Multiple steroid receptors (SR) have been proposed to localize to the plasma membrane. Some structural elements for membrane translocation of the estrogen receptor α (ERα) have been described, but the mechanisms relevant to other steroid receptors are entirely unknown. Here, we identify a highly conserved 9 amino acid motif in the ligand binding domains (E domains) of human/mouse ERα and ERβ, progesterone receptors A and B, and the androgen receptor. Mutation of the phenylalanine or tyrosine at position −2, cysteine at position 0, and hydrophobic isoleucine/leucine or leucine/leucine combinations at positions +5/6, relative to cysteine, significantly reduced membrane localization, MAP and PI 3-kinase activation, thymidine incorporation into DNA, and cell viability, stimulated by specific SR ligands. The localization sequence mediated palmitoylation of each SR, which facilitated caveolin-1 association, subsequent membrane localization, and steroid signaling. Palmitoylation within the E domain is therefore a crucial modification for membrane translocation and function of classical sex steroid receptors.

Steroid receptors (SR) exist predominantly in the nucleus and mediate gene transcription, both in ligand-independent and -dependent fashion (1). However, evidence also supports distinct pools of functional membrane-localized SR, binding estrogen (2), progesterone (P), or androgen (reviewed in Ref. 3). Overall, very little is understood regarding the mechanisms of translocation of SR to the plasma membrane (PM).

From a structure/function relationship for membrane localization, estrogen receptors (ER) have been best studied. Serine 522 and cysteine 447 in the E domain are necessary for strong physical association of ERα with caveolin-1, and subsequent membrane translocation (4, 5). Cysteine 447 has been reported to be a site of palmitoylation (5), a modification that generally increases protein hydrophobicity and membrane association of proteins (6). Other regions, such as the N terminus (A/B) region of ERα have also been reported to participate in membrane localization (7), but this has been disputed (4).

ER have been localized to caveola rafts in the membrane (8–10). Here, ER closely associate with signaling proteins, leading to rapid activation of G proteins and subsequent multiple transducer signals (reviewed in Ref. 11). Evidence strongly supports the idea that the membrane and nuclear ERα and ERβ proteins are distinct pools of the classical receptor proteins in several cells (12, 13).

Regarding other steroid receptors, virtually nothing is known about the mechanisms of membrane localization. Related to this, a report identified a new family of membrane heptahelical G protein-coupled receptors that bind progesterone (P) and are products of genes distinct from the gene encoding the classical progesterone receptor(s) (PR) (14). It is not clear whether these proteins or the classical PR mediate rapid actions of P at the membrane (15).

In the studies here, we identified a highly conserved sequence and mechanism required for membrane localization of multiple SRs. Mutational analysis established that a 9 amino acid motif in the E domains of ERα and ERβ, PR, and the androgen receptor (AR) mediates membrane translocation via palmitoylation, events necessary for rapid signaling by these receptors.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructs—**Mouse ERα (pcDNA3–ERα kindly provided by K. Korach), ERβ (pSG5–ERβ from J. Gustafsson), human PRB (pLEM–PR) and human AR (pCMV5–AR kindly provided by D. Tindall) were used as wild-type (wt) controls and templates for creating mutations by polymerase chain reaction (PCR)-based, site-directed mutagenesis (kit from Stratagene). Primers used and the alanine mutants created are shown in Table 1, and all PCR products were sequenced for mutation verification. CHO cells or steroid receptor-negative breast cancer cells were transfected with wt or mutant SR using lipopectamine (4), recovered overnight, then exposed for 2 h to 10 nM E2, 100 nM P, or 10 nM T, and immunofluorescent microscopy was carried out for receptor localization, as described (4). First antibodies were against the receptor C terminus (Santa Cruz Biotechnology), and second antibody was conjugated to FITC (Molecular Probes). For each condition, 300 cells were counted to compare the PM localization of wt versus mutant SR-expressing CHO cells, in two separate experiments. Protein expression was controlled by limiting the
amount of plasmid used, to simulate the expression of endogenous SRs expressed at the membrane of MCF-7 and LnCap cells, determined by Western blot (data not shown). To create a fusion protein containing the 9 amino acid motif from ERα (FVCLKSIIL) C terminus to the GFP protein, we used the oligonucleotides (5'-aattctttgtgtgcctcaaatctattttgg and gatcccttcagtttcccaggtcagacacaaag) to form double-stranded DNA containing the myristoylation sense sequence flanked by sticky ends recognizing EcoRI and BamHI enzymes. The DNA was ligated to pEGFP-C2 (Clontech), linearized by EcoRI and BamHI. To test whether key residues of the ERα palmitoylation motif dictated translocation to a palmitoyl acyltransferase, or promoted palmitoylation, we constructed a plasmid with additional membrane targeting sequences. A myristoylation sequence to mutant ER vectors containing amino acid changes, F-A or IL-AA. The constructs were verified by sequencing.

