Introduction

Steroid receptors, in addition to regulating the transcription of specific genes (Mangelsdorf et al., 1995), trigger rapid effects in the extra nuclear compartment (for review see Migliaccio et al., 2007a). It is expected that hormone action depends on the integration of these different receptor activities (Vincent et al., 2006).

Localization and action of estradiol receptor α (ERα) are regulated by multiple factors, including interaction with signaling effectors or other proteins, such as the metastatic tumor antigen or the modulator of nongenomic action of steroid receptors (MNAR; Kumar et al., 2002; Vadlamudi et al., 2005; Gururaj et al., 2006). Deregulation of these processes causes ERα mislocalization and may trigger tumor progression. Overexpression of the EGF receptor is a hallmark of aggressive human breast cancers (Slamon et al., 1989), and cross talk between ERα and growth factor signaling has emerged as a critical factor in endocrine resistance through the control of the subcellular localization of ERα signaling components (Saporita et al., 2003; Gururaj et al., 2006). ER association with MNAR may sequester ER in the cytoplasm/membrane (Vadlamudi et al., 2005), thus enhancing the nongenomic effects of ERα and resulting in tumorigenesis, as well as the anti-hormone resistance of breast cancer cells (Vadlamudi et al., 2005; Gururaj et al., 2006). Expression of a shortened form of the metastatic tumor antigens sequesters ERα in the cytoplasm and leads to malignant phenotypes by enhancing ERα cytoplasmic functions in hormone-dependent breast cancer cells (Kumar et al., 2002). These data imply that cytoplasmic localization of ERα provides a mechanism to control signal transduction–dependent functions, such as DNA synthesis and anchorage-dependent growth of target cells. They suggest that the ERα localization has functional implications in breast cancer progression.

Steroid receptors undergo nucleocytoplasmic shuttling (for review see DeFranco, 2001). In the presence of a ligand, glucocorticoid receptors (GR), androgen receptors (AR), thyroid hormone receptors (TR), and progesterone receptors (PgR) rapidly shuttle between the nuclei and cytoplasm (DeFranco, 1995).
stances, LMB treatment does indeed inhibit the nuclear export of a NES on this basis alone (Nigg, 1997). Under some circumstances that the ER is associated to FKHR export, the estradiol-induced CRM1-directed NES in the ER rhabdomyosarcoma (FKHR) nuclear export, the estradiol-induced hormone-dependent and (PI3K) – AKT pathway and depends on CRM1. By combining different approaches, we identified a hormone-dependent and CRM1-dependent nuclear export (Kudo et al., 1998). Conflicting data have been reported on inhibition of steroid receptor export by LMB treatment. Although it is generally accepted that steroid receptors lack classical leucine-rich NESs, they do have sequences with limited homology to NESs (Liu and De Franco, 2000). Because the exact spacing of the leucine/hydrophobic residues in the NES of each protein is subject to variation, it can be difficult to define a NES on this basis alone (Nigg, 1997). Under some circumstances, LMB treatment does indeed inhibit the nuclear export of steroid receptors (Savery et al., 1999; Prüfer and Barsony, 2002; Maruvada et al., 2003; Saporita et al., 2003).

Here, we find that in MCF-7 cells, ERα is a nucleocytoplasmic shuttling protein whose traffic out of nuclei is regulated by estradiol activation of the phosphatidylinositol-3-kinase (PI3K)–AKT pathway and depends on CRM1. By combining different approaches, we identified a hormone-dependent and CRM1-directed NES in the ERα hormone-binding domain. The potential interest of these findings is highlighted by the observation that the ERα NES shows significant homology with sequences of other steroid receptors. A peptide mimicking the NES-ERα specifically sequesters the receptor in the nuclei and interferes in hormone-triggered S phase entry in these cells. Site-directed mutagenesis of the ERα NES inhibits forkhead in rhabdomyosarcoma (FKHR) nuclear export, the estradiol-induced cytoplasmic relocalization of receptor, and DNA synthesis. Targeting ERα by siRNA retains FKHR in the nuclear compartment of estradiol-treated MCF-7 cells.

This study provides a new link between the rapid extra-nuclear action of estradiol and ERα nuclear export, as well as a proliferative role of receptor nuclear export, which is closely associated to FKHR export.

Results

Estradiol regulates ERα shuttling in MCF-7 cells

Localization of endogenous ERα in quiescent MCF-7 cells was verified by immunofluorescence using two different antibodies directed against either a C-terminal (H222 mAb; Katzenellenbogen et al., 1987) or N-terminal epitope of ERα (A314 mAb; Abbondanza et al., 1998). Fig. 1a shows that, regardless of the antibody used, estradiol treatment of MCF-7 cells induces nuclear translocation of ERα after 30 min, followed by a decrease of the nuclear receptor to the basal level by 1 h. Representative images of one of these experiments are presented in the supplemental materials, together with the parallel change in cytoplasmic redistribution of the receptor (Fig. S1a and Fig. S1b, left, available at http://www.jcb.org/cgi/content/full/jcb.200712125/DC1). Immunoblot analysis of cell lysates does not reveal any significant change in ERα level (Fig. S1c), which suggests that the observed decrease in ERα nuclear localization is caused by nuclear export of the receptor.

