Analysis of estrogen receptor α signaling complex at the plasma membrane

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Abstract There is accumulating evidence that the estrogen receptor (ER) can transduce specific signals at the plasma membrane. We tried to clarify the biological function of ER as a signaling molecule by identifying proteins that interact with the membrane-localized ER. The activation function 1 and 2 (AF-1 and AF-2) domains of ERα with or without the membrane-targeting sequence were stably expressed in the breast cancer cell line, MCF-7. The level of tyrosine phosphorylation of AF-2 was significantly elevated by the membrane localization. By mass-spectrometry analysis, α- and β-tubulins and heat shock protein 70 were identified as the AF-1-associated proteins. Of these, tubulins are associated only with membrane-targeted AF-1.

Keywords: Estrogen receptor; Non-genomic action; Breast cancer; Tubulin; Heat shock protein 70; Tyrosine phosphorylation

1. Introduction

Estrogen plays an important role not only in physiological processes such as the development of the female organs, reproduction, bone metabolism, and vascular dilatation, but also in pathological processes such as the development of breast cancer. Human estrogen receptor α (ERα) is a member of the nuclear receptor superfamily and functions as a ligand-dependent transcription factor [1,2]. A typical nuclear receptor contains a variable N-terminal region called activation function 1 (AF-1), a conserved DNA binding domain (DBD), a hinge region, and a C-terminal ligand binding domain called activation function 2 (AF-2). Both the AF-1 and AF-2 domains of ERα are shown to have a transcriptional activation function [3], and interact with transcriptional mediators and co-factors [4–10]. More than 80% of ERα is shown to localize in the nucleus in the absence of estrogen, and more than 95% is shown to localize in the nucleus upon estrogen stimulation [11]. Thus, it has been believed that the action of estrogen is mediated by nuclear-localizing ER through the regulation of target gene transcription.

However, there are accumulating evidences that the ER can transduce specific signals through association with other molecules outside the nucleus. In vascular endothelial cells, estrogen rapidly induces nitric oxide production by activating the PI3-kinase-Akt pathway [12]. In this process, ERα is shown to interact with the p85 subunit of PI3-kinase in a ligand-dependent manner [13]. In osteoblasts, ERα is shown to mediate the anti-apoptotic effect by activating the Src/Shc/Erk (extracellular signal-related kinase) pathway, and only the AF-2 domain expressed in the cytoplasm is necessary for this function [14]. These phenomena, called non-genomic actions, are not explained by the classical genomic action of ERα because of their rapid time course and the localization of interacting molecules, and are recognized as novel functions of the ER.

Independent laboratories have reported the non-genomic action of ERα in the breast cancer-derived cell line, MCF-7 [15,16]. They all observed that estrogen rapidly induces the phosphorylation of Erk, although different modes of action of ERα are proposed. Migliaccio et al. [15] showed that the association of ERα with non-receptor tyrosine kinase c-Src is the upstream event of ERα activation and the association of two molecules is dependent on the phosphorylation of Tyr 527 in the AF-2 domain of ERα. On the other hand, Song et al. demonstrated the direct association of ERα and adaptor protein Shc. This association was shown to use the AF-1 domain of ERα and does not require the phosphorylation of Tyr 527 [16]. It seems that the paucity of endogenous ERα at the plasma membrane makes it difficult to analyze the role of ERα at the membrane. In this study, we tried to clarify the components of the signaling complex in MCF-7 cells by the targeted expression of the ERα domain fragments at the plasma membrane.

2. Materials and methods

2.1. Plasmids

A FLAG epitope-tagged ERα AF-1 domain construct was generated by amplifying the coding sequence of human ERα by PCR using primers 5′-CGTACCTCGAGATGACCATGACCCCTCCACAC-3′

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Abbreviations: AF-1 and AF-2 domains, activation function 1 and 2 domains; BSA, bovine serum albumin; DBD, DNA binding domain; DMEM, Dulbecco's Modified Eagle's medium; EGF, epidermal growth factor; ER, estrogen receptor; Erk, extracellular signal-related kinase; FBS, fetal bovine serum; Hsp70, heat shock protein 70; LC/MS/MS, liquid chromatography tandem mass spectrometry; WCL, whole cell lysate

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and 5′-CGGGATCCTATTTGTCATCGTCGTCCTTGTAGTCCT-TGGCAGATTCCATAGCC- 3′. This resulted in a fragment with an *Xho*I site (underlined), a sequence that encodes the FLAG epitope (DYKDDDDK), followed by a termination codon and a *Bam*HI site (underlined). This fragment was then digested with *Xho*I and *Bam*HI sequentially, and ligated into mammalian expression vector pcDNA3.1(−)/Myc-His B (Invitrogen).

