bear particular hormone response elements in their promoters [6].

Oestrogen receptor α (ERα) is a nuclear receptor that has been studied not only to understand the functions of oestrogens in growth and development, but also to gain insights into how this family of transcription factors operate [7]. Two separate transactivation domains, activation function (AF)-1 and AF-2, have been identified in ERα [8]. Although AF-1 and AF-2 are physically separate, they are believed to synergize *in vivo* through overlapping binding of co-regulators [9–12].

AF-1 is found in the N-terminal portion of the molecule and is ligand-independent; whereas AF-2 is in the C-terminal portion of the molecule, near the ligand-binding pocket, and requires hormone binding for transactivation [8,13,14]. Phosphorylation of several serine residues by an array of kinases, including extracellular signal-regulated kinase (ERK)1/2, cyclin-A/cyclin-dependent kinase 2, $pp90^{rsk}$ and p38 mitogen-activated protein (MAP) kinase, governs AF-1 activation [15–24]. This post-translational modification regulates recruitment of key co-activators [25]. ERα AF-2 can also be activated in the absence of ligand: cyclin-D1 (independent of cyclin-dependent kinases) facilitates activation of ERα by recruiting co-activators and transcriptional machinery to DNA-bound, but unliganded, ERα AF-2 [26–28].

We recently reported that expression of caveolin-1 in the oestrogen-dependent human mammary adenocarcinoma cell line MCF-7 results in hormone-independent nuclear concentration and activation of ERα [29]. Caveolin-1 expression antagonizes the inhibitory effect of hydroxy-tamoxifen, and synergizes with a constitutively active ERα AF-2 mutant, ERα($Tyr^{537} \rightarrow Ser$) [30]. Finally, caveolin-1 co-immunoprecipitates with ERα when both proteins are expressed in 293T cells [29].

In the present study we show that caveolin-1 binds directly to ERα. The contact is mediated by the caveolin scaffolding domain (i.e. residues 82–101) and residues 1–282 of ERα. Deletion of the caveolin scaffolding domain abolishes caveolin-1 binding to ERα. Because caveolin-1 interacted with the N-terminus of ERα, we hypothesized that caveolin-1 is an AF-1 activator. In support of this, we found that co-expression of caveolin-1 and ERα(1–282), a truncation mutant that has intact AF-1 and DNA-binding domains, resulted in potentiation of oestrogen response element-driven transcription. Furthermore, caveolin-1 increased the basal level of ERα(1–282) phosphorylation. However, caveolin-1 blocked epidermal growth factor (EGF)-stimulated AF-1 phosphorylation, and dramatically inhibited the activation of ERK1/2 MAP kinases. Interestingly, caveolin-1 potentiated the transactivation of an ERα mutant lacking the ERK1/2 phosphorylation site. Thus caveolin-1 regulates AF-1 through modulation of phosphorylation via an ERK1/2-independent pathway.

Abbreviations used: AF, activation function; AR, androgen receptor; ERα, oestrogen receptor α; C/D FBS, charcoal/dextran-stripped fetal bovine serum; GFP, green fluorescent protein; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody.

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MATERIALS AND METHODS

Materials

All cell culture materials were obtained from Life Technologies, except for charcoal/dextran-stripped fetal bovine serum (C/D FBS; Gemini Bioproducts, Woodland, CA, U.S.A.) and phosphate-free Dulbecco's modified Eagle's medium (DMEM; ICN). BSA (fraction V), calyculin A, Ponceau S and 17-β-oestradiol were obtained from Sigma. Powdered non-fat milk was from Carnation and okadaic acid was purchased from Calbiochem. Complete MINI™ and pepstatin A (protease inhibitors), Fugene 6 transfection reagent and n-octyl glucoside were from Roche Molecular Biochemicals. Rabbit anti-ERα (H184), anti-(caveolin-1) (N-20) and anti-[green fluorescent protein (GFP)] (FL) antibodies, and mouse anti-(c-Myc) IgG_1 [monoclonal antibody (mAb) clone 9E10] were all obtained from Santa Cruz Biotechnology, and anti-ERK1/2 (p44/p42 MAP kinases) rabbit IgG and anti-(phospho-ERK1/ERK2) rabbit IgG were purchased from New England Biolabs. Recombinant human ERα (expressed in baculovirus-infected insect cells) was from Affinity Bioreagents. GSH- and Protein A-conjugated Sepharose were purchased from Amersham Pharmacia Biotech, and human EGF was obtained from Upstate Biotechnology. Anti-(caveolin-1) mouse IgG_1 (mAb clone 2297) was a gift from Dr Roberto Campos González (Transduction Laboratories, Lexington, KY, U.S.A.).

