MAP kinase mediates growth factor-induced nuclear translocation of estrogen receptor α

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Received 7 January 2001; revised 6 February 2002; accepted 6 February 2002

First published online 5 March 2002

Edited by Jacques Hanoune

Abstract In addition to mediating the classical transcriptional effects of estrogen, estrogen receptors (ERs) are now known to regulate gene expression in the absence of estrogen by ligand-independent activation pathways, and to mediate the rapid, non-genomic effects of estrogen as well. ERs have been shown to associate with the cell membrane, and recent studies demonstrate that this subpopulation of membrane-associated ER mediates the rapid effects of estrogen. To date, however, little is known regarding the pathways that regulate the distribution of the ER between the nuclear and membrane fractions. In the current study, we demonstrate membrane localization of transiently transfected ERα in human vascular smooth muscle cells, and translocation of ERα from the membrane to the nucleus in response to both estrogen-dependent and estrogen-independent stimulation. Mutational analyses identified serine 118 as the critical residue regulating nuclear localization following estrogen-independent stimulation, but not following estrogen stimulation. Induction of nuclear localization of ERα by estrogen-independent, but not estrogen-dependent stimulation was blocked by both pharmacologic and genetic inhibition of mitogen-activated protein (MAP) kinase activation. Furthermore, constitutive activation of MAP kinase resulted in nuclear translocation of ERα. These overexpression studies support that MAP kinase-mediated phosphorylation of ERα induces nuclear localization of the ER in response to estrogen-independent, but not estrogen-dependent stimulation, demonstrating stimulus-specific molecular pathways regulate the nuclear localization of the ER. These findings identify a previously unrecognized pathway that regulates the intracellular localization of the ER, and represent the first demonstration that the distribution of the ER between membrane and nuclear compartments is regulated by physiologic stimuli.

Key words: Ligand-independent activation; Gene expression; Estrogen receptor

1. Introduction

The diverse biological effects of estrogen are mediated by estrogen receptors (ERs) of which two are known, ERα and ERβ [1–4]. Classically, ERs function as ligand-activated transcription factors [5,6] and as such are considered to be confined to the nucleus where they regulate gene transcription in response to hormone binding. Recent reports, however, have defined two additional molecular pathways for ERα-mediated effects in cells (reviewed in [7–9]). First, ERα can be transcriptionally activated in the absence of estrogen, a process referred to as ligand-independent activation (reviewed in [8]). Ligand-independent activation of ERα has been reported in response to a variety of stimuli (e.g. serum [10], dopamine [11], cAMP [12], caveolin-1 [13], Akt kinase [14], epidermal growth factor (EGF) [15] and specific cyclins [16–18]). The most completely studied pathway for ligand-independent ERα activation involves mitogen-activated protein (MAP) kinase-mediated activation of ERα in tumor-derived cell lines [15,19,20]. In *Cos*1 cells, for example, growth factor-induced activation of ERα results from MAP kinase-mediated phosphorylation of serine 118 in the A/B domain of the ER [15].

The second recently described alternative mechanism for ERα-mediated signaling in cells involves rapid effects of estrogen (reviewed in [7,9]). The rapid effects of estrogen occur within 5–15 min of estrogen exposure without alterations in gene expression. Given the importance of estrogen's effects on the cardiovascular system, non-genomic effects of estrogen have recently been examined in vascular endothelial cells. In these cells, short-term stimulation with 17β-estradiol (E2) results in activation of both MAP kinase and Akt kinase, with subsequent activation of endothelial cell nitric oxide synthase [21–25]. Despite its rapid, non-genomic nature, estrogen-induced nitric oxide release from endothelial cells is mediated by ERα [24–26]. Rapid effects of estrogen have also been reported in vascular smooth muscle cells (VSMC) [27], though the mechanisms that mediate these effects are less well understood. In vivo studies demonstrating that administration of estrogen acutely enhances vasodilation in both animal and human subjects supports a physiologically relevant role for non-genomic activation of the ER [22,28–31].

Recent studies exploring further the non-genomic activation of ERα in endothelial cells have shown that ERα, localized to caveolae associated with the cell surface membrane, is capable of mediating E2-induced nitric oxide release [23]. Rapid, estrogen-mediated activation of several signaling pathways has also been reported recently in CHO cells, and in these ERα overexpression studies, ERα was also detected in cell membranes [32]. These studies confirmed previous reports [33–35,40] suggesting that though the majority of ER protein is found within the nucleus, a subpopulation of ERα can also be detected in the cell membrane.

Taken together, these studies suggest that ERs are not constitutively confined to the nucleus, as is widely believed, but rather support that the receptor is present in the cell in at least

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0014-5793/02/$22.00
PII: S0014-5793(02)02432-8

two populations: (i) a cell membrane population that mediates the rapid, non-genomic effects of estrogen; and (ii) a nuclear population that mediates changes in gene transcription. We hypothesized that the distribution of the ER between the membrane and nuclear compartments is regulated by physiologic stimuli and thus undertook the current series of experiments to investigate the molecular pathways that regulate the intracellular localization of ERα. The results presented below demonstrate that hormone-dependent and hormone-independent stimuli lead to nuclear localization of ERα via distinct molecular pathways.