A membrane-targeted AF-1 domain construct was generated by PCR-based site-directed mutagenesis using the AF-1 construct as a template and primers 5′-GGTAGCAACAAGAGCAAGCCCAAG-GATGCCAGCCAGCGGACCATGACCCTCCACACC- 3′ and 5′-CATCTCGAGTCTAGAGGGC. The PCR product was then digested by *Dpn*I that degrades only methylated template plasmids. As a result, the N-terminal sequence of Src kinase (MGSNKSKPKDASQ encoded by underlined 39 bp) was inserted between the *Xho*I site and the N-terminal of AF-1.

A FLAG epitope-tagged ERα AF-2 domain construct was generated using the same procedure as that of the AF-1 construct by using primers 5′-CGTACCTCGAGGCCACCATGGGCAAGAAGAACA-GCCTGGCCTT-3′ and 5′-CGGGATCCTATTTGTCATCGTCGT-CCTTGTAGTCGACTGTGGCAGGGA-3′ (*Xho*I site and *Bam*HI site are underlined). A membrane-targeted AF-2 construct was also generated by PCR-based site-directed mutagenesis using the AF-2 construct as a template and primers 5′-GGTAGCAACAA-GAGCAAGCCCAAGGATGCCAGCCAGCGGAAGAAGAACA-GCCTGGCCTT- 3′ and 5′-CATGGTGGCCTCGAGTCTAG-3′.

2.2. Cell culture and transfection

MCF-7 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS at 37 °C with 5% CO_2. When estrogen stimulation was necessary, MCF-7 cells were cultured in phenol red free DMEM with 5% Charcoal/Dextran Treated FBS (HyClone) for two days. Transfection was performed using FuGENE 6 (Roche) according to the manufacturer's instructions. Clones were selected using geneticin (Sigma) at a concentration of 800 μg/ml.

2.3. Antibodies and reagents

Anti-FLAG M2 monoclonal antibody and anti-α-tubulin antibody (B-5-1-2) were purchased from Sigma. Anti-β-tubulin monoclonal antibody (D-10) was from Santa Cruz. Anti-Hsp70 monoclonal antibody was purchased from Stressgen. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology. Anti-ERα antibody (H-184) was purchased from Santa Cruz. FITC-conjugated anti-mouse antibody was from Santa Cruz. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG were purchased from Molecular Probe. HRP-conjugated anti-mouse antibody was purchased from Amersham Pharmacia. Protein-G Sepharose was purchased from Amersham Pharmacia, β-estradiol was purchased from Sigma. Nocodazole was from Sigma.

2.4. Immunocytochemistry

Cells were grown on microscope slides in 24-well plates, washed three times with PBS, fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 4 min at room temperature, washed once with PBS, and permeabilized with 0.2% Triton X-100 in PBS. After another washing with PBS and blocking with 2% (bovine serum albumin, BSA)/TBST (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 30 min, the cells were incubated with appropriate first antibodies in 2% BSA/TBST for 1 h at room temperature. The cells were washed three times with PBS and incubated with FITC-conjugated anti-mouse antibody (1:40) in 2% BSA/TBST. After the cells were washed three times with PBS, microscope slides were mounted in 1.25% DABCO and 50% PBS and 50% glycerol and visualized using a Radiance 2100 confocal microscope (Bio-Rad). In Fig. 4B, 3% BSA, 5% goat serum in TBS were used instead of 2% BSA in TBST. Alexa Fluor 488 goat anti-mouse IgG (l:2000) and Alexa Fluor 594 goat anti-rabbit IgG (1:2000) in 3% BSA and 5% goat serum/TBS were used as the secondary antibodies in Fig. 4B.

