Characterization of the Physical Interaction between Estrogen Receptor α and JUN Proteins*

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Activated estrogen receptor α (ERα) modulates transcription triggered by the transcription factor activator protein-1 (AP-1), which consists of Jun-Jun homodimers and Jun-Fos heterodimers. Previous studies have demonstrated that the interference occurs without binding of ERα to DNA but probably results from protein-protein interactions. However, involvement of a direct interaction between ERα and AP-1 is still debated. Using glutathione S-transferase pull-down assays, we demonstrated that ERα bound directly to c-Jun and JunB but not to FOS family members, in a ligand-independent manner. The interaction could occur when c-Jun was bound onto DNA, as shown in a protein-protein-DNA assay. It implied that ERα and AP-1 interaction could be crucial for the stability of this complex. VP16-ERS and c-Jun, which both interact with GRIP1, had synergistic effect on GAL4-GRIP1-induced transcription in the presence of estradiol, and this synergistic effect was not observed with the ERα mutant VP16-ER241G or when c-Fos, which bound GRIP1 but not ERα, was used instead of c-Jun. Finally, ER241G was inefficient for regulation of AP-1 activity, and an ERα truncation mutant encompassing the hinge domain had a dominant negative effect on ERα action. These results altogether demonstrate that ERα can bind to c-Jun in vitro and in intact cells and that this interaction, by stabilizing a multiprotein complex containing p160 co-activator, is likely to be involved in estradiol regulation of AP-1 responses.

Estrogens play a pivotal role in the control of growth and differentiation of estrogen target tissues. Their action is mediated through estrogen receptors (ER), which belong to a superfamily of nuclear receptors that act as ligand-activated transcription factors and can be subdivided to six regions (A–F) exhibiting different degrees of evolutionary conservation (1). Domain C encompasses the highly conserved DNA binding domain (DBD). The moderately conserved region E contains the ligand binding domain (LBD) and a ligand-dependent transactivation function (AF-2). The quite divergent A/B domains contain, in some nuclear receptor members such as ER, a transcription activation function (AF-1), which can activate transcription constitutively in the absence of ligand. Upon binding to their cognate ligands, nuclear receptors activate transcription by interacting with specific DNA sequences present in target gene promoters (reviewed in Refs. 2, 3). Coactivators, among them the CAMP-response element-binding protein CBP/p300 and a group of highly related molecules called p160 proteins, comprising SRC-1, TIF2/GRIP1, and RAC3/pCIP/AIB1/ACTR, associate with receptors in a ligand- and AF-2-dependent manner to enhance their transactivation potential. They function as bridging proteins to the components of the basal transcriptional machinery, and some of them, such as CBP/p300, SRC-1, and ACTR, possess an intrinsic histone acetyltransferase activity that could influence the accessibility of transcription factors to the chromatin template (reviewed in Refs. 4, 5).

Nuclear receptors also modulate transcription without receptor-DNA interaction by functional interference with other transcription factors such as activating protein-1 (AP-1) (reviewed in Refs. 6–9). AP-1, which is implicated in diverse cellular processes, including differentiation, cell proliferation, and transformation (reviewed in Ref. 10), predominantly consists of various combinations of JUN (c-Jun, JunB, JunD) and FOS (c-Fos, Fra-1, Fra-2, FosB) proteins. JUN proteins can form homodimers or more stable heterodimers with proteins of the FOS family that do not homodimerize. Jun-Jun and Jun-Fos dimers regulate gene transcription through interactions with a specific DNA sequence, the TPA-responsive element (TRE) (11–13). We and others have previously shown that estradiol could modulate AP-1-dependent transcription (14–18). Estrogen regulation of AP-1 activity is generally positive (14–17), although it can also be negative in breast cancer cells expressing high Fra-1 level (18). ERα whose expression is necessary for the estradiol effect, does not bind to TRE (14). In addition, ERα bearing a point mutation in the first zinc finger (18) or complete deletion of the DBD (14, 17) was shown to be efficient in regulating AP-1 responses, thus demonstrating that modulation of AP-1-dependent transcription is directly induced by the activated receptor. These data together suggest that the

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† The abbreviations used are: ER, estrogen receptor; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; GST, glutathione S-transferase; DCC, dextran-coated charcoal-stripped serum; DBD, DNA binding domain; LBD, ligand binding domain; TRE, TPA-responsive element; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; IC1i64,384, N-buty1-11-(3,17 β-dihydroxyestra-1,3,5(10)-trien-7-α-y1); GR, glucocorticoid receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Because deletion of ER donated by S. Fuqua, was constructed by inserting human ER data.