In some studies, siRNAs to caveolin-1 or control (GFP) were obtained from Qiagen, cat. no. Si00299635. siRNA, 3 μg in 1 ml of buffer, per 500,000 cells in a single well, was transfected at 0 h with Oligofectamine as described (13), after time course studies showed maximal protein knockdown at 48 h. wtERα was transfected at 24 h, and microscopy of ERα localization in CHO cells was performed at 48 h. For other studies, CHO were transfected and recovered, incubated with 10 μM 2-bromopalmitate for 6 h, followed by 2 h of incubation with steroid. Transcription studies utilized a triple, tandem estrogen response element/luciferase reporter (kindly provided by John Couse/Ken Korach), transiently transfected into CHO cells, with or without the various ERα constructs. Luciferase activity was determined after incubation of the cells with 10 nM E2 for 24 h, as described (16).

**Estradiol-specific Binding**—CHO cells were transfected to express wt or mutant ERα, and whole cell binding of 17β-[3H]E2 (1 nM) to equilibrium was carried out in the presence or absence of 100 nM unlabeled E2 (nonspecific binding) (13).

**Palmitoylation of SRs Expressed in CHO-K1 Cells**—CHO-K1 cells were grown on 100-mm-diameter dishes in Dulbecco’s modified Eagle’s medium/nutrient mixture F12 Ham without phenol red. Twenty-four hours after transfection with wild-type or mutant SRs, the cells were synchronized without serum overnight, then labeled with [3H]palmitic acid (0.5 mCi/ml) for 1 h. 10 nM 17β-E2 or other sex steroids were added for another 4 h, the cells washed, then lysed in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 50 mM NaF, 100 μM phenylmethylsulfonyl fluoride, protease inhibitor mixture, and 0.2% Triton X-100).
Nuclear pellets were collected by low speed centrifugation (6000 rpm) and solubilized by sonication. Equal amounts of solubilized cell extracts (supernatant and nuclear fractions) were immunoprecipitated for 2 h at 4 °C with antibodies to ERα (MC-20, Santa Cruz Biotechnology), ERβ (51-7700, directed against the C terminus, Zymed Laboratories Inc.), PR (SC-539, C-20, Santa Cruz Biotechnology), or AR (SC-815, C-19, Santa Cruz Biotechnology) each conjugated to protein A-Sepharose. The immunoprecipitates were washed with lysis buffer, then eluted with 4× Laemmli sample buffer containing 2-mercaptoethanol after boiling for 5 min. Samples were subjected to 10% SDS-PAGE, followed by fluorography and autoradiography. Some aliquots underwent counting by β-scintillation. Data were analyzed by analysis of variance plus Schefe’s test, and incorporated counts were normalized per milligram of protein from each condition. To determine whether palmitoylation occurred at internal site(s) via thioester linkage, CHO cells expressing wtERα were incubated with 1 μM unlabeled palmitic acid for 2 h. The cells were washed, then lysed, and ER was immunoprecipitated. The supernatant containing isolated ER was exposed to buffer containing or not containing 1 M hydroxylamine at pH 7.2 for 2 h. Hydroxylamine cleaves thioester linkage of the fatty acyl group to cystine (17). The buffers were centrifuged, and the supernatants sent for analysis by electrospray ionization mass spectrometry (HT Laboratories Inc., San Diego).