2.5. Immunoprecipitation and immunoblotting

Cells were lysed in 1% Triton X-100 buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 1 mM EGTA, 100 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and the protein concentration was measured using BCA Protein Assay (Pierce).

For immunoprecipitation with anti-FLAG M2 antibody, cell lysate (2 mg/ml) was mixed with anti-FLAG affinity gel (Sigma) and rotated for 2–12 h at 4 °C. After the affinity gel was washed four times with 1% Triton X-100 buffer, FLAG-tagged proteins were eluted with 0.3 M glycin HCl at pH 3.5. The elutant was removed, neutralized with 2 M Tris–HC1 (pH 8.8), and then boiled in the sample buffer (0.1 M Tris–HCl, pH 6.8, 2% SDS, 0.1 M dithiothreitol, 10% glycerol, and 0.01% bromophenol blue) for 5 min and analyzed by SDS–PAGE. The gels were transferred onto a polyvinylidene difluoride membrane (Millipore) and probed by immunoblotting. Immnunoreactive proteins were visualized with a chemiluminescence reagent (Western Lighting, Perkin Elmer).

2.6. Silver staining and LC/MS/MS analysis

The SDS–PAGE gels were silver stained according to a method used previously [17].

For liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis, 12 ml of cell lysate containing 36 mg of total protein was mixed with 200 μl of anti-FLAG affinity gel and rotated for 8 h at 4 °C. The affinity gel was loaded onto a chromatography column (Bio-Rad), washed with 4 ml of 1% Triton X-100 buffer, and then eluted with 0.1 M glycin HCl at pH 3.5. The elutant was separated into several fractions in chronological order and then neutralized with 2 M Tris–HCl (pH 8.8). Aliquots of these fractions were subjected to SDS–PAGE analysis, stained with SYPRO Ruby protein gel stain (Bio-Rad), and fractions with a high concentration of FLAG-tagged protein were selected for further analysis. The selected fractions were dialyzed using EasySep (TOMY) in diluted (10%) PBS and then semi-lyophilized using a SpeedVac concentrator. The concentrated samples were subjected to SDS–PAGE analysis and the gel was stained with CBB (Bio-Rad). The bands to be analyzed were dissected, digested in gel with trypsin, and subjected to LC/MS/MS analysis.

3. Results

3.1. Establishment of cell lines stably expressing a series of domain fragments of ERα in MCF-7

To analyze molecules associated with ERα at the plasma membrane, a series of domain constructs were generated and named as shown in Fig. 1A. The FLAG epitope was attached to the C-terminus of the separated AF-1 domain and the AF-2 domain of ERα. They were named AF1 and AF2, respectively. To generate membrane-targeted constructs, the membrane-localizing sequence of c-Src kinase was added to the N-terminus of AF1 and AF2. Src kinase is a non-receptor tyrosine kinase localizing at the plasma membrane with its myristoylated N-terminal sequence. The membrane-targeted ERα domain constructs were named mAF1 and mAF2, respectively. ERα has a DBD and a hinge region between the AF-1 and AF-2 domains. Nuclear localizing signals are shown to reside in the DBD and hinge region [11]; therefore, these regions were excluded from our domain constructs to ensure cytoplasmic localization.

Stable transfectants that express each domain of ERα were then established using the breast cancer cell line, MCF-7. The expression and localization of the domain fragments were confirmed by the anti-FLAG staining of cells. It was observed that AF1 and AF2 localized diffusely in the cytoplasm (Fig. 1B: b and d), whereas mAF1 and mAF2 localized at the periphery of the cells (Fig. 1B: c and e), showing the obvious effect of the membrane-targeting sequence.

