Caveolin-1 Potentiates Estrogen Receptor α (ERα) Signaling

Caveolin-1 drives ligand-independent nuclear translocation and activation of ERα*

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Estrogen receptor α (ERα) is a soluble protein that mediates the effects of the gonadal estrogens such as 17β-estradiol. Upon ligand binding, a cytoplasmic pool of ERα translocates to the nucleus, where it acts as a transcription factor, driving the expression of genes that contain estrogen-response elements. The activity of ERα is regulated by a number of proteins, including cytosolic chaperones and nuclear cofactors. Here, we show that caveolin-1 potentiates ERα-mediated signal transduction. Coexpression of caveolin-1 and ERα resulted in ligand-independent translocation of ERα to the nucleus as shown by both cell fractionation and immunofluorescence microscopic studies. Similarly, caveolin-1 augmented both ligand-independent and ligand-dependent ERα signaling as measured using a estrogen-responsive element-based luciferase reporter assay. Caveolin-1-mediated activation of ERα was sensitive to a well known ER antagonist, 4-hydroxytamoxifen. However, much higher concentrations of tamoxifen were required to mediate inhibition in the presence of caveolin-1. Interestingly, caveolin-1 expression also synergized with a constitutively active, ligand-independent ERα mutant, dramatically illustrating the potent stimulatory effect of caveolin-1 in this receptor system. Taken together, our results identify caveolin-1 as a new positive regulator of ERα signal transduction.

Caveolae are flask-shaped vesicular invaginations of the plasma membrane (1). So far, these structures have been implicated in three overlapping areas of cell physiology, i.e. endocytosis (2, 3), cholesterol trafficking (4–9), and signal transduction (reviewed in Ref. 10). To engage in these processes, caveolae have a protein and lipid composition that is distinct from the plasma membrane proper. More specifically, they are enriched in cholesterol, glycosphingolipids, and sphingomyelin as well as lipid-modified signaling proteins (10, 11).

The principal coat proteins of caveolae are the caveolins. Thus far, three distinct mammalian caveolin genes have been identified, and their 20–25-kDa gene products are broadly expressed in a variety of tissues and cell types (12–16). In addition to interacting with an array of integral membrane, lipid-modified, and soluble signaling molecules, the caveolins share the ability to self-oligomerize, to bind cholesterol, and to cross-link cell-surface gangliosides (10, 17–20).

In general, caveolins bind to and inactivate signaling molecules. Such examples include, but are not limited to, the following: receptor tyrosine kinases (e.g. epidermal growth factor receptor and c-Neu) and their downstream targets (e.g. Ha-Ras, MEK1, and ERK2), serpentine receptors (e.g. endothelin receptor) and their attendant enzymes (e.g. various Gα subunits, adenylyl cyclase, and protein kinase A), and regulated enzymes (e.g. endothelial nitric-oxide synthase). All these signaling components are inhibited by their interaction with caveolins (reviewed in Ref. 21).

The interaction of caveolin-1 with many of the proto-oncogene products described above has important consequences for cellular transformation and, perhaps, cancer. Several experimental lines of evidence support this hypothesis. First, caveolin-1 is down-regulated in a variety of oncogenically transformed cells (22). Second, when the caveolin-1 cDNA is reintroduced into Rasα12V-transformed NIH 3T3 cells, anchorage-independent growth is abrogated (23). Third, disruption of caveolae by antisense-mediated down-regulation of caveolin-1 protein expression in normal NIH 3T3 cells results in (i) hyperactivation of the p42/44 mitogen-activated protein kinase cascade, (ii) anchorage-independent growth, and (iii) tumor formation in nude mice (24). Fourth, pharmacological depletion of cellular cholesterol with a concomitant morphologic loss of caveolae also results in p42/44 mitogen-activated protein kinase activation (25). Finally, the caveolin-1 and -2 genes are co-localized to a known tumor suppressor locus in mice and humans (7q31.1/D7S522) (reviewed in Ref. 26).

