Formation of an hER α –COUP-TFI complex enhances hER α AF-1 through Ser118 phosphorylation by MAPK

Raphaël Métivier^{1,2}, Frédérique A. Gay^{3,4}, Michael R. Hübner⁵, Gilles Flouriot¹, Gilles Salbert³, Frank Gannon⁵, Olivier Kah¹ and Farzad Pakdel^{1,6}

¹Equipe d'Endocrinologie Moléculaire de la Reproduction (EMR) and

³Equipe d'Information et Programmation Cellulaire (IPC), UMR CNRS 6026, Université de Rennes I, 35042 Rennes Cedex, France and

⁵EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

²Present address: EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

⁴Present address: Harvard Medical School, Department of Pathology, WAB 120, 200 Longwood Avenue, Boston, MA 02115, USA

⁶Corresponding author

e-mail: Farzad.Pakdel@univ-rennes1.fr

The enhancement of the human estrogen receptor α (hER α , NR3A1) activity by the orphan nuclear receptor COUP-TFI is found to depend on the establishment of a tight hER α –COUP-TFI complex. Formation of this complex seems to involve dynamic mechanisms different from those allowing hER α homodimerization. Although the hER α –COUP-TFI complex is present in all cells tested, the transcriptional cooperation between the two nuclear receptors is restricted to cell lines permissive to hER α activation function 1 (AF-1). In these cells, the physical interaction between COUP-TFI and hER α increases the affinity of hER α for ERK2/p42^{MAPK}, resulting in an enhanced phosphorylation state of the hER α Ser118. hER α thus acquires a strengthened AF-1 activity due to its hyperphosphorylation. These data indicate an alternative interaction process between nuclear receptors and demonstrate a novel protein intercommunication pathway that modulates hER α AF-1.

Keywords: AF-1/ERK2/estrogen receptor/COUP-TFI/physical interaction

Introduction

Estrogens (estradiol, E2) are small lipophilic steroid hormones, predominantly synthesized by ovaries. Although commonly recognized as the pivotal hormone for female reproductive tract physiology, E2 is also involved in homeostasis of other tissues, and also performs important roles in males (Lombardi *et al.*, 2001; Nilsson *et al.*, 2001). Critical roles of E2 involve a control of the cell proliferation/differentiation balance (Feigelson and Henderson, 1996). Most of the physiological functions of E2 are exerted by specific nuclear receptors, termed estrogen receptors (ER α and ER β ; NR3A1 and NR3A2; Nuclear Receptor Committee, 1999). These proteins

involved in controlling endocrine and reproductive functions (Couse and Korach, 1999) are evolutionarily widely distributed from chordates to mammals, and belong to the transcription factor superfamily of nuclear receptors (NRs; Laudet, 1997). This superfamily includes ligand-inducible transcription factors, such as the steroid, retinoid and thyroid hormone receptors, as well as orphan receptors for which no ligand has been identified so far (Giguere, 1999). ERs bind as dimers to DNA, on specific estrogen response elements (EREs; Parker, 1995). ERs activate transcription of target genes through an N-terminal activation function (AF-1) in the B domain, and a C-terminal AF-2 region (Parker, 1995; see Figure 1A). These AFs exhibit distinct properties, which depend on both cell and promoter contexts (Metzger *et al.*, 1992). AF-2-mediated activation requires ligand-induced conformational changes, which allow interaction with co-activators that possess histone acetyltransferase activity and multiprotein complexes that interact with the general transcription machinery (Freedman, 1999). Extracellular signals influence ER functions by modulating the receptor phosphorylation state (Weigel, 1996). ER actions are also influenced by other transcription factors (Gaub *et al.*, 1990; Salvatori *et al.*, 2000), and orphan receptors such as SF-1 (NR5A1; Le Dréan *et al.*, 1996).