\[-\text{GGAATGATGAAAGGTGGGATACGAAAA-3}\]

/H11032

\[5\]

/H11032

\[AAGAAGAACAGCCTG-3\]

In addition, some coactivators likely posed that the mutual inhibition observed between some

AP-1 cross-talk. It has been proposed that the mechanism by which estrogens regulate AP-1 activity (29).2 Some of these coactivators, whole cell extracts of untreated cells were transfected with 5 μg of pCMV-\(\gamma\)-galactosidase expression plasmid PCH110 (Amersham Pharmacia Biotech) for COS cells, were used for internal control of transfection efficiency. One microgram of the \(\gamma\)-galactosidase expression plasmid pCMV \(\beta\) (CLONTECH Laboratories, Palo Alto, CA) for MCF7 cells, and 2 μg of the \(\beta\)-galactosidase expression plasmid PCH110 (Amersham Pharmacia Biotech) for COS cells, was used for internal control of transfection efficiency. Luciferase assays were performed with a Fuji BAS1000 Bioimaging Analyzer (Raytest, Paris, France). For luciferase assays, cells were lysed for 15 min in the cell culture lysis reagent from Promega. Luciferase activity was measured using an LKB luminometer (LKB Instruments, Rockville, MD) and normalized for \(\beta\)-galactosidase activity as described by Roux et al. (44).

Expression, Purification of GST Fusion Proteins, and GST Pull-Down—Overnight cultures of \(E.\ coli\) transformed with parental or recombinant pGEX plasmids were diluted 1:10 in L-broth with 50 μg/ml ampicillin and incubated at 37 °C with shaking to an \(A_{600}\) of 0.5. Isopropyl-\(\beta\)-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM. After a further 3–5 h of growth, cells were pelleted at 5000 × g for 10 min at 4 °C and resuspended in 1:1.5 (v/v) solution for plasmids recombinants and in 1:10 (v/v) for parental plasmids of the original culture volume of NETN (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris, pH 8, 100 mM NaCl) containing protease inhibitors (Complete, Roche Molecular Biochemicals). Cells were then sonicated and centrifuged at 10 000 × g for 5 min at 4 °C. GST fusion protein suspensions (100 μg) were incubated overnight at 4 °C with \(\gamma\)-labeled proteins generated by the Taq in vitro transcription-coupled translation system from Promega. After three washes with NETN, samples were boiled in 2 × SDS sample buffer and analyzed by SDS-PAGE. Signals were amplified by fluorography (Amplify, Amersham Pharmacia Biotech) and gels exposed at ~80 °C. Quantification of labeled proteins was performed with a Fuji BAS1000 Bioimaging Analyzer (Raytest, Paris, France).

Protein-Protein-DNA Assays—Protein-Protein-DNA assay was performed as described by Thénot et al. (45). The double-stranded oligonucleotide, corresponding to the collagenase TRE (18), was labeled with Klenow enzyme in the presence of \(^{32}\)PdCTP. C-Jun-primed reticuloocyte lysate (15 μl) was preincubated with the TRE (2 nM) in TKE buffer (10 mM Tris, 75 mM KCl, 0.5 mM EDTA) plus 0.5 mM dithiothreitol, 0.1 μg/ml poly(dIdC) and protease inhibitors. GST fusion proteins preloaded on glutathione-Sepharose and resuspended in TKE were then added, and binding reactions were performed overnight at 4 °C. After two washes in TKE, bound molecules were analyzed on a 12% polyacrylamide denaturing gel and visualized by autoradiography.

Immunoprecipitation and Immunoblotting—For immunoprecipitation, transfected COS cells were harvested in lysis buffer containing 20 mM HEPES (pH 7.5), 0.4 M KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, and a mixture of protease inhibitors (Complete, Boehringer). Cell lysates were clarified by centrifugation before incubation overnight at 4 °C with monoclonal antibodies (clone 1D5, Dako, Glostrup, Denmark) reacting with the A/B domain of ERα (dilution 1:40). Pre-washed protein G-Sepharose (Amersham Pharmacia Biotech) was then added and incubation continued for 2 h at 4 °C. Immunoprecipitates were separated by centrifugation, washed four times in lysis buffer, and resolved by SDS-PAGE. Proteins were analyzed by Western blotting using polyclonal anti-c-Jun rabbit antibodies (N-G, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:3000) followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma-Aldrich, Saint Quentin Fallavier, France, dilution 1:4000). Signals were visualized by

2 C. Teysier, K. Belguise, F. Galtier, and D. Chalbos, unpublished data.
**Estrogen Receptor αAP-1 Interaction**

*Chemiluminescence* (Renaissance, PerkinElmer Life Sciences, Le Blanc Mesnil, France).