Plasmids

The caveolin-1 cDNAs [31,32], constructs encoding glutathione S-transferase (GST) fused to select caveolin-1 domains [33,34], the wild-type human ERα cDNA, ERE_2-tk81-luc and pSV-β-galactosidase control vector [29,35] have been described previously. ERα(Ser^{118} $\rightarrow$ Ala) [18] and ERβ [36] cDNAs were gifts from Benita S. Katzenellenbogen (Department of Molecular and Integrative Physiology, University of Illinois, Urbana, IL, U.S.A.). ERα(1–530) [37] and ERα(1–282) [13] were generated by PCR using the wild-type ERα cDNA as a template. ERα(1–530) was cloned into pCMV5, and ERα(1–282) was cloned into pCI-neo (Promega, Madison, WI, U.S.A.).

Cell culture and transfection methods

Human embryonic kidney 293T and COS-7 cells were propagated in DMEM containing 10% (v/v) FBS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in Phenol Red-free DMEM supplemented with 10% (v/v) C/D FBS for 12–24 h before each experiment. 293T cells were transfected by calcium phosphate precipitation. COS-7 cells were grown to 60% confluence in 6-well dishes. Following 12 h of culture in Phenol Red-free DMEM supplemented with 10% (v/v) C/D FBS, cells were transfected with Fugene 6 reagent. More specifically, DNA [600 ng of ER expression vector, 600 ng of caveolin-1 expression vector (or empty vector, pCB7), 1 μg of ERE_2-tk81-luc and 50 ng of pSV-β-galactosidase control vector] was mixed with 4 μl of transfection reagent (diluted in 96 μl of serum-free medium) as directed by the manufacturer. Cells were incubated with lipid–DNA complexes for 12 h. Following transfection, cells were washed twice with PBS, and cultured in medium containing 0.5% serum. Vehicle (ethanol) or 10 nM 17-β-oestradiol was added. Lysates were prepared and assayed for luciferase activity and β-galactosidase activity 24 h later [28]. In all experiments, the final concentration of ethanol was 0.1%. To correct for transfection efficiency, luciferase activity was divided by β-galactosidase activity. Results represent means $\pm$ S.D. for three separate experiments.

Pull-down assay

GST fusion proteins were purified as described previously [31,34]. GST fusion proteins (2 μg) were immobilized on GSH-conjugated Sepharose beads. Beads were washed three times in TNET [10 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100 and 1 mM EDTA], and made up to 0.5 ml with TNET. An equal mass of recombinant human ERα was added, and the mixture was incubated at 4 °C for 1 h. Beads were collected by centrifugation and washed five times with 1 ml of TNET. Proteins were liberated by boiling in 1% (w/v) SDS, separated by SDS/PAGE, transferred on to nitrocellulose and detected by immunoblotting as described below.

Immunoblotting

Samples were subjected to SDS/PAGE under reducing conditions. Proteins were transferred on to nitrocellulose membranes and stained with Ponceau S (Sigma). Membranes were then washed in 10 mM Tris (pH 8.0), 150 mM NaCl and 0.05% Tween-20 (wash buffer), blocked in wash buffer supplemented with 2% (w/v) non-fat milk and 1% (w/v) BSA (blocking buffer), incubated with primary antibody, washed again, and incubated with a secondary antibody conjugated to horseradish peroxidase. Bound IgGs were detected using a chemiluminescent substrate according to the manufacturer's instructions (Pierce). Monoclonal mouse IgG and donkey anti-(mouse IgG) were diluted in wash buffer supplemented with 1% (w/v) BSA. Polyclonal rabbit IgG and donkey anti-rabbit IgG were diluted in blocking buffer. In experiments where blots were re-probed, the blots were stripped by incubating in 50 mM Tris (pH 6.8), 200 mM 2-mercaptoethanol and 1% (w/v) SDS for 10 min at 50 °C, and then blocked again before incubating with the next antibody.

Immunoprecipitation

293T cells, grown to confluence in a 100-mm diameter dish, were washed twice with cold PBS (36 h after transfection), and were scraped into 1 ml of immunoprecipitation buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, 60 mM n-octyl glucoside, 0.1 mM Na_3VO_4, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.1 μg/ml okadaic acid and 1 mM EDTA, supplemented with protease inhibitors]. After sonication on ice, debris was removed by centrifugation at 21000 ***g*** for 10 min. Lysates were precleared by incubation with 30 μl of a 1:1 slurry of Protein A–Sepharose for 45 min at 4 °C and then transferred to tubes containing fresh Protein A–Sepharose and immunoprecipitation buffer. Anti-ERα IgG [2 μg; rabbit IgG H184 or mouse mAb clone TE111.5D11 (Lab Vision Corporation, Fremont, CA, U.S.A.)] was added to the mixture. For co-immunoprecipitation studies, rabbit anti-GFP IgG (FL) or mouse anti-(c-Myc) IgG (mouse mAb 9E10) were used as negative controls. Following a 4 h incubation at 4 °C, immune complexes were collected by centrifugation, washed six times in 1 ml of immunoprecipitation buffer, washed four times with 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA and 1% (v/v) Triton X-100, and disrupted by boiling in 1% (w/v) SDS.