COUP-TFs are orphan NRs that are highly expressed in the developing nervous system, where they exert crucial roles in specific cell fates (Qiu *et al.*, 1997). In addition, COUP-TFs are expressed in a wide variety of tissues such as liver, uterus and mammary gland, where they may regulate vital biological functions and correct organogenesis (Pereira *et al.*, 1995). Several of these tissues also express ERs, and cross-talk between both NRs was suggested in the context of genes expressed in estrogen-sensitive tissues such as the uterus. In agreement with a role for COUP-TFs as transcriptional repressors (Cooney *et al.*, 1993), these orphan receptors were shown to repress the estrogen-regulated expression of the oxytocin and hepatocyte growth factor (HGF) genes (Jiang *et al.*, 1997; Chu *et al.*, 1998). On the other hand, a positive transcriptional effect of COUP-TFI on ER α activity was demonstrated for the first time on the rainbow trout ER α (rtER α) promoter (Lazennec *et al.*, 1997). The aim of this current study was to investigate the mechanisms underlying the positive action of COUP-TFI on ER α activity. First, we extend this positive interplay to other promoters and to the human ER α (hER α). Then, we describe a new pathway where the formation of a tight ER α –COUP-TFI intermediate complex leads to an increased recruitment of the ERK2/p42^{MAPK}, phosphorylation of the hER α on Ser118 and enhancement of its transcriptional activity.

Fig. 1. Physical and transcriptional interrelationships between hERα and COUP-TFI. **(A)** Schematic illustration of the hERα sequence: domains and associated functions. **(B)** Transfection experiments in CHO-K1 cells were performed using the 0.2 basic, ERE-TK or chicken vitellogenin (Vg) reporters, pCH110 internal control and 25 ng of each expression vector (pCMV5/hERα and pCDNA/COUP-TFI). Cells were treated with ethanol (EtOH) or 10^{-8} M estradiol (E2). Values are shown as the fold induction of normalized luciferase reporter activity (mean $\pm$ SEM). **(C)** Co-immunoprecipitation of the endogenous hERα-COUP-TFI complex in MCF-7 cells using anti-COUP-TFI-specific antibody. A goat pre-immune serum (PreI) was the control. The presence of each component of the complex was checked by western blots. **(D)** GST pull-down experiments performed using 250 ng of the GST-COUP-TFI 57-423 fusion protein or GST as a control, with *in vitro* labeled hERα. Input is 40% of the labeled protein used in the assay.

Results

Transcriptional cooperation and physical interaction between hERα and COUP-TFI

Although previously described as a repressive factor, COUP-TFI enhances the hERα activity on the rERα promoter gene, an ERE-TK synthetic promoter, the chicken vitellogenin (Vg) promoter in CHO-K1 cells (Figure 1B) and, hence, presumably on other target genes *in vivo*. One way that COUP-TFI could influence hERα

activity is by a direct interaction between the two proteins, the resulting complex exhibiting properties that differ from those of each NR. The occurrence of an interaction between both endogenous proteins was examined in MCF-7 cells that express these NRs (Figure 1C). A co-immunoprecipitation experiment performed with a specific antibody directed against COUP-TFI revealed that endogenous hERα and COUP-TFI are found in the same protein complex, with or without E2 treatment. To demonstrate a physical interaction between the two NRs, we used a GST fusion protein with the longest COUP-TFI sequence possible [residues 57-423, encompassing half of the N-terminal region, the DNA-binding domain (DBD) and the ligand-binding domain (LBD)]. Subsequent *in vitro* GST pull-down assays confirm the occurrence of a ligand-independent interaction between COUP-TFI and *in vitro* labeled hERα (Figure 1D).

Molecular dissection of the physical interaction between hERα and COUP-TFI

To map the domains of both NRs involved in their interaction, we performed two-hybrid assays, whose accuracy was tested by fusing the full-length COUP-TFI and hERα to the activation domain (AD) or DBD of the yeast transcriptional factor Gal4. Subsequent assays shown in Figure 2A confirm the ligand independence of the interaction between the two NRs. A dominant-negative form of COUP-TFI (Adam *et al.*, 2000), harboring a C141S point mutation within its DBD, is unable to interact with hERα (Figure 2A). On the contrary, this mutant maintains its ability to interact with wild-type COUP-TFI. This indicates that the mechanisms underlying the interaction between COUP-TFI and hERα are probably different from those involved in COUP-TFI homodimerization.

