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ERs Associate with and Regulate the Production of Caveolin: Implications for Signaling and Cellular Actions

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Recent evidence supports the existence of a plasma membrane ER. In many cells, E2 activates signal transduction and cell proliferation, but the steroid inhibits signaling and growth in other cells. These effects may be related to interactions of ER with signal-modulating proteins in the membrane. It is also unclear how ER moves to the membrane. Here, we demonstrate ER in purified vesicles from endothelial cell plasma membranes and colocalization of ERα with the caveolae structural coat protein, caveolin-1. In human vascular smooth muscle or MCF-7 (human breast cancer) cell membranes, coimmunoprecipitation shows that ER associates with caveolin-1 and -2. Importantly, E2 rapidly and differentially stimulates ER-caveolin association in vascular smooth muscle cells but inhibits association in MCF-7 cells. E2 also stimulates caveolin-1 and -2 protein synthesis and activates a caveolin-1 promoter/luciferase reporter in smooth muscle cells. However, the steroid inhibits caveolin synthesis in MCF-7 cells. To determine a function for caveolin-ER interaction, we expressed caveolin-1 in MCF-7 cells. This stimulated ER translocation to the plasma membrane and also inhibited E2-induced ERK (MAPK) activation. Both functions required the caveolin-1 scaffolding domain. Depending upon the target cell, membrane ERs differentially associate with caveolin, and E2 differentially modulates the synthesis of this signaling-inhibitory scaffold protein. This may explain the discordant signaling and actions of E2 in various cell types. In addition, caveolin-1 is capable of facilitating ER translocation to the membrane. (*Molecular Endocrinology* 16: 100–115, 2002)

STEROID HORMONES, INCLUDING E2, act by binding nuclear receptors, which then transactivate target genes (1). There is also emerging evidence that E2 has rapid, nongenomic actions (2–4), often originating from the cell membrane. The latter findings are in concert with the identification of plasma membrane ERs that signal when activated by E2 binding (5–7). When ERα or ERβ is expressed in Chinese hamster ovary (CHO) cells, multiple signaling pathways are rapidly activated by E2 despite only 3% of total ERs residing in the cell membrane (6). These same pathways are rapidly activated by E2 in a variety of target cells expressing endogenous ER, and some have been linked to important cellular actions of the steroid (8–10).

Although the plasma membrane ER has not been physically isolated and sequenced, it appears to be very similar to the nuclear protein. Both membrane and nuclear receptors can originate from a single transcript (6), and membrane ER can be identified by antibodies raised against various epitopes of the nuclear receptor (11). The process whereby the ER localizes to the membrane is not known. Also, examination of the nuclear ER sequence does not identify a motif that is homologous to a kinase or catalytic domain of growth factor receptors, binding proteins that are typically inserted into the plasma membrane. Thus, it is not clear how ER enacts signal transduction, but this probably occurs through physical interactions with proteins that functionally modulate signaling. As a mitogen, E2 activates signaling in some target cells (2, 3, 7) but also inhibits signaling and proliferation induced by vascular growth factors in other cells (12, 13). These dichotomous findings illustrate an important but unknown mechanism whereby cytokines can act either in a positive or negative fashion, depending upon the cellular context.

Signaling cascades are activated when a growth factor binds its transmembrane receptor, causing the translocation of proximal signaling molecules to the plasma membrane (14). This often results in the localization of these molecules to subdomains within the membrane bilayer, including rafts and caveolae. Caveolae are ω-shaped, invaginated microstructures that are found in most mammalian cell types and communicate with the cell surface while residing within the membrane (15). They function in vesicular transport, endocytosis, and transcytosis (16), but also play a role in signal transduction (17). Caveolae are predominantly structurally composed of a family of proteins, known as caveolins. Caveolin 1 and 2 are found in

Abbreviations: CHO, Chinese hamster ovary; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; 5′NT, 5′-nucleotidase; NTF2, nuclear transport factor 2; VSMC, vascular smooth muscle cells.

many cells expressing caveolae (18), whereas caveolin 3 is restricted to muscle cells (19). Growth factor receptors are enriched within the caveolae (20) where they bind to caveolin proteins (17) and form complexes with signaling molecules localized to this structure (21). Caveolin-1 contains a cytosolic, N-terminal juxtamembrane domain (scaffolding domain), which binds to signaling molecules and inhibits their usual activation after growth factor ligation of receptors (17, 22). Thus, within the caveolae, an ER interaction with other signaling molecules may be important for the propagation of signal transduction. In the studies reported here, we examined E2-ER interactions with caveolin proteins and the implications for the rapid, nongenomic effects of estrogen.

RESULTS

Endogenous ERα Localizes to Caveolae in the Plasma Membrane

EC were selected because they have high concentrations of endogenous caveolae in the membrane and also express membrane ER (23). To determine the ERα localization within plasma membranes in whole cells, we examined cultured rat lung endothelial cells for possible colocalization of ERα with caveolin-1 by confocal Immunofluorescence microscopy. As shown in Fig. 1A, there is significant colocalization of ERα (*red*) with caveolin-1 (*green*). *Yellow* labeling indicates an extensive but not complete colocalization of the two proteins. This is especially evident in a more three-dimensional depiction of the whole cell (*top*). Thus, by these techniques, we detect the presence of ERα at the cell surface, extensively colocalized with caveolin-1.

We then carried out immunoblots of cell fractions from the endothelial cells. Rat lung tissue was subfractionated to isolate first the luminal endothelial cell plasma membranes (P) and then their caveolae (V) (15, 24, 25). As shown in Fig. 1B, immunoblot analysis of silica-coated plasma membrane (P) displayed ample enrichment for plasma membrane markers such as 5′ nucleotidase (5′NT, a glycosyl phosphatidyl inositol-anchored protein) and caveolin-1 (caveolae coat protein) relative to the starting whole lung homogenate (H). ERα is easily detected in P, but unlike 5′NT and caveolin-1, it is not greatly enriched in this fraction relative to the whole lung homogenate. Molecular mass markers (not shown) indicate that ER migrates at approximately 62 kDa. Because ERα is mostly found located in the nuclear membrane, we tested for markers of intracellular organelles including nuclear membrane proteins. P was markedly depleted of the nuclear membrane proteins, transportin and nuclear transport factor 2 (NTF2), as well as the endosomal/Golgi marker β-Cop. Thus, ERα appears to be present at the cell surface using these fractionation techniques.

The caveolae attached on the cytoplasmic side of the membranes opposite to the silica coating were stripped by shearing and then were isolated by sucrose density centrifugation to yield a low buoyant density fraction of intact caveolar vesicles (V). This was well separated from the membranes stripped of the caveolae (P-V). As show in Fig. 1B, ERα is enriched in V relative to P. It is also detected in P-V, but to a lesser extent than in V. This result is consistent with the confocal localization, where ER colocalized extensively but not completely with caveolin-1. Note that the nuclear membrane proteins, transportin and NTF2, are not detected in V. As we previously reported (15, 24, 25), V was enriched in caveolin-1 but not 5′NT. Little caveolin-1 signal remained in P-V.

We also took advantage of another technique (albeit not our preferred method) that does not use silica coating for caveolae isolation (25). Here, the homogenized lung is subjected to percoll gradient centrifugation to yield a plasma membrane-rich fraction (PM) that contains the plasma membrane markers 5′NT and caveolin-1 but also, unfortunately, β-Cop and one of the two nuclear membrane proteins (transportin, but not NTF2). Sonication of PM followed by flotation via sucrose density centrifugation yielded a low buoyant density fraction (AC) quite enriched in caveolin-1. 5′NT, transportin, and β-Cop, are readily detected in AC, whereas NTF2 is only found in the homogenate (H) with little to no signal elsewhere. We subjected AC to further subfractionation to isolate the caveolae more selectively by immunoaffinity separation. 5′NT, transportin, and β-Cop are all detected in the unbound fraction (U) with little to no signal in the immunoisolated, caveolin-coated caveolae bound to the magnetic beads (B). Both ERα and caveolin-1 are found enriched in the immunoisolated, caveolin-coated caveolae bound to the beads (B). Thus, ERα and caveolin-1 are in the same vesicles. Past work shows that B and V appear equivalent in molecular composition and both methods agree here. Thus, ERα is contained within low-density, caveolin-coated plasmalemmal vesicles, namely caveolae.

