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# CRM1-mediated nuclear export of estradiol receptor $\alpha$ depends on estradiol and controls cell cycle progression of breast cancer cells

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# CRM1-mediated nuclear export of estradiol receptor $\alpha$ depends on estradiol and controls cell cycle progression of breast cancer cells

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## LIST OF PUBLICATIONS

### This dissertation is based upon the following publications:

- Lombardi M, Di Stasio R, Castoria G, Migliaccio A, Barone MV, Bottero D, Yamaguchi H, Appella E and Auricchio F. Hormone-dependent nuclear export of estradiol receptor and DNA synthesis of breast cancer cells. *Submitted*

- Castoria G, Migliaccio A, D'Amato L, Di Stasio R, Ciociola A, Lombardi M, Bilancio A, Di Domenico M, de Falco A and Auricchio F. Integrating signals between cAMP and MAPK pathways in breast cancer. Front. Biosci. 2008; 13: 1318-1327.

- Migliaccio A, Varricchio L, de Falco A., Castoria G, Arra C, Yamaguchi H, Ciociola A, Lombardi M, Di Stasio R, Barbieri A, Baldi A, Barone MV, Appella E and Auricchio F. Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth. Oncogene 2007; 26: 6619-6629

- Cosentino C, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G, Santillo MR, Agnese S, Di Stasio R, Feliciello A, Migliaccio A and Avvedimento EV. p85 regulatory subunit of PI3-K mediates cAMP-PKA and estrogen biological effects on growth and survival. Oncogene 2007; 26: 2095-2103.

- Migliaccio A, Castoria G, Di Domenico M, Ciociola A, Lombardi M, de Falco A, Nanayakkara M, Bottero D, Di Stasio R, Varricchio L and Auricchio F. Crosstalk between EGFR and extranuclear steroid receptors. Ann. N. Y. Acad. Sci. 2006; 1089: 194-200.

## ABSTRACT

In breast cancer cells, estradiol induces in rapid succession nuclear translocation and nuclear exit of the estradiol receptor (ERα). Receptor export depends on CRM1. Experiments with constructs expressing a receptor-derived sequence reveal the presence of a nuclear export sequence (NES) within the hormone-binding domain of ERα. This sequence restores the CRM1-dependent export of a Rev-HIV mutant protein in an *in vivo* export assay. A peptide mimicking the ERα export sequence disrupts *in vitro* the ER/CRM1 interaction and sequesters the receptor in the nuclear compartment of MCF-7 cells. Remarkably, it inhibits estradiol-induced DNA synthesis. Furthermore, by site-directed mutagenesis of full-length ERα, we identified the ERα-NES. Mutant of ERα-NES ectopically expressed in NIH 3T3 fibroblasts does not exit nuclei and is unable to mediate estradiol-induced S-phase entry. This mutant, however, leaves unaltered receptor-dependent gene transcription.

Confocal microscopy analysis shows that ERα colocalizes with forkhead in nuclei of estradiol-treated MCF-7 cells. A forkhead mutant, unphosphorylatable by Akt, is trapped together with ER wt in nuclei and blocks estradiol-induced S-phase entry in MCF-7 cells. In turn, the ERα NES mutant fails to exit nuclei and prevents forkhead wt nuclear export.

Findings presented in this thesis identify for the first time an ERα NES, which depends on estradiol and is directed by CRM1. Moreover, they show that ERα nuclear export is associated with forkhead exit and regulates G1-S transition in breast cancer cells.

# 1. BACKGROUND

## 1.1 Nucleocytoplasmic shuttling of proteins: a general overview

The most important features that differentiate eukaryotic cells from prokaryotic cells is the presence of distinct intracellular compartments, organelles and nucleus. The spatial separation of mRNA synthesis, occurring in the nucleus, from translation of proteins, occurring in the cytoplasm, provides eukaryotes with the possibility to achieve higher complexity. Many molecules continuously move between the nuclear and the cytoplasmic compartments through nuclear pore complexes (NPCs), which essentially serve as gatekeepers spatially and temporally segregating the genomic material from all cytoplasmic processes (Sebastian et al. 2004).

NPCs constitute high-order octagonal diffusion channels that penetrate the double bilayer of membranes surrounding the nucleoplasm. These remarkable structures are estimated to weigh 125 MDa and to contain 50-100 different proteins, called nucleoporins (Nups), most of which are characterized by hydrophobic phenilalanine/glycine (FG)-rich repeat motifs (Meyer and Vinkemeier 2005). The derived channels have a diameter of roughly 9 nm, which is equivalent to a globular protein of 50-60 kDA. Somehow, however, the NPCs change conformation to allow active transport of substrates (cargoes) greater than 25 nm in diameter, which is equivalent to a ribosomal diameter. Therefore, some small molecules (i.e. ions and metabolites) passively diffuse across the nuclear pore complexes through a concentration gradient, whereas macromolecules are, in most cases, actively transported across the nuclear channels (Yoneda 2000). Thus, NPCs function as selective filters because they restrict the transport of some macromolecules, while allowing the rapid translocation of others.

Fig. **1** depicts the structure of the nuclear-pore complex.

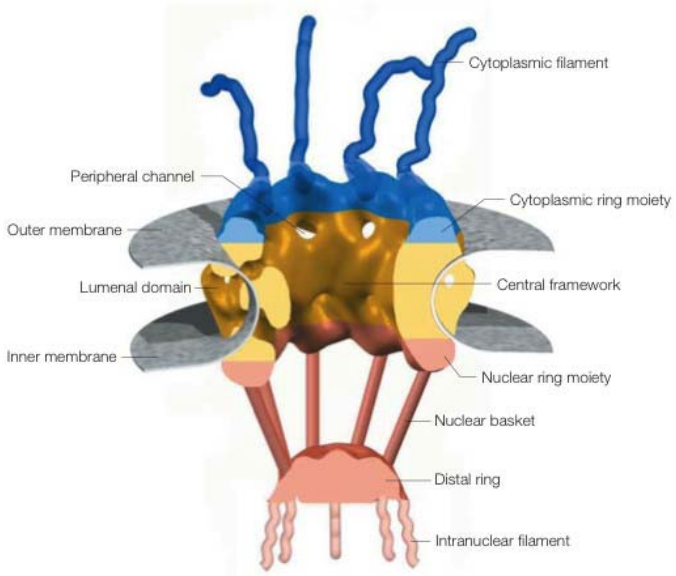



**Fig. 1 Schematic structure of nuclear-pore complex.**
The nuclear-pore complex consists of an eightfold symmetric central framework. The cytoplasmic ring moiety of the central framework is decorated with eight cytoplasmic filaments, whereas the nuclear ring moiety is topped with eight tenuous filaments that join distally into a massive distal ring and thereby form a distinct nuclear basket (from *Fahrenkrog and Aebi 2003*).

Active transport between nucleus and cytoplasm involves primarily three classes of macromolecules: substrates, adaptors and receptors (Sebastian et al. 2004). The movement of macromolecules into and out of the cell nucleus is usually mediated by soluble transport receptors that recognize specific sequences or structural characteristics of their cargoes. Particularly, these receptors recognize the nuclear localization signal (NLS), which contains a cluster of basic amino acids (i.e. lysine and arginine), as well as the nuclear export signal (NES), which is rich in hydrophobic amino acids (i.e. leucine or isoleucine). Both these sequences give specificity to the transport (Nigg 1997). The majority of known transport receptors mediating interactions with the NPC belong to the importin β super-family of RanGTP binding proteins, also called karyopherins. Karyopherins act as chaperones during nucleocytoplasmic translocation (Rogue 2002). Passage through the pore requires weak and transient binding of importin β receptors to the nucleoporin FG repeats (Meyer and Vinkemeier 2005). Furthermore, energy consumption confers

directionality to this process, which is therefore also termed active transport (Meyer and Vinkemeier 2005).

Ran is the small Ras-like GTPase crucial for maintaining the direction of transport. It controls the assembly of the karyopherin-cargo complex and ensures the directionality of nucleocytoplasmic protein trafficking (Izaurralde et al. 1997). Ran switches from a GDP- to a GTP-bound state, thus regulating the binding of substrates to the transport receptors. However, its intrinsic activity of GDP/GTP exchange or GTP hydrolysis is very low. Therefore, Ran GTPase cycle is modulated by various interacting proteins that regulate its guanine nucleotide-bound state. The guanine nucleotide exchange factor, Ran-GEF (also called RCC1, regulator of chromosome condensation 1) accelerates the dissociation of guanine nucleotide from Ran and facilitates the conversion of RanGDP to RanGTP. Since RCC1 is exclusively localized in the nuclear compartment, the generation of the GTP-bound form of Ran occurs in the nucleus. Conversely, Ran GTPase activity is stimulated by the GTPase-activating protein, Ran-GAP, which is exclusively localized in the cytoplasm. Consequently, the binding of Ran-GTP to an importin (import receptor) triggers the release of bound cargo. On the other hand, the binding of Ran-GTP to an exportin (export receptor) facilitates the assembly of the exportin-cargo complex. This asymmetry ensures the efficient release of the import cargo within the nucleus and that of the export cargo in the cytoplasm (Yoneda 2000).

After traversing the nuclear pore complex, importins and exportins release their cargo and recycle, moving back to the other side of the nuclear envelope to begin a new transport event. Thus, transport receptors and Ran shuttle continuously between nucleus and cytoplasm, moving rapidly back and forth across the NPCs (Gama-Carvalho and Carmo-Fonseca 2001). A schematic representation of the import/export mechanism is shown in Fig. **2**.

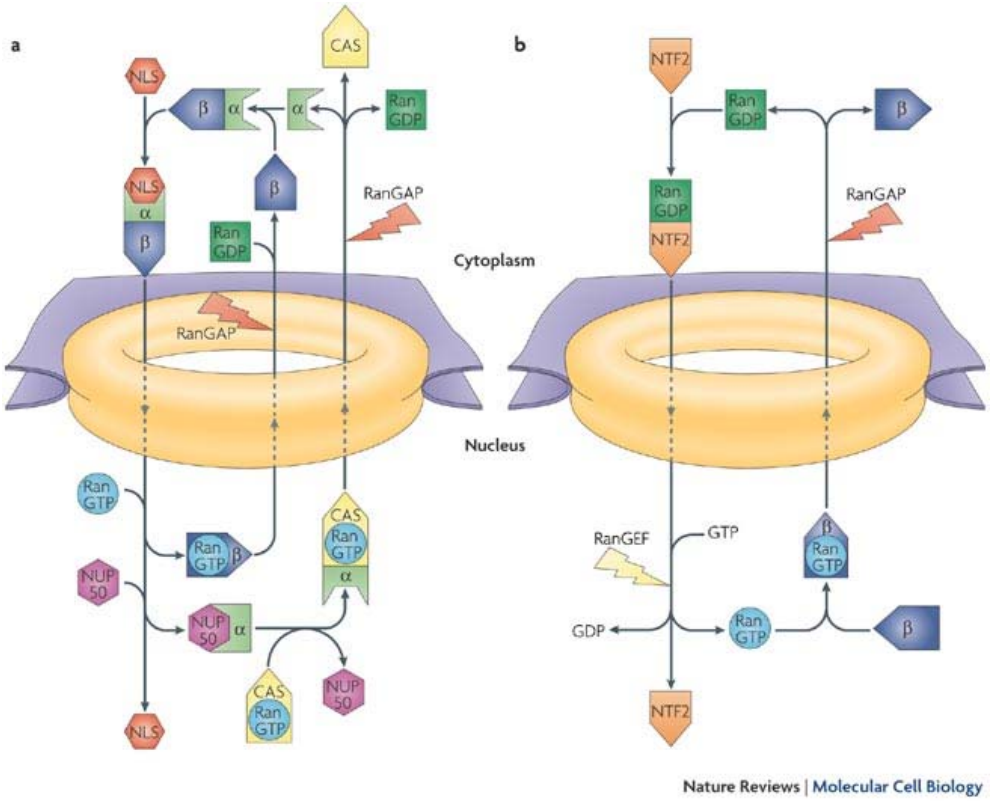



**Fig. 2 Nucleocytoplasmic transport of proteins.**
In **a,** the complex NLS cargo/importin $\alpha$/importin β is translocated through the nuclear pore complex into the nucleus. After entering the nucleus, the NLS cargo is dissociated by Ran-GTP. Importin β and Ran-GTP are recycled back out through the pore to the cytoplasm, where Ran-GTP is hydrolyzed to Ran-GDP. Importin $\alpha$ is carried out of the nucleus by the nuclear export receptor CAS. This export involves complex formation with Ran-GTP, and then CAS is transported back into the nucleus. NUP50 is a Ran-binding protein and a co-factor for importin α/β-mediated import. In **b**, NES cargoes bind to the exportin β/Ran-GTP complex before they are exported out of the nucleus. Hydrolysis of Ran-GTP to Ran-GDP by RanGAP promotes complex dissociation in the cytoplasm. Thus, the exportin is transported back into the nucleus, where it can re-associate with a NES cargo and Ran-GTP to start the process over. NTF2 binds specifically to Ran-GDP and mediates efficient interaction with the nuclear pore complex and translocation into the nucleus (from *Kau et al. 2004*).

The mechanism regulating the nuclear import/export of proteins has been dissected using the Simian Virus 40 (SV40) T-antigen NLS as a model for the import and the Human Immunodeficiency Virus (HIV) Rev protein or Protein Kinase I (PKI) NES for the export. It took approximately thirty years for the first shuttling protein, the nucleolin, to be identified and scientists have today learned that a protein previously believed to be

confined to the cell nucleus actually shuttles between the nucleus and the cytoplasm (Gama-carvalho and Carmo-Fonseca 2001). Furthermore, a variety of transport pathways simultaneously operating in cells have been identified (Gorlich and Mattaj 1996). Importin β receptors need in many cases an adaptor, importin α, which binds both, the substrate proteins and importin β. In such a way, importin α forms a bridge between the import receptor and the protein cargo (Sebastian et al. 2004). In addition, other adapter molecules have been identified. They include snurportin 1, which specifically functions as NLS receptor for small-nuclear ribonucleoproteins (snRNPs), and Xenopus RPA Interacting Protein alpha (XRIPα), which is required for the nuclear import of replication protein A (RPA; Yoneda 2000). It has also been demonstrated that importin β can by itself mediate the nuclear import of certain karyophiles without the aid of adapter molecules, such as the viral protein Rev (Yoneda 2000). However, certain proteins are translocated through the NPC in a Ran-independent manner, as occurs in the nuclear translocation of β-catenin (Xu and Massaguè 2004).

The best characterized protein export mechanism is mediated by Chromosome Region Maintenance 1 (CRM1 or exportin1), a protein originally identified in the fission yeast *Schizosaccharomices pombe* (Ossareh-Nazari et al. 1997). The sequence similarity between exportin1 and importin β provided an initial lead to the discovery that CRM1 is a receptor for nuclear export. The role of CRM1 in nuclear export of protein is now well established. By a highly conserved mechanism, it recognizes leucine-rich NES-containing cargoes thus exporting them to the cytoplasm (Fukuda et al. 1997; Kuersten et al. 2001). Leptomycin B (LMB) is a potent inhibitor of CRM1-mediated nuclear export (Kudo et al. 1998). It was isolated from *Streptomyces sp* strain ATS 1287 and initially implicated in a screen for compounds that block the export of HIV Rev protein. To date, LMB is the best characterized small-molecule inhibitor of CRM1-dependent nuclear transport (Kau and Silver 2003). The nuclear export inhibition mechanism involves the direct binding of LMB to CRM1 and the consequent inhibition of the binding of CRM1 to its cargoes. NES-CRM1 interaction was identified on a conserved region near cysteine-529 residue of human exportin1 and this residue provides LMB sensitivity (Kudo et al. 1998). When LMB binds CRM1, the NES-bearing nuclear export cargo fails to bind the receptor and nuclear export is impaired.

Cells constantly exchange a variety of information between the nucleus and the cytoplasm through the modulation of subcellular localization of proteins. Therefore, nuclear trafficking is regulated in several ways. The first is the number of pores, which can be shifted up or down depending on the transcriptional activity in the nucleus. In growing cells, for instance, the number of nuclear pores increases concomitantly with S-phase entry (Yoneda 2000). Traffic across the nuclear envelope may also be regulated by masking NLSs and NESs on the cargoes (Yoneda 2000). An example of this is the nuclear factor κB (NFκB). In unstimulated cells, it is generally localized in the cytoplasm as an inactive form complexed with its own inhibitor (IκB). Upon cell challenging with TNF-$\alpha$ (tumor necrosis factor-alpha), or CD 40 (cluster of differentiation 40) ligand, or IL-1 (interleukin-1), IκB is phosphorylated and then unmasking of NFκB-NLS occurs. Thus, the nuclear factor translocates into the nucleus and activates the target genes that promote cell proliferation (Kau and Silver 2003).

Another control mechanism of import/export is represented by the anchoring of cargoes on one side of the nuclear membrane, as occurs for the ribonucleoproteins (RNPs) that hold immature RNAs in the nucleus until their processing is completed (Kuersten et al. 2001).

It has also been demonstrated that the hetero- or homodimerization of proteins can regulate the nuclear import of proteins. This is the case of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. The "canonical" model of JAK-STAT pathway proposes that cytokine-binding to its own receptor induces activation of the JAK family tyrosine kinase, which phosphorylates the STAT molecules on tyrosine. Once phosphorylated, STATs dimerize in the cytoplasm and in such a way they translocate into the nucleus, thus activating gene transcription (Yoneda 2000; Meyer and Vinkemeier 2005). This is just one example of how the phosphorylation/dephosphorylation process regulates nucleo-cytoplasmic transport of proteins.

Furthermore, it has also been observed that cyclin B1 is phosphorylated at G2/M transition of cell cycle. This event inhibits the interaction between cyclin B1 and CRM1. As a consequence, reduction of cyclin B1 nuclear export occurs and cell cycle is fostered (Jones et al. 2000). It has also been reported that cytoplasmic relocalization of the cdk inhibitor, p27, is regulated by the serine-threonine kinase Akt (Shin et al. 2002). According to these data, Akt-mediated phosphorylation of p27 inhibits its nuclear re-

entry. This event facilitates cell cycle progression and cell transformation in various cell types, including breast cancer cells (Viglietto et al. 2002). In agreement with these data, impairment of p27 localization has been reported in many human cancers (Slingerland and Pagano 2000; Blain and Massaguè 2002).

Altogether, these data demonstrate the key role of phosphorylation/ dephosphorylation in the import/export process. Moreover, they show that impairment of nucleocytoplasmic shuttling of proteins is involved in deregulation of cell cycle and cell transformation (Kau et al. 2004). A list of proteins mislocalized in different cancers is presented in **Table 1**.

**Table 1. Protein mislocalization in cancers**

| Target protein | Location in normal cells | Location in cancer cells | Result of mislocalization | Implicated cancers |
|---|---|---|---|---|
| NF-κB | cytoplasm | nucleus | Inhibition of apoptosis | Breast, ovary, colon, pancreas and thyroid |
| FOXO | nucleus | cytoplasm | Cell cycle arrest and inhibition of apoptosis | Renal, colon and glioblastoma multiforme |
| p27 | nucleus | cytoplasm | E2F1 activation and cell cycle progression | Esophagus, thyroid, colon and breast |
| p53 | nucleus | cytoplasm | No DNA-damage response | Neuroblastoma, retinoblastoma, colorectal, ovarian and breast |
| APC | cytoplasm | nucleus | β-catenin nuclear retention | Colorectal |
| β-catenin | cytoplasm | nucleus | Binding and activation of LEF1 | Colorectal |
| INI1 | nucleus | cytoplasm | Activation of cyclin D1 and E2F targets | Atypical teratoid rhabdoid |
| ERα | nucleus | cytoplasm | Enhancement of non-genomic functions of ERα | Breast |

Abbreviations: **NF-κB,** nuclear transcription factor of immunoglobulin light-chain κ in B cells; **FOXO**, forkhead related family of transcription factors, sub-group O; **p27**, cyclin-dependent kinase inhibitor that runs as a 27 kilodalton protein on SDS-PAGE; **E2F1**, transcription factor, member of E2F family and activator of the adenovirus E2 promoter; **p53**, transcription factor that runs as a 53 kilodalton protein on SDS-PAGE; **APC**, adenomatous polyposis coli; **β-catenin**, a subunit of the cadherin protein complex; **LEF1**, lymphoid enhancing factor 1; **INI1**, a component of the ATP-dependent chromatin remodelling hSWI/SNF complex; **ERα**, estradiol receptor alpha (modified by *Kau et al. 2004*).

Because of mislocalization of various proteins in human cancer, it is now accepted that interference in the nucleocytoplasmic shuttling of proteins represents a new approach to manipulate cellular commitment. Methods that re-direct the proteins to the correct cellular compartment have been developed. They include inhibitors of the transport machinery as well as small-molecules or lipid-kinase inhibitors specifically modulating the transport of target proteins (Kau and Silver 2003).

## 1.2 Shuttling of Steroid Receptors

Steroid receptors belong to a superfamily of nuclear receptors (NRs). These ligand-activated transcription factors are involved in normal physiology and in a variety of human diseases (Deroo and Korach 2006). The members of this family share common structural features. Particularly, they are characterized by three principle distinct domains: the most variable N-terminal transactivation domain; the most highly conserved central DNA-binding domain (DBD); and the relatively conserved C-terminal ligand-binding domain (LBD; Tata 2002). A schematic representation of nuclear receptors is depicted in Fig. 3.

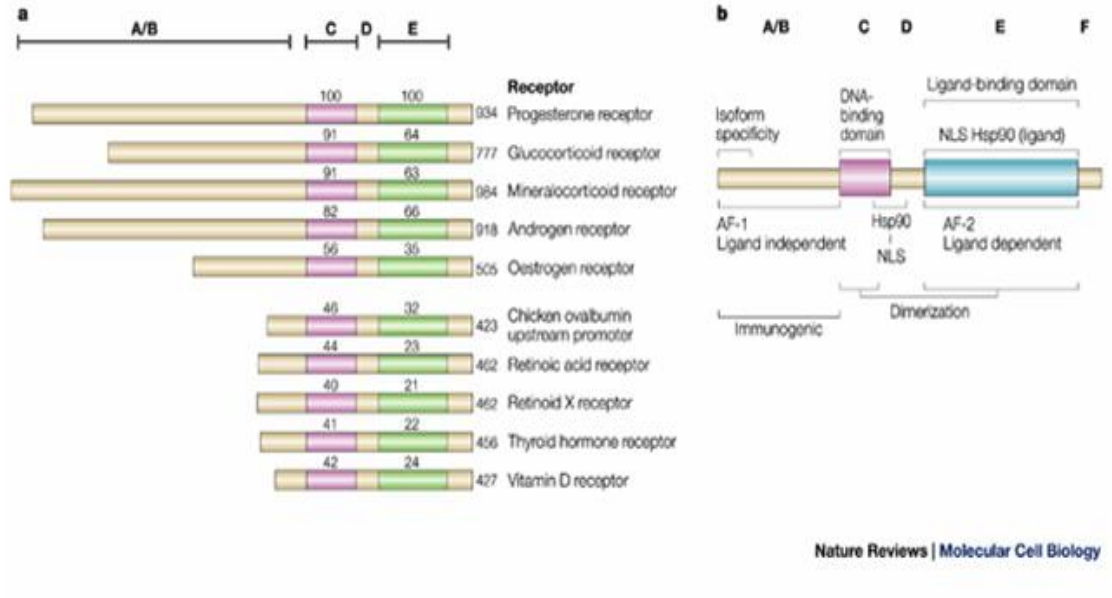



**Fig. 3 Structure and function of nuclear receptors.**
In **a**, the number of amino acids at the amino terminus indicates the relative sizes of the human receptors. The modular structure is represented by the domains, A/B, C, and E. The numbers above the bars indicate the percentage amino acid homology of the consensus regions of the DNA- and ligand-binding domains. In **b**, some important functions that are associated with the different domains of nuclear receptors are depicted (AF, activation function; Hsp, heat shock protein; NLS, nuclear localization signal). The three principle domains are presented in the figure. They include the transactivation, ligand-independent domain, endowed in N-terminal region, the DNA-binding domain, endowed in the central region, and the transactivation, ligand-dependent domain, endowed in C-terminal region (from *Tata 2002*).

The "classical" mechanism of steroid action proposes that unliganded NRs are associated with a chaperone protein complex, made of above all heat shock proteins (hsp70, hsp90) and immunophilins (Ylikomi et al. 1992). Binding to the hormone leads to a conformational change in the receptor acting as an on-switch. It therefore induces the release of NRs from chaperone molecules, the dimerization and entrance of NRs into the nucleus, where they bind to palindromic hormone response elements (HREs) within the regulatory regions of target genes (Rochette-Egly 2005). The mechanism of gene transcription activation by liganded NRs, however, relies on a complex network of interactions between NRs and co-regulatory proteins (Mangelsdorf et al. 1995). Depending on the complexes that NRs form with co-activators or co-repressors, they function both as activators or inhibitors of transcription (Tata 2002). For most NRs, this network is directed by a specific domain, the activation function 2 (AF-2) domain, located in the LBD. Upon ligand binding, transactivation domain undergoes major structural rearrangements. The AF-2 domain cooperates with a second, ligand-independent, activation domain residing in the N-terminus, the AF-1 domain. It recruits multiple complexes to alter the chromatin structure surrounding the promoter of target genes. Finally, these events pave the way for recruitment of RNA polymerase II (RNA Pol II) and the general transcription factors (GTFs) (Rochette-Egly 2005).

In addition to their well-studied nuclear function, steroid receptors participate in extranuclear or membrane-initiated signaling events. Such a non genomic action has been linked to the rapid responses elicited by steroid hormones. These events are independent of RNA or protein synthesis and occur within minutes or seconds. They involve activation of Src, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K), protein kinase C (PKC) and etherotrimeric G-proteins in cytoplasm or membrane of target cells (Migliaccio et al. 2007). Interestingly, important biological responses such as DNA synthesis and cytoskeleton changes occur in the absence of transcriptional activity or nuclear localization of steroid receptors (Castoria et al. 2008). However, depending on the cell type and experimental conditions, steroid action may depend on integration between extranuclear and nuclear activities of their receptors (Vicent et al. 2006). For these and other reasons, analysis of intracellular distribution of nuclear receptors may contribute to a better understanding of their action in target tissues.

Steroid receptors continuously shuttle between cytoplasm and nucleus (De Franco 2001). Although the import mechanism of steroid receptors has been extensively dissected, their export mechanism is still obscured. All NRs contain nuclear localization sequences in the "hinge" region between the DBD and the LBD. Glucocorticoid, estrogen and progesterone receptors-NLSs, for instance, share similar position and sequence (Picard and Yamamoto 1987; Guiochon-Mantel et al. 1991; Ylikomi et al. 1992). In contrast, no classical nuclear export sequences have so far been identified in NRs and conflicting data have been reported on inhibition of steroid receptor nuclear export by LMB treatment (De Franco 2001). Although it is generally accepted that NRs lack classic leucine-rich NESs, they have sequences with limited homology to NESs (Liu and De Franco 2000). In addition, since the exact spacing of the hydrophobic residues (i.e. leucine) in NES of each protein is subject to variation, it is difficult to define a nuclear export sequence on this basis alone (Nigg 1997). Under some circumstances, indeed, LMB treatment inhibits the nuclear export of NRs (Savory et al. 1999; Prufer and Barsony 2002; Maruvada et al. 2003).

ERα shuttles from nucleus to cytoplasm and the antiestrogen ICI 182780 disrupts this process (Dauvois et al. 1993). In addition, ligand binding and protein-protein interactions significantly influence the nucleocytoplasmic shuttling of the chimeric protein GFP-ER (Maruvada et al. 2003). In the presence of ligand, glucocorticoid, androgen, thyroid hormone and progesterone receptors (GR, AR, TR and PR) rapidly shuttle between nuclei and cytoplasm (De Franco 2001). Hormone withdrawal induces accumulation of GR and AR in the cytoplasm of target cells (Tyagi et al. 2000). Furthermore, a classic mouse AR permanently localized in the cytoplasmic compartment does not activate gene transcription, even if it recruits several signaling effectors allowing S-phase entry and cytoskeletal changes in fibroblasts (Castoria et al. 2003). In addition, the molecular mechanism responsible for cytoplasmic cross-talk between ER, AR and epidermal growth factor receptor (EGFR) has been recently dissected in breast and prostate cancer cells (Migliaccio et al. 2005). Besides, much evidence shows that a cross-talk occurs between p53 and GR in mediating responses to stress (i.e. hypoxia) in normal endothelial cells (HUVEC). The two proteins form a trimeric complex with the E3 ubiquitin ligase Hdm2 (human double minute 2) in the cytoplasm. Upon dexamethasone stimulation of HUVEC cells, cytoplasmic sequestration of GR and p53 occurs. This event leads to increased degradation of the two proteins

through recruitment and activation of the proteasome pathway (Sengupta and Wasylyk 2001).

Moreover, expression of a shortened form of the metastatic tumor antigen (MTA1s) sequesters estradiol receptor in the cytoplasm and leads to malignant phenotypes by enhancing ER extranuclear signaling in hormone-dependent cancer cells (Kumar et al. 2002). On the other hand ER association with the modulator of non genomic action of steroid receptors (MNAR) may help to sequester the receptor in the cytoplasm or membrane of breast cancer cells (Vadlamudi et al. 2005). Thus, tumorigenesis and hormone-resistance of breast cancer cells follow (Gururaj et al. 2006).

Altogether, these findings point to the critical role of SR extranuclear localization in steroid action. Therefore, further research into steroid receptor localization might lead to a better understanding of their roles in the normal physiology of cells. These studies could also provide new insights into the diagnosis, treatment and prevention of steroid-related diseases, mainly hormone-dependent tumors.

## 2. AIM

It is well established that steroid receptors shuttle between the nuclear and the cytoplasmic compartments of cells. Nevertheless, the nuclear export mechanism of estradiol receptor is still obscure. Therefore, this thesis focuses on the study of the molecular mechanism underlying ERα nuclear export and its role in the estradiol action.

To this end we used MCF-7 cells, which derive from human breast cancer and express the isoform alpha of estradiol receptor (ERα). They represent a model for the study of estradiol-induced biological effects.

We firstly attempt to identify the sequence responsible for nuclear export of the receptor, NES-ERα, and then to establish whether the export mechanism of ER depends on CRM1.

Finally, we attempt to gain more insight in the functional role of ER nuclear export in breast cancer cells. We previously reported that, once activated by the hormone, cytoplasmic ER recruits various signalling effectors with mitogenic effect. Therefore, we address the role of ER subcellular localization in cell cycle progression of MCF-7 cells.

# 3. MATERIALS AND METHODS

## 3.1 Constructs

The constitutive active Ran Q69L was in pQE plasmid (Izaurralde et al., 1997). The pCDNA/HA-CRM1 (Alt et al., 2000) was digested with KpnI and BamH1 and subcloned into pSG5. cDNAs encoding the wild type of hERα (HEG0) or its deletion mutants (HEG14, HEG15, HE241G and NLS-HEG14) were in pSG5 expression vector (Tora et al., 1989). They were subcloned into EcoRI-pEGFP (C2, Clontech) or into EcoRI-pSG5 Myc, as reported (Castoria et al. 2004). The Myc-tagged pSG5 was prepared as described (Castoria et al. 2004). Forkhead (FKHR), either wild type (wt) or its mutated form (AAA-FKHR), was in pcDNA-GFP (Addgene). For the export assay, the putative export sequences of ERα were cloned into the BamHI and AgeI sites of pREV(1.4)-GFP and inserted between the REV and GFP coding sequence (eGFP-N1, Clontech). To insert the putative export ER*α* sequence into pREV(1.4)-GFP, a PCR strategy was adopted for the following fragments: *NLS sequence* (241-306 aa) was amplified by forward primer:
5′-GACTGGATCCAATGATGAAAGGTGGGATACGAAAAGACCG-3′
and reverse primer:
5′-GCGACCGGTGGCAGGCTGTTCTTCTTAGAGCGTTTGATCA-3′;
*286 sequence* (286-311 aa) forward primer:
5′-GATCGGATCCAATGAGAGCTGCCAACCTTTGGCCAAGCCCG-3′ and reverse primer:
5′-GCGACCGGTGGGGCCGTCAGGGACAAGGCCAGGCTGTTCT-3′;
*286 sequence* (286-382 aa) reverse primer:
5′-GCGACCGGTGGGGCACATTCTAGAAGGTGGACCTGATCATG-3′;
*296 sequence* (296-335aa) forward primer:
5′-GATCGGATCCAATGATCAAACGCTCTAAGAAGAAC-3′
and reverse primer:
5′-GCGACCGGTGGGGGTCTGGTAGGATCATACTCGGAATAGA-3′;
*316 sequence (*316-335aa*)* forward primer:
5′-GATCGGATCCAAGTGCCTTGTTGGATGCTGAGCCCCCCAT-3′;
*341 sequence* (341-361 aa) forward primer:
5′GATCGGATCCATCGATGATGGGCTTACTGACCAACCTGGC-3′
and reverse primer:

5′-GCGACCGGTGGCGCCCAGTTGATCATGTGAACCAGCTCCC-3′;
*368 sequence* (368-394 aa) forward primer:
5′-GATCGGATCCAGTGGATTTGACCCTCCATGATCAGGTCCA-3′
and reverse primer
5′-GCGACCGGTGGGCGCCAGACGAGACCAATCATCAGGATCT-3′;
*381 sequence* (381-412 aa) forward primer:
5′-GATCGGATCCATGTGCCTGGCTAGAGATCCTGATGATTGGT-3′
and reverse primer:
5′-GCGACCGGTGGGTTCCTGTCCAAGAGCAAGTTAGGAGCAA-3′;
*395 sequence* (395-412 aa) forward primer
5′GATCGGATCCATCCATGGAGCACCCAGTGAAGCTACTGTT-3′;
*427 sequence* (427-457 aa) forward primer:
5′-GATCGGATCCAATGCTGCTGGCTACATCATCTCGGTTCCG-3′
and reverse primer
5′-GCGACCGGTGGTCCAGAATTAAGCAAAATAATAGATTTGAG
GCACAC-3′;
*381-457 sequence*;
*458-552 sequence* forward primer:
5′-GATCGGATCCAGTGTACACATTTCTGTCCAGCACCCTGAAGTC
T-3′ and reverse primer:
5′-GCGACCGGTGGGGGCGCATGTAGGCGGTGGGCGTCCAGCATC
TC-3′.

All these fragments were amplified using Platinum Pfx (Invitrogen), according to the manufacturer's instructions. PCR products were purified, digested with BamHI/AgeI and finally subcloned into pREV(1.4)-GFP. The ER 444-456 sequence was subcloned in the same vector, as a short fragment generated by annealing of specific oligonucleotides. The same strategy was utilized to generate the ER 444-456 mutant. All putative ER NESs were verified by sequencing to confirm the exact reading frame between REV and GFP. The site-directed mutations of ERα were introduced into the pSG5-HEG0 using a standard PCR methodology. Two mutants were generated: pSG5-HEG4A (Ile 451 and 452 changed with Ala; Leu 453 and 454 changed with Ala) and pSG5-HEGIL (Ile 452 changed with Ala; Leu 454 changed with Ala). The cDNAs coding for the ER$\alpha$ mutants (HEG4A and HEGIL) were subcloned into EcoRI-pEGFP. All junctions were verified by sequencing.

### 3.2 Cell culture

Human breast cancer-derived MCF-7 cells and low passage embryo mouse NIH 3T3 fibroblasts were grown and made quiescent as described (Castoria et al. 1999; Castoria et al. 2003).

### 3.3 Transfection experiments, nuclear export and transactivation assays

Unless otherwise stated, quiescent MCF-7 cells on coverslips were transfected with 1 μg of each purified plasmid using the SuperFect reagent (Qiagen). After transfection, the cells were incubated at 37°C for 24 h, and then used for the indicated experiments. The nuclear export assay was performed as described (Henderson and Eleftheriou, 2000). Briefly, each putative NES-ER sequence was subcloned into the Rev 1.4-GFP and then transfected ( at 2 μg) by SuperFect into growing MCF-7 cells. After 18 h the cells were incubated with cycloheximide (at 15 μg/ml; Calbiochem) and left untreated or treated for 8 h with actinomycin D (at 5μg/ml; Calbiochem). When indicated, leptomycin B (at 5 ng/ml; Calbiochem) was added 30 min before the addition of actinomycin D. Growing NIH3T3 fibroblasts, plated on Petri dishes or coverslips, were transfected by SuperFect using 2 μg of either pEGFP or pEGFP-HEG0 or pEGFP-HEGIL. For transactivation assay, 2 μg of pGL2 ERE-Luc were cotransfected. Twenty-four hours after transfection, the cells were made quiescent and then left untreated or treated with 10 nM estradiol for 16 h. Lysates were prepared and luciferase activity was measured using a luciferase assay system (Promega). The results were corrected using CH110-expressed β-galactosidase activity (Amersham Biosciences).

### 3.4 DNA synthesis analysis and peptides

Quiescent MCF-7 cells and NIH 3T3 fibroblasts on coverslips were left unstimulated or stimulated with the indicated compounds for 24 h and 18 h, respectively. Estradiol was added at 10 nM and epidermal growth factor was added at 100 ng/ml. When indicated, the Tat peptide conjugated to the 444-456 amino acid sequence of ER$\alpha$ (Tat-pep; Ac-EFVCLKSIILLNS-AAA-RKKRRQRRR-NH2) as well as the Tat control peptide (ctrl) were added at 1 μM. The peptides were N-terminal acetylated and C-terminal amidated. For S-phase entry analysis, the

cells were made quiescent and then left unstimulated or stimulated with the indicated compounds. Estradiol was used at 10 nM, epidermal growth factor was used at 100 ng/ml and serum was added at 20%. After a 6-h pulse with 100 μM BrdU (Sigma, St.Louis, MO), BrdU incorporation was analyzed by immunofluorescence using mouse monoclonal anti-BrdU antibody (Amersham Biosciences, UK), as reported (Castoria et al. 1999).