Metabolic labelling

[^{32}P]P_i incorporation was monitored as follows: 36 h post-transfection, cells were washed twice with PBS and cultured for 2 h in 2 ml of phosphate-free DMEM supplemented with 1 mCi

of [^{32}P]P_i. Cells were washed again, treated with 100 ng/ml EGF or vehicle (DMEM) for 10 min, and then subjected to lysis in immunoprecipitation buffer (100 nM calyculin A was substituted for okadaic acid to achieve broader inhibition of serine/threonine phosphatases). Proteins were immunoprecipitated as described above. Following immunoprecipitation, proteins were separated by SDS/PAGE and transferred on to nitrocellulose. Radiolabelled proteins were detected using a PhosphorImager, and incorporation of [^{32}P]P_i was quantified using ImageQuant software (Molecular Dynamics/Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Proteins were then detected by immunoblotting as described above. Immunoreactivity (chemiluminescence) was quantified with ChemiImager 4000 software (Alpha Innotech, San Leandro, CA, U.S.A.).

RESULTS

Recombinant ERα and the caveolin scaffolding domain interact *in vitro*

We previously found that caveolin-1 is precipitated by anti-ERα IgG when both proteins are co-expressed in 293T cells [29]. To assess whether the interaction between caveolin-1 and ERα is direct, we performed a pull-down assay with GST fusion proteins to select caveolin-1 domains and recombinant purified human ERα. Figure 1 shows that ERα and caveolin-1 interact directly *in vitro*, and that caveolin-1 residues 82–101 (i.e. the caveolin scaffolding domain) are required for binding ERα. It should be noted that in this assay, approx. 10% of total ERα was recovered using GST–caveolin-1 fusion proteins.

To corroborate the above binding results *in vivo*, 293T cells were co-transfected with ERα cDNA and either wild-type caveolin-1 cDNA or a cDNA encoding a caveolin-1 deletion mutant that lacks the caveolin scaffolding domain (Figure 2A). Cells were then subjected to immunoprecipitation with anti-ERα IgG. Note that expression of either full-length caveolin-1 or

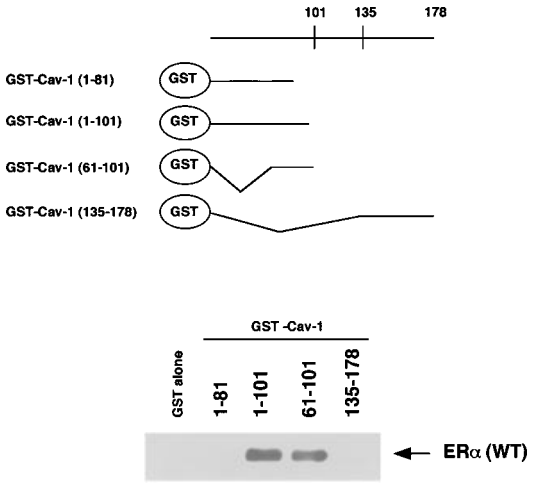

Figure 1 Caveolin-1 binds directly to ERα *in vitro*

Top panel: diagram of a panel of GST–caveolin-1 (Cav-1) fusion proteins. Residues 101 and 135 represent the boundaries of the central hydrophobic segment believed to anchor the protein to membranes. Both the N- and C-terminal domains are directed toward the cytoplasm. Bottom panel: GST fusion proteins (2 μg), immobilized on GSH–Sepharose beads, were mixed with an equal mass of wild-type (WT) ERα. Beads were recovered and washed extensively. Proteins were liberated in 1% (w/v) SDS, separated by SDS/PAGE, transferred on to nitrocellulose and immunoblotted with anti-ERα IgG. Note that caveolin-1 residues 82–101 were required for binding ERα.

