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Direct interaction between estrogen receptor α and NF-κB in the nucleus of living cells

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Keywords: ERα; NF-κB; FRET; interaction; in vivo

Abstract

Inhibition of NF-κB transcriptional activity by steroid receptors is the basis for the antiinflammatory actions of steroid hormones and the molecular mechanism underlying this cross-talk is thought to involve direct protein-protein interactions. In this study, we show that estrogen receptor (ER)α and NF-κB interact in vivo by using fluorescence resonance energy transfer (FRET) and coimmunoprecipitation. U2-OS cells were used to study direct interactions between fluorescent fusion proteins of ERα and the NF-κB subunits p50 and p65. Interactions were observed only in the nucleus and maximal FRET signal was detected when ERα is coexpressed with both NF-κB subunits and cells were stimulated with estrogen. This is in agreement with the induction of nuclear colocalization of the proteins under this condition. Moreover, in a U2-OS clone stably expressing ERα, interaction with NF-κB was confirmed. A p65 deletion mutant lacking the Rel homology domain was strongly impaired in its interaction with ERα showing the importance of this domain. Taken together, these findings provide a strong basis for the direct protein-protein interaction model for cross-talk between ERα and NF-κB.
1. Introduction

The estrogen receptor (ER), like other members of the nuclear receptor family, is a ligand-inducible transcription factor capable of regulating gene expression in several ways. In the classical pathway, the ligand-activated receptor binds directly to specific hormone response elements in the promoters of target genes and recruits co-regulators to enhance transcription (Metivier et al., 2003). In an alternative pathway, nuclear receptors modulate gene transcription without directly binding DNA by changing the activity of other transcription factors such as activator protein-1 (AP-1), Sp1 and nuclear factor-κB (NF-κB) (Paech et al., 1997; Saville et al., 2000; Kalaitzidis and Gilmore, 2005). A variety of steroid hormones, including estrogens, glucocorticoids, androgens and progestins, are known to inhibit NF-κB activity upon binding to their cognate steroid receptor (Caldenhoven et al., 1995; Kalkhoven et al., 1996; McKay and Cidlowski, 1999).

NF-κB is well-known for its key role in tissues of the immune system, but it is also important in development and physiology of bone, skin, mammary gland and central nervous system. Although NF-κB activity is essential in the regulation of many processes in normal physiology, dysregulation is known to be involved in various diseases including inflammatory diseases, autoimmune diseases and cancer. Inhibiting the NF-κB pathway has proven to be an effective treatment for several of these diseases (Baldwin, Jr., 2001). Therefore, glucocorticoids are widely used to treat a variety of inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease (IBD) (Franchimont, 2004). Furthermore, estrogen is used to prevent postmenopausal osteoporosis and also relieves symptoms in animal models of atherosclerosis, rheumatoid arthritis and IBD (Yamasaki et al., 2001; Pfeilschifter et al.,...
NF-κB is expressed as a homo- or heterodimer formed between the five members of the NF-κB protein family consisting of p65 (RelA), RelB, c-Rel, p50 and p52. Each protein contains a Rel homology domain (RHD) at the N-terminus which is responsible for DNA binding, dimerization, and association with the inhibitor of NF-κB (IκB). In most cell types NF-κB is present as a heterodimer between p50 and p65 and in unstimulated cells it remains inactive in the cytoplasm due to association with IκB. The classical pathway of activation involves a phosphorylation cascade and subsequent degradation of IκB, which can be induced by various signals including cytokines, growth factors and UV radiation. Once NF-κB becomes liberated from IκB it rapidly translocates to the nucleus where it can bind to specific NF-κB binding sites in the promoters of target genes to enhance transcription (Chen and Greene, 2004).

Although different theories have been proposed to explain the molecular mechanism of NF-κB transrepression, models involving direct protein-protein interactions between steroid receptors and NF-κB are most likely (De Bosscher et al., 2003; Kalaitzidis and Gilmore, 2005). In this study, we used fluorescence resonance energy transfer (FRET) to investigate the interaction between ERα and homo- or heterodimers of p65 and p50 in living osteoblastic U2-OS cells. Interactions are observed only in the nucleus and are strongly enhanced by estrogen. Maximal interaction is observed when ERα is coexpressed with both p50 and p65 in the presence of E2. This is consistent with the observation that all three proteins are colocalized under this condition. Interaction between ERα and NF-κB is confirmed in cells stably expressing fluorescently-labeled ERα. A deletion mutant of p65 lacking the RHD, necessary for interaction with ERα in vitro, also loses its capacity to interact in vivo. Therefore our findings provide a solid basis for the direct interaction model for cross-talk
between ER$\alpha$ and NF-kB.
2. Materials and methods