Caveolin Proteins Differentially Associate with Membrane ERs in MCF-7 and Vascular Smooth Muscle Cells (VSMC)

Caveolin proteins constitute an important structural component of caveolae but are also found to a smaller extent in noncaveolar fractions of the membrane. We next determined whether ERα and caveolin proteins could associate in the membrane. This is potentially important to begin to understand the organization of signaling molecules within this membrane domain. We turned to cell systems in which we have previously characterized E2 signaling through the membrane and the attendant effects on cell biology (9, 12). Thus, MCF-7 and VSMC serve as potential models by which to investigate how E2 can differentially stimulate or inhibit signaling. We first immunoprecipitated ERα in

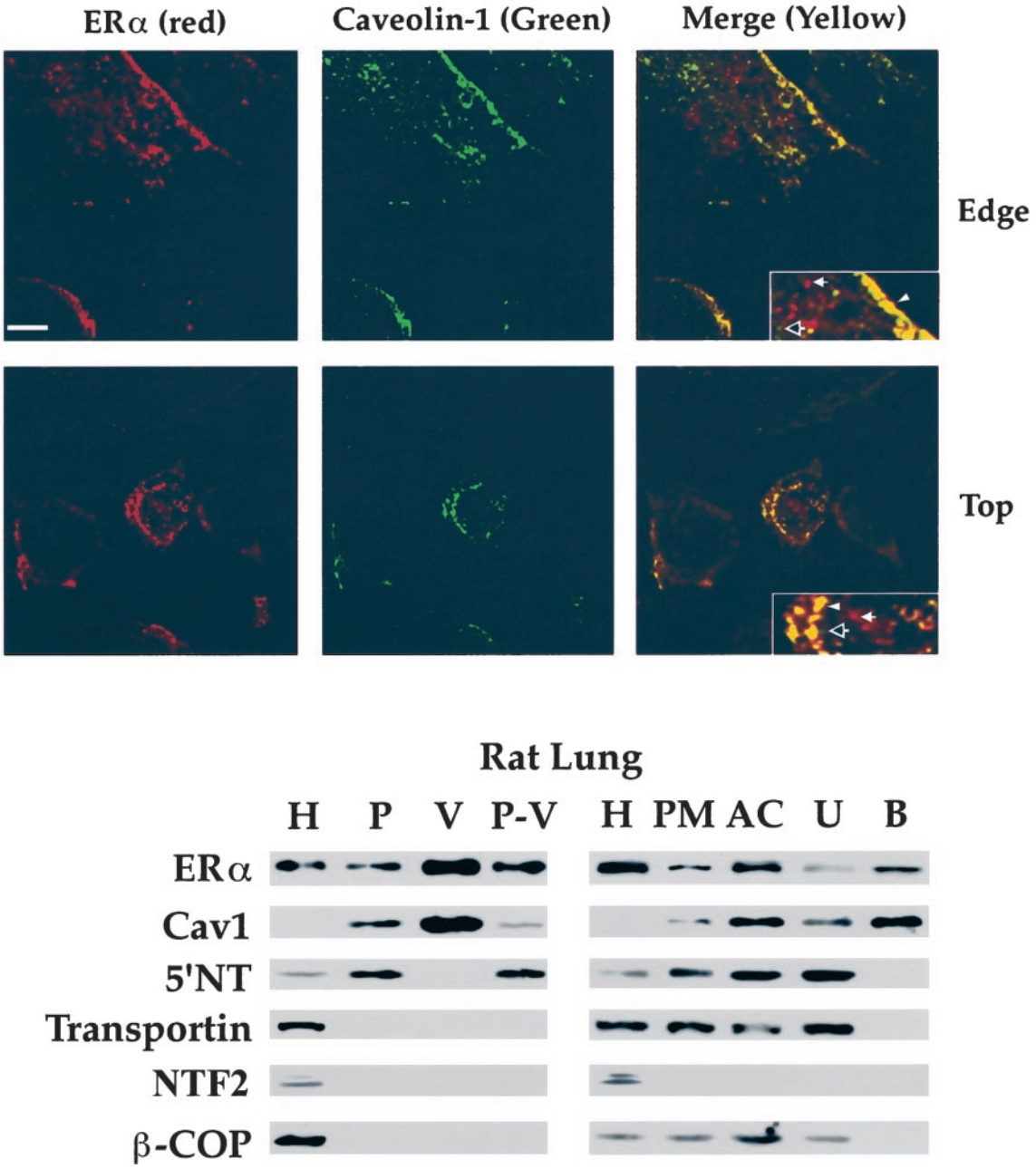


Fig. 1. ERα Colocalizes with Caveolin-1 in the Plasma Membrane of Endothelial Cells

A, Immunofluorescence microscopy shows significant ERα and caveolin-1 colocalization. Antibodies to ERα and caveolin-1 were used to determine colocalization between the two proteins in rat lung endothelial cells, followed by the appropriate reporter antibodies (goat antirabbit-Texas Red (*red*, ERα) and goat antimouse-Bodipy FL (*green*, cav-1). Merge is an overlay of the ERα (*red*) and cav1 (*green*) signal. Edge is the flattened leading edge of the cell, whereas Top refers to the top crest of the cell. *Arrowhead* indicates ERα and cav-1 colocalization, *white arrow* indicates ERα localization without colocalization with cav-1, and *black arrow* indicates caveolin-1 without colocalization with ERα. The *insets* are 3× magnification. B, ERα is contained within caveolae (V & B) fraction of EC membranes. The *left panel* shows the colloidal silica-coating technique to subfractionate rat lung into total homogenate (H), silica-coated luminal endothelial cell plasma membranes (P), caveolae (V), and the repelleted silica-coated membranes stripped of caveolae (P-V). The *right panel* shows the immunoisolation technique for total lung homogenate (H), plasma membrane-rich fraction (PM) by percoll gradient, and caveolin-rich fraction (AC) by sucrose gradient centrifugation. Immunoisolated caveolae were recovered using antibodies to caveolin-1, which fractionates material bound (B) to the beads (caveolae) or unbound (U) in the supernatant. Antibody to the C terminus of ERα was used, along with other appropriate antibodies to the indicated proteins (see *Results*), for Western blot of each fraction (2 μg/lane). The experiments were repeated three times.