Quiescent MCF-7 cells were next transiently transfected with a full-length ERα cDNA subcloned into the GFP plasmid (pEGFP-HEG0; HEG0 represents the wtERα). In Fig. 1b, the quantitative count of cells with nuclear fluorescence shows that hormone treatment triggers nuclear accumulation of GFP-ERα after 30 min in the absence of any significant change in GFP-wtERα expression (Figs. 1b and S1c). By 1 h, the nuclear GFP-wtERα decreases to the basal level, whereas no trafficking of GFP-wtERα was detectable in untreated cells (Fig. 1b). Representative images of one of these experiments are presented in Fig. S1b (right). In addition, GFP alone was insensitive to estradiol treatment when overexpressed in MCF-7 cells (unpublished data). This pattern of trafficking is similar to that observed for endogenous ERα. Furthermore, the partial antagonist, 4-OH-tamoxifen, prevents the observed nucleocytoplasmic shuttling of GFP-wtERα in response to hormonal treatment (Fig. 1b).

To exclude the potential contribution of de novo GFP-ERα synthesis to its reemergence in the cytoplasm 1 h after estradiol treatment, we included actinomycin D in the medium during hormone stimulation. No significant change in the estradiol-induced redistribution of GFP-wtERα (Fig. 1c) or in protein expression was observed (Fig. S1c). Therefore, the chimeric protein in cytoplasm is from nuclear export rather than from de novo synthesis. Because actinomycin D inhibits the nuclear import of the NES-containing REV protein (Henderson, 2000), and the GFP-wtERα dynamic redistribution is not modified by actinomycin D (Fig. 1c), it is likely that cytoplasmic relocalization of the chimera depends on nuclear export rather than inhibition of nuclear import.

Because data in Fig. 1 (a–c) show that ERα export short occurs upon estradiol treatment of MCF-7 cells, we hypothesized a role for a rapid action of estradiol in the ERα intracellular traffic. Activation of the PI3K–AKT pathway, for instance, modulates the trafficking of various proteins, including FKHR, nuclear factor κB, glycogen synthase kinase-3 β, Mdm2, p27 (for review see Kau and Silver, 2003), and PTEN (phosphatase and tensin homologue deleted on chromosome; Liu et al., 2007). Because of the rapid activation of this pathway by estradiol treatment of breast cancer cells (Fig. S1d; Castoria et al., 2001, 2004), we verified its contribution to the ERα nuclear export. Quiescent MCF-7 cells were then transiently transfected with the dominant-negative form of the regulatory subunit of PI3K, p85α (Δp85α; Dhand et al., 1994), or the catalytically inactive version of AKT (K179M; dominant-negative AKT). In Fig. 1d, the count of transfected cells showing ERα nuclear fluorescence indicates that overexpression of either Δp85α or dominant-negative AKT induces nuclear retention of ERα in 60-min estradiol-treated MCF-7 cells. Transfection of cells with the Myc-tagged pSG5 control
plasmid does not interfere in the trafficking of ERα. Images of one experiment in Fig. 1 d are presented in Fig. S1 e.

To determine if CRM1 plays a role in ERα nuclear exit, MCF-7 cells were treated with LMB in addition to estradiol. This antifungal compound blocks the nuclear export of NES-containing proteins by preventing their association with the CRM1 export receptor (Kudo et al., 1998). A quantitative analysis of transfected cells shows that LMB treatment results in maintenance of nuclear GFP-wtER