Kinase Signaling—ERK activity in CHO cells expressing wt or mutant SR was determined at 8 min after exposure to steroid ligand. Activity of immunoprecipitated and protein normalized ERK was directed against the myelin basic protein substrate in an in vitro assay, as described (4). PI 3-kinase activity was determined as phosphorylation of AKT at serine 473 (13), after 15 min of exposure of the cells to steroid. p38 activity was determined...
similarly to ERK, immunoprecipitating this kinase from ERβ-expressing cells incubated with/without 10 nM E2 for 30 min. Protein-normalized immunoprecipitates were used in an in vitro assay with the substrate ATP2 protein. p38β antibody (Santa Cruz Biotechnology) was also used to immunoblot total p38β protein (for normalization). All studies were repeated.

**Cell Biology Studies**—CHO or HCC-1569 cells (ER/PR/AR-negative breast cancer cells) expressing the various SR constructs were recovered, synchronized overnight in serum and steroid-free medium. Progression from G1 to S phase was determined at 24 h as described (12). In some experiments, 2-Br was added prior to assay. Cell viability was separately quantified by the colorimetric MTT assay (Sigma). The conversion of MTT to MTT-formazan crystal by mitochondrial enzymes occurs in viable cells. In brief, CHO cells were transiently transfected with different SR constructs, and recovered for 24 h. Cells were exposed or sham exposed to brief UV radiation (50 J/M2), and incubated in the presence or absence of specific steroid ligands for 24 h. The cells were then washed in medium without phenol red and incubated with medium containing MTT 1 mg/ml for 4 h at 37 °C. MTT/formazan was extracted by overnight incubation at 37 °C with 100 ml of extraction buffer. Optical densities at 570 nm were measured using buffer as a blank. The optical density of the control vector alone (sham UV) was converted to 100% viability, and all other densities were normalized to this value as a percent of control.

**RESULTS**

**Functional Analysis of Mutated ERα**—Many heptahelical G-protein-coupled receptors (GPCR) contain a conserved F(X)LL sequence, where X is any amino acid and L is leucine or isoleucine (18). F, LL, and the precise 6 amino acid spacing between F and LL are required for protein export from the endoplasmic reticulum. Upon sequence mutation, membrane localization of several GPCRs was not found (18).

In the E domain of human/mouse ERα proteins, a sequence highly homologous to F(X)L is present. In contrast to typical GPCRs, the third amino acid of this motif in ERα is cysteine (amino acid 447 in the human) (Fig. 1A). We expressed wtERα or F449A, C451A, and IL453/454AA mutant ERα in ER-null CHO cells. Wild-type receptor was detected in a discontinuous pattern at the PM in 95% of successfully transfected cells, but no mutant receptors were observed at this location (Fig. 1B and Table 2). Similar results were obtained after expression in ER-null breast cancer cells (data not shown). Whole cell and cytoplasmic ER protein was comparable by immunoblot in wild-type and mutant receptor-expressing cells (Fig. 1D). All receptors comparably localized to the nucleus (Fig. 1D) but each mutant ERα protein was significantly less abundant at the PM. We previously established the purity of our subcellular fractionation approach (4, 13, 19). E2 rapidly activates ERK and PI 3-kinase via membrane-localized ER in various target cells (4, 20, 21). WT compared with mutant ERα supported rapid and robust ERK activation by E2 (Fig. 1E), and stronger PI3K activation. Specific binding of labeled E2 was similar for wt and mutant ERα (Fig. 1F), and the transcriptional activities of the various ERα constructs were comparable in ERE-luciferase-transfected CHO cells (Fig. 1G).

**Palmitoylation and Function of Membrane ER**—How does this motif promote the PM localization of functional ERα? The cysteine residue has been reported to be a site for palmitoylation, potentially mediating ERα translocation to the PM (5). Here we determined whether cysteine and other amino acids in our identified motif affect ERα lipiddation and PM localization/function.