**3.5 Immunofluorescence and confocal microscopy**

Cells on coverslips were fixed for 10 min with Para formaldehyde (3%, w/v in PBS), washed with phosphate buffer serum (PBS), and then analyzed by fluorescence microscopy for the subcellular localization of GFP-HEGO, its mutants as well as putative ER-NES 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 (Castoria et al. 1999). Endogenous ER$\alpha$ was visualized using either the A314 mouse monoclonal antibody or the H222 rat monoclonal antibody (Castoria et al. 1999). The Myc-tagged pSG5 or HEG0 or HEGIL and p27 were detected as described (Castoria et al. 2004). The mouse monoclonal anti p53 antibody (D0-1 Santa Cruz) was used to detect p53 (Bai and Merchant 2001). Coverslips were finally stained with Hoechst 33258, inverted and mounted in Mowiol (Calbiochem, CA). Fields were analyzed with a DMBL Leica (Leica) fluorescent microscope using 40, 63 and 100x objectives. Images were processed using IMI1000 or FW4000 (Leica) software. When indicated, the distribution of fluorescence was analyzed by a confocal LSM 510 Zeiss microscope.

**3.6 Purified proteins and *in vitro* protein-protein interactions**

Ran Q69L expressed in JM109 bacteria was purified using the Ni-NTA agarose (Qiagen). The recombinant human ER$\alpha$ (2800pmol/ml) was from Panvera. $S^{35}$-labeled HA-CRM1 was produced *in vitro* using rabbit reticulocyte lysate (Promega). *In vitro* protein-protein interaction was performed as described (Migliaccio et al. 2000). When indicated, the purified peptide 427 NH2-MLLATSSRFRMMNLQGEEFVCLKSIILLNS-COOH (from Primm; Milan) was used at 200-fold excess. Immunoprecipitation was performed incubating the mixture at 4°C for 1 h using the rabbit polyclonal anti-ER Ab (clone G-20, Santa Cruz; at

1μg/ml) or the mouse monoclonal anti-HA Ab (Covance; at 2μg/ml) antibodies. Immunocomplexes were reduced using Laemmli sample buffer and eluited proteins were separated by SDS-PAGE and revealed by immunoblotting or fluorography respectively.

**3.7 Lysates, electrophoresis and immunoblotting**

The cellular lysates were performed as described (Migliaccio et al. 2000) and protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad CA). The rabbit polyclonal anti-ER antibody (clone G-20, Santa Cruz) was used to detect ER$\alpha$. Expression and production of Ran Q69L was verified using the mouse monoclonal anti-His tag antibody (Qiagen). ERα and the chimera GFP-ERα were detected using either the rat monoclonal (H222 from Abbott Laboratories) anti-ERα antibody or the mouse monoclonal anti-GFP antibody (Clontech). Immunoreactive proteins were revealed by the ECL detection system (Amersham Biosciences).

# 4. RESULTS AND DISCUSSION

## 4.1 Estradiol regulates ER shuttling in MCF-7 cells

Localization of ERα in quiescent MCF-7 cells was determined by immunofluorescence, using two different antibodies directed against either a C-terminal, H222 MAb (Katzenellenbogen et al. 1987), or an N-terminal epitope of ERα, A314MAb (Abbondanza et al. 1998). Fig. 4**a** shows that, regardless of the antibody used, 30 minutes of estradiol treatment (10 nM) of cells induces nuclear translocation of ERα, which is followed by a decrease in the nuclear localization of the receptor towards the basal level upon 1-hour hormone stimulation. Immunoblotting of cell lysates with anti-ER monoclonal antibodies does not reveal any significant change in ERα amount (Fig. 4**a**$_1$), thus showing that the observed decrease in ERα nuclear localization at 1 h is due to nuclear exit of the receptor.

We then applied a widely used approach in studying sublocalization of proteins. A full-length ERα cDNA was subcloned into a green fluorescent protein encoding plasmid (pEGFP). Quiescent MCF-7 cells were transiently transfected with the obtained chimera (GFP-HEG0; HEG0 represents the wild type ERα). By quantitative score of cells showing nuclear fluorescence, we observe in Fig. 4**b** that in the absence of any significant change in GFP-ERα expression (Fig. 4**b**$_1$), hormone treatment triggers nuclear accumulation of GFP-ERα after 30 minutes. Thirty minutes later the nuclear GFP-ERα is reduced towards the basal levels. It is noteworthy that the estradiol-regulated trafficking of ERα we observe is reminiscent of ERα cycling onto and off the cathepsin D promoter in response to the estradiol treatment of MCF-7 cells (Shang et al. 2000). In addition, no trafficking of GFP-ERα was observed in untreated cells.

Since ERα nuclear uptake is inhibed by the pure antiestrogen ICI 182780 (Dauvois et al. 1993), we analyzed the effect of a partial antiestrogen, the 4-hydroxy-tamoxifen (4-OH TX). Fig. 4**b** shows that

this antagonist prevents the observed nucleocytoplasmic shuttling of GFP-ERα in response to hormone treatment. Fig. 4**c** shows that in the absence of any significant change in GFP-ER expression (Fig. 4**c**$_1$), actinomycin D (Act D) does not modify the estradiol-induced import-export of GFP-ER. Therefore, the observed cytoplasmic GFP-ER increase detectable after 60 minutes of hormone treatment arises from nuclear export rather than from *de novo* synthesis. Since actinomycin D also inhibits nuclear import of the NES-containing REV protein (Henderson, 2000) and GFP-ER dynamic redistribution is not modified by actinomycin D (Fig. 4**c**), we conclude that cytoplasmic relocalization of chimera depends on nuclear export rather than inhibition of nuclear import.

Ultimately, this set of data shows that estradiol modulates the nucleocytoplasmic shuttling of its own receptor in MCF-7 cells.

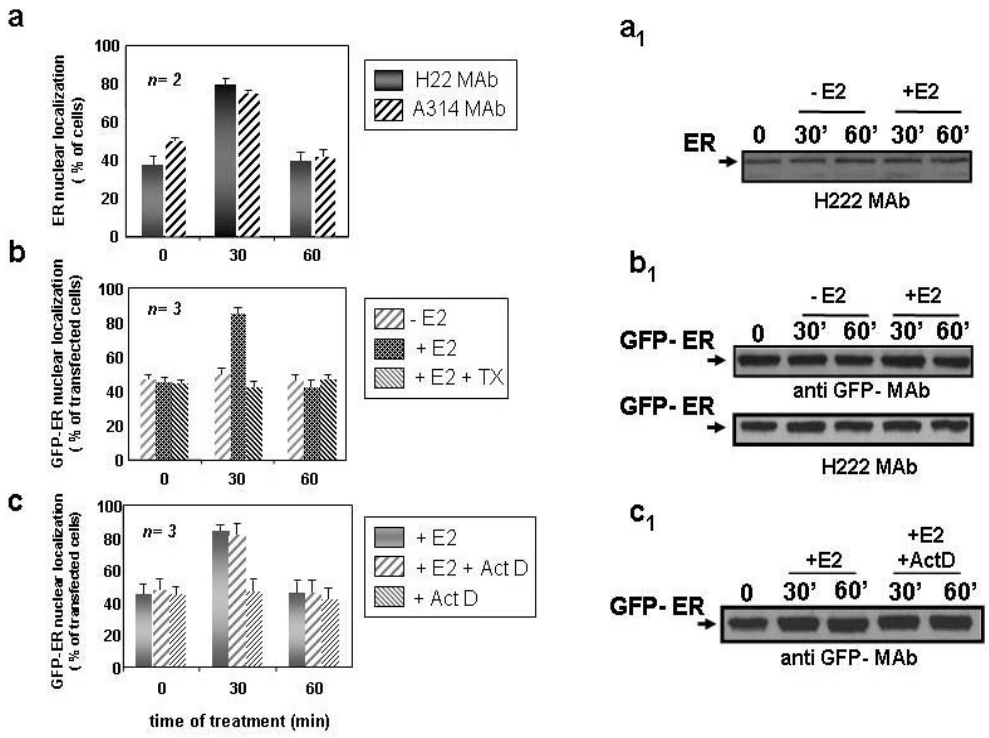



**Fig. 4 Estradiol induces rapid nucleocytoplasmic shuttling of its own receptor in MCF-7 cells.**
Quiescent MCF-7 cells were used. In **a**, cells were untreated or treated with 10 nM estradiol ($E_2$) for the indicated times (min). ER$\alpha$ localization was analyzed by fluorescence microscopy using the indicated antibodies. In **b** and **c**, cells were transfected with pEGFP-HEG0, then left untreated or treated with the indicated compounds. 4-OH-Tamoxifen (TX) was used at 0.1 μM; actinomycin D (Act D; at 5 μg/ml) was added 1 h before estradiol stimulation. The GFP-HEG0 localization was determined by fluorescence. Cells that fell into the category of exclusively nuclear fluorescence were scored and data expressed as a percentage of total cells (in **a**) or transfected cells (in **b** and **c**). For each experiment, data were derived from at least 1000 scored cells. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. Right panels (**a**$_1$, **b**$_1$ and **c**$_1$) show the Western blot of lysates obtained from one experiment in **a**, **b** or **c**, respectively. Analysis was performed using the antibodies against the indicated proteins.

**4.2 Leptomycin B blocks the nuclear export of GFP-ER**

LMB is an anti-fungal compound which blocks the nuclear export of NES-containing proteins by preventing their association with the CRM1 export receptor (Kudo et al. 1998). We tested the effect of this export inhibitor on the estradiol-induced shuttling of GFP-ER. The quantitative score of transfected cells shows that 1 hour after hormone stimulation, LMB causes nuclear accumulation of GFP-ERα in estradiol-stimulated cells. Interestingly, LMB is ineffective when added alone to the resting cells (Fig. 5**a**). Images from one experiment in **a** are presented in panels **b**. Thus, the LMB effect on ERα localization indicates that this receptor is exported from nuclei through the CRM1/exportin pathway.

To address this question, an *in vitro* protein-protein interaction assay was performed. Fig. 5**c** shows that estradiol induces a strong interaction between the recombinant proteins ER*α* and CRM1 when active Ran (Ran-GTP; Askjaer et al. 1999) was included as a GTP-ase deficient mutant (RanQ69L; Bischoff et al. 1994).

Altogether, data in Fig. 5 show that the CRM1 pathway definitely contributes to ER nuclear export triggered by estradiol in MCF-7 cells.

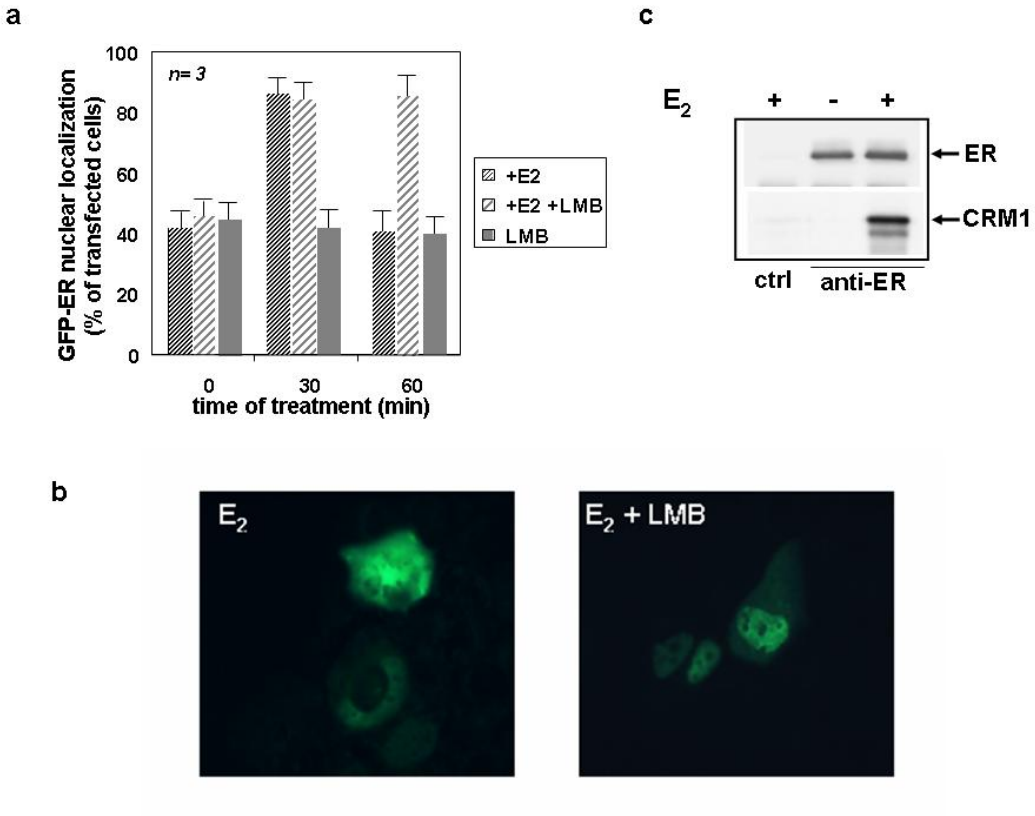



**Fig.5 The estradiol-induced nuclear exit of ERα depends on CRM1.**
In **a**, quiescent MCF-7 cells were transfected with pEGFP-HEG0. Cells were left untreated or treated for the indicated times with estradiol (10 nM) in the absence or presence of LMB (5 ng/ml). LMB was added 30 min before the hormone. Cells were also treated with LMB alone. The GFP-HEG0 localization was determined by fluorescence and data were derived from at least 600 scored cells. The results of several independent experiments were averaged (*n* represents the number of experiments); means and SEM are shown. In **b**, images from one experiment in **a** were captured. They show the GFP-ER localization in MCF7 cells stimulated for 60 min with estradiol ($E_2$), in the absence (left panel) or presence (right panel) of LMB. In **c**, $^{35}$S-labeled HA-CRM1 was incubated with recombinant ERα from baculovirus in the absence or presence of estradiol (10 nM). The purified recombinant RanQ69L (1 μM) was included in the incubation mixture of each sample. Proteins were immunoprecipitated with the rabbit polyclonal antibody (ctrl) or the rabbit polyclonal anti-ERα antibody (anti-ER). Immunocomplexes were revealed by immunoblot analysis (upper panel) or fluorography (lower panel).

### 4.3 Identification of domains involved in the hormone-regulated nucleocytoplasmic shuttling of ER

Two ER mutants, HEG15 and HEG14, were subcloned into pEGFP. HEG15 (Δ282-595 HEG0) contains highly conserved nuclear localization signals, NLS 2 and 3 (Guiochon-Mantel et al. 1991). It binds DNA but does not bind estradiol (Ylikomi et al. 1992). Meanwhile, HEG14 (Δ1-281 HEG0) contains NLS1. It binds the hormone, but does not bind DNA (Ylikomi et al., 1992). A schematic representation of resulting chimeras, GFP-HEG15 and GFP-HEG14, is shown in Fig. 6**a**. The constructs were transiently transfected into quiescent MCF-7 cells. In agreement with previous results (Ylikomi et al., 1992), data in Fig. 6**b** show that neither GFP-HEG15 nor GFP-HEG14 shuttle between the nucleus and the cytoplasm. Regardless of hormone 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 (Fig. 6**b**). From these data, we speculated that the hormone-binding domain endowed in the HEG14 mutant as well as NLSs (2 and 3) of ER$\alpha$ are both required for estradiol-induced nuclear import of the receptor. We next subcloned the HE241G into pEGFP. This mutant contains the hormone-binding domain and lacks the three NLSs (Fig. 6**a**). It is in fact prevalently localized in the cytoplasm regardless of estradiol treatment (Fig. 6**c**) and similar results have been previously obtained by ectopic expression of this mutant in NIH3T3 fibroblasts (Castoria et al. 1999). The behavior of this mutant confirms that in the absence of NLSs, hormone binding is not sufficient to induce nuclear translocation of the receptor.

Since HEG15 is localized in the nuclear compartment and its localization is unaffected by hormone treatment of cells, we reasoned that addition of NLSs to HEG14 might restore estradiol-induced import of ER$\alpha$. Analysis of transfected cells with the resulting chimera, GFP-NLS/HEG14, shows not only nuclear import but also export similar to GFP-HEG0 in response to the estradiol treatment (Fig. 6**c**).

This set of experiments shows that the 241-595 aa sequence of ER$\alpha$ is responsible for the estradiol-regulated nucleocytoplasmic shuttling of ER in MCF-7 cells.

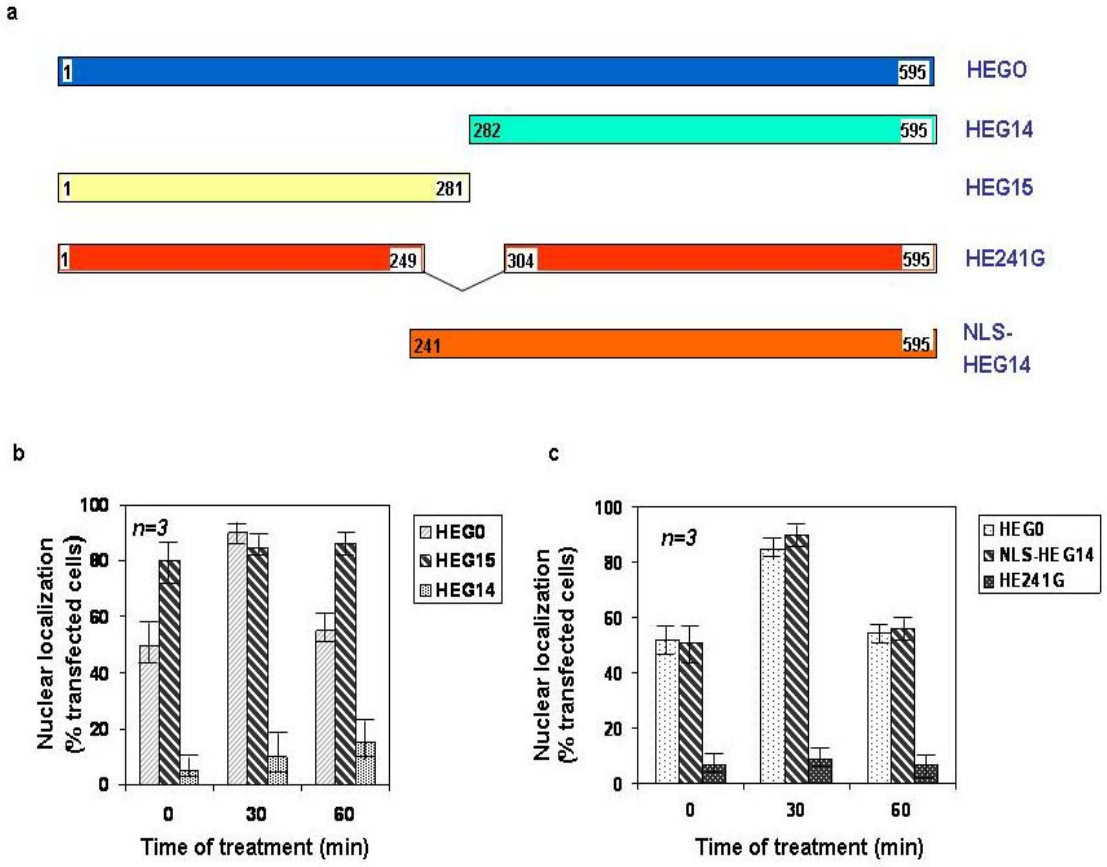



**Fig. 6 The 291-595 ERα domain is responsible for the import/export of estradiol receptor in MCF-7 cells.** In **a**, a schematic representation of cDNAs encoding wild type ERα (HEG0) and its deletion mutants (HEG14, HEG15, HE241G and NLS-HEG14) is shown. In **b** and **c**, quiescent MCF-7 cells were transfected with the indicated constructs. Cells were untreated or treated for the indicated times with estradiol (10 nM). The localization of chimeras was determined by fluorescence microscopy. Cells that fell into the category of exclusively nuclear fluorescence were scored and data expressed as a percentage of transfected cells. The results of several independent experiments (*n* represents the number of experiments) were averaged. For each experiment data were derived from at least 600 scored cells. Means and SEM are shown.

### 4.4 Identification of an ERα nuclear export sequence

Findings in Fig. 6 point to the presence of nuclear export sequence(s) in the NLS/HEG14 construct, which is made up of the nuclear localization signals and the hormone-binding domain of ERα.

It has been reported that bi-directional transport of RNA-associated proteins depends on M9 motif, which functions as both NLS and NES (Nigg 1997) and that nucleocytoplasmic shuttling of proteins can also be controlled by the emerging class of bi-directional transport signals, called nucleocytoplasmic shuttling signals (NSs) (Michael 2000). In addition, it has been suggested that nuclear localization signals of the progesterone receptor are also responsible for the nuclear exit of this receptor (Guiochon-Mantel et al. 1994). Therefore, we verified by an *in vivo* export assay (Henderson and Eleftheriou 2000) the possibility that NLSs are involved in both the import and export of ERα.

According to this assay, nuclear export sequences are identified by their ability to restore the export activity of the chimera NES-deficient Rev1.4-GFP at levels similar to those observed with the wild-type Rev-GFP or the Rev1.4-GFP NES (NES is the canonical export sequence of the Rev protein). Therefore, NLS sequences of estrogen receptor were subcloned into pRev1.4-GFP and transfected in MCF-7 cells. After transfection, cells were incubated in the absence or presence of actinomycin D, since it causes cytoplasmic accumulation of the putative NES-containing Rev protein by preventing nuclear import of Rev (Henderson 2000). Fig. 7**a** shows that, irrespective of experimental conditions, Rev1.4-GFP NLS is localized in nuclei of MCF-7 cells. In the same experiment, the Rev1.4-GFP NES, used as a positive control, completely shifts to the cytoplasm in the presence of actinomycin D, whereas the mutant Rev1.4-GFP shows a nuclear/nucleolar staining. In conclusion, the NLS sequences of ERα are ineffective in this assay.

We then subcloned different sequences of ERα containing leucine residues into pRev1.4-GFP. These constructs were transfected into MCF-7 cells and then analyzed for their ability to restore the nuclear

export of the mutant Rev1.4-GFP. By means of this assay, we identified a 427-457 amino acidic sequence of ERα that, although less active than the Rev1.4-GFP NES positive control, shifts the Rev1.4-GFP mutant from nuclei to cytoplasm in cells treated with actinomycin D (Fig. 7**a**). Interestingly, LMB treatment blocks the export activity of 427-457 ERα signal. Altogether, these data show that the 427-457 region is involved in the export of ERα through CRM1/exportin binding. Furthermore, a peptide corresponding to the 427-457 ERα sequence (427) specifically displaces the estradiol-induced interaction between recombinants CRM1 and ERα (Fig. 7**b**). In contrast, a control peptide (ctrl) does not interfere in ER*α*/CRM1 interaction.

In conclusion, data in Fig. 7 show that the 427-457 sequence is responsible for CRM1-dependent ERα export in MCF-7 cells.

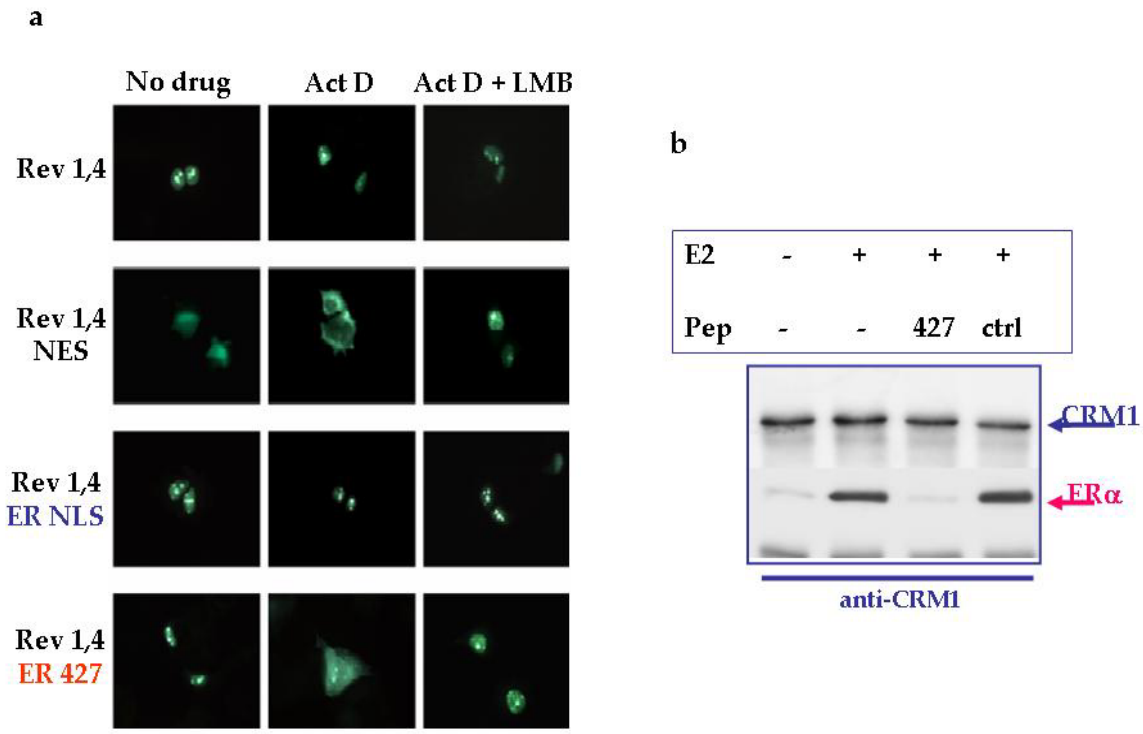
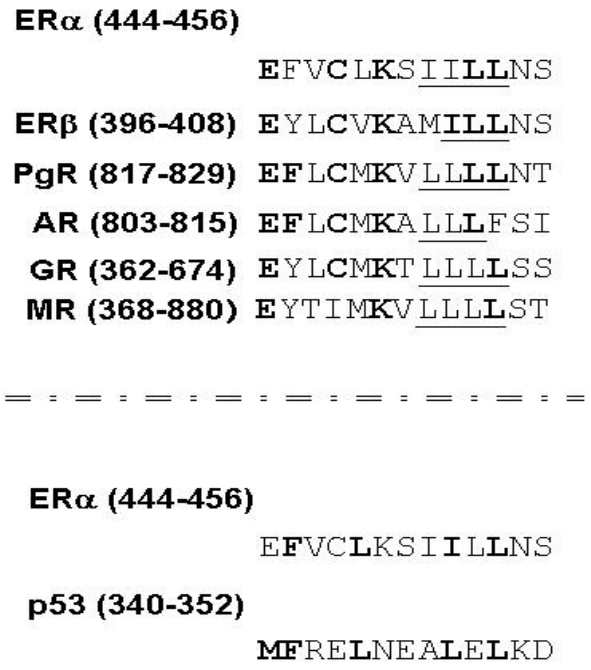



**Fig. 7 The 427-457 ERα sequence restores the export activity of NES-deficient Rev 1.4-GFP.**
MCF-7 cells were used and nuclear export assay was performed in growing cells as described in Methods. In **a**, the cells were transfected with the indicated constructs (GFP-Rev 1.4, GFP-Rev 1.4 NES, GFP-Rev 1.4 ER NLS and GFP-Rev 1.4 ER 427). After transfection, cells were left untreated (no drug) or treated with actinomycin D (Act D; 5 μg/ml) alone or together with leptomycin B (LMB; 5 ng/ml). The subcellular distribution of GFP proteins was analyzed by fluorescence microscopy and images were captured and shown. They are representative of three independent experiments showing that, in the presence of actinomycin D, the ERα 427-457 sequence rescues the nuclear export of GFP-Rev 1.4 by 70%. In **b**, $^{35}$S-labeled HA-CRM1 was incubated with recombinant ERα in the absence or presence of estradiol (10 nM), alone or together with a 200-fold excess of the ERα 427-457 peptide (427). A 200-fold excess of a non-specific peptide was used as control (ctrl). CRM1 was immunoprecipitated with the anti-HA monoclonal antibody and proteins in immunocomplexes were revealed by fluorography (upper panel) or immunoblotting with the rabbit polyclonal anti-ERα antibody (lower panel).

A limited sequence analysis showed homology between the 444-456 amino acids of ERα and the putative NES sequences of other steroid receptors. Furthermore, the putative NES-ERα sequence also showed homology with the conserved leucine-rich and Rev-like NES of p53, 340-352 (Stommel et al. 1999). The alignment between the sequences is presented in Table 2.

**Table 2. The putative NES-ERα is conserved in steroid receptor family and shows homology with a NES of p53**




The **upper** section shows the sequence alignment between the indicated regions of ERα and other steroid receptors. Sequences were obtained from the National Center for Biotechnology Information Data Library: ERα (estrogen receptor alpha) accession no. NP000116; ERβ (estrogen receptor beta) no. NP001035366; PgR (progesterone receptor) no. NP000917; AR (androgen receptor) no. NP000035; GR (glucocorticoid receptor) no. P04150; MR (mineralcorticoid receptor) no. P08235. Conserved amino acids are in bold. The core of ER-NES sequence and the putative core of NES sequences in the indicated steroid receptors are underlined. The **lower** section shows the sequence alignment between the indicated regions of ERα and p53, with the conserved amino acids in bold.

The possibility that the 444-456 amino acids of ER*α* sequence actually represents the ER-NES was then investigated. For this purpose, the 444-456 ER sequence as well as its mutated version (containing 4 residues of alanine instead of IILL amino acids in position 451-454; Fig. 8**a**) were subcloned in pRev1.4-GFP. Interestingly, the ER wild type 444-456 sequence is able to shift the Rev1.4-GFP into the cytoplasm of MCF-7 cells, whereas its mutated form fails to do so (Fig. 8**b**). This difference is even more evident in the presence of actinomycin D.

Taken together, results in Fig. 8 show that a very limited sequence of the ER$\alpha$ hormone-binding domain is responsible for CRM1-mediated ER$\alpha$ nuclear export.

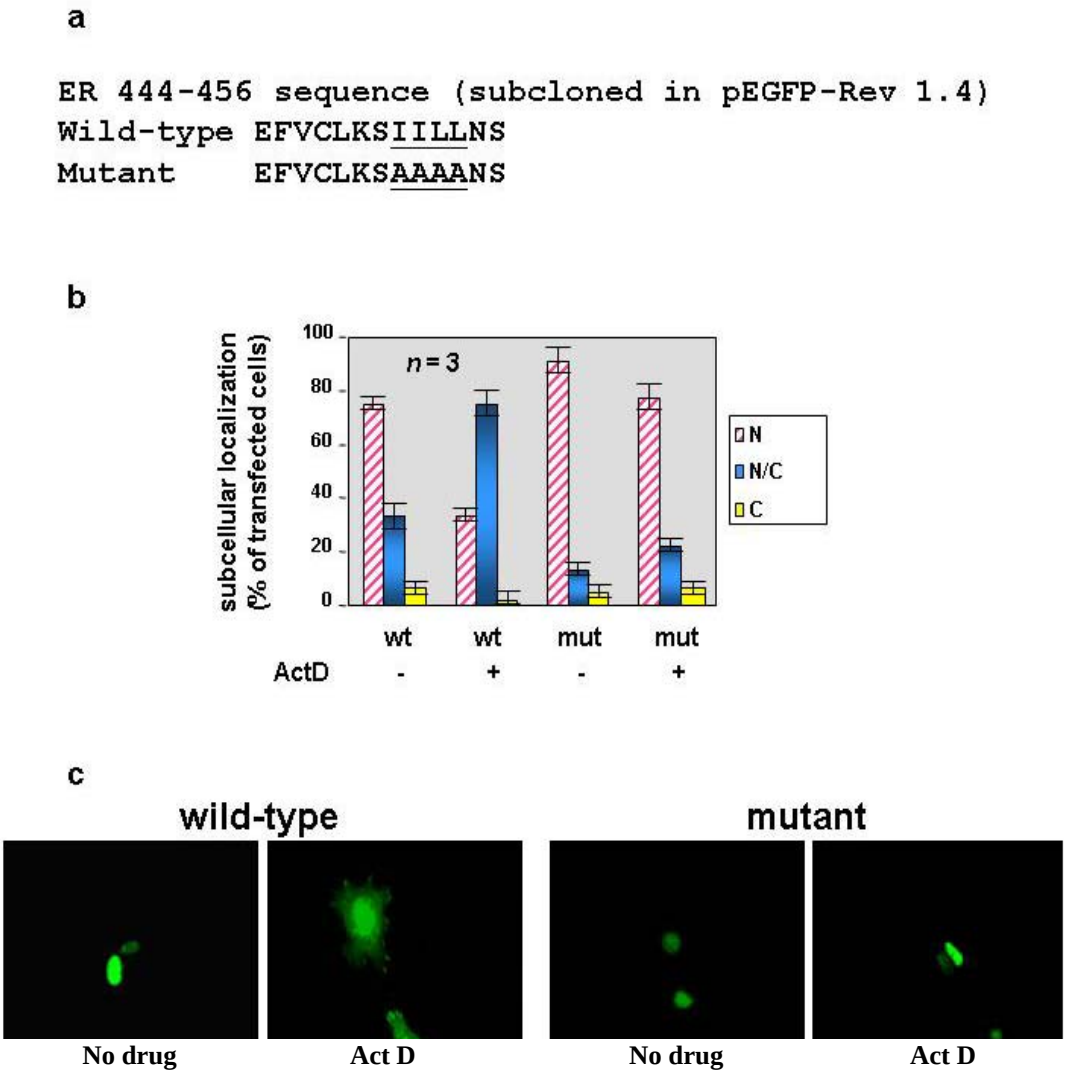



**Fig. 8 The limited ER 444-456 sequence is the NES-ERα sequence.**
In **a**, a schematic representation of ER 444-456 sequence and its mutant version is shown. The two limited sequences were subcloned into pEGFP-Rev 1.4 and transfected into growing MCF-7 cells. In **b**, nuclear export assay was performed as desribed in Methods. After transfection, the cells were left untreated or treated with actinomycin D (Act D; at 5μg/ml) for 8 h. The subcellular distribution of GFP proteins was analyzed by fluorescence microscopy. Number of cells that fell into the categories of nuclear (N), nucleocytoplasmic (N/C) or cytoplasmic (C) respectively were scored and data were expressed as a percentage of transfected cells. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. For each experiment data were derived from at least 600 cells. Images from one experiment in **b** are captured and shown in **c**.

**4.5 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.**

Following recent approaches (Joliot and Prochiantz 2004), a Tat-conjugated peptide construct corresponding to the 444-456 ERα sequence was synthesized (Fig. 9**a**). The carboxyfluorescein-conjugated peptide translocated across plasma membrane and reached the nuclei of MCF-7 cells within 30 minutes, as assessed by confocal microscopy experiments (data not shown). In addition, the specificity of Tat-conjugated peptide was analyzed monitoring its ability to modify the nuclear export of either p27 or a classic leucine-rich NES-containing protein, such as p53 (Stommel et al. 1999). Fig. 9**b** shows that addition of peptide does not modify p27 nuclear export as assessed by immunofluorescence analysis of NIH 3T3 cells treated with EGF. Moreover, the peptide does not induce nuclear accumulation of p53 in estradiol-treated MCF-7 cells (Fig. 9**c**). It is also ineffective on subcellular localization of pREV1.4-GFP NES ectopically expressed in MCF-7 cells (data not shown).

Interestingly, data in Fig. 9**d** and 9**e** show that that pre-incubation of MCF-7 cells with the peptide specifically blocks the 60 min estradiol-induced nuclear export of GFP-ERα, thus sequestering the receptor in nuclei (Fig. 9**d** and 9**e**). Similar results were obtained when the dynamic redistribution of endogenous ERα was followed by indirect immunofluorescence (data not shown).

It is known that the extranuclear activity of ERα triggers hormone-dependent DNA synthesis (Castoria et al., 1999). We therefore investigated the effect of Tat-peptide on S-phase entry in MCF-7 cells. Panel **f** in Fig. 9 shows that treatment of MCF-7 cells with the Tat-conjugated peptide inhibits BrdU incorporation induced by estradiol by 60%, whereas addition of Tat alone, as a control, does not interfere in DNA synthesis. Moreover, the Tat-conjugated peptide does not inhibit serum-induced DNA synthesis in MCF-7 cells (Fig. 9**f**), indicating the specificity of the peptide in interference with ERα action.

Interestingly, addition of the Tat-conjugated peptide after 60 min of estradiol stimulation, when ERα is almost completely exported from cell nuclei, does not affect S-phase entry in MCF-7 cells (Fig. 9**g**), thus reinforcing the view that the 60-min estradiol-induced nuclear export of ER plays a role in cell cycle progression in breast cancer cells.