We recently uncovered a reciprocal relationship between Neu tyrosine kinase activity and caveolin-1 expression in mammary adenocarcinomas (27). An increase in Neu kinase activity correlated with a decrease in caveolin-1 expression both in vitro and in vivo. Conversely, overexpression of caveolin-1 inhibited Neu kinase activity in vivo. As the c-Neu proto-oncogene is mutationally activated in human breast cancers, these results may have implications for understanding the functional role of caveolin expression in the prevention of mammary tumorigenesis.

In this report, we address the possible role of caveolin-1 in
estrogen receptor (ER)\(^1\) signal transduction, another major pathway that is thought to be involved in the development of human breast cancers. Here, we show that caveolin-1 re-expression in MCF-7 cells, an estrogen-dependent human breast cancer cell line, promotes nuclear translocation of ER\(_a\). The possible implications of these findings for understanding ER\(_a\) signaling and breast carcinogenesis are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**— Estradiol (E\(_2\)) was purchased from Sigma. (Z)-4-Hydroxytamoxifen (OHT) was from Calbiochem. Anti-caveolin-1 IgG (rabbit anti-peptide, N20-directed against residues 2–21) and anti-ER\(_a\)-IgG (H-9) were purchased from Santa Cruz Biotechnology. Anti-caveolin-1 IgG (mouse monoclonal antibody cl 2234) (28) and anti-caveolin-2 IgG (mouse monoclonal antibody cl 65) (29) were the generous gifts of Dr. Roberto Campos-González (Transduction Laboratories, Lexington, KY). Dr. Perry E. Bickel (Washington University, School of Medicine, St. Louis, MO) kindly provided rabbit anti-guanine nucleotide dissociation inhibitor (GDI) IgGs (30). Charcoal/dextran-treated fetal bovine serum (CDS) was from JRH Biosciences. All other cell culture materials were from Life Technologies, Inc.

**Expression Constructs**—Wild-type human estrogen receptor \(_a\) (ER\(_a\)/HEGO) and a constitutively active form, ER\(_a\) Y537S (31), were subcloned into the mammalian expression vector pCMV5. The reporter expression vector pSV-\(_a\)-galactosidase control vector was from Promega. The canine caveolin-1 cDNA was cloned into the pCEB expression vector as we described previously (28, 34).

**Cell Culture**—MCF-7 cells (ATCC-HTB-22) were obtained from the American Type Collection and were propagated in Dulbecco’s modified Eagle’s medium, 10% donor bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin.

**Cell Fractionation**—Thirty-six hours post-transfection, cells from one 60-mm diameter plate were harvested by gentle scraping into phosphate-buffered saline and collected by centrifugation at 1000 \(\times\) g. Cells were then subjected to hypotonic lysis in 10 mM Tris (pH 7.5) and 20 mM Na\(_2\)MoO\(_4\), passed through a 26-gauge needle, and then sonicated. Cells were fractionated into cytoplasmic and nuclear fractions as we described in detail previously (24). Samples were brought to the same volume, and equal-volume aliquots from each fraction were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis (35).

**Immunoblotting**—Proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and then transferred to nitrocellulose. Protein bands were visualized by staining with Ponceau S. Blots were washed with Tris-buffered saline (10 mM Tris (pH 8.0) and 150 mM NaCl) and 0.05% Tween 20 and then placed in blocking solution (Tris-buffered saline, 0.05% Tween 20, 2% nonfat milk, and 1% bovine serum albumin) for 1 h. Blots were incubated for 1 h with primary antibodies, washed with Tris-buffered saline and 0.05% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibodies (Transduction Laboratories). Bound IgGs were visualized using an enhanced chemiluminescence detection system (Pierce) according to the manufacturer’s protocol.

**Immunolocalization Studies**—Immunofluorescent labeling was performed as described previously (28). Briefly, cells were fixed in 2% paraformaldehyde and doubly immunostained with mouse anti-caveolin-1 IgG (cl 2234) and rabbit anti-ER\(_a\)-IgGs (H-184). Bound primary antibodies were visualized with fluorescein isothiocyanate-conjugated donkey anti-mouse and lissamine rhodamine-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Cells were viewed with an Olympus IX70 inverted microscope using a 60\(\times\) objective, and images were collected with a Photonics cooled CCD camera.