To obtain further information on the specific mechanisms of interaction between the two NRs, we identified their contacting surfaces. The different domains of COUP-TFI were fused to the Gal4AD, while the C-F (CF) or D-F (DF) domains of hERα fused to the Gal4DBD were used as bait proteins (Figure 2B, left). These hERα proteins exhibit no intrinsic transcriptional activity that might have interfered in these assays. This property allowed us to show the ligand independence of all detected interactions (data not shown). The hERα DBD + LBD bait (hERα CF/Gal4DBD) associates with COUP-TFI N-terminal (amino acids 1-57), C-terminal (amino acids 153-423) and DBD (amino acids 57-153) regions. Performing the two-hybrid assays with the hERα LBD alone (hERα DF/Gal4DBD) showed that it associates with COUP-TFI N- and C-terminal regions (Figure 2B, right). The absence of the DBD in this hERα DF/Gal4DBD bait and its failure to bind the COUP-TFI DBD demonstrate that the DBDs of the two NRs form direct contacts (Figure 2B, right). We next wanted to see if the hERα N-terminal domain was able to contact COUP-TFI. When fused to the Gal4DBD, the hERα N-terminal domain activates transcription in yeast through its AF-1 (Métivier *et al.*, 2001). Since such a protein is not informative in two-hybrid assays, we therefore conducted inverse assays. hERα AB domains were fused to the Gal4AD, and COUP-TFI domains to the Gal4DBD. These assays demonstrate that the hERα N-terminal region associates

Fig. 2. The hER α -COUP-TFI complex is required for transcriptional cooperation but is destabilized by DNA. (A) Yeast two-hybrid assays using full-length hER α , wild-type and dominant-negative COUP-TFI (C141S point mutation) fused to the Gal4AD or Gal4DBD. Yeast transformants were grown in media including ethanol (EtOH) or 10^{-8} M estradiol (E2). Liquid β -galactosidase assays were then performed. (B and C) The different domains of COUP-TFI were fused to the Gal4AD or Gal4DBD for two-hybrid assays, which were performed using as bait the complementary fusion proteins indicated at the top of each panel. Results are expressed as the mean $\pm$ SD from three independent experiments. (D) Ability of COUP-TFI mutants to enhance hER α activity on the ERE-TK reporter gene, in CHO-K1 cells treated with EtOH or 10^{-8} E2. Results (mean $\pm$ SEM) are expressed as the reporter fold stimulation in the presence of E2. (E) Pull-down experiments were performed with *in vitro* labeled hER α and COUP-TFI using the GST-COUP-TFI 57-423 or GST-hER α DF proteins. During the incubation, increasing amounts of DR-1, ERE (25–200 ng) and AP-1 as non-specific control (50–200 ng) oligonucleotides were added.

with the C-terminal part of COUP-TFI (Figure 2C). In conclusion, three surfaces of interaction are identified within the two NRs: one mediated by their DBD, one by their C-terminal region and one involving their N- and C-terminal regions. *In vitro* pull-down assays confirmed the yeast two-hybrid data (see below), indicating that the interaction surfaces used are specific.

Correlation between physical interaction and transcriptional cooperation between hER α and COUP-TFI

We next identified the domains of COUP-TFI involved in the enhancement of hER α transcriptional activity to look for a relationship between physical interaction and transcriptional cooperation. This direct relationship is suggested first by the fact that the COUP-TFI C141S

mutant is not able to enhance hER α activity (Figure 2D). The partial or total deletion of the COUP-TFI N-terminal region (57–423 and 87–423 constructs) does not affect the increase in hER α activity (Figure 2D). In contrast, deleting the COUP-TFI C-terminal region (57–153 protein) reduces the transcriptional cooperation by 60%. This points to the involvement of the COUP-TFI DBD and LBD in the transcriptional cooperation with hER α . This is demonstrated further by the hER α activity enhancement by the COUP-TFI DBD alone (residues 57–153) with hER α . In the case of the COUP-TFI LBD, we had to fuse the COUP-TFI 153–423 region to the SV40 nuclear localization signal (NLS; Figure 2D) to obtain a correct localization of the protein, as revealed by green fluorescent protein (GFP; not shown). Expression of this GFP-NLS/COUP-TFI 153–423 protein increases hER α -mediated

transcription on the ERE-TK-Luc reporter by 2-fold. In conclusion, both an intact DBD and LBD are required for COUP-TFI to interact physically with hER α and to enhance its activity.