**a** **Tat-pep** Ac-EFVCLKSIILLNS-AAA-RKKRRQRRR-$NH_2$

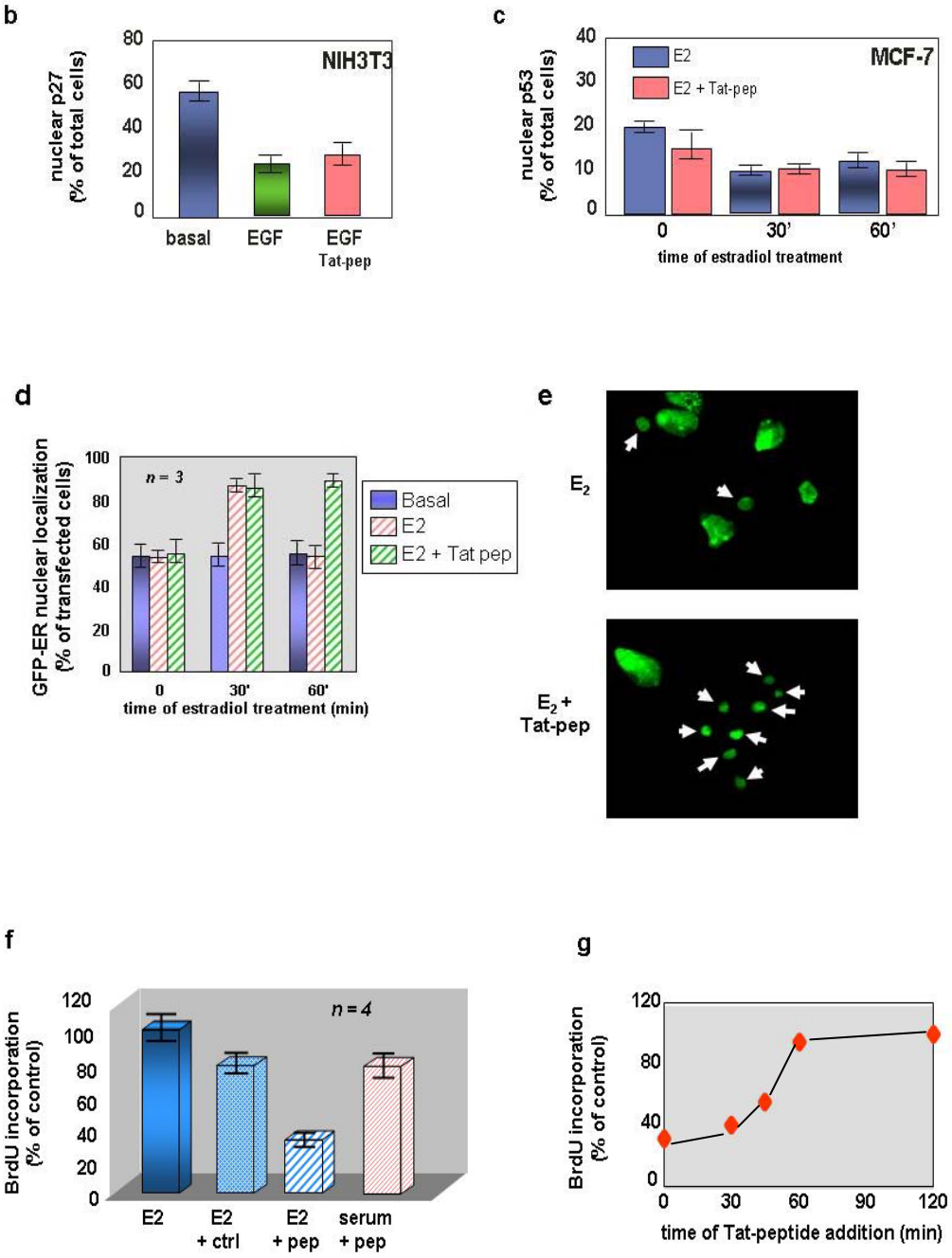


**Fig. 9 The specific peptide mimicking the (444-456)-ERα sequence sequesters estradiol receptor in the nucleus and inhibits hormone-dependent S-phase entry in MCF-7 cells.**

In **a**, the sequence of the Tat-conjugated peptide mimicking the 444-456 ERα sequence is shown. In **b** and **c**, NIH 3T3 fibroblasts and MCF-7 cells were used. Quiescent cells were left untreated or treated with EGF (100 ng/ml; panel **b**) and $E_2$ (10 nM; panel **c**), in the absence or presence of the Tat-conjugated ERα peptide (Tat-pep). The peptide (1μM) was added to the cell medium 1 hour before stimulation. p27 and p53 were detected by immunofluorescence. The percentage of cells with prevalent nuclear p27 or p53 was determined by fluorescence microscopy and graphically shown. For each experiment, at least 200 cells were scored. The results of two independent experiments were averaged; means and SEM are shown. In **d**, quiescent MCF-7 cells were transfected with GFP-HEG0. After transfection cells were left untreated or treated with $E_2$ (10 nM) for the indicated times (min), in the absence or presence of the Tat-conjugated ERα-peptide. The peptide was added at 1μM to cell medium 1 hour before cell stimulation. The percentage of cells with prevalent nuclear GFP protein was analyzed by fluorescence microscopy and graphically shown. For each experiment, at least 200 cells were scored. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. Images of one experiment in **d** are presented in panel **e**. It shows the intracellular distribution of GFP-ER in MCF-7 cells treated for 60 min with $E_2$ (10 nM) in the absence (upper microphotograph) or presence (lower microphotograph) of Tat-peptide. The arrows indicate the cells showing nuclear distribution of GFP-ER. In **f**, quiescent MCF-7 cells were stimulated with either $E_2$ (10 nM) or 20% serum for 24 hours. When indicated, the Tat- peptide (pep) or the control peptide (ctrl) were added at 1μM to the cell medium 1 hour before cell stimulation. After *in vivo* pulse with BrdU, DNA synthesis was analyzed by immunofluorescence and BrdU incorporation expressed as a percentage of control. In the control cells, BrdU incorporation induced by estradiol was approximately 70%. The basal BrdU incorporation (almost 10%) was in each case calculated and subtracted. For each experiment, at least 300 cells were scored. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. In **g,** quiescent MCF-7 cells were stimulated with $E_2$ (10 nM) for 24 hours and the Tat-conjugated ERα peptide was added after hormone stimulation at the time points indicated in the figure. After *in vivo* pulse with BrdU, DNA synthesis was analyzed as in **f**. For each experiment, at least 300 cells were scored. For each experiment, at least 300 cells were scored. The results of two independent experiments were averaged.

**4.6 ERα NES mutants do not exit nuclei and fail to induce DNA synthesis stimulated by estradiol.**

Based on the findings in Fig. 8 and in Fig. 9, we prepared by site-directed mutagenesis two mutants of full-length ERα-GFP. A schematic representation of the GFP-HEG0 NES and its mutated versions is shown in Fig. 10**a**. The constructs were transiently transfected in MCF-7 cells. As expected, estradiol treatment induces nuclear export of GPF-HEG0. In contrast, both mutants, GFP-HEG4A and GFP-HEGIL, are unable to exit nuclei upon hormone stimulation (Fig. 10**b**). Images in **c** show a diffuse, sometimes extranuclear localization of GFP-HEG0 in MCF-7 cells stimulated for 60 minutes with estradiol. Under these conditions, a nuclear/nucleolar localization of both GFP-HEG4A and GFP-HEGIL is detected.

The ability of the mutant GFP-HEGIL to activate gene transcription and induce S-phase entry in response to estradiol treatment was then verified. ERα-negative NIH 3T3 fibroblasts (Castoria et al., 1999 and 2003) were transiently transfected with GFP-HEG0 or GFP-HEGIL or GFP alone, as a control. ERE-Luc reporter gene was cotransfected and its activity assayed. Data in Fig. 10**d** show that the constructs GFP-HEG0 and GFP-HEGIL are equally efficient in activating gene transcription upon estradiol stimulation of the cells, with an induction of the ERE-Luc activity ranging from 6- to 6,3-fold. Transcriptional activation is almost undetectable in unstimulated cells or cells expressing GFP alone as a control.

In another set of experiments, we assessed the ability of GFP-HEG0 and GFP-HEGIL to mediate DNA synthesis induced by estradiol in transfected NIH 3T3 fibroblasts. In agreement with our previous findings (Castoria et al. 1999), we observe that GFP-HEG0 is a potent inducer of S-phase entry in cells stimulated with estradiol. Conversely, the mutant GFP-HEGIL fails to do so. Nevertheless, the mutant is still able to commit the cells towards the S-phase upon serum stimulation (Fig. 10**e**). Here again, the Tat-conjugated peptide inhibits estradiol-induced S-phase entry of GFP-HEGO transfected fibroblasts, whereas it

does not interfere in serum-induced BrdU incorporation (bars with asterisks in Fig. 10**e**).

Altogether, data in Fig. 10 show that the leucine rich 444-456 sequence of ERα contains a functional NES. Mutations in the **IILL** core of this sequence affect estradiol-induced nuclear export of ER as well as DNA synthesis, without interfering in gene transcription. These data point to the critical role of ERα nuclear export in controlling cell cycle progression modulated by estrogens in breast cancer cells.

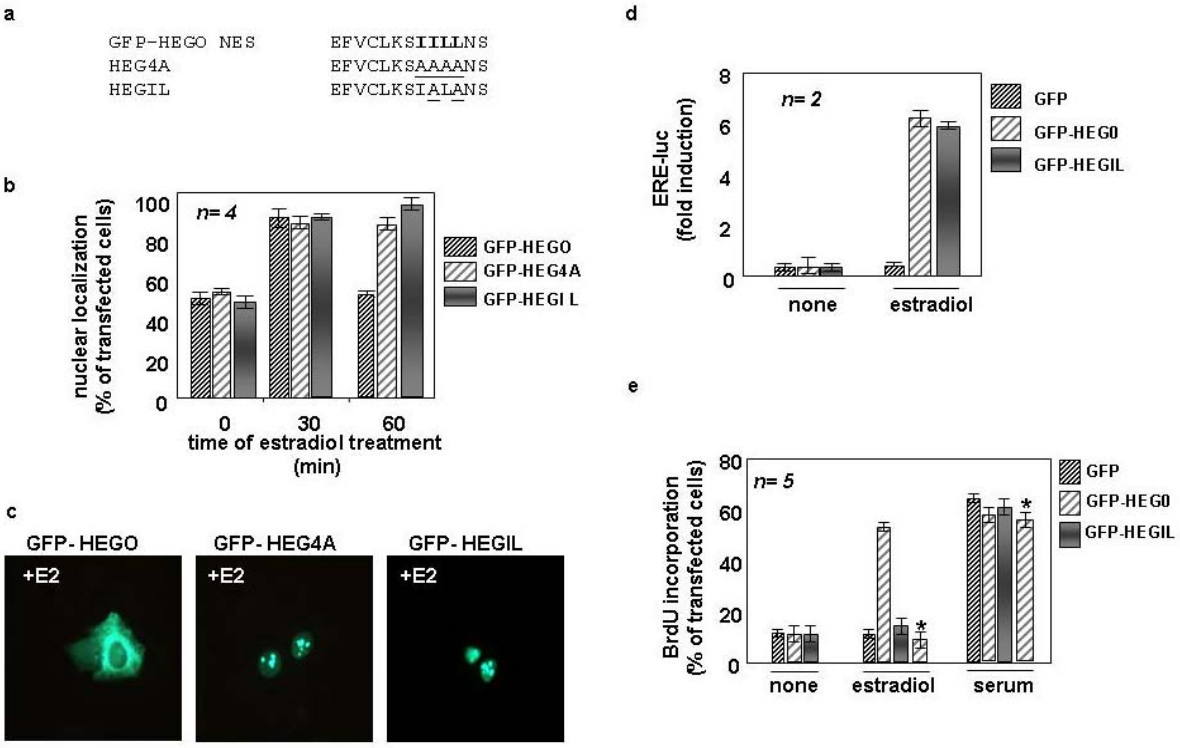



**Fig. 10 Mutations in the core of NES-ERα sequence affect the estradiol-induced nuclear export of ERα and DNA synthesis.**

In **a,** a schematic representation of NES-ERα sequence and its point-mutated versions is shown. In **b**, the cDNAs encoding ER wt (HEG0) as well as the mutants (HEG 4A and HEGIL), subcloned into the pEGFP plasmid, were transfected in MCF-7 cells. After transfection, the cells were left untreated or treated with $E_2$ (10 nM) for the indicated times. The percentage of cells with prevalently nuclear GFP proteins was determined by fluorescence microscopy and graphically shown. Data were derived from at least 600 scored cells. The results of several independent experiments (*n re*presents the number of experiments) were averaged; means and SEM are shown. Images of one experiment in **b** are captured and shown in panels **c**. They show the intracellular distribution of GFP proteins in MCF-7 cells treated with $E_2$ (10 nM) for 60 min. In **d**, growing NIH 3T3 fibroblasts were transfected with the ERE-Luc construct along with the indicated plasmids. After transfection, the cells were made quiescent and then left unstimulated or stimulated with $E_2$ (10 nM) for 16 hours. Luciferase activity was assayed, normalized using β-gal as an internal control, and expressed as fold induction. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. In **e**, growing NIH 3T3 fibroblasts were transfected with the indicated constructs. After transfection, the cells were made quiescent and left unstimulated or stimulated with either $E_2$ (10 nM) or serum (20%) for 18 hours. When indicated by an asterisk, the Tat-conjugate ERα peptide (444-456) was added (1 μM) to the cell medium 1 hour before cell stimulation. After *in vivo* pulse with BrdU, DNA synthesis was analyzed by immunofluorescence. In transfected cells, BrdU incorporation was calculated by the formula: percentage of BrdU-positive cells = (number of transfected-positive cells/number of transfected cells) x 100; the percentage of BrdU-positive cells was then compared with the percentage of BrdU incorporation of untransfected cells from the same coverslip. For each plasmid, data were derived from at least 300 scored cells. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown.

### 4.7 Estradiol simultaneously regulates nuclear export of ERα and FKHR thereby modulating S-phase entry in MCF-7 cells

It is well established that cellular localization of many proteins controls different biological responses. These proteins include cell cycle regulators and transcription factors, such as NFκB, p53 and mammalian members of the FKHR transcription factor family (Kau and Silver 2003). Therefore, it is not unexpected to find that permanent localization of transcription factors in the cell nucleus can stop cell cycle.

Stimulation of Pak-1 and ERα by estrogen treatment of breast cancer cells promotes cell survival by inducing phosphorylation and nuclear exclusion of FoxO1 (Mazumdar and Kumar 2003). In addition, an estradiol-dependent interaction between ERα and FKHR has been observed using both a yeast two-hybrid screen and immunoprecipitation analysis in MCF-7 cells (Schuur et al. 2001). Extension of these investigations to other nuclear receptor (NR) family members has shown that, depending on the receptor type, FKHR represents a bi-functional NR intermediary protein acting as either a co-activator or co-repressor (Zhao et al. 2001). It is noteworthy that FKHR nuclear export depends on its phosphorylation by Akt (Biggs et al. 1999). Interestingly, a triple alanine mutant of FKHR (FKHR AAA), which cannot be phosphorylated by Akt, localizes in the nucleus thus inducing G1 arrest in cells (Nakamura et al. 2000; Birkenkamp and Coffer, 2003). Moreover, our data have shown that estradiol activation of the PI3K-dependent pathway is required to drive cell cycle progression in breast cancer cells (Castoria et al. 2001). Therefore, we hypothesized a role for FKHR, a downstream effector of PI3-K pathway, in both estradiol-regulated ERα nuclear export and cell cycle arrest mediated by the ERα-NES mutant, GFP-HEGIL.

To address this question, we transiently transfected quiescent MCF-7 cells with the wild-type FKHR (GFP-FKHR wt) or its mutated version, containing a triple alanine substitution (GFP-FKHR AAA). Data in Fig. 11**a** show that expression of this FKHR mutant greatly reduces estradiol-induced S-phase entry in MCF-7 cells. Expression of GFP alone or GFP-FKHR wt does not interfere with estradiol-induced BrdU incorporation of cells. In the same experiments we also analyzed by confocal microscopy the role of FKHR in sub-cellular distribution of ERα. Data in Fig. 11**b** show that expression of GFP-FKHR wt or GFP alone does not modify the 60-min estradiol-induced nuclear export of

ERα. Interestingly, expression of the mutant GFP-FKHR AAA sequesters ERα in the nuclear compartment of 60-min estradiol-treated MCF-7 cells. Conversely, over-expression of the tagged ERα-NES mutant, Myc-HEGIL, retains GFP-FKHR wt in the nuclear compartment of 60-min estradiol-treated MCF-7 cells (Fig. 11**c**). Representative fields from one experiment in **b** and **c** are presented in Fig. 11**d**. Left and central panels show the staining of endogenous ERα (red) in MCF-7 cells expressing the GFP-FKHR wt (left panels) or the mutant GFP-FKHR AAA (middle panels), treated for 60 minutes with estradiol. Right panels show the staining of Myc-tagged NES-ERα mutant (red) in 60-min hormone-treated MCF-7 cells co-expressing GFP-FKHR wt. The lower microphotographs in panel **d** show the merged images.

Taken together, the data in Fig. 11 show that estradiol simultaneously regulates ERα and FKHR nuclear export in MCF-7 cells. Such mutual interplay seems to be crucial in G1-S transition, thus facilitating S-phase entry induced by estradiol in breast cancer cells.

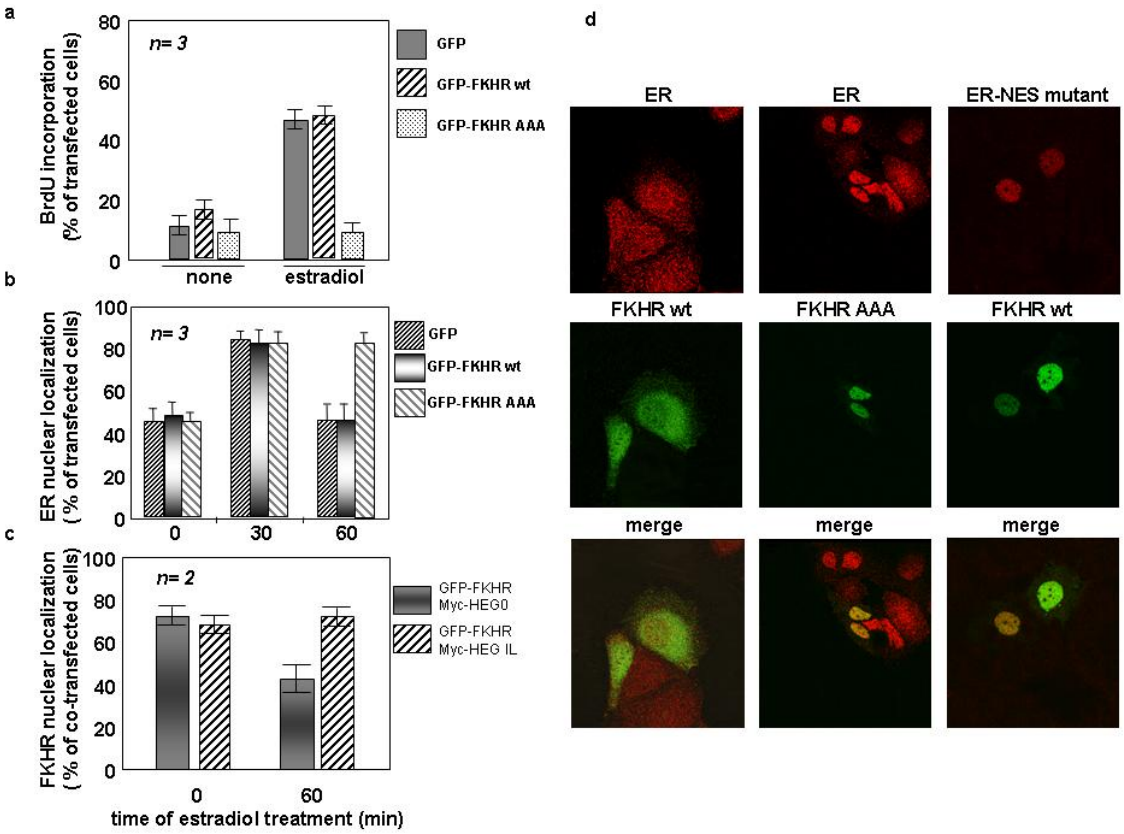



**Fig. 11 Estradiol concomitantly regulates ERα and FKHR nuclear export.**
Quiescent MCF-7 cells on coverslips were used. In **a**, cells were transfected with the indicated plasmids (GFP, GFP-FKHR wt and GFP-FKHR AAA) and then left unstimulated or stimulated with 10 nM estradiol for 24 hours. After *in vivo* pulse with BrdU, DNA synthesis was analyzed by immunofluorescence and BrdU incorporation was calculated as in Fig.10e. In **b**, cells were transfected with the indicated plasmids (GFP, GFP-FKHR wt and GFP-FKHR AAA) and then left unstimulated or stimulated with 10 nM estradiol for the indicated times. Endogenous ERα localization as well as expression of GFP, or GFP-FKHR wt or GFP-FKHR AAA mutant was monitored by confocal microscopy. Cells that fell in the category of exclusively ER nuclear fluorescence were scored and data expressed as a percentage of transfected cells. In **c**, the cells were cotransfected with the indicated plasmids (GFP-FKHR wt plus myc-HEG0 or myc-HEGIL) and 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 in the category of exclusively FKHR nuclear fluorescence were scored and data expressed as a percentage of cotransfected cells. For each experiment in **a**, **b** and **c** data were derived from at least 500 scored cells. The results of several independent experiments (*n* represents the number of experiments) were averaged; means and SEM are shown. In **d**, images from one experiment in **b** or **c** are shown. They represent the staining of endogenous ERα (red) in MCF-7 cells expressing the GFP-FKHR wt (green in left panels) or the mutant, GFP-FKHR AAA (green in middle panels) treated for 60 min with estradiol. Right panels show the staining of Myc-tagged ERα-NES mutant (red) in MCF-7 cells co-expressing the GFP-FKHR wt (green in right panels) treated with 10 nM estradiol for 60 min. Merged images are also shown in the lower pictures of the panel.

## 5. CONCLUSIONS

We analyzed the nucleocytoplasmic shuttling of estradiol receptor in MCF-7 cells focusing on its export from the nucleus. We observe that estradiol induces nuclear translocation followed by nuclear exit of endogenous ER or GFP-ER, during the initial 60 min of treatment. Leptomycin B inhibits ER export, which depends on CRM1 as shown by *in vitro* interaction experiments. Estradiol strongly enhances this *in vitro* interaction. Identification of ERα sequence responsible for its nuclear export was then assessed by a nuclear export assay. Our data show that an ER-derived leucine-rich sequence (444-456), which is highly conserved among the members of nuclear receptor family, restores the nuclear exit of a GFP-Rev mutant viral protein in an *in vivo* export assay. Furthermore, a Tat-peptide mimicking the same sequence prevents *in vitro* estradiol-dependent ER/CRM1 interaction, inhibits GFP-ER exit and greatly reduces estradiol-induced DNA synthesis in MCF-7 cells. Significantly, addition of the Tat-peptide to the cells after 60 min of hormone treatment, when ER exit is complete, does not affect S-phase entry. Interestingly, site-directed mutagenesis of the NES core in full-length ERα impairs nuclear export of receptor as well as DNA synthesis induced by estradiol, but does not affect receptor-mediated gene transcription. Altogether, these data identify and characterize for the first time a functional NES in ER. Moreover, the homology of the NES-ER sequence with sequences of several other nuclear receptors suggests that a similar export occurs in these receptors.

Furthermore, by confocal microscopy we observe that estradiol induces the simultaneous release of ER and FKHR from nuclear compartment in MCF-7 cells. ER-NES mutant traps FKHR in nuclei and *viceversa* a FKHR mutant, which cannot be phosphorylated by Akt, retains ER in nuclei. Altogether these data point to a novel link between rapid estradiol action and ER export, together with a new estradiol action on DNA synthesis in MCF-7 cells.

Present findings suggest a novel mechanism of estrogen action that involves simultaneous regulation of ER and FKHR nuclear export. Although other possibilities can be envisaged, we propose that the two proteins modulate the effectiveness of their NESs by interacting with each other. Association of FKHR with ERα, implicated by their

colocalization, might favor FKHR nuclear exit by masking its NLS. A similar action on FKHR has been reported for 14-3-3 protein (Van Der Heide et al. 2004).

Cell cycle progression is fostered by such mutual interplay between ER and FKHR, thus offering an attractive tool to manipulate cell proliferation in human breast cancers.

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# APPENDIX

*Running Title: ERα nuclear export and S-phase entry of estradiol-stimulated cells*

# HORMONE-DEPENDENT NUCLEAR EXPORT OF ESTRADIOL RECEPTOR AND DNA SYNTHESIS OF BREAST CANCER CELLS

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**ABSTRACT**

In breast cancer cells, estradiol induces in quick succession nuclear translocation and nuclear exit of the estradiol receptor (ERα). The receptor export depends on the PI3K/Akt pathway and is directed by CRM1. Experiments with construct expressing a receptor-derived sequence reveal the presence of a nuclear export signal (NES) within the hormone binding domain. This sequence restores the CRM1-dependent export of a REV-HIV mutant protein in an *in vivo* export assay. A Tat-peptide with the ERα export sequence disrupts **in vitro** the ER/CRM1 interaction and sequesters the receptor in the nuclei of MCF-7 cells. Remarkably, it inhibits the estradiol-induced DNA synthesis. In addition, ERα NES mutant obtained by site directed mutagenesis does not exit nuclei and is unable to mediate the estradiol-induced S-phase entry, leaving unaltered the receptor-dependent gene transcription. Confocal microscopy analysis shows that ERα colocalizes with forkhead in nuclei of estradiol-treated MCF-7 cells. A forkhead mutant, unphosphorylatable by Akt, is trapped together with ERwt into nuclei and blocks the estradiol-induced S-phase entry of MCF-7 cells. In turn, the ERα NES mutant fails to exit nuclei and prevents the forkhead wt nuclear export. Present findings for the first time identify a ERα NES, which is dependent on the estradiol-regulated PI3K/Akt pathway and it is CRM1-mediated. In addition, they show that nuclear export of ERα and forkhead is associated and such an association regulates G1-S transition of breast cancer cells.

## INTRODUCTION

There is a growing body of evidences that steroid receptors in addition to regulate the transcription of specific genes (Mangelsdorf et al., 1995) trigger rapid effects in the extranuclear compartment (*reviewed by* Migliaccio et al., 2007). Therefore, it is expected that hormone action depends on integration of these different receptor activities (Vicent et al., 2006).

Localization and action of ER is regulated at multiple levels, including interaction with signaling effectors or other proteins, such as the metastatic tumor antigen (MTA1) or the modulator of non genomic action of steroid receptors (MNAR; Kumar et al., 2002; Vadlamudi et al., 2005; Gururaj et al., 2006). Deregulation of these processes causes ER mis-localization and might trigger tumor progression. Overexpression of EGFR, for instance, is a hallmark of aggressive human breast cancers (Slamon et al., 1989), and a cross talk between ER and growth factor signaling has emerged as a critical factor in endocrine resistance by controlling the subcellular localization of ER signaling components (Gururaj et al., 2006). In addition, ER association with MNAR may help to sequester ER in the cytoplasm/membrane (Vadlamudi et al., 2005). Thus, enhancement of non-genomic effects of ER occur, and tumorigenesis as well as anti-hormone resistance of breast cancer cells follow (Vadlamudi et al., 2005; Gururaj et al., 2006). Furthermore, expression of a shortened form of the metastatic tumor antigen 1 (MTA 1s) sequesters ER in the cytoplasm and leads to malignant phenotypes by enhancing the ER non-genomic functions in hormone-dependent breast cancer cells (Kumar et al., 2002). These and other similar data imply that cytoplasmic localization of ER provides a mechanism to control signal transducing-dependent functions, such as DNA synthesis and anchorage-dependent growth of target cells. Moreover, they strongly suggest that ER localization compartment has functional implications in breast cancer progression.

Steroid receptors undergo nucleo-cytoplasmic shuttling (*reviewed by* De Franco, 2001). In the presence of ligand, glucocorticoid, androgen, thyroid hormone and progesterone receptors rapidly shuttle between nuclei and cytoplasm (De Franco, 2001). Hormone withdrawal from cells induces accumulation of GR and AR in the cytoplasm (Tyagi et al., 2000). ERα also shuttles from nuclei to cytoplasm, and the antiestrogen ICI 182, 780 disrupts this process (Dauvois et al., 1993). In addition, ligand binding and protein-protein interactions can

significantly influence the nucleo-cytoplasmic shuttling of the chimeric GFP-ER (Maruvada et al., 2003). However, while the mechanism of nuclear import of steroid receptors is well documented, how these receptors are exported is still unclear (De Franco, 2001).

The best-characterized nuclear export pathway uses CRM1 as a receptor for proteins with leucine-rich NESs (Fornerod et al., 1997; Fukuda et al., 1997). Leptomycin B (LMB), by covalent binding to CRM1, inhibits the 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 classic leucine-rich NESs, they have sequences with limited homology to NESs (Liu & De Franco, 2000). In addition, since the exact spacing of the leucine/hydrophobic residues in NES of each protein is subject to variation, it can be difficult to define a NES signal on this basis alone (Nigg, 1997). Under some circumstances, LMB treatment, indeed, inhibits the nuclear export of steroid receptors (Savory et al., 1999; Prufer & Barsony, 2002; Maruvada et al., 2003).

Here, we report that in MCF-7 cells ERα is a nucleo-cytoplasm shuttling protein whose trafficking out of nuclei is regulated by estradiol and depends on CRM1. Combining different approaches, we identified in the ERα hormone-binding domain a hormone-dependent and CRM1-directed NES. The 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 ERα-NES specifically sequesters the receptor in cell nuclei and interferes in the hormone-triggered S-phase entry of these cells. Site-directed mutagenesis of ERα NES also inhibits estradiol-induced cytoplasm re-localization of the receptor and DNA synthesis. Moreover, we observe that the transcription factor Forkhead (FKHR) and ERα are tightly associated. Activation by estradiol of the PI3K/Akt pathway induces nuclear export of ERα and FKHR, under conditions of its association with FKHR. This event favors cell cycle progression of breast cancer cells.

## 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 an N-terminal epitope of ERα (A314MAb; Abbondanza et al., 1998). Fig. 1**a** shows that, regardless of the antibody used, 30 min of estradiol treatment of MCF-7 cells induces nuclear translocation of ERα, which is followed by a decrease of the nuclear receptor to basal level after 1h of hormone stimulation. This decrease parallels cytoplasmic redistribution of the receptor (not shown). Immunoblot of cell lysates with anti-ER monoclonal antibodies does not reveal any significant change in ERα level (Supplemental data), thus suggesting that the observed decrease of ERα nuclear localization is due to nuclear exit of the receptor.

Quiescent MCF-7 cells were next transiently transfected with a full-length ERα cDNA subcloned into the green fluorescent protein plasmid (pEGFP-HEG0). In Fig. 1**b**, the quantitative count of cells with nuclear fluorescence shows that, in the absence of any significant change of GFP-ERα expression (Supplemental data), 30 min hormone treatment triggers nuclear accumulation of GFP-ERα. Thirty min later, the nuclear GFP-ERα decreases to the basal levels, whereas no trafficking of GFP-ERα was observed in untreated cells (Fig. 1**b**). In addition, GFP was insensitive to estradiol treatment when overexpressed alone in MCF-7 cells (not shown). The partial antagonist, 4-OH-tamoxifen, prevents the observed nucleo-cytoplasmic shuttling of GFP-ERα in response to hormonal treatment (Fig. 1**b**).

To exclude the potential contribution of *de novo* GFP-ERα synthesis for its reemergence in the cytoplasm 1 hour after estradiol treatment, we included actinomycin D in the medium during hormone stimulation. Here again, we did not observe any significant change in the estradiol-induced redistribution of GFP-ERα (Fig. 1 **c**), as well as protein expression (Supplemental data). Therefore, the chimeric protein in cytoplasm arose from nuclear export rather than from *de novo* synthesis.

Since actinomycin D inhibits the nuclear import of the NES-containing REV protein, (Henderson, 2000) and the GFP-ERα dynamic redistribution is not modified by actinomycin D (Fig. 1**c**), we conclude that cytoplasmic re-localization of the chimera depends on nuclear export rather than inhibition of nuclear import.

We next transiently transfected quiescent MCF-7 cells 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; AktK$^-$). In Fig. 1**d**, the quantitative count of transfected cells showing ER nuclear fluorescence shows that overexpression of both, Δp85α or Akt K$^-$ induces nuclear retention of ERα in 60 min estradiol-stimulated MCF-7 cells. Transfection of cells with the Myc-tagged pSG5 control plasmid does not interfere in the trafficking of ERα. Altogether, these data point to the regulatory role of PI3K/Akt pathway in estradiol-triggered nuclear export of its own receptor.

To verify the role of CRM1 export receptor in ERα nuclear exit we used LMB. This anti-fungal compound blocks the nuclear export of NES-containing proteins by preventing their association with the CRM1 export receptor (Kudo et al., 1998). Therefore, we tested the effect of this inhibitor on the estradiol-induced shuttling of GFP-ER. Quantitative analysis of transfected cells shows that LMB abolishes the 1h nuclear decrease of GFP-ERα in estradiol-stimulated cells (Fig. 1**e**). LMB is ineffective when added alone to the resting cells (Fig. 1**f**). Representative images from one experiment in panel **e** are presented in panel **f**. Thus, the LMB effect on ERα localization suggests that this receptor, when bound to the hormone, is exported from nuclei through the CRM1/exportin pathway. To further address this question, an **in vitro** protein-protein interaction assay was used. Fig. 1**g** shows that estradiol induces a strong interaction between recombinants ERα and CRM1 when active Ran (Ran-GTP; Askjaer et al., 1998) was included as a GTP-ase deficient RanQ69L mutant (Bischoff et al., 1994).

Altogether, findings in Fig 1 show that estradiol activation of PI3K/Akt pathway modulates the nuclear export of ERα. They also demonstrate that CRM1 definitely contributes to ER nuclear export triggered by estradiol in MCF-7 cells.

**Identification of ERα nuclear export sequence**

Our findings as reported in Supplemental Data point out to the presence of nuclear export sequence(s) in the NLS/HEG14 construct, which is made up by the nuclear localization signals and the hormone binding domain of ERα. Since it has been suggested that nuclear localization signals of the progesterone receptor are responsible for the nuclear exit of this receptor (Guiochon-Mantel et al., 1994), we verified by an *in vivo* export assay (Henderson & Eleftheriou, 2000) whether NLSs are involved in the ERα export. According to this assay, nuclear export sequences are identified by their ability to restore the export activity of the NES-deficient REV1.4-GFP at levels similar to those observed with the wild-type pREV-GFP or the REV1.4-GFP NES (NES is the canonical export sequence of the REV protein). Therefore, NLS

sequences of estrogen receptor were subcloned into pREV1.4-GFP and expressed in MCF-7 cells. After transfection, cells were incubated in the absence or presence of actinomycin D, since it causes cytoplasmic accumulation of the putative NES-containing REV protein by preventing the nuclear import of REV (Henderson, 2000). Irrespective of experimental conditions, pREV1.4-GFP NLS is localized in the nuclei of MCF-7 cells (Fig. 2**a** and **b**). In the same experiment, the pREV1.4-GFP NES, used as a positive control, completely shifted to the cytoplasm in the presence of actinomycin D, whereas the mutant pREV1.4-GFP 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 pREV1.4-GFP. These constructs were transfected into MCF-7 cells and then analyzed for their ability to restore the nuclear export of the mutant pREV1.4-GFP. By this assay we identified a 427-456 amino acidic sequence of ERα that, although less active than the pREV1.4-GFP NES positive control, shifted the pREV1.4-GFP mutant from nuclei to cytoplasm of cells treated with actinomycin D (Fig. 2**a** and **b**). Interestingly, LMB treatment blocked the export activity of 427-456 ERα signal (Fig. 2 **a** and **b**). Regardless of the experimental conditions, the leucine-rich 296-335 ERα sequence was unable to drive the REV mutant into cytoplasm (Fig. 2**a** and **b**). Taken together, these data show that the 427-456 region is involved in the export of ERα through the CRM1/exportin binding.

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 (340-351; Stommel et al., 1999). Moreover, a comparison of the homologous amino acids in position 444-456 of ERα across the family of steroid receptors revealed a high level of sequence homology with ERβ, PgR, AR and glucocorticoid receptor. The alignment between the sequences is shown in Supplemental Data. The possibility that the 444-456 amino acids of ERα sequence contains the ER-NES was then investigated. For this purpose, the 444-456 ER sequence as well as the mutated version of the sequence (containing 4 residues of alanine instead of IILL amino acids in position 451-454; Fig. 2**c**) were subcloned into the pREV1.4-GFP. Interestingly, the ER wild type 444-456 sequence was able to shift the pREV1.4-GFP into the cytoplasm of MCF-7 cells, whereas its mutated form failed to do it (Fig. 2**d**). This difference is even more evident in the presence of actinomycin D. Pictures in panel **e** are representative of one experiment in **d**.

These results provide direct evidence that a small sequence of the ERα hormone-binding domain is responsible for the CRM1-mediated ERα nuclear export.

**A peptide mimicking the 444-456 ERα sequence sequesters the receptor into the nuclear compartment and inhibits the estradiol-induced S-phase entry of breast cancer cells.**

Following recent approaches (Joliot & Prochiantz, 2004), a Tat-conjugated peptide construct corresponding to the 444-456 ERα sequence was synthesized (Fig. 3**a**). In a preliminary experiment (not shown), we observed that the carboxyfluorescein-conjugated peptide translocated across the plasma membrane and within 30 min accumulated in the nuclei of MCF-7 cells. This peptide displaced the estradiol-induced interaction between recombinants CRM1 and ERα, while the Tat alone (ctrl) did not affect such an interaction (Fig. 3**b**). Moreover, the Tat-conjugated peptide blocked the 60 min estradiol-induced nuclear export of GFP-ERα, thus sequestering the receptor in the nuclei (Fig. 3**c** and **d**). Here again, the Tat alone used as a control did not modify the trafficking of GFP-ERα (not shown). Findings in Supplemental data show the specificity of the Tat-conjugated peptide action. In immunofluorescence analysis, it did not affect the p27 nuclear export, or the p53 subcellular localization.

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

**ERα NES mutants do not exit nuclei and fail to induce DNA synthesis stimulated by estradiol.**

Based on previous findings (Fig. 2), we prepared by site-directed mutagenesis two mutants of ERα-GFP. Fig. 4**a** shows a schematic representation of the GFP-HEG0

NES and its mutated versions. We transiently transfected these constructs in MCF-7 cells. Expectedly, estradiol treatment induced nuclear export of GPF-HEG0 (HEG0 is the wild type human ERα). In contrast, both mutants, GFP-HEG4A and GFP-HEGIL, were unable to exit nuclei upon hormonal stimulation (Fig. 4**b**). Images in **c** show a diffuse, sometime extranuclear localization of GFP-HEG0 in MCF-7 cells stimulated for 60 min with estradiol. Under these conditions, a nuclear/nucleolar localization of both GFP-HEG4A and GFP-HEGIL was detected.

We then verified the ability of the mutant GFP-HEGIL to activate estradiol-induced gene transcription. To this end, ERα-negative NIH3T3 fibroblasts (Castoria et al., 1999 and 2003) were transiently transfected with GFP-HEG0 or GFP-HEGIL or GFP alone, as a control. The ERE-Luc reporter gene was cotransfected and its activity assayed. Fig. 4**d** shows that the constructs GFP-HEG0 and GFP-HEGIL are equally efficient in activating gene transcription upon estradiol stimulation of the cells, with an induction of the ERE-Luc activity ranging from 6 to 6,3 fold. Transcriptional activation was almost undetectable in unstimulated cells or in cells expressing GFP alone, as a control.

In another set of experiments, we assessed the ability of GFP-HEG0 and GFP-HEGIL to mediate the estradiol-induced DNA synthesis in transfected NIH3T3 fibroblasts. In agreement with previous data (Castoria et al., 1999), the GFP-HEG0 was a potent inducer of the S-phase entry in cells challenged with estradiol. In contrast, the mutant GFP-HEGIL failed to do it, but it did not prevent the serum-induced S-phase entry (Fig. 4**e**). Here again, the Tat-conjugated peptide inhibited the estradiol-induced S-phase entry of GFP-HEGO transfected fibroblasts, whereas it did not interfere in the serum-induced BrdU incorporation (bars with asterisks in Fig. 4**e**).