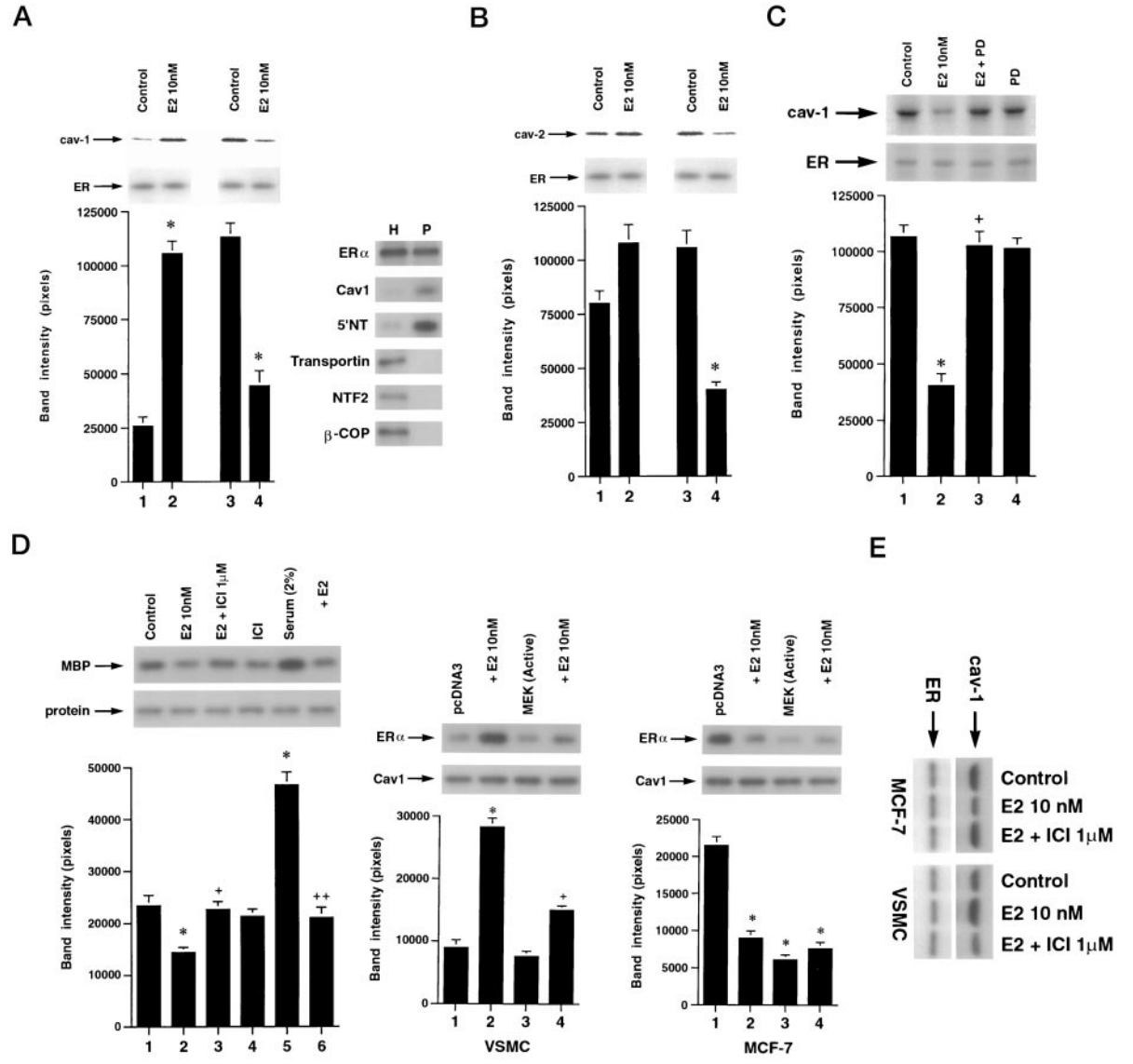


Fig. 2. Caveolin-ERα Association Is Regulated by E2 and ERK

A, *Top*, E2 differentially modulates the association of caveolin-1 and ERα in membranes from VSMC (lanes 1 and 2) and MCF-7 (lanes 3 and 4). Cells were treated, or not, with 10 nM E2 for 30 min, after which the cells were lysed and membranes were prepared as described in *Materials and Methods*. ER was immunoprecipitated, followed by Western blot for caveolin-1. ER protein immunoprecipitated from each condition is also shown. *Bottom,* Immunoblot detection of ERα, caveolin-1, 5′NT (NT), transportin (Trans), NTF2, and β-Cop from whole MCF-7 cell homogenate (H) or sucrose gradient-fractionated plasma membranes (P). B, Differential association of caveolin-2 with ERα. C, E2 inhibition of ER/caveolin-1 association in MCF-7 cells is ERK dependent. Cells were incubated for 30 min with 10 nM E2 ± PD98059, a soluble MEK inhibitor. The *bar graphs* reflect three experiments combined. *, $P < 0.05$ for control *vs.* E2, +$P < 0.05$ for E2 *vs.* E2 + PD98059 by ANOVA plus Scheffe's test. D, *Left*, E2 inhibits ERK activity in VSMC. Cells were incubated with or without 10 nM E2 for 8 min, sometimes in the presence of 2% serum, and ERK activity was determined using the substrate protein myelin basic protein. ERK protein as loading controls is seen *below* each condition, and the *bar graph* represents three combined experiments.*, $P < 0.05$ for control *vs.* E2 or serum; +, $P < 0.05$ for E2 *vs.* E2 + ICI182,780; ++, $P < 0.05$ for serum *vs.* E2 + serum. *Right*, Expression of active MEK-1 differentially modulates ER-caveolin-1 association. MEK-1 expression inhibits E2-induced ER-caveolin-1 association in VSMC, and (independently of E2) inhibits basal ER-caveolin-1 association in MCF-7. The cell lysates were immunoprecipitated for caveolin-1 and then immunoblotted for ERα. Equal amounts of protein and immunoprecipitated caveolin are shown *above* the *bar graph*. The data are from three experiments combined; *, $P < 0.05$ for expressed pcDNA3 control *vs.* condition; +, $P < 0.05$ for pcDNA3 + E2 *vs.* MEK + E2 in VSMC. E, E2 modulation of ER-caveolin association occurs through binding to ER. Cells were incubated with E2 ± ICI 182,780, a specific ER antagonist, and three studies were combined.

the membranes of both VSMC and MCF-7 cells, followed by blotting against caveolins, and found that ERα can associate with either caveolin-1 or -2 (Fig. 2). Similar results were found by immunoprecipitating caveolin, followed by ER blotting (data not shown). Interestingly, ligation of ER with E2 for 30 min strongly alters the above association, and this occurs differentially in the two cell types. In VSMC, there was relatively little association of ER and caveolin-1 in basal cells, but in response to E2, the association increased 3-fold (Fig. 2A, *top*, lanes 1 and 2). However, in the basal MCF-7 cells, there was a relatively strong association of these two proteins in the membrane, but E2 addition caused a 67% down-regulation of this interaction (Fig. 2A, *top*, lanes 3 and 4). Similar results were found for caveolin-2, although the E2 stimulation of ER-caveolin-2 association was not as strong (only 50% increased) in the VSMC (Fig. 2B). To support the purity of the sucrose gradient-isolated plasma membranes, immunoblots for ER and caveolin-1, 5′NT (integral membrane protein), transportin, and NTF2 (nuclear proteins), and the endosomal/Golgi marker β-Cop were carried out. As seen in Fig. 2A (*bottom*), 5′NT and caveolin-1 were enriched in the plasma membrane fraction (P). However, the other three proteins were not found, despite their presence in whole cell homogenates. ERα was detected in both samples, as expected.

We then investigated whether the ability of E2 to activate ERK (MAPK) influenced steroid association with caveolin. As shown in Fig. 2C, abolishing ERK activation with the soluble MAPK kinase (MEK) inhibitor, PD98059, strongly prevented the inhibition of ER-caveolin-1 association in MCF-7 cells. We previously showed that E2 rapidly activates ERK in this human breast cancer cell line, and PD98059 blocks this action (9). These data suggest that downstream signal transduction through ERK feeds back to inhibit the association of ER and caveolin-1 proteins in the membrane after E2 treatment. To corroborate a role for ERK, we transiently transfected/expressed a constituitively active MEK-1 into the MCF-7 cells and determined ER and caveolin-1 association. This kinase construct is well known to directly stimulate ERK activity. We found that independent of exposing the cells to E2, active MEK could also stimulate the dissociation of the two proteins in the membrane (Fig. 2D, *right*).

We then assessed the situation in VSMC. First, we determined the ability of E2 to affect ERK activity in VSMC (Fig. 2D, *left*). The low basal ERK activity was inhibited 44% by 10 nM E2, and the specific ER antagonist, ICI 182,780, prevented this. Furthermore, serum-induced ERK activity was almost completely blocked by E2. When we expressed an activated MEK construct in the VSMC, and incubated the cells with 10 nM E2, the augmented ER-caveolin-1 association seen with E2 alone was significantly prevented (Fig. 2D, *right*). Thus, the ability of E2 to differentially modulate ER-caveolin-1 association in the two cell types is consistent with its differential effects on ERK activity. This, in turn, is consistent with the known differential effects of E2 on cell proliferation, positive in MCF-7 and negative in VSMC, where proliferation/antiproliferation is related to the appropriate modulation of ERK activity (7, 12). We also determined that ICI 182,780 blocked the association-modulating actions of E2 in both cell types (Fig. 2E), consistent with ICI 182,780 effects on E2-modulated ERK activity. Overall, this indicates that E2 specifically acts through ER to signal, and thus influence, the association of ER-caveolin.

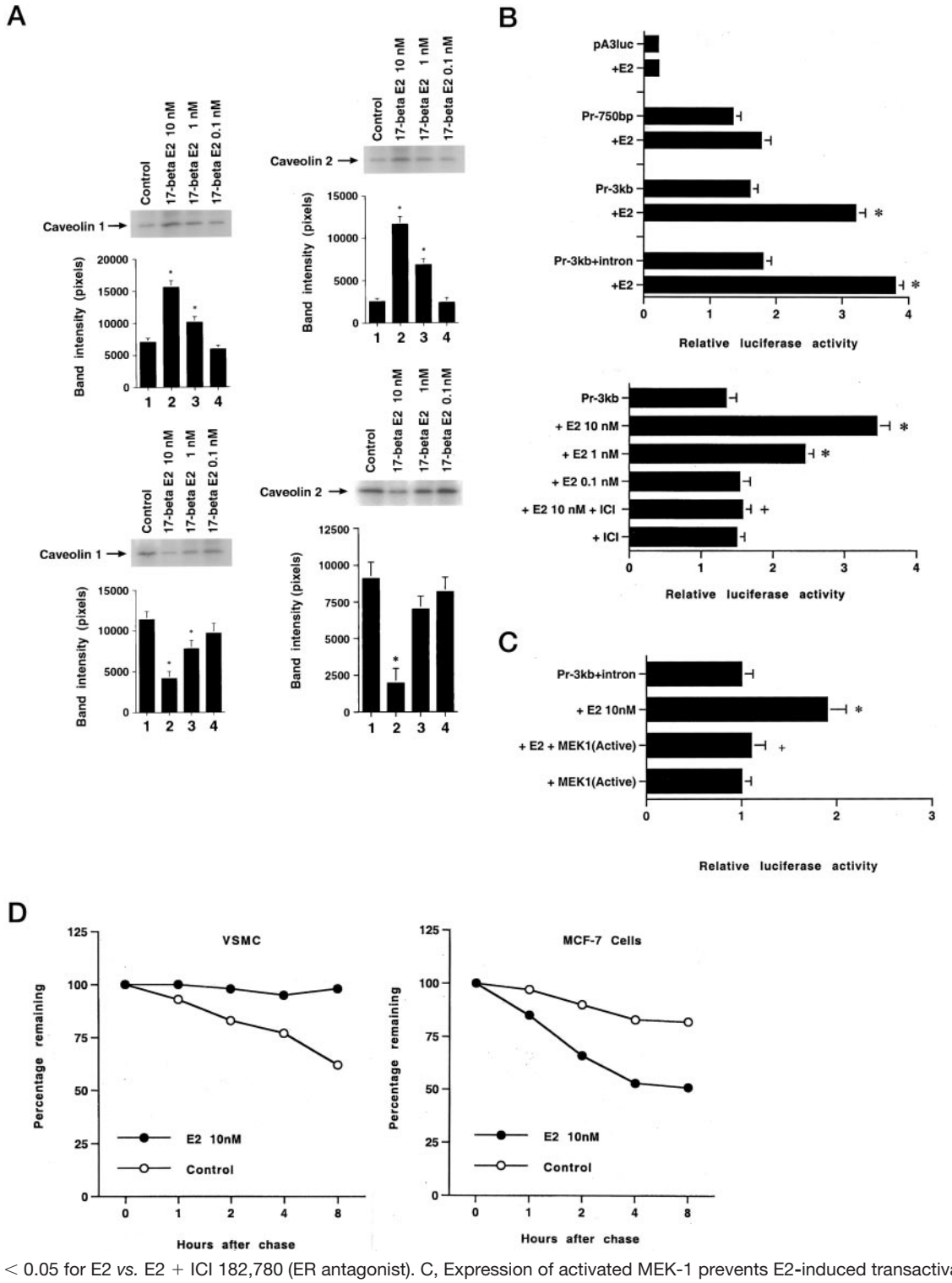
E2 Differentially Modulates Caveolin Production in MCF-7 and VSMC