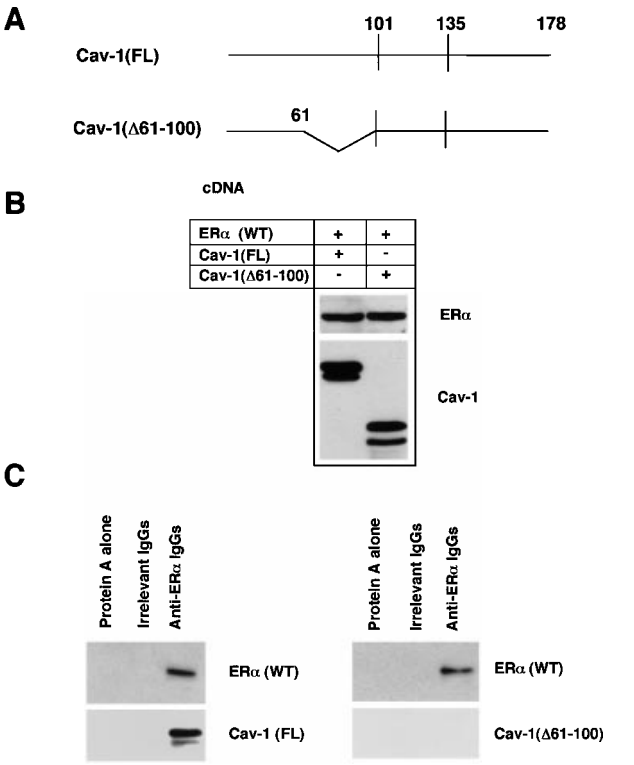

Figure 2 The caveolin scaffolding domain is required for ERα binding *in vivo*

(**A**) Diagram of caveolin-1 (Cav-1) constructs. Full-length (FL) caveolin-1, and a deletion mutant lacking the caveolin scaffolding domain, caveolin-1(Δ61–100), were used to confirm that residues 82–101 are required for interaction with ERα *in vivo*. (**B** and **C**) 293T cells were co-transfected with the indicated expression vectors. Lysates were then subjected to immunoprecipitation with the indicated IgG. Proteins were separated by SDS/PAGE, transferred on to nitrocellulose and detected by immunoblot analysis. Note that caveolin-1(Δ61–100) did not bind wild-type (WT) ERα. Importantly, both caveolin-1 constructs were expressed to equivalent levels (**B**). The irrelevant IgG used was anti-GFP IgG.

caveolin-1(Δ61–100) did not alter the level of ERα (Figure 2B). Similarly, co-transfection of the empty expression vector pCB7 with the ERα expression vector did not alter the level of ERα (results not shown). Figure 2(C) shows that deletion of the caveolin scaffolding domain prevents the caveolin-1–ERα interaction.

Caveolin-1 binds to ERα AF-1 *in vivo*, and potentiates its ligand-independent transactivation of a reporter gene

Further immunoprecipitation studies were performed to map the portion of ERα required for caveolin-1 interaction. Two C-terminal truncations, ERα(1–530) and ERα(1–282) (Figures 3A and 3B) were used to define the minimal caveolin-interacting domain. Figure 3(C) shows that caveolin-1 co-immunoprecipitated with ERα(1–282) when both proteins were expressed in 293T cells.

Although ERα(1–282) lacks a ligand-binding domain, this truncation mutant still bears the ligand-independent transactivation domain AF-1 [8]. Therefore we determined if caveolin-1 regulates AF-1 signalling by using an oestrogen response element-based gene reporter assay. Figure 4(A) shows that caveolin-1 expression increases ERα(1–282)-driven transcriptional acti-