Data in Figs. 2 and 4 conclusively show that the leucine rich 444-456 sequence of ERα contains a functional NES. Interestingly, mutations in the core (IILL) of this sequence impair the estradiol-induced nuclear export of ER as well as DNA synthesis, without interfering in gene transcription.

**Estradiol regulates the nuclear export of FKHR**

A sustained estradiol activation of PI3-K is required to drive MCF-7 cells into the S-phase (Migliaccio et al., 2002). In addition, previous findings reported that an estradiol-dependent interaction between ERα and FKHR occurs in MCF-7 cells (Schuur et al., 2001). Therefore, we hypothesized a role for this downstream effector of the PI3-K pathway in both estradiol-regulated ERα nuclear export and cell cycle

arrest mediated by the NES-ERα mutant. Therefore, we analyzed the role of FKHR in estradiol-induced DNA synthesis and ERα nuclear export. To this end, we transiently transfected quiescent MCF-7 cells with the wild-type FKHR (GFP-FKHR wt) or its mutant containing a triple alanine substitutions (GFP-FKHR AAA), a version of FKHR that cannot be phosphorylated by Akt and localizes into nuclei, thereby inducing G1 arrest of cells (Nakamura et al., 2000). Fig. 5 **a** shows that expression of this mutant greatly reduced the estradiol-induced BrdU incorporation in MCF-7 cells, whereas expression of GFP-FKHR wt did not interfere in the DNA synthesis induced by the hormone. In the same set of experiments we also analyzed by confocal microscopy the role of FKHR in the sub-cellular distribution of ERα. While expression of 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 of 60 min hormone-treated cells (Fig. 5**b**). Conversely, over-expression of the tagged NES-ERα mutant, Myc-HEGIL, retained GFP-FKHR wt in the nuclear compartment of 60 min estradiol-treated MCF-7 cells (Fig. 5**c**). Representative fields from one experiment in **b** and **c** are presented in panel **d**. Left and central panels show the staining of endogenous ERα (red) in MCF-7 cells expressing the GFP-FKHR wt (left panels) or the mutant GFP-FKHR AAA (middle panels), treated for 60 min with estradiol. Right panels show the staining of Myc-tagged NES-ERα mutant (red) in 60 min hormone-treated MCF-7 cells co-expressing GFP-FKHR wt. The lowest microphotographs in panel **d** show the merged images. Altogether, data in Fig. 5 show that estradiol simultaneously regulates ERα and FKHR nuclear export in MCF-7 cells.

## DISCUSSION

Nucleo-cytoplasmic shuttling of proteins plays a critical role in cell function (*reviewed by* Kau & Silver, 2003). Most of the estradiol receptor (ER) is localized in the nuclei of hormone target cells (Stenoien et al., 2001). Indeed, 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 ER (Migliaccio et al., 2007). Ligand stimulation of different cell types recruits to ERα or ERβ different extranuclear signaling effectors, which leads to signal transduction pathway activation (Migliaccio et al. 2000; Castoria et al., 2001). In addition, a transcriptionally inactive ERα mutant, permanently residing in the cytoplasm, mediates the S-phase entry triggered by estradiol activation of the Src/Ras/ERK and PI3-K/Akt pathways (Castoria et al., 1999 and 2004). These findings implicate a proliferative function of the extranuclear receptor. This view is further supported by the observation that a classic mouse AR, which is expressed at a very low level in NIH3T3 fibroblasts, does not enter nuclei and does not activate gene transcription in hormone-stimulated cells. Nevertheless, it recruits several signaling effectors that control the androgen-induced S-phase entry and cytoskeletal changes of fibroblasts (Castoria et al., 2003). In addition, rat uterine stromal cells, although expressing low levels of transcriptional incompetent PgR, respond to progestins with active proliferation (Vallejo et al., 2005). Furthermore, the extranuclear cross-talk between ER/AR and epidermal growth factor receptor (EGFR) regulates the EGF-elicited responses, such as actin changes and DNA synthesis in breast and prostate cancer cells (Migliaccio et al., 2005). These and other similar findings point to a critical role of steroid receptor extranuclear localization in steroid hormone or growth factor action.

We have now analyzed in MCF-7 cells the nucleo-cytoplasmic shuttling of the estradiol receptor focusing on its export from the nucleus. We observed that estradiol induces a CRM1-dependent nuclear export of ERα. Thus, identification of ERα sequence responsible for its nuclear export has been assessed by a nuclear export assay. Previous findings reported that the outward movement of PgR from nuclear compartment is mediated by its NLSs (Guiochon-Mantel et al., 1994). Therefore, we firstly assayed the ability of ERα NLSs to shift the REV mutant into cytoplasm. Our data show that NLSs are not involved in the nuclear export of ERα. It has also been hypothesized that nuclear export of ERα is regulated by Thr311 phosphorylation (Lee & Bai, 2002). This amino acidic residue is present in a putative

leucine-rich ER nuclear export signal (305-322 of ERα), which shares homology with the p53 amino-terminal nuclear export signal (Zang & Xiong, 2001). In contrast with this hypothesis, the leucine-rich 296-335 ERα sequence is unable to drive the REV mutant into cytoplasm. Using the same approach, we observe that the leucine rich 444-456 sequence of ERα contains a functional NES, which is sensitive to LMB treatment. Furthermore, a Tat-conjugated peptide mimicking this amino acidic sequence displaces the **in vitro** interaction between ERα and CRM1. Altogether, these data support each other the conclusion that the 444-456 sequence contains a putative NES. We further verified this conclusion by site-directed mutagenesis. Mutations in the core of this sequence impair the estradiol-induced nuclear export of full-length ERα in MCF-7 cells. The 444-456 sequence of ERα shows homology with the conserved leucine-rich and REV-like NES of p53 (340-351, Stommel et al., 1999; see also Supplemental data). Remarkably, the 444-456 region of ERα is conserved in other steroid receptors, such as ERβ, PgR, AR, GR and MR. This corroborates the hypothesis that this sequence is responsible for CRM1-dependent nuclear export of most of the steroid receptors. In agreement with this hypothesis, inhibition of nuclear export of different steroid receptors by LMB treatment has been shown by different groups and under different experimental conditions (Savory et al., 1999; Prufer & Barsony, 2002; Maruvada et al., 2003).

We have also modulated the ERα localization using a Tat-conjugated peptide mimicking the ERα-NES sequence. It specifically traps ERα in the nuclear compartment of MCF-7 cells and greatly reduces the estradiol-induced S-phase entry of these cells, thus corroborating the hypothesis that ERα nuclear export plays a role in DNA synthesis of target cells. This view is reinforced by the finding that addition of peptide to the cell medium after 1h of hormonal stimulation, when the ERα nuclear exit is almost complete, does not affect the estradiol-induced DNA synthesis of MCF-7 cells. Furthermore, experiments in NIH3T3 fibroblasts show that an NES-ERα mutant fails to induce S-phase entry of cells, whereas it is able to activate gene transcription. These data further point to the role of ERα nuclear export in cell cycle progression modulated by estrogens in breast cancer cells. On the other hand, the function of many proteins is regulated by their subcellular localization. These proteins include cell cycle regulators and transcription factors, such as NFkB, p53 and mammalian members of the FHKR transcription factors (Kau & Silver, 2003). A triple alanine mutant of FKHR localizes into the nucleus and induces G1 arrest of cells (Nakamura et al., 2000). Therefore, it is not new that permanent localization of transcription factors into the cell nucleus can stop cell cycle. Furthermore,

stimulation of Pak-1 and ERα by estrogen treatment of breast cancer cells promotes cell survival by inducing phosphorylation and nuclear exclusion of FoxO1 (Birkenkamp & Coffer, 2003). In addition, an estradiol-dependent interaction between ERα and FKHR has been observed using both a yeast two-hybrid screen and MCF-7 cells (Schuur et al., 2001). Extension of these investigations to other nuclear receptor (NR) family members has shown that, depending on the receptor type, FKHR represents a bi-functional NR intermediary protein acting as either a co-activator or co-repressor, (Zhao et al., 2001). Our data suggest a novel mechanism of estrogen action that involves regulation of FKHR nuclear export, a crucial step in G1-S transition (Birkenkamp & Coffer, 2003). The triple alanine FKHR mutant, which cannot be phosphorylated by Akt, sequesters ERα in the nuclei and inhibits S-phase entry of cells. Conversely, an NES-ERα mutant, unable to exit nuclei, retains FKHR in the nuclear compartment of estradiol-treated breast cancer cells and in this way blocks the cell cycle. In addition to the previous described effects (Castoria et al., 2001), we now show that estradiol stimulation of the PI3K/Akt pathway facilitates the export of the two transcription factors in breast cancer cells. Our findings for the first time demonstrate that estradiol cross-regulates the nuclear export of its own receptor and FKHR in target cells, thus facilitating the cell cycle progression.

In conclusion, our results highlight the studies on regulation of ERα intracellular trafficking and point to a key role of estradiol in modulating this process. They also identify for the first time a functional NES of ERα. Targeting of this motif by either synthetic peptide or site-directed mutagenesis modulates the subcellular localization of ER and impairs the estradiol-induced S-phase entry of target cells. Therefore, these observations offer a powerful tool to modify the intracellular distribution of ER and reveal much about the biological functions of this receptor. On this basis, the strategy of trapping ER in nuclear compartment might offer a potential approach to the therapy of human breast cancers.

## MATERIALS AND METHODS

### Constructs

The constitutive active Ran Q69L was into pQE plasmid (Izaurralde et al., 1997). The pCDNA/HA-CRM1 (Alt et al., 2000) was digested with KpnI and BamH1 and subcloned into pSG5. cDNA encoding the wild type of hERα (HEG0) was in pSG5 expression vector (Tora et al., 1989). It was subcloned into EcoRI-pEGFP (C2, Clontech) or EcoRI-pSG5Myc as reported (Castoria et al., 2004). The dominant negative form of p85α (Δp85α) was in pSG5 (Dhand et al., 1994). Myc-His tagged dominant negative form of Akt (K179M) in pUSEAmp plasmid was from UBI (Lake Placid, NY). The Myc-tagged pSG5 was prepared as reported (Castoria et al., 2004). Forkhead (FKHR), either wild type (wt) or its mutated form (AAA-FKHR) were in pcDNA-GFP (Addgene). For the export assay, the putative export sequences of ERα were cloned into the BamHI and AgeI sites of pREV(1.4)-GFP and inserted between the REV and GFP coding sequence (eGFP-N1, Clontech). To insert the putative export ERα sequence into pREV(1.4)-GFP, a PCR strategy was adopted for the following fragments: *NLS sequence* (241-306 aa) was amplified by forward primer 5′-GACTGGATCCAATGATGAAAGGTGGGATACGAAAAGACCG-3′ and reverse primer 5′-GCGACCGGTGGCAGGCTGTTCTTCTTAGAGCGTTTGATCA-3′; *286 sequence* (286-311 aa) forward primer 5′-GATCGGATCCAATGAGAGCTGCCAACCTTTGGCCAAGCCCG-3′ and reverse primer 5′-GCGACCGGTGGGGCCGTCAGGGACAAGGCCAGGCTGTTCT -3′; *286 sequence* (286-382 aa) reverse primer 5′-GCGACCGGTGGGGCACATTCTAGAAGGTGGACCTGATCATG3′; 296 sequence (296-335aa) forward primer 5′-GATCGGATCCAATGATCAAACGCTCTAAGAAGAAC-3′ and reverse primer 5′-GCGACCGGTGGGGGTCTGGTAGGATCATACTCGGAATAGA-3′; *316 sequence (316-335aa)* forward primer 5′-GATCGGATCCAAGTGCCTTGTTGGATGCTGAGCCCCCCAT-3′; *341 sequence* (341-361 aa) forward primer 5′GATCGGATCCATCGATGATGGGCTTACTGACCAACCTGGC-3′and reverse primer 5′-GCGACCGGTGGCGCCCAGTTGATCATGTGAACCAGCTCCC-3′; *368 sequence* (368-394 aa) forward primer 5′-GATCGGATCCAGTGGATTTGACCCTCCATGATCAGGTCCA-3′ and reverse primer 5′-GCGACCGGTGGGCGCCAGACGAGACCAATCATCAGGATCT-3′; *381 sequence* (381-412 aa) forward primer 5′-

GATCGGATCCATGTGCCTGGCTAGAGATCCTGATGATTGGT-3′ and reverse primer 5′-GCGACCGGTGGGTTCCTGTCCAAGAGCAAGTTAGGAGCAA-3′; *395 sequence* (395-412 aa) forward primer 5′GATCGGATCCATCCATGGAGCACCCAGTGAAGCTACTGTT-3′; *427 sequence* (427-457 aa) forward primer 5′-GATCGGATCCAATGCTGCTGGCTACATCATCTCGGTTCCG-3′ and reverse primer 5′-GCGACCGGTGGTCCAGAATTAAGCAAAATAATAGATTTGAGGCACAC-3′; *381-457 sequence*; *458-552 sequence* forward primer 5′-GATCGGATCCAGTGTACACATTTCTGTCCAGCACCCTGAAGTCT-3′ and reverse primer 5′-GCGACCGGTGGGGGCGCATGTAGGCGGTGGGCGTCCAGCATCTC-3′. All these fragments were amplified using the Platinum Pfx (Invitrogen), according to the manufacturer's instructions. The PCR products were purified, digested with BamHI/AgeI and finally subcloned into pREV(1.4)-GFP. The ER 444-456 sequence was subcloned in the same vector, as a short fragment generated by annealing of specific oligonucleotides. The same strategy was utilized to generate the ER 444-456 mutant. All putative ER NES were verified by sequencing to confirm the exact reading frame between REV and GFP. The site-directed mutations of ERα were introduced into the pSG5-HEG0 using a standard PCR methodology. Two mutants were generated: pSG5-HEG4A (Ile 451 and 452 changed with Ala; Leu 453 and 454 changed with Ala) and pSG5-HEGIL (Ile 451 changed with Ala; Leu 454 changed with Ala). The cDNAs coding for the ERα mutants (HEG4A and HEGIL) were subcloned into EcoRI-pEGFP. All junctions were verified by sequencing.

**Cell culture**

Human breast cancer-derived MCF-7 cells and low passage mouse embryo NIH3T3 fibroblasts were grown and made quiescent as reported (Castoria et al., 1999 and 2003).

**Transfection experiments, nuclear export and transactivation assays**

Unless otherwise stated, quiescent MCF-7 cells on coverslips were transfected with 1 μg of each purified plasmid using the SuperFect reagent (Qiagen). After transfection, the cells were incubated at 37°C for 24h, and then used for the indicated experiments. The nuclear export assay was performed as described (Henderson & Eleftheriou, 2000). Briefly, each putative NES-ER sequence was subcloned into the

REV (1.4)-GFP and then transfected (at 2 μg) by SuperFect into growing MCF-7 cells. After 18 h the cells were incubated with cycloheximide (at 15 μg/ml; Calbiochem) and left untreated or treated for 8 h with actinomycin D (at 5μg/ml; Calbiochem). When indicated, leptomycin B (at 5 ng/ml; Calbiochem) was added 30 min before the actinomycin D addition. Growing NIH3T3 fibroblasts were plated on Petri dishes or coverslips. They were then transfected by SuperFect using 2 μg of either pEGFP or pEGFP-HEG0 or pEGFP-HEGIL. For trans-activation assay, 2 μg of pGL2 ERE-Luc were co-transfected. Twelve hours after transfection, cells were made quiescent and then left untreated or treated with 10 nM estradiol for 16 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 (Amersham Biosciences). For DNA synthesis analysis, the fibroblasts were made quiescent and then left unstimulated or stimulated with the indicated compounds.

**DNA synthesis analysis.**

Quiescent cells on coverslips were left unstimulated or stimulated for 18 h with the indicated compounds. Estradiol was added at 10 nM. The Tat peptide conjugated to the 444-456 amino acid sequence of ERα (Tat-pep) as well as the Tat control peptide (ctrl) were added at 1 μM. After a 6 h-pulse with 100 μM BrdU (Sigma, St.Louis, MO), BrdU incorporation was analyzed as described (Castoria et al., 1999).

**Immunofluorescence and confocal microscopy**

Cells on coverslips were fixed for 10 min with Para formaldehyde (3%, w/v in PBS), washed with PBS, and then analyzed by fluorescence microscopy for the subcellular localization of GFP-HEGO, its mutants as well as putative ER-NES constructs. Similar analysis was done 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 (Castoria et al., 1999). Endogenous ERα was visualized as described (Castoria et al., 1999), using the A314 mouse monoclonal antibody or the H222 rat monoclonal antibody. The dominant negative Δp85α and Myc-His tagged Akt $K^-$ were visualized as reported (Castoria et al., 2001). The Myc-tagged pSG5 or HEG0 or HEGIL were detected as reported (Castoria et al., 2004), using the mouse anti-Myc tag monoclonal antibody (Clontech). Coverslips were finally stained with Hoechst 33258, inverted and mounted in Mowiol (Calbiochem, CA). Fields were analyzed with a DMBL Leica (Leica) fluorescent microscope using 40, 63 and 100x objectives. Images were processed using IMI1000 or FW4000 (Leica) software. When indicated,

the distribution of fluorescence was analyzed by a confocal LSM 510 Zeiss microscope.

**Purified proteins and in vitro protein-protein interactions**

Ran Q69L expressed in JM109 bacteria was purified using the Ni-NTA agarose (Qiagen), and recombinant human ERα (2800pmol/ml) was from Panvera. $S^{35}$-labeled HA-CRM1 was produced in rabbit reticulocyte lysate (Promega) and protein-protein interaction was done as described (Migliaccio et al., 2000). Immunoprecipitation was done using the rabbit polyclonal anti-ER Ab (G-20 Santa Cruz, at 1μg/ml) or the mouse monoclonal anti-HA antibody (Covance, at 2μg/ml). Eluted proteins were analyzed by immunoblotting or fluorography.

**Lysates, electrophoresis and immunoblotting**

All performed as described (Migliaccio et al., 2000). The rabbit polyclonal anti ER antibody (G-20; Santa Cruz) was used to detect ERα. Expression and production of Ran Q69L was verified using the mouse monoclonal anti-His tag antibody (Qiagen). ERα and the chimera GFP-ERα (see the Supplemental data) were detected using either the rat monoclonal (H222 from Abbott Laboratories) anti ERα antibody or the mouse monoclonal anti GFP antibody (Clontech). Immunoreactive proteins were revealed by the ECL detection system (Amersham Biosciences).

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**FIGURE LEGENDS**

**Fig. 1. Estradiol-induced nuclear export of ERα is modulated by PI3K/Akt pathway and depends on CRM1.**

Quiescent MCF-7 cells were used. In **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. In **b** and **c**, cells were transfected with pEGFP-HEG0, then left untreated or treated with the indicated compounds. OH-Tamoxifen (TX, from Zeneca) was used at 0.1 μM; actinomycin D (Act D) was added at 5 μg/ml, 1h before the estradiol stimulation. The GFP-HEGO localization was determined by fluorescence. In **d**, cells were transfected with the indicated plasmids and left untreated or treated with estradiol (10 nM) for the indicated times (min). The Myc-tagged pSG5, or Δp85α or Myc-His-tagged dominant negative Akt ectopically expressed in MCF-7 cells were visualized by immunofluorescence, as described in Methods. ERα localization was analyzed by immunofluorescence using the rat anti-ER monoclonal antibody (H222 MAb). In **e**, cells were transfected with pEGFP-HEG0 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 treatment. Cells were also treated with LMB in the absence of hormone. The GFP-HEGO localization was determined by fluorescence. In **a**, **b**, **c**, **d**, and **e**, cells that fell into the category of exclusively nuclear fluorescence were scored and data expressed as a percentage of total cells (in **a**) or transfected cells (in **b**, **c, d, e**). Data were derived from at least 1000 scored cells. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. In **f**, images from one experiment in **e** were captured. Panels show the GFP-ER localization in MCF-7 cells stimulated for 60 min with estradiol ($E_2$), in the absence (left picture) or presence (right picture) of LMB. In **g**, $^{35}$S-labeled HA-CRM1 was incubated with recombinant ERα in the absence or presence of estradiol (10 nM). The purified recombinant RanQ69L (at 1 μM) was included in the incubation mixture of each sample. Proteins were immunoprecipitated with the rabbit polyclonal anti ERα antibody. Eluted proteins were immunoblotted with the anti ERα antibody (upper panel) or revealed by fluorography (lower panel).

**Fig. 2. The ERα 444-456 sequence restores the export activity of the NES-deficient REV1.4-GFP.**

Growing MCF-7 cells were used. In **a** and **b**, cells transfected with the indicated constructs. After transfection, the cells were left untreated (no drug), or treated with actinomycin D (5 μg/ml), alone or together with leptomycin B (5 ng/ml). The subcellular distribution of GFP

proteins was determined by fluorescence microscopy and graphically shown in panel **a**, with mean values taken from at least three experiments, and a standard deviation of less than 15%. For each experiment, at least 600 cells were scored. Panel **b** shows representative images of one experiment in **a**. Panel **c** shows the ERα wild type 444-456 sequence as well as its mutated version. The putative NES-ERα sequence is indicated by the underlined amino acids, which were substituted with alanine residues in the mutant sequence. The ER-444 wt (wt) as well as the ER-444 mutant (mut) sequences were subcloned into the REV (1.4)-GFP as described in Methods and then transfected (**d** and **e**) in growing MCF-7 cells. After transfection, the cells were left untreated (-), or treated with 5 μg/ml actinomycin D (+). The percentage of cells with nuclear (N), nuclear/ cytoplasmic (N/C) and cytoplasmic (C) GFP protein was determined by fluorescence microscopy and graphically shown in **d**. Data were derived from at least 600 scored cells. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. Panel **e** shows representative images of one experiment in **d**.

**Fig. 3. A peptide mimicking the putative NES sequence of ERα displaces the CRM1/ERa interaction, sequesters the receptor into the nuclei and inhibits the S-phase entry of estradiol-treated MCF-7.**

Panel **a** shows the aminoacidic sequence of the Tat-conjugated ERalpha (444-456) peptide (Tat-pep). In **b**, $^{35}$S-labeled HA-CRM1 was incubated with recombinant ERα from baculovirus in the absence or presence of estradiol (10 nM), alone or together with a 200-fold excess of the Tat-conjugated peptide (Tat-pep). A 200-fold excess of a non-specific peptide (Tat) was used as a control (ctrl). CRM1 was immunoprecipitated with the anti-HA monoclonal antibody and proteins in immunocomplexes were revealed by fluorography (upper panel) and immunoblotting with the anti ERα antibody (lower panel). In **c**, quiescent MCF-7 cells transfected with GFP-HEGO were incubated for 1h with the Tat-conjugated peptide (Tat-pep). Thereafter, the cells were left untreated or treated for the indicated times with 10 nM estradiol. The proportion of cells with nuclear GFP protein was determined by fluorescence microscopy and graphically shown. For each experiment, at least 200 cells were scored. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. Images of one experiment in **c** are presented in panel **d**. It shows the intracellular distribution of GFP-ER in MCF-7 cells treated for 60 min with estradiol in the absence (left picture) or presence of Tat-pep (right picture). The arrows indicate the cells showing nuclear distribution of the GFP-ER. In **e**, MCF-7 cells on coverslips were made quiescent as described in Methods or serum starved (maintained for 24 h in 0, 5% FCS). The cells were incubated for 24 h with 10 nM estradiol or 20% serum,

in the absence or presence of the Tat-conjugated ERalpha (444-456) peptide (Tat -pep) or Tat alone (ctrl). After **in vivo** pulse with BrdU, DNA synthesis was analyzed and BrdU incorporation expressed as percent of control. In estradiol-stimulated cells ($E_2$), BrdU incorporation ranged from 55 to 65% of total cells, whereas in serum-stimulated cells (serum) it ranged from 65 to 70%. The basal BrdU incorporation (7%) was in each case calculated and subtracted. For each experiment, at least 200 cells were scored. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. In **f**, quiescent MCF-7 cells were stimulated with 10 nM estradiol for 24 h and the Tat-conjugated peptide was added after the hormone addition, at the time points indicated in Figure. After **in vivo** pulse with BrdU, DNA synthesis was analyzed as in **e**. For each experiment, at least 300 cells were scored. The results of two independent experiments have been averaged. Means and SEM are shown.

**Fig. 4. Mutations in the NES sequence prevent nuclear export of full-length ERα and the S-phase entry in MCF-7 cells stimulated by estradiol.**

In **a,** aligned sequences of the wild type and NES mutants of ERα are shown. The NES-ERα sequence between amino acids 444-456 is highlighted in bold. Mutated amino acids are underlined. The NES mutants, GFP-HEG4A and GFP-HEGIL, were prepared from GFP-HEGO as described in Methods. In **b**, quiescent MCF-7 cells were transfected with the indicated plasmids and then left unstimulated or stimulated with 10 nM estradiol for the times indicated in the Figure. The percentage of cells with nuclear GFP protein was determined by fluorescence microscopy and graphically shown. For each experiment, at least 150 cells were scored. The results of different independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. Images of one experiment in **b** are presented in panel **c**, which shows the intracellular distribution of GFP-HEGO or GFP-HEG 4A or GFP-HEG IL in MCF-7 cells treated for 60min with estradiol. In **d** and **e**, growing NIH3T3 fibroblasts were used. In **d,** cells were transfected with an ERE-Luc construct, along with the indicated plasmids. After transfection, the cells were made quiescent and then left unstimulated or stimulated with 10 nM estradiol. The luciferase activity was assayed, normalized using β-gal as an internal control, and expressed as fold induction. In **e**, cells on coverslips were transfected with the indicated plasmids and made quiescent. The cells were left unstimulated or stimulated for 18 h with either 10nM estradiol or 20% serum. When indicated by the asterisk, the Tat-conjugated ERalpha peptide was included at 1μM to the cell medium. After **in vivo** pulse with BrdU, DNA synthesis was analyzed by immunofluorescence. In transfected cells, BrdU incorporation was calculated by the formula: percentage of BrdU-positive cells= number of transfected-positive cells/number

of transfected cells)x 100 and compared with BrdU incorporation of untransfected cells from the same coverslips. For each plasmid, data are derived from at least 500 scored cells. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments.

**Fig. 5. Estradiol regulation of FKHR nuclear export and role of FKHR in the hormone-induced DNA synthesis of MCF-7 cells.**

Quiescent MCF-7 on coverslips were used. In **a,** cells were transfected with the indicated plasmids and 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. In **b,** cells were transfected with the indicated plasmids and then left unstimulated or stimulated with 10 nM estradiol for the indicated times. Endogenous ERα localization as well as expression of GFP, or GFP-FKHR wt or GFP FKHR AAA mutant was monitored by confocal microscopy. Cells that fell into the category of exclusively ER nuclear fluorescence were scored and data expressed as a percentage of transfected cells. In **c**, cells were co-transfected with the indicated plasmids and 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 data expressed as a percentage of co-transfected cells. For each experiment in **a**, **b** and **c**, data were derived from at least 500 scored cells. The results of several independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments. In **d**, images from one experiment in **b** or **c** are shown. They represent the staining of endogenous ERα (red) in MCF-7 cells expressing the GFP-FKHR (green in left panels) or the mutant, GFP-FKHR AAA (green in middle panels) and treated for 60 min with estradiol. Right panels show the staining of Myc-tagged NES-ERα mutant (red) in MCF-7 cells co-expressing the GFP-FKHR wt (green) and treated for 60 min with estradiol. Merged images are also shown in the lowest pictures of the panel.

Fig. 1

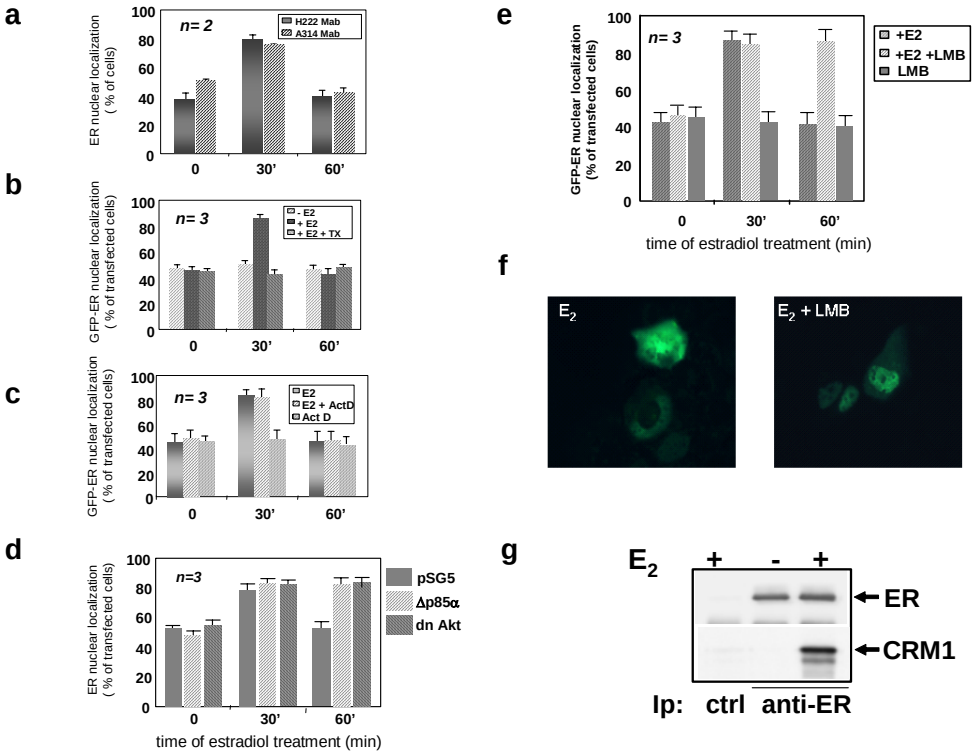

a
ER nuclear localization ( % of cells)
n= 2
H222 Mab
A314 Mab
b
GFP-ER nuclear localization ( % of transfected cells)
n= 3
- E2
+ E2
+ E2 + TX
c
GFP-ER nuclear localization ( % of transfected cells)
n= 3
E2
E2 + ActD
Act D
d
ER nuclear localization ( % of transfected cells)
n=3
pSG5
Δp85α
dn Akt
0
30'
60'
time of estradiol treatment (min)
e
GFP-ER nuclear localization (% of transfected cells)
n= 3
+E2
+E2 +LMB
LMB
time of estradiol treatment (min)
f
E2
E2 + LMB
g
E2 + - +
ER
CRM1
Ip: ctrl anti-ER

Fig. 2

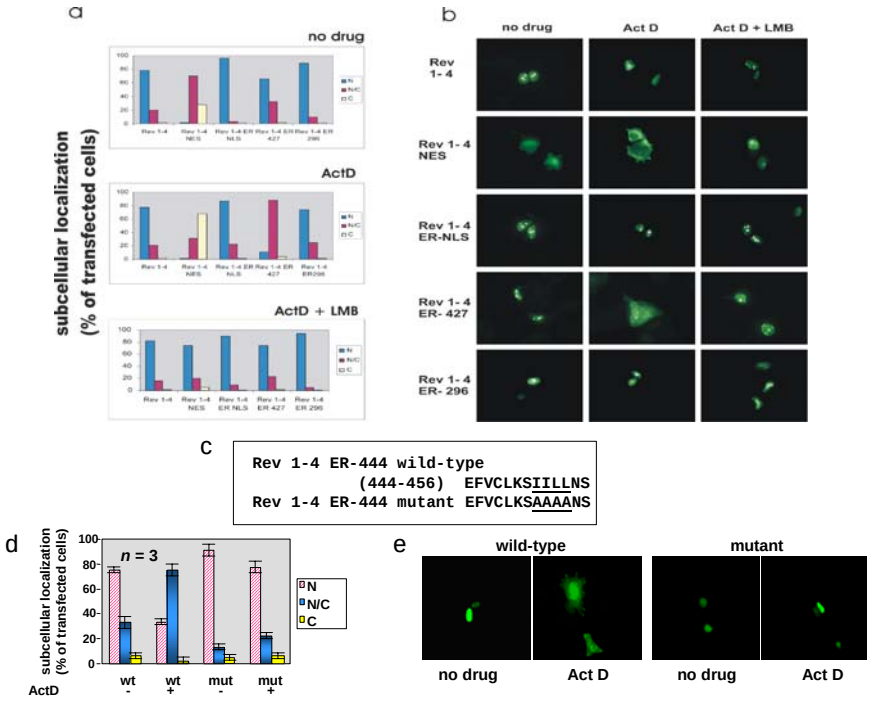

a
no drug
ActD
ActD + LMB
subcellular localization
(% of transfected cells)
b
no drug
Act D
Act D + LMB
Rev 1-4
Rev 1-4 NES
Rev 1-4 ER-NLS
Rev 1-4 ER-427
Rev 1-4 ER-296
c
Rev 1-4 ER-444 wild-type
(444-456) EFVCLKSIILLNS
Rev 1-4 ER-444 mutant EFVCLKSAAAANS
d
n = 3
subcellular localization
(% of transfected cells)
N
N/C
C
wt
wt
mut
mut
ActD
e
wild-type
mutant
no drug
Act D
no drug
Act D

Fig. 3

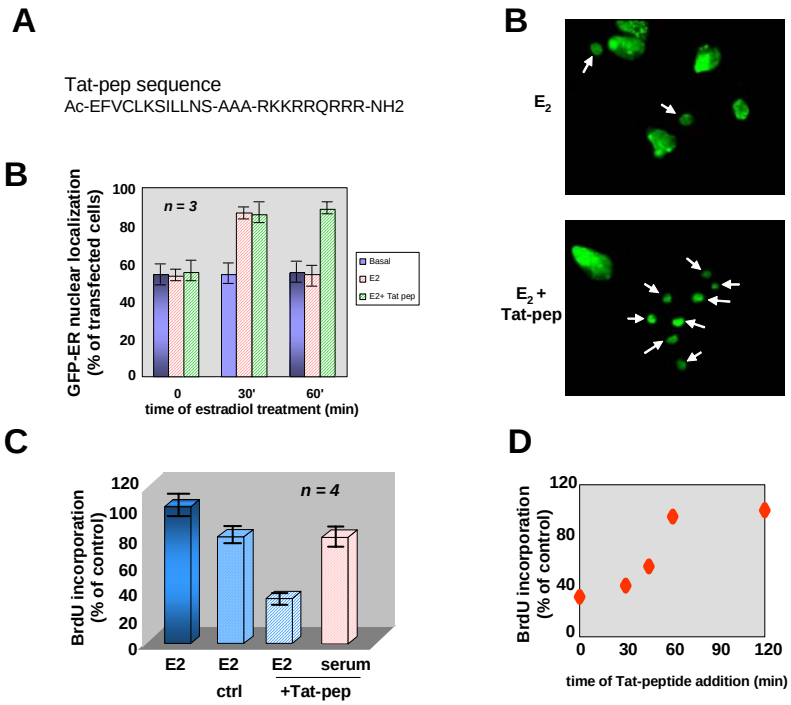

A
Tat-pep sequence
Ac-EFVCLKSILLNS-AAA-RKKRRQRRR-NH2
B
GFP-ER nuclear localization
(% of transfected cells)
n = 3
Basal
E2
E2+ Tat pep
time of estradiol treatment (min)
B
E2
E2 +
Tat-pep
C
BrdU incorporation
(% of control)
n = 4
E2
E2
E2
serum
ctrl
+Tat-pep
D
BrdU incorporation
(% of control)
time of Tat-peptide addition (min)

Fig. 4

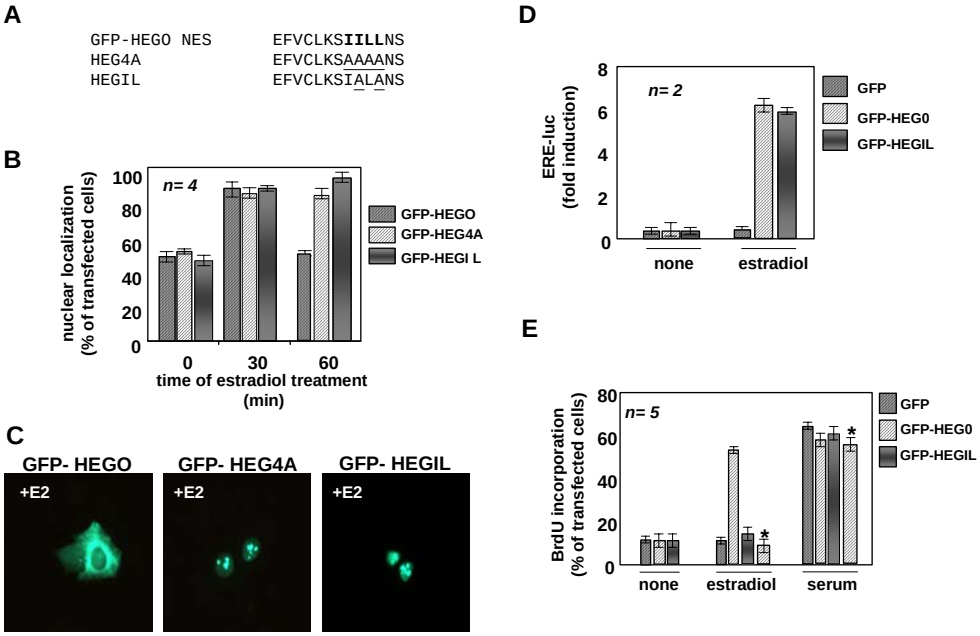
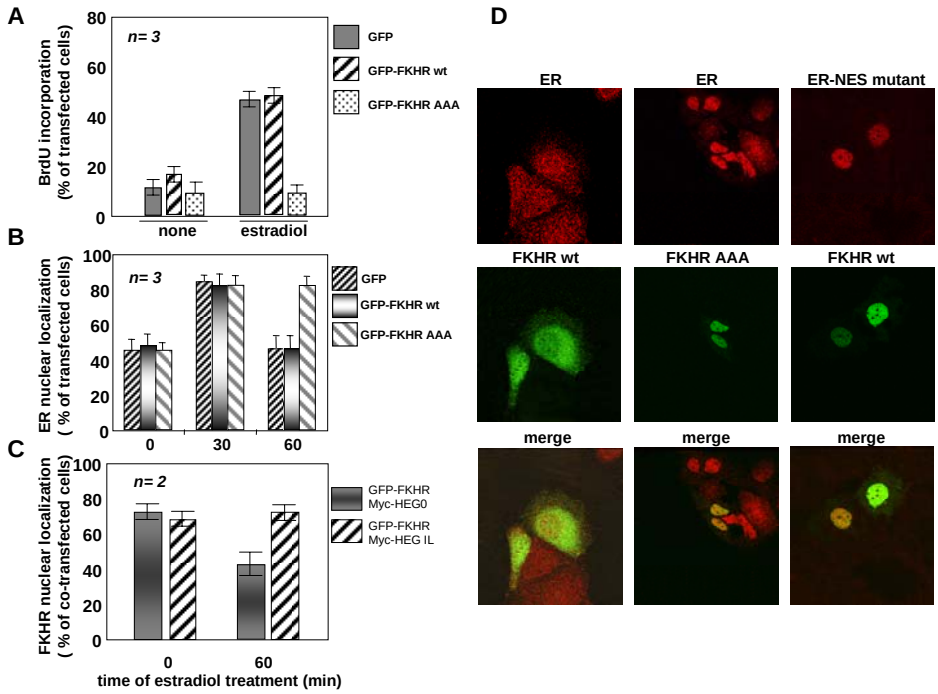

A
GFP-HEGO NES EFVCLKSIILLNS
HEG4A EFVCLKSAAAANS
HEGIL EFVCLKSIALANS
B
n= 4
nuclear localization (% of transfected cells)
100
80
60
40
20
0
0
30
60
time of estradiol treatment (min)
GFP-HEGO
GFP-HEG4A
GFP-HEGI L
C
GFP- HEGO
GFP- HEG4A
GFP- HEGIL
+E2
+E2
+E2
D
n= 2
ERE-luc (fold induction)
8
6
4
2
0
none
estradiol
GFP
GFP-HEG0
GFP-HEGIL
E
n= 5
BrdU incorporation (% of transfected cells)
80
60
40
20
0
none
estradiol
serum
*
*
GFP
GFP-HEG0
GFP-HEGIL

Fig. 5


A
BrdU incorporation (% of transfected cells)
n= 3
GFP
GFP-FKHR wt
GFP-FKHR AAA
none
estradiol
B
ER nuclear localization ( % of transfected cells)
n= 3
GFP
GFP-FKHR wt
GFP-FKHR AAA
C
FKHR nuclear localization ( % of co-transfected cells)
n= 2
GFP-FKHR Myc-HEG0
GFP-FKHR Myc-HEG IL
time of estradiol treatment (min)
D
ER
ER
ER-NES mutant
FKHR wt
FKHR AAA
FKHR wt
merge
merge
merge

## SUPPLEMENTAL DATA

## EXPERIMENTAL PROCEDURES

### Constructs

The wild type ERα (HEGO) and its mutants, HEG14, HEG15 and HEG241 into pSG5 (Tora et al. 1989; Ylikomi et al. 1992) were digested with the EcoRI and ligated into the pEGFP plasmid (C2 from Clontech, CA). pEGFP-NLS/HEG14 was obtained by digestion of pEGFP-HEGO with MspI and BamHI and subcloned into SmaI/BamHI pEGFP. All junctions were verified by DNA sequencing. All other experimental procedures have been described in the text.