2. Materials and methods

Unless otherwise specified, all chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Cell culture techniques

Human VSMC were obtained by standard explant techniques as described [36]. Rad91 cells are a spontaneously immortalized VSMC line derived from a human radial artery. Cells were grown in phenol red-free Dulbecco's modified Eagle's medium with 10% estrogen-deficient fetal bovine serum (ED-FBS) as described [36]. VSMC were used at passages 4–6 and Rad91 cells were used at passages 6–18. GH3B6 cells (rat pituitary tumor cells; kind gift of Cheryl Watson) [35] were grown in F-10 with 2.5% calf serum and 12.5% horse serum.

2.2. Plasmids

pCMV3ERα, an expression plasmid for full-length, wild-type human ERα, was constructed by cloning the ERα cDNA into the pCDNA3.1 vector as described [10]. The plasmid pCMV3ER-NTF was produced by inserting the coding sequence for an eight-amino acid epitope tag recognized by a commercially available monoclonal antibody (M2) at the 5′ end of the ERα coding sequence in pCMV3ER as described [10]. To facilitate studies of the subcellular distribution of ERα, we next constructed an expression plasmid for an N-terminal chimeric green fluorescent protein (GFP)-human ERα protein in the backbone vector pEGFP. The transcriptional integrity of the chimeric protein was studied extensively in preliminary experiments using an estrogen response element reporter plasmid [36], and the chimeric GFP-ERα behaved identically in all instances to the wild-type ERα (data not shown), confirming previous reports with a similar construct [37]. The plasmid pCMV3ER-S118A which contains an alanine for serine substitution at amino acid 118 was constructed by the Kunkle method as described [10]. The plasmid pCMV3ER-S118E was constructed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The cDNA inserts of all plasmids with mutant ER constructs were sequenced in their entirety to ensure that no inadvertent mutations were introduced during the mutagenesis or cloning procedures. The plasmid pCMV3ER-ΔAB, which expresses a truncation mutation of ERα lacking the N-terminal transcription activation domain, and the plasmids pGST-ERα and pGST-ERβ which express GST-ER fusion proteins were the kind gift of Myles Brown [38]. The plasmids pLNX-MAPKK and pLNX-DN-MAPKK which express full-length wild-type and dominant negative MAP kinase were the kind gift of Yukiko Gutah.

2.3. Transient transfections

Forty-eight hours prior to each experiment, cells were arrested by serum starvation and then transfected by electroporation as described previously [36]. Cells were plated on coverslips in 12 well plates at approximately 80% confluence and allowed 6 h for attachment. The cells were rinsed and then maintained for 24 h in serum-free medium (SFM), in the presence or absence of 10^{-8} M E2, 10^{-7} M ICI 182780 (a pure ER antagonist; ICI), EGF (100 ng/ml), 10% ED-FBS, or charcoal-stripped 10% FBS (charcoal-stripped to remove any residual E2, as described [10]; S-FBS). In some experiments, the cells were also grown in the presence or absence of the MEK1 inhibitor PD98059 (60 μM) as described [10]. We have previously shown that this concentration completely inhibits MAP kinase activation in VSMC [10].

2.4. Immunostaining

Twenty-four hours after stimulation as described above, the cells were fixed in 3.7% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100, and stained with primary antibody (M2 1:1000 (Sigma); AER 320 1:100 (Neo Markers, Fremont, CA, USA); AER 304 1:100 (Neo Markers)) for 1 h at 25°C, rinsed and then incubated with either an FITC- or Cy3-labeled secondary antibody. Cells were DAPI-stained (0.1 μg/ml for 15 min at 25°C), and then examined by fluorescence microscopy. For experiments in which expression of endogenously expressed ER was examined, the specificity of the staining was investigated by pre-absorbing the antibody with either GST-ERα or GST-ERβ protein. GST fusion proteins were incubated with the appropriate antibody, Sepharose beads were added and the mixture was centrifuged at $14\,000\times g$ for 20 min at 4°C. The supernatants were then used for immunostaining. In additional subsets of immunostaining experiments, the cells were (a) not permeabilized with any detergent prior to staining to prevent entry of the antibody into the cell's interior; or (b) pre-treated with trypsin for 2 min at 25°C to remove extracellular epitopes.

2.5. Cell counting

For each coverslip, 50 cells were counted and ERα distribution for each cell (detected either by GFP or immunostaining) was categorized as nuclear staining only, mild extra-nuclear staining (1+), moderate extra-nuclear staining (2+) or predominantly extra-nuclear staining (3+). The reader was blinded to cell treatments during all counting procedures. For each experiment coverslips were counted in duplicate and each experiment was independently repeated three times or more.