2.1. Generation of fluorescent fusion constructs

To create an expression vector containing enhanced cyan fluorescent protein (ECFP) fused to the C-terminus of human ERα, PCR was performed using the forward primer 5'-GGAATTCATGGTGAGCAAGGCGAGGAG-3' and the reverse primer 5'-TCC-CTCGAGTTAGATATCCTTGACAGCTCGTGCCATGCC-3' to generate the ECFP fragment with an EcoRI (5') and a XhoI (3') restriction site (underlined in primer sequence). After digestion of this fragment with EcoRI and XhoI restriction enzymes it was cloned into the modified mammalian expression vector PSG5(neo) containing a multiple cloning sequence and the neomycin resistance gene (gift of Dr. E. Sonneveld, Amsterdam, The Netherlands). An ERα fragment with a SpeI (5’) and an EcoRI (3’) restriction site was made by using the forward primer 5’-AAACTAGTATGACCATGACCCTCCACACCAAG-3’ and the reverse primer 5’-AAGAATTCGACTGTGGCAGGGAAACCCTCTG-3’ and after digestion it was cloned into PSG5(neo)-ECFP. Expression vectors for human p50 and p65 with enhanced yellow fluorescent protein (EYFP) fused to the N-terminus, were made by made by cutting the EYFP PCR fragment with EcoRI and EcoRV (forward primer 5’-GGAATTCACCACCATGGTGAGCAAGGAGGC-3’ and reverse primer 5’-TCCCCGGGTTAGATATCCTTGACAGCTCGTGCCATGCC-3’) and cloning into PSG5(neo)-EYFP. pBluescript SK containing p50 was cut with EcoRI and after filling-in with the Klenow enzyme, the p50 fragment was cut with NotI and cloned into PSG5(neo)-EYFP. PGEX-3X containing p65 was partially cut with SmaI and cloned into PSG5(neo)-EYFP. The expression vector for YFP-p65Δ22-248 was made partially cutting PGEX-3X containing p65Δ22-248 with SmaI and cloning into PSG5(neo)-EYFP.
2.2. Transfection and reporter assays

U2-OS cells were cultured and transiently transfected with DNA constructs, using calcium-phosphate precipitation, as described previously (Quaedackers et al., 2001). For studying NF-κB transcriptional activity, cells were cotransfected with 100 ng 4xNF-κB(HIV)-tk-Luc and expression vectors encoding p50 or p65 (10 ng and 2.5 ng, respectively) or empty expression vectors. Repressive effects of ERα on NF-κB activity were assessed by cotransfecting 50 ng ERα expression vector. ERα transcriptional activity was analyzed by cotransferring 1.0 μg 3xERE-tata-Luc and 200 ng ERα or empty expression vector. Each transfection also contained 200 ng SV2-LacZ plasmid as an internal reference for transfection efficiency; pBluescript SK- was added to obtain a total amount of 2 μg DNA/well. After 16 h, cells were treated with vehicle (0.1% EtOH), 10 nM E2 and/or 5 ng/ml TNFα. Cells were harvested 24 h later and assayed for luciferase and β-galactosidase activity. Data are represented as mean values ± SEM from at least three independent experiments assayed in triplicate. For FRET analysis cells were transfected as described above, with 60 ng ER-CFP in combination with 70 ng YFP-p50 alone, 75 ng YFP-p65 alone or 40 ng YFP-p50 together with 50 ng YFP-p65. pBluescript SK- was added to obtain a total amount of 3.3 μg DNA/well. Methods to obtain stable transfectants of U2-OS expressing ER-CFP were described previously (Quaedackers et al., 2001).