## EXPERIMENTAL DATA SUPPORTING THE TEXT

### Identification of domains involved in the hormone regulated nucleo-cytoplasmic shuttling of ER

Two ER mutants, HEG15 and HEG14, were subcloned into pEGFP. HEG15 (Δ282-595 HEG0) contains highly conserved nuclear localization signals (NLS 2 and 3; Guiochon-Mantel et al., 1991). It binds DNA but is unable to bind estradiol (Ylikomi et al. 1992). In turn, HEG14 (Δ 1-281) 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. In agreement with previous results (Ylikomi et al., 1992), our data show that neither GFP-HEG15 nor GFP-HEG14 shuttle between the nucleus and the cytoplasm. Irrespective of the hormone 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 the HE241G into pEGFP. This mutant contains the hormone binding domain and lacks the three NLSs. It is indeed prevalently localized in the cytoplasm regardless of estradiol treatment. Similar results were obtained by ectopic expression of this mutant in NIH3T3 fibroblasts (Castoria et al., 1999). The behavior of this mutant confirms that in the absence of NLS, hormone binding is not sufficient to induce nuclear translocation of the receptor. Since HEG15 is localized in the nuclear compartment and its localization is unaffected by hormonal treatment of cells, we reasoned that 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-HEG0 in response to the estradiol treatment.

Data collected in Fig. 2 show that the 241-595 AA sequence of ERα is responsible for the estradiol-regulated nucleo-cytoplasmic shuttling of ER in MCF-7 cells.

**Legend to Fig. 1-** MCF-7 cells were used. In **A**, untransfected cells were untreated or treated with estradiol (10 nM), for the indicated times. Lysates were prepared and analysed by Western blot using the rat monoclonal H222 anti ER antibody. In **B** and **C**, cells were tnasfected with GFP-HEGO and untreated or treated with 10 nM estradiol for the indicated times. Lysates were analysed by Western blot using the antibodies against the indicated proteins.

**Legend to Fig. 2-** Wild-type and mutants of ERα were subcloned into pEGFP as described in Supplemental Experimental Procedures. Fig. 2 **a** shows a schematic representation of the mutants. As shown in Fig. 2 **b** and **c**, quiescent MCF-7 cells were transfected with the indicated plasmids and then left untreated or treated for the indicated times with estradiol (10 nM). The localization of the indicated mutants as well as GFP-HEG0 was determined by fluorescence. The score was performed as described in the text (see the legend to Fig.1). For each plasmid, data were derived from at least 500 cells. The results of more independent experiments have been averaged; means and SEM are shown. ***n*** represents the number of experiments.

**Legend to Fig. 3-**

**The Tat-peptide does not affect the EGF-induced p27 nuclear exit in mouse fibroblasts NIH3T3 (A), and does not interfere in sub-cellular distribution of p53 in MCF-7 cells (B).**

**In A,** quiescent NIH3T3 fibroblasts on coverslips were unstimulated or stimulated for 14h with EGF (100 ng/ml). When indicated the Tat-peptide (Tat-pep) was added at 1 μM together with EGF. Nuclear localization of p27 was revealed by immunofluorescence (Castoria et al., 2004) and expressed as % of total cells.

In B, quiescent MCF-7 cells on coverslips were unstimulated or stimulated with 10 nM estradiol for the indicated times. When indicated, the Tat-peptide (Tat-pep) was added at 1 mM, together with estradiol. Nuclear localization of p53 was revealed by immunofluorescence and expressed as % of total cells.

**The putative NES sequence of ERα shows homology with the p53 NES and is shared by other steroid receptors.**

In **C**, the sequence alignment between the indicated regions of ERα and p53 is shown, with the conserved amino acids in bold.

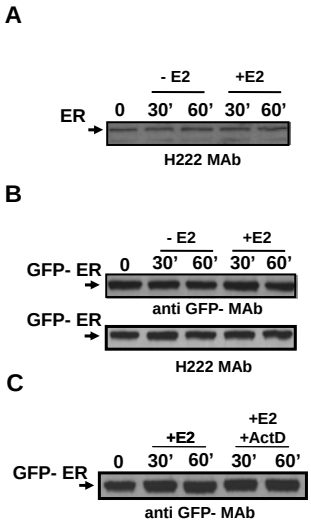
In **D**, the sequence alignment between the indicated regions of ERα and other steroid receptors is depicted. Sequences were obtained from The National Center for Biotechnology Information Data Library: ERα (estrogen receptor α) accession no. NP000116; ERβ (estrogen revìceptor β) no. NP001035366; PgR (progesterone receptor) no. NP000917; AR (androgen receptor) no. NP000035; GR (glucocorticoid receptor) no. P04150; MR (mineralocorticoid receptor) no.P08235. Conserved amino acids are in bold; the core of ER-NES sequence and the putative core of NES sequences in the indicated steroid receptors are underlined.

**References not cited in the text**

Guiochen-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M., and Milgrom, E. (1991) Nucleocytoplasmic shuttling of the progesterone receptor. EMBO J. 10, 3851-3859.

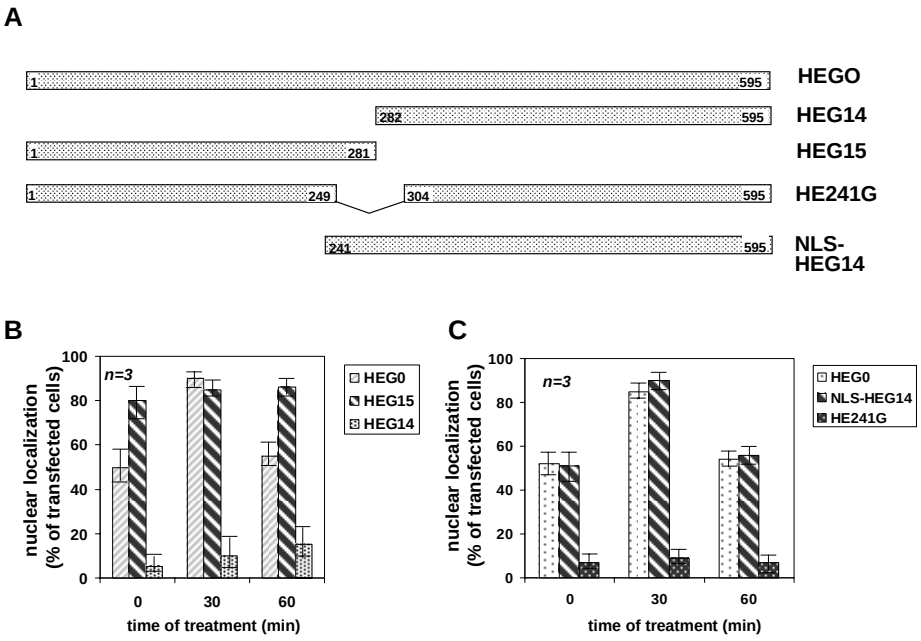
Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H. and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. EMBO J. 11, 3681-3694.

Supplemental materials 1



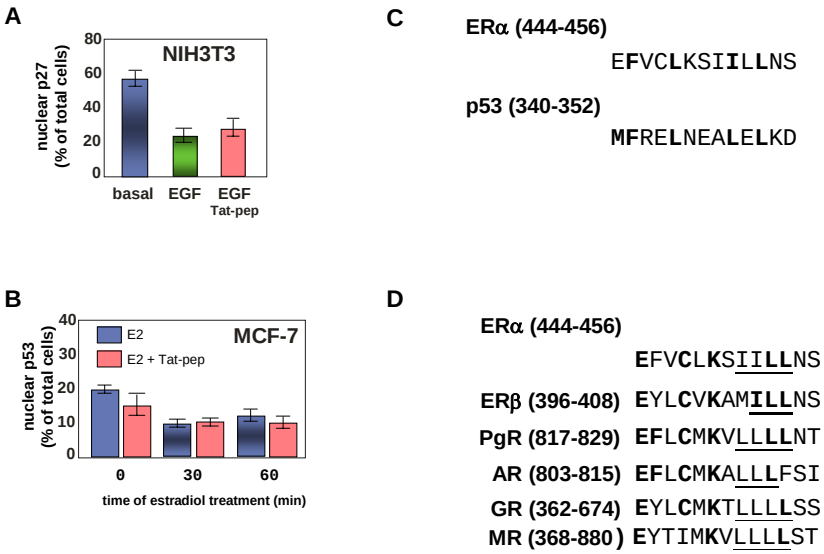

Supplemental Data- Fig. 1

Supplemental materials 2




Supplemental Data- Fig. 2

Supplemental materials 3




Supplemental Data- Fig. 3

[Frontiers in Bioscience 13, 1318-1327, January 1, 2008]

# Integrating signals between cAMP and MAPK pathways in breast cancer

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## 1. ABSTRACT

Breast cancer is one of the most common malignancies in Western society. Localized breast cancer, before it spreads, can be cured by surgery. However, the high mortality rate associated with breast cancer is due to a propensity of the tumor to metastasize when the primary tumor is small or undetectable. Although steroid receptor status has been recognized as the most precise predictor of response to hormone therapy, a significant number of tumors expressing these receptors metastasize and patients do not respond to the antihormone therapy. The mechanism leading to breast cancer progression and resistance to the hormone therapy is not completely understood at the present time. Compelling evidence shows that hormone-bound steroid receptors in breast cancer cells activate complex signaling networks, which include MAPK- and G protein-dependent pathways. These responses, which occur within seconds or minutes after steroid administration, are not due to changes in gene expression. Depending on cell systems, steroid activation of these networks leads to different and profound effects on extra nuclear and nuclear events. In such a way steroids foster cell cycle, reduce apoptosis and stimulate cell migration of target cells. All these processes are deregulated in breast cancer. In this review we will discuss new aspects of signaling pathways activated by steroids and their integration with other pathways in breast cancer. Recent findings on the discovery of compounds specifically interfering in such a complex network will be presented.

## 2. INTRODUCTION

Breast cancer is very common in developed countries, with one in ten women developing the disease and half of those dying of it. The status of steroid receptors is a well-established prognostic marker in breast cancer. Estradiol receptor alpha (ER alpha) has been implicated in the progression of breast cancer, and this is corroborated by the finding that about 60-70% of human breast cancers are ER alpha-positive (1). ER alpha status predicts a favorable disease outcome. Most patients with ER alpha-positive breast cancer receive tamoxifen as adjuvant endocrine therapy (2). Survival of tamoxifen-treated patients is longer for women with cancer with ER alpha amplification than for women with ER alpha expressing cancer without amplification (3). However, although tamoxifen treatment has improved the outcome from breast cancer, many patients become resistant to the hormone therapy and develop metastatic breast tumors. Several mechanisms have been proposed to explain the causes of breast cancer resistance to endocrine therapy. These include expression of steroid receptor variants, ligand-independent activation of steroid receptors, over-expression and activation of tyrosine kinases, most notably ErbB2 (4), and signaling effectors, such as AKT (5).

Steroid hormones control proliferation and survival of breast epithelial cells. This activity has been so far attributed to the interaction of steroids with their cognate

receptors and the consequent regulation of gene transcription (6). In addition to the well-studied nuclear function, ERs, progesterone receptor (PgR) and androgen receptor (AR) participate in extranuclear and membrane-mediated signaling events (7). Such a non genomic action has been linked to rapid responses elicited by steroid hormones and involves activation of Src, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K), protein kinase C (PKC) and etherotrimeric G-proteins in cytoplasm or membrane of target cells (8). Furthermore, the extra-nuclear mechanism regulating the cross talk between ER alpha and EGFR in cytoplasm of breast cancer cells has been recently analyzed (9). Interestingly, important biological responses such as DNA synthesis and cytoskeleton changes leading to cell migration can occur in the absence of transcriptional activity or nuclear localization of steroid receptors (10-12). Depending on the cell type and experimental conditions, steroid action may depend on integration between extranuclear and nuclear receptor activities (13).

In this review, we discuss new concepts of cross talk between steroid receptor and signaling effectors accounting for the non genomic actions of steroids. In particular, we highlight recent developments unraveling the intricate signaling network regulated by steroids in breast cancer and the integration of these pathways in the cell. Elucidating the details of these programs should provide a more rational approach to breast cancer therapy.

## 3. BREAST CANCER: A GENERAL OVERVIEW

Breast cancer remains a widespread disease. In 2004, there were 371,000 new cases of breast cancer diagnosed and 129,900 breast cancer-related deaths in Europe (14). Nevertheless, a decline in mortality rate has been observed during the last few years (15). This decline is due to mammographic screening, more precise diagnosis, and an increase in the number of women receiving the best treatment for their condition, like the extensive use of tamoxifen (16).

The causes leading to breast cancer and the identification of prevention strategies are still elusive. Association of the risk of breast cancer with age at first birth and parity was proposed several years ago (17) and confirmed by subsequent studies (15). Additional risk factors have been added in recent years. These include genetic factors, geographical location, exposure to ionising radiation, particularly during puberty, absence or short lifetime duration of breastfeeding (typical of women in developed countries), use of oral contraceptives, hormone-replacement therapy, high body-mass index and dietary factors, such as alcohol abuse. Progression from healthy mammary tissue to invasive carcinoma is still a debated process. The pre-neoplastic potential of benign, proliferative lesions of breast and dysplastic changes present in different non-malignant breast diseases is not defined. To date, *in situ* carcinomas (either ductal or lobular) are morphologically identifiable as neoplastic transformation, whereas stromal invasion and metastasis to regional lymph nodes or distant organs are the hallmarks of developed breast cancer.

The best approach to breast cancer therapy remains targeting the disease at the earliest stages of development. Tamoxifen, a selective estrogen receptor modulator (SERM), has been largely used because of the data from laboratory models and its ability to prevent contro-lateral breast cancer (18). Although the role of tamoxifen as a chemopreventive for women with high risk of breast cancer is generally accepted, what degree of risk is appropriate for its use remains unclear. In addition, tamoxifen induces increased risk of endometrial cancer and other side effects because of its partial agonist activity (19). Thus, other molecules such as Raloxifene and aromatase inhibitors have been developed. Raloxifene is also a SERM largely used in the treatment of osteoporosis in postmenopausal women. It reduces the incidence of breast cancer in osteoporosis trials and does not exert estrogen-like activity in uterus of rodents. Unfortunately, like tamoxifen, it increases thromboembolic events. Aromatase inhibitors are more effective than tamoxifen in preventing contro-lateral breast cancer and in the adjuvant treatment of early-stage disease. Aromatase inhibitors, however, do not suppress the levels of estradiol in premenopausal patients (18). It is noteworthy that tamoxifen acts through ER, and that only ER-positive breast cancers were reduced in the tamoxifen prevention trials. Its use is not suitable for women with BRCA1 mutations who develop ER-negative breast cancer or in patients with ER-negative breast cancers overexpressing ErbB2/HER2/neu. Drugs targeting other pathways involved in breast carcinogenesis, such as trastuzumab (Herceptin), an antibody against ErbB2, or oral tyrosine kinase inhibitors are used in therapy and new molecules with more specific action are being investigated in laboratory models of breast cancer (8, 20).

In conclusion, the general trend is now to identify new molecular targets in tumors and their neighboring cells and to increase targeted therapy of breast cancer.

### 3.1. Steroid receptors in breast cancer

Several years ago, Beatson observed that oophorectomy caused tumor regression in advanced breast cancer (21). This seminal finding opened the way to the study of the role of estradiol in this disease. After 70 years, an ER was identified (22) and purified by affinity chromatography (23). This receptor was detected in breast tumors (24) and is now an established prognostic marker. Its expression determines whether or not tamoxifen should be given as adjuvant endocrine therapy.

A second ER was later identified (25) and named ER beta to distinguish it from the original receptor, ER alpha. Two human ER beta isoforms of 530 and 485 amino acids have been described (26-28). The 530 amino acid form is generally believed to be the mature full-length ER beta (27-29).

Although estradiol is the main steroid implicated in breast cancer progression, much evidence points to progesterone as an important factor in the progression and maintenance of the neoplastic phenotype in the mammary gland (30). In fact, clinical data have demonstrated a higher risk of breast cancer in patients under hormone replacement

therapy using a combination of estrogens and progestins as compared with those using estrogens alone (31, 32). PgR, like ER, represents a target in the therapeutic approach to breast cancer (33). Accordingly, recent data raise the possibility that anti-progesterone treatment may be useful for breast cancer prevention in individuals with BRCA1 mutations, which predispose to breast and ovarian cancers (34).

PgR in rodents and humans exists as two isoforms, PgR-A and PgR-B. The two isoforms are produced from a single gene by translation initiation at two distinct start codons under the control of separate promoters (35). PgR-A is a truncated form of PgR-B. In humans, the N-terminal 164 amino acids of PgR-B are missing in PgR-A. Although the two forms of PgR have similar structures and identical DNA and ligand binding domains, *in vitro* studies using a progesterone-responsive transcription system reconstituted in mammalian cells revealed that PgR-A and PgR-B are functionally different. In most cases, PgR-B acts as a potent activator of transcription of target genes, whereas PgR-A acts as a dominant repressor of transcription regulated by PgR-B as well as other nuclear receptors (36).

Although a multitude of molecules involved in breast cancer biology, particularly ErbB2 and mutated BRCA1, are used as markers, determination of steroid receptor status remains an important prognostic assay. Overexpression of ER alpha is a well-established prognostic and predictive factor in breast cancer patients (2). More importantly, a large subset of breast cancers shows a single-gene amplification of the ER alpha gene, thus suggesting that this amplification may be a common mechanism in proliferative breast disease and a very early genetic alteration in breast cancer progression (3). Expression of PgR serves as a functional assay because it indicates that the ER transcriptional pathway is intact. When biochemical ligand binding assays indicate concentrations of 10 fmol/mg cytosol protein or more, breast cancers are generally considered ER-positive and PgR-positive for clinical purposes. ER and PgR status can also be evaluated using immunohistochemistry (IHC). Unlike chemical assays, IHC does not require destruction of tissue specimens; in addition, it shows ER tissue distribution. For these reasons, it has become the preferred method for determining ER/PgR status in breast cancer specimens. Quantitative methods using computer-aided image analysis are being developed to improve the accuracy of IHC.

## 4. SIGNALING BY STEROID RECEPTORS

### 4.1. Classical and rapid response models of steroid action

Steroid hormones influence many processes in mammals, including cell growth, cardiovascular health, bone integrity, immunity, cognition, and behavior. Evidence collected in the last few years indicates that regulation of these effects may be mediated by a complex interface between modulation of signaling cascades and control of gene expression. Receptors in the cell nucleus regulate gene expression, whereas classical receptors localized in close proximity to the cell membrane or in the extranuclear compartment of cells activate signal transduction (7, 10). Transcriptional effects of steroid hormones usually occur via ligand-dependent binding of receptors to target gene promoters as part of a pre-initiation transcription complex, which leads to chromatin remodeling and ultimately regulates gene expression (37). The resulting fluctuations in mRNAs and the proteins they encode take place within hours following hormonal exposure. In contrast, steroid activation of signal transducing pathways occurs within seconds or minutes. These rapid effects are insensitive to RNA and protein synthesis inhibitors. Almost all the members of the steroid hormone family, from the corticosteroids (glucocorticoids and mineralocorticoids) to the sex steroid hormones (estrogens, progestins, and androgens), exhibit rapid, non genomic actions. These range from activation of Src, MAPKs, adenylyl cyclase and PI3-K to rises in intracellular-calcium concentrations (38-45).

Much evidence shows that steroid stimulation of breast cancer cells rapidly induces G-protein activation and generation of a second messenger such as cAMP and cGMP (46). Although controversial findings have been reported about the nature of receptors mediating these responses, G protein activation by steroids leads to stimulation of various signaling effectors (46) and release of growth factors with consequent activation of their cognate receptors (47). Depending on the cell context, these signals are related to different effects of steroid hormones, such as proliferation, survival, migration and differentiation.

### 4.2. Role of cAMP pathway in breast cancer

Several years ago, Szego & Davis reported a rapid, acute elevation of uterine cAMP by estradiol treatment of rats (48). Subsequent studies indicated that estradiol treatment of human prostate tissue greatly increases the intracellular cAMP (49), and findings in ER-positive MCF-7 breast cancer cells showed that estradiol enhances intracellular cAMP production through adenylyl cyclase activation and stimulates cAMP response element (CRE)-mediated gene expression (50). In agreement with these observations, a role for cAMP/protein kinase A (PKA)-dependent pathway in the estradiol-regulated cyclin D1 transcription of ER-positive ZR-75 breast cancer cells has been proposed (51). Altogether, these studies show that signals resulting from activation of G-protein and cAMP-signaling pathways contribute to gene regulation by estradiol.

In addition to being produced in response to steroids and to regulating CRE-mediated genes, cAMP plays a role in the ligand-independent activation of steroid receptors. In fact, 8-Br cAMP treatment of cells transfected with a chicken PgR expression vector and a PgR-responsive reporter causes hormone-independent, but receptor-dependent activation of the reporter (52). These findings have been explained by the observations that cAMP increases phosphorylation of the steroid receptor coactivator-1 (SRC-1; 53-54). In addition, cAMP is also involved in resistance to steroid antagonists that frequently develops in breast cancer, since it enhances the ability of antiprogestin to activate gene transcription mediated by PgR-B in T47D breast cancer cells (55, 56).

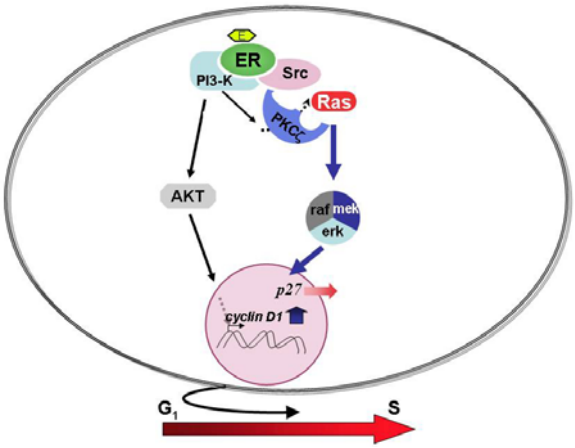


**Figure 1.** Estradiol activation of signaling effectors is responsible for cell cycle progression in ER-positive breast cancer cells. In breast cancer cells, estradiol rapidly induces the assembly of a complex made up by ER, Src and PI3-K. Through PKC zeta, Ras is also recruited to the complex and the Ras-dependent kinase cascade activated. Stimulation of PI3-K and Ras-dependent cascade leads to increased cyclin D1 transcription and p27 nuclear exclusion. These events are responsible for the G1/S transition of cells.

The role of cAMP in mammary carcinoma cell proliferation has also been investigated. Initial reports indicated that dibutryl-cAMP in conjunction with arginine suppresses the proliferation of MCF-7 cells (57). Subsequently, it was confirmed that elevation of cAMP levels produces substantial effects in MCF-7 cells. Addition of 8Br-cAMP or expression of mutant (Q227L)-activated G alpha$_s$ in MCF-7 cells did indeed block the ability of these cells to grow in an anchorage-independent manner, and stable transfection of activated-G alpha$_s$ in MCF-7 cells reduced the ability of these cells to form tumors in athymic mice (58). These findings indicate that cAMP may be crucial in preventing the expression of transformed phenotype in mammary epithelial cells. In addition, G protein coupled receptor 30 (GPR30) expression correlates with progestin-induced growth inhibition in different breast cancer cells and GPR30 is critical for progestin-induced growth inhibition (59).

It is now largely accepted that estradiol and progestin treatment of breast cancer cells rapidly generates cAMP. This action results from G protein activation and signaling is then transmitted to various effectors, including PKA, PKC, MAPK and PI3-K (46). Although the importance of these signals in the cellular action of sex steroids *in vitro* and *in vivo* is well documented, the nature of receptors mediating these events is still debated. Some models propose the involvement of classical steroid receptors, which initiates signaling cascades by association with the scaffold protein, caveolin-1 (60) and a variety of proximal signaling molecules, including G proteins (61-63), Src (39, 42, 64), PI3-K (11, 43, 65), MNAR (66), PKC zeta (45) and Shc (67). Other candidates in mediating these events are represented by traditional G protein-coupled receptors (GPRs). One of these receptors has recently been identified by different groups as GPR30, an orphan GPR (68, 69).

### 4.3. MAPK signaling in breast cancer

MAPK circuits transmit and amplify signals involved in a plethora of cell responses. These pathways are indicators of the intensity and length of signals induced by growth factor, steroid hormones, and ligands of G protein coupled receptors. Three major MAPK pathways exist in human tissues, but ERK-1 and -2 are the most relevant to breast cancer, and several studies demonstrate that they are frequently activated in breast cancer (70). A number of investigators have now studied the expression of activated MAPK in human breast cancer tissues by enzymatic assay and immunohistochemical techniques. In one half of breast tumors MAPK is more active than in the surrounding benign tissue. Studies also show a trend toward higher MAPK activity in primary tumors of node-positive than in node-negative patients; this up-regulation is not caused by Ras mutations, but results from enhancement of growth factor pathway activation (70).

Estradiol, progesterone and androgens very rapidly activate MAPK in breast, prostate and colon cancer cells (39, 40, 42, 64, 71, 72). This activation depends on the stimulation of the Src/Ras cascade by sex steroids and has a proliferative role as demonstrated by experiments with chemical inhibitors and signaling effector mutants (10, 12, 42). In breast cancer cells, estradiol triggers direct interaction of classical ER alpha with the SH2 domain of Src, whereas androgens trigger AR interaction with the SH3 domain of Src (42). Estradiol activation of the Src axis occurs alongside PI3-K. Hormone stimulation of MCF-7 cells induces the assembly of a multi-molecular complex made up by ER, Src and p85 alpha, the regulatory subunit of PI3-K, which triggers activation of the Src and PI3-K-dependent pathways. Hormone-activated PI3-K targets Akt and PKC zeta. Once activated, Akt increases cyclin D1 transcription, whereas PKC zeta controls Ras recruitment to the ER/Src/PI3-K complex, Erk-2 nuclear translocation and the consequent release of p27 from cell nuclei. By this interplay between signaling effectors and cell cycle regulators, cells enter the S-phase (43, 44). These conclusions have been highlighted by recent findings showing that specific interference in the sex steroid receptor/Src interaction by new, cell-permeable molecules inhibits the growth of mammary tumor and prostate tumor cells *in vitro* and in nude mice (8, 20).

Figure 1 depicts the estradiol control of cell cycle progression through signaling effectors in breast cancer cells.

Progesterone activation of MAPK was initially reported in T47D breast cancer cells (40). Progesterone stimulation of cells induces cross talk between cytoplasmic PgR-B and ER alpha, which in the absence of estradiol triggers ER alpha/Src association with consequent activation of the Src/Ras/MAPK pathway (40). Activation of MAPK by progestins is needed for the S-phase entry of T47D cells (10). Subsequent studies in *in vitro* reconstituted systems further clarified the molecular mechanism underlying progesterone activation of MAPK cascade by cross talk between PgR-B and ER alpha (41). Such cross talk is also

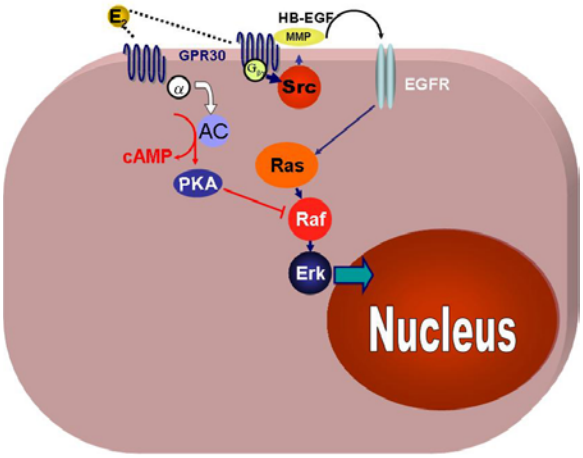


**Figure 2.** Model of estradiol action through GPR30 and cross talk between cAMP and MAPK pathways in ER-negative breast cancer cells. In ER-negative breast cancer cells, estradiol ($E_2$) directly binds to GPR30 and induces, through Gβγ-subunit protein activation, a Src-mediated activation of metalloproteinase (MMP) and release of HB-EGF. Transactivation of EGFR then occurs and Erk activation is triggered. Estradiol binding to GPR30 also activates adenylyl cyclase (AC) and increases cAMP levels. PKA activation occurs and Raf is blocked. Erk signaling is then switched off.

responsible for progestin stimulation of endometrial stromal cell proliferation mediated by non genomic pathway activation (73). More recently, it has been shown that activation of MAPK cascade by progesterone through the PgR-B and ER alpha cross talk leads to phosphorylation of histone H3 with the consequent induction of progesterone target genes, thus pointing to the regulatory role of MAPK in the integration between non genomic and genomic signaling activated by steroids (13). Under different experimental conditions, it has been observed that PgR can directly activate Src, without the contribution of ER (64). Rapid activation of MAPK by steroids has been observed in different cell systems, including *in vivo* models (74, 75).

MAPK are also implicated in the ligand-independent activation of ER alpha, as shown by findings demonstrating that activation of MAPK by growth factors phosphorylates and potentiates the transactivation function of ER alpha (76). In addition, expression of constitutively activated MEK-1 in MCF-7 breast cancer cells increases ER alpha-mediated transcriptional activation and accelerates tumor growth *in vivo* (77). Altogether, these data indicate that MAPK pathway can also intersect with steroid receptors at the transcriptional level.

### 4.4. Integration between cAMP and MAPK pathways in breast cancer

The complexity of signaling pathways, the cross talk between multiple pathways and the presence of feedback loops occurring within the circuits has been actively investigated (78). Integration between different signaling pathways activated by steroid hormones in breast cancer has been explored. As described in the previous section, estradiol treatment of MCF-7 cells triggers activation of PI3-K and Src-dependent pathways with a proliferative final effect. Signaling of steroid hormones can also be regulated by adenylyl cyclase. Traditionally, adenylyl cyclase activity is modulated by receptors that couple to GPRs, and data from different groups have shown that GPR30, an orphan GPR, plays a critical role in steroid signaling (68-69). It binds estradiol and regulates MAPK activation in a transient way, since it is involved in both the rapid activation of MAPK and its subsequent inactivation. These findings indicate that the estradiol control of MAPK axis occurs even in the absence of classical ER. In fact, estradiol treatment of ER-negative cells triggers GPR30 activity that, through Gβγ-subunit protein activation, induces the Src-mediated release of heparin- bound EGF (HB-EGF) from the cell surface. Once released, HB-EGF activates EGFR, which, in turn, triggers MAPK activation (79). A similar pathway, however, can be activated by estradiol occupancy of the classical ER (46 and refs therein). Furthermore in cells lacking ER, estradiol also through GPR30 activation and Gα-subunit protein, stimulates adenylyl ciclase and increases cAMP levels. This event leads to activation of PKA and PKA-mediated block of Raf. In this way, MAPK inactivation follows to the initial MAPK activation (80, 81). Recent work supports such a model. Addition of cAMP in MCF-7 cells activates PKA, which, in turn, phosphorylates the regulatory subunit p85 of PI3-K in serine 83. In this way, cAMP intersects with estradiol by facilitating the binding of ER to PI3-K. This results in a selective increase in Ras/PI3-K association and a net decrease in the Ras/Raf-1 complex. Thus, Ras signaling is mainly channeled to PI3-K rather than to Raf-1/MAPK (82). These data offer an example of how cAMP may act as an inhibitor of MAPK.

The cross talk between cAMP and MAPK signaling pathways is involved in cell transformation. In fibroblasts, elevation of cAMP blocks signaling through the Ras/Raf/MEK pathway and therefore blocks Ras-induced transformation through PKA. Thus, Raf appears to be the major target of PKA in inhibiting signal transmission to MAPK. In this regard, it has been described that elevation of cAMP levels reduces both EGF stimulation of MAPK in MCF-7 cells and the ability of the same cells to form tumors in nude mice (58). Subsequent studies have shown that expression of G protein alpha inhibits the growth of established human tumors of breast cancer cells in athymic mice by inhibiting the MAPK pathway (83). In addition to indicating that interactions between the cAMP and MAPK signaling pathways regulate proliferation of breast cancer *in vivo*, these data also imply that targeting of the cAMP/MAPK axis (i.e. by continuous elevation of cAMP) could be used to block tumor formation.

Figure 2 illustrates the GPR30-mediated actions of estradiol and the cross talk between adenylyl cyclase and MAPK (Erk) in ER-negative breast cancer cells. The initial estradiol activation of Erk is followed by PKA/Raf-mediated inactivation of the same enzyme.

## 5. SUMMARY AND PERSPECTIVES

To date, most of the studies investigating the non genomic action of steroid hormones have been conducted *in vitro* using cancer-derived cells, and only a small number of these studies concern non-reproductive cells, mainly stromal cells, which strongly contribute to cancer progression. We have to learn much more about the role of steroid-activated pathways as well as their integration *in vivo* with pathways activated by different ligands, such as non-steroid hormones and growth factors. The proteomic approach, in association with the use of animals expressing genetically modified signaling effectors, will be of great help in this complex analysis. Another promising line of research has been initiated by laboratories seeking for ER ligands that preferentially act on the transcriptional or non-transcriptional signaling of ERs. A synthetic compound termed estren mainly induces the non-transcriptional actions of ER, whereas another pyrazole compound induces the transcriptional activity of ER, with minimal effects on its rapid signaling action (84, 85). It is expected that other similar receptor ligands will be found and employed in the study of steroid receptor action as well as in the therapy of receptor-associated diseases.

The emerging field of steroid receptor-mediated signaling activation in breast cancer is very promising and one of the reasons for this mounting interest is offered by the potential use of signalosoma-based approaches to cancer therapy. Recently, new molecules have been identified and used to inhibit the proliferation of breast and prostate cancer cells *in vitro* as well as in immune-depressed mice (20, 45 and submitted). These molecules act at nano-molar concentrations by specifically interfering in the interaction of steroid receptors and Src. They leave unaltered the receptor-mediated gene transcription as well as the signaling transduction that does not depend on steroid receptors. Further investigation is required to validate these approaches to cancer therapy in preclinical and clinical studies and find new strategies to contrast breast cancer.

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**Abbreviations:** SR, steroid receptor; ER, estradiol receptor, PgR, progesterone receptor; AR, androgen receptor; EGF, epidermal growth factor; HB-EGF, heparin-bound EGF; EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinases; MEK-1, mitogen-activated kinase kinase; MMP, metalloproteinase; PI3-K, phosphatidylinositol-3-kinase; GPRs, G protein coupled receptors; PKA, protein kinase A; PKC, protein kinase C.