Figure 1. Estradiol induces nuclear export of ERα, which is regulated by the PI3K-AKT pathway and depends on CRM1. Quiescent MCF-7 cells were used. (a) Cells were untreated or treated with 10 nM estradiol (E2) for the indicated times (min). ERα localization was analyzed by immunofluorescence using the indicated antibodies. (b and c) Cells were transfected with GFP-wtERα then left untreated or treated with the indicated compounds. OH-tamoxifen (Tx; AstraZeneca) was used at 0.1 μM; actinomycin D (Act D) was added at 5 μg/ml, 1 h before estradiol stimulation. GFP-wtERα localization was determined by fluorescence. (d) Cells were transfected with the indicated plasmids and left untreated or treated with 10 nM estradiol for the indicated times (min). The Myc-tagged pSG5, Δp85α, or Myc-His-tagged dominant-negative AKT ectopically expressed in MCF-7 cells were visualized by immunofluorescence, as described in Materials and methods. ERα localization was analyzed by immunofluorescence using the rat H222 anti-ERα mAb. (e) Cells were transfected with GFP-wtERα and then left untreated or treated with 10 nM estradiol in the absence or presence of LMB (at 5 ng/ml). LMB was added 30 min before the hormone. Cells were also treated with LMB in the absence of hormone. GFP-wtERα localization was determined by fluorescence. (a, b, c, and e) Cells that fell into the category of exclusively nuclear fluorescence were scored, and data was expressed as a percentage of total cells (in a) or transfected cells (in b, c, d, and e). Data were derived from at least 1,000 scored cells. The results of several independent experiments were averaged; means and SEM are shown. n represents the number of experiments. (f) Images from one experiment in panel e were captured. Panels show GFP-wtERα localization in MCF-7 cells stimulated for 60 min with estradiol (E2) in the absence or presence of LMB. Bar, 5 μm. (g) 35S-labeled HA-CRM1 was incubated with recombinant ERα in the absence or presence of 10 nM estradiol. The purified recombinant RanQ69L (at 1 μM) was included in the incubation mixture of each sample. Proteins were immunoprecipitated with rabbit polyclonal anti-ERα antibody. Eluted proteins were immunoblotted with anti-ERα antibody (top) or revealed by fluorography (bottom).
Figure 2. The ERα 444–456 sequence restores export activity of the NES-deficient REV1.4-GFP. Growing MCF-7 cells were used. (a and b) Cells were transfected with the indicated constructs. After transfection, the cells were left untreated (no drug) or treated with actinomycin D (ActD) at 5 μg/ml, alone or together with 5 ng/ml LMB. The subcellular distribution of GFP proteins was determined by fluorescence microscopy, and cells that fell into the category of exclusively nuclear fluorescence were scored. Data are expressed as a percentage of transfected cells, with mean values taken from at least three experiments. For each experiment, at least 600 cells were scored. (b) Images of one experiment in panel a. (c) The wt ERα 444–456 sequence (NES ERα wt) as well as its mutated version (NES ERα mutant). The putative NES-ERα sequence is indicated by the underlined amino acids, which were substituted with alanine residues in the mutant sequence. The NES-ERα wt as well as the NES-ERα mutant subcloned into the Rev mutant were transfected (d and e) in growing MCF-7 cells. After transfection, the cells were left untreated (−) or treated with 5 μg/ml Act D (+). The percentage of cells with nuclear GFP protein was determined by fluorescence microscopy and graphically shown in panel d. Data were derived from at least 600 scored cells. The results of several independent experiments were averaged. (a and d) Means and SEM are shown. n represents the number of experiments. (e) Images of one experiment in panel d. Bars, 5 μm.
on the localization of GFP-wtERα (Fig. 1, e and f). Thus, the effect of LMB treatment on ERα localization suggests that this receptor, when bound to the hormone, is exported from nuclei through the CRM1–exportin pathway. Estradiol induces a strong interaction between recombinant ERα and in vitro translated CRM1 (Fig. 1g) when active Ran (Ran-GTP; Askjaer et al., 1999) was included as a GTPase-deficient RanQ69L mutant (Bischoff et al., 1994).

This set of experiments shows that estradiol activation of the PI3K–AKT pathway modulates nuclear export of ERα and that CRM1 contributes to estradiol-mediated ERα nuclear export in MCF-7 cells.

Identification of an ERα nuclear export sequence

Two ERα mutants, HEG15 and HEG14, were subcloned into pEGFP. HEG15 (Δ282–595 HEG0) contains highly conserved NLSs (2 and 3; Guiochon-Mantel et al., 1991). It binds DNA but is unable to bind estradiol (Ylikomi et al., 1992). In turn, HEG14 (Δ1–281) contains NLS1. It binds the hormone but does not bind DNA (Ylikomi et al., 1992). The resulting chimeras, GFP-HEG15 and GFP-HEG14, were transiently transfected into quiescent MCF-7 cells. Consistent with previous findings (Ylikomi et al., 1992), results in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200712125/DC1) show that neither GFP-HEG15 nor GFP-HEG14 shuttle between the nucleus and the cytoplasm. Irrespective of the hormonal treatment, the first mutant mostly resides in the nuclear compartment. Although GFP-HEG14 contains the NLS1 sequence and binds the hormone, it does not enter nuclei even after estradiol addition. From these data, we speculated that the hormone-binding domain localized in the HEG14 mutant as well as NLSs (2 and 3) of ERα are both required for the estradiol-induced nuclear import of the receptor. We next subcloned HE241G into pEGFP. This mutant contains the hormone-binding domain and lacks the three NLSs. It is, in fact, prevalently localized in the cytoplasm regardless of estradiol treatment. The behavior of this mutant confirms that in the absence of NLS, the hormone-binding domain is not sufficient to induce nuclear translocation of the receptor. Because HEG15 is localized in the nuclear compartment and its localization is unaffected by hormonal treatment of cells, we reasoned that the addition of NLSs to HEG14 might restore the estradiol-induced import of ERα. Analysis of transfected cells with the resulting chimera, GFP-NLS/HEG14, showed not only nuclear import but also export similar to GFP-wtERα in response to estradiol treatment. These findings collected in Fig. S2 point to the presence of nuclear export sequences in the NLS–HEG14 construct, which is made up of the NLSs and the hormone-binding domain of ERα.