3.2. Tyrosine phosphorylation of the AF-2 domain at the plasma membrane

We next investigated the relationship between the tyrosine phosphorylation of the AF-2 domain and membrane locali-

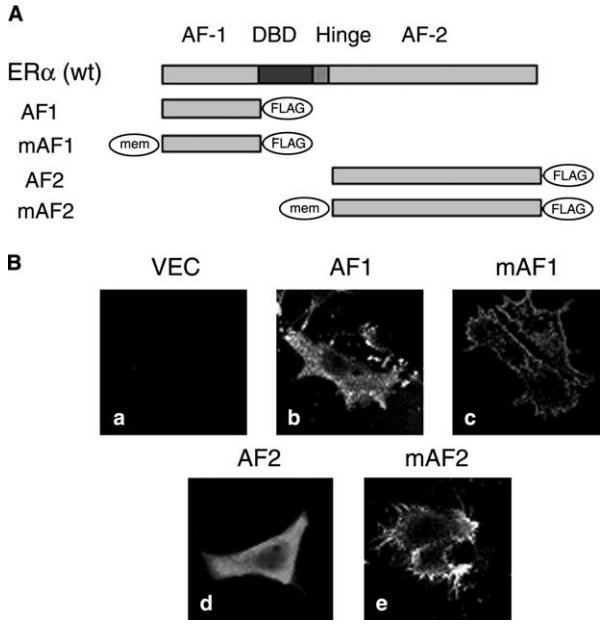

Fig. 1. Establishment of cell lines stably expressing a series of domain fragments of Erα in MCF-7. (A) Schematic representation of wild-type full-length ERα (wt), FLAG epitope-tagged AF-1 domain constructs with and without the membrane-targeting sequence (mem), and FLAG epitope-tagged AF-2 domain constructs with or without the membrane-targeting sequence. (B) Stable transfectants were immunostained with anti-FLAG antibody. (a) Empty vector; (b) AF1; (c) mAF1; (d) AF2; (e) mAF2. Cells were visualized with a confocal microscope at a magnification of 600×.

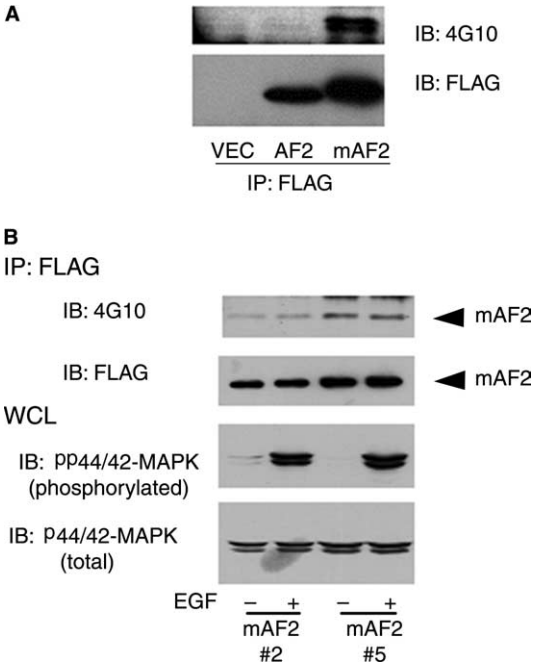

Fig. 2. Tyrosine phosphorylation of AF-2 domain at the plasma membrane. (A) Cell lysates of AF2 and mAF2 were immunoprecipitated with anti-FLAG antibody. Cells transfected with an empty vector (VEC) were used as a negative control. Each immunoprecipitate was subjected to immunoblotting analysis with anti-phosphotyrosine antibody 4G10 and anti-FLAG antibody. (B) Two independent clones of mAF2 (clones #2 and #5) were treated with either EGF (100 ng/ml) or distilled water for 5 min. mAF2 fragments were immunoprecipitated with anti-FLAG antibody and immunoblotted for 4G10 and anti-FLAG antibody. Whole cell lysates (WCLs) were immunoblotted with anti-phosphorylated p44/42 MAPK antibody and anti-p44/42 MAPK antibody.

zation using the stable transfectants we had established. The tyrosine phosphorylation of the mAF2 domain fragment was observed using phosphotyrosine specific antibody; however, almost no phosphorylation was detected in AF2 (Fig. 2A). This indicates that the AF-2 domain is phosphorylated exclusively at the plasma membrane.

To further characterize the tyrosine phosphorylation of the AF-2 domain, two independent clones of mAF2 cells were stimulated by EGF. In breast cancer cells, several tyrosine kinases, such as src family kinases [18,19] and Her-2 [20], have been reported to be transactivated upon EGF stimulation. However, no remarkable difference in the level of phosphorylation of mAF2 was observed upon EGF stimulation (Fig. 2B), suggesting that ERα is not the substrate of tyrosine kinases that is activated by EGF stimulation.