**Key words:** Breast Cancer, Steroids, Steroid Receptor Signaling, cAMP, MAPK, Review

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ORIGINAL ARTICLE

# Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth

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**In human mammary and prostate cancer cells, steroid hormones or epidermal growth factor (EGF) trigger association of the androgen receptor (AR)-estradiol receptor (ER) (α or β) complex with Src. This interaction activates Src and affects the G1 to S cell cycle progression. In this report, we identify the sequence responsible for the AR/Src interaction and describe a 10 amino-acid peptide that inhibits this interaction. Treatment of the human prostate or mammary cancer cells (LNCaP or MCF-7, respectively) with nanomolar concentrations of this peptide inhibits the androgen- or estradiol-induced association between the AR or the ER and Src the Src/Erk pathway activation, cyclin D1 expression and DNA synthesis, without interfering in the receptor-dependent transcriptional activity. Similarly, the peptide prevents the S phase entry of LNCaP and MCF-7 cells treated with EGF as well as mouse embryo fibroblasts stimulated with androgen or EGF. Interestingly, the peptide does not inhibit the S phase entry and cytoskeletal changes induced by EGF or serum treatment of AR-negative prostate cancer cell lines. The peptide is the first example of a specific inhibitor of steroid receptor-dependent signal transducing activity. The importance of these results is highlighted by the finding that the peptide strongly inhibits the growth of LNCaP xenografts established in nude mice.**
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**Keywords:** androgen receptor; estrogen receptor; receptor antagonist; Src; xenografts

## Introduction

Prostate and mammary cancers are among the most frequently diagnosed cancers and are major causes of cancer death. These cancers are frequently androgen receptor (AR) positive (Lopez-Otin and Diamandis, 1998), and express the α- or β-forms of the estradiol receptor (ER). AR and ER expression is maintained even in hormone-independent cancers, although their role under these conditions is still poorly understood. More than 80% of clinically androgen independent human prostate tumors show high levels of AR (Zhao *et al*., 2000). There appear to be different possible mechanisms for acquiring androgen-independence in the presence of AR expression. An increased level of AR could sensitize cancers to low levels of residual androgens, and antiandrogens could promote androgen activation rather than inhibition (Chen *et al*., 2004). Also, growth factors, such as epidermal growth factor (EGF), could bypass the hormone requirement by directly activating the AR (Culig *et al*., 1994, Migliaccio *et al*., 2005). Alternatively, non-androgenic steroids could promote androgen-independent growth of prostate cancer cells through a mutated AR (Zhao *et al*., 2000). Estrogen independence in the presence of ER might arise via other mechanisms including the ligand-independent ER activation, expression of ER variants, and increased expression of co-activators (Gururaj *et al*., 2006). In addition, alteration of the complex interactions between ER and membrane-associated or cytoplasmic effectors might be involved in breast tumor progression as well as resistance to hormonal therapy (Vadlamudi *et al*., 2005).

Previous studies have shown that sex steroid hormones activate signal transducing pathways that are frequently upregulated in human breast tumors (Cato *et al*., 2002; Levin, 2003). In human prostate and mammary cancer-derived cells, treatment with androgen or estrogen rapidly induces association of Src with AR and ER, leading to Src activation and stimulation of DNA synthesis (Migliaccio *et al*., 2000). AR association with Src has also been observed in androgen-stimulated immortalized fibroblasts (Castoria *et al*., 2003), raising

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the possibility that, in response to androgens, stromal cells facilitate the malignant outgrowth and the metastatic spread of prostate epithelial cells. In addition, EGF signaling, which induces S phase entry and cytoskeletal changes of prostate and breast cancer cells, requires AR/ER association with Src (Migliaccio *et al*., 2005).

On the basis of our previous findings (Migliaccio *et al*., 2000) and establishment of new functional assays for AR deletion mutants, in this report, we define the amino-acid sequence responsible for the human AR (hAR) interaction with Src. A synthetic 10 amino-acid peptide that mimics this sequence prevents the AR/ER complex from associating with Src. This peptide inhibits the G1 to S transition of androgen- or estradiol-stimulated prostate and mammary cancer cells *in vitro* as well as the growth of LNCaP prostate cell xenografts established in nude mice. Because of the role of AR in EGF-elicited signaling (Migliaccio *et al*., 2005), the peptide also prevents EGF-dependent DNA synthesis in normal and cancer cell lines. Use of this peptide, alone or in association with other compounds, potentially offers a novel approach to specifically inhibit human prostate and breast cancers.

## Results

Deletion of the 371–422 sequence of the hAR abolishes the *in vitro* association of the receptor with the Src SH3 domain (Migliaccio *et al*., 2000). To better define the AR-interacting sequence, different human AR-deletion mutants were prepared. Three deletions were separately introduced within the proline-rich 372–379 region and the contiguous sequence including arginine (Figure 1a). Arginine is either contiguous or adjacent to proline in Src, Yes and Lyn SH3-binding peptides (Kay *et al*., 2000). The wild-type (wt) AR and its three mutants were transiently transfected in Cos cells, which do not express endogenous AR, and the ectopically expressed AR was detected by immunoblotting the cell lysates with an anti-AR antibody (Figure 1b, upper panel). To assess the interaction of Src with AR, lysates from Cos cells treated with the synthetic androgen R1881 for 3 min were incubated with anti-Src antibodies and the immunocomplexes blotted with antibodies against either Src or AR. In the presence of the synthetic androgen R1881, a strong association of wt AR with Src was detected, whereas a much weaker association, similar to that found in the absence of hormone, was observed in cells expressing the AR mutants (Figure 1b, lower panel). As expected (Migliaccio *et al*., 2000), Src immunoprecipitated with the wt AR from the lysate of Cos cells treated with R1881 for 3 min actively phosphorylated acidified enolase. In contrast, no increase of Src activity over the basal level was observed when the AR constructs with a deletion of either the 372–385 residues (AR−Δ1) or the 372–378 sequence (AR−Δ2) were overexpressed in the same cells (Figure 1c). The AR deleted of the 380–386 (AR−Δ3) sequence only weakly activated Src (Figure 1c).

In contrast to Src activation, Cos cells transfected with the deleted AR did not show reduction of AR-dependent gene transcription. To verify this issue, an androgen enhancer (the 3416 construct; Verrijdt *et al*., 2000) was transiently co-transfected in Cos cells and the effect of R1881 on reporter gene transcription was evaluated (Figure 1d).

To characterize further the functional role of the AR proline-rich region, four peptides derived from the sequences deleted in the AR mutants were chemically synthesized. The peptides were N-terminal acetylated and C-terminal amidated (Figure 2, upper panel). They were analysed for the ability to reduce the DNA synthesis induced by R1881 in LNCaP (Figure 2, middle panel) or estradiol in MCF-7 (Figure 2, lower panel) cells. A peptide corresponding to the amino acids 377–386 of the hAR (Ac-PPPHPHARIK-NH2; S1 peptide) was finally selected because of its slightly stronger inhibitory action and its smaller size. In addition, a peptide containing the same amino-acid composition of the S1 peptide, but with a shuffled sequence (Ac-HPKPARIPHP-NH2) was synthesized (Ss peptide) and analysed in parallel. The uptake of both carboxy-fluorescein-conjugated S1 and Ss peptides was firstly analysed by confocal microscopy in quiescent, unfixed LNCaP cells. Figure 3a shows that in 30 min at 37°C the peptides are delivered similarly into the cells (upper panels). No dependence on the temperature was observed, thus excluding an energy-dependent mechanism of peptide internalization (lower panels in Figure 3a). Similar results were obtained in fixed LNCaP cells (not shown). To assess the effect of the peptides on association between Src and AR, or ER, we used quiescent LNCaP and MCF-7 cells. As expected, R1881 or estradiol triggered the interaction of the corresponding receptor with Src in LNCaP or MCF-7 cells, respectively, (Figure 3b and c, upper panels). Notably, treatment of these cells with the S1 peptide or the steroid antagonists Casodex (Figure 3b) or ICI 182 780 (Figure 3c) prevented the receptor/Src interaction (Figure 3b and c, upper panels). The Ss peptide did not abolish the hormonal-induced assembly of Src with AR or ER, although a decrease of the complex assembly was observed. The S1 peptide also prevented the hormonal activation of Src and Erk assayed as enolase (en) and myelin basic protein (MBP) phosphorylation, respectively. Such an effect supports the evidence that the S1 peptide action on the association of the steroid receptors with Src is responsible for the inhibition of the mitogenic Src/Erk pathway triggered by hormones. The Ss peptide effect on the activity of Src and Erk is negligible or absent (Figure 3b and c, upper and lower panels).

In agreement with our previous findings (Castoria *et al*., 2001), LNCaP or MCF-7 cells treated with R1881 or estradiol for 8 h show an increase in cyclin D1 expression and the Src-kinase inhibitor, PP2, abolished this hormonal effect (Figure 4a and c). The increased expression of cyclin D1 was clearly prevented by the S1 peptide and slightly decreased by the Ss peptide (Figure 4a and c). At this point it is clear that the S1

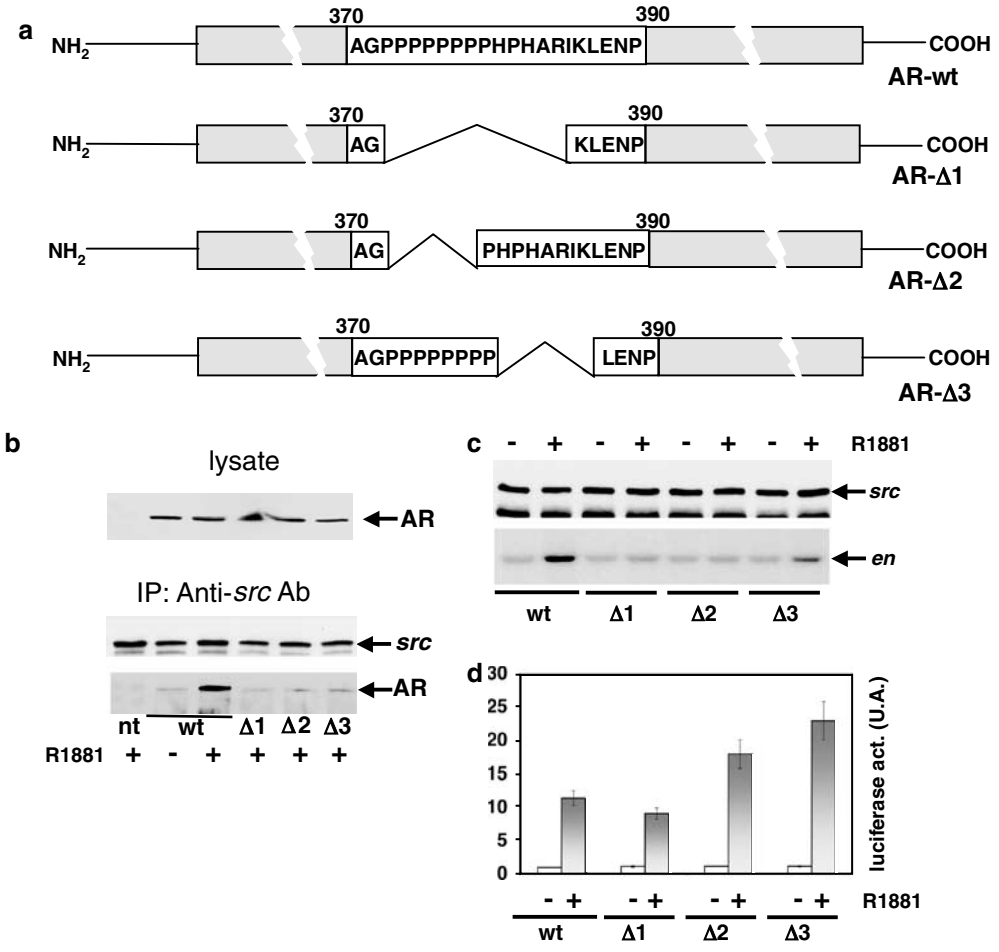


**Figure 1** Identification of the AR domain responsible for the receptor association with Src. (**a**) Diagram of the wt hAR as well as its mutants engineered as described in Materials and methods. The wt hAR (wt) or its mutants (Δ1, Δ2 and Δ3) were transiently transfected in Cos cells. In (**b** and **c**), cells were made quiescent and then left untreated or treated for 3 min with 10 nM R1881. In (**b**), lysates were either analysed for expression of AR and its mutants using the anti-AR antibody (upper panel) or immunoprecipitated with anti-Src MAb (lower panels). Immunocomplexes were then immunoblotted with the antibodies against the proteins indicated by the arrows. In (**c**), lysates were immunoprecipitated with the anti-Src MAb and the Src kinase activity assayed using acidified enolase as a substrate (lower panel). Immunocomplexes were also blotted with the anti-Src MAb to control the protein loading (upper panel). In (**d**), cells were co-transfected along with ARE3416 construct, then left unstimulated or stimulated for 18 h with 10 nM R1881. The luciferase activity was assayed, normalized using $\beta$-gal as internal control and expressed as fold induction. Wt, wild-type.

peptide prevents an entire sequence of events: AR/Src association, Src/Erk activation, cyclin-D1 expression. This conclusion suggested that AR/Src interaction might be a target for inhibition of the AR/Src-dependent cell proliferation by this peptide. Such a possibility was verified by treating LNCaP or MCF-7 cells with either R1881 or estradiol (Figure 4b and d), in the presence or absence of the corresponding steroid antagonists and comparing the inhibitory response to that observed in the presence of the peptide. The S1 peptide and the steroid antagonists inhibited to a similar extent DNA synthesis in LNCaP and MCF-7 cells, whereas the scrambled peptide slightly reduced the hormonal effect.

It has been recently reported that, in LNCaP and MCF-7 cells, EGF-induced signaling requires the association of AR and ER with Src (Migliaccio *et al*., 2005). In addition, AR or ER inhibition by steroid antagonists or siRNA prevents EGF-triggered DNA synthesis and cytoskeletal changes (Migliaccio *et al*., 2005). This is confirmed by the experiments in Figure 5 showing that steroid antagonists inhibit EGF-induced DNA synthesis in LNCaP and MCF-7 cells. Interestingly, the S1 peptide also abolished the EGF effect in these cells, whereas the Ss peptide had no effect (Figure 5a and b). Since the NIH3T3 fibroblasts express AR (Castoria *et al*., 2003), the effect of the S1 peptide on the S phase entry of NIH3T3 fibroblasts stimulated with R1881 or EGF was also evaluated (Figure 5c). The S1 peptide substantially reduced the R1881- and EGF-induced DNA synthesis in NIH3T3 fibroblasts, while the Ss peptide showed again little or no effect (Figure 5c). Experiments in Figure 6a show that serum-induced DNA synthesis in NIH3T3 fibroblasts, LNCaP or MCF-7 cells is unaffected by the S1 peptide, thus implicating that the inhibitory effect of the peptide is restricted to AR-dependent DNA synthesis. This conclusion is further reinforced by data in panels b and c

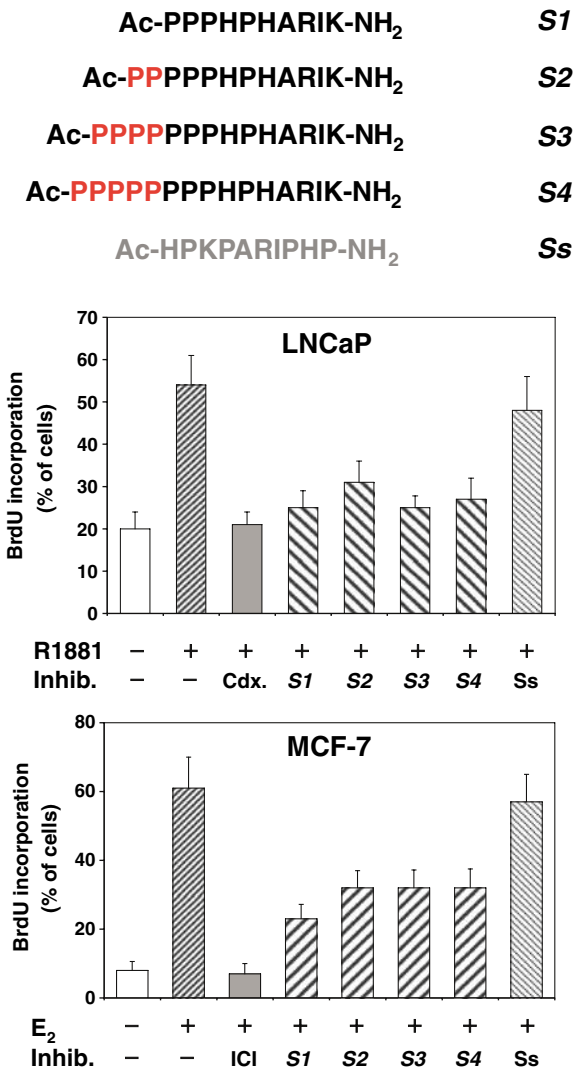


**Figure 2** Identification of a small peptide mirroring the AR 377–386 sequence. The upper panel is a diagram of peptides mimicking the AR sequences deleted in the AR mutants shown in (**a**) of Figure 1; a peptide with the scrambled sequence of the peptide S1 (Ss) is also shown. Quiescent LNCaP (middle panel) or MCF-7 (lower panel) cells on coverslips were left unstimulated or stimulated with either R1881 (10 nM) or estradiol (10 nM) in the absence or presence of the indicated peptides (at 1 nM) for 24 h. After *in vivo* pulse with BrdU, the cells were stained for BrdU incorporation. DNA synthesis was calculated by the formula: percentage of BrdU-positive cells (number of BrdU-positive cells/number of total cells) × 100. Data are derived from at least 1000 scored cells. The results of two independent experiments have been averaged; means and s.e.m. are shown. BrdU, bromodeoxyuridine.

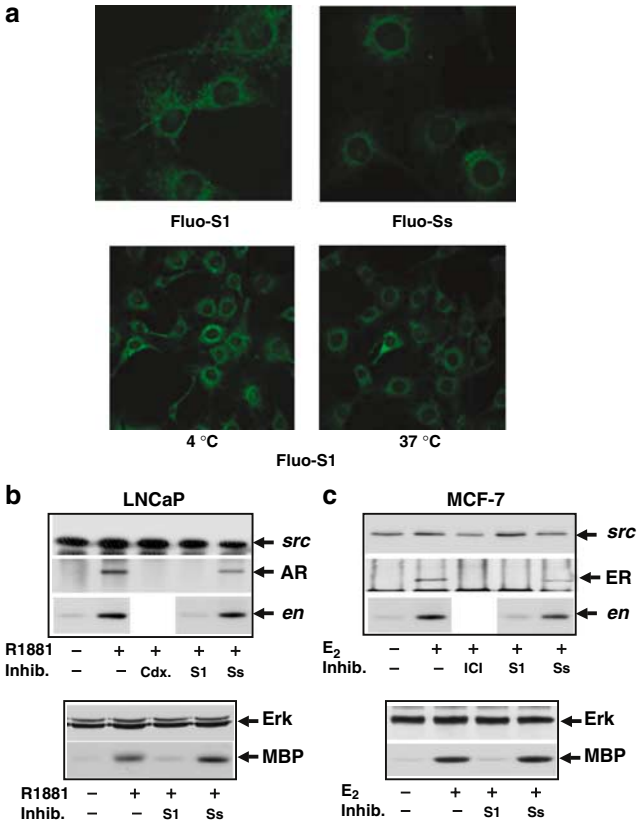


**Figure 3** A peptide mimicking the AR 377–386 sequence inhibits the association between the receptor and Src and the Src/Erk pathway in prostate and breast cancer-derived cells. (**a**) The 30 min uptake of fluorescein-conjugated S1 or Ss (upper microphotographs) peptides in quiescent, unfixed LNCaP cells analysed by confocal microscopy is shown. Lower images of (**a**) show the fluorescent conjugated S1 peptide incubated at 4°C (left microphotograph) or 37°C (right microphotograph). In (**b**), quiescent LNCaP cells were left unstimulated or challenged for 3 min with 10 nM R1881, in the absence or presence of the indicated compounds. The antiandrogen Casodex (Cdx) was used at 10 $\mu$M, whereas the S1 (S1) and the shuffled (Ss) peptides were used at 1 nM. In (**c**), quiescent MCF-7 cells were left unstimulated or stimulated for 3 min with 10 nM estradiol in the absence or presence of the indicated compounds. The antiestrogen ICI (ICI) was used at 10 $\mu$M; the S1 (S1) and the shuffled (Ss) peptides were used at 1 nM. Lysates in (**b** and **c**) were immunoprecipitated with either anti-Src MAb (upper panels) or anti-Erk-2 Ab (lower panels). In the upper panels, immunocomplexes were either blotted with antibodies against the proteins indicated by the arrows or assayed for Src activity, using enolase as a substrate (en). In the lower panels, immunocomplexes were either blotted with anti-Erk-2 antibody, or assayed for Erk-2 activity using MBP as a substrate. AR, androgen receptor; ER, estradiol receptor; MBP, myelin basic protein.

of the same figure, showing that addition of the S1 peptide does not affect EGF or serum-induced DNA synthesis of AR-negative PC3 or DU145 cells. Another finding against the possibility that the effect of the S1 peptide is due to interference in intrinsic Src activity is shown in Figure 6d. Clearly, the peptide has no effect on a typical Src-dependent effect, such as the cytoskeletal changes in EGF-treated DU 145 cells. In contrast, the Src inhibitor PP2 completely abolishes the induction of fan-like protrusions by EGF (Figure 6d). These data together with previous results clearly demonstrate that the S1-peptide specifically targets Src activation dependent on AR, but does not affect the intrinsic activity of this kinase.

The effect of the S1 peptide on steroid receptor-dependent transcription was then analysed. Therefore, the stimulatory effect of R1881 on the AR-dependent transcriptional activity in LNCaP cells transfected with an ARE-luciferase gene reporter (Figure 7a) and the

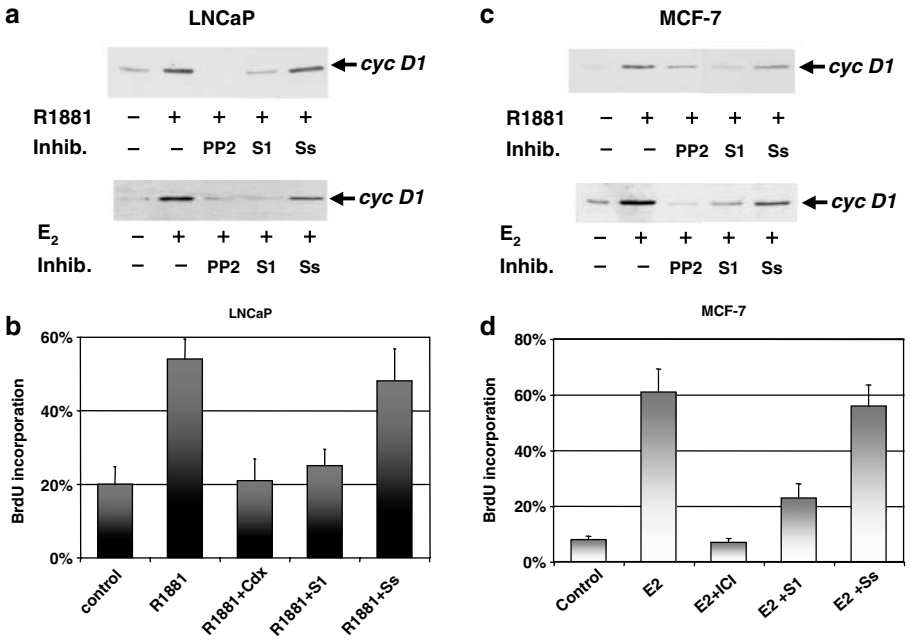


**Figure 4** The S1 peptide inhibits the hormone-stimulated G1 to S progression of human prostate and breast cancer-derived cells. LNCaP (**a** and **b**) and MCF-7 (**c** and **d**) cells were made quiescent, and then left untreated or treated with the indicated compounds at the following concentrations: 10 nM of R1881 or estradiol, 10 $\mu$M of the Src inhibitor, PP2 (Calbiochem), 1 nM of the S1 or Ss peptides. The antiandrogen Casodex and the antiestrogen ICI were used at 10 $\mu$M. In (**a** and **c**), lysates were collected after 8 h and analysed for cyclin D1 expression. In (**b** and **d**), quiescent cells on coverslips were left unstimulated or stimulated with the indicated compounds for 24 h. After *in vivo* pulse with BrdU, the cells were stained for BrdU incorporation. DNA synthesis was calculated as in Figure 2. Data are derived from at least 3000 scored cells. The results of six independent experiments have been averaged; means and s.e.m. are shown.

estradiol effect on the ER transcriptional activity in MCF-7 cells transfected with an ERE-luciferase gene reporter (Figure 7b) were examined. In contrast with classic androgen (Casodex) or estradiol (ICI) antagonists, the S1 or the Ss peptide did not significantly affect the hormonal action in both cell lines. This supports the view that the S1 peptide inhibits only the non-genomic action of AR or ER on DNA synthesis but has no effect on transcriptional activation mediated by these receptors.

Next, we analysed the effect of the S1 peptide on LNCaP xenografts established in nude mice. Interestingly, treatment with S1 peptide strongly inhibited tumor growth of LNCaP cells, whereas a negligible effect was observed upon treatment with the Ss peptide (Figure 8a). At the conclusion of the experiments, tumor tissues were analysed by immunohistochemistry for cell proliferation and apoptotic index. The proliferation status of cancer cells was determined by Ki67 positivity, which was significantly decreased ($P<0.005$) in LNCaP tumors treated with the S1 peptide (Figure 8b and C[2]). In addition, the number of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-positive nuclei was also significantly increased ($P<0.001$) in the same tumors (Figure 8b and 8d[2]), indicating that the peptide treatment acts on both proliferation and apoptosis. The peptide-treated mice did not show a decrease in weight (not shown).

## Discussion

The current therapy of prostate and mammary cancers is based on the use of steroid antagonists. However, this approach is limited by the mixed, antagonist and agonist action of these molecules, their side effects, and the appearance of hormone resistance. Estradiol, progestins and androgens induce G1 to S transition of cells derived from human mammary and prostate cancers through activation of signal transducing pathways (Castoria *et al*., 1999; Migliaccio *et al*., 2000). These findings open potential new approaches to the therapy of hormone-dependent cancers. In principle, such new therapy could be more specific than the classical therapy with steroid antagonists. This is because the classic antagonists, in addition to abolish the receptor-dependent signaling pathways, also inhibit the receptor transcriptional action. This action is not required for the steroid-stimulated DNA synthesis of mammary and prostate cancer-derived cells *in vitro* (Castoria *et al*., 1999; Migliaccio *et al*., 2000) but is relevant to positive actions of sex-steroid hormones, such as neuroprotection and bone preservation.

Simultaneous ER and AR association with Src is the initial event triggered during sex steroid-induced G1 to S transition of MCF-7 and LNCaP cells (Migliaccio *et al*., 2000). The same association is required in the same cells for EGF-induced DNA synthesis (Migliaccio *et al*., 2005). Recent work indicates that association of the two

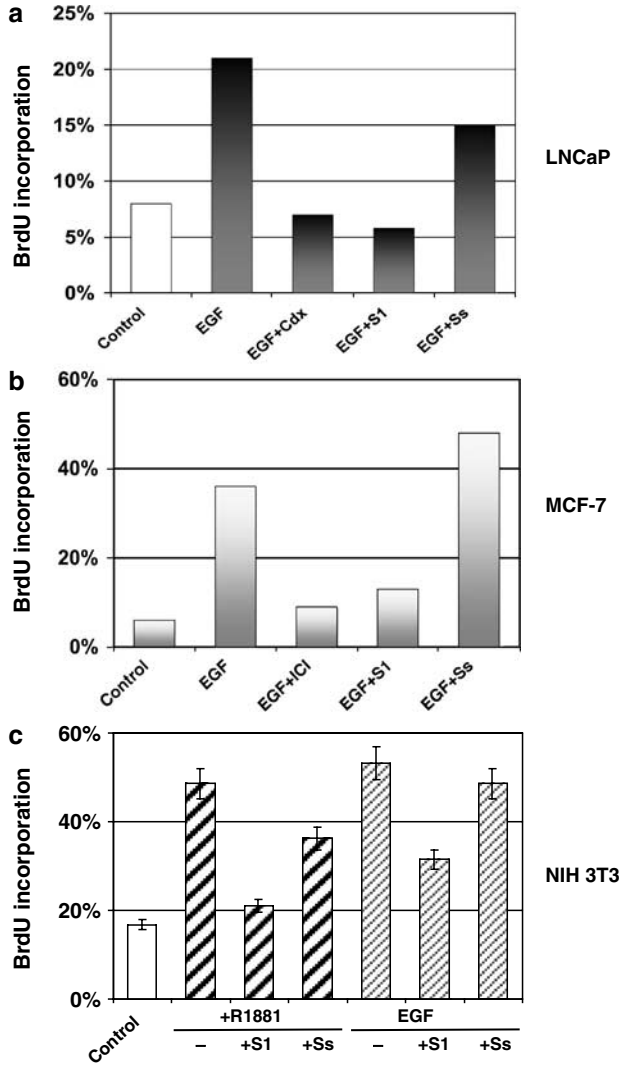


**Figure 5** The S1 peptide inhibits the S phase entry induced by EGF in LNCaP, MCF-7 and NIH3T3 cells. LNCaP (**a**), MCF-7 (**b**) and NIH3T3 (**c**) cells on coverslips were made quiescent, then left untreated or treated with the following compounds: R1881 (1 pM), EGF (Roche, Basel, Switzerland; 100 ng/ml), S1 and Ss peptides (1 nM). The antiandrogen Casodex and the antiestrogen ICI were used at 10 $\mu$M. After *in vivo* labeling with BrdU, the cells were stained for BrdU incorporation. DNA synthesis was calculated as in Figure 2. Data are derived from at least 2000 scored cells. For LNCaP and MCF-7 cells, the results of two independent experiments have been averaged. For NIH3T3 fibroblasts, the results of four independent experiments have been averaged. Means and s.e.m. are shown. BrdU, bromodeoxyuridine; EGF, epidermal growth factor.

receptors with Src in response to a single signal is due to the presence of an ER/AR complex in unstimulated mammary and prostate cancer-derived cells (Migliaccio *et al*., 2005). Therefore, a single steroid antagonist (antiestrogen or antiandrogen) can equally block the androgen or the estradiol-induced association of the ER/AR complex with Src (Migliaccio *et al*., 2000, 2005). Because of the crucial role of receptor/Src interaction in steroid- or growth factor-triggered cell proliferation (Migliaccio *et al*., 2000, 2005), we speculated that this association might be a target for specific anticancer drugs. These drugs, in contrast to general signal transducing inhibitors, should spare signal-transducing pathways not involved in the steroid receptor action. Parallel use of deletion AR mutants and synthetic peptides mirroring the deleted sequences in functional assays show that the entire 372–379 proline stretch or a part of it and the adjacent sequence including the arginine amino acid plays an important role in the AR/Src association. Use of the S1 peptide directly proves the link between the association, Src/Erk activation, cyclin D1 expression and the S phase entry. Similar concentrations of a peptide with the same composition but a shuffled sequence show a weak inhibition of the same processes suggesting that, although many peptides containing prolines might be slightly inhibitory, only peptides exactly repeating the AR proline-rich sequence can exert a strong negative effect. Remarkably, experiments in AR-negative cells stimulated by EGF show that the peptide does not interfere in the DNA synthesis. In the same cells, EGF-induced cytoskeletal changes, which also depend on Src activity, are unaffected by the peptide. These data show that AR is required for the observed inhibitory effects of the peptide treatment. Furthermore, the steroid receptor-dependent transcriptional activity is unaffected by the peptide. All together, these findings show that this compound is a specific antagonist of the signaling activation dependent on steroid receptors.

NIH3T3 cells as well as mouse embryo or mouse female adult fibroblasts express a very low amount of AR (Castoria *et al*., 2003). This receptor mediates a robust DNA synthesis induced by androgens in these cells. Such a response also requires AR/Src association and Src activation. Interestingly, NIH3T3 cells, unlike the mammary and prostate cancer cells, do not respond to androgen with receptor dependent transcriptional activity. Therefore, they represent a model useful for the analysis of receptor antagonist action independently of the receptor transcriptional activity. We are additionally interested in these cells since stromal cells, through a cross talk with epithelial cells, have a role in the development and carcinogenesis of both the prostate and the mammary gland (Coussens and Werb, 2002; Cunha *et al*., 2002). For this reason, interference of the inhibitory peptide in AR/Src complex assembled upon androgen or growth factor stimulation of fibroblasts might also affect the interactions between stromal and epithelial cells, and in such a way contribute to slow down cancer growth. The ability of the peptide to inhibit the G1 to S transition of NIH3T3 fibroblasts stimulated by androgen is additional evidence that this inhibitory effect does not require the receptor transcriptional activity.

Furthermore, the S1 peptide inhibits DNA synthesis induced by EGF in prostate and breast cancer cells. This is an important point, since many human malignancies express high levels of growth factors and their receptors. Activation of growth factor receptors by autocrine or paracrine mechanisms has also been described in human cancers. The inhibition of stromal cells and growth

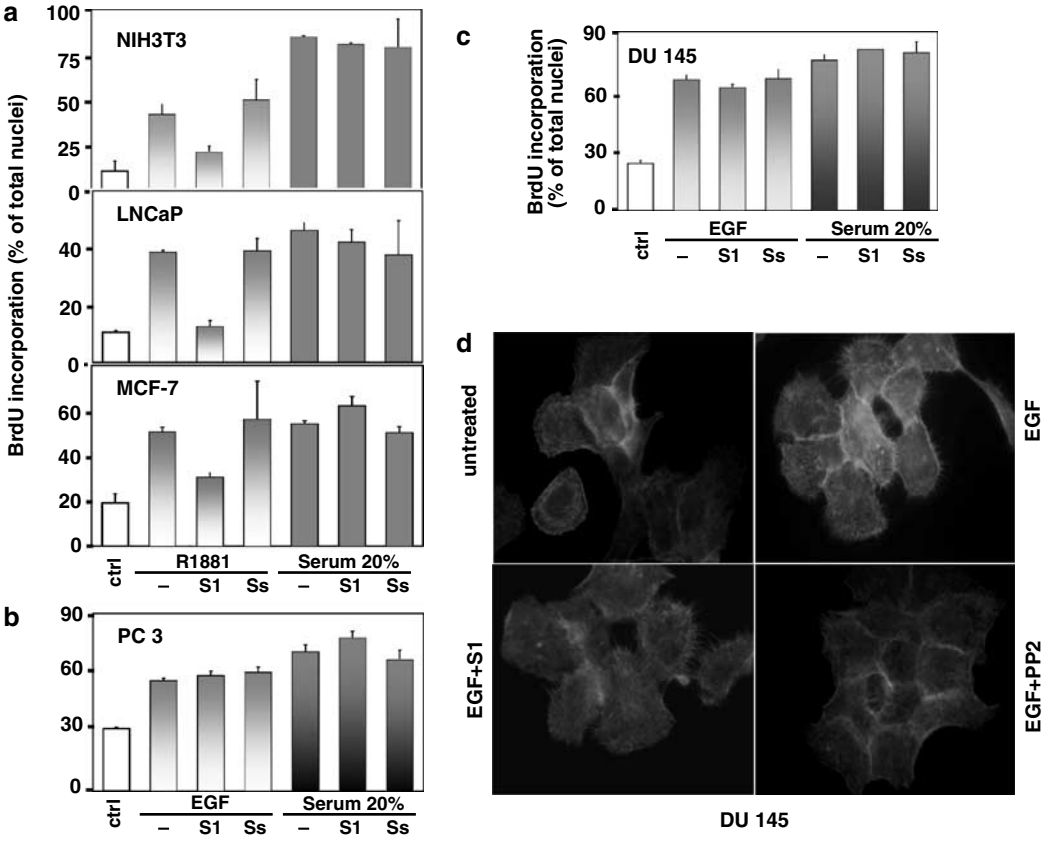


**Figure 6** The S1 peptide does not affect the serum-induced S phase entry of AR-positive cells and the EGF- or serum-induced DNA synthesis and the EGF-induced cytoskeletal changes of AR-negative cells. NIH3T3, LNCaP and MCF-7 cells on coverslips were made quiescent and then left untreated or treated with the following compounds: 1 pM (NIH3T3) or 10 nM (LNCaP) R1881, 20% serum, 1 nM of S1 or Ss peptide (**a**). AR-negative PC3 (**b**) or DU145 (**c**) cells on coverslips were made quiescent by serum starvation (0.5% serum) and then untreated or treated with the following compounds: 100 ng/ml EGF, 20% serum, 1 nM S1 or Ss peptide. After *in vivo* labeling with BrdU, DNA synthesis was evaluated as in Figure 2. Data are derived from at least 500 scored cells. The results of two independent experiments have been averaged. Means and s.e.m. are shown. DU145 cells (**d**) on coverslips were made quiescent by serum starvation (0.1% serum) and then untreated or treated for 30 min with 100 ng/ml EGF in the absence or presence of 1 nM S1 peptide or 5 $\mu$M PP2. PP2 was added 10 min before EGF stimulation. Cells were labeled with Texas red-conjugated phalloidin and then visualized by fluorescence microscopy. BrdU, bromodeoxyuridine; EGF, epidermal growth factor.

factor action by the S1 peptide may contribute to the observed strong inhibition of the xenografts growth. The peptide treatment significantly increases the number of cells undergoing apoptosis in LNCaP tumor xenografts in agreement with the view that hormonal activation of Src protects target cells from apoptosis (Singer *et al*., 1999; Kousteni *et al*., 2001).

In recent years, much evidence has shown that in multiple cell types under different experimental conditions, steroid receptors directly interact with several signaling effectors and trigger various biological effects. In addition to Src, these effectors include calmodulin (Castoria *et al*., 1988), the regulatory subunit of the phosphoinositide 3-kinase, p85$\alpha$ (Simoncini *et al*., 2000; Castoria *et al*., 2001), Shc (Song *et al*., 2002), modulator of non genomic activity of receptor (Wong *et al*., 2002), protein kinase C$\zeta$ (Castoria *et al*., 2004), EGF receptor (Marquez *et al*., 2001; Migliaccio *et al*., 2005), and many other signaling or signaling-related proteins. Therefore, approaches similar to those followed in this report, that is, recognition of new receptor/signaling effector interactions and identification of new inhibitors of such interactions, could enable us to specifically inhibit different hormone actions mediated by signal transducing pathways in multiple cell types and in different pathological conditions.