It has been found that NLSs of the PgR are responsible for its nuclear export (Guiochon-Mantel et al., 1994). Therefore, we studied whether NLSs are involved in ERα export using in vivo export assay (Henderson and Eleftheriou, 2000). In this assay, nuclear export sequences are identified by their ability to restore export activity of the NES-deficient REV1.4-GFP (Rev mutant) to levels similar to those observed with the wild-type (wt) pREV-GFP or the REV1.4-GFP NES (Rev positive control), in which the NES is the canonical export sequence of the REV protein. The NLS sequences of ERα were subcloned into the Rev mutant and expressed in MCF-7 cells. After transfection, cells were incubated in the absence or presence of actinomycin D because it causes cytoplasmic accumulation of the putative NES-containing REV protein by preventing nuclear import of REV (Henderson, 2000). Irrespective of experimental conditions, the Rev mutant NLS localizes in nuclei of MCF-7 cells (Fig. 2, a and b). In the same experiment, the Rev-positive control completely shifted to the cytoplasm in the presence of actinomycin D, whereas the Rev mutant NLS sequences from the ERα showed nuclear, sometimes nucleolar, staining. These data indicate that the NLS sequences of ERα are inactive in this export assay.

We then subcloned different sequences of ERα containing leucine residues into a Rev mutant. These constructs were transfected into MCF-7 cells and then analyzed for their ability to restore the nuclear export of the Rev mutant. Using this assay, we verified that ERα residues 427–456 induced a nucleocytoplasmic redistribution of Rev mutant in cells treated with actinomycin D, whereas the Rev mutant NLS sequences from the ERα showed nuclear, sometimes nucleolar, staining. These data indicate that the NLS sequences of ERα are inactive in this export assay.

A thorough sequence analysis showed homology between the 444–456 amino acids of ERα and the conserved leucine-rich and REV-like NES of p53 (residues 340–351; Stommel et al., 1999). A comparison of the homologous amino acids across the family of steroid receptors revealed a high level of sequence homology with ERβ, PgR, AR, and GR. The alignment between the sequences is shown in Fig. S3 (a and b, available at http://www.jcb.org/cgi/content/full/jcb.200712125/DC1).

The possibility that residues 444–456 of the ERα sequence contain the NES-ERα was further investigated. The 444–456 ERα sequence were subcloned into the Rev mutant, as they were with a mutated version in which residues 444–456 were mutated from IILL to alanines (Fig. 2c). The ERα wt sequence (NES-ERα wt) was able to shift the Rev mutant into the cytoplasm of MCF-7 cells, whereas the mutated form of ERα (NES-ERα mutant) failed to do so (Fig. 2, d and e). This difference is even more evident in the presence of actinomycin D.

These results show that a small sequence of the ERα hormone-binding domain contains a CRM1-mediated ERα NES.

A peptide mimicking the 444–456 ERα sequence sequesters the receptor in the nuclear compartment and inhibits estradiol-induced S phase entry in breast cancer cells

Small cationic peptides (also called protein transduction domains) derived from nucleic acid–binding proteins, such as HIV-Tat protein, deliver a myriad of peptides into animal models and represent a biological and potentially therapeutic tool for targeting proteins into the cells (Joliot and Prochiantz, 2004).
To trap ERα in the nuclear compartment of cells, we synthesized a Tat-conjugated peptide construct corresponding to the residues 444–456 of ERα (Fig. 3 a). Confocal microscopy analysis revealed in preliminary experiments that the carboxyfluorescein-conjugated peptide translocated across the plasma membrane and, within 30 min, accumulated in nuclei of MCF-7 cells (unpublished data). This peptide displaced the estradiol-induced interaction between recombinant CRM1 and ERα (Fig. 3 b) and blocked the 60-min estradiol-induced nuclear export of GFP-wtERα, thus sequestering the receptor in nuclei (Fig. 3, c and d). The Tat alone did not affect both the ERα–CRM1 interaction (Fig. 3 b) as well as the trafficking of GFP-wtERα (unpublished data). Furthermore, the Tat-conjugated peptide did not affect p27 nuclear export or p53 subcellular localization (Fig. S3, c and d), which indicates that it specifically interferes in ERα localization.