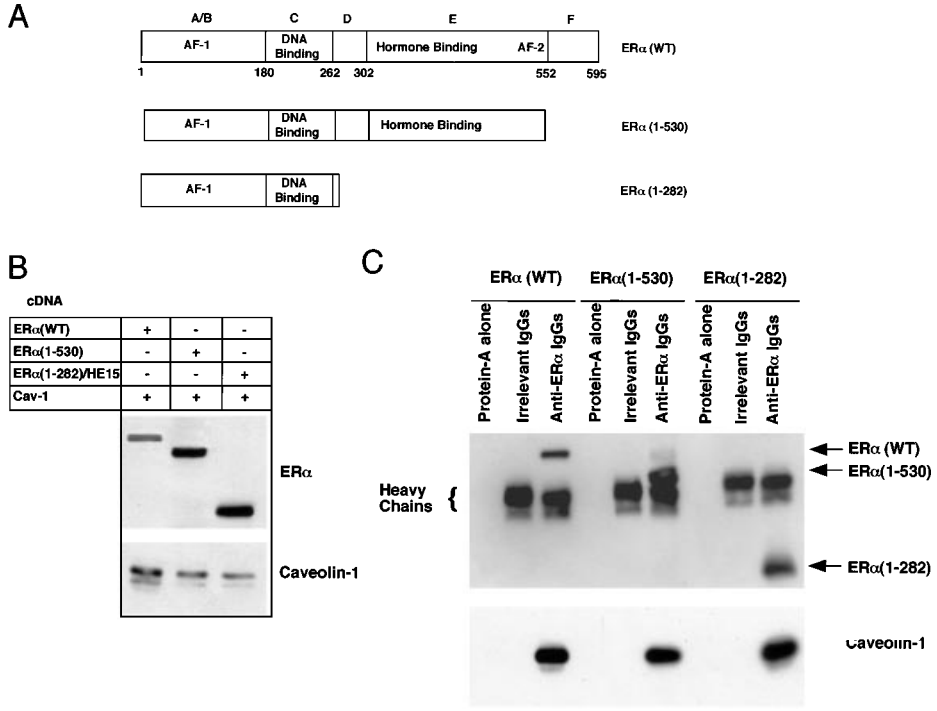

Figure 3 ERα AF-1 binds to caveolin-1

(**A**) Diagram of ERα constructs used to map the caveolin-1 interaction domain. Six conserved domains (A–F) have been described in most nuclear receptors. The boundaries of these domains are indicated below the wild-type (WT) ERα. AF-1 is found in the N-terminal portion (A/B domains) of ERα (residues 51–149, minimally [14]), while AF-2 is in the C-terminal ligand-binding domain (E domain). ERα (1–530) is a C-terminal truncation missing the F domain and part of the E domain [37]. ERα(1–282) contains AF-1 and the DNA-binding domain (C domain) [13]. (**B**) 293T cells were co-transfected with caveolin-1 (Cav-1) cDNA and the indicated ERα cDNAs. Aliquots of each lysate were subjected to SDS/PAGE. Proteins were transferred on to nitrocellulose and blotted with anti-ERα IgG (top panel) or anti-(caveolin-1) (bottom panel) antibodies. (**C**) Detergent lysates were precleared with Protein A–Sepharose, and split into three equal volume portions. Each fraction was subjected to immunoprecipitation with Protein A-conjugated Sepharose (Protein-A), with 2 μg of irrelevant IgG (anti-GFP IgG) or with 2 μg of anti-ERα IgG. Immunoprecipitates were recovered by centrifugation, washed extensively and disrupted in 1% (w/v) SDS. Samples were then subjected to SDS/PAGE. Proteins were transferred on to nitrocellulose and immunoblotted as in (**A**). Note that wild-type ERα, ERα(1–530) and ERα(1–282) each co-immunoprecipitated caveolin-1. The same rabbit anti-ERα IgG (H184) was used for immunoprecipitation and immunoblotting. Thus the heavy chains were detected in the immunoblot.

vation by 70%. As expected, 17-β-oestradiol treatment did not affect the activity of this ligand-binding domain-deficient mutant [14,19]. Thus caveolin-1 modulation of ERα activity requires AF-1.

Importantly, these studies were performed using cells cultured in a low concentration of serum (0.5%) to avoid the confounding effect of growth factor activation of AF-1 [19,20]. Likewise, these results are not due to caveolin-1 alteration of ERα(1–282) expression level (Figure 4B), or to caveolin-1 modulation of basal ERE_2-tk81-luc transcriptional activation (results not shown).

Caveolin-1 increases the basal level of ERα AF-1 phosphorylation, but blocks AF-1 phosphorylation induced by EGF treatment

We hypothesized that caveolin-1 potentiates AF-1 by altering the phosphorylation level of this transactivation domain, as this post-translational modification is the major switch regulating AF-1 [38]. Of the many kinases known to catalyse AF-1 phosphorylation, ERK1/2 MAP kinases are the best characterized [19,20]. Therefore we assessed whether caveolin-1 would alter basal or EGF-induced phosphorylation of AF-1. To this end, cells were transfected with ERα(1–282) and caveolin-1 (or vector alone). After the intracellular phosphate pool was labelled with [^{32}P]P_i, under serum-free conditions, cells were treated with EGF (or vehicle). ERα(1–282) was recovered by immunoprecipitation after EGF treatment, and the level of [^{32}P]P_i incorporation was determined by PhosphorImager analysis. Phosphorylation levels were corrected for the amount of protein recovered from the lysate by quantitative immunoblotting, and the ensuing ratio of phosphorylation level to protein level was determined.

Figure 5 shows that caveolin-1 expression increased the basal ERα(1–282) phosphorylation level by approx. 50%. However, caveolin-1 expression reduced the EGF-induced increase in ERα(1–282) phosphorylation. Using activation state-specific antisera, we also found that caveolin-1 expression markedly inhibited EGF-induced activation of ERK1/2 MAP kinases (Figure 5B). This finding is consistent with previous reports that caveolin-1 negatively regulates receptor tyrosine kinase/Ras/MAP kinase signalling *in vitro* [39–41] and *in vivo* [41–43]. Notably, the increase in ERα(1–282) phosphorylation observed in pCB7 transfected cells is identical to the levels previously reported for wild-type ERα [20].

Caveolin-1 potentiates the activity of ERα mutants that cannot be activated by ERK1/2

Growth factor treatment and expression of constitutively active Ras signalling can activate of ERα through ERK1/2-catalysed phosphorylation of ERα Ser^{118}. Conversely, mutation of ERα Ser^{118} into alanine blocks growth factor and Ras activation of