3.3. Screening of the membrane-specific binding partners of the AF-1 and AF-2 domains

In order to identify the interacting molecules with each domain of ERα, the lysates of the stable transfectants were purified with the anti-FLAG immunoaffinity column and analyzed by subsequent silver staining. Several candidates for mAF1-associated proteins were visualized by these procedures (Fig. 3). The bands around 200 kDa (arrowhead a), 50 kDa (arrowhead c) and 40 kDa (arrowhead d) were specifically seen in cells expressing mAF1, while the band around 70 kDa (arrowhead b) was observed in both cells expressing AF1 and cells expressing mAF1. None of these bands was co-purified with mAF2. These four bands were visible in the CBB gel stain from large-scale sample preparation. Two of these were successfully identified by LC/MS/MS analysis. The 50-kDa band that was specific to membrane-targeted AF1 was identified as β-tubulin (arrowhead c in Fig. 3). The 70-kDa band which was seen in both AF1 and mAF1 but not in mAF2 was identified as heat shock protein 70 (Hsp70) (arrowhead b in Fig. 3). Proteins specifically bound to mAF2 were not detected by this protocol (Fig. 3), although larger-scale purification was further attempted.

The association of mAF1 with β-tubulin was confirmed by immunoprecipitation followed by detection with β-tubulin antibody (Fig. 4A). This association was not detected in cells expressing AF1, indicating that this association is localized only at the plasma membrane. The absence of association between mAF2 and β-tubulin suggests that the association with β-tubulin was AF-1 domain-dependent and the membrane-targeting sequence did not serve as binding site for β-tubulin. A similar manner of association between mAF1 and α-tubulin was also detected (Fig. 4A), which is supported by the fact that β-tubulin forms heterodimers with α-tubulin.

To investigate physiological cooperation of tubulin and full-length endogenous ERα, immunocytostaining of MCF-7 cells was performed (Fig. 4B). Estrogen-dependent translocation of ERα to the plasma membrane was observed in 15 min on estrogen stimulation (Fig. 4B: h and k). At the same time, redistribution of α-tubulin was also observed at the plasma membrane (Fig. 4B: g and j). These two molecules showed co-localization in the superimposed image (Fig. 4B: i and l), which indicates possible association of these molecules in intact

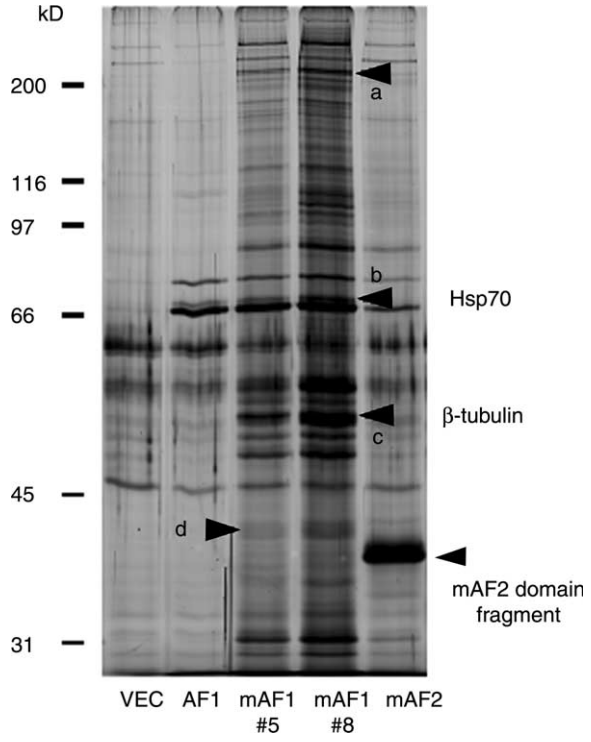

Fig. 3. Associated molecules to each domain fragment of ERα detected by silver staining. Each FLAG epitope-tagged domain fragment was immunoprecipitated by anti-FLAG antibody. One AF1 clone, two independent mAF1 clones (clones #5 and #8), and one mAF2 clone were analyzed. Immunoprecipitates were subjected to SDS–PAGE, and the gel was silver-stained. The two bands indicated by the arrowheads are Hsp70 and β-tubulin, which were identified later by LC/MS/MS analysis.