The extranuclear activity of ERα triggers hormone-dependent DNA synthesis (Castoria et al., 1999), and ERα sequestration in the cytoplasm increases its nongenomic actions and drives neoplastic transformation (Kumar et al., 2002). Fig. 3 e shows that the Tat-conjugated peptide reduced estradiol-induced DNA synthesis in MCF-7 cells by 60%. A negligible effect was observed in cells treated with Tat alone. Furthermore, the Tat peptide did not interfere in serum-induced DNA synthesis in MCF-7 cells (Fig. 3 e), which indicates that the peptide specifically interferes in estradiol action. Addition of the Tat-conjugated peptide at different times after estradiol stimulation shows that after 60 min, when ERα is almost completely exported from nuclei, the peptide does not affect S phase entry in MCF-7 cells (Fig. 3 f), thus reinforcing the view that the estradiol-induced nuclear export of ERα plays a role in DNA synthesis.

**ERα NES mutants do not exit nuclei and fail to mediate estradiol-induced DNA synthesis**

Based on our findings (Fig. 2), two mutants of GFP-wtERα were prepared by site-directed mutagenesis to identify the NES-ERα sequence and analyze the biological effects induced by these mutations. Fig. 4 a shows a schematic representation of the GFP-wtERα NES and its mutated versions, which were transiently transfected into MCF-7 cells. Estradiol treatment induced nuclear export of GFP-wtERα, which contains the wild-type human ERα. In contrast, both mutants, GFP-ERα 4A and GFP-ERα IL, were unable to be exported from nuclei upon hormonal stimulation (Fig. 4 b). Images in Fig. 4 c show the diffuse, sometimes extranuclear localization of GFP-wtERα in MCF-7 cells stimulated for 60 min with estradiol. Under these conditions, a nuclear/nucleolar localization of both GFP-ERα 4A and GFP-ERα IL was detected.

We then verified the ability of the GFP-ERα IL mutant to activate estradiol-induced gene transcription. To this end, we used ERα-negative NIH3T3 fibroblasts (Castoria et al., 1999, 2003). The Western blot in Fig. 4 d (inset) confirms that NIH3T3 fibroblasts are indeed ERα negative. The cells were then transiently transfected with GFP-wtERα, GFP-ERα IL, or GFP alone. TheERE-Luc reporter gene was cotransfected and its activity assayed. Fig. 4 d shows that the constructs GFP-wtERα and GFP-ERα IL are equally efficient in activating gene transcription upon estradiol stimulation of the cells, with a sixfold induction of ERE-Luc activity. Transcriptional activation was almost undetectable in unstimulated cells or in cells expressing GFP alone.

In another set of experiments, we assessed the ability of GFP-wtERα and GFP-ERα IL to mediate estradiol-induced AKT activation as well as DNA synthesis when ectopically expressed in ERα-negative NIH3T3 fibroblasts. We first verified that GFP-wtERα and its export mutant GFP-ERα IL both activate AKT upon estradiol stimulation of NIH3T3 cells (Fig. 4 e). Although able to activate the nongenomic pathway usually engaged by estradiol to transmit its mitogenic signal, the mutant GFP-ERα IL failed to induce S phase entry in fibroblasts treated with estradiol (Fig. 4 f). The mutant did not affect S phase entry induced by the receptor-independent serum stimulation (Fig. 4 f).

In contrast, the GFP-wtERα induced a robust BrdU incorporation in hormone-treated cells (Fig. 4 f). As observed in MCF-7 cells (Fig. 3 e), addition of Tat-conjugated peptide inhibited the estradiol-induced S phase entry in fibroblasts transfected with GFP-wtERα. Here again, the peptide did not interfere in DNA synthesis induced by serum stimulation of the same cells (Fig. 4 f, asterisks).

These experiments demonstrate that the leucine-rich sequence 444–456 of ERα contains a functional NES. Mutations in the core (III/II) of this sequence impair the estradiol-induced nuclear export of ERα and DNA synthesis without affecting the signal transduction pathway or gene transcription regulation by estradiol.

**Estradiol regulates FKHR/ERα export**

Estradiol activation of PI3K is required to drive MCF-7 cells into S phase (Castoria et al., 2001). Activation of the PI3K–AKT pathway by estradiol regulates ERα nuclear export (Fig. 1), and FKHR nuclear export depends on its phosphorylation by AKT (Biggs et al., 1999). In addition, previous studies reported that an estradiol-dependent interaction between ERα and FKHR occurs in vitro (Schuur et al., 2001). Thus, we hypothesized a role for FKHR in both estradiol-regulated ERα nuclear export and cell cycle arrest mediated by the NES-ERα mutant. To analyze the role of FKHR in estradiol-induced DNA synthesis and ERα nuclear export, quiescent MCF-7 cells were transiently transfected with wt FKHR (GFP-FKHR wt) or a mutant containing a triple alanine substitution (GFP-FKHR AAA) that localizes in nuclei and blocks FKHR phosphorylation by AKT, thereby inducing G1 arrest of cells (Nakamura et al., 2000). Fig. 5 a shows that expression of this mutant reduced estradiol-triggered BrdU incorporation in MCF-7 cells, whereas expression of GFP-FKHR wt did not. We then used confocal microscopy to analyze the role of FKHR in the estradiol-regulated subcellular distribution of ERα. Although ectopically expressed GFP-FKHR wt or GFP alone did not modify the 60-min estradiol-induced nuclear export of ERα, the mutant GFP-FKHR AAA sequestered ERα in the nuclear compartment at that time (Fig. 5, b and d). Conversely, overexpression of the tagged NES-ERα mutant, Myc-ERα IL, resulted in retention of GFP-FKHR wt in the nuclear compartment.
ERα-positive breast cancer cells, FKHR moves from nucleus to the cytoplasm by a mechanism depending on ERα.