**Purification of HEXAHISTidine Fusion Proteins**—Overnight cultures of *E. coli* transformed with pDS56-c-Jun and pDS56-c-Fos (46) were diluted 1:20 in L-broth and incubated at 37 °C with shaking to an *A*<sub>550</sub> of 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM. After a further 4 h of growth, cells were pelleted at 5000 × g for 10 min at 4 °C and resuspended in a 1:20 (v/v) solution of NETN. Cells were then sonicated and centrifuged at 10,000 × g for 5 min at 4 °C. The pellet was then dissolved in the same volume of inclusion body solubilization reagent (Pierce, Rockford, IL). After 20-min incubation at 4 °C, the solution was centrifuged for 30 min at 15,000 × g, and the supernatant was incubated with nickel-nitrotriacetic acid silica (Qiagen, Courtaboeuf, France) for 1 h at room temperature. After three washes with DWB buffer (6 M urea, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, pH 8), recombinants proteins were eluted in the same buffer in the presence of 0.5 M imidazole. The purified proteins were then dialyzed against 6 M urea for 6 h, and 25 mM Tris, pH 7.5, was added, eight times every 2 h, until an urea concentration of 2 M was reached. Finally, proteins were dialyzed for another 6 h against 25 mM Tris, 150 mM NaCl. The purity of histidine-tagged Fos and Jun was ~95% as determined by SDS-PAGE. Protein renaturation was verified by gel retardation assay using a consensus collagenase TRE, as previously described (18).

**RESULTS**

**ERα Directly Interacts with c-Jun in Vitro**—To test whether c-Jun associates directly with ERα in vitro, we performed glutathione S-transferase (GST) pull-down experiments in which GST and GST-c-Jun fusion proteins, preloaded on glutathione-coupled beads, were incubated with *in vitro* translated ERα. As shown in Fig. 1B, 35S-labeled ERα, which was not retained by GST, associated with the bead-bound GST-c-Jun fusion protein. The ability of 35S-labeled *in vitro* translated c-Jun to interact with a GST-ERα fusion protein was also tested in a reciprocal experiment. Contrary to GST-c-Jun, only a small fraction of the hybrid ERα protein was expressed as a full-length protein. Although efficacy of interaction between c-Jun and ERα was lower than in the reciprocal experiment, the assay confirmed the direct *in vitro* binding between the two proteins (data not shown).

*In vitro* translated ERα mutant proteins (Fig. 1A) were then analyzed for binding to GST-c-Jun to localize the ERα domain(s) required for interaction with c-Jun (Fig. 1B). Deletion of the C-terminal part of ERα (mutant HE15) totally abolished binding with the fusion protein. On the contrary, the ERα mutant protein HE19, deleted of the N-terminal part of ERα, was still able to interact with GST-c-Jun. HE11, deleted of the entire DBD, also bound to GST-c-Jun although less efficiently than HE0 or HE19. To assess the role of ERα domains in ERα-mediated regulation of AP-1 activity, increasing concentrations of the same ERα deletion constructs (1) were transfected in MCF7 cells together with the AP-1:4-TK-CAT reporter plasmid (Fig. 1C). Mutant HE19, lacking AF-1, was as efficient as HE0 in increasing the hormonal effect. In contrast, mutant HE15, deleted of the LBD and AF-2, and, in agreement with previous results (18), mutant HE11, lacking the DBD, had no effect on AP-1 activity in MCF7 cells. Both LBD and DBD therefore appeared to be important in ERα-mediated regulation of AP-1 activity in these cells.

**The ERα Hinge Domain Is Implicated in the Protein-Protein Interaction**—To more accurately define the borders of c-Jun binding sites on ERα, a series of GST-ERα deletion mutants were tested in pull-down experiments (Fig. 2). The GST-ERα(2–184) fusion protein, which only contains the A/B ERα domain, did not interact with radiolabeled c-Jun, in agreement with results obtained in the reciprocal experiment (Fig. 1B). In fact, deletion of 250 amino acids from the ERα N terminus (hybrid protein GST-ERα (251–595)) did not impair the interaction. By contrast, deletion of amino acids 251–312 (compare results obtained with GST-ERα (251–595) and GST-ERα (313–599)) totally abolished c-Jun binding, indicating that an important motif is localized in the ERα hinge region (domain D). Conversely, binding of c-Jun to GST-ERα (251–312) and GST-ERα (251–595) demonstrated that the C terminus of ERα was also dispensable. The fact that the binding efficiency of c-Jun to both fusion proteins was equivalent also showed that DBD did not participate in the protein/protein interaction. Finally, c-Jun binding was retained by protein GST-ERα (259–595) but not by GST-ERα (283–530).