Binding to DNA response elements specific for both NRs destabilizes the hER α -COUP-TFI complex

The above results indicate that the transcriptional cooperation between hER α and COUP-TFI reflects their physical interaction. COUP-TFI can bind EREs (Klinge *et al.*, 1997). One can thus postulate that the hER α -COUP-TFI complex might possess a higher affinity for EREs, compared with hER α homodimers, hence explaining the transcriptional cooperation. Electrophoretic mobility shift assays (EMSAs) were performed using various types of response elements, but in all cases we failed to detect a complex other than each of the NRs homodimers on DNA (data not shown). On the other hand, adding DNA-binding sites for COUP-TFI (DR-1) or ER (ERE), but not unspecific (AP1) sequence, destabilizes the hER α -COUP-TFI complex. As a control, the COUP-TFI-COUP-TFI 57–423 heterodimer remains unaffected by these DNA sequences (Figure 2E). These results suggest that the mechanisms that allow COUP-TFI to enhance hER α activity occur before hER α interacts with its target genes.

Physical interaction with COUP-TFI increases hER α AF-1 activity and phosphorylation of the hER α N-terminal region

In yeast cells, the hER α N-terminal AF-1 is required for the enhancement of hER α transcriptional activity by COUP-TFI (Petit *et al.*, 1999). Moreover, deleting the hER α N-terminal region (hER α CF) abrogates the transcriptional cooperation between the two NRs in CHO-K1 cells (Figure 3A). We thus hypothesized that the actions of COUP-TFI might target specifically hER α AF-1. To test this assumption, the effect of COUP-TFI on hER α activity was checked in different cell lines, as it has been shown that the relative involvement of AF-1 and AF-2 in hER α transcriptional activity depends upon the cell type (Metzger *et al.*, 1992). Cell contexts can thus be defined as AF-1 or AF-2 permissive, depending upon which AF is involved principally in hER α activity. The relative sensitivity of different cell lines to AF-1 and AF-2 was evaluated by comparing the transcriptional activity of full-length hER α with that of a mutant lacking AF-1 (hER α CF). A similar transcriptional activity of the two proteins would indicate AF-2-permissive cell contexts; no activity of the mutant would indicate AF-1-permissive contexts; and a reduced activity would indicate a mixed context permissive to both AF-1 and AF-2. Based on this classification, data obtained demonstrate that COUP-TFI increases hER α activity in AF-1-permissive cells (mixed and exclusive; Figure 3B). This was not due to cell-specific differences in COUP-TFI or hER α expression, as detected by western blots (see below).

A cell-specific formation of the hER α -COUP-TFI complex might be one basis for the cell-dependent action of COUP-TFI on hER α activity. However, co-immunoprecipitation experiments detect a protein complex containing the two NRs even in cells restricted to AF-2

Fig. 3. Cell-specific enhancement of hER α AF-1 by COUP-TFI. (A) Transfection of CHO-K1 cells with 0.2 basic reporter, internal control pCH110 and expression plasmids for COUP-TFI, full-length or N-terminally truncated hER α (hER α CF). (B) Nine cell lines exhibiting a differential sensitivity to ER α AFs were transfected with vectors encoding hER α alone or with COUP-TFI. Cells were treated with ethanol (EtOH) or 10^{-8} M estradiol (E2). Values are the fold induction of normalized luciferase reporter activity (mean $\pm$ SEM).

activation (see below). Thus, physical interactions alone do not account for the effect of COUP-TFI on hER α activity in certain cells.

AF-1-mediated transcription stimulation is strongly modulated by cell-specific phosphorylation events (Ali *et al.*, 1993; Le Goff *et al.*, 1994). We therefore postulated that phosphorylation could mediate the cell-specific effect of COUP-TFI on hER α AF-1. To obtain information about a direct impact of COUP-TFI interaction on the hER α phosphorylation state, we developed an *in vitro* assay in which proteins are first subjected to a GST pull-down procedure. This step is followed by *in vitro* phosphorylation using whole cell extracts (WCEs) as a donor of kinases. As shown above, two regions of COUP-TFI allow a transcriptional cooperation with hER α : the LBD (amino acids 153–423) and the DBD (amino acids 57–153). These regions were used in the phosphorylation assays, in conjunction with either GST-hER α B or GST-hER α BD domain fusion proteins, with which they can interact (Figure 4A). Data shown in Figure 4B illustrate a typical result obtained after *in vitro* phosphorylation of the protein complexes. We have shown previously that one of the consequences of the direct interaction between the hER α N- and C-terminal domains is an increase in hER α B domain phosphorylation (Métivier *et al.*, 2001). This provided a positive control for COUP-TFI actions. The quantification of these assays performed with several