Finally, we observed by Western blot analysis that estradiol stimulation of quiescent MCF-7 cells rapidly induces FKHR phosphorylation in Ser256 (Fig. 6a). To complement colocalization experiments, we next analyzed the interaction compartment 60 min after estradiol treatment of MCF-7 cells (Fig. 5, c and d). Thus, estradiol simultaneously regulates ERα- and FKHR-associated nuclear export.

We then verified that silencing of ERα by siRNA retains GFP-FKHR wt in the nuclear compartment of 60-min hormonal-treated MCF-7 cells (Fig. 5e). These findings confirm that, in
Discussion

Nucleocytoplasmic shuttling of proteins plays a critical role in cell function (for review see Kau and Silver, 2003). Most of the ERα is localized in nuclei of hormone target cells (Stenoien et al., 2001), and its best-known function as a ligand-activated transcription factor requires nuclear localization (Mangelsdorf et al., 1995). Much evidence, however, has demonstrated rapid, extranuclear action of ERα (for review see Migliaccio et al., 2007a). Ligand stimulation of different cell types recruits different

between ERα and FKHR in coimmunoprecipitation experiments. Data in Fig. 6 b show that 30-min hormonal stimulation increases the association of FKHR wt with ERα in MCF-7 cells (Fig. 6 b, left). Whatever the experimental condition, the mutant FKHR AAA does not coimmunoprecipitate with ERα (Fig. 6 b, right), therefore implicating that phosphorylation of FKHR induced by estradiol is required for its association with the receptor.

These findings led us to hypothesize the model of ERα export and its interplay with FKHR depicted in Fig. 6 c.
signaling effectors to ERα or ERβ, which in turn leads to signal transduction pathway activation (Migliaccio et al., 2000; Castoria et al., 2001). A transcriptionally inactive ERα mutant, permanently residing in the cytoplasm, mediates S phase entry triggered by estradiol activation of the Src–Ras–ERK and PI3K–AKT pathways (Castoria et al., 1999, 2004). These findings suggest a proliferative function for this extranuclear receptor that is independent of its transcriptional activity. This view is supported by the observation that a classical mouse AR, which is expressed at a very low level in NIH3T3 fibroblasts, does not enter nuclei and does not activate gene transcription in androgen-stimulated cells. Nevertheless, it recruits several signaling effectors that control hormone-induced S phase entry and cytoskeleton changes in fibroblasts (Castoria et al., 2003). Rat uterine stromal cells, although they express low levels of transcriptionally incompetent PgR, respond to progesterin with

![Figure 5](image_url)

**Figure 5.** FKHR nuclear export: regulation by estradiol and a role in hormone-induced DNA synthesis in MCF-7 cells. Quiescent MCF-7 cells on coverslips were used. (a) Cells were transfected with the indicated plasmids then left unstimulated or stimulated for 24 h with 10 nM estradiol. After in vivo pulse with BrdU, DNA synthesis was analyzed by immunofluorescence and BrdU incorporation was calculated as in Fig. 4. (b) Cells were transfected with the indicated plasmids then left unstimulated or stimulated with 10 nM estradiol for the indicated times. Endogenous ERα localization was observed by expression of GFP, GFP-FKHR wt, or GFP-FKHR AAA mutant monitored by confocal microscopy. Cells that fell into the category of exclusively ERα nuclear fluorescence were scored, and data were expressed as a percentage of transfected cells. (c) Cells were cotransfected with the indicated plasmids then left unstimulated or stimulated with 10 nM estradiol for 60 min. Localization of GFP-FKHR wt, Myc-HEG0, or Myc-HEGIL mutant was monitored by confocal microscopy. Cells that fell into the category of exclusively FKHR nuclear fluorescence were scored and the data were expressed as a percentage of cotransfected cells. For each experiment in panels a–c, data were derived from at least 500 scored cells. The results of several independent experiments were averaged; n represents the number of experiments. (d) Images from one experiment in b or c are shown. They represent the staining of endogenous ERα (red) in MCF-7 cells expressing GFP-FKHR wt (left, green) or the mutant, GFP-FKHR AAA (middle, green), and treated for 60 min with estradiol. (right) The staining of Myc-tagged NES-ERα mutant (red) in MCF-7 cells coexpressing GFP-FKHR wt (green) and treated for 60 min with estradiol. Merged images are also shown on the bottom. Bar, 5 μm. (e) The cells were cotransfected with ERα siRNA (ERα siRNA) or nontargeting siRNA (nt siRNA) and GFP-FKHR wt. The cells were then left unstimulated or stimulated with 10 nM estradiol for the indicated times. GFP-FKHR wt localization was monitored by confocal microscopy. Cells that fell into the category of exclusively FKHR nuclear fluorescence were scored and data were expressed as a percentage of cotransfected cells. Data were derived from at least 200 scored cells. The results of two independent experiments were averaged. The blot in panel e confirms the silencing of ERα in MCF-7 cells transfected with ERα siRNA (top). The bottom shows the blot of loading proteins revealed using the anti-tubulin antibody. (a, b, c, and e) Means and SEM are shown.
The 444–456 sequence of ERα contains a functional NES, which is sensitive to LMB treatment. A Tat-conjugated peptide mimicking this amino acid sequence displaces the in vitro interaction between ERα and CRM1. These findings support the conclusion that the 444–456 sequence contains a putative NES.