**ERα Interacts with the C-Terminal Domain of c-Jun but Not with Fos Proteins**—To specify the c-Jun domain(s) involved in the interaction with ERα, several c-Jun deletion mutants were translated *in vitro* in the presence of [35S]methionine and tested in GST pull-down assays for their ability to bind GST-ERα (251–595) (Fig. 3). C-Jun mutants deleted of amino acids 6–194 or 146–221 still bound to the GST-ERα fusion protein. In agreement with these results, the c-Jun N terminus (mutant Δ224–334) alone did not interact with GST-ERα (251–595). By contrast, deletion of the 238 residues from the N terminus only moderately affected this interaction. We therefore conclude.

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**FIG. 1.** Physical and functional interactions between c-Jun and ERα mutants. The ability of ERα and ERα mutants to bind to bacterially expressed GST-c-Jun fusion protein was investigated by pull-down assays. A, schematic representation of ERα deletion mutants used in B and C. Mutants HE19, HE15, and HE11 are derived from HE0, which differs from the wild-type ERα by a Gly-400→Val mutation in the LBD of the ERα protein. B, GST pull-down. In *in vitro* translated radiolabeled HE0 and ERα deletion mutants (300,000 cpm per sample) were incubated overnight with GST and GST-c-Jun fusion proteins preloaded on glutathione-Sepharose beads, as described under “Experimental Procedures.” After extensive washes, proteins were eluted and subjected to SDS-PAGE and fluorography. Ten percent inputs of the different radiolabeled proteins used in the assays are shown on the left. C, effect of ERα mutants on estradiol modulation of AP-1 activity. Steroid-stripped MCF7 cells were transfected with 1 μg of (AP-1)/4-TK-CAT and increasing concentrations (0, 0.2, 0.4, and 0.8 μg) of expression vector coding for an ERα mutant as indicated. Cells were then incubated for 28 h with 10 nM 17β estradiol (E2) or vehicle (C). CAT activity was evaluated in whole cell extracts as described under “Experimental Procedures.” Results represent the mean (± S.D.) of three independent experiments.
that the C-terminal part of c-Jun containing the bZIP region, i.e., the basic region and the leucine zipper, was sufficient for ERα binding. Multiple bands were observed after migration of in vitro translated c-Jun in SDS gels (Figs. 2B, 3B, 4, and 5). These bands most likely correspond to Ser-63, Ser-73, or both phosphorylated forms of c-Jun, as previously described by Bannister et al. (26). In fact, they were only detected for c-Jun mutants containing the N terminus part of the protein (Fig. 3). All phosphorylated forms of c-Jun bound to the same extent to GST-ER-(251–595) (Fig. 3) and to all GST-ERα fusion proteins containing residues 259–302 (Fig. 2), indicating that, at least in our in vitro GST pull-down assay, phosphorylation of Ser-63 or Ser-73 did not modify the interaction with ERα. The bZIP region is highly conserved between members of the Jun family (38). Because our results showed that the c-Jun C terminus was implicated in the in vitro interaction with ERα, we analyzed the ability of JunD and JunB to bind ERα. As shown in Fig. 4, JunB was as efficiently retained by GST-ER-(251–595) as c-Jun (for both proteins, 15–25% of the total input was specifically bound to the hybrid protein in at least four experiments). On the contrary, JunD only weakly hybridized with the fusion protein (1–4% of the total input in four independent experiments). The potential of Fos proteins to physically interact with ERα was also examined. Neither c-Fos nor FosB, Fra-2 or Fra-1 significantly interacted with the GST-ER-(251–595) fusion protein. We also used pull-down experiments with GST-

ER(2-184) or GST-ER-(179–312) to investigate the possibility that Fos proteins bind an ERα domain other than that bound by Jun proteins. We did not detect any specific interaction of Fos proteins with both fusion proteins (data not shown).