2.3. Coimmunoprecipitation and Western blotting

Cells were transiently transfected with a 1:5 mixture of DNA and linear polyethylenimine MW 25K (L-PEI; Polysciences, Warrington, PA). This mixture
consisted of serum free medium with 2.5 µg pFlag-CMV-2-p65 (Wang and Baldwin, 1998), 5µg pSG5-ERα and 2.5 µg CMV-4 p50 or empty vectors, as indicated, and 52.5 µl L-PEI (1 mg/ml) and was incubated at room temperature for 15 minutes before adding to the cells. After two days cells were treated with 10 nM E2 for 3 h before harvesting. Whole cell extracts were made by resuspending pelleted cells in hypertonic buffer (20 mM HEPES pH 8, 630mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 % NP-40, 1x proteinase inhibitor cocktail). Samples were freeze-thawed three times, incubated on ice for 30 min and cellular debris was removed by centrifugation. Supernatants were diluted ten times in IP buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and E2 was added to a final concentration of 10 nM. After preclearing with protein G-agarose, extracts were incubated for 5 h at 4°C with 4 µg of M2 anti-Flag antibody (Sigma, St. Louis, MO) preabsorbed to protein G-agarose. Immunoprecipitates were washed four times with wash buffer (24 mM Tris-HCl pH 7.5, 2mM KCl, 163mM NaCl) and the pellets were boiled in Laemmli sample buffer. Proteins were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies against p65, p50 (sc-109 and sc-114, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) or ERα (Ab-15; Lab Vision, Fremont, CA) according to manufacturer’s protocols. After incubation with peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using enhanced chemiluminescence.

2.4. FRET analysis

A Leica DMR upright microscope (Leica Microsystems, Wetzlar, Germany) was used, equipped with a fluorescence system, a 63x/0.9 U-V-I long distance water-immersion
objective and a temperature-controlled specimen holder at 37°C. Cells were kept in DMEM without phenol-red, supplemented with 10 mM HEPES (pH 7.4). As an excitation source, a SPEX Fluorolog (Spex Industries, Edison, NJ) was used. The light from the excitation monochromator was coupled into the microscope via an UV fiber optic cable (Schott, Mainz, Germany). The emission fluorescence from the microscope was collected (via another fiber optic cable) and fed back into the emission monochromator of the SPEX Fluorolog. Spectral analysis of CFP and the FRET (430 nm excitation) was performed with a >455 nm dichroic mirror (Chroma Technology, Rockingham, VT). Separate YFP spectra (excitation 490 nm) were collected with a dichroic mirror >510 nm. Spectral data were recorded with an integration time of 0.5 second/nm, 1 nm step resolution, 8 nm slit width. Emission wavelength ratios \( R_{525/480} \) of a single experiment are averages of 10-20 individual single cell spectra. Each experiment was repeated at least two times. For every single cell FRET spectrum (excitation 430 nm), a separate spectrum of YFP (excitation 490 nm) from the same cell was collected as a control for expression levels of CFP and YFP constructs. The molar extinction coefficients, \( \varepsilon_{\text{CFP}(430)} \), \( \varepsilon_{\text{YFP}(490)} \) are respectively, 32.5 and 55.3 \( \times 10^3 \) M\(^{-1}\)cm\(^{-1}\). As a consequence only cells with intensity ratios CFP/YFP within 1.6–2.2 were taken into account in order to avoid the formation of low donor/acceptor pairs (R<1.6) and to avoid unspecific bleed-through of 430 nm excited YFP at ratios R> 2.2. FRET images of Supplementary Figure S1A were captured with an Image Intensifier (Delft Electronic Products, Roden, The Netherlands) coupled to a progressive scan CCD camera CV-M10RS (JAI, Copenhagen, Denmark) with a frame capture board AG5 (Scion, Frederick, MD). The images were processed with a program (written in the Pascal macro language of Scion Image) based on the algorithms of Xia et al (Xia and Liu, 2001).
2.5. **Cellular localization of fluorescent proteins**

Images of CFP and YFP fluorescence were collected with a Leica NT confocal system (Leica Microsystems, Mainz, Germany) with a 40x objective (same detection photomultiplier settings in all experiments). Maximum projections of Z-series (10 slices) were made. For each experimental condition, 100-120 cells were counted. Values are expressed as percentage of fluorescence present in either cytosol or nucleus of the total number of cells found in one image.
3. Results