**In Vivo ER\(_a\) Signalizing Assay**—Twenty-four hours prior to transfection, \(\sim 1 \times 10^6\) cells were seeded into 12-well tissue culture plates and cultured in phenol red-free Dulbecco’s modified Eagle’s medium containing charcoal/dextran-treated fetal bovine serum. Cells were then transfected with 1 \(\mu\)g of vector (pCB7) or vector containing caveolin cDNA (Cav-1/pCB7), 1 \(\mu\)g of ER\(_{Z357S}\)TK81LUC reporter, and 200 ng of pSV-\(_a\)-galactosidase by calcium phosphate precipitation. One microgram of the wild-type human ER\(_a\) cDNA or a constitutively active (Y357S) ER\(_a\) mutant cDNA was also cotransfected. Twelve hours after addition of calcium phosphate precipitates, cells were washed twice with phosphate-buffered saline and incubated for an additional 24 h in medium containing E\(_2\) or an equivalent volume of vehicle (ethanol). In all experiments, the final concentration of ethanol was 0.1% (v/v). In antagonist studies, OHT dissolved in dimethyl sulfoxide was added from stock solutions such that the final concentration of solvent was 0.1% (v/v). Lysates were prepared 24 h after pharmacological treatment and assayed for luciferase and \(\beta\)-galactosidase activities. Results represent the mean \(\pm\) S.D. of luciferase activity normalized to \(\beta\)-galactosidase activity (\(n = 3\)).

**RESULTS AND DISCUSSION**

**Caveolin-1 Expression Induces Ligand-independent Nuclear Translocation of ER\(_a\)**—Mammary epithelial cells normally express both caveolin-1 and -2, whereas many mammary adenocarcinoma cell lines such as MCF-7 show selective down-regulation of caveolin-1 (reviewed in Ref. 26). However, MCF-7 cells continue to express wild-type ER\(_a\). Thus, we employed MCF-7 cells as a model system to study the effects of recombinant caveolin-1 expression on the behavior of endogenous ER\(_a\).

Fig. 1A (upper panel) shows that transient expression of caveolin-1 in MCF-7 cells resulted in a dramatic decrease in the cytoplasmic pool of ER\(_a\). To ensure equal protein loading, the same blots were reprobed with antibodies against both membrane (caveolin-2) and cytosolic (GDI) proteins (Fig. 1A, lower panels).

To examine whether the decrease in the cytoplasmic pool of ER\(_a\) was due to its degradation, total cellular proteins were recovered by lysis in 1% SDS; the amount of ER\(_a\) was determined by immunoblot analysis. Fig. 1B shows that ER\(_a\) levels were identical in caveolin-1 transfectants and mock-transfected control cells both in the presence and absence of estradiol. As expected, estradiol treatment decreased the steady-state levels of ER\(_a\) expression, as ligand binding results in nuclear translocation and subsequent ubiquitination, followed by proteosomal degradation (37). Thus, the decrease in cytoplasmic ER\(_a\) levels following caveolin-1 transfection is most likely due to enhanced nuclear translocation.

Next, we tested whether caveolin-1 expression can influence ER\(_a\) translocation from the cytoplasm to the nucleus using subcellular fractionation techniques. In unstimulated control cells, ER\(_a\) resided in both the nucleus and cytoplasm; the cytoplasmic pool underwent translocation to the nucleus when stimulated with estradiol (Fig. 2A, left panel) (38). Interestingly, in unstimulated cells, caveolin-1 caused a shift in the subcellular localization from the cytoplasm to the nucleus (Fig. 2A, right panel). As a control, we verified that caveolin-1 expression did not alter the location of a known cytosolic protein, GDI (Fig. 2B). Thus, it appears that caveolin-1 expression can

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1 The abbreviations used are: ER, estrogen receptor; E\(_2\), estradiol; OHT, (Z)-4-hydroxytamoxifen; GDI, guanine nucleotide dissociation inhibitor; ERE, estrogen-response element.
cause ligand-independent nuclear translocation of ERα.