**ERα Interacts with c-Jun Bound onto DNA**—To determine whether ERα could interact with c-Jun when AP-1 complexes were bound onto DNA, a protein-protein-DNA binding assay was then performed. 35S-Labeled in vitro translated c-Jun was preincubated with a 32P-labeled double-stranded oligonucleotide containing the collagenase TRE before it was tested for its ability to bind GST-ER-(251–595). As shown in Fig. 5, in the presence of c-Jun, the labeled TRE was retained onto GST-ER-(251–595) but not onto GST. Retention of TRE by the fusion protein was c-Jun mediated, because no specific binding of TRE to GST-ER-(251–595) was observed using unprogrammed reticulocyte lysate. Moreover, addition of 50 nM cold TRE did not significantly modify the in vitro interaction between ERα and c-Jun (not shown) demonstrating that, at least in vitro, binding of c-Jun on DNA did not influence the ERα-c-Jun physical interaction.

**In Vitro Effect of ERα Ligands**—Because ERα regulation of AP-1 activity was dependent on the presence of ligand, we then examined the ability of estradiol and antiestrogens to modulate the in vitro physical interaction between ERα and c-Jun. Interaction of ERα with the coactivator SRC-1 (47), which was reported to be hormone-dependent, was tested in parallel as a control. In vitro-translated c-Jun or SRC-1 were incubated with GST-ER-(251–595) in the presence of 1 μM 17β estradiol, 4-hydroxytamoxifen, or ICI164,384, or in the absence of ligands. As shown in Fig. 6, only modest binding of SRC-1 to ERα was observed when the receptor...
was either free or occupied with either antiestrogen as compared with the strong binding detected in the presence of estradiol. In contrast, significant amounts of c-Jun bound to ERα irrespective of whether the receptor was unoccupied or occupied with agonist or antagonists.

Interaction between ERα and c-Jun in Mammalian Cells—

The interaction between ERα and c-Jun in intact cells was evaluated using a mammalian cell two-hybrid system. Full-length human ERα was fused to the transcriptional activator VP16 (VP16-ERα) and c-Jun to the DNA binding domain of GAL4 (GAL4-c-Jun). Expression vectors for the hybrid proteins were cotransfected with a GAL4-responsive luciferase reporter (pG5-luc) in COS cells (Fig. 7A). As expected, c-Jun increased luciferase activity when tethered to DNA by the GAL4 DBD, due to the intrinsic c-Jun transactivating activity. VP16-ERα did not have any significant effect either in the absence or presence of estradiol or antagonists 4-hydroxytamoxifen and ICI164,384. However, when VP16-ERα and GAL4-c-Jun were coexpressed, GAL4-c-Jun transcriptional activity was enhanced after estradiol addition but not after antihormone treatment or in control cells. Moreover, both antiestrogens inhibited estradiol-induced luciferase activity. The two-hybrid system does not distinguish whether the c-Jun:ERα interaction is direct or mediated by another unknown factor in assembling a multiprotein complex with c-Jun and ERα. To evaluate the importance of the direct interaction between ERα and c-Jun in the observed enhancement of luciferase activity, an ERα mutant deleted of amino acids 250–303 (ER241G (32)) and unable to bind c-Jun in vitro (not shown) was fused to the transcriptional activator VP16 and used in the same experiment. This ERα mutant, which was mostly nuclear in the presence of estradiol (not shown), was totally inefficient in increasing GAL4-c-Jun transcriptional activity. The association between ERα and c-Jun was further investigated by coimmunoprecipitation. COS cells were cotransfected with c-Jun and ERα expression vectors. Proteins associated with ERα were first precipitated with monoclonal antibodies directed against the A/B domain of the receptor and subsequently analyzed by immunoblotting with c-Jun-specific antibodies. As shown in Fig. 7B, c-Jun protein was detected in immunoprecipitates from cells transfected with the wild-type ERα expression vector (HEGO) and cultivated in the presence but not in the absence of estradiol. The same experiment was also performed using ER241G
were subjected to immunoprecipitation (IP) with mouse monoclonal anti-ERα and were then incubated with vehicle (C) and VP16 activating domain (VP16) alone or in combination as indicated. Luciferase activities calculated from triplicate wells from one experiment were then subjected to SDS-PAGE and fluorography as described under “Experimental Procedures.” Ten percent inputs of radiolabeled proteins used in the assays are shown on the left.