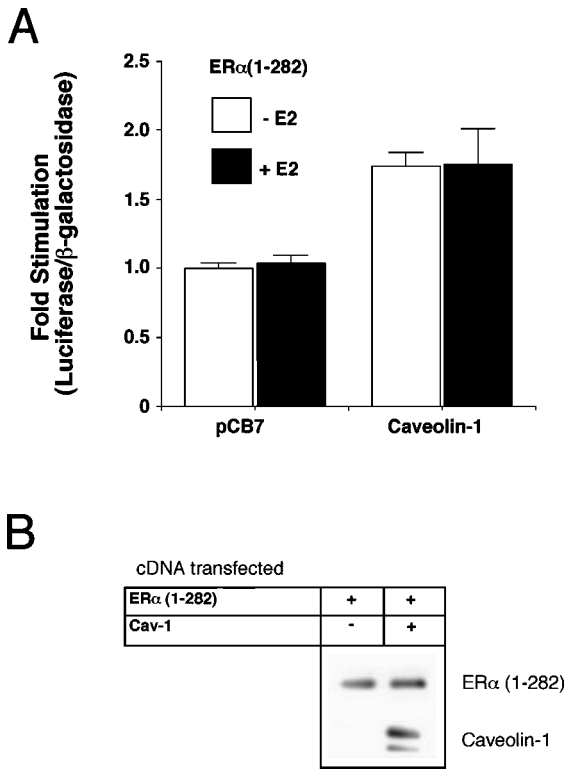

Figure 4 Caveolin-1 potentiates ERα AF-1 transcriptional activation

(**A**) COS-7 cells were transfected as described in the Materials and methods section with ERα(1–282) expression vectors and either caveolin-1 cDNA or the corresponding empty vector. Cells were washed twice (12 h post-transfection), cultured in medium containing 0.5% serum and treated with 10 nM 17-β-oestradiol (+E2) or vehicle (−E2), as indicated. After 24 h, detergent lysates were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. The resulting ratio for vehicle-treated, pCB7-transfected cells was set to unity. Results represent the means ± S.D. for three experiments. (**B**) Cells were transfected with the indicated combination of cDNAs. Lysates were prepared (36 h post-transfection), and equal masses of protein were separated by SDS/PAGE and transferred on to nitrocellulose . Blots were probed with antibodies raised against caveolin-1 (Cav-1) and ERα(1–282). Note that caveolin-1 expression had no effect on ERα(1–282) levels. As an additional control we transfected cells with ERE_2-tk81-luc reporter plasmid and either pCB7 or caveolin-1 cDNA. A β-galactosidase control vector was co-transfected. After 36 h, detergent lysates were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. Caveolin-1 expression did not alter basal ERE_2-tk81-luc transcriptional activation (results not shown).

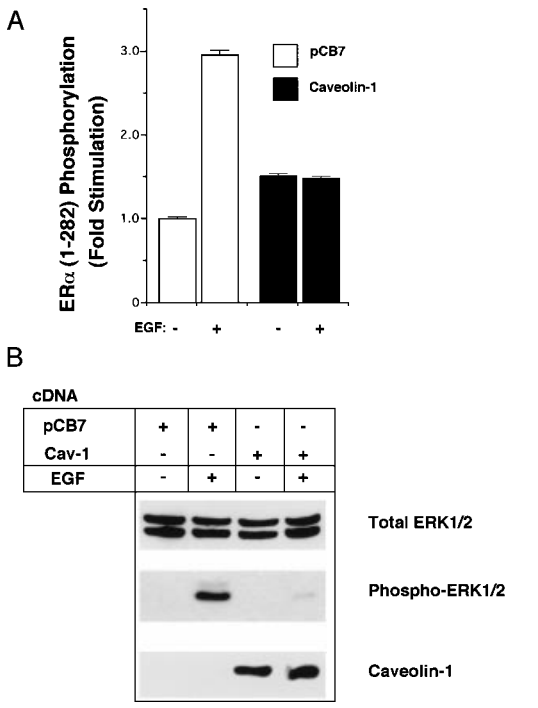

Figure 5 Caveolin-1 increases basal AF-1 phosphorylation, but inhibits EGF-induced phosphorylation of AF-1

293T Cells were transfected as indicated. The medium was replaced with phosphate-free medium supplemented with 150 mCi/ml [^{32}P]P_i 24 h after transfection. Cells were then treated with 100 ng/ml EGF for 10 min. Lysates were then prepared in immunoprecipitation buffer. (**A**) ERα(1–282) was immunoprecipitated, subjected to SDS/PAGE and blotted on to nitrocellulose membranes. Phosphate incorporation was determined by PhosphorImager analysis. The membranes were then subjected to immunoblotting with anti-ERα IgG. Immunoreactivity (chemiluminescence) was detected using ChemiImager software, and used to normalize the phosphate incorporation. Note that caveolin-1 expression increased basal ERα(1–282) phosphorylation, but it inhibited EGF-induced phosphorylation. Results reflect the means ± S.D. from three experiments. (**B**) Equal masses of protein derived from the lysates were separated by SDS/PAGE. Proteins were transferred on to nitrocellulose and detected by immunoblot analysis. Note that caveolin-1 expression markedly inhibited MAP-kinase activation (phospho-ERK1/2 blot) when cells were treated with EGF, without altering MAP-kinase levels (total ERK1/2 blot). Cav-1, caveolin-1.