2.6. Immunoblotting

Cells were grown, transfected, plated in 100 mm dishes at approximately 80% confluence, and treated as described above. After an overnight incubation, the cells were rinsed three times with ice-cold phosphate-buffered saline, and then scraped from the dish in TLB lysis buffer (20 mM Tris (pH 7.5), 0.14 M NaCl, 2 mM EDTA, 1% Triton X-100, 25 μm β-glycerol phosphate, and 10% glycerol). The crude lysates were centrifuged at $3000\times g$ for 10 min at 4°C and the nuclear pellet was recovered. The supernatant from this first spin was next centrifuged at $100\,000\times g$ for 1 h at 4°C and the cell membrane pellet was recovered. The remaining supernatant after the high-speed spin contained the cytosolic fraction. The fractionated pellets were resuspended in TLB and protein concentration was determined using the Micro BCA Protein Assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Equal quantities of protein from the nuclear pellet, the membrane pellet and the soluble fraction were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted for ERα using either the M2 antibody (1:1000 dilution) or AER 320 (1:200 dilution). Prior to initiation of these studies, extensive pilot studies were undertaken to exclude the possibility that the cell membrane fraction was contaminated by nuclear proteins. Thus, the fractionated lysates were immunoblotted for nuclear proteins with an anti-Ku86 antigen antibody (1:500 dilution, Sigma). The presence of cell membrane proteins in the membrane fraction was confirmed by immunoblotting for the EGF receptor (1:500 dilution, Upstate Biotechnology, Lake Placid, NY, USA). To ensure that the determination of total protein concentration resulted in equal loading of fractionated proteins on the gel, each membrane was probed with these fraction-specific antibodies and analyzed by densitometry. This then allowed for at least semi-quantitative determination of the relative abundance of ER in each fraction.

2.7. Statistical analysis

All data are presented as mean ± S.E.M. Two-way comparisons were made with the Student's *t*-test; multiple group comparison were made by one-way analysis of variance followed by a postdoctoral Student–Neuman–Keuls test. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Cell surface ERs in human VSMC

Quiescent Rad91 cells, transfected with pCMV3ERα and maintained in SFM were immunostained for ERα. A subpopulation of cells exhibited significant extra-nuclear staining

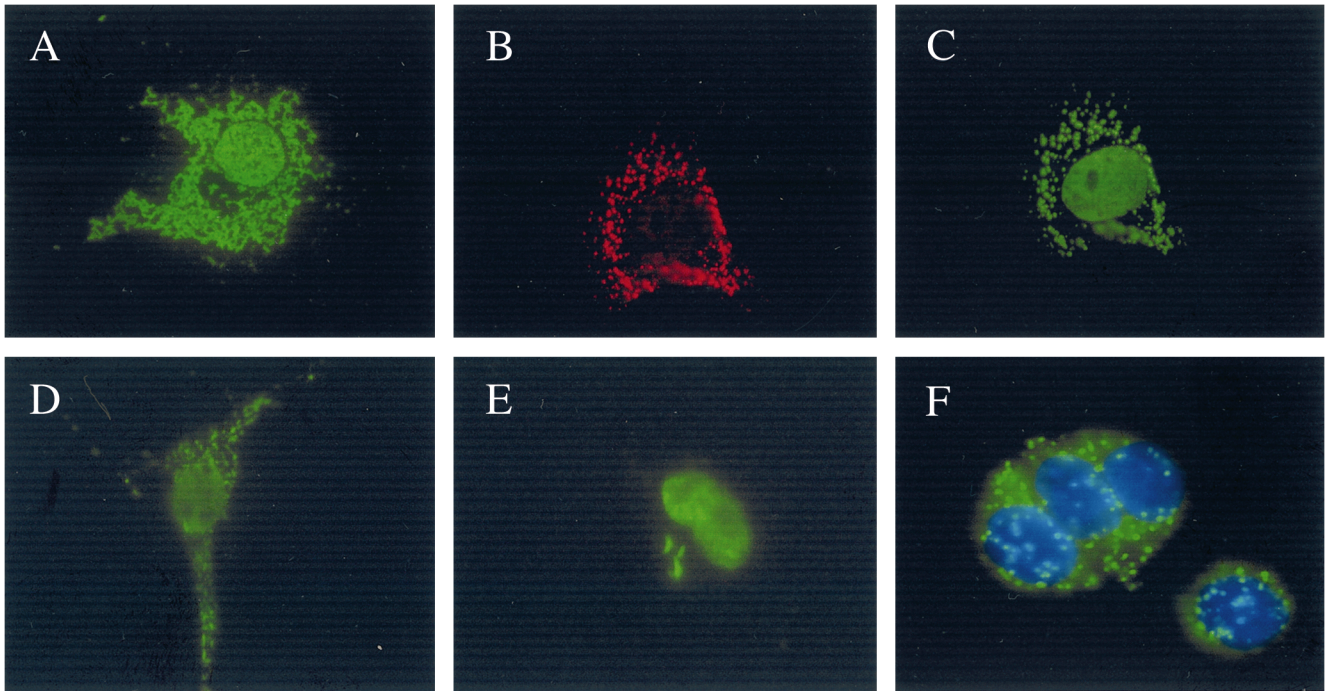

Fig. 1. Immunocytochemical detection of cell surface ERs. Quiescent, human radial artery VSMC (Rad91 cells) were transfected with either GFP-ERα or ERα and ER localization was visualized by fluorescent microscopy. A: GFP-ERα is distributed in both nuclear as well as extra-nuclear compartments. B: Immunostaining of non-permeabilized Rad91 cells for ERα demonstrates only extra-nuclear staining (red). C: Visualization of the ER by the GFP signal (green) demonstrates the presence of ER in the nucleus of the same cell as shown in B which was not accessible to the anti-ER antibody. D: Extra-nuclear ER immunostaining is also detected in cells in the absence of pre-treatment with trypsin. E: Following pre-treatment with trypsin, extra-nuclear ER is no longer detectable. F: Endogenously expressed extracellular ER is also detected by immunostaining in non-permeabilized GH3B6 cells (DAPI-stained nucleus shown in blue).