**Membrane Localization and Signaling**—We found that cytoplasmic/membrane wtERα underwent palmitoylation, not influenced by steroid ligand (Fig. 2A). Importantly, wtERα in the nucleus did not show this acylation. This supports the idea that palmitoylation drives the SR specifically to the PM. Furthermore, virtually no palmitoylation of the cysteine mutant receptor was detected in this cell fraction. Thus, Cys447 is apparently the only palmitoylation site in ERα. Scant lipiddation occurred in cells expressing the F or IL mutants (Fig. 2A).

**TABLE 2**

Localization of steroid receptors expressed in CHO cells

Data are derived from counting 300 CHO cells per receptor condition in two separate experiments. Transfected cells were counted if they expressed the nuclear receptor, determined by immunofluorescent microscopy. Lack of nuclear expression was always accompanied by lack of membrane expression. In some transfected cells, it was technically difficult to determine if membrane localization occurred.

| Receptor | Cells showing receptor percentages |
|----------|----------------------------------|
| wtERα    | Nuclear | Plasma membrane |
| F449A    | 100     | 0 |
| C451A    | 100     | 0 |
| IL453–4AA| 100     | 0 |
| wtERβ    | 100     | 93 |
| Y416A    | 100     | 0 |
| C418A    | 100     | 0 |
| IL423–4AA| 100     | 0 |
| wtPR     | 100     | 92 |
| FB18A    | 100     | 0 |
| C820A    | 100     | 0 |
| LL1825–6AA| 100    | 0 |
| wtAR     | 100     | 95 |
| FB305A   | 100     | 0 |
| C607A    | 100     | 0 |
| LL1812–3AA| 100    | 0 |

**FIGURE 1. Membrane ER localization.** A, highly conserved amino acid sequence in the ligand binding domains of ER, AR, and PR. B, immunofluorescent localization of wt or mutant ERα expressed in CHO cells. Arrows point to membrane ER expression. C, 2-bromopalmitate (2-Br) prevents membrane localization of endogenous ERα in MCF-7 cells. D, Western blots of whole cell, nuclear, cytoplasmic, or PM fractions from ER-transfected CHO cells (n = 3). E, kinase activation in cells expressing wild-type or mutant ERα. ERK activity directed against the myelin basic protein (MBP) substrate or PI3 kinase activity (serine 473 phosphorylation of AKT kinase) was accomplished in transfected CHO cells. F, whole cell specific binding of estradiol to cells expressing wild-type or mutant ERα. CHO cells were transfected to express wt or mutant ERαs, and total specific binding of [3H]E2 was determined at 1 h (in the presence or absence of 100 nM unlabeled E2). The study was repeated. G, wild-type and mutant ERαs comparably stimulate transcription. ER-transfected CHO cells were co-transfected with an ERE-luciferase reporter plasmid, and luciferase activity was measured as described under “Experimental Procedures.” The bar graph values are mean ± S.E. from three experiments combined. *, p < 0.05 by analysis of variance plus Schefes’ test for any ER expressed versus same plus E2.
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We also incubated CHO-wtERα cells with an inhibitor of palmitoylation, 2-bromopalmitate (2-Br) (22). This compound prevents wtER palmitoylation (Fig. 2A, lane 5), the membrane localization of expressed wtERα (Fig. 1B), and the localization of endogenous ERα to the PM in MCF-7 cells (Fig. 1C). 2-Br also dampened E2-induced ERK and PI 3-kinase activities via endogenous membrane ER in MCF-7 cells (Fig. 2B), or ERK in CHO-wtERα cells (Fig. 2C).

Palmitoylation is the co- or post-translational attachment of palmitic acid to a cysteine, promoted by a palmitoyl acyltransferase enzyme (PAT) (6). This most often occurs in the N-terminal region of membrane-bound signaling molecules by amide linkage. However, internal cysteine residues can be reversibly S-palmitoylated via thioester linkage. To support the idea that palmitoylation occurred by thioester bonding of palmitic acid to the designated cystine, a plasmid encoding a GFP wtERα fusion protein was expressed in CHO cells, incubated with 3H-labeled or unlabeled palmitic acid, then immunoprecipitated from the cells. Isolated GFP-ER was then incubated in buffer with or without hydroxylamine. Hydroxylamine cleaves thioester bonding of fatty acyl groups (17). Here, hydroxylamine blocked palmitoylation of ERα (Fig. 2D, left) and liberated palmitic acid (right). The latter was identified by electrospray ionization mass spectrometry as a 256 Da peak, compatible with palmitic acid. This was found released in the buffer from hydroxylamine-treated ER but not in the control buffer (latter not shown).