MCF-7 cells. These phenomena were not observed in the cells treated only by ethanol (Fig. 4B: d–f).

To further characterize the association of mAF1 and tubulins, cells expressing AF1 or mAF1 were treated with nocodazole, an inhibitor of tubulin polymerization. As a result, nocodazole treatment significantly blocked the association of mAF1 and α-tubulin (Fig. 4C). This indicates that mAF1 has higher affinity with polymerized microtubule filaments than with the depolymerized heterodimeric tubulin subunit.

Hsp70 was immunoprecipitated with both the AF1 and mAF1 domain fragments, but not with mAF2 (Fig. 4D). This suggests that the AF-1 domain is associated with Hsp70 outside the nucleus, but this association is not a membrane-specific event, in contrast with the case of tubulin.

4. Discussion

This study identifies polymerized tubulins as specific binding partners of the AF-1 domain of ERα at the plasma membrane using a breast cancer cell line, MCF-7. Hsp70 was also found to associate with the AF-1 domain although this association is not restricted at the plasma membrane. It was demonstrated that tyrosine phosphorylation of the AF-2 domain occurred within the plasma membrane, while the membrane-localized AF-2 domain failed to characterize any significant binding partners.

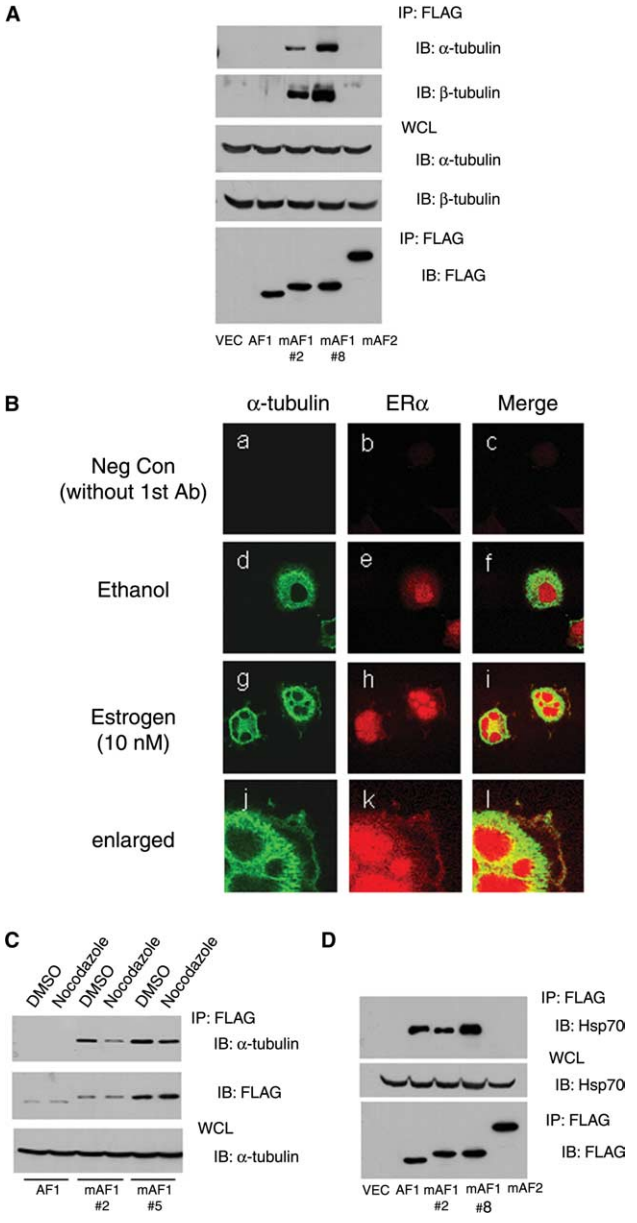

Fig. 4. Association of tubulins and Hsp70 with the AF-1 domain of ERα. (A) Cell lysates were immunoprecipitated and immunoblotted with anti-FLAG antibody, or anti-α- or β-tubulin antibodies as indicated. One AF1 clone, two independent mAF1 clones (clones #2 and #8), and one mAF2 clone were analyzed. (B) MCF-7 cells were treated with estrogen (10 nM) or ethanol (same concentration used in dilution of estrogen) for 15 min, then they were immunostained with anti-α-tubulin antibody (a, d, g, and j: green), and anti-ERα antibody (b, e, h, and k: red). Cells were visualized with a confocal microscope at a magnification of 600×. Superimposed confocal images (c, f, i, and l: merge) are also shown. Images without first antibodies (a–c) are shown as negative controls. Membranous area of estrogen stimulated MCF-7 cells was shown in magnified views (j–l: enlarged). (C) One AF1 clone and two independent mAF1 clones (clones #2 and #5) were treated with Nocodazole (33-μmol solution in DMSO) or DMSO (negative control) for 1 h. Cell lysates were immunoprecipitated and immunoblotted as indicated. (D) Cell lysates were immunoprecipitated and immunoblotted with anti-FLAG or anti-Hsp70 antibodies as indicated. One AF1 clone, two independent mAF1 clones (clones #2 and #8), and one mAF2 clone were analyzed.

It has been suggested by several independent laboratories that a subpopulation of ERα is associated with the plasma membrane and is responsible for the rapid effects of estrogen [15,16]. However, it appears that the subpopulation of membrane-associated ERα is considerably small, while the majority of ERα translocates into the nucleus upon estrogen stimulation. Therefore, we targeted the AF-1 and AF-2 domains of ERα to the plasma membrane to overcome the small amount of endogenous receptors at the plasma membrane.