Similar results were obtained by immunofluorescence microscopy. In caveolin-1-transfected cells and in the absence of ligand, the cytoplasmic pool of endogenous ERα shifted to the nucleus (Fig. 3A). More important, this effect was cell autonomous, as adjacent untransfected cells did not show enhanced nuclear concentration of ERα (Fig. 3, A and C). Caveolin-1 transfectants to the left with the corresponding untransfected cells to the right in the same field). These data independently confirm our results obtained via cell fractionation (Fig. 2).

Potentiation of ERα Signaling by Caveolin-1—Since caveolin-1 causes ligand-independent ERα translocation from the cytoplasm to the nucleus, we wondered whether caveolin-1 expression also results in ERα-mediated transcriptional activation of estrogen-responsive genes. To evaluate this possibility, we employed an established luciferase-based reporter system that has been used extensively by other investigators to monitor ERα-mediated signal transduction in vivo. This reporter contains two EREs linked 5’ to a minimal thymidine kinase promoter that drives luciferase expression (32).

Fig. 4 shows that when unstimulated cells were transfected with caveolin-1, an ~2-fold increase was observed in ERα reporter activity. In addition, when caveolin-1-transfected cells were stimulated with a range of estradiol concentrations (from 5 to 100 nM), caveolin-1 induced a dramatic increase (up to ~7–8-fold) in ERα reporter activity. Thus, caveolin-1 expression is sufficient to induce ligand-independent activation of ERα, and caveolin-1 can potentiate ERα signaling in the presence of ligand.

We next evaluated whether caveolin-1-mediated potentiation of ERα signaling is sensitive to ER antagonists (Fig. 5). For this purpose, we treated cells with estradiol (10 nM) in the absence or presence of OHT (0.1–100 nM). Note that in cells transfected with vector alone, OHT had an IC_{50} of <0.1 nM. In contrast, in cells transfected with caveolin-1, the IC_{50} for OHT was increased >5-fold to ~0.5 nM. These results indicate that caveolin-1 expression can prevent OHT-mediated inhibition of ERα signaling in vivo. In addition, these data may have clinical implications for understanding the development of tamoxifen resistance in breast and ovarian cancer cells, as tamoxifen is routinely used in a variety of cancer chemotherapy regimens.

As caveolin-1 expression can potentiate both ligand-dependent and ligand-independent ERα signaling, we next determined if caveolin-1 can influence signal transduction mediated by a mutated, constitutively activated form of ERα. For this purpose, we utilized a well characterized constitutively active mutant (ERα^{Y573S}) that is known to dramatically increase ERα-dependent transcription in the absence of ligand (39). Fig. 6 shows that caveolin-1 expression augmented ERα^{Y573S} activation of estrogen signaling, resulting in an ~7–8-fold increase in transcription both in the presence and absence of estradiol. As compared with wild-type ERα, in the absence of caveolin-1 (Fig. 6, see vector-alone controls (open bars)), ERα^{Y573S} plus caveolin-1 resulted in an ~150-fold increase in ERα ligand-independent...
ent signaling. These findings directly support our observation that caveolin-1 causes nuclear translocation and activation of wild-type ERα (Figs. 2–4).

**FIG. 3.** Immunofluorescent localization of endogenous ERα in caveolin-1-transfected MCF-7 cells. Cells were transfected with the caveolin-1 cDNA and incubated for 30 min with E2 (10 nM; B and D) or with vehicle alone (A and C). Cells were then fixed and doubly immunostained with rabbit anti-ERα IgG (A and B) and mouse anti-caveolin-1 IgG (monoclonal antibody cl 2234; C and D). Note that in both C and D, in the same field, the cells to the left expressed caveolin-1, whereas the cells to the right did not. In unstimulated cells, caveolin-1 expression induced nuclear translocation of endogenous ERα (A). More importantly, note that only the caveolin-1-expressing cells showed nuclear concentration of ERα. When cells were stimulated with E2, both caveolin-1-transfected cells (to the left) and untransfected cells (to the right) showed nuclear concentration of ERα (B).