Next, we determined cell transition through G1 to S phase of the cell cycle. Compared with wtERα, each mutant receptor resulted in ~50% less E2-stimulated thymidine incorporation (TI) (Fig. 2E). 2-Br also significantly but incompletely blocked the ability of wtERα to support this action. 2-Br further decreased E2-induced TI when the palmitoylation inhibitor was combined with F449A mutant expression, perhaps further impairing palmitoylation. Finally, wtERα-induced TI was significantly but partially prevented by inhibitors of MEK (PD98059) or PI3 kinase (wortmannin) (Fig. 2F). Thus, membrane localization of palmitoylated ERα leads to signal transduction that contributes to cell cycle progression.

Palmitoylation Facilitates ER Association with Caveolin—We previously showed that membrane translocation of ERα requires the physical association of the steroid receptor with caveolin-1 protein, mediated in part through serine 522 of ERα (4). Further, the scaffolding domain of caveolin-1 (amino acids 80–100) was necessary for the membrane localization of both caveolin and ER.

We asked whether palmitoylation promotes caveolin-1/ERα association, leading to membrane transport. CHO cells contain endogenous caveolin-1 but lack ER. Transfection of wtERα led to membrane ERα association with caveolin-1, this aspect promoted by E2 (Fig. 3A). This was substantially diminished from expression of each mutant receptor (top), and wtERα association with caveolin-1 was inhibited by 2-Br (Fig. 3A, bottom). This protein-protein interaction was important to membrane localization because knockdown of caveolin-1 with siRNA prevented wtERα localization at the membrane (Fig. 3B).

Is the identified 9 amino acid motif in ERα sufficient to dictate membrane localization? To address this issue, we attached the E domain motif to a protein that normally does not translocate to the membrane. We cloned the ERα motif C terminus
to green fluorescent protein in the pEGFP-C2 plasmid. Transfection of the parent vector, pEGFP-C2, resulted in diffuse cellular fluorescence in CHO cells (Fig. 3C). In contrast, expression of the fusion protein resulted in membrane localization, although a weaker fluorescent intensity was seen. The fusion protein was palmitoylated, and this was reversed by treatment of the precipitated protein with hydroxylamine (Fig. 3D). Thus, the 9 amino acid sequence bestows protein localization at the membrane and palmitoylation.

The F and IL amino acids in the E domain motif might localize steroid receptors to the modifying PAT in a subcellular partition, and/or promote the physical interaction of the enzyme(s) with the SRs. To investigate this, we cloned a myristoylation sequence from Src to wt, F445A, and IL453/454AA mutant ERα. wtERα without the myristoylation fusion sequence was expressed in CHO cells and revealed predominantly a nuclear distribution, with cytoplasmic and membrane localization also seen (data not shown). In contrast, wt and mutant ERα containing the myristoylation sequence localized only at the cytoplasmic/membrane interface (Fig. 3E). wtER but not mutant ER underwent palmitoylation despite myristolate-induced membrane localization of all constructs. This suggests that the amino acids flanking cystine facilitate the physical and functional interaction of ERα with a palmitoyl acyltransferase.

Analysis of Other SRs—ERβ contains a comparable 9 amino acid motif except that tyrosine is present rather than phenylalanine at 416 in the native protein (Fig. 1A). Expressed wtERβ localized to the cell membrane in 93% of transfected cells, not seen with the mutant forms (Fig. 4A and Table 2). All mutations, especially at cysteine 418, impaired cytoplasmic/membrane ERβ palmitoylation (Fig. 4B). As with ERα, wild-type nuclear ERβ was not palmitoylated. Further, 2-Br prevented both the palmitoylation and membrane localization of cyto/memb wtERβ. Activation of p38 MAP kinase by E2 contributes to cell survival in several cell types (16, 23). We
report the novel finding that wtERβ supports E2-induced activation of p38β, prevented by 2-Br. In contrast, mutant (s) ERβ does not support this signaling (Fig. 4C).