Mutations in the core of this sequence impair estradiol-induced nuclear export of full-length ERα in MCF-7 cells. The 444–456 sequence shows homology with the conserved leucine-rich and REV-like NES of p53 (340–351; Fig. S3 a; Stommel et al., 1999). Remarkably, the 444–456 region of ERα is conserved in other steroid receptors, such as ERβ, PgR, AR, GR, and MR (Fig. S3 b), which suggests that such a sequence is responsible for the CRM1-dependent nuclear export of most steroid receptors. This view is in agreement with the inhibition of nuclear export of different steroid receptors by LMB treatment described by several groups under different experimental conditions (Savory et al., 1999; Prufer and Barsony, 2002; Maruvada et al., 2003).

ERα localization was also modulated by a Tat-conjugated peptide mimicking the ERα 444–456 sequence. It specifically traps ERα in the nuclear compartment of MCF-7 cells and reduces the estradiol-induced S phase entry in these cells, thus confirming that ERα nuclear export plays a key role in DNA synthesis of target cells. This view is reinforced by the finding that addition of the peptide to the cell medium 1 h after hormonal stimulation, when ERα nuclear export is almost complete, does not affect estradiol-induced S phase entry in MCF-7 cells. Experiments with NIH3T3 fibroblasts show that an NES-ERα mutant fails to induce S phase entry in these cells, whereas it active proliferation (Vallejo et al., 2005). In addition, the extranuclear cross talk between ERα–AR and the EGF receptor regulates EGF-elicited responses such as actin changes and DNA synthesis in breast and prostate cancer cells (Migliaccio et al., 2005). Direct evidence of the proliferative role of the ERα–AR complex association with Src in mammary and prostate cancer cell xenografts has been recently described (Migliaccio et al., 2007b; Varricchio et al., 2007). These and other similar findings point to the critical role of steroid receptor extranuclear localization in steroid hormone or growth factor action.

In this study, we analyzed the nucleocytoplasmic shuttling of ERα in MCF-7 cells, focusing on its export from the nucleus. We observed that estradiol induces CRM1-dependent nuclear export of ERα. Thus, identification of the ERα sequence responsible for its nuclear export was initially performed by a nuclear export assay (Henderson and Eleftheriou, 2000). Previous studies found that the outward movement of PgR from the nuclear compartment is mediated by its NLSs (Guiochon-Mantel et al., 1994). We then assayed the ability of ERα NLSs to shift the REV mutant into the cytoplasm. Our data show that NLSs are not involved in the nuclear export of ERα. It has also been hypothesized that the nuclear exit of ERα is regulated by its phosphorylation on Thr311 (Lee and Bai, 2002). This residue is present in a putative leucine-rich ERα NES (305–322 of ERα), which shares homology with the p53 N-terminal NES (Zhang and Xiong, 2001). We found that the leucine-rich 296–335 ERα sequence does not drive the REV mutant into the cytoplasm. Using the same approach, we observed that the leucine-rich 444–456 sequence of ERα contains a functional NES, which is sensitive to LMB treatment. A Tat-conjugated peptide mimicking this amino acid sequence displaces the in vitro interaction between ERα and CRM1. These findings support the conclusion that the 444–456 sequence contains a putative NES.

Mutations in the core of this sequence impair estradiol-induced nuclear export of full-length ERα in MCF-7 cells. The 444–456 sequence shows homology with the conserved leucine-rich and REV-like NES of p53 (340–351; Fig. S3 a; Stommel et al., 1999). Remarkably, the 444–456 region of ERα is conserved in other steroid receptors, such as ERβ, PgR, AR, GR, and MR (Fig. S3 b), which suggests that such a sequence is responsible for the CRM1-dependent nuclear export of most steroid receptors. This view is in agreement with the inhibition of nuclear export of different steroid receptors by LMB treatment described by several groups under different experimental conditions (Savory et al., 1999; Prufer and Barsony, 2002; Maruvada et al., 2003).