FIG. 8. Multiprotein complex between ERα, c-Jun, and GRIP1 in vitro. GST-ER-(251–595) protein preloaded on glutathione-Sepharose beads was preincubated for 4 h with 10 μg of histidine-tagged c-Fos (c-Fos*) or c-Jun (c-Jun*) purified on nickel-chelating resin as described under “Experimental Procedures.” After extensive washes, the fusion protein was incubated overnight with in vitro translated radiolabeled c-Jun (A) or GRIP1 (B) (300,000 cpm per sample) in the presence of estradiol (E2, 1 μM), ICI 164,384 (ICI, 1 μM), 4-hydroxytamoxifen (OHT, 1 μM), or vehicle (C). Proteins were then eluted and subjected to SDS-PAGE and fluorography as described under “Experimental Procedures.” Ten percent inputs of radiolabeled proteins used in the assays instead of HEGO. Although expression level of the two proteins was comparable, no immunoprecipitation of c-Jun was detected with the mutant protein. These results altogether demonstrate that ERα and c-Jun could interact in mammalian cells in a ligand-dependent manner.

Tripartite Complex between ERα, GRIP1, and c-Jun—ERα mutants unable to bind coactivators drastically decrease estradiol regulation of AP-1-mediated transcription and overexpression of the coactivator GRIP1 (43) enhanced the estradiol effect on AP-1 activity (29 and not shown). Moreover, the closely related p160 protein SRC-1 was reported to interact with both c-Jun and c-Fos in vitro (27). We therefore analyzed whether GRIP1 could participate in a multiprotein complex containing ERα and c-Jun. Binding of GRIP1 on pre-formed ER-c-Jun complexes was first tested in vitro, in GST pull-down assays. GST-ER-(251–595) preloaded on glutathione-coupled beads was preincubated with an excess of purified unlabeled c-Jun protein and unlabeled c-Fos, which does not bind to ERα, was used as a control. After extensive washes, beads were incubated with in vitro, 35S-labeled c-Jun or GRIP1 in the absence or the presence of ERα ligands (Fig. 8). Preincubation with c-Jun drastically decreased the consecutive interaction of ERα with labeled c-Jun demonstrating that most GST-ER-(251–595) molecules were bound to unlabeled c-Jun in these experimental conditions (Fig. 8A). In contrast, 35S-labeled GRIP1 efficiently interacted with the bead-bound ER-c-Jun complexes in a ligand-dependent manner (Fig. 8B). GRIP1 binding, which was increased by the presence of c-Jun in control conditions, in agreement with a direct interaction of GRIP1 with c-Jun, was further enhanced by estradiol addition whereas antiestrogens had no effect.

The direct interaction between GRIP1 and c-Jun was confirmed in intact cells. c-Jun or c-Fos overexpression increased

(a-c-Jun) as described under “Experimental Procedures.” As a control, 5% cell extracts used in immunoprecipitations were analyzed by Western blotting to monitor the amounts of ERα and c-Jun expressed in transfected cells.
luciferase activity driven by GRIP1 fused to the GAL4 DBD (GAL4-GRIP1) in MCF7 cells cotransfected by a GAL4-responsive luciferase gene reporter (Fig. 9A). The same experiment was then performed in the absence or presence of the hybrid protein VP16-ERα. As shown in Fig. 9A, and as expected, an enhancement of GAL4-driven luciferase activity was measured when GAL4-GRIP1 and VP16-ERα alone were coexpressed in estradiol-stimulated cells. Note that estradiol had no effect in the absence of VP16-ERα indicating that endogenous ERα concentration was likely negligible compared with that of overexpressed proteins. The addition of VP16-ERα together with GAL4-GRIP1 and c-Jun or c-Fos, did not significantly modify reporter gene transcription, in the absence of hormone. However, it had a synergistic effect in estradiol-treated cells in the presence of c-Jun. In contrast, in the same experimental conditions, an additive rather than a synergistic effect was observed when c-Fos was used instead of c-Jun. As we had shown that ERα interacted with c-Jun but not with c-Fos (Fig. 4), these results suggested that binding of ERα to c-Jun was important for the synergy. To try to confirm this hypothesis, VP16-ER241G mutant, deleted of the ERα part interacting with c-Jun, was therefore used in the same experiment. As shown in Fig. 9B, in the presence of GAL4-GRIP1 alone, VP16-ER241G increased reporter gene transcription as efficiently as the VP16 fusion protein containing wild-type ERα. However, contrary to the results obtained with VP16-ERα, no synergistic effect was detected on luciferase activity induced by GAL4-GRIP1 and c-Jun with VP16-ER241G, thus demonstrating the role of the ERα-c-Jun interaction in the observed phenomenon.