## Materials and methods

*Constructs*

The cDNA coding hAR was cloned into the pSG5 expression vector, as reported previously (Chang *et al*., 1988). The hAR deletion mutants $\Delta$1, $\Delta$2 and $\Delta$3, lacking part of the proline-rich region spanning from 372 to 386 AA, were generated using *in vitro* site-directed mutagenesis. The mutants $\Delta$2 ($\Delta$372–378) and $\Delta$3 ($\Delta$380–386) were constructed from the hAR using the Gene Tailor Site-direct Mutagenesis System (Invitrogen, Carlsbad, CA, USA) and the mutagenic primers 5′-TTTCCACTGGCTCTGGGCGGACCCCATCCCC-3′ and 5′-CCCCCTCCGCCGCCTCCCCTGGAGAACCC-3′, respectively. The mutant $\Delta$1 ($\Delta$372–385) was generated from AR mutant $\Delta$2, using the mutagenic primer 5′-CCACTGGCTCTGGGCG

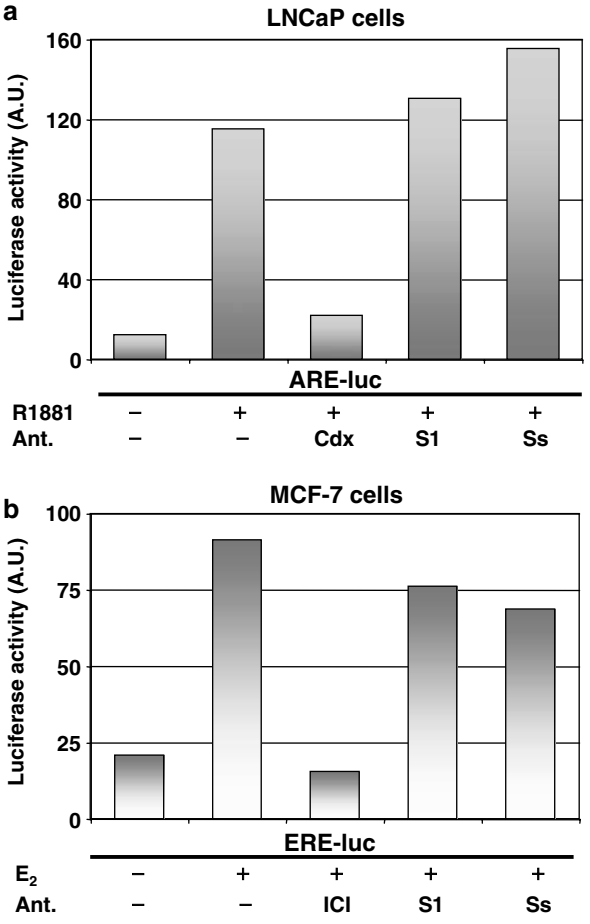


**Figure 7** The S1 peptide does not interfere in the steroid receptor-dependent transcriptional activity in human prostate and breast cancer-derived cells. ARE 3416 or ERE/luc constructs were transiently transfected into LNCaP (**a**) or MCF-7 (**b**) cells. Cells were made quiescent, and then left unstimulated or stimulated for 18 h with 10 nM of either R1881 or estradiol, in the absence or presence of the indicated compounds. The S1 and Ss peptides were used at 1 nM. The antiandrogen Casodex and the antiestrogen ICI were used at 10 $\mu$M. The luciferase activity was assayed, normalized using $\beta$-gal as an internal control and expressed as arbitrary units of luciferase activity.

GAAAGCTGGAGAAC-3′. All constructs were verified by sequencing. The 3416 construct, containing four copies of the wt *slp*-HRE2 (5′-TGG-TCAgccAGTTCT-3′), was cloned in the *Nhe*I site in pTK-TATA-Luc (Verrijdt *et al*., 2000).

*Cell culture, transfection and transactivation assay*

Human mammary cancer MCF-7 cells, fast growing human prostate cancer LNCaP cells, Cos cells and low-passage mouse embryo NIH3T3 fibroblasts were grown and made quiescent as reported (Castoria *et al*., 1999, 2003; Migliaccio *et al*., 2000). Human prostate carcinoma-derived PC3 and DU145 cells were cultured as described (Pandini *et al*., 2005; Bonaccorsi *et al*., 2006). Cos cells were made quiescent and transfected as reported (Migliaccio *et al*., 2000), using 2 $\mu$g of purified plasmids. Twenty-four hours later, transfected cells were left unstimulated or stimulated with the indicated compounds. The androgen-stimulated transcriptional assay in Cos cells was carried out as described (Castoria *et al*., 2003). For androgen- and estradiol-stimulated transcriptional analysis, LNCaP and MCF-7 cells were made quiescent and then transfected by Superfect (Qiagen, Hilden, Germany) with 4 $\mu$g of purified plasmids. Twenty-four hours later, transfected cells were left unstimulated or stimulated with the indicated compounds. Lysates were prepared and the luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, USA). The results were corrected using CH110-expressed $\beta$-galactosidase activity (Amersham Bioscience, Bucks, UK).

*Mouse xenografts*

LNCaP cells suspended in 50% (v/v) Matrigel solution in phosphate-buffered saline (PBS) (pH 7.4) were subcutaneously injected in the dorsal posterior region at $2.5 \times 10^6$ cells/male athymic mice (CD mice, Charles-River Italia, Milano, Italy) without hormone priming. After 14–21 days, animals with tumors of similar size were randomly selected for the treatment with the Src-S1 peptide (S1-peptide) or the Ss peptide or vehicle alone for an additional 5 weeks. Treatment was initiated with tumors at approximately 200–400 mm$^3$ in size. Tumor volumes of LNCaP cells xenografts were measured by a caliper and recorded according to the formula $D \times d^2 \times 0.5$ ($D$ represents the length and $d$ the width of tumor). For the peptide treatment of each animal, 200 $\mu$l of 20 nM S1 peptide dissolved in 0.1% dimethylsulfoxide or the same amount of Ss peptide or vehicle alone were intraperitoneally administered on alternate days to the mice. No difference in body weight was detected between control mice or peptide-treated mice.

*Ki67 antigen and TUNEL assays in tumor specimens*

At the end of the treatments, the mice were killed and tumor specimens assayed for Ki67 antigen and apoptotic index. Sections from each specimen were cut at 3–5 $\mu$m, mounted on glass and dried overnight at 37°C. They were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in PBS, which was used for all the subsequent washes as well as the antibody dilution. After the staining with hematoxylin/eosin and hematoxylin/Van Gieson, the sections were analysed by light microscopy. For immunohistochemistry, tissue sections were heated two times in a microwave oven for 5 min at 700 W in citrate buffer (pH 6), and then processed with the standard streptavidin–biotin–immunoperoxidase method (DAKO Universal Kit, DAKO Corporation, Carpinteria, CA, USA). Rabbit anti-human Ki67 (DAKO) was used (at final dilution 1:100) for 1 h at room temperature. Diaminobenzidine was used as chromogen, and hematoxylin as the nuclear counterstain. For each tissue section, negative and positive controls were performed, either leaving out the primary antibody or using tissue expressing the antigen of interest. TUNEL reaction was performed using the peroxidase-based Apoptag kit (Oncor, Gaithersburg, MD, USA). TUNEL-positive cells were detected with diaminobenzidine and $H_2O_2$, according to the supplier's instructions. For each specimen, the staining pattern as well as the score of Ki67 or apoptotic cells was evaluated by scanning the entire section and estimating the number of positive cells visible for high-power field $10 \times 20$.

*DNA synthesis analysis and cytoskeletal changes*

For bromodeoxyuridine (BrdU) incorporation analysis, quiescent cells on coverslips were left unstimulated or stimulated for 24 h with the indicated compounds. After a 6 h pulse with 100 $\mu$M BrdU (Sigma, St Louis, MO, USA), BrdU incorporation was analysed as described (Castoria *et al*.,

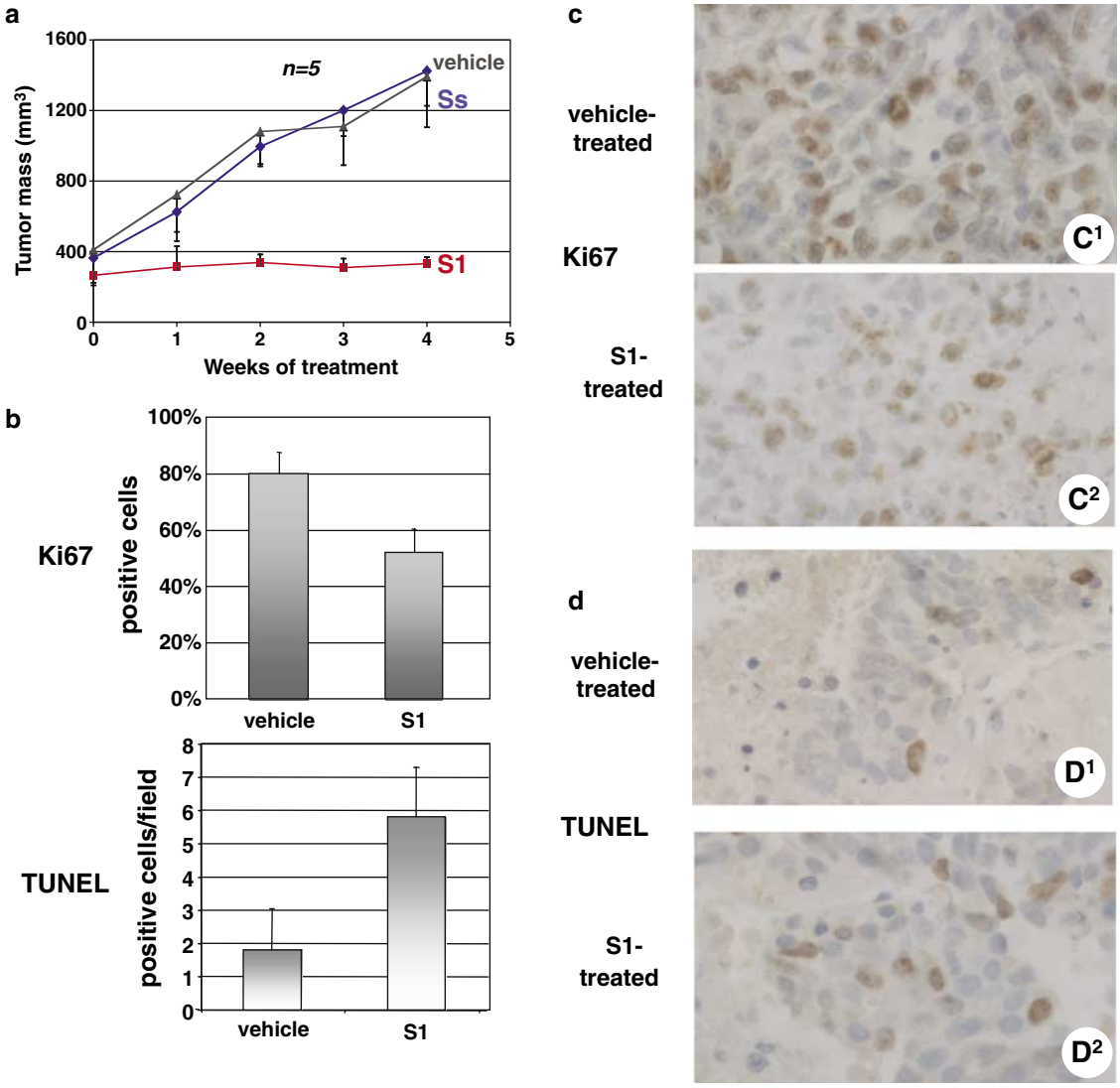


**Figure 8** The S1 peptide inhibits the growth of LNCaP xenografts. In (**a**), LNCaP xenografts were established in nude mice as described in Materials and methods. Tumors were treated with either S1 peptide (S1) or the shuffled sequence peptide (Ss) or vehicle alone (vehicle) and tumor volume was measured. *n* represents the number of experiments. Means and s.e.m. are shown. Tumor specimens in (**a**) were analysed by immunohistochemistry for Ki67 and apoptotic index by TUNEL. In (**b**), the score of Ki67 was evaluated (upper panel) and expressed according to the formula: number of Ki67-positive cells (number of Ki67-positive cells/number of total nuclei) $\times$ 100. The score of apoptotic cells (lower panel) was evaluated by estimating the number of TUNEL-positive cells/field. In (**c**), photomicrographs of Ki67 immunoreactivity of the mice treated with vehicle alone ($C^1$) or S1 peptide ($C^2$) are shown. In (**d**), photomicrographs of apoptotic cells of the mice treated with vehicle alone ($D^1$) or S1 peptide ($D^2$) are presented. TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

1999) using Alexa Fluor 594-conjugated mouse monoclonal anti-BrdU antibody (Molecular Probes, Eugene, OR, USA). The coverslips were finally stained with Hoechst 33258, inverted and mounted in Mowiol (Calbiochem, CA, USA). For cytoskeletal analysis, cells on coverslips were made quiescent by serum starvation (0.1% serum) for 24 h. They were then left unstimulated or stimulated for 20 min with the indicated compounds. F-actin was visualized using Texas red-labeled phalloidin as reported (Castoria *et al*., 2003). Coverslips were mounted in Mowiol (Calbiochem, Darmstadt, Germany). The fields were analysed with a DMLB fluorescent microscope (Leica) equipped with $\times 40$ and $\times 63$ objectives. Images were generated using FW4000 (Leica) software.

*Uptake analysis of labeled peptides*

For this analysis, exponentially growing cells were dissociated with a non-enzymatic cell dissociation medium (Sigma). About $2.5 \times 10^5$ cells were plated and cultured overnight on 30 mm plates on a glass coverslips. The cells were then made quiescent. The medium was discarded, and the cells were washed with NaCl/Pi (pH 7.3). NaCl/Pi was discarded, and the cell monolayer was incubated with the S1 and the Ss peptides conjugated to 5-(6)-carboxyfluorescein succinimidyl ester (Molecular Probes). Fluorescein-conjugated peptides were dissolved in Opti-MEM and added (at 1 nM) at 37°C or 4°C for 30 min to the cell medium. For direct detection of fluorescein-labeled peptides, the cells were washed three times with NaCl/Pi before being processed in Vectashield mounting

medium (Vector Laboratories, Burlingame, CA, USA). The distribution of fluorescence was analysed by a confocal LSM 510 Zeiss microscope.

*Lysates, immunoprecipitation and kinase assays*
Lysates were prepared as described previously (Migliaccio *et al*., 1996), and protein concentration was measured with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of cell lysates (at a protein concentration of 2 mg/ml) were immunoprecipitated with either mouse monoclonal anti-Src antibody (clone 327; Calbiochem) or rabbit polyclonal anti-Erk-2 antibody (C-14; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Src and Erk-2 kinase assays were performed as described (Migliaccio *et al*., 1996, 1998).

*Electrophoresis and immunoblotting*
The electrophoresis and immunoblotting procedures were performed as described elsewhere (Migliaccio *et al*., 1998). Src was revealed using the mouse monoclonal anti-Src antibody (clone 327; Calbiochem), and Erk-2 was detected using rabbit polyclonal anti-Erk-2 antibody (C14; Santa Cruz). Cyclin D1 was detected using the mouse monoclonal antibody (AM20; Zymed, San Francisco, CA, USA). The rabbit polyclonal anti-AR antibodies (either C-19 or N-20; Santa Cruz) were used to reveal AR. ERα was immunoblotted using the rabbit polyclonal anti-ER (HC-20; Santa Cruz) antibody. Immunoreactive proteins were revealed with the ECL detection system (Amersham Bioscience).

## Abbreviations

BrdU, bromodeoxyuridine; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estradiol receptor; hAR, human androgen receptor.

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ORIGINAL ARTICLE

# p85 regulatory subunit of PI3K mediates cAMP–PKA and estrogens biological effects on growth and survival

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**Cyclic adenosine 3′5′ monophosphate (cAMP) and protein kinase A (PKA) cooperate with phosphatidylinositol 3′ kinase (PI3K) signals in the control of growth and survival. To determine the molecular mechanism(s) involved, we identified and mutagenized a specific serine (residue 83) in p85α[PI3K], which is phosphorylated *in vivo* and *in vitro* by PKA. Expression of p85α[PI3K] mutants (alanine or aspartic substitutions) significantly altered the biological responses of the cells to cAMP. cAMP protection from *anoikis* was reduced in cells expressing the alanine version p85α[PI3K]. These cells did not arrest in G1 in the presence of cAMP, whereas cells expressing the aspartic mutant p85D accumulated in G1 even in the absence of cAMP. S phase was still efficiently inhibited by cAMP in cells expressing both mutants. The binding of PI3K to Ras p21 was greatly reduced in cells expressing p85A in the presence or absence of cAMP. Conversely, expression of the aspartic mutant stimulated robustly the binding of PI3K to p21 Ras in the presence of cAMP. Mutation in the Ser 83 inhibited cAMP, but not PDGF stimulation of PI3K. Conversely, the p85D aspartic mutant amplified cAMP stimulation of PI3K activity. Phosphorylation of Ser 83 by cAMP–PKA in p85α[PI3K] was also necessary for estrogen signaling as expression of p85A or p85D mutants inhibited or amplified, respectively, the binding of estrogen receptor to p85α and AKT phosphorylation induced by estrogens. The data presented indicate that: (1) phosphorylation of Ser 83 in p85α[PI3K] is critical for cAMP–PKA induced G1 arrest and survival in mouse 3T3 fibroblasts; (2) this site is necessary for amplification of estrogen signals by cAMP–PKA and related receptors. Finally, these data suggest a general mechanism of PI3K regulation by cAMP, operating in various cell types and under different conditions.**
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**Keywords:** cAMP; PI3K; p21Ras; growth

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## Introduction

Cyclic adenosine 3′5′ monophosphate (cAMP) regulates the growth of many cells types (Pastan *et al*., 1975). Although cAMP can promote the growth of some cells, such as Swiss 3T3 fibroblasts and thyrocytes (Lee *et al*., 1998; Ariga *et al*., 2000), it inhibits proliferation in most cells (Magnaldo *et al*., 1989). However, the precise mechanism by which cAMP inhibits cell cycle entry and progression remains undefined (Houslay and Kolch, 2000). cAMP prevents cells from entering S phase and arrests the cells in G1 (Kato *et al*., 1994). Furthermore, cAMP inhibits proliferation stimulated by either G protein-coupled receptors or receptor tyrosine kinases (Magnaldo *et al*., 1989), suggesting that it targets a signaling pathway that is central to cell cycle progression.

Stimulation of growth by cAMP–protein kinase A (PKA) in selected cell types, as thyroid cells, is tightly dependent on Ras and phosphatidylinositol 3′ kinase (PI3K) (Ciullo *et al*., 2001). Also, cAMP and PKA are powerful survival signals in several cell types (Affaitati *et al*., 2003). To date the mechanism(s) and the relevant players mediating cAMP effects on growth and survival are not completely known.

We have previously shown that cAMP and PKA regulate Ras signaling, by selectively stimulating Ras–PI3K complex. Also, we have found that p85α[PI3K] was an efficient PKA substrate *in vitro* (Ciullo *et al*., 2001).

Here we report that Ser 83 in the p85α[PI3K] is phosphorylated *in vivo* by PKA and that the corresponding mutants (p85A or p85D) inhibit or amplify cAMP biological effects on growth and survival. This site is also relevant for the interaction of p85α[PI3K] with estrogen receptor α, as the mutants inhibit or amplify estrogen signaling to PI3K. Finally, we demonstrate that cAMP in the absence of serum stimulates PI3K and that p85α[PI3K] mutants selectively interfere with cAMP stimulation of PI3K activity.

These data provide a molecular framework that explains the synergism between cAMP and different types of receptors, and add another level of complexity to the regulation of PI3K and Ras p21 by cAMP–PKA.

## Results

*PKA phosphorylates Ser 83 in p85α$^{PI3K}$*

p85α subunit of PI3K can be efficiently phosphorylated by PKA *in vitro* (Ciullo *et al*., 2001). A possible PKA target sequence is Ser 83, which is highly conserved in evolution and is preceded by basic residues in bovine/human (KKIS) and mouse/rat (KRIS) sequences.

To determine the relevance of this site *in vivo*, we have substituted Ser 83 with alanine (p85A) to prevent phosphorylation or with aspartic acid (p85D) to mimic the phosphorylated residue. The tagged recombinant proteins were transiently expressed in HeLa or NIH3T3 cells and immunoprecipitated with anti-flag antibody. Immunoprecipitates were incubated *in vitro* with purified catalytic PKA subunit in the presence of [$^{32}\gamma$-P]ATP. p85α$^{PI3K}$ wild type was efficiently phosphorylated by PKA whereas p85A was not, indicating that Ser 83 was the site phosphorylated by PKA *in vitro* (Figure 1a). PKA-phosphorylated p85α$^{PI3K}$ was also identified *in vivo* in breast cancer cells MCF7 extracts immunoblotted with an antiphosphoserine antibody. Figure 1b shows that p85α$^{PI3K}$, not p85A, was recognized by the phosphoserine antibody in extracts of MCF7 cells treated with cAMP. Figure 1c shows the same experiment performed in NIH3T3 cells expressing p85α$^{PI3K}$ and p85A. The p85A protein was not recognized by the antiphosphoserine antibody. Phosphorylation of p85α$^{PI3K}$ was prevented by incubating the cells with the PKA kinase inhibitor H89 (Figure 1c). The endogenous protein p85α$^{PI3K}$ was also phosphorylated in NIH3T3 cells stimulated with cAMP. Phosphorylation was inhibited by pretreating the cells with H89 (Figure 1d).

Taken together these data indicate that PKA phosphorylates p85α$^{PI3K}$ at Ser 83.

*p85α$^{PI3K}$ mutants alter cAMP cytoprotection and growth inhibition*

To test the biological relevance of Ser 83 phosphorylation of p85α$^{PI3K}$, we determined in mouse fibroblasts the rate of *anoikis*, a specific apoptotic pathway triggered by loss of cell adhesion to the extracellular matrix (Frisch and Francis, 1994; Khwaja *et al*., 1997). We co-transfected NIH3T3 cells with a GFP encoding vector and the wild type or the mutant versions of p85α$^{PI3K}$ (Figure 2a and b). At 48 h after transfection, the cells were plated on 2% agarose in a medium containing 0.1% calf serum in presence or absence of 200 $\mu$M cAMP. After 5 h, we determined the fraction of apoptotic cells by fluorescent activated cell sorter (FACS) analysis. Figure 2b shows that treatment with cAMP significantly reduced the number of apoptotic cells. cAMP cytoprotection was abolished by wortmannin, a PI3K inhibitor (data not shown and Khwaja *et al*., 1997). Expression of wild-type p85α$^{PI3K}$ amplified cAMP response (see ∗ in Figure 2b), whereas expression of p85A completely eliminated cAMP amplification, displayed by wild-type p85α$^{PI3K}$ (Figure 2b). Cells expressing p85D, on the other hand, were significantly more resistant to *anoikis* in the absence of cAMP (see ∗∗ in Figure 2b). The low response to cAMP of these cells was probably dependent on the low levels of p85D protein (Figure 2a). These data indicate that p85α$^{PI3K}$

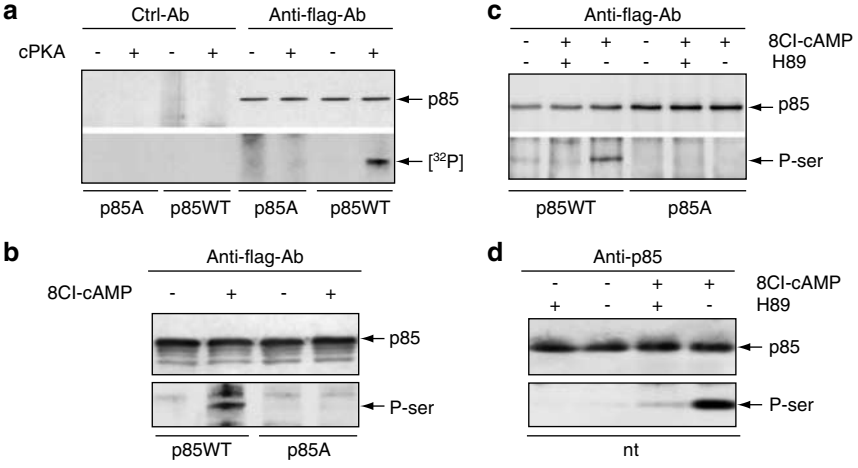


**Figure 1** Phosphorylation *in vivo* and *in vitro* of Ser 83 in p85α$^{PI3K}$ by cAMP–PKA. (**a**) *In vitro* phosphorylation of wild-type p85α$^{PI3K}$ or p85A. HeLa cells were transiently transfected with p85α$^{PI3K}$-flag wild type or p85A; 48 h after transfection, cell lysates were immunoprecipitated with non-immune IgG or anti-flag antibody for 15 h. A/G bound immunoprecipitates were *in vitro* phosphorylated with cPKA as described in Material and methods. Sample aliquots were run on SDS–PAGE and subjected to immunoblot (upper panel) or autoradiography (bottom panel). (**b**) *In vivo* phosphorylation of wild-type p85α$^{PI3K}$ or p85A. MCF7 cells were made quiescent using charcoal-treated serum and medium lacking phenol-red for 3 days. Quiescent cells were transfected with p85α$^{PI3K}$ or p85A. At 24 h after the transfection cells were treated with 100 $\mu$M cAMP. Protein lysates were immunoprecipitated with anti-flag antibody for 15 h, separated on 10% SDS–PAGE and immunoblotted with anti-p85$^{PI3K}$ (upper panel) or antiphosphoserine (lower panel) antibodies. The arrow indicates p85α$^{PI3K}$ band. (**c**) NIH 3T3 were transfected with p85α$^{PI3K}$ or p85A. At 24 h after transfection, the cells were treated with 100 $\mu$M cAMP in the presence or absence of the protein kinase A inhibitor, H89 (10 $\mu$M). In parallel cultures (**d**) un-transfected (nt) NIH3T3 cells, starved in DMEM 0.1% calf serum for 16 h, were treated with 100 $\mu$M cAMP for 10 min in the presence or absence of 10 $\mu$M H89. Cell lysates were immunoprecipatated with anti-p85α antibody and immunoblotted with anti-p85α (upper panel) or anti-phosphoserine (lower panel) antibodies.

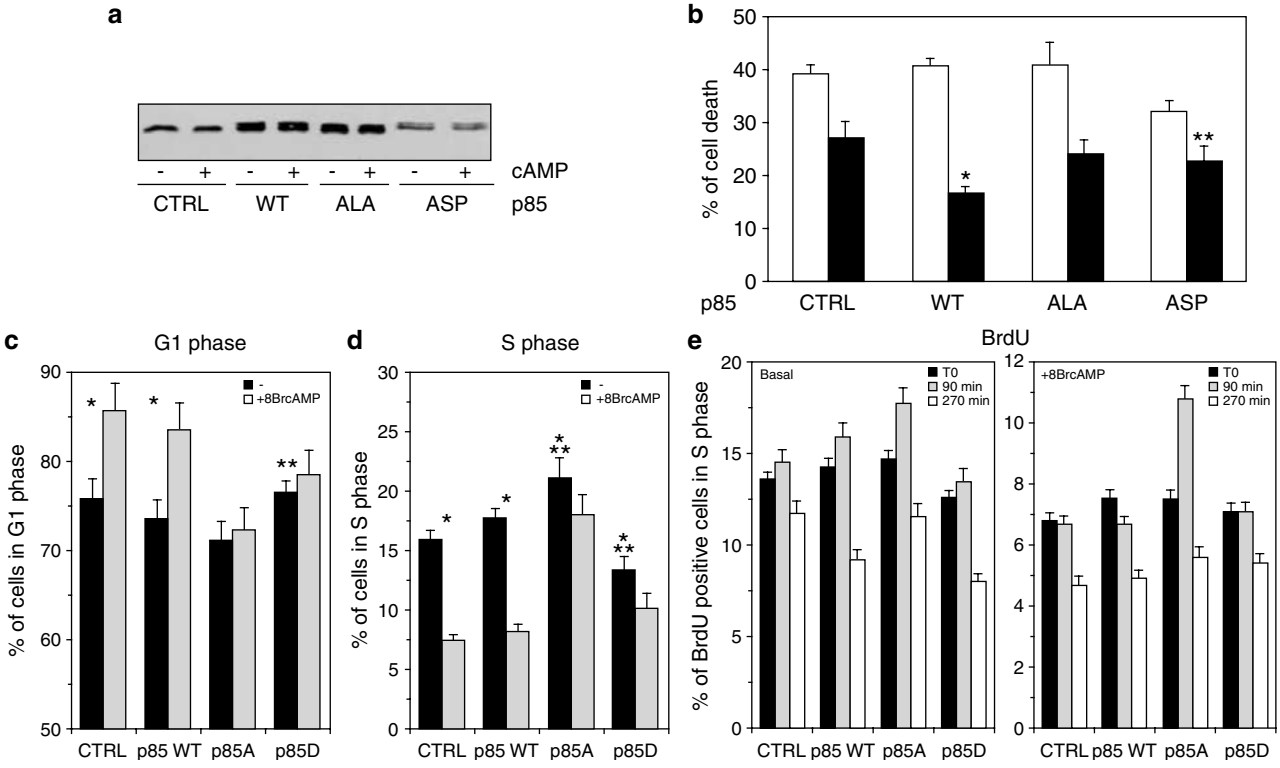


**Figure 2** Phosphorylation of p85α$^{PI3K}$ Ser 83 mediates cAMP biological effects on growth and survival. NIH3T3 cells were transiently transfected with p85α$^{PI3K}$-flag wild type or mutant versions. A GFP expression vector encoding green fluorescence protein was used as reporter. At 48 h after transfection, cells were plated on 2% agarose in DMEM 0.1% CS without or with cAMP 200 $\mu$M for 5 h and the fraction of apoptotic cells was determined by FACS analysis. (**a**) Extracts of transfected cells immunoblotted with anti-p85α$^{PI3K}$ antibody. Exogenous p85α$^{PI3K}$ is represented by the upper band in the doublet. (**b**) Quantitative analysis of FACS data. White and black columns represent, respectively, untreated or cAMP-treated samples. The data are the mean of four independent experiments; * indicates $P \leqslant 0.01$ comparing cell death in the presence of cAMP in all samples. The p85A (ALA) sample is significantly different from p85α$^{PI3K}$ (WT), but not from CTRL or p85D (ASP). To analyse the growth, NIH3T3 cells were transiently transfected with the constructs indicated above. At 24 h after the transfection, the cells were serum-starved 15 h and induced into the cycle with 2% serum in the presence or absence of cAMP (200 $\mu$M for 6 h). After 12 h the cells were subjected to FACS analysis. The quantitative analysis of FACS data relative to G1 phase (**c**) or S phase (**d**) was derived from three independent experiments and analysed by Student paired *T*-test. Black and gray columns represent respectively untreated or cAMP-treated samples. * indicates $P \leqslant 0.01$ basal versus cAMP; **$P \leqslant 0.01$ basal p85A versus p85D; ***$P \leqslant 0.01$ basal p85α wild type, A or D-transfected cells versus control plasmid-expressing cells. (**e**) Pulse (30′)-chase of BrdU incorporation in transfected cell lines in the presence or absence of cAMP. The columns represent the fraction of labeled cells stained with propidium iodide at 0 (black columns), 90 (gray columns) and 270 min (white columns) from the initial cell cycle induction (10% serum). The data are the mean of three independent experiments.

mediates cAMP inhibition of *anoikis* and that phosphorylation of Ser 83 is an important signal for cAMP-induced-survival.

To test if p85α$^{PI3K}$ mediated also cAMP inhibition of growth in mouse NIH3T3 fibroblasts, we analysed the biological effects of p85α$^{PI3K}$ mutants on growth arrest induced by cAMP. NIH3T3 fibroblasts were cultured in low serum in the presence or absence of cAMP. Under these conditions, cAMP induced a robust and reversible inhibition of cell growth by accumulating the cells in G1 phase and reducing the fraction of cells entering S phase (see * in Figure 2c and d). Cells expressing p85A did not efficiently arrest in G1 in the presence of cAMP (Figure 2c). Conversely, in cell lines expressing the aspartic mutant, cAMP response was lost and the fraction of cells accumulating in G1 was significantly higher than in cells expressing p85A (see ** in Figure 2c). The fraction of cells arrested in S phase in the presence of cAMP was robustly stimulated in the control and cells expressing the wild-type p85α$^{PI3K}$ (see * in Figure 2d). Cells expressing the mutant versions of p85α$^{PI3K}$ did not respond to cAMP and the fraction of cells transiting S increased (see Figure 2d, p85A***) or decreased (see Figure 2d, p85D***) relative to control or wild-type p85α$^{PI3K}$ cells. These data indicate a higher (p85D) or lower rate of G1 arrest (p85A) of these cell lines, independently on cAMP presence in the medium. The high statistical significance of S phase values, relative to G1, is due to the lower value of S phase cell fraction. To discriminate more precisely the effects of cAMP in G1 or S phases in cells expressing p85α$^{PI3K}$ mutants, we performed a kinetic analysis of the cell cycle by labeling the cells with BrdU. Under these conditions, the entry and the progression in S phase of transfected cells can be precisely determined. Figure 2e shows that p85A or p85D did not alter S phase progression,

although p85A-expressing cells transited S phase very rapidly, compared to control or p85D expressing lines in the presence of cAMP. Cells expressing p85A were unrestrained in G1 in the presence of cAMP and entered S phase more efficiently than control or p85D-expressing cells. Taken together, these data indicate that Ser 83 in the p85$\alpha^{PI3K}$ is critical for cAMP-induced G1 arrest on cell cycle progression. Alanine substitution of Ser 83 abolished G1 arrest by cAMP. p85D, on the other hand, increased the number of cells arrested in G1 in the absence of cAMP (see ⁂ in Figure 2d).

*p85$\alpha^{PI3K}$ (ser83) phosphorylation increases association of to p21 Ras*

We have previously shown that cAMP PKA selectively increased the association between PI3K and p21 Ras (Ciullo *et al*., 2001). As this complex can mediate cAMP–PI3K effects on growth and survival, we set out to determine the formation of p21 Ras–PI3K complex in cells expressing wild-type p85$\alpha^{PI3K}$ or p85A or p85D. NIH3T3 were transfected with p85$\alpha^{PI3K}$ wild type or p85A or p85D and 36 h after transfection, the cells were serum starved for 16 h before 10 min treatment with cAMP. Cell lysates were immunoprecipitated with anti-flag antibody and then analysed by Western blot with anti-Ras antibody. In the presence of cAMP, p85$\alpha^{PI3K}$ wild type efficiently was found associated with p21 Ras. This association was significantly inhibited in cells expressing p85A and stimulated in p85D expressing cells. In the absence of cAMP, Ras–PI3K complex was barely detectable in all the samples. In p85D-expressing cells, however, cAMP stimulated robustly association of PI3K to p21 Ras, suggesting that Ser 83 in p85$\alpha^{PI3K}$ cooperates with another cAMP–PKA signal to regulate PI3K association with p21 Ras (Figure 3a and b). We also tested the effects of H89 on the formation of the Ras–PI3K complex. H89 treatment reduced the complex Ras–PI3K to the levels found in p85A-expressing cells (Figure 3c). These data indicate that under conditions of cAMP stimulation, the interaction Ras–PI3K is significantly favored.

*p85$\alpha^{PI3K}$ (Ser 83) phosphorylation selectively amplifies PI3K signaling by cAMP*

To directly test the hypothesis that cAMP stimulation amplified Ras–PI3K signalling, we stimulated the cells with cAMP or PDGF and determined AKT and GSK phosphorylation, downstream targets of PI3K. Figure 4a shows that cAMP and PKA stimulated the phosphorylation of AKT and GSK$\alpha$ and that H89 reversed cAMP effects. To test if p85$\alpha^{PI3K}$ mutants interfere also with other pathways that signal to PI3K, we determined PDGF stimulation of AKT and ERK1/2, the two major kinases downstream to PDGFR. Figure 4b shows that expression of p85A and p85D did not significantly influence PDGF stimulation of ERK1/2 or P-AKT accumulation. p85A-expressing cells, on the other hand, poorly phosphorylated AKT or GSK$\alpha$ in response to cAMP. Conversely, expression of p85D increased the basal P-AKT and P-GSK$\alpha$ and robustly amplified cAMP response (Figure 4c and d).

So far we have analysed the effects of p85$\alpha^{PI3K}$ variants on indirect effectors of PI3K signaling. To directly test the relevance of cAMP induced phosphorylation of p85$\alpha^{PI3K}$ on PI3K activity, we have measured the activity of the enzyme in cells expressing wild type or the mutant versions of p85$\alpha^{PI3K}$. We have previously reported that cAMP in the presence of serum did not stimulate PI3K activity (Ciullo *et al*., 2001). As the presence of serum can mask some important biological effects of cAMP–PKA (Porcellini *et al*., 2003) and (De Gregorio *et al*., 2006, in press), we carried out the

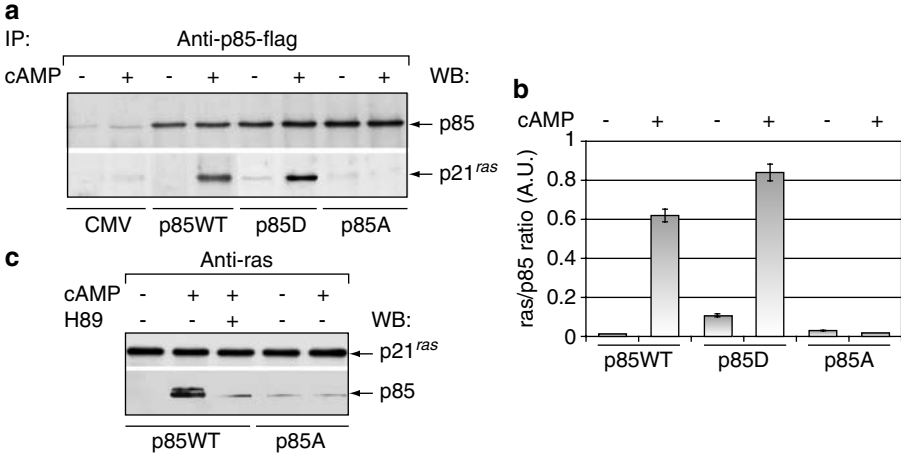


**Figure 3** cAMP stimulates the formation of PI3K/Ras complex. NIH-3T3 cells were transiently transfected with wild type or mutant versions of p85$\alpha^{PI3K}$-flag. At 36 h after transfection, the cells were starved for 16 h in DMEM, 0.1% calf serum and treated with cAMP 200 $\mu$M for 10 min. (**a**) Cells lysates were immunoprecipitated with anti-flag antibody and blotted with anti-Ras or anti-p85$\alpha^{PI3K}$ antibodies. (**b**) The histograms represent the ratio of Ras-p85$\alpha^{PI3K}$ bands in the p85$\alpha^{PI3K}$ immunoprecipitates, derived from three independent experiments. (**c**) NIH-3T3 were transiently transfected with wild-type p85$\alpha^{PI3K}$-flag or p85A. At 36 h after transfection, the cells were starved for 16 h in DMEM, 0.1% calf serum and treated with cAMP 200 $\mu$M for 10 min in the presence or absence of H89. Cells lysates were immunoprecipitated with anti-Ras antibody and blotted with anti-Ras or anti-flag antibodies.