3.1. Coimmunoprecipitation of ERα and NF-κB

Previously, we compared the repressive effect of both ER subtypes, ERα and ERβ, on NF-κB transcriptional activity in the human osteoblast-like cell line U2-OS. From this study it became clear that ERα, and not ERβ, is the major ER through which transcription of NF-κB-regulated genes is inhibited in U2-OS cells (Quaedackers et al., 2001). Therefore, in this study we focused on ERα in order to investigate the protein-protein interaction model in U2-OS cells. First, we performed a luciferase reporter assay in order to analyze the effect of ERα on p65 and p50/p65 induced transcriptional activity. U2-OS cells were cotransfected with the NF-κB responsive reporter 4xNF-κB-Luc and an expression vector encoding p65 alone or in combination with an expression vector for p50. In both conditions NF-κB reporter activity was efficiently induced (~ 6 fold). Cotransfection of ERα resulted in 50% reduction of p65 and p50/p65 induced transcriptional activity. Upon stimulation with 17β-estradiol (E2), NF-κB transcriptional activity was almost completely abolished, reducing reporter activity to basal activity of the empty vector control (Fig.1A). Next, we investigated complex formation between ERα and p50/p65 in U2-OS cells by coimmunoprecipitation. In order to achieve this we used an expression construct encoding p65 with an N-terminal FLAG-tag, which does not interfere with the ability to activate transcription (Wang and Baldwin, 1998) and transrepression by ERα (data not shown). Immunoprecipitation of Flag-p65, from cell extracts coexpressing ERα and treated with E2, resulted in efficient pull down of ERα. When Flag-p65 and p50 were coexpressed, p50 was also coimmunoprecipitated showing that p50/p65 heterodimers are formed. Since the affinity between homodimers...
of p50 or p65 is weaker than between heterodimers, it can be assumed that when p50 and p65 are coexpressed, mainly the heterodimer will be present (Chen et al., 1998). When ERα was coexpressed with both Flag-p65 and p50, immunoprecipitation of Flag-p65 still resulted in efficient pull down of ERα after E2 stimulation (Fig.1B). As a control, immunoprecipitations were performed in the absence of Flag-p65 and this did not result in pull down of ERα, showing that ERα is specifically coimmunoprecipitated with p65 (data not shown). Taken together, these results show that ERα interacts directly with p65, present as homodimer or heterodimer with p50, in extracts from mammalian U2-OS cells treated with E2.

3.2. Expression and functionality of CFP and YFP fusion proteins of ERα and NF-κB

In addition to coimmunoprecipitation, we were interested in investigating direct protein-protein interactions between ERα and NF-κB in living cells by using Fluorescence Resonance Energy Transfer (FRET). Therefore, we generated fluorescent fusion constructs encoding ERα with cyan fluorescent protein fused to the C-terminus (ER-CFP) and p50 and p65 with yellow fluorescent protein fused to the N-terminus (YFP-p50 and YFP-p65, respectively). Expression of correct molecular weight of the fusion proteins was investigated by Western blot analysis of whole cell extracts from U2-OS cells overexpressing wild type protein (Fig.2A; left lane) or fusion protein (Fig.2A; right lane). To study the ability of the fusion proteins to activate transcription compared to wild type proteins, ER-CFP or ERα was cotransfected with the estrogen responsive reporter 3xERE-Luc (Fig.2B) and YFP-p65 or p65 with 4xNF-κB-Luc (Fig.2C). ER-CFP strongly induced transcription of 3xERE-Luc after E2 stimulation, although less efficiently than with wild type ERα. This could be due to sterical hindrance of CFP with ER-
coactivator interactions or increased protein turnover. YFP-p65 was equally potent in inducing transcription as wild type p65. Similar to wild type ER, cotransfection of ER-CFP resulted in 50% reduction of YFP-p65 transcriptional activity and E2 stimulation completely abolished transcriptional activity. Although, cotransfection of YFP-p50 resulted in reduced transcriptional activity (60% compared to YFP-p65 alone), it was still repressed to basal activity by E2-stimulated ER-CFP (Fig.2D). Therefore, we can conclude that the functional characteristics of the fusion proteins are comparable to wild type proteins and can be used for studying in vivo interactions by FRET analysis.

3.3. Colocalization of fluorescent fusion proteins

To examine the cellular expression and dynamic behavior of the fluorescent proteins we studied their localization upon transient transfection in U2-OS cells. When ER-CFP was coexpressed with YFP-p50, we observed that both proteins were exclusively present in the nucleus, with and without E2 stimulation (Fig.3A). Coexpression of ER-CFP with YFP-p65 revealed that ER-CFP remained localized in the nucleus, while YFP-p65 was present in both the cytosol and the nucleus, and this did not change upon E2 treatment (Fig.3B). However, when ER-CFP was coexpressed with both YFP-p65 and YFP-p50 we observed that upon E2 treatment, YFP fluorescence was almost completely translocated to the nucleus resulting in colocalization of all three proteins (Fig.3C). These observations enabled us to study the interaction between ERα, p50 and p65 in the nucleus of living U2-OS cells by FRET.
3.4. Interaction between ERα and NF-κB in living U2-OS cells