The ERα Hinge Domain Contributes to the Regulation of AP-1 Activity—We further questioned whether the physical interaction between ERα and c-Jun actually took part in estradiol regulation of AP-1-dependent transcription. On a first approach, the contribution of the ERα hinge domain on AP-1-directed transcription was tested in MCF7 cells transfected with the (AP-1)-4-TK-CAT reporter plasmid and increasing concentrations of the ERα mutant expression vector ER241G. As shown in Fig. 10A, overexpression of the hinge deleted mutant had no significant effect on AP-1 activity compared with the transfection of HE6 and HE19 in a same experiment (Fig. 1). We then constructed an ERα mutant encompassing the interaction domain with c-Jun as determined by in vitro protein-protein assays. If the protein-protein interaction was important in vivo, this truncated ERα, by competing with the endogenous receptor for binding to c-Jun, should act as a dominant negative mutant on AP-1 activity. Increasing concentrations of pCI-ERα(-249–306) were therefore transfected in MCF7 cells with the (AP-1)-4-TK-CAT reporter plasmid (Fig. 10B). In the absence of estradiol, ERα(-249–306) overexpression had no significant effect on basal AP-1 activity. However, ERα(-249–306) inhibited the estradiol effect on AP-1-mediated transcription. Estradiol induction of CAT activity decreased by more than 2-fold with the highest amount of pCI-ERα(-249–306). In all experiments and irrespective of the amount of pCI-ERα(-249–306) used, total inhibition of the estradiol effect was, however, never achieved. To determine whether ERα(-249–306) overexpression specifically inhibited estradiol-induced AP-1 activity, the effect of increasing ERα(-249–306) expression was tested in parallel in cells cotransfected by an ERE-containing reporter plasmid. Neither basal transcription nor estradiol induction of the ERE-β-globin-luciferase construct was significantly altered by ERα(-249–306) overexpression. These results altogether suggested that physical interaction between activated ERα and c-Jun participated in estradiol regulation of AP-1 responses.

DISCUSSION

Previous transfection experiments using ERα mutants demonstrated that ERα could modulate AP-1 responses without binding to DNA, therefore indicating that cross-talk between the two transcription factors resulted from protein-protein interactions (14, 17, 18). However, involvement of a direct interaction between ERα and AP-1 complexes in this regulation is still debated (48).

We evaluated whether AP-1 family members could interact in vitro with ERα and showed that some of them do indeed bind to ERα. ERα efficiently bound to c-Jun and JunB but only weakly interacted with JunD. ERα did not directly bind to any Fos family members. ERα thus behaved like other nuclear receptors for which an interaction with c-Jun has been described (19–23). In most studies, no interaction between GR or retinoic acid receptor and c-Fos was detected in the absence of c-Jun (19, 21–23). Only the group of Tournay et al. (20) reported an interaction of GR with c-Fos, which was, however, less stable than with c-Jun. The C-terminal part of c-Jun containing
both the DBD and the leucine zipper was implicated in the association with ERα. However, c-Jun-ERα homodimers bound on TRE were still retained by ERα in vitro, demonstrating that the interaction did not prevent either dimerization or binding onto DNA (Fig. 5). We dissected ERα to determine the region of interaction with c-Jun. In contrast with the findings of Webb et al. (17), which showed that a GST fusion protein harboring the N-terminal part of ERα (residues 1–185) bound to c-Jun, no or only a very weak interaction could be detected with this ERα domain (Figs. 1 and 2). In fact, our data demonstrated that ERα amino acids 259–302 located in the hinge D domain were sufficient for binding to c-Jun. The fact that neither ERα residues 1–282 (Fig. 1C) nor residues 283–330 (Fig. 2B) hybridized with c-Jun in GST pull-down assays also demonstrated that an important motif for the in vitro interaction was localized around amino acid 282. This region belongs to one of the less conserved domain of nuclear receptors, which might suggest that different regions are implicated in interactions between other receptors and c-Jun.

In agreement with the in vitro studies, ERα truncation mutant HE19 (amino acids 179–595) functionally interacted with AP-1 whereas HE15 (amino acids 1–282) did not. Although deletion mutant HE11 harbors the 259–302 ERα region, it repeatedly bound to c-Jun with a lower efficiency than wild-type ERα or mutant HE19. This may suggest that residues present in the DBD directly participate in the protein-protein interaction, as already suggested for other nuclear receptors. However, this is not consistent with results obtained with a series of truncated ERα GST fusion proteins (Fig. 2B). Conversely, deletion of the DBD could induce conformational changes in the hinge region, leading to a reduced affinity for c-Jun. It is worth mentioning that HE11 has been reported to increase (14, 17) or to have no effect (14, 16, 17) on AP-1 activity in different cellular or promoter contexts in which ERα was a potent activator. In the case of the ovalbumin promoter (14), mutant HE11 coactivated when cotransfected with c-Fos but not c-Jun, which may suggest that the Jun partner in AP-1 complexes could modulate the strength of the interaction with ERα. Further experiments are, however, needed to definitively answer this question.