(Fig. 1A). The specificity of the immunodetection of ERα was supported by control experiments demonstrating lack of staining in cells transfected only with an empty vector plasmid, and also by abolishment of the immunostaining by pre-absorption of the antibody with GST-ERα, but not with GST-ERβ fusion proteins (data not shown). To facilitate examination of the intracellular distribution of the ER, a GFP-ERα expression plasmid was constructed and a series of studies were undertaken to confirm that the subcellular distribution of the chimeric GFP-ERα was identical to that of the wild-type ER. For these studies, the GFP-ERα protein was visualized by detecting the GFP signal and by staining with each of two different ERα-specific antibodies (AER 304 and AER 320 which recognize the N-terminal and C-terminal domains of ERα respectively). Preliminary studies established that the subcellular distribution of GFP-ERα did not differ from that of the wild-type protein (data not shown). Furthermore, the transcriptional competence of the GFP-ERα construct was confirmed using transient transfection assays with an estrogen response element reporter plasmid as described [10,36] (data not shown). To determine whether the extra-nuclear ER observed above represented cell surface ER, two approaches were taken. First, unstimulated Rad91 cells transfected with GFP-ERα were permeabilized with Triton, or left with their membranes intact, followed by immunostaining with AER 320. In unpermeabilized cells, only extra-nuclear ER was detected (Fig. 1B). In these same cells, however, the presence of nuclear ERα was confirmed by the detection of GFP in the nucleus (Fig. 1C). Lack of immunodetection of nuclear ER in the unpermeabilized cells supports the hypothesis that the cell membrane-associated ER is exposed to the extracellular space since the antibody was not able to enter the cell's interior. As a second step to investigate the presence of cell surface ER, unstimulated Rad91 cells were immunostained with or without gentle pre-treatment with trypsin to cleave off extracellular epitopes. In the absence of trypsin both extra-nuclear and nuclear ERα was detected (Fig. 1D), whereas only nuclear ERα was detected following trypsin treatment (Fig. 1E). As shown in Fig. 1F immunostaining of GH3B6 cells using ERα-specific antibody confirmed the presence of extra-nuclear ERα, as has previously been reported [32–35]

The presence of cell membrane ER was next examined by Western blotting of subcellular fractions produced by differential centrifugation. In untransfected cells, no ER was detected in the cell membrane fraction (Fig. 2A). In contrast, ER was readily detected in the cell membrane fraction of unstimulated Rad91 cells transfected with ER, and this was intensified by treatment with the ER antagonist ICI 182781 (Fig. 2A). Membrane ER was also detected by immunoblotting with two additional ERα-specific antibodies (Fig. 2B). The membrane fraction was confirmed as such by staining for EGF receptor protein (Fig. 2C), and lack of contamination of the membrane fraction with nuclear proteins was demonstrated by the absence of detection in the cell membrane fraction of the nuclear protein Ku86 (Fig. 2D). Taken together, these data demonstrate that a subpopulation of ERα in human VSMC is membrane-associated.

3.2. *Physiologic stimuli regulate the intracellular localization of the ER*

Quantification of the proportion of VSMC expressing cell surface ER, and the effects of various physiologic stimuli on

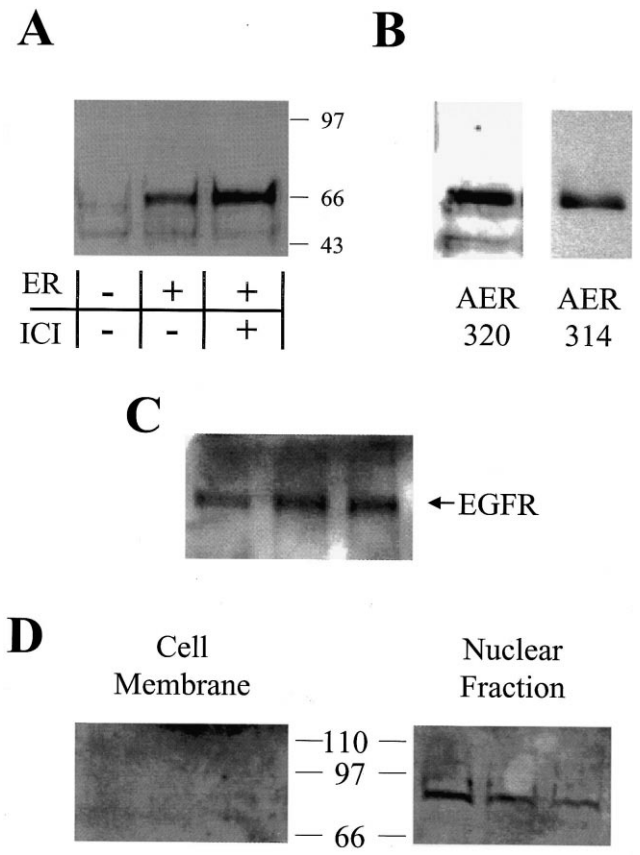

Fig. 2. Immunoblot detection of ERs in the cell membrane. Nuclear and cell membrane fractions were isolated from quiescent Rad91 cells and analyzed for the presence of ER by immunoblotting. A: ER was detected in the cell membranes of Rad91 cells transfected with ERα, but not control cells transfected with an empty vector. Furthermore, treatment with the anti-estrogen ICI 182780 (ICI) increased the abundance of membrane ER. B: ER was also detected in the cell membrane fraction using an antibody directed against the C-terminus (AER 320) and the N-terminus (AER 314) of the ER. C: EGF receptor was detected in the cell membrane fractions but not in the nuclear fractions (data not shown). D: Lack of contamination of the membrane fraction with nuclear proteins is demonstrated by the absence of the nuclear protein Ku86 in the membrane fractions, whereas it was readily detected in the nuclear fractions. One representative example from three independent experiments with similar results is shown.