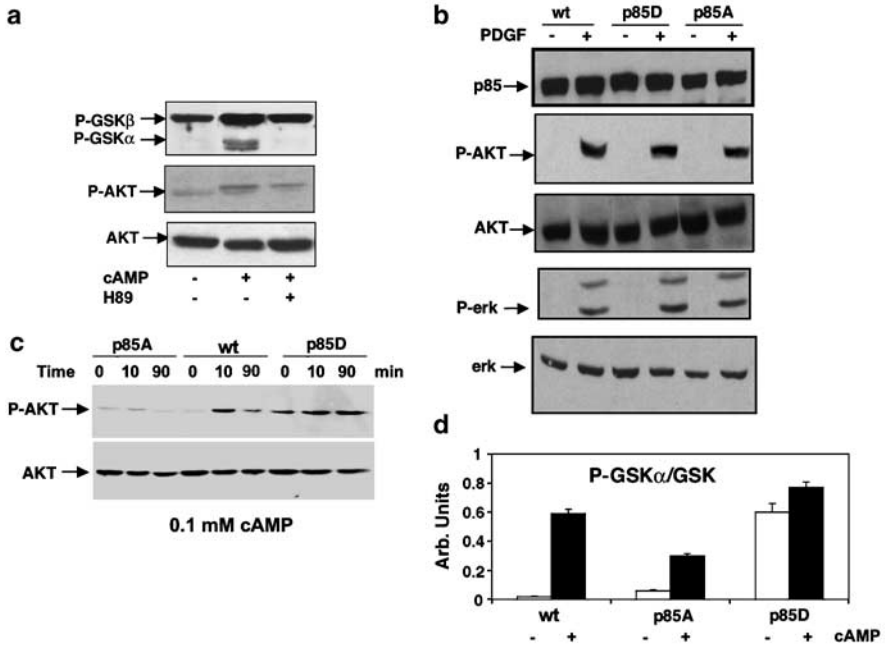


**Figure 4** Serine phosphorylation of p85 selectively amplifies cAMP signaling to AKT and GSK. (**a**) NIH3T3 cells were stimulated with 8-Cl-cAMP (100 $\mu$M) in the presence or absence of H-89 (10 $\mu$M) for 10 min. Total extracts were prepared and immunoblotted with the specific antibodies indicated. (**b** and **c**) Immunoblots of extracts derived from cells transiently transfected with the p85$\alpha^{PI3K}$ expression vectors indicated. In (**b**) the cells were stimulated with PDGF (100 ng/ml) for 15 min, 48 h after the transfection; in (**c**) the cells were stimulated with 8-Cl-cAMP (100 $\mu$M) for 10 and 90 min, respectively. (**d**) The histograms of the pGSK$\alpha$/GSK ratio, evaluated by densitometric analysis of Western blot with appropriate antibodies of extracts derived from cells transfected with the indicated p85$\alpha^{PI-K}$ plasmids and stimulated with (black) or without (white) cAMP (100 $\mu$M) for 10 min. Basal P-AKT in cells expressing p85D is lower in (**b**) than in (**c**), because the cells were starved 24 h (**b**), instead of 16 h (**c**).

stimulation of transfected cells with cAMP in low serum (0.2%). Figure 5 shows that cAMP stimulated PI3K activity. The activation of PI3K was inhibited by treatment of the cells with the PKA inhibitor, H89. Moreover, cells expressing p85A did not activate PI3K in response to cAMP, whereas efficiently activated PI3K, when treated with PDGF. Conversely, p85D amplified PI3K activity induced by cAMP and did not significantly influence PI3K stimulated by PDGF (Figure 5, lower inset).

To determine if p85A altered the structure of the protein and inhibited the formation of the PI3K complex, we tested the ability of *in vitro* synthesized p85$\alpha^{PI3K}$ to interact and form a stable complex with p110$\alpha^{PI3K}$. Supplementary Figure 1S shows that co-translated p85$\alpha^{PI3K}$ and p110$\alpha^{PI3K}$ interact very efficiently. When tested under the same conditions, p85A ability to interact with p110$\alpha^{PI3K}$ was comparable to that of the wild-type p85$\alpha^{PI3K}$ (Supplementary Figure 1S). p85D, on the other hand, formed a complex with p110$\alpha^{PI3K}$ synthesized *in vitro*, with a higher affinity than p85$\alpha^{PI3K}$ wild type or p85A. These data indicate that p85A does not disrupt the folding of p85$\alpha^{PI3K}$ protein or the ability to interact with p110$\alpha^{PI3K}$, whereas p85D induces a conformational change that improves the interaction with p110$\alpha^{PI3K}$. As p85D stimulates cAMP-induced PI3K activity, we suggest that this conformational change activating the enzyme is mediated by phosphorylation of p85$\alpha^{PI3K}$ in Ser 83 by PKA.

*cAMP–PKA amplify estrogen binding and signaling to PI3K*

PI3K mediates also AKT activation by estrogens (Simoncini *et al*., 2000; Castoria *et al*., 2001). As p85$\alpha^{PI3K}$ was efficiently phosphorylated by PKA in MCF7, the breast carcinoma cell line, we asked whether p85$\alpha^{PI3K}$ phosphorylation on Ser 83 also affected PI3K interaction with upstream effectors such as estrogen receptor. Therefore, we analysed the phosphorylation of AKT stimulated by estrogen**.** Cells expressing p85$\alpha^{PI3K}$ wild type or p85A or p85D were treated with estrogens and stimulation of AKT was monitored with specific P-AKT antibodies. Figure 6a shows that p85D amplified AKT phosphorylation induced by estrogens and that p85A abolished this stimulation. Also, induction of GSK phosphorylation by estrogens was inhibited by p85A expression (data not shown).

As estrogen stimulation of AKT was inhibited in cells expressing p85A, we measured the fraction of the estrogen receptor $\alpha$ bound to wild type and mutant versions of p85$\alpha^{PI3K}$. Figure 6b shows that wild-type p85$\alpha^{PI3K}$ immunoprecipitated efficiently the receptor. The binding of estrogen receptor to p85$\alpha^{PI3K}$ was stimulated by estrogens, as expected. cAMP did not stimulate the binding *per se* in the absence of estrogens, but amplified the action of estrogens on p85$\alpha^{PI3K}$ binding. H89, a PKA inhibitor, eliminated the binding of the receptor to p85$\alpha^{PI3K}$. The p85A protein did not bind the estrogen receptor in the presence or in the absence of estrogens. Expression of p85D substituted

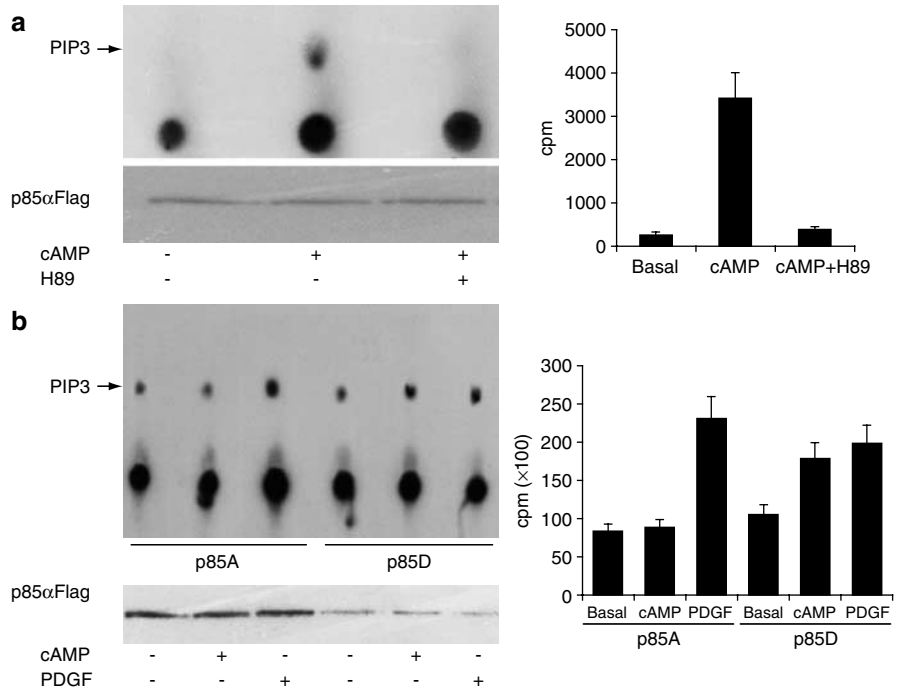


**Figure 5** cAMP stimulates PI3K activity in the absence of serum. Effects of p85α$^{PI3K}$ mutants on PI3K catalytic activity. NIH3T3 cells were transfected with the p85α$^{PI3K}$ expression vectors indicated and stimulated with cAMP (100 μM) 20 min in the presence or absence of H89 (10 μM) or PDGF (100 ng/ml) 15 min, 48 h after transfection. Total extracts were prepared and subjected to immunoprecipitation with anti-flag antibody. Immunoprecipitates were quantified by immunoblot with anti-p85α$^{PI3K}$ antibodies (p85α$^{PI3K}$-flag) and assayed for PI3K activity by using a mixture of lipids containing PI4-5 (see Materials and methods). PI-4-5 labeled at position 3 is indicated by the arrow. The upper inset (**a**) shows PI3K activity associated with p85α$^{PI3K}$-flag wild type and the immunoblot with anti-p85α$^{PI3K}$ antibody of the p85α$^{PI3K}$-flag immunoprecipitate. The lower inset (**b**) shows PI3K activity associated with p85A and p85D, immunoprecipitated with the anti-flag antibody. The immunoblot with the p85α$^{PI3K}$ antibody is shown below. The histograms on the right show the statistical analysis of (**a**) and (**b**) derived from three experiments.

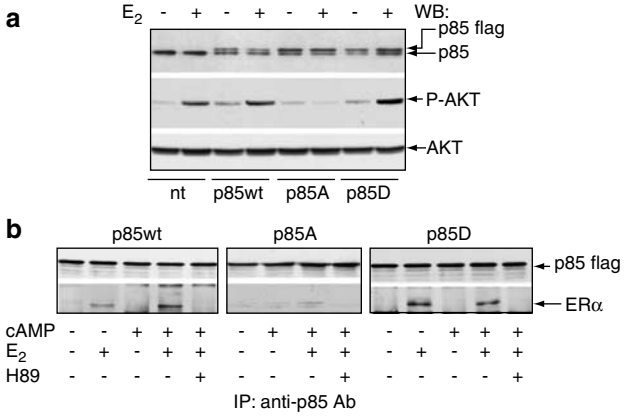


**Figure 6** cAMP phosphorylation of p85α$^{PI3K}$ amplifies estrogen binding and signaling to PI3K. MCF-7 cells were made quiescent by charcoal-treated serum and medium lacking phenol-red for 3 days. (**a**) Quiescent cells were transfected with p85α$^{PI3K}$ wild type or p85A or p85D. After 24 h, the cells were treated with 10 nM $E_2$ for 3 min. Cell lysates were analysed by Western blot with anti-p85α$^{PI3K}$ (upper panel), P-AKT (middle panel) or AKT (lower panel). (**b**) Quiescent cells were transfected with the wild type or the mutant versions of p85α$^{PI3K}$. After 24 h, the cells were treated with 10 nM $E_2$ for 3 min or for 10 min with 200 μM cAMP, or with 10 nM $E_2$ for 3 min following a 10-min stimulation with 200 μM cAMP. The cell lysates were immunoprecipitated with anti-flag antibody 15 h, as described above. Sample aliquots were immunoblotted with anti-ERα and anti-p85α$^{PI3K}$ antibodies to detect the PI3K/ER complex. Lanes 5 and 14 show the complex estrogen receptor-p85α$^{PI3K}$ in cells pretreated with 10 μM H89 for 30 min.

cAMP by amplifying estrogen stimulated binding of the receptor to p85α$^{PI3K}$. H89 inhibited the binding of p85D to estrogen receptor, indicating that another phosphorylation PKA-dependent was necessary for the formation of the complex estrogen-p85α$^{PI3K}$.

These data indicate that phosphorylation by cAMP–PKA of Ser 83 in p85α$^{PI3K}$ is required for estrogen binding and signalling to PI3K.

## Discussion

The data presented here indicate that the phosphorylation of p85α$^{PI3K}$ in a critical serine residue mediates cAMP action on growth and survival. More specifically, phosphorylation of Ser 83 in p85α$^{PI3K}$ shifts the signaling flow towards PI3K-driven pathways. Such a conclusion is supported by several observations. The substitution of Ser 83 with alanine inhibited cAMP effects on cell survival and G1 arrest in NIH 3T3 fibroblasts. Conversely, the aspartic mutant of Ser 83 in p85α$^{PI3K}$ stimulated cell survival in the absence of cAMP and slowed down cell cycle cycle progression, replicating the effects of cAMP. Ras binding to PI3K and AKT phosphorylation were inhibited by expressing p85A and were stimulated by p85D.

cAMP effects on growth and cell cycle progression are very selective. cAMP–PKA act on at least three phases

of cell cycle. cAMP slows down G1, blocks S phase and G2–M transition (Kato *et al*., 1994; Kurokawa and Kato, 1998; Houslay and Kolch, 2000). Our data indicate that p85$\alpha^{PI3K}$ phosphorylation of Ser 83 controls selectively G1–S transition (Figure 2).

PI3K is an important regulator of cell growth as it links cell size, growth and cycle progression. For example, constitutive expression of p110$\alpha$ increases cell mass and size by stimulating the biosynthetic capacity of the cells. However, if not restrained, p110 activity impairs exit from the cell cycle (Alvarez *et al*., 2003). We suggest that Ras–PI3K signals regulate the length of G1 phase. cAMP by amplifying and stabilizing PI3K, lengthens G1 and slows down G1–S transition. The net result is an apparent G1–S block, which results in increased biosynthetic activity and cell mass prior to division. This is better shown in thyroid cells, FRTL5, which are exquisitely dependent on TSH and cAMP for growth and differentiation. In these cells, Ras and PI3K are selectively required for G1 phase progression (Ciullo *et al*., 2001). In thyroid cells, TSH induces PI3K activity and stabilizes the complex Ras–PI3K. Also, expression of p85 mutant (p85A) inhibits selectively the stimulation of growth and PI3K activity induced by TSH (De Gregorio *et al*., in press). In thyroid cells and in cells expressing high levels of the regulatory subunit of PKA, RII$\beta$, p85$\alpha^{PI3K}$ binds and anchors PKA. This binding is amplified in cells expressing p85D and it is H89 resistant (De Gregorio *et al*., in press). This suggests that anchoring PKA to the membrane is a prerequisite for cAMP–PKA biological effects.

Also, cAMP–PKA stimulate in the absence (Figure 5), not in the presence of serum (Ciullo *et al*., 2001), PI3K activity. This stimulation requires phosphorylation of Ser 83 in in p85$\alpha^{PI3K}$, probably because phosphorylated p85$\alpha^{PI3K}$ induces a conformational change in the p110$\alpha^{PI3K}$ molecule (Supplementary Figure 1S) or increases its affinity to PKA (De Gregorio *et al*., in press). The stimulation of PI3K activity by cAMP appears to be very selective, as p85A or p85D mutants did not interfere with PDGF (Figure 4) or EGF (De Gregorio *et al*., in press) induced PI3K activity.

Although the cell lines we have used are not robustly dependent on cAMP signaling for growth or differentiation, our data indicate that PI3K-Ras signals are amplified by cAMP both in cAMP-independent (NIH3T3 fibroblasts or breast MCF7 cells) or dependent (thyroid) cells. In these latter cells, amplification of PI3K by cAMP is mainly triggered by TSH and PKA, which binds p85$\alpha^{PI3K}$. The amplification of the pathway Ras–PI3K induced by cAMP is redundant in 3T3, but not in thyroid cells, because in these latter cells expression of p85A triggers apoptosis (De Gregorio *et al*., in press).

We suggest that Ser 83 in p85$\alpha^{PI3K}$ is an important point of convergence of two parallel pathways: cAMP–PKA and PI3K signals. In this framework, it is worth noting that in *Saccharomyces cerevisiae* inactivation of PI3K–TOR signalling is partly compensated by iper-activation of cAMP–PKA (Rohde *et al*., 2004; Zurita-Martinez and Cardenas, 2005).

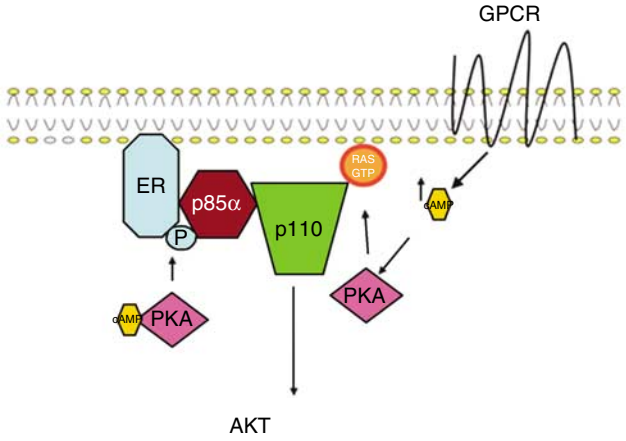


**Figure 7** Amplification of estrogen and Gs protein-coupled receptors (GPCR) signaling to PI3K. A scheme outlining the effects of cAMP–PKA activation by Gs protein coupled receptors on PI3K signaling by estrogens. PKA and cAMP arrows on the right side indicate other cAMP–PKA targets that amplify Ras–PI3K interaction. p85$\alpha^{PI3K}$ links receptors coupled to G proteins to estrogens and Ras–PI3K signals.

Our data indicate that estrogen receptor signalling was profoundly altered when p85A was expressed. Accordingly, p85$\alpha^{PI3K}$ binding to estrogen receptor was severely impaired. Although PKA phosphorylates directly AKT and estrogen receptor (Cui *et al*., 2004), the effects we described were mainly dependent on p85$\alpha^{PI3K}$, as they were inhibited or amplified by expressing the p85$\alpha^{PI3K}$ alanine or aspartic mutants, respectively. Moreover, we have data indicating that also induction of estrogen responsive genes is inhibited by H89 and it is significantly altered in p85A-expressing cells (data not shown). Estrogens stimulate adenylyl cyclase (Aronica *et al*., 1994) and cAMP–PKA stimulate differentiation of granulosa cells (Knecht *et al*., 1984). Our data indicate that phosphorylation of Ser 83 in p85$\alpha^{PI3K}$ by cAMP PKA stabilizes the binding of activated receptor to PI3K and stimulates PI3K activity.

*General implications*

The data presented above have broad implications because they point to p85$\alpha^{PI3K}$ as the physical link between Ras, estrogen receptor and cAMP–PKA (Figure 7). Also, we find in many and unrelated cell types that cAMP amplification of PI3K is essential for negative or positive cAMP effects on growth and survival. Apparently, all receptors binding p85$\alpha^{PI3K}$ can cooperate with cAMP–PKA signals via phosphorylation of Ser 83 in p85$\alpha^{PI3K}$. This may explain the pleiotropic nature of the effects exerted by cAMP–PKA on several, apparently unrelated, signaling cascades and illustrates how cAMP–PKA can influence Ras p21 effectors at multiple levels.

## Materials and methods

*Plasmid construction*

The cDNA encoding for p85$\alpha^{PI3K}$-flag wild type was generated by fusing the flag sequence (MDYKDDDDK) to the C-

Terminus of bovine p85$\alpha^{PI3K}$ cDNA in the pSG5 vector (gift of Dr J Downward). The region of p85$\alpha^{PI3K}$ from the *Xho*I restriction site in position 1014 (Acc. No: 163476) was amplified by PCR with a 3′ end primer containing the flag sequence and a *Bam*HI restriction site. The PCR product was subcloned in the II-TOPO vector (Invitrogen), digested with *Xho*I and *Bam*HI, extracted and ligated to a pSG5-p85$\alpha^{PI3K}$ vector previously digested with the same restriction enzymes. The vector encoding p85$\alpha^{PI3K}$-flag was then used as template for site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) to convert Ser 83 in alanine or aspartic acid. All the plasmid constructs were verified by DNA sequence analysis.

*Cell culture and transfections*

HeLa and NIH3T3 cells were grown in RPMI with 10% fetal calf serum or Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum, respectively. MCF-7 cells were grown in DMEM with 5% fetal bovine serum, supplemented with: penicillin/streptomycin 100mU/ml, 2 mM glutamine, 6 ng/ml insulin and 3.75 ng/ml hydrocortisone. Cells were transfected with lipofectamine (Gibco-Invitrogen, Carlsbad, CA, USA). In all transfections, RSV-LacZ was included to determine and normalize transfection efficiency. Experiments varying in the transfection efficiency above 20% were discarded.

*Antibodies, immunoprecipitation and immunoblot*

The antibodies used were: anti-Ras (mouse monoclonal pan-Ras clone 10, UBI (an affiliate of Millipore, Billerica, MA, USA)), anti-p85PI3K (rabbit polyclonal, UBI), anti-P-Serine (rabbit polyclonal, Zymed, San Francisco, CA, USA), anti-Erk 1/2 (rabbit polyclonal, Santa Cruz, Santa Cruz, CA, USA), anti-P-Erk 1/2 (mouse monoclonal, Santa Cruz), anti-P-Akt Ser 473 (rabbit polyclonal, Cell Signaling, Beverly, MA, USA), anti-Akt (rabbit polyclonal, Cell Signaling), anti-P-Gsk Ser 21/9(rabbit polyclonal, Cell signaling), anti-GSK$\alpha/\beta$ (mouse monoclonal, UBI). The anti-flag antibody was the mouse monoclonal Sigma M2 antibody.

*Immunoprecipitation*

Total extracts were prepared by lysing the cells on ice 5 min with 50 mM Tris-HCl pH 7.4, 1% Nonidet NP-40, 100 mM NaCl, 2 mM EDTA 50 mM NaF, 0.1 mM $NaVO_3$ 1 mM $\beta$-glycerophosphate, 2.5 mM sodium pyrophosphate and a protease inhibitor cocktail. Cell lysates (2 mg/ml) were incubated with 4 $\mu$g of antibody/0.5–1 mg of protein at 4°C in gentle rock agitation 15 h. At the end of incubation, 20 $\mu$l of A/G plus were added to samples and the immunoprecipitates were collected by centrifugation. SDS–PAGE and immunoblots were performed as previously described (Feliciello *et al*., 2000).

*Anoikis*

At 48 h after transfection, the cells were collected and maintained in suspension on dishes covered with a thin layer of 2% agarose. Cells were plated in medium containing 0.1% serum with or without 200 $\mu$M CPT-cAMP (Sigma) for 5 h. At the end of incubation, the cells were collected, washed twice with phosphate-buffered saline (PBS) and incubated 5 min with propidium iodide and analysed by FACS.

*Analysis of DNA content and 5′-bromo-2′-deoxyuridine (BrdU) incorporation*

$2 \times 10^6$ transfected cells (48 h after transfection) were plated in 100 mm dishes and grown in low serum (0.5% fetal bovine serum) for 18 h in the presence or in absence of 50 $\mu$M 8-Cl cAMP. Cells were labelled for 30 min with BrdU to a final concentration of 20 $\mu$g/ml and harvested at 0, 90 and 270 min. After treatment, the cells were fixed in ice-cold 70% ethanol for 4 h at +4°C and washed three times in PBS. Cells were re-suspended in 0.25 ml of 1 N HCl and maintained 20 min at room temperature. After acidic denaturation of DNA, the cells were washed two times in phosphate/citric buffer (0.2 M $Na_2HPO_4$; pH 7.4). BrdU incorporation was revealed by anti-BrdU-FITC (Becton Dickinson, Franklin Lakes, NJ USA) and then stained for 30 min at room temperature in 0.1% Triton X-100, 0.2 mg/ml DNase-free RnaseA, 20 $\mu$g/ml propidium iodide. Fluorescence was determined by using the FACScan Flow Cytometer (Becton Dickinson). Experiments were performed in triplicate. The data were acquired and analysed by CELLQuest software (Becton Dickinson) and by Cell Fit Cell-Cycle Analysis Version 2 for bivariate analysis of DNA content versus BrdU incorporation.

In vitro *phosphorylation*

HeLa cells were transiently transfected with p85$\alpha^{PI3K}$-flag and p85A. At 48 h after transfection, cell lysates were immunoprecipitated with non-immune IgG or anti-flag antibody 15 h at 4°C. Protein A/G bound immunoprecipitates were washed twice with lysis buffer and finally with kinase buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES) 20 mM, $MgCl_2$ 10 mM, pH 7.4). The washed immunoprecipitates were treated with 0.4 $\mu$g of partly purified catalytic subunit of protein kinase A. Each aliquot was incubated in a final volume of 30 $\mu$l of kinase buffer containing $10^{-5}$ M cAMP, 100 $\mu$M ATP and 10 $\mu$Ci[$\gamma^{32}$P-ATP] for 30 min at 30°C. The reaction was terminated by adding 2 × Laemmli buffer.

*Lipid kinase assay*

Lipid kinase activity was determined as described by Maier *et al*. (1999). Briefly, the assays were carried out in a final volume of 50 $\mu$l containing 0.1% bovine serum albumin, 1 mM EGTA (ethylene glycol-bis($\beta$-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid), 120 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM glycerophosphate, 7 mM $MgCl^2$ (buffer E). Lipid vesicles (30 $\mu$l containing 320 $\mu$M phosphatidylethanolamine, 300 $\mu$M phosphatidylserine, 140 $\mu$M phosphatidylcholine, 30 $\mu$M sphingomyelin, supplemented with 40 $\mu$M PI-4,5-P2 in buffer E) were sonicated 1 h and incubated on ice 10 min. Some aliquots were preincubated with LY294002, 0.5 $\mu$M (Calbiochem (EMD Biosciences, Inc, an Affiliate of Merck KGaA, Darmstadt, Germany)) at 37°C. The immunoprecipitates were added to the lipid mixture and incubated for 10 min at 4°C in a final volume of 40 $\mu$l. The reaction was started by adding 40 $\mu$M ATP (1 $\mu$Ci of [-$^{32}$P]ATP in 10 $\mu$l of the assay buffer at 30°C. After 15 min, the reaction was stopped with ice-cold 150 $\mu$l HCl (1 N) on ice. The lipids were extracted by vortexing the samples with 500 $\mu$l of chloroform/methanol (1:1). After centrifugation the organic phase was washed twice with 200 $\mu$l of 1 N HCl. Phosphorylated lipids were separated by TLC, detected by autoradiography and quantified with Phosphor-Imager.

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# Crosstalk between EGFR and Extranuclear Steroid Receptors

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**ABSTRACT: Epidermal growth factor (EGF) stimulates DNA synthesis and cytoskeletal rearrangement in human breast cancer (MCF-7) and human prostate cancer (LNCaP) cells. Both effects are inhibited by estrogen (ICI 182,780) and androgen (Casodex) antagonists. This supports the view that crosstalk exists between EGF and estradiol (ER) and androgen (AR) receptors and suggests that these receptors are directly involved in the EGF action. Our recent work shows that EGF stimulates ER phosphorylation on tyrosine and promotes the association of a complex between EGFR, AR/ER, and the kinase Src. The complex assembly triggers Src activity, epidermal growth factor receptor (EGFR) phosphorylation on tyrosine, and the EGF-dependent signaling pathway activation. In these cells, the AR/ER/Src complex is required for the EGF action, as the growth factor effects are abolished upon receptor silencing by specific SiRNAs and steroid antagonists or Src inhibition by the kinase inhibitor PP2.**

**KEYWORDS: androgen receptor; estrogen receptor; signal transduction; epidermal growth factor**

## INTRODUCTION

Crosstalk between growth factors and steroid receptors in the nuclear compartment leads to ligand-independent activation of the steroid receptor–dependent transcription. Serine phosphorylation of steroid receptors by growth factor–activated signaling kinases triggers this activation. Recent data and findings described herein reveal that a crosstalk between growth factors and steroid

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receptors occurs in a bidirectional way also at a nontranscriptional level. Interestingly, epidermal growth factor (EGF) signaling is strongly enhanced by the functional interplay between the EGF receptor/erb-B2 heterodimer and the estradiol receptor (ER)–androgen receptor (AR) complex in MCF-7 cells. ER tyrosine phosphorylation, triggered by growth factors, causes the assembly of the EGFR/ER/AR/Src signaling complex, which, in turn, induces EGFR phosphorylation on tyrosine. Since growth factors and steroid receptors are known to dramatically contribute to breast cancer progression, these findings contribute to the understanding of their action.

EGF activity converges on the ER in human mammary cancer–derived cells as well as in uterus, thereby triggering DNA synthesis and cell proliferation. EGF can also activate genes regulated by estrogen-responsive elements.[1–3] An increase in uterine weight and proliferation of the uterine epithelial cells follows EGF or insulin-like growth factor-I (IGF-I) treatment of ovariectomized mice. Interestingly, these effects are not observed in ER-α knockout mice, indicating that ER-α is required in this growth factor activity.[3] Elevated Src activity has been found in breast tumor specimens and cell lines.[4] Moreover, both EGFR and Src are overexpressed in a subset of human breast tumors and a wealth of evidence indicates physical and functional associations between EGFR and Src (reviewed in Ref. 5). Expression of dominant negative Src in murine fibroblasts interferes with EGF-induced mitogenesis and cytoskeletal changes.[6] Therefore, steroid receptors and Src seem to be components of the signaling pathway elicited by EGF.

In MCF-7 and LNCaP cells, ER and AR, once activated by steroid hormones, stimulate a mitogenic signaling network known to be engaged by growth factors.[7,8] We recently observed in these cells an unexpected crosstalk between EGFR and the extranuclear steroid receptors, which will be analyzed in the course of this report.

## *Epidermal Growth Factor Signaling in Mammary Cancer Cells is Upregulated by Steroid Receptors and Src*

Recent evidence from our laboratory[9] indicates that steroid receptors and Src play a key role in EGF-triggered DNA synthesis and stress fiber breakdown. EGF stimulates the S-phase entry of MCF-7 cells maintained in phenol red–free medium supplemented with charcoal-treated serum. In accordance with a previous report,[1] the effect of EGF is abolished by ICI 182,780, a pure antiestrogen. Interestingly, the pure antiandrogen Casodex also abolishes the growth hormone effect. Effects similar to those of the two steroid antagonists are observed in the presence of the Src kinase family inhibitor, PP2, as well as in cells transiently transfected with siRNA silencing ER-α or AR. EGF rapidly induces fan-like membrane protrusions and ruffles in MCF-7 cells. Also in this case, both steroid antagonists prevent EGF-induced cytoskeletal changes. Src

activity is also involved in these responses as indicated by the PP2 inhibitory effect on EGF-induced cytoskeletal changes. In conclusion, the two steroid receptors and Src have a key role in the EGF-elicited responses in MCF-7 cells.

### *EGF-Triggered Src Activation in MCF-7 Cells is Inhibited by Steroid Antagonists*

Src family tyrosine kinases are involved in signaling of different growth factor receptors including EGFR. They can promote initiation of signaling pathways required for DNA synthesis and actin cytoskeleton rearrangements.[10] In MCF-7 cells EGF activates Src, whereas ICI 182,780 prevents this activation as well as the EGF-induced Ras and Erk-2 activities. Therefore, ER-α plays a major role in the regulation of EGF-elicited signal transducing pathway in MCF-7 cells. Casodex also prevents the EGF-induced activation of Src in MCF-7 cells, which express AR. Lack of effect of ICI 182,780 on EGF signaling in ER-negative MDA-MB231 cells indicates that inhibition of EGF-induced Src activation by ICI 182,780 in human mammary cancer cells requires ER-α expression.

### *EGF Triggers Association of ER-α and AR with Src and EGFR*

Simultaneous to Src activation, EGF induces association of ER-α and AR with Src and EGFR in MCF-7 cells. A selective inhibitor of the EGFR tyrosine kinase, Iressa (ZD 1839), and the anti-erb-B2 antibody herceptin (Trastuzumab), block the EGF-elicited Src activation and ER-α tyrosine phosphorylation and prevent the association of Src with ER and AR. Transient transfection of Cos cells with hAR and either hER-α (HEG0) cDNAs or the HEG537 cDNA mutant, lacking the only phosphorylatable tyrosine, that in position 537,[11] shows that this phosphotyrosine is required for the coexpressed receptors/Src complex assembly.

### *AR and ER-α are Associated with Unstimulated MCF-7 and LNCaP Cells*

Co-immunoprecipitations of either AR or ER indicate that 8% of the two receptors are associated in MCF-7 cells under basal conditions. Interestingly, a similar association occurs in LNCaP cells between ERβ and AR. Pull-down experiments with glutathione S-transferase (GST) fusion protein constructs show that the association between the two receptors is direct. Association was previously observed between ERα and progesterone receptor-B in human mammary cancererived cells under basal conditions and *in vitro*. It is required in different cell lines for progestin stimulation of signal transducing pathway, which triggers G1-S transition.[12,13,14]

### *Ligand-Stimulated Epidermal Growth Factor Receptor Tyrosine Phosphorylation in MCF-7 Cells is Uupregulated by Steroid Receptors and Src*

The above described experiments show that EGF induces ligand-independent extranuclear steroid receptor activation. Remarkably, steroid receptors, in turn, regulate the EGFR. In fact, EGFR phosphorylation in EGF-stimulated MCF-7 cells is strongly reduced when the cells are stimulated by the growth factor in the presence of either ER or AR antagonists. This finding suggests a novel, steroid-independent regulatory role of steroid receptors on EGFR. This conclusion is supported by the strong inhibitory effect on EGFR phosphorylation observed after the knockdown of ERa or AR gene in MCF-7 cells. In addition to the steroid receptors, Src is required for EGFR phosphorylation in MCF-7 cells. EGFR tyrosine phosphorylation triggered by EGF is much weaker in cells expressing kinase-inactive Src. On this basis we propose that Src kinase activity plays a key role in the EGF-dependent EGFR phosphorylation in MCF-7 cells and that this activity is under the control of the Src-associated steroid receptors. This possibility is strongly corroborated by the experiments in Cos cells transiently co-transfected with hAR and the wild-type hER$\alpha$, or its mutant HEG537. In fact, EGF strongly stimulates EGFR tyrosine phosphorylation in Cos cells expressing HEG0, whereas a much weaker stimulation was detected in cells expressing HEG537, which is not able to interact with Src and induce association of AR/ER/Src. Remarkably, comparison of EGFR tyrosine phosphorylation in cells transfected with the empty vector or ER$\alpha$- and AR-expressing plasmids shows that in the presence of the two steroid receptors a much stronger EGFR phosphorylation is triggered by EGF. This provides additional evidence that the expression of the two steroid receptors upregulates EGFR phosphorylation.

### *EGF-Elicited Effects Are Inhibited by Steroid Antagonists in LNCaP Cells*

In LNCaP cells, as in MCF-7 cells, EGF induces DNA synthesis. Antiandrogen and antiestrogen abolish this stimulation. The antagonists also prevent EGF-induced cytoskeletal changes and the growth factortimulated Src activation. These findings indicate that also in prostate cancer cells EGF signaling is regulated by the two steroid receptors.

## CONCLUSIONS

On the basis of the results presented, one may envisage a new model of crosstalk between extranuclear steroid receptors and EGFR. a central role being played by the physical and functional interactions between EGFR, steroidal

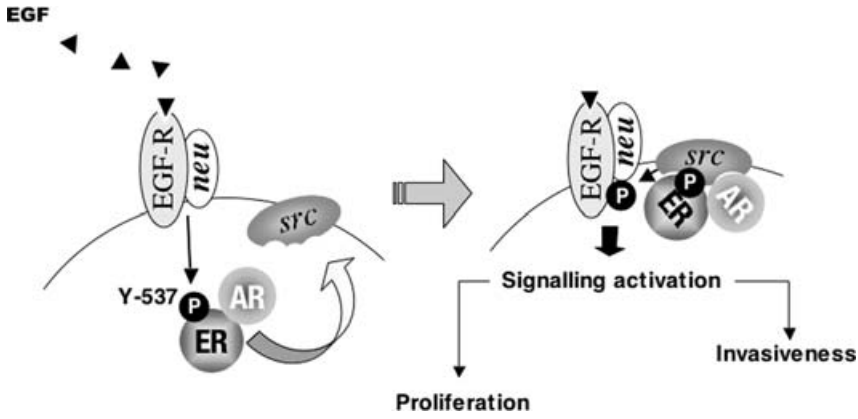


**FIGURE 1.**

receptors, and Src (FIG. 1). The EGF-activated Src, which is associated with the ER/AR complex, strongly acts on the EGFR phosphorylation. Conversely, when ER and/or AR are locked in an inactive conformation by hormone antagonists or when the steroid receptor levels are downregulated by siRNA, their action on Src and EGFR is missing or heavily impaired and EGF-induced EGFR tyrosine phosphorylation is minimal. Interestingly, in MCF-7 cells, silencing of steroid receptor genes abolishes the EGF-elicited DNA synthesis, further indicating that such an effect requires steroid receptors. Similarly, ER-α is required for EGF-triggered DNA synthesis in uterine epithelial cells *in vivo*.[3] The complexity of the described crosstalk between EGF and the steroid receptor/Src complex is underlined by the observation that steroid receptors also control, through Src, the EGF-elicited cytoskeletal changes, a classic nongenomic effect in breast and prostate cancer cells. Association of AR with ER in MCF-7 and LNCaP cells under basal conditions represents a novel and important crosstalk between the two receptors, which are linked in their responses to growth factors or steroid hormones. This study also reveals other aspects of the molecular assembly that regulates nongenomic steroid receptor action. In the ER/Src complex triggered by estradiol or androgen in MCF7, LNCaP or T47D cells,[8] phosphotyrosine in position 537 of ER-α is crucial for the hormone-induced association of ER-α with Src-SH2 and consequent Src activation and mitogenesis.[8] The same phosphotyrosine residue is required for the association of ER-α with Src triggered by EGF. On the basis of previous and present findings, the ER/AR/Src association is crucial for proliferation triggered by steroid hormone[8] or EGF in hormone-responsive cells. This is a point with important implications since a large number of mammary and prostate cancers respond to steroid hormones and growth factors. This association represents a target for a novel, rational, and specific therapy of cancers expressing steroid receptors.

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