E2 could modulate the chronic interactions of ER-caveolin in part by affecting caveolin production. This would likely occur through the actions of the nuclear receptor. We therefore determined whether E2 could regulate caveolin protein synthesis. In VSMC, E2 stimulated the production of newly synthesized caveolin-1 and -2 proteins after 8 h incubation in a dose-responsive manner (Fig. 3A, *upper*). These effects were significant at physiologically relevant concentrations of 1 and 10 nM E2. In contrast, E2 significantly inhibited caveolin-1 and -2 new protein production in MCF-7 cells (Fig. 3A, *lower*). These findings could have important implications for the ability of E2 to signal, since caveolin often serves as an inhibitory scaffold protein (17, 22).

As postulated, the modulation of caveolin protein synthesis demonstrated here may result from the ability of the nuclear ER to regulate the transcription of caveolin. We therefore examined the effect of E2 on caveolin-1 promoter/luciferase reporters, expressed in VSMC. Compared with the empty vector, pA3-Luc, basal reporter activity increased from the expression of the −750-bp Cav-1/Luc construct (26), but this reporter was not estrogen responsive (Fig. 3B, *top*). In

Fig. 3. E2 Modulates the Expression of Caveolin-1

A, E2 stimulates the synthesis of caveolin-1 (*upper left*) and caveolin-2 (*upper right*) in VSMC, while inhibiting caveolin-1 (*lower left*) or caveolin-2 (*lower right*) protein synthesis in MCF-7 cells. Cells were first labeled with ^{35}S-methionine and then incubated with or without E2 for 8 h. Caveolin proteins were immunoprecipitated and resolved by PAGE. *Bar graph* data are combined from three experiments. *, $P < 0.05$ for control *vs.* E2. B, *Top*, E2 stimulates the activity of caveolin-1 promoter/luciferase reporters. pA3-Luc (control), and Pr-750bp, Pr-3 kb, and Pr-3 kbandInt 1/pA3 Luc, which contain various lengths of the caveolin-1 promoter, were transfected into VSMC, as described in *Materials and Methods*. The cells were then incubated with 10 nM E2 for 8 h. *Bottom*, Dose responsiveness of the Pr-3 kb caveolin-1 promoter/luciferase reporter activity to E2. Experiments were similarly carried out. Concentrations of E2 are expressed as molar. Experiments were carried out four times, each condition in triplicate during each experiment, and the data were combined. *, $P < 0.05$ for control plasmid expression (pA3-Luc or Pr-3 kb) *vs.* E2-treated cells;



+, $P < 0.05$ for E2 *vs.* E2 + ICI 182,780 (ER antagonist). C, Expression of activated MEK-1 prevents E2-induced transactivation of the caveolin-1 promoter/luciferase reporter. VSMC were first transfected to express the −3 kb + intron reporter ± MEK-1, recovered overnight, and then incubated with 10 nM E2. A representative study with triplicate determinations per condition, repeated twice, is shown. *, $P < 0.05$ for control plasmid expression (Pr-3 kb plus intron) *vs.* E2-treated cells; +, $P < 0.05$ for E2 *vs.* E2+MEK-1. D, E2 differentially modulates the stability of the newly synthesized caveolin-1 protein in cell-specific fashion. After labeling with ^{35}S-methionine, followed by unlabeled methionine, caveolin-1 was immunoprecipitated from lysed VSMC (*left*) or MCF-7 (*right*) over an 8-h time course, in cells exposed or not exposed to 10 nM E2. Caveolin-1 proteins were immunoprecipitated, proteins were normalized before gel loading, and caveolin-1 was resolved by PAGE, followed by densitometry. The data are from a single representative experiment, repeated twice.

contrast, the −3 kb, and −3 kb and intron Cav-1/luciferase reporters were significantly and equally responsive to E2. Further, E2 at 1 and 10 nM stimulated the activity of this reporter (Fig. 3B, *bottom*), and ICI 182,780, the ER-specific antagonist, strongly prevented this action of 10 nM E2. In scanning the 3 kb of promoter sequence, there is no palindromic estrogen response element, but there are numerous transcription factor-binding sites that could potentially mediate the action of E2. These data suggest that the E2-induced synthesis of caveolin-1 likely results from the stimulation of transcription.

Does the ability of E2 to inhibit ERK (see Fig. 2D, *left*) lead to the up-regulation of the reporter fusion gene activity in theses cells? We found that expression of active MEK-1 significantly inhibited the stimulatory effect of E2 (Fig. 3C). These results support overall the ideas that E2 inhibition of ERK stimulates ER-caveolin association (Fig. 2D, *right*) and the transcriptional transactivation of caveolin-1 in the VSMC.

We then asked whether E2 might also modulate the stability of the caveolin-1 protein. To examine this, we carried out pulse-chase labeling studies in both MCF-7 and VSMC, in the presence and absence of E2 (Fig. 3D). In VSMC, there was a 35% degradation of newly formed caveolin-1 protein over an 8-h period in the absence of E2. In contrast, the steroid completely prevented this degradation. Thus, the increased levels of VSMC caveolin-1 protein in the presence of E2 reflect both the stimulation of transcription/synthesis, as well as the inhibition of protein loss. In MCF-7 cells, the loss of newly produced, labeled caveolin-1 over 8 h was only 13% in the absence of E2 (Fig. 3D, *right*). However, the sex steroid accelerated caveolin degradation, yielding a 45% decrease during this same time period. Therefore, E2 inhibits the concentration of caveolin-1 protein in these cells through several mechanisms.

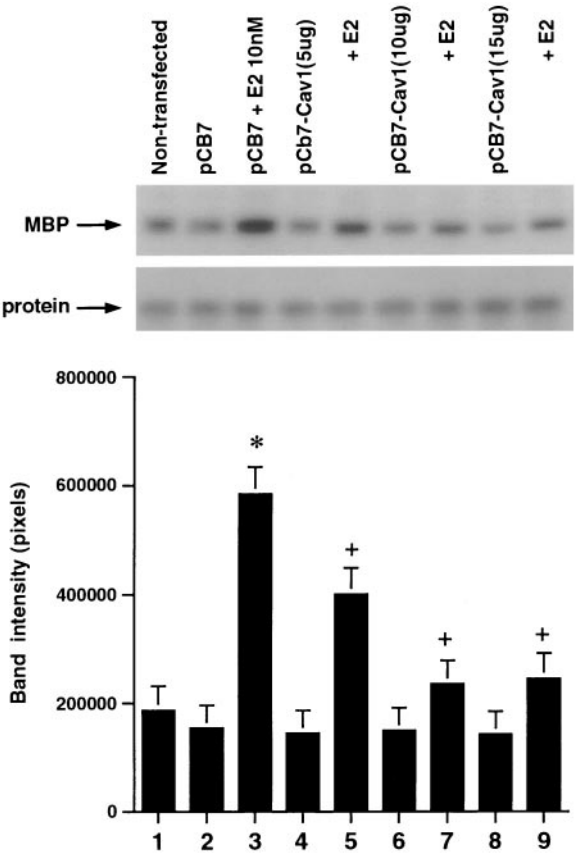


Fig. 4. Concentration-Related Inhibition by Caveolin-1 of E2 Activation of ERK in MCF-7 Cells

Increasing amounts of full-length caveolin-1 cDNA in pCB7 were expressed after transient transfection. The cells were then treated with or without E2 for 8 min and then lysed, and ERK activity was immunoprecipitated for an *in vitro* assay using myelin basic protein as substrate (see *Materials and Methods*). Immunoblot of ERK protein is shown *below* a representative study. Three experiments were combined for the *bar graph*.

Caveolin Expression Inhibits E2-Induced ERK Activity

What might be the role of caveolin-ER interactions in the membrane? In MCF-7 cells, E2 rapidly stimulates signaling to ERK activation, which is necessary for DNA synthesis or cell survival (7, 9). In contrast, E2 inhibits growth factor activation of ERK and the proliferation of VSMC (12). We hypothesized that the differential modulation of caveolin production/association that we have shown here might explain these divergent actions in different cell types.