the distribution of ER were examined next. Representative cells are shown in Fig. 3, and summary data are shown in Fig. 4. Quiescent Rad91 cells in SFM consisted of a mixed population of cells expressing varying degrees of both nuclear and extra-nuclear ER (Fig. 3A). E2 stimulation for 24 h resulted in nuclear localization of ERα in most cells, and E2-induced nuclear translocation was blocked by co-incubation with ICI (Fig. 3B,C). Ligand-independent activators of ERα, ED-FBS, S-FBS, or EGF also resulted in nuclear translocation of the ER, that was also blocked by ICI (Fig. 3D,F). The Rad91 cells used in the above experiments are a spontaneously immortalized smooth muscle cell line, and so next we sought to confirm these results in non-immortalized cells derived from human saphenous veins. Nuclear localization by estrogen-independent activators, and blockade of this by ICI, was observed in human saphenous vein-derived VSMC as it was in the Rad91 cells (Fig. 3G–I). Taken together, these experiments support that membrane-associated ER in human VSMC translocates to the nucleus following either hormone-dependent or hormone-independent stimulation and that this can be inhibited by ICI.

3.3. Distinct pathways mediate nuclear translocation of ERα following estrogen-independent and estrogen-dependent stimuli

As a first step toward elucidating the molecular mechanisms that mediate the nuclear translocation of ERα in response to ligand-independent stimulation, the intracellular distribution of a series of mutant ERs was examined. Rad91 cells, treated as described above, were transfected with wild-type ERα or the deletion mutant ER-ΔAB (which lacks the A/B domain consisting of amino acids 1–180, but contains the known E2-dependent nuclear localization signal at amino acids 256–263). Like WT ER, ER-ΔAB was predominantly extra-nuclear in SFM and localized to the nucleus following E2 treatment. In contrast, however, ER-ΔAB did not localize to the nucleus following stimulation with ED-FBS, EGF, or S-FBS (Fig. 5A). Additional mutational analyses were then conducted focusing on residues within the A/B domain previously identified as potential phosphorylation sites. As shown in Fig. 5B, the single base pair mutant in which alanine is substituted for serine at amino acid 118 also localized to the nucleus in response to E2, but not with ED-FBS or EGF stimulation.

3.4. Estrogen-independent stimuli cause nuclear localization of ERα by a MAP kinase-dependent pathway

Based on previous studies in non-vascular cells demonstrating MAP kinase-mediated ligand-independent activation of ERα via phosphorylation of serine 118 in the A/B domain of the ER, we next sought to determine whether MAP kinase mediates ED-FBS-induced nuclear translocation of the ER in VSMC. Pharmacologic inhibition of MAP kinase with the MEK1 inhibitor PD98059 completely abolished ED-FBS-induced nuclear translocation of ERα (Fig. 6A). PD98059 also blocked EGF-induced nuclear translocation (Fig. 6A). In contrast, PD98059 had no effect on E2-induced nuclear translocation (Fig. 6A). Inhibition of MAP kinase activation by overexpression of a dominant negative MAP kinase kinase (DN-MAPKK) also inhibited ED-FBS-induced and EGF-induced ER translocation, but had no effect on E2-induced translocation (Fig. 6A). Consistent with the above findings, co-transfection of a constitutively activated MAPKK plasmid enhanced nuclear localization of wild-type ERα even in quiescent cells (Fig. 6A). Overexpression of the activated MAPKK was unable to cause nuclear localization of the ER-S118A mutant (data not shown).

Based on the findings presented above, we hypothesized that substitution of serine 118 of ERα with a charged amino acid such as glutamate would mimic the effects of MAP kinase-mediated phosphorylation of the ER and result in a mutant ER that would translocate to the nucleus even in the absence of growth factor stimulation. As hypothesized, ER-S118E constitutively localized to the nucleus even in SFM (Fig. 6B). Taken together, these data support a role for MAP kinase in mediating mitogen-, but not E2-induced ER nuclear localization.