We applied the method of spectral analysis to detect transfer of energy from the excited donor fluorophore (CFP) to the acceptor fluorophore (YFP), which occurs if the two fluorophores are in very close vicinity of each other (less than 60Å) (Pollok and Heim, 1999). The quantitative parameter for donor/acceptor interaction is depicted by the ratio between emission intensities at 525nm and 480nm (R525/480) of the spectra obtained from cells excited with 430 nm. Increased efficiency of energy transfer between CFP- and YFP-fusion proteins, leads to decreased donor emission intensity at 480 nm and a subsequent increase in acceptor intensity at 525 nm and thus an increased R525/480. As a negative control, cells were transfected with equal amounts of expression vectors encoding CFP and YFP alone, which did not result in a FRET signal (R525/480=0.84). When cells were transfected with ER-CFP in combination with YFP alone or YFP-p50 and YFP-p65 in combination with CFP alone and next stimulated with or without E2, FRET signals were all within the same range as the negative control (data not shown). As a positive control cells were transfected with an expression construct encoding the fusion protein CFP-D2-YFP (Blanchetot et al., 2002) resulting in a strong FRET signal (R525/480=3.44) (Fig.4A).

To study interactions between ERα and NF-κB, U2-OS cells were transfected with ER-CFP in combination with YFP-p50 or YFP-p65 alone or in combination. In all three transfection conditions, R525/480 was increased in absence of ligand, compared to the negative control, and this was induced further upon stimulation with E2. It appeared that maximal FRET signal was observed when ER-CFP was coexpressed with both YFP-p50 and YFP-p65 after stimulation with E2 (Fig.4A and 4B). We used a second method to quantify FRET by determining the FRET-to-donor fluorescence ratio (nF/ICFP)
as described by Xia et al (Xia and Liu, 2001). Upon E2 treatment $nF/I_{CFP}$ was strongly enhanced and the signal was only detected in the nucleus (Fig.4C, top). This is consistent with colocalization of the interacting proteins in the nucleus (Fig.3 and Fig.4C, bottom). Moreover, it was determined that the interaction induced by E2 is dose-dependent (Fig.4D), which is consistent with our observations of functional reporter assays in U2-OS (Quaedackers et al., 2001). In addition to overnight (16 h) E2 incubation, the condition used in reporter assays, interaction between ER$\alpha$ and NF-$\kappa$B was observed already after 4h and longer incubations resulted only in a minor increase in $R_{525/480}$ (Fig.4E).

Because the use of transiently transfected cells can lead to complications arising from overexpression, we isolated an U2-OS clone stably expressing ER-CFP (Fig.4F), which retained functional characteristics (Fig.5). It appeared that when cells were cultured in charcoal-stripped serum, necessary for studying hormone effects, the expression of ER-CFP was reduced beneath levels required for spectral analysis. This effect is also observed in U2-OS clones stably expressing wild type ER$\alpha$ (data not shown). This problem was overcome by performing incubations in combination with TNF$\alpha$, which results in higher ER$\alpha$ expression. Upon transient coexpression of YFP-p50 and YFP-p65 we were able to detect E2-induced interaction with stably expressed ER-CFP (Fig.4G).

In a previous study we showed that the Rel homology domain (RHD) of p65 is essential for in vitro interaction with the glucocorticoid receptor (Wissink et al., 1997). Moreover, it has been reported that the RHD of p65 was sufficient for the in vitro interaction with ER (Stein and Yang, 1995). Therefore, we generated a deletion construct of YFP-p65, lacking amino acids 22 to 248 comprising a large part of the RHD (YFP-p65$\Delta$22-248). Expression and functionality of p65$\Delta$22-248 has been published
previously (Wissink et al., 1997) and fusion of YFP to the N-terminus did not change its functionality (Fig.6A-C). Coexpression of YFP-p65Δ22-248 instead of wild type YFP-p65 resulted in a strong reduction of the E2-induced interaction between ERα and NF-κB in our FRET analysis (Fig.4H). Moreover, the effect of estrogen on colocalization was not observed with the RHD deletion mutant YFP-p65Δ22-248 (Fig.6D).

Taken together, our FRET analysis shows that ERα directly interacts with NF-κB in living U2-OS cells. This interaction is observed in the nucleus and is strongly enhanced in the presence of E2, which is in agreement with functional data obtained from reporter assays in U2-OS.
4. Discussion

Repression of NF-κB transcriptional activity by ER has been described to occur in a variety of cell types, including osteoblasts, macrophages, hepatoma cells, breast cancer cells and cardiac myocytes. Moreover, in human diseases and in several animal models for specific diseases, estrogen has antiinflammatory activity which is attributed to ER-mediated inhibition of NF-κB activity (Kalaitzidis and Gilmore, 2005). Furthermore, it has been established that estrogen plays an important role in bone physiology by maintaining bone mass, which is thought to be due to downregulation of NF-κB mediated cytokine production. This becomes evident in postmenopausal women where estrogen deficiency results in rapid bone loss and consequently a higher risk for developing osteoporosis, which appears to be associated with increased levels of circulating proinflammatory cytokines (Pfeilschifter et al., 2002).