Rapid signaling by progestins and androgens has previously been demonstrated, indicating that these sex SRs probably exist at the PM (15, 24–26). The identity of membrane PR is unclear,
endogenous protein was localized to the membrane (and nucleus and cytoplasm) of MCF-7 breast cancer cells (Fig. 5A). PRA and PRB isoforms were found at the PM of MCF-7 cells, with PRB localized in excess of PRA (Fig. 5A).

We also found membrane, nuclear, and cytoplasmic AR in LnCap prostate cancer cells, identified with antibodies to the “classical” receptor (Fig. 5B). Mouse and human AR and PRB contained sequences in their E domains almost identical to ERα (Fig. 1A), and comparable mutations were created. While expressed wtAR and PR localize to the PM in 95 and 92% of cells, respectively, each mutation prevents this (Fig. 5C and Table 2). 2-Br prevented membrane localization of wtAR and PR (Fig. 5C), and palmitoylation was seen in wt but not mutant AR or PRB-expressing cells (Fig. 5D). Signaling to ERK and PI3K (Fig. 5E) and DNA synthesis (Fig. 5F) was significantly stimulated by specific steroid ligands, more so in cells expressing wtSRs compared with mutant SRs. We also determined cell viability after UV-radiation of transfected CHO cells (Fig. 5G). Cell viability was reduced by ~50% in irradiated cells expressing the wtSRs in the absence of steroid, when compared with sham radiation of these cells. The specific ligands E2, T, and P, respectively, prevented 55, 52 and 40% of the cell death caused by UV exposure, seen from wtSR expression. In contrast, mutant SR expression and steroid exposure failed to significantly prevent UV-induced cell death. Previous studies have proposed that rapid signaling by SRs contributes to cell viability (27–29).

**DISCUSSION**

Rapid signaling effects of sex steroids E2, P, and T have been increasingly recognized (20, 24–26), and ascribed to membrane-localized ER (28), PR (14, 25), and AR (30). Recently, it was shown that the membrane ER (31) in human breast cancer cells is the classical ERα (13). The exact nature of membrane P and T binding proteins is not clearly defined, and the mechanisms required for membrane localization are unknown.

Here we identify a unifying mechanism as to how classical sex steroid receptors translocate to the PM to enact rapid signaling. A signature, 9 amino acid motif was found to be highly conserved in the ligand binding domains of all sex steroid receptors, identical in both mouse and human genes. Interestingly, the glucocorticoid receptor but not the mineralocorticoid receptor also contains the identified motif. Thyroid receptors A and B contain a comparable sequence (lpcedqiil) but peroxisome proliferative-activated receptors, and the retinoic acid receptor do not. In addition, this motif was 1) similar to a sequence in typical GPCRs that are not palmitoylated because they do not contain the cysteine residue, and 2) the GPCR motif was found necessary for the angiotensin II and alpha adrenergic receptors to be exported from the endoplasmic reticulum (17).

The motif mediated SR palmitoylation, and blocking palmitoylation of expressed or endogenous wtSRs with 2-Br prevented membrane localization and signaling. SRs mutated in key motif residues (F or T, C, IL/LL) were not normally palmitoylated, and PM localization and rapid kinase signaling to downstream functions were decreased. Mammalian PATs that interact with many membrane-bound signaling proteins (Fyn,
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Rho, Src, G proteins, etc.) have not been isolated to date. Recently, a conserved family of proteins (DHHC) has been found in yeast-through mammals, promoting palmitoylation of a few identified membrane proteins (32). Whether these PATs interact with steroid receptors is unknown.

In contrast, mutated receptors translocated to the nucleus comparably to wild-type receptors. Importantly, only wild-type SRs in the cytoplasmic/membrane (but not the nuclear) fraction of the cell underwent palmitoylation. Thus, a limited number of receptors outside the nucleus undergo this post-translational modification, facilitating PM translocation. We observed that palmitoylation increases the physical association of ERs with caveolin-1. This protein-protein interaction was required for membrane localization, demonstrated by knocking down caveolin-1 with siRNA. Caveolin-1-null cancer cells that contain endogenous ER show only a nuclear pool of sex steroid receptor; upon introducing caveolin-1, ER translocates to the PM (4).