In addition to the convergent in vitro evidences, we demonstrated using a mammalian two-hybrid system or performing coimmunoprecipitation assays, that c-Jun and ERα form a protein complex in intact cells. Direct interaction between ERα and c-Jun appeared crucial in the complex formation, because it was not observed when the ER241G mutant (Fig. 7), which is unable to bind c-Jun and inefficient in regulating AP-1 activity (29 and this study) could bind preformed ERα-GST fusion proteins (Fig. 2B). Moreover, the dominant negative effect of ERα-(249–306) (an ERα truncation mutant encompassing the c-Jun-ERα interaction region) on estradiol regulation of AP-1-dependent transcription is further evidence in favor of a direct interaction between the two proteins within cells, strongly suggesting that this physical interaction actually participated in estradiol-induced AP-1 activity. However, total inhibition of ERα-mediated regulation of AP-1 activity was never obtained. This observation and the low amplitude of the effect in the two-hybrid system (Fig. 7A) suggested that one or more additional factors could also take part in this regulation and stabilize c-Jun-ERα complexes.

We show that an additional partner, i.e. the nuclear receptor coactivator GRIP1, which increased estradiol-regulated AP-1 activity (29 and this study) could bind preformed ERα-c-Jun complexes (Fig. 8). Moreover, in a modified two-hybrid system, c-Jun and ERα had a synergistic effect on GAL4-GRIP1-driven transcription (Fig. 9). Synergy was not observed when c-Fos was present instead of c-Jun or when an ERα mutant unable to bind c-Jun was used, thus enlightening the crucial role of the ERα-c-Jun interaction in the tripartite complex formation. Therefore, our results altogether indicate that ERα does not only link the pre-existing Jun-coactivator complexes via contacts with p160s (48) but could stabilize the c-Jun-GRIP1 interaction through binding to the coactivator and c-Jun. Interestingly, similar stabilization of a protein-protein complex by a third factor has recently been described (49) concerning the progesterone receptor-SRC-1 complex and JAB1, a c-Jun coactivator. JAB1 potentiates the transactivation properties of most receptors, among them ERα (49), reflecting the high complexity of the cross-talk between ERα and c-Jun. Moreover, it has been suggested that stabilization by CBP/p300 could mediate the observed cooperation between Myb and the b-Zip protein NF-M, which both bind directly to the same target DNA sequence (50), and also the positive cross-talk between thyroid
hormone and retinoic acid receptors and the bZIP protein p45/ NF-E2 (51). CBP/p300, which associates with c-Fos (52), c-Jun (26), and ERα and ERAP1/SRC-1 (28, 53) and cooperatively enhances AP-1-mediated transcription (27), may also participate in the multiprotein complex recruited by c-Jun.

Neither estradiol nor the estrogen antagonists 4-hydroxytamoxifen and IC1164,384 influenced the in vitro binding of ERα to Jun (Fig. 6). This was not the case in vivo: Mammalian two-hybrid experiments revealed the interaction in the presence of estradiol but not in steroid-stripped cells or after treatment with antiestrogens (Fig. 7A). Similar differences in hormone dependence in vivo and in vitro have been reported for interactions between nuclear receptors and some corepressors (54, 55) or coactivators (43, 49). It has been suggested that in vitro translated nuclear receptors could be in an active conformation, even in the absence of ligand (43). It is, however, tempting to speculate that the interaction between ERα and c-Jun is labile or weak in vivo in the absence of hormone, but enhanced by estradiol, which promotes the recruitment of nuclear receptor coactivators and further stabilizes the multiprotein complex. In addition, the fact that a coactivator is required for a stable interaction may explain the different efficiencies of ERα and ERβ in regulating AP-1 activity (56), whereas both proteins bound to c-Jun in vitro (data not shown). SRC-3, which belongs to the same coactivator family as GRIP1, was reported to differentially interact with the two receptors and enhance ERα- but not ERβ-stimulated gene transcription (57). Moreover, some LXXLL peptides were shown to selectively interact with both ERs (58).

In conclusion, our present study demonstrates that the ERα hinge domain binds to c-Jun in vitro. This interaction also occurs in intact cells and is likely to be involved in the regulation of AP-1-induced responses. Whereas direct ERα-c-Jun binding may not be sufficient by itself to trigger estradiol regulation of AP-1 activity, it could be crucial for the stability of a multiprotein complex containing c-Jun, ERα, and a nuclear receptor activator such as GRIP1.

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