To explain the molecular basis by which ER and other steroid receptors, mediate repression of NF-κB activity, both indirect and direct mechanisms have been proposed. It appears that the mechanisms involved vary between studies and depend on promoter context, cell type and stimuli that were investigated. Indirect effects include competition for a limited amount of coactivators shared by both pathways (Harnish et al., 2000) and induction of IκB expression levels (Nakshatri et al., 1997). In this study, we use U2-OS cells to investigate ER/NF-κB cross-talk. Since previous work has shown that this cross-talk is not the result of indirect mechanisms (Stein and Yang, 1995; Quaedackers et al., 2001), it must be the result of a direct mechanism such as inhibition of NF-κB DNA binding or interference with components of the NF-κB transcriptional complex (Nissen and Yamamoto, 2000; De Bosscher et al., 2003; Kalaitzidis and Gilmore, 2005; Luecke and Yamamoto, 2005). These direct mechanisms are believed to be the result of
physical interaction between NF-κB and ER. Although, it has been found that ERα can directly bind to p65, p50 and c-Rel \textit{in vitro} via GST pull down (Stein and Yang, 1995; Kalaitzidis et al., 2004), the only \textit{in vivo} evidence for an interaction between ERα and NF-κB was seen in the use of a fusion protein between c-Rel and the ligand binding domain of ERα that is functionally inhibited by estrogen, presumably through an intramolecular interaction (Kalaitzidis et al., 2004).

Here, we report that ERα is coimmunoprecipitated with p65 when expressed as either a homodimer or a heterodimer with p50 from E2 treated U2-OS cell extracts showing that ERα can interact with NF-κB \textit{in vivo}. This is in agreement with data obtained from reporter assays showing a functional interaction between ERα and NF-κB in U2-OS cells. Moreover, we determined \textit{in vivo} interaction between ERα and the NF-κB subunits p50 and p65 in living U2-OS cells by using FRET.

It has been shown before that FRET is an effective method for detecting ligand-induced interactions between ERα and other proteins including the transcription factor Sp1 and the coactivators SRC1 and PBP (Llopis et al., 2000; Kim et al., 2005). In this study, we measured FRET by applying spectral analysis, which is a quantitative and extremely sensitive method. However, several precautions must be taken into account to avoid misinterpretation of results. Most importantly, it is necessary to establish equal expression levels of interacting donor and acceptor proteins. Under all circumstances this was carefully controlled and confirmed by measuring the ratio of CFP and YFP emission intensities for every individual single cell. We also confirmed that expression and transcriptional properties of the fluorescent fusion proteins ER-CFP, YFP-p65 and YFP-p50 were comparable to wild type proteins in U2-OS cells. Using FRET we observed maximal interaction when ER-CFP was coexpressed with both YFP-p65 and YFP-p50 and cells were treated with E2. This is in agreement with the observation that
all three proteins are colocalized only under this condition, indicating that this is the result of interaction between ERα and NF-κB. In contrast, when the RHD deletion mutant YFP-p65Δ22-248 is coexpressed with ERα-CFP and YFP-p50 and cells are treated with E2, we do not observe colocalization and the FRET signal is strongly reduced. From this we can conclude that the RHD of p65 is required for the in vivo interaction between ERα and NF-κB. This domain was previously described to be sufficient for the interaction between ER and NF-κB as observed by GST pull down (Stein and Yang, 1995). In contrast to GST pull down assays where the interacting proteins are exclusively expressed, in our FRET analysis it is possible that the ER-NF-κB complex formation involves the presence of additional proteins that are expressed in U2-OS cells.

We generated a stable U2-OS cell line expressing ER-CFP at a level equal to endogenously expressed ERα in breast cancer cells. In this cell line we were able to verify the interaction with YFP-p50 and YFP-p65. Therefore, we can conclude that the observed interaction between ERα and NF-κB also occurs when expressed at physiological expression levels and is not caused by overexpression.

In summary, we demonstrate for the first time that ERα and NF-κB interact in vivo by using coimmunoprecipitation and FRET analysis. We show that the interaction is observed in the nucleus upon estrogen treatment in living U2-OS cells expressing ERα at physiological levels. Using ERα as a model, these data provide a strong basis for the protein-protein interaction model for cross-talk between steroid receptors and NF-κB. In addition, our observations enable in vivo testing of newly developed ER ligands for their ability to induce interaction between ERα and NF-κB. This could be an important contribution to the development of estrogen-like compounds with antiinflammatory properties for the treatment of diseases, such as osteoporosis, without side effects.
observed with current estrogen therapy (Turgeon et al., 2004; Chadwick et al., 2005).
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Figure legends

Fig. 1. ERα represses NF-κB transcriptional activity and directly interacts with the NF-κB subunit p65.