4. Discussion

ERs mediate both the long-term genomic effects, and the

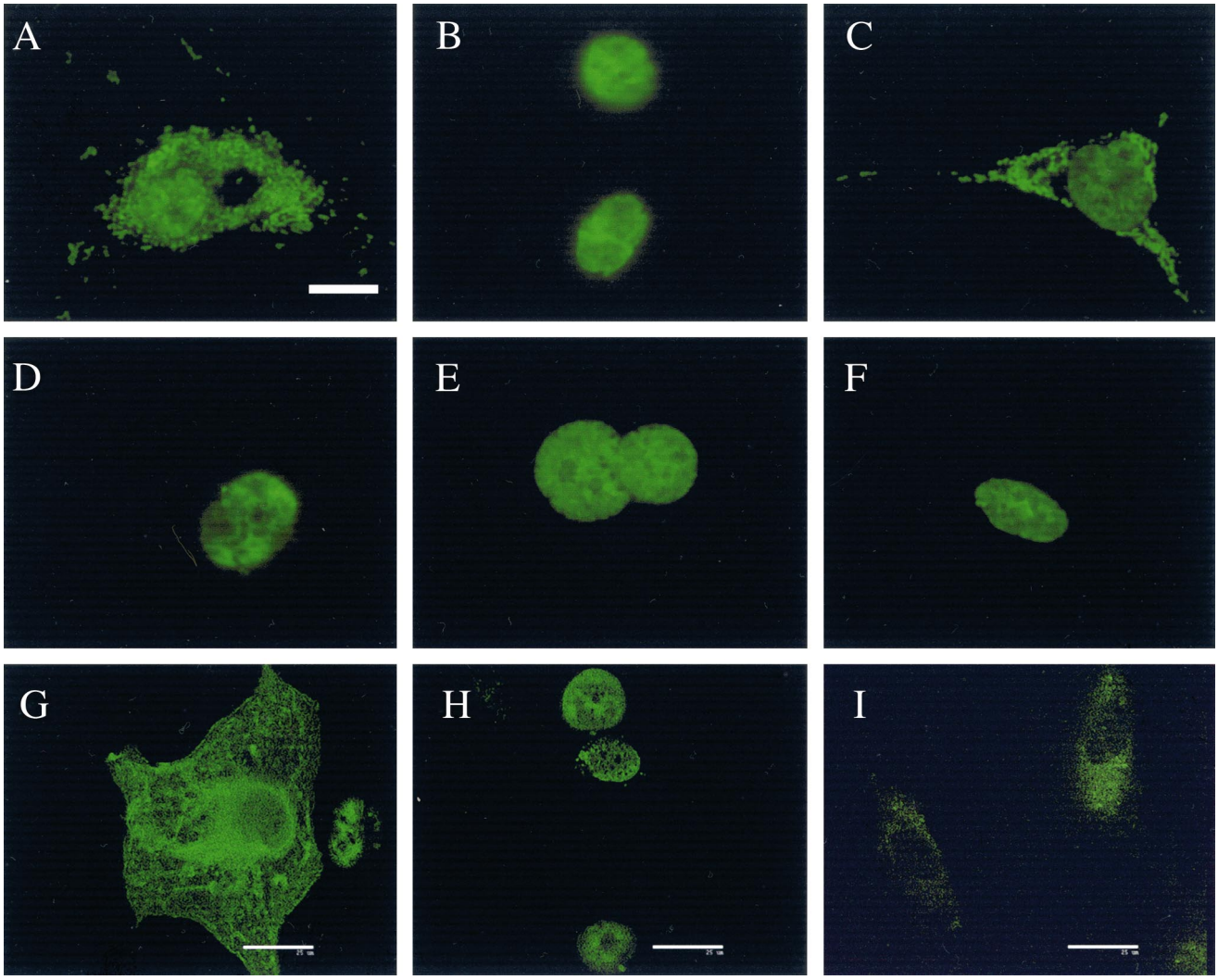

Fig. 3. Physiologic stimuli regulate the intracellular distribution of ERα in human VSMC. Rad91 cells and human saphenous vein derived VSMC (HSVSMC) were transfected with GFP-ERα and visualized either in the quiescent state or after overnight stimulation with various agents. A: Quiescent Rad91 cells express extensive extra-nuclear ER. B: Nuclear localization of the ER was induced following estrogen stimulation. C: Estrogen-induced nucleus localization was inhibited by co-incubation with ICI 182780. D–F: Treatment of Rad91 cells with ED-FBS (D) EGF (E), or S-FBS (F) also induced nuclear localization of the ER. G–I: Similarly, in HSVSMC the ER re-distributed from an extra-nuclear pattern in quiescent cells (G) to a nuclear pattern following stimulation with ED-FBS (H) and the nuclear translocation was blocked by ICI 182780 (I).

short-term non-genomic effects of estrogen. Though ERs have classically been thought of as being confined to the nucleus, previous reports support the existence of membrane-associated ERs. More recent work confirms the existence of membrane-associated ERs, and further suggests that this population of ER mediates the non-genomic effects of estrogen [23,32]. These data demonstrate that cells contain at least two subpopulations of ERα, nuclear and membrane-associated, that likely regulate different cellular processes. To date, however, little is known regarding the molecular mechanisms that regulate the distribution of the ER between nuclear and membrane fractions. Furthermore, though rapid effects of estrogen have also been reported in VSMC, there are few data examining whether these cells also express membrane-associated ERs. Therefore, in the present study we sought (1) to determine whether human VSMC can express membrane ER; and (2) to examine the molecular mechanisms that regulate the intracellular localization of ER in these cells.

The data presented above demonstrate in quiescent human VSMC, transient transfection of the full-length, wild-type ERα results in expression of a subpopulation of ER that is membrane-associated. This conclusion is supported by immunocytochemical detection of ERα using a variety of antibodies, and by studies using fluorescent-tagged ERα. This conclusion is supported further by immunoblot analysis of membrane fractions demonstrating ER in the membrane fraction and lack of contamination of the membrane fraction with other nuclear proteins. Additional studies using non-permeabilized cells, and cells treated with gentle trypsinization suggest that at least a portion of the membrane ER is exposed to the exterior of the cell. Given the lack of a putative transmembrane domain in the ER, the mechanism by which ERα localizes to the extracellular space is unclear, and additional studies are needed to explore this question further.