ERα [19,20]. Interestingly, 17-β-oestradiol treatment also activates ERK1/2 [19] and stimulates phosphorylation of Ser^{118} *in vivo* [18–20,44]. Since caveolin-1 expression blocks EGF-driven activation of ERK1/2 and phosphorylation of ERα(1–282), we compared 17-β-oestradiol stimulation of wild-type ERα and ERα($Ser^{118} \rightarrow$ Ala). Figure 6(A) shows that co-transfection with caveolin-1 potentiated ERα($Ser^{118} \rightarrow$ Ala) transactivation 3.4-fold over co-transfection with vector (pCB7). Caveolin-1 also potentiated wild-type ERα (1.7-fold over vector co-transfection). Notably, caveolin-1 also co-immunoprecipitates with ERα($Ser^{118} \rightarrow$ Ala) (Figure 6B). The absolute transcriptional activity of ERα($Ser^{118} \rightarrow$ Ala) was approx. 70% of the transcriptional activity of wild-type ERα (383 106 ± 3 488 relative light units for the wild-type, and 272 249 ± 6224 relative light units for the Ser^{118} mutant). This result is similar to that obtained by Katzenellenbogen and co-workers [18].

Caveolin-1 does not potentiate ERβ activity

AF-1 of ERβ shows < 25% sequence similarity with the AF-1 of ERα [45]. We predicted, therefore, that caveolin-1 would not potentiate ERβ-driven transcription. Figure 7 shows that caveolin-1 does not potentiate ERβ transcriptional activation, and may even inhibit both ligand-dependent and ligand-independent transcriptional activation. This modest inhibition probably reflects caveolin-1 antagonism of ERK1/2-dependent phosphorylation of ERβ AF-1 [25]. As a control, we verified that caveolin-1 potentiated ligand-independent and ligand-dependent activation of ERα.

DISCUSSION

Three functions have been assigned to caveolins. First, they are the main coat proteins of caveolae membranes [46], and are involved in attaching the cytoskeleton to the extracellular matrix [47–49]. Secondly, caveolins are cholesterol-binding proteins [34,50] that actively contribute to the intracellular traffic of cholesterol [51–53]. Thirdly, they are regulators of an array

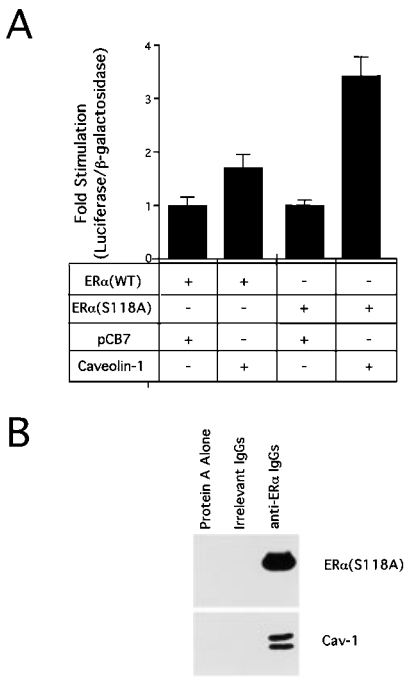

Figure 6 Caveolin-1 potentiates the activity of an ERα mutant that cannot be phosphorylated by ERK1/2

(**A**) COS-7 cells were transfected with wild-type (WT) ERα or ERα(Ser^{118} → Ala) cDNA, ERE$_2$-tk81-luc reporter, pSV-β-galactosidase control vector and caveolin-1 cDNA (or empty vector). Cells were washed 12 h post-transfection, and cultured in medium supplemented with 0.5% serum. Cells were then treated with 10 nM 17-β-oestradiol (or vehicle) for 24 h. Lysates were then prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. The resulting ratio for vehicle-treated, pCB7-transfected (i.e. no caveolin-1 cDNA) cells was set to unity for both wild-type ERα and ERα(Ser^{118} → Ala). Results reflect the means ± S.D. for three experiments. (**B**) ERα(Ser^{118} → Ala) cDNA was co-transfected with the caveolin-1 (Cav-1) cDNA. Lysates were prepared in immunoprecipitation buffer, split into three equal aliquots and subjected to immunoprecipitation with the indicated antibodies as described in the legend of Figure 2. Note caveolin-1 is co-immunoprecipitated by anti-ERα IgG, but not an irrelevant IgG (anti-GFP).