The effect of ERα, in the absence and presence of E2, on p65 and p65/p50 induced transcriptional activity determined by luciferase reporter assay in U2-OS cells. NF-κB reporter activity induced by p65 was set at 100% (A). Coimmunoprecipitation assay in U2-OS cell extracts coexpressing Flag-p65, ERα and/or p50 in absence and presence of E2, as indicated (top). Flag-p65 was immunoprecipitated using M2 anti-Flag antibody (IP). Precipitated proteins and input levels were visualized by Western blot analysis (WB) using the indicated antibodies (B).

Fig. 2. Expression and functional activity of fluorescent fusion proteins ER-CFP, YFP-p65 and YFP-p50 compared to wild type proteins.

Schematic representation of fusion proteins and Western blots showing the expression of the wildtype protein (left lane) and fusion protein (right lane) expressed in U2-OS cells. The expected size (kDa) is indicated on the right and verified using a molecular weight marker (A). Transcriptional activity of ER-CFP, in absence and presence of E2, compared to wild type ERα determined by luciferase reporter assay in U2-OS cells. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with empty vector (B). Experiment performed the same as in B, for comparison of transcriptional activity of YFP-p65 and wild type p65 on NF-κB reporter (C). Experiment performed the same as in 1A, except ER-CFP, YFP-p65 and YFP-p50 were tested (D).
Fig. 3. Colocalization of fluorescent proteins in U2-OS cells.

Representative images of a cell coexpressing ER-CFP (excitation 430 nm) and YFP-p50 (excitation 490 nm), before and after treatment with E2. The graph represents the percentage of cells positive for YFP expression in the cytosol (white bars) and YFP expression in the nucleus (black bars). Total number of YFP positive cells analyzed was set at 100% (A). The same is depicted as in A, for a cell coexpressing ER-CFP and YFP-p65 (B). The same is depicted as in A, for cells coexpressing ER-CFP, YFP-p50 and YFP-p65 (C).

Fig. 4. FRET analysis of the interaction between ERα and the NF-κB subunits p50 and p65.

Emission spectra, after excitation with 430 nm, of U2-OS cells coexpressing ER-CFP in combination with YFP-p50 and/or YFP-p65 and treated overnight (16 h) with or without 10^{-8} M E2. Negative control (CFP/YFP): dark blue line; positive control (CFP-D2-YFP): black line. Insert: separate CFP spectrum (excitation 430 nm) and YFP spectrum (excitation 490 nm) emitted by the same cell (A). Graphical representation of the protein interactions between ER-CFP and YFP-p50 or YFP-p65 alone or in combination, with or without E2, expressed as R_{525/480} (B). FRET images depicting FRET-to-donor fluorescence ratio (nF/ICFP) of U2-OS cells coexpressing ER-CFP, YFP-p50 and YFP-p65, treated with or without E2 after excitation with 430 nm of ER-CFP (top). False-colored pictures of a U2-OS cell coexpressing ER-CFP, YFP-p50 and YFP-p65, treated overnight with E2, after excitation with 430 nm of ER-CFP and after excitation with 490 nm of YFP-p50 and YFP-P65 (bottom) (C). Dose-dependent increase in R_{525/480} of cells coexpressing ER-CFP, YFP-p50 and YFP-p65 after E2 treatment (ranging from 10^{-8} M to 10^{-14} M) (D). Time-dependent increase of R_{525/480} in cells coexpressing ER-CFP, YFP-
p50 and YFP-p65 after E2 treatment (4h, 6h or 16h) (E). Images of the U2-OS clone stably expressing ER-CFP (U2-OS#208). Upper panel: differential interference contrast (DIC) image. Lower panel: Emission after excitation with 430 nm (F). Interaction between stably expressed ER-CFP and transiently coexpressed YFP-p50 and YFP-p65 in U2-OS#208 upon treatment with 5 ng/ml TNFα, with or without 10⁻⁸ M E2 (G). The interaction between ER-CFP, YFP-p50 and YFP-p65 is greatly reduced when YFP-p65Δ22-248 is coexpressed instead of wild type YFP-p65 (H).

In all graphs values were significantly different (P<0.05) from control values of unstimulated cells, unless indicated (*) as determined by two-tailed Student's t test.