The data presented above also demonstrate stimulus-specific regulation of the intracellular localization of the ER. Estrogen-dependent and estrogen-independent stimuli capable of transcriptional activation of ERα both result in nuclear local-

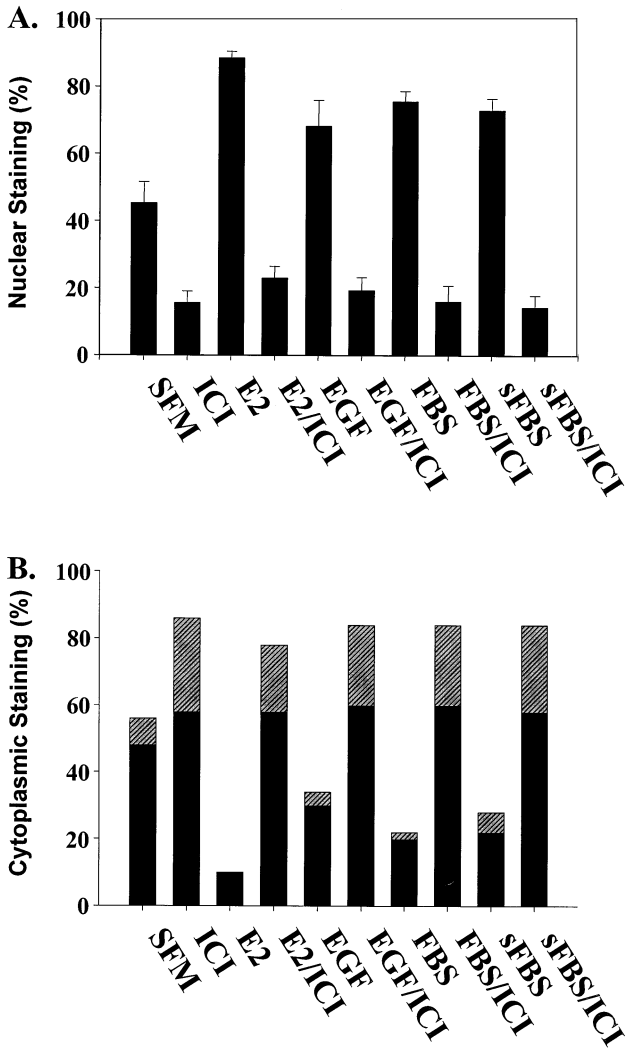

Fig. 4. Summary data demonstrating re-distribution of the ER in human VSMC in response to physiologic stimuli. A: Cells with exclusively nuclear staining are shown. B: The distribution of cytoplasmic staining is shown. Bars represent means ± S.E.M.; $n = 6$ independent experiments. $P < 0.01$ for all treatments vs. SFM.

ization of the receptor, but they do so by different molecular mechanisms. Mutational analyses demonstrate that a deletion mutant of the ER lacking the N-terminal A/B domain remains responsive to estrogen, but not to estrogen-independent stimuli. More detailed mutational analyses suggested the hypothesis that mitogen-induced activation of MAP kinase mediates hormone-independent nuclear localization of the ER, as ER mutants lacking the MAP kinase phosphorylation consensus site no longer become nuclear-localized following ED-FBS or EGF stimulation. These mutants did however remain estrogen responsive, underscoring the distinct molecular pathways that mediate estrogen-dependent and estrogen-independent effects. A role for MAP kinase in regulating ER nuclear localization is supported further by experiments demonstrating that inhibition of MAP kinase activation, either by pharmacologic or genetic means, inhibits mitogen-induced ER nuclear localization, and that constitutive activation of MAP kinase leads to nuclear localization of the ER even in quiescent cells. The mechanism by which ICI blocks nuclear translocation of the ER in response to hormone-independent stimulation is unclear. It is compatible with our previous demonstration that ligand-independent ER activation is blocked by ICI, but not by tamoxifen, presumably because ICI blocks activation of both AF-1 and AF-2, whereas the effects of tamoxifen are limited primarily to AF-2 alone. The role of signaling pathways such as receptor tyrosine kinase-mediated cascades in regulating the intracellular localization of ERα is unknown, but worthy of additional investigation.

Several limitations of the current study deserve mention. First, the majority of these studies were conducted in transiently transfected cells expressing high levels of ERα. Thus, it remains to be seen whether endogenously expressed ER in VSMC also localizes to the cell membrane, and if so, whether similar molecular mechanisms regulate its intracellular localization. We undertook a series of preliminary experiments to address these issues in untransfected VSMC but were unable to convincingly demonstrate membrane ER. This likely results from the very low abundance of total ERα in these cells at baseline [36,39], making detection of a membrane fraction technically difficult. Endogenously expressed membrane ERα