To support this hypothesis, we transfected different amounts of caveolin-expressing plasmid (pCB7Cav-1) and assessed the ability of E2 to stimulate ERK activity. In MCF-7 cells expressing the empty vector (Fig. 4, lane 2), E2 caused a 3-fold increase in MAPK activity (lane 3). Transfecting increasing quantities of caveolin-1 plasmid led to a concentration-related decrease in the ability of E2 to activate ERK. The maximal 83% inhibition was seen with 10 μg of caveolin-1 plasmid. (lanes 6 and 7, compared with lanes 2 and 3). These results directly indicate that caveolin impairs E2 signaling through ERK, consistent with its reported negative signaling function (17, 22). In VSMC, E2 stimulates caveolin production and enhances ER-caveolin association. These findings provide a mechanism for our previous observations that E2 inhibits ERK activation and ERK-mediated, growth factor-induced cell proliferation *in vitro* (12).

Caveolin Expression Facilitates the Translocation of ER to the Membrane

What other ER functions might caveolin modulate? The events that result in ER being localized to the plasma membrane are unclear. One possibility is that ER translocation from cytoplasm to the membrane is facilitated by caveolin-1, because this protein can move from the cytosol, in and out of the

plasma membrane (18). To test this idea, we expressed caveolin-1 and determined the relative ratios of ER in the cytosol and the cell membrane (Fig. 5A). The plasma membranes were found to be free of several nuclear or cytoplasmic proteins, determined by immunoblotting as shown in Fig. 2A. In nontransfected cells or in cells expressing the empty vector, pCB7, more ER resides in the cytoplasm, relative to the cell membrane (lanes 1–4). Caveolin-1 expression caused a clear increase in the localization of ER at the plasma membrane, as well as a decrease in the cytosolic content of this protein (lanes 3 and 4 *vs.* 7 and 8). In the absence of exogenous caveolin expression, incubation of the cells with E2 modestly stimulated the translocation of ER to the membrane with a concurrent diminution in cytoplasmic steroid receptor (lanes 3 and 4 *vs.* 5 and 6). Expression of caveolin plus E2, however, was not additive.

We then compared the ability of wild type and a scaffolding domain-deleted caveolin-1 (Δ 60–100) as to their ability to translocate ER to the membrane (Fig. 5B). It has been established previously that caveolin-1 that lacks this domain is incapable of reaching the cell surface (27). The mutant caveolin-1 was nearly 60% less efficient in reducing the cytoplasmic pool and increasing the membrane localization of ER [lanes 3 and 4, compared with lanes 5 and 6 (wild type), then lanes 7 and 8 (Δ 60–100)]. Equal amounts of caveolin protein were expressed using the two constructs, determined by immunoblot (data not shown). Our findings indicate 1) a novel property of caveolin to promote membrane localization of ER, and 2) that the scaffolding domain of caveolin-1 appears to be important for this effect.

To further support the role of caveolin-1 to facilitate ER localization at the membrane, we examined Caco-2 rat intestinal epithelial cells. These are among the few mammalian cells that lack caveolae and do not produce caveolin-1 protein (28). We confirmed the lack of caveolin-1 by immunoblot studies in the native cells. These cells are reported to produce a small population of ER when confluent, which we confirmed by our binding studies (see below). In the control or pcDNA3 transfected cells, there was virtually no specific binding in the membrane, and a small amount of binding in the nucleus, consistent with a modest expression of endogenous ER detected in the latter location (Fig. 3C). Upon expression of ERα, specific binding was clearly detectable but was somewhat modest at the membrane (Fig. 3 C, *left*). In part, this reflected our transfection efficiency in these cells, which was approximately 20%, determined by using a green fluorescent protein-ERα construct. However, coexpression of caveolin-1 increased the specific binding by labeled E2 at the membrane nearly 70%. Expression of caveolin-1 in the absence of ER was similar to that of control (data not shown). In the nucleus, there was much more specific binding after ER transfection (Fig. 5C, *right*). This likely reflected the predominance of ER at this location, similar to what we have shown in CHO cells (6). However, caveolin expression did not further enhance the binding of E2 at this site, indicating that there was no facilitation of ER moving to this location in this model. The results from the nuclear fraction rule out an effect of caveolin to enhance E2 binding through a mechanism apart from facilitating receptor translocation/number. The differential effects at the two sites also support the lack of perinuclear membranes contaminating our plasma membrane fractions. These findings in a non-overexpression model support our contention that caveolin significantly facilitates ER translocation to the plasma membrane.

Role of the Scaffolding Domain in ER-Caveolin Interactions

If caveolin-1 facilitates ER translocation to the membrane, then it is probably necessary for the two proteins to associate in the cytoplasm. Furthermore, it is not clear whether the scaffolding domain of caveolin-1 is needed for the protein-protein interaction with ER or whether it serves to target the steroid receptor to the membrane. The latter role would be consistent with a known function of the scaffolding domain (17). To examine this, we transfected full-length or scaffolding domain mutant caveolin-1 into MCF-7. We then immunoprecipitated ER from cytosol, followed by blotting for caveolin-1, and also carried this out in reverse order. As seen in Fig. 6A (*upper*), there is a strong association of caveolin-1 with ERα, and the association is the same whether expressing full-length or scaffolding domain-deleted mutant caveolin-1. Upon transfection with either construct, Western blot for caveolin-1 revealed comparable amounts of caveolin-1 protein (*lower bands*). Furthermore, the same amount of endogenous ER protein was expressed across the experimental conditions. We therefore conclude that that these two proteins associate both in cytosolic and membrane compartments of the cell, and the scaffolding domain is not generally required for the physical association of caveolin-1 with endogenous ER.

This led us to propose that the ability of caveolin to inhibit E2/ER signaling to ERK is dependent upon membrane localization of the two molecules. This is an important issue because it is likely that ER interacts in the membrane with components of the signal cascade that activate this MAPK. Based upon the previous experiments (Fig. 5), we suggest that membrane targeting of ER is dependent, in part, upon the scaffolding domain of caveolin-1. We therefore determined whether full-length caveolin-1, but not the scaffolding domain mutant, was capable of inhibiting E2 activation of ERK in MCF-7. Consistent with the overall data, we found this to be the situation, in that only the full- length caveolin protein significantly inhibited E2 activation of ERK (Fig. 6B). Thus,

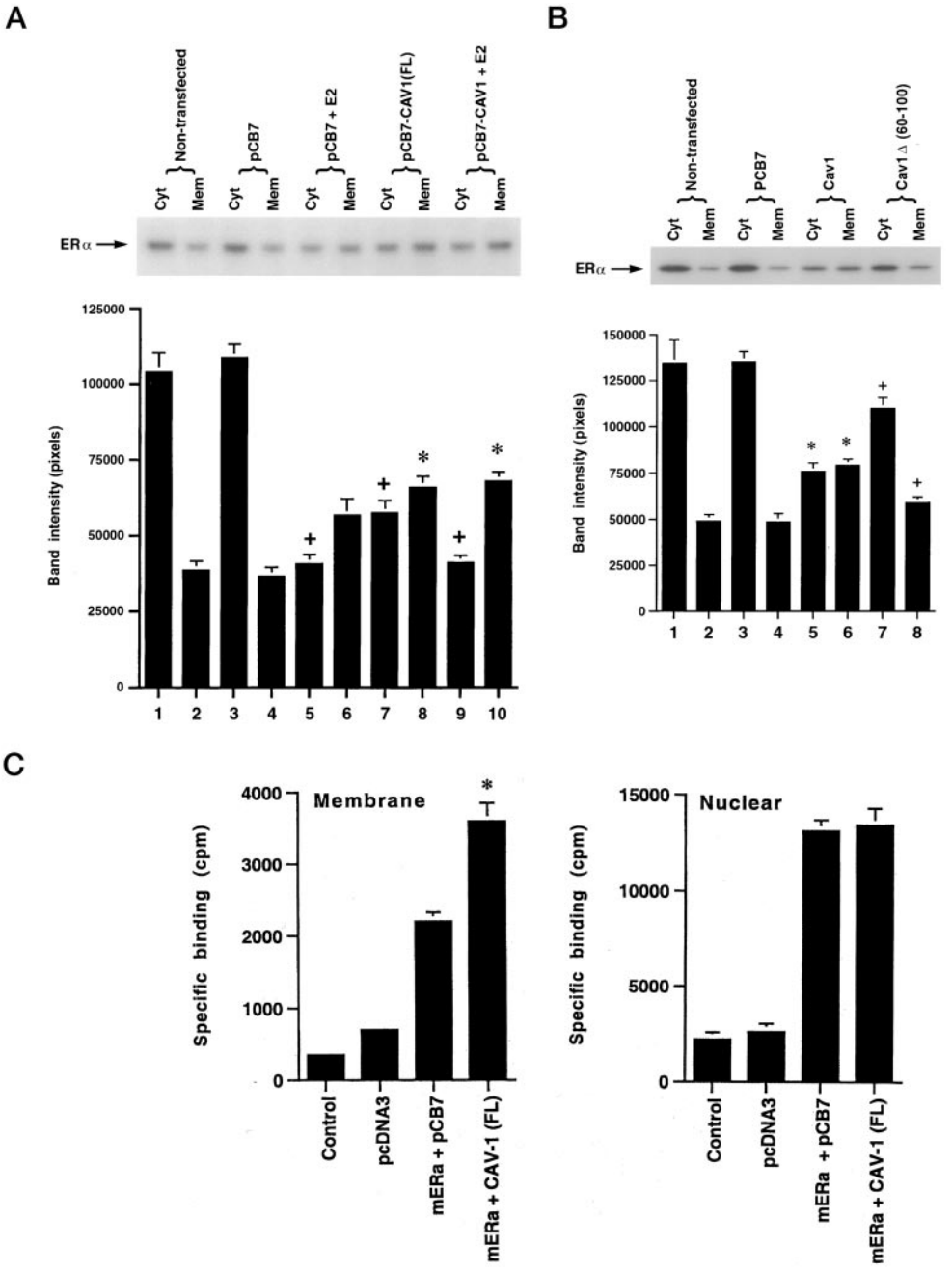


Fig. 5. Caveolin-1 Expression Facilitates ERα Translocation to the Plasma Membrane