**FIG. 4.** Caveolin-1 expression is sufficient to induce ligand-independent activation of ERα, and caveolin-1 can potentiate ERα signaling in the presence of ligand. Cells were cotransfected with the caveolin-1 cDNA (Cav-1/pCB7; closed bars) or with vector alone (pCB7; open bars), an ERα expression vector, the ERE2TR81LUC reporter, and a β-galactosidase expression vector as described under “Experimental Procedures.” Twelve hours post-transfection, cells were washed with phosphate-buffered saline and cultured for 24 h in medium containing the indicated concentrations of E2. Lysates were then prepared and assayed for luciferase and β-galactosidase activities. To correct for transfection efficiency, luciferase activity (raw light units) was divided by the corresponding β-galactosidase activity (absorbance at 574 nm). The resulting ratios were then expressed as fold stimulation relative to vehicle-treated, vector-transfected cells normalized to 1. Note that cotransfection with caveolin-1 stimulated ERα signaling activity ~2-fold in cells treated with vehicle alone. In addition, when caveolin-1-transfected cells were stimulated with a range of estradiol concentrations (from 5 to 100 nM), caveolin-1 induced a dramatic increase (up to ~7–8-fold) in ERα reporter activity. Data represent the mean ± S.D. of luciferase activity normalized to β-galactosidase activity (n = 3).

**FIG. 5.** Caveolin-1 expression can prevent tamoxifen-mediated inhibition of ERα signaling in vivo. Cells were transfected and processed as described in the legend to Fig. 4. Twelve hours post-transfection, cells were washed and cultured further in medium containing E2 (10 nM) and the indicated concentrations of OHT (0.1–100 nM). Twenty-four hours after pharmacological treatment, cells were subjected to lysis and assayed for luciferase and β-galactosidase activities. To correct for transfection efficiency, luciferase activity (raw light units) was divided by the corresponding β-galactosidase activity (absorbance at 574 nm). The resulting ratios were then expressed as fold stimulation relative to vector-transfected cells without OHT treatment normalized to 1. Note that in cells transfected with vector alone, OHT had an IC50 of ~0.1 nM (open bars). In contrast, in cells transfected with caveolin-1, the IC50 for OHT was increased ~5-fold to ~0.5 nM (closed bars). These results indicate that caveolin-1 expression can prevent OHT-mediated inhibition of ERα signaling in vivo. Data represent the mean ± S.D. of luciferase activity normalized to β-galactosidase activity (n = 3).

**Interaction of Caveolin-1 and ERα in Vivo**—One possible mechanism by which caveolin-1 potentiates ERα signaling is through a direct or indirect interaction between caveolin-1 and ERα itself. Although caveolin-1 is an integral membrane pro-
Caveolin-1 Potentiates Estrogen Receptor α Signaling

![Graph: Caveolin-1 also potentiates signaling via a constitutively active form of ERα.](image)

**Fig. 6.** Caveolin-1 also potentiates signaling via a constitutively active form of ERα. Cells were cotransfected with the wild-type (WT) or constitutively active (CA; Y537S) ERα cDNA and with vector alone (pCB7; open bars) or with the caveolin-1 cDNA (Cav-1; pCB7; closed bars). Twelve hours post-transfection, the cells were washed with phosphate-buffered saline and cultured in vehicle alone (−) or with E2 (++; 10 nM) for 24 h. Cells were then subjected to lysis and assayed for luciferase and β-galactosidase activities. To correct for transfection efficiency, luciferase activity (raw light units) was divided by the corresponding β-galactosidase activity (absorbance at 574 nm). The resulting ratios were then expressed as fold stimulation relative to vehicle-treated, vector-transfected cells normalized to 1. Note that caveolin-1 expression augmented ERαY537S activation of estrogen receptor signaling, resulting in an ~7–8-fold increase in transcription both in the presence and absence of estradiol. Values are plotted logarithmically on the ordinate. Data represent the mean ± S.D. of luciferase activity normalized to β-galactosidase activity (n = 3).