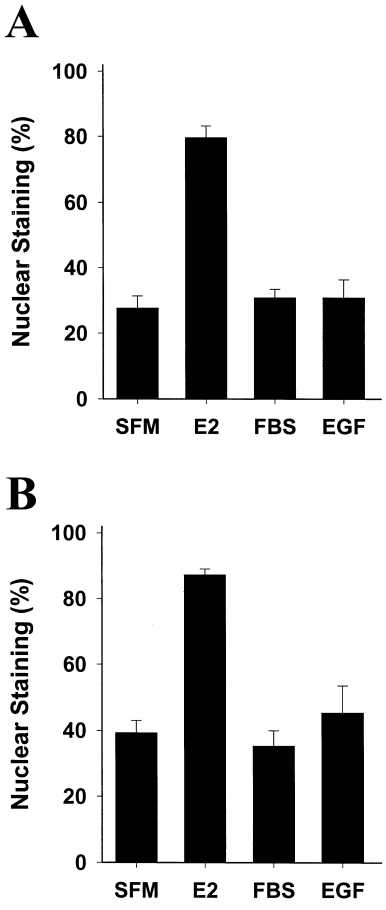

Fig. 5. ER mutants demonstrate that distinct molecular pathways mediate E2-dependent and E2-independent nuclear localization of the ER in human VSMC. A: ER-ΔAB, an N-terminal truncation that lacks the AF-1 transcription activation domain, transfected into Rad91 cells translocates to the nucleus in response to estrogen (E2) treatment, but not in response to stimulation with either ED-FBS or EGF. B: Similarly, ER-S118A, an alanine for serine substitution at amino acid 118, transfected into Rad91 cells also translocates to the nucleus in response to estrogen (E2) treatment, but not in response to stimulation with either ED-FBS or EGF. Bars represent means ± S.E.M.; $n = 6$ independent experiments.

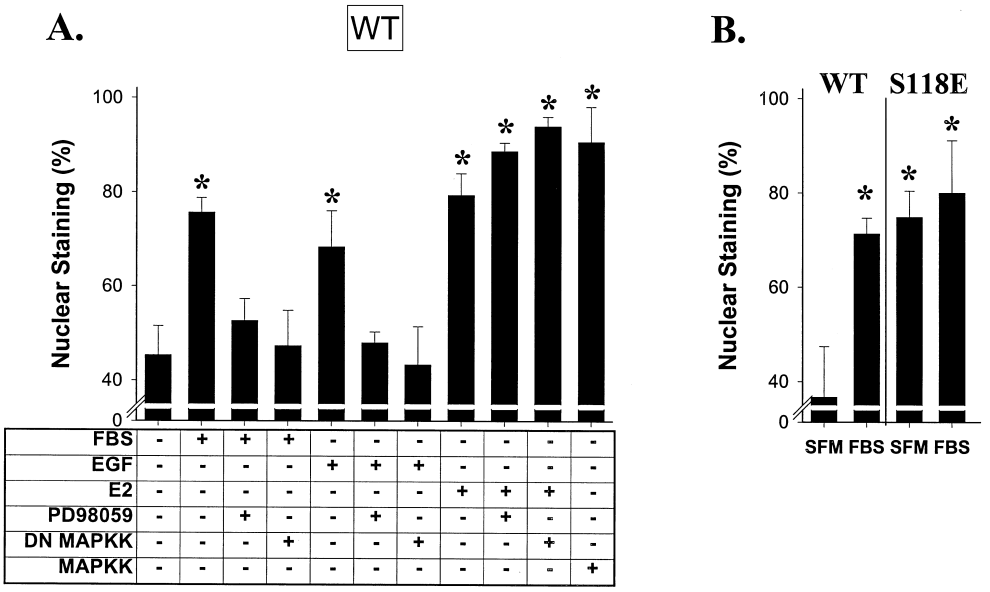

Fig. 6. MAP kinase mediates estrogen-independent, but not estrogen-dependent nuclear localization of the ER. A: Pharmacologic inhibition of MAP kinase activation with PD98059, or inhibition of MAP kinase activation by overexpression of a DN-MAPKK, blocked FBS- and EGF-induced nuclear translocation of the ER. In contrast, inhibition of MAP kinase activation by either of these methods had no significant effect on E2-induced nuclear translocation of the wild-type ER. Overexpression of a constitutively active MAPKK resulted in nuclear localization of the wild-type ER in SFM. B: ER-S118E, which has a glutamate for serine substitution at amino acid 118 (a known site for MAP kinase phosphorylation), transfected into Rad91 cells is nuclear-localized in quiescent cells, even in the absence of stimulation. Bars represent means ± S.E.M.; $n = 6$ independent experiments.

was demonstrated in GH3B6 cells, however, confirming previous observations of endogenous membrane-associated ER from other laboratories. In addition, all of our studies were conducted in cultured vascular cells, and thus the presence of membrane ER and the mechanisms that regulate ER localization in cells in the intact vessel remain to be examined. Finally, the current investigation did not include examination of ERβ though ERβ also likely regulates important physiologic processes within vascular cells.

In summary, the data presented above demonstrate that in immortalized human VSMC: (1) ERα localizes to the cell membrane in quiescent, unstimulated cells; (2) both estrogen-dependent and estrogen-independent activators induce nuclear localization of ERα; (3) distinct molecular mechanisms mediate estrogen-dependent and estrogen-independent ER nuclear localization; (4) MAP kinase mediates hormone-independent, but not hormone-dependent nuclear localization of ERα.

Acknowledgements: The authors thank Sharon Lynch and Patti Griffiths for expert preparation of the manuscript. We also thank Michael Mendelsohn for numerous helpful discussions. This work was supported in part by NIH RO1 HL61298 (R.H.K.). J.M. was supported by a grant for the Deutsche Forschungsgemeinschaft.

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