A, Expression of caveolin or incubation of MCF-7 with E2 each stimulates translocation of ER from the cytosol to the plasma membrane. Cells were transfected with wild-type caveolin-1 or incubated with E2 without exogenous caveolin, and after ultracentrifugation and percoll gradient isolation, ER localization in cytoplasm and membrane was determined by Western blot. Total protein from cell lysate fractions was determined, and equal aliquots from the conditions were loaded on the gel. A representative study is shown, repeated three times for the *bar graph*. +, $P < 0.05$ for cytoplasmic ER control (lane 3) *vs.* E2-treated cells (lane 5), caveolin-expressing (lane7), or both (lane 9). *, $P < 0.05$ for membrane ER control (lane 4) *vs.* caveolin-expressing cells (lane 8), or caveolin + E2 (lane 10). B, A scaffolding domain-deleted caveolin-1 protein is ineffective in translocating ER to the membrane. MCF-7 were transfected with wild-type or Δ (60–100)cav-1, and ER localization in cytoplasm and membrane was determined by Western blot. *, $P < 0.05$ for cytoplasmic or membrane ER control (lanes 3 and 4) *vs.* wild-type caveolin-expressing cells (lanes 5 and 6, respectively); +, $P < 0.05$ for wild-type caveolin-expressing cells (lanes 5 and 6) *vs.* scaffolding domain-deleted caveolin-expressing cells (lanes 7 and 8). C, Expression of caveolin-1 in Caco-2 cells enhances E2 binding only at the membrane. Caco-2 cells were not transfected (control) or transfected to express ERα without or with cotransfection of caveolin-1. Specific binding of ^{3}H-E2 was determined in membrane (*left*) and nuclear (*right*) fractions. The data are from duplicate determinations/condition, in each of two experiments. *, $P < 0.05$ for ERα-expressing *vs.* cotransfected ERα plus caveolin-1.

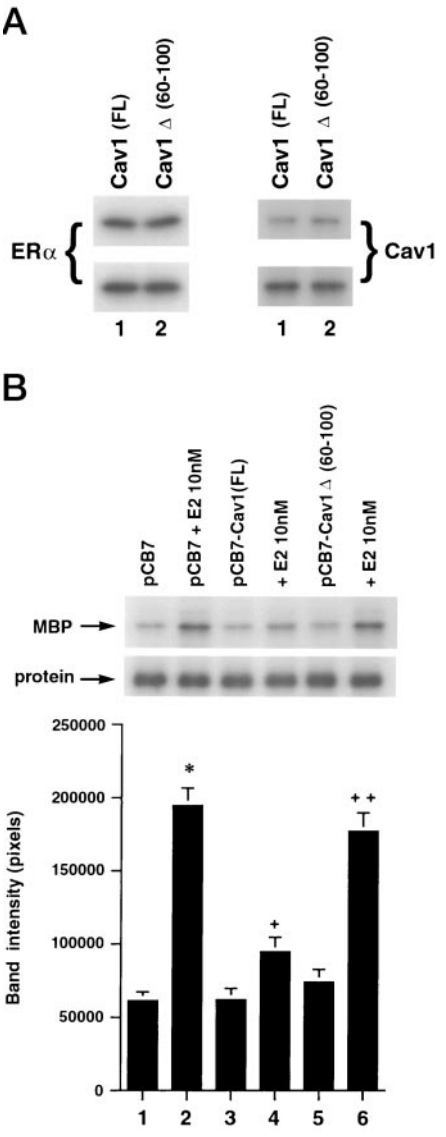


Fig. 6. Full-Length Caveolin-1 Associates with Cytosolic ERα and Is Necessary for Membrane Localization

A, Caveolin-1 and ERα associate in the cytosol of MCF-7 cells. MCF-7 were transiently transfected to express either full-length (lanes 1) or scaffolding domain-deleted (lanes 2) caveolin-1, and then the cytosol fractions were isolated by centrifugation of whole-cell lysates. Either caveolin-1 or ERα was immunoprecipitated, followed by immunoblot for ERα (*left upper*) or caveolin-1 (*right upper*). Western blot for total ERα or cav-1 protein is seen in the *lower* bands. The data are representative of two separate experiments. B, Expression of full-length but not the scaffolding domain mutant caveolin-1 inhibits E2/ER activation of ERK. MCF-7 cells were transfected as above and then ERK activity was determined as described. Data are from three separate experiments combined for the *bar graph*.

the scaffolding domain promotes ER targeting to the membrane, and this also allows the functional interaction with wild-type caveolin-1 that down-regulates E2/ER signaling from this location.

Targeted Expression of ERα E Domain to the Plasma Membrane Activates ERK

To this point, we have demonstrated the localization of ER in the plasma membrane and have presented data to support the proposal that it is the membrane, and not the nuclear ER, that participates to activate ERK. Another approach to this latter, important issue is to target ER to each of the two cell locales and determine the effects of E2. Recently, Kousteni *et al.* (29) showed that E2 acts through the cell membrane ER to prevent etoposide-induced osteoblast cell death. This occurred after E2 activated a signal transduction cascade that ultimately resulted in ERK activation, and this signaling was required for cell survival. These authors also targeted just the E domain of ERα to the plasma membrane and showed that this was sufficient to prevent HeLa cell death. Signaling to ERK was not determined in the targeting model.

We therefore asked whether targeting the E domain to the plasma membrane could result in the ability of E2 to activate ERK. We found that in CHO cells transfected to express this domain at the plasma membrane (E-Mem-ECFP) (29), E2 strongly activated ERK activity (Fig. 7). In contrast, when the E domain was targeted to the nucleus (E-Nuc-ECFP), there was no activation of this MAPK by E2. E2 also could not activate ERK in the native CHO cells, which we previously showed do not express endogenous ER (6). Expression of the membrane-targeted E domain did not result in E2 activating the −3 kb caveolin-1/luciferase reporter (data not shown). This was expected, since in the VSMC, expression of active MEK (and hence ERK) suppressed E2-induced caveolin-1/luciferase reporter activation. Thus, it is likely that, in response to steroid, the E domain portion of the membrane ERα activates the signaling to MAPK activation.

DISCUSSION

The existence of ER in the plasma membrane is now supported by studies from several laboratories (5, 23) in addition to our own. E2 stimulates a variety of signal transduction molecules that localize to the membrane (2–5, 7, 10, 14), potentially mediated through membrane ER. Signaling could also result from not well defined interactions of E2 or E2/ER with other membrane-localized proteins, such as growth factor receptor tyrosine kinases (epidermal growth factor receptor or IGF-1 receptor) (30–32), or through complex interactions with SHBG (33). A related and developing story is the contributions of signaling by E2/ER to cell biological actions (8–10). However, it is unclear how ER signals and where the endogenous receptor resides within the plasma membrane. The latter is critical information because the spatio-temporal arrangement of signaling molecules is important to the modulation of these cascades.

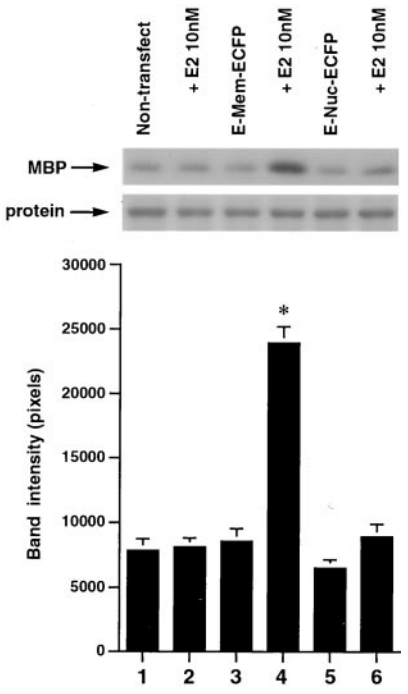


Fig. 7. Transient Expression and Targeting of the E Domain of ERα to the Plasma Membrane of CHO Cells Results in E2 Activation of ERK

CHO cells were not transfected or were transiently transfected to express either E-Mem-ECFP or E-Nuc-ECFP, as described in *Materials and Methods*. ERK activity was then determined in response to an 8-min incubation with 10 nM E2. ERK protein is shown *below* the activity studies, and the *bar graph* data represent three combined experiments. *, $P < 0.05$ for nontransfected CHO (+E2) or E-Mem-ECFP CHO (−E2), *vs.* E-Mem-ECFP CHO (+E2).