Caveolin-1, a soluble cytoplasmic pool of caveolin-1 has been reported (7). This is consistent with the finding that caveolin-1 can move in and out of membranes (existing as a soluble protein) depending on the oxidative state of caveolin-bound membrane cholesterol.

To evaluate the potential interaction of caveolin-1 with ERα, we cotransfected 293T cells with their corresponding cDNAs. We chose 293T cells for these studies as they do not express either caveolin-1 or ERα endogenously. Cell lysates were then prepared and subjected to immunoprecipitation with antibodies directed against ERα. Fig. 7 shows that when cells were cotransfected with ERα and caveolin-1, caveolin-1 co-immunoprecipitated with antibodies directed against ERα (third lane). In contrast, when cells were transfected with the caveolin-1 cDNA alone, anti-ERα antibodies did not coprecipitate caveolin-1 (Fig. 7, first lane). These results indicate that the observed caveolin-1 co-immunoprecipitation with ERα is highly specific, as it was strictly dependent on ERα expression.

**Possible Functional Significance of the Caveolin-1/ERα Interaction—**Caveolins are known to interact with a diverse group of signaling molecules. However, it remains unknown whether caveolins influence steroid receptor signaling pathways. Here, we have provided several independent lines of evidence that suggest that caveolin-1 acts as a positive modulator of estrogen receptor signaling in vivo. (i) We found that caveolin-1 directly potentiated estrogen signaling by inducing translocation of ERα from the cytoplasm to the nucleus, even in the absence of ligand. (ii) Caveolin-1-driven ERα nuclear translocation resulted in increased transcription from an ERα-dependent reporter gene. (iii) Caveolin-1 conferred resistance to the anti-estrogen tamoxifen (with a >5-fold increase in IC50). (iv) Caveolin-1 augmented the transcriptional activation of a constitutively active form of the estrogen receptor, ERαY537S.

Finally, we observed that caveolin-1 interacted with ERα in vivo, as evidence by co-immunoprecipitation studies.

The interaction of signaling molecules with caveolins is mediated largely by the caveolin scaffolding domain, a 20-amino-acid residue membrane proximal domain (40). Using a phage display-based approach, we have previously defined a conserved caveolin-binding motif, i.e., ΦXΦXXXΦ and ΦXXXΦXΦ, where Φ is an aromatic residue and X is any residue (41). However, analysis of the protein sequence of ERα failed to identify a putative caveolin-binding motif.

We recently reported that G protein-coupled receptor kinase 2 interacts with caveolin-1 through a motif that diverges slightly from the above-described consensus, 6GYYLFLRDF71, where Leu substitutes for an aromatic amino acid (42). Interestingly, Gαq has similar substitutions for aromatic residues in its caveolin-binding motif, but Gαq still co-immunoprecipitates caveolin-1 (43). Thus, ERα may interact with caveolins through a divergent caveolin-binding motif or may be recognized by other caveolin domains that have been shown to interact with signaling molecules (44, 45).

Finally, we consider the significance of the caveolin-1/ERα interaction. ERα-expressing breast cancer cells show enhanced tumorigenicity; however, these ERα-positive cells have less of a propensity to metastasize (reviewed in Refs. 46 and 47). One possibility is that caveolin-1 expression may help prevent metastasis by potentiating estrogen-mediated transcription in these cells. This view is consistent with the suggestion that caveolin-1 may function as a tumor suppressor gene whose expression is down-regulated during cell transformation (23, 24, 27, 48). Ultimately, gene ablation studies with model animals will be needed to elucidate the exact physiologic role of the caveolins during the development of mammary adenocarcinomas.

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