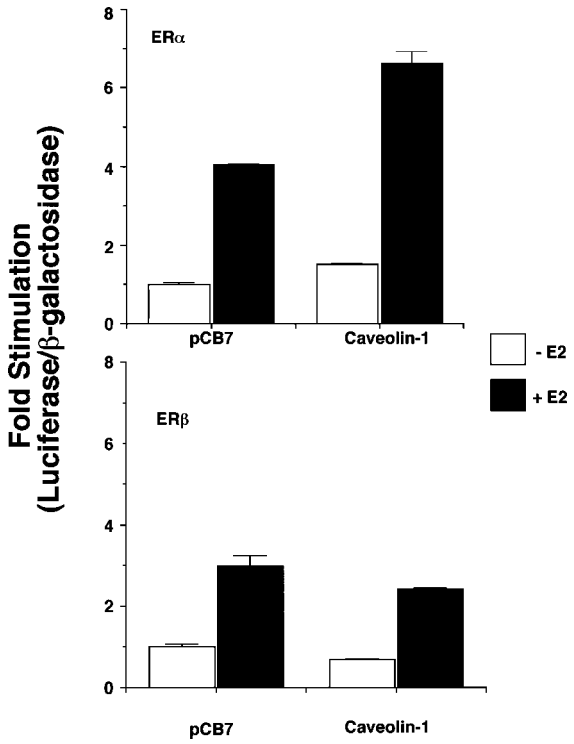

Figure 7 Caveolin-1 does not potentiate ERβ activity

COS-7 cells were transfected with ERα or ERβ cDNA, ERE$_2$-tk81-luc reporter, pSV-β-galactosidase control vector and caveolin-1 cDNA (or empty vector). Cells were washed 12 h post-infection and cultured in 0.5% serum. Cells were treated with vehicle (− E2) or 10 nM 17-β-oestradiol (+ E2) for 24 h. Lysates were then prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. The resulting ratio for vehicle-treated, pCB7-transfected (i.e. no caveolin-1 cDNA) cells was set to unity for both ERα and ERβ. Note that caveolin-1 expression potentiated ligand-independent and ligand-dependent ERα activation. However, caveolin-1 expression did not potentiate ligand-independent or ligand-dependent ERβ activation. Results represent the means ± S.D. from three experiments.

of soluble, lipid-modified and integral membrane signalling molecules [54].

Following our initial observations that diverse signalling proteins bind to caveolin-1 residues 82–101 [40,55,56], we termed this portion of the protein the 'caveolin scaffolding domain,' and identified binding motifs for this domain using phage display [57]. The consensus caveolin binding motifs are ΦXΦXXXXΦ, ΦXXXXΦXXΦ, and ΦXΦXXXXΦXXΦ, where Φ is a phenylalanine, tyrosine or tryptophan residue, and X is any aminoacyl residue. Analysis of the ERα primary sequence reveals two potential caveolin-binding motifs in the N-terminal portion of the molecule: ^{52}**Y**N**Y**PEGA**A**A**Y**^{60} and ^{89}**F**GSNG**L**GG**F**^{97}. Although these motifs have aliphatic-for-aromatic substitutions (underlined residues) in the '**Φ**' positions two known caveolin-interacting proteins, $G_q\alpha$ [58] and GRK2 [59], have similarly divergent caveolin-binding motifs (**F**D**L**QSVI**F**RM**V** and **L**GYLL**F**RD**F** respectively). Notably, ERβ lacks potential caveolin-binding motifs [45], consistent with our finding that caveolin-1 does not potentiate the activity of this ER isoform (Figure 7).

In the present paper we provide a possible mechanism for caveolin-1 potentiation of ERα activity [29]. First we demonstrated that caveolin-1 binds to ERα directly. The domain required for interaction (ERα residues 1–282) with caveolin-1 contains the two putative caveolin-binding motifs. Since ERα(1–282) includes the ligand-independent transactivation domain AF-1, we verified that caveolin-1 potentiated the transcriptional activity of this truncation mutant. We also determined that caveolin-1 raised the basal phosphorylation level of ERα(1–282), but blocked the ERK1/2-dependent increase in AF-1 phosphorylation following EGF treatment. Furthermore, removal of the ERK1/2 phosphorylation site (i.e. Ser^{118} → Ala) did not undermine caveolin-1 potentiation of ERα.

Thus caveolin-1 potentiation of AF-1 function may involve facilitating phosphorylation of this transactivation domain by another kinase [15–24]. Conversely, caveolin-1 binding may prevent dephosphorylation of AF-1. In either case, our present results, showing that caveolin-1 potentiates ERα AF-1 transactivation, provide a satisfying explanation for our previous observations that caveolin-1 expression in MCF-7 cells confers resistance to hydroxy-tamoxifen, an AF-2 antagonist, and that caveolin-1 dramatically potentiates the activity of ERα(Tyr^{537} → Ser), a constitutively active AF-2 mutant [29]. We also note that caveolin-1 does not appear to function like known cytosolic chaperone proteins that facilitate AF-2 function through increasing ligand binding capacity [60,61], as we show in the present study that caveolin-1 potentiates the transactivation of a ligand-binding domain-deficient mutant, ERα(1–282).

Recently, Lu et al. reported that caveolin-1 interacts with the androgen receptor (AR) [62]. Some important differences between the association of ERα and AR with caveolin-1 were found. Notably, Lu et al. reported that caveolin-1 residues 1–60 are required for AR binding [62]. Although androgens were not required for this interaction, dihydrotestosterone enhanced the association of AR and caveolin-1 [62]. Thirdly, caveolin-1 binds AR via two regions: (1) the AR N-terminal domain; and (2) the AR ligand-binding domain [62]. The interaction of caveolin-1 with the AR-ligand binding domain was enhanced by addition of ligand, but not by the antagonist, bicalutamide (Casodex) [62]. Future experiments should address the significance of the differences between how these two nuclear receptors associate with caveolin-1.

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