Here we report that ER is found in the plasma membrane. Immunocolocalization shown by confocal microscopy indicated extensive, but not complete, overlap of ER with caveolin-1 in the whole-cell plasma membrane. Regarding our immunoblot studies, we determined that ER exists in noncaveolar compartments of the membrane, but predominantly is associated with isolated caveolae vesicles. The latter finding is consistent with a recent report that ER can be localized to caveolar subfractions of the endothelial cell (EC) plasma membrane (34). We additionally illustrate novel findings that ER associates with caveolin proteins in the plasma membrane and that E2 modulates this association differentially, dependent upon cell context. Furthermore, the differential modulation by E2 occurs through signal transduction to ERK via ER. In the MCF-7 cells, association of ER and cav-1 is down-regulated due to ERK activation, whereas in the VSMC, E2 inhibits basal or growth factor-induced ERK and up-regulates ER/cav-1 association. We also show that E2 differentially modulates the production of caveolin, and that this can occur in part through effects on transcription. The latter finding implicates an action of the nuclear receptor. E2 also modulates the stability of caveolin-1, occurring in cell-specific fashion. Finally, we demonstrate that caveolin-1 expression down-regulates E2-induced signal transduction and facilitates ER translocation to the membrane. Both of these functions require the caveolin scaffolding domain for full efficiency.

Caveolin appears to organize the association of signaling molecules within the caveolae (17), including Ras, Src, and PI3K. These molecules move to the membrane from cytoplasm to be activated (35) and are found in caveolae, where they complex and attach to caveolin proteins. Growth factor receptors also localize to the caveolae, where downstream signaling, *e.g*. to ERK, may be facilitated (17). Caveolae dynamics have been elucidated for the activation of endothelial nitric oxide synthase (eNOS). Caveolin-1 attaches to eNOS, keeping the enzyme in a relatively inactive state in the caveolae (36). Activators of eNOS trigger a calcium- and calmodulin-dependent displacement of caveolin-1 from eNOS, leading to the increased activity of the enzyme (37). Also as a result, caveolin translocates out of the plasma membrane. Recently, Chambliss *et al.* (38) reported that ERα and eNOS exist together in EC membrane caveolar fractions, and this facilitates eNOS activation by E2. The results of Chambliss *et al.* seem to be at odds with the aforementioned studies (36, 37), which show that caveolin protein inhibits E2 activation of signaling. However, we believe that the caveolin proteins have a dual purpose in this setting. Serving as a scaffold protein, caveolin helps assemble and localize the signaling molecules into a complex that is capable of being activated. Nevertheless, caveolin itself can inhibit signal activation and may need to dissociate from the assembled complex, as for instance, to allow eNOS activation (36, 37). There is a precedent for this idea with other scaffold proteins. The kinase suppressor of Ras (ksr) purportedly acts as a scaffold protein, forming a complex with MEK, 14–3-3 proteins, ERK, and heat shock proteins 70 and 90, but itself inhibits Ras signaling (39). Similarly, the Jip family of proteins assemble signal molecules in the c-Jun N-terminal kinase (JNK) pathway, thereby modulating JNK activation in the cytoplasm, yet they restrain JNK from translocating to the nucleus and thus inhibit the function of this kinase (40, 41).

E2 has been previously shown to rapidly stimulate NO production in the caveolae (23) through the activation of ERK (10), although it is not clear how ERK participates. We show that blocking E2 activation of ERK prevents the dissociation of ER and caveolin-1 within the MCF-7 membranes. Thus, we propose that the ability of E2 to activate eNOS (10) may result from an ERK-dependent dissociation of ER and caveolin, leading to the activation of NO synthase, perhaps through the recently described activation of PI3K and AKT (42). We also note that, because E2 can regulate caveolin transcription/production, this could influence the longer term interaction of membrane ER and caveolin, promoting localization of ER at the membrane and modulating signal transduction. This likely

represents an example of the coordinated cellular actions of the nuclear and membrane pools of ER, a general concept that has a precedent in other cell models (9, 43).

In our studies, transfection of caveolin-1 cDNA in MCF-7 cells prevented E2 activation of ERK. Overexpression of caveolin-1 inhibits ERK activation by epidermal growth factor (44) and breast cancer cell migration and anchorage-independent growth (45). Transformation of cells by oncogenes is associated with a reduction or loss of caveolin-1 expression (45). Furthermore, stable expression of caveolin-1 antisense in NIH 3T3 cells leads to transformation that is reversed by restoring caveolin-1 protein to normal levels. In this model, hyperactivation of p42/p44 isoforms of ERK resulted from caveolin down-regulation (46). In human breast cancer specimens, endogenous ERK activity is consistently hyperexpressed (47). ERK activation in response to E2/ER action at the membrane significantly contributes to breast cancer cell growth and survival *in vitro* (7, 9). Therefore, the ability of E2 to down-regulate caveolin synthesis and association with ER in MCF-7, leading to the activation of signaling molecules such as ERK, is likely to be important in this regard.

In these same cells, we found that expression of exogenous caveolin-1 caused the loss of ER in the cytosol and an increased amount of ER expressed in the membrane. Although the effects were moderate, this probably reflects the presence of endogenous caveolin protein in the MCF-7 that may have limited the functional effects of overexpression on ER translocation to the membrane. More importantly, the total membrane pool of endogenous ER is only approximately 3% (6, 12), and therefore a limited number of ERs are available to move to the membrane under most circumstances. In a non-overexpression model for ER and caveolin-1, we found binding of E2 at the cell surface and that expressing caveolin-1 into cells that normally do not produce this protein greatly increased this binding, but only at the membrane. Importantly, the lack of enhanced E2 binding to ER in the nuclear fraction of Caco-2 cells rules out an alternative effect of caveolin to enhance E2 binding, through a mechanism apart from facilitating receptor translocation. In all, these data support our proposal that caveolin-1 facilitates the translocation of ER to the membrane, after binding to this receptor in the cytoplasm. In MCF-7, our results also extend the recent findings of Schlegel *et al.* (48), who demonstrated that overexpression of caveolin-1 results in translocation of ER from the cytoplasm to the nucleus. In a similar model in prostate cells, Lu *et al.* (49) have recently shown that caveolin-1 interacts with the AR and facilitates androgen transcriptional activity. We also found translocation of ER from the cytosol to the nucleus after overexpression in MCF-7 (our unpublished results). However, at steady state, 90% of caveolin-1 is at the plasma membrane (23) and, therefore, endogenous caveolin may be most important to facilitate ER movement to this location. Supporting this idea, caveolin-1 enhances the transport of the caveolin-2 protein to the plasma membrane (50).

Recently, Schlegel and Lisanti (51) determined that the plasma membrane attachment and caveolae targeting regions of caveolin-1 reside within the scaffolding domain (residues 82–101) and the first 16 nucleotides of the C-terminal region (135–150). In fact, caveolin-1 that lacks the scaffolding domain is incapable of reaching the cell surface (27). We found that the scaffolding domain is an important contributor to the ability of exogenous caveolin-1 to promote ER translocation, which is consistent with the aforementioned studies. Furthermore, these two proteins associate in the cytoplasm, but this is not dependent upon the presence of this domain. Therefore, we propose that the scaffolding domain facilitates ER localization at the membrane and, therefore, the full-length caveolin protein (but not the scaffolding domain-deleted protein) inhibits ERK activation. Importantly, these data further support the idea that it is the membrane-localized ER that modulates signal transduction cascades to MAPK (see below). The scaffolding domain may also serve a second role to restrain signaling, in some way, at the membrane (52, 53). A detailed analysis of other caveolin, as well as ER, domains that contribute to this process, and the mechanisms facilitating ER translocation and modulation of signal transduction in caveolae, is underway.

A still controversial issue is whether the membrane ER is responsible for the activation of signaling (for instance to ERK) after E2 ligation of endogenous steroid receptors. Previous results suggest that the membrane receptor is important. This is based upon 1) the rapid effects of E2 to stimulate a variety of signaling pathways, 2) the activation of these pathways by a membrane-impermeable E2-BSA compound, and 3) the linkage of the membrane ER to G protein activation and subsequent signaling, an event that is known to occur only at the plasma membrane. Here, we have taken a different approach and found that targeting the expression of the E domain of ERα to the plasma membrane allowed the activation of ERK but did not result in the transactivation of an estrogen response element/luciferase reporter by E2. For comparison, targeting of the E domain to the nucleus did not result in E2-induced MAPK activation. These results suggest that the E domain of the native membrane receptor is important to activate the signaling molecules at the plasma membrane that result in the subsequent activation of ERK (29).

One important finding is that E2 can differentially modulate the production of caveolins and association with ER in the membrane, depending upon cell type. It is well appreciated that a variety of growth-modulating cytokines, including E2, can stimulate proliferation in one cell while inhibiting this process in another. Although this is perhaps related to modulating different signaling cascades in cell autonomous fashion (54–56), the exact details of these responses to proteins

such as TGFβ, platelet-derived growth factor, or angiotensin II is unknown. We propose that, at least for E2, this occurs in part via the differential modulation of caveolin production and association/function. It has been demonstrated that endothelial cell proliferation factors, such as vascular endothelial growth factor and basic fibroblast growth factor, inhibit caveolin-1 synthesis in EC (57), consistent with the effects of E2 reported here. Our findings allow us to hypothesize that the positive or negative modulation of caveolin production and association with signaling molecule complexes may contribute to the differential actions of other growth-regulatory cytokines as well.