Our findings extend results from Acconcia et al. (5) who showed that human ER is palmitoylated at cysteine 447, promoting caveolin-1 interaction, and membrane translocation. We corroborate these findings and report that 1) the nuclear-localized ER pool is not palmitoylated, 2) additional sequences flanking the cysteine residue are needed for optimal palmitoylation, and 3) thymidine incorporation and cell survival are stimulated in part from membrane-localized ER action. Most importantly, we show that ER, PR and AR utilize this same mechanism and conserved motif to facilitate PM translocation. Both Acconcia et al. (5) and we do not find that E2 promotes palmitoylation; in fact, the earlier report suggests that the sex steroid promotes de-palmitoylation, perhaps a function of the timing of the studies. Upon attaining membrane localization, de-palmitoylation of many proteins rapidly ensues (6). We speculate that de-palmitoylation of PM-localized ER allows important protein-protein interactions to occur in subcellular domains, necessary for membrane signaling. Previous studies from Li et al. (33) showed that a 46-kDa membrane ER produced in immortalized endothelial cells undergoes post-translational palmitoylation at an undetermined residue, leading to membrane localization.

**FIGURE 5.** A, membrane localization of endogenous PR in MCF-7 cells (left) and immunoblot of PR in MCF-7 cell fractions (right). B, AR distribution in LnCap cells. C, intact but not mutant AR (left) and PR (right) localize to the PM in CHO cells. D, palmitoylation of cytoplasmic/membrane AR (left) and PR (right) expressed in CHO cells. E, ERK and PI 3-kinase activity in CHO cells expressing wild-type or mutant AR or PR. F, wild-type AR and PR support ligand-stimulated thymidine incorporation. CHO cells were transfected to express wt or mutant AR (left) or PR (right), recovered and incubated with 10 nM T or 100 nM P, and thymidine incorporation was carried out as described. Bar graph data are the mean ± S.E. of three experiments, duplicate determinations per condition in each. *, p < 0.05 for pCMV5 versus wtPR (plus P), or wtCMVAR (plus T); +, p < 0.05 for PR + or P or AR + T versus mutant PR or AR + ligand. G, cell viability of SR-expressing CHO cells subjected to UV radiation. wt or mutant ERα, AR, or PR-expressing cells were exposed or sham-exposed to UV radiation (50 J/M²), cells incubated with specific steroid receptor ligand, and viability was determined by the MTT assay. Bar graphs are from n = 3. *, p < 0.05 for vector control versus same plus UV or other condition; +, p < 0.05 for UV plus wtSR versus same plus SR ligand.
We provide novel evidence of endogenous, PM-localized, classical PR and AR in cancer cells. It is controversial as to the nature of PR at the PM. Membrane-localized PR (mPR) have been identified as typical GPCRs in fish through humans (14). In contrast, other data (including shown here) indicate that classical, “nuclear PR” can localize and function at the membrane (24). We report that endogenous PRA and PRB isoforms are present at the PM of MCF-7 cells with PRB the most abundant form. Further work will determine if endogenous, heptahelical P-binding proteins exist at the membrane, and function either separately or co-operatively with classical PR. Nevertheless, we showed that classical wt PR expression results in membrane localization and rapid P signaling to enhanced thymidine incorporation and cell viability. Regarding AR, our findings support the idea that the classical “nuclear receptor mediates rapid signaling by male sex steroids at the PM. To date, the endogenous membrane-localized AR has not been isolated and analyzed in a manner comparable to ER (13).

Our results suggest that mutations selectively preventing membrane localization will help sort out the distinct but integrated functions of the various SR pools. In this regard, blocking membrane localization or signaling only partially prevents cell cycle progression. This indicates the important role in this regard for nuclear SR (4), and suggests collaboration of subcellular receptor pools to effect cellular actions.

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