Fig. 5. Characterization of U2-OS clone stably expressing ER-CFP (U2-OS#208).

Comparison of expression level of ER-CFP in U2-OS#208 with the expression level of ERα endogenously expressed in the breast cancer cell line MCF-7. The expected size of the proteins is indicated on the left and was verified using a molecular weight marker (A).

E2-induced transcriptional activity of stably expressed ER-CFP and repression by TNFα determined by luciferase reporter assay in U2-OS#208. Fold induction indicates luciferase activity of the transiently transfected ERE reporter induced by the stimuli over untreated cells (B). Repression of TNFα-induced NF-κB activity by stably expressed ER-CFP, upon E2 treatment, determined by luciferase reporter assay in U2-OS#208. Fold induction indicates luciferase activity of the transiently transfected NF-κB reporter induced by stimuli over untreated cells (C).

Fig. 6. Deletion of the RHD of p65 abolishes transcriptional activity and the ability to repress ERα transcriptional activity.

Schematic representation and expression of YFP-p65 and YFP-p65Δ22-248 in U2-OS
cells. The expected size of the proteins (kDa) is indicated on the right and was verified by using a molecular weight marker (A). The effect of deleting the RHD of p65 (p65del and YFP-p65del) on transcriptional activity determined by luciferase reporter assay in U2-OS cells. Fold induction indicates reporter activity induced by expression vectors for p65 over cells transfected with empty vector (B). Repression of ERα and ERα-CFP transcriptional activity by p65 analyzed by luciferase reporter assay in U2-OS cells. Cells were cotransfected with empty vector and left untreated (white bars) or treated with E2 alone (black bars) or in combination with 5 ng/ml TNFα (hatched bars) or cells were co-transfected with expression vectors for p65 or YFP-p65 (grey bar) or with p65Δ22-248 or YFP-p65Δ22-248 (double hatched bar) and treated with E2. Fold induction indicates ERE reporter activity induced by ER over untreated cells transfected with only empty vectors (C). Localization of YFP fusion proteins in U2-OS cells coexpressing ER-CFP and YFP-p65Δ22-248 alone (left) or in combination with YFP-p50 (right), before and after treatment with E2. Graphs represent the percentage of cells positive for YFP expression in the cytosol (grey bars) and YFP expression in the nucleus (black bars). Total number of YFP positive cells analyzed was set at 100% (D).
Figure 1

A

![Bar graph showing relative activity of 4xNF-kB-Luc with or without E2](image)

B

<table>
<thead>
<tr>
<th></th>
<th>Flag-p65</th>
<th>ERα</th>
<th>p50</th>
<th>E2</th>
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<tr>
<td></td>
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![Western Blot images showing protein expression](image)
Figure 2

A

ERα CFP
1 595

YFP p50
1 399

YFP p65
1 551

WB:anti-ERα
95 68

WB:anti-p50
77 50

WB:anti-p65
92 65

B

Fold Induction

3xERE-Luc □ E2

Fold Induction

4xNF-κB-Luc

C

- ER ER-CFP

- p65 YFP-p65

D

Relative Activity (%)

4xNF-κB-Luc


- ER-CFP - YFP-p50 - YFP-p50
Figure 4

A

Normalized intensity (cps x 1000)

B

Ratio 525 nm/480 nm

C

nF / ICFP

D

Ratio 525 nm/480 nm

E

Ratio 525 nm/480 nm

F

G

H

Ratio 525 nm/480 nm

U2-OS#208

DIC

430 nm

490 nm

430 nm

1.0

480 nm

525 nm

400 450 550 500 600

Wavelength (nm)

ER+p50
ER+p50+E2
ER+p65
ER+p65+E2
ER+p50+p65
ER+p50+p65+E2
CFP-D2-YFP
CFP+YFP

400 450 550 500 600

Wavelength (nm)

1.0

1.2

1.4

1.6

Log concentration E2 (M)

14 13 12 11 10 9 8

- Log concentration E2 (M)

1.0

1.2

1.4

1.6

1.8

2.0

2.2

2.4

430 nm 490 nm

U2-OS#208

DIC

430 nm

0

1.0

nF / ICFP E2

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

D22-248

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

D22-248

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65

ER-CFP
YFP-p50
YFP-p65
Figure 5

A

B

C

3xERE-Luc

4xNF-κB-Luc

Fold induction

Fold induction

U2-OS #208  MCF-7

ERα-CFP  ERα

-  E2  TNF+E2

-  TNF  TNF+E2

0  100  200  300  400

0  2  4  6