The association of the AF-1 domain with polymerized tubulins or microtubules was specifically detected with the membrane-localized type of AF1, despite the wide distribution of microtubules throughout the cytoplasm. This result led us to conjecture that the association of ERα and microtubules is mediated by other molecules that reside at the membrane. Some signaling molecules are reported to associate with microtubules at the plasma membrane. p190RhoGEF (guanine nucleotide exchange factor) has a PH (pleckstrin homology) domain and localizes at the plasma membrane. This RhoA-activating molecule, which affects transcription and actin reorganization, has also been shown to interact directly with microtubules [21]. A small GTPase K-Ras, which can transduce signals to Erk, is another molecule shown to associate with microtubules at the plasma membrane [22]. It is possible that ERα forms a complex with K-Ras, microtubules and other unidentified molecules and affects the Ras signaling pathway. This may explain the rapid elevation of phosphorylated Erk on estrogen stimulation. The anti-tumor drug, paclitaxel, which is often used in the treatment. of breast cancer, has been shown to associate with polymerized microtubules and affect Ras-dependent signaling events [23]. This suggests that paclitaxel might also function by dissociating the signaling complex that involves ERα, K-Ras, and microtubules.

Association between the AF-1 domain and microtubules also tells us that microtubules are not used as tracks when ERα is transported to the plasma membrane, because the AF-1 domain needs to associate with microtubules at the cytoplasm before translocation if microtubules are used as tracks. Although membrane translocation is an important step for the non-genomic action of ERα, the precise mechanism is still elusive.

Ligand-dependent redistribution of both tubulins and ERα toward plasma membrane strongly suggests physical association between these molecules. To show the interaction of full-length ERα and tubulins, immunoprecipitation of tubulins or ERα was attempted several times using MCF-7 and Cos-7 cells. However, no association between tubulins and endogenous ERα was observed (data not shown). Immunocytostaining revealed that most of the full-length ERα was expressed in the nucleus and this situation was not improved even when membrane localizing sequence was attached to full-length ERα. Therefore, we concluded that our antibodies are not sensitive enough to detect the small population of membrane-localizing full-length ERα by immunoprecipitation.

It remains to be elucidated whether translocation of the small part of tubulins is a consequence of ERα translocation or an independent event. Nevertheless, this observation may indicate biological cooperation between ERα and tubulins.

It is also possible that the association of ERα and tubulins might contribute to the stabilization of microtubules, since mAF1 has higher affinity with polymerized tubulin and tubulin was well visualized at the plasma membrane when ERα was co-localized.