As mentioned, the down-regulation of caveolin, and the subsequent activation of signaling, could contribute significantly to E2-induced growth and survival of breast cancer (7, 9). Furthermore, E2-related inhibition of VSMC proliferation and migration has been demonstrated *in vivo* after acute vascular injury (58). This may result from the up-regulation of caveolin dynamics at the membrane, as shown here. Increased caveolin would inhibit growth factor signaling to ERK, providing a mechanism by which E2 inhibits VSMC proliferation (12), and the reactive hyperplasia that results in the injured vessel wall *in vivo* (58, 59).

MATERIALS AND METHODS

Materials

Antibodies and substrate for kinase activity were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD 98059 was a gift from Dr. Alan Saltiel (Parke-Davis, Morris Plains, NJ). Caveolin reporter expression reagents were kindly provided by Dr. M. Lisanti (Albert Einstein College of Medicine, New York, NY). Primary cultures of human VSMC were prepared and used as previously described (12). MCF-7 cells were obtained from ATCC (Manassas, VA).

Subcellular Fractionation of Rat Lung Homogenates to Isolate Endothelial Cell Plasma Membranes and Caveolae

The luminal EC plasma membranes and caveolae were isolated directly from rat lung tissue using an *in situ* silica-coating procedure (15, 24). Briefly, the rat lungs were perfused via the pulmonary artery with a colloidal silica solution to coat the surface endothelium. This allowed selective isolation of the EC plasma membranes from the lung homogenate by centrifugation. The caveolae were separated from the membrane by shearing and then isolated by sucrose density centrifugation in a low buoyant density fraction, well separated from the membrane pellet stripped of caveolae.

For translocation and other membrane studies, cells were washed three times with PBS, then lysed in buffer A (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 100 nM NaCl; 50 mM NaF; 100 μM phenylmethylsulfonyl fluoride; protease inhibitor cocktail; and 0.2% Triton X-100) and sonicated, after which nuclear pellets were collected through low-speed centrifugation. The supernatants were centrifuged at 100,000 $\times$ *g* for 30 min to pellet cell membranes. The cell membranes were then further separated by sucrose gradient overlay, and [in accordance with our extensive past experience (15, 16, 24)], fractions 3–5 contained the buoyant membranes (with caveolae and rafts) that were pooled for experiments. Briefly, membrane samples were placed into a tube with an equal volume of 85% (wt/vol) sucrose/25 mM A-morpholine-ethanesulfonic acid and 0.15 M NaCl solution, then overlaid with 8.5 ml of 35% sucrose, topped up with 16% sucrose, and centrifuged at 35,000 rpm for 18 h at 4 C. Ten fractions (1 ml each) were obtained and further processed, or separated on SDS-PAGE followed by membrane transfer for immunoblotting. The membrane fractions were immunoblotted with antibodies to caveolin-1 and 5′NT (plasma membrane proteins), transportin and NTF-2 (nuclear proteins), and β-COP (endosomal/Golgi marker protein). Only caveolin and 5′NT were enriched and found in the early pooled fractions.

Kinase Activity Assays

For ERK activity assays, the cells were synchronized for 24 h in serum, phenol red, and growth factor-free medium. The cells were then exposed to E2, 10 nM, for 8 min with or without additional substances, as previously described (12). Immunoprecipitated kinases were then added to substrate myelin basic protein for *in vitro* assays (6, 9). In some studies, the E domain of ERα was transiently expressed in CHO cells, from plasmid vectors that targeted this portion of the receptor to either the nucleus (E-Nuc-ECFP) or the plasma membrane (E-Mem-ECFP) (29). After recovery, the cells were synchronized without serum for 12 h, after which 10 nM E2 was added to the cells for 8 min, and ERK activity was then determined.

Transient Transfections

VSMC (passage 1–2) or MCF-7 were grown to 50–60% confluence, and then transiently transfected with LipofectAMINE and 1.5 μg of fusion plasmid when cells were cultured in each well of six-well plates (luciferase reporter studies). For all other studies, 10 μg total DNA/100-mm dishes of cells were used. For reporter assays, the plasmids included pA3-Luc, and Pr-750bp, Pr-3kb, and Pr-3 kb and Int 1/pA3 Luc, which contain various lengths of the caveolin-1 promoter, upstream of the ATG site and cloned into a luciferase reporter (pA3-luciferase) (26). In other experiments, 10 μg pCB7Cav-1 (full length), which expresses canine caveolin-1, or pCB7Cav-1 (Δ 60–100), which is missing the scaffolding domain segment, were used, except for ERK studies in which 5, 10, and 15 μg plasmid were used. The cells were synchronized and incubated with E2 as previously described (6, 12). Cell extract supernatants were assayed by the dual-luciferase reporter assay system (Promega Corp., Madison, WI). To correct for transfection efficiency, cells were cotransfected with 0.1 μg of pRL-SV40 expressing the Renilla luciferase (Promega Corp.). In other experiments VSMC, MCF-7, or CHO cells were transfected with plasmids expressing a constituitively active MEK-1 (Upstate Biotechnology, Inc., Lake Placid, NY).

Caveolin Synthesis

Cells were serum deprived for 24 h and then incubated in methionine-free DMEM with dialyzed 10% FBS for 1 h before experimentation. The cells were then incubated in the absence of serum or unlabeled methionine, but with 250 μCi of ^{35}S-methionine in the presence or absence of E2 for 8 h. Caveolin-1 or -2 protein was immunoprecipitated from lysed cells, and the proteins were denatured in SDS and resolved by PAGE, followed by fluorography and autoradiography. In additional pulse-chase studies, the cells were labeled for 1 h with ^{35}S-methionine, and then the medium was replaced with 10-fold excess unlabeled methionine, in the presence or absence of 10 nM E2. At intervals over 8 h, the cells were lysed, and caveolin-1 was immunoprecipitated and resolved by PAGE.

Coimmunoprecipitation and Immunoblot Protein Analysis

Membrane and cytosolic fractions were incubated with protein A-Sepharose for 1 h, after which supernatants were transferred to fresh tubes containing protein A-Sepharose conjugated to caveolin proteins and incubated for 4 h at 4 C. Immune complexes were washed and boiled and then separated by SDS-PAGE. After transfer to nitrocellulose, the proteins were washed with blocking solution and incubated with primary antibody to ERα for 2 h and then with horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Inc.). Bound IgGs were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) and autoradiography. In other experiments, as described previously (15, 24, 25), rat lung protein subfractions were solubilized and separated by SDS-PAGE and transferred to nitrocellulose filters, followed by immunoblotting. Primary antibody (diluted from 1:500 to 1:5,000 in Blotto, Sigma, St. Louis, MO), was followed by the appropriate horseradish peroxidase-labeled reporter antibodies (diluted 1:1,000). Reactivity was visualized using enhanced chemiluminescence and quantified densitometrically using ImageQuant (Quantum Images, San Diego, CA). Protein concentrations were measured using the micro BCA protein assay kit with BSA as a standard following a protocol previously described (24, 25).

Immunofluorescence Microscopy

Bovine aortic endothelial cells were grown on coverslips and then methanol fixed before dual immunofluorescence confocal microscopy was performed (60). Cells were then stained with monoclonal antibody to caveolin (1:250 dilution of clone Z034; Zymed Laboratories, Inc., South San Francisco, CA) and polyclonal antibody to ERα (1:250 dilution of MC20; Santa Cruz Biotechnology, Inc.). Binding of primary antibody was detected by a reporter IgG conjugated to Texas Red (antimouse IgG) and Bodipy (antirabbit IgG) (Molecular Probes, Inc., Eugene, OR).

Binding Studies

Caco-2 cells (rat intestinal epithelial cells from ATCC) were grown on 100-mm petri dishes in DMEM-F12 without phenol red. Twenty four hours after transfection with 5 μg of pcDNA3-ERα (plus 5 μg of backbone vector), or with 5 μg each of both ERα and caveolin-1 constructs, the cultures were washed, and lysed in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 nM NaCl, 50 mM NaF, 100 μM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and 0.2% Triton X-100). Nuclear pellets were collected through low-speed centrifugation. The supernatants were centrifuged at 100,000 $\times$ *g* for 30 min to pellet cell membranes. Both pellets were washed twice, once without detergent. Fifty microliters of membrane proteins from the cells were incubated with/without 1 μM unlabeled E2 but always with 50 μl ^{3}H-E2 (specific activity, 80 Ci/mmol, pH 7.5) (Perkin-Elmer Corp., Norwalk, CT) (1.4 pmol of labeled steroid) at 37 C for 45 min, as previously described by us (6). The pellets were washed three times and then quantified by β-scintillation counting. The differences in the presence and absence of unlabeled E2 constituted specific binding.

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