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activates gene transcription. These findings further point to the role of ERα nuclear export in cell cycle progression modulated by estrogens in breast cancer cells.

The function of many proteins is regulated by their subcellular localization. These proteins include cell cycle regulators and transcription factors, such as nuclear factor κB, p53, and mammalian members of the FKHR transcription factors (for review see Kaul and Silver, 2003). A triple alanine mutant of FKHR localizes in the nucleus and induces G1 arrest of cells (Nakamura et al., 2000; Birkenkamp and Coffer, 2003). Consistent with the hypothesis that permanent localization of transcription factors in the nucleus can stop the cell cycle, it has been shown that estradiol stimulation of Pak-1 and ERα promotes cell survival by inducing phosphorylation and nuclear exclusion of FoxO1 in breast cancer cells (Mazumdar and Kumar, 2003). Relevant to the observed association between estradiol-regulated export of ERα and FKHR, an estradiol-dependent interaction between ERα and FKHR has been observed using a yeast two-hybrid screen and in vitro pull-down experiments (Schuur et al., 2001). Extension of these investigations to other nuclear receptor family members has shown that, depending on the receptor type, FKHR represents a bifunctional intermediary protein acting as either a coactivator or corepressor of these receptors (Zha et al., 2001).

Estradiol stimulation of MCF-7 cells triggers DNA synthesis through PI3K- and Src-dependent pathway activation, which causes an increase in cyclin D1 transcription and p27 nuclear release (Castoria et al., 2001, 2004). We observe here that estradiol-activated AKT phosphorylates FKHR, thus triggering the FKHR–ERα association and facilitating the nuclear export of the complex. By removing the inhibitory action of FKHR, this event triggers cell cycle progression. Increased FKHR–Ser256 phosphorylation by estradiol activation of AKT has been recently described in neuronal cells (Won et al., 2006). Association of FKHR with ERα, as indicated by their colocalization and co-immunoprecipitation, might favor FKHR nuclear exit by masking a regulated export of ERα by estrogens in breast cancer cells. Relevant to the observed association between estradiol-motors cell survival by inducing phosphorylation and nuclear export of ERα by estrogens in breast cancer cells. Relevant to the observed association between estradiol-motors cell survival by inducing phosphorylation and nuclear export of ERα (Van Der Heide et al., 2004).

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Materials and methods

Constructs

The constitutive active Ran G69L was in the pQE plasmid was a gift of I.W. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany; Izzaurralde et al., 1997). The pCDNA/HA-CRM1 (Ali et al., 2000) was di-
After transfection, the cells were incubated at 37°C for 24 h and then used for the indicated experiments.

**Nuclear export and transactivation assays**

The nuclear export assay was performed as described previously (Castoria et al., 2003). Endogenous ERα was visualized as described previously (Castoria et al., 1999). The putative NES-ERα sequence was confirmed using the rabbit polyclonal anti-ERα antibody (Jackson ImmunoResearch Laboratories). Lysates were analyzed by indirect immunofluorescence microscopy for the subcellular localization of GFP-wtERα or its mutant, as well as putative NES-ERα constructs. Similar analysis was performed to detect GFP-FKHR wt or its mutant GFP-FKHR AAA. In all other experiments, the cells on coverslips were fixed and permeabilized as described previously (Castoria et al., 1999). Endogenous ERα was visualized as described previously (Castoria et al., 1999) using the mouse monoclonal anti-ERα antibody (clone BU1-1 from GE Healthcare). Diluted (1:200 in PBS) Texas red–conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) was used to detect the primary antibody.

**DNA synthesis analysis and peptides**

Quiescent MCF-7 or NIH3T3 cells on coverslips were left unstimulated or treated with 10 nM estradiol for 18 h. Lysates were prepared and the luciferase activity was measured using a luciferase assay system (Promega). The results were corrected using CH110–expressed β-galactosidase activity (GE Healthcare).

**Immunofluorescence and confocal microscopy**

Cells on coverslips were fixed for 10 min with paraformaldehyde (3% wt/vol in PBS), washed with PBS, and then analyzed by fluorescence microscopy for the subcellular localization of GFP-wtERα, its mutants, as well as putative NES-ERα constructs. Similar analysis was performed to detect GFP-FKHR wt or its mutant GFP-FKHR AAA. In all other experiments, the cells on coverslips were fixed and permeabilized as described previously (Castoria et al., 1999). Endogenous ERα was visualized as described previously (Castoria et al., 1999) using the mouse monoclonal anti-ERα antibody (clone BU1-1 from GE Healthcare). Diluted (1:200 in PBS) Texas red–conjugated goat anti–mouse antibody (Jackson Immunoresearch Laboratories) was used to detect the primary antibody.
cell cycle progression without inducing ERK-1 and -2 MAP kinase acti-

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