Hsp70 was shown to be another molecule that associates with the AF-1 domain expressed outside the nucleus. This association was not restricted at the membrane. The physiological role of the association between the AF-1 domain and Hsp70 is unclear. One possibility is that this association is involved in the degradation of cytoplasmic ERα. The glucocorticoid receptor, another member of the nuclear receptor superfamily, is shown to associate with Hsp90. CHIP, a U-box protein that has ubiquitin ligase activity, was shown to interact directly with Hsp90 and promote the degradation of glucocorticoid receptor [24]. CHIP was originally found as a Hsp70 interacting protein, and is also shown to localize outside the nucleus and to promote the degradation of Hsp70 bound protein [25]. Therefore, it is conceivable that in the case of ERα, Hsp70 mediates the degradation of cytoplasmic population of ERα. The degradation of ERα inside the nucleus was shown to be regulated differently depending on whether ERα is liganded or unliganded, and this mode of degradation generates a cyclic rhythm in the recruitment of ERα on estrogen-responsive promoters [26]. Hsp70 may be involved in the different regulation of ERα turnover outside the nucleus, which reflects the different functions of ERα outside the nucleus.

We also showed that tyrosine phosphorylation of the AF-2 domain occurs at the plasma membrane. It was previously reported that the AF-2 domain of ERα is phosphorylated on estrogen stimulation [15]. However, it was not clear whether the AF-2 domain is phosphorylated before or after membrane translocation. Our results favor the latter scenario. As the AF-2 domain without the membrane-targeted signal was not phosphorylated, the tyrosine kinases responsible for AF-2 phosphorylation localize strictly in the membranous area. Therefore, we assume that AF-2 phosphorylation does not regulate membrane translocation itself and that estrogen stimulation elicits the membrane translocation of ERα through interaction independent of phosphotyrosine.

So, what is the role of tyrosine phosphorylation of the AF-2 domain at the plasma membrane? Does phosphotyrosine mediate specific signals when the receptor translocates to the plasma membrane? Despite several attempts at purification, we have not yet identified the AF2-associated proteins. One of the factors that prevented the identification of the associated proteins was that the tyrosine-phosphorylated population of the AF-2 domain fragment was extremely small. The phosphorylation of the membrane-localized AF-2 domain was hardly detected by transient transfectants (data not shown). We managed to detect tyrosine phosphorylation of the membrane-localized AF-2 domain using stable transfectants. As long as the phosphorylated population of the membrane-localized AF-2 domain is small, only a small amount of molecules is supposed to bind to the AF-2 domain in a phosphotyrosine-dependent manner. This may be the reason that the previously reported association of membrane-localized AF-2 and c-Src [15] was not detected using our method (data not shown). Another possible factor that prevented the identification of the associated proteins is that AF-2 domain-bound proteins require the other domains of ERα to stabilize their association. In this case, longer ERα constructs would be needed to purify AF-2-associated proteins.

However, this is technically difficult because strong nuclear localization signals in the DBD and hinge region prevent the membrane localization of longer constructs, even though the membrane-targeting signal was attached to them (data not shown).

Another limitation of this purification method was that only proteins existing abundantly in the cytoplasm, such as tubulins and Hsp70, were identified as AF-1 domain-associated proteins. It is possible that signaling molecules, which are not expressed abundantly enough to be detected using our method, interact with the AF-1 or AF-2 domain at the plasma membrane and play an important role. Further improvement in our purification and identification method is necessary to characterize these molecules.

In conclusion, we have shown that the AF-1 domain of ERα interacts with microtubules at the plasma membrane, and Hsp70 outside the nucleus. We also demonstrated that the AF-2 domain of ERα is phosphorylated at the plasma membrane. We hypothesize that ERα forms a complex with microtubule-associated signaling molecules and phosphotyrosine-dependent AF-2-associated molecules. However, the whole view of the ERα complex is still unclear. To understand the pathological function of ERα in breast cancers, further investigation is required. This will lead to further improvement in breast cancer therapy and also bring about a deeper understanding of the physiological processes in which nuclear receptors are involved.

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