Fig. 4. Cell-specific enhancement of hER α phosphorylation upon interaction with COUP-TFI. (A) GST pull-down experiments performed with 250 ng of the GST-hER α B or 150 ng of the GST-hER α BD with *in vitro* labeled COUP-TFI and hER α regions. Controls were made using 250 ng of GST alone, and inputs (I) are 20% of the labeled protein used. (B) *In vitro* phosphorylation assays were performed using similar amounts of the fusion proteins as in (A), with retained D-F (DF; 153-423) or C-D (DBD; 57-153) domains of hER α or COUP-TFI, in the presence of 5×10^{-6} M estradiol (E2) or ethanol (EtOH). Controls were made using an *in vitro* translation performed with empty pSG5 vector (Unpg Lys). Phosphorylation was ensured using 10 μ Ci of [γ - 32 P]ATP and 10 or 20 μ g of CHO-K1 and HepG2 or HeLa WCEs, respectively. (C and D) Phosphoimager quantification of the *in vitro* phosphorylation assays performed using the same conditions as above. The values shown, normalized by the basal 32 P incorporation detected with Unpg Lys, were reproducible ($\pm 1\%$).

WCEs as donors of kinases are shown in Figure 4C and D, and demonstrate that (i) COUP-TFI LBD and DBD enhance the phosphorylation of the hER α B or BD domains by 2- to 3.5-fold in a ligand-independent manner; and (ii) this increase occurs with WCEs from CHO-K1 and HepG2 cells (AF-1-permissive) but not from HeLa cells (AF-2-permissive). These actions of COUP-TFI on hER α B domain phosphorylation are specific, since (i) as previously reported, the interaction of hER α LBD with its own B domain requires E2 to affect AF-1 phosphorylation (Métivier *et al.*, 2001); and (ii) hER α DBD interaction with the hER α BD domains does not reproduce the phosphorylation enhancement observed with COUP-TFI DBD.

COUP-TFI targets hER α Ser118 phosphorylation by MAPK

To identify the amino acids within the hER α B domain targeted by this phosphorylation process, we conducted transfection experiments with hER α mutated at the known phosphorylated serines (Ali *et al.*, 1993; Arnold *et al.*,

1994; Le Goff *et al.*, 1994; Rogatsky *et al.*, 1999). COUP-TFI enhanced the activity of the receptors mutated at residues S167, S154, S104 and S106, but not that of S118A. Further, placing a negative charge at the 118 position (S118E) abrogated COUP-TFI action, but also enhanced the activity of the receptor to the same extent that COUP-TFI does on wild-type hER α (Figure 5A). These differences in transactivation are not due to an altered interaction of COUP-TFI with the two S118 mutants (Figure 5B). COUP-TFI is also unable to enhance the phosphorylation state of hER α domains mutated at residue S118 *in vitro* (Figure 5C). These results suggest that the basis of the transcriptional cooperation mechanism between hER α and COUP-TFI is the provision of a negative charge at the hER α S118 position, which is an essential residue for the hER α AF-1 activity.

S118 is a target of the mitogen-activated protein kinase (MAPK) pathway (Kato *et al.*, 1995). We thus performed transfection experiments in the presence or absence of an inhibitor of this pathway, PD098059. Inhibiting the MAPK pathway markedly decreases COUP-TFI DBD-

Fig. 5. COUP-TFI interaction with hERα targets Ser118 phosphorylation. (A) CHO-K1 cells were transfected with the ERE-TK reporter, pCH110 and plasmids expressing hERα point mutated on each of the residues indicated. (B) GST pull-down assays using *in vitro* labeled wild-type or point-mutated hERα with 250 ng of each of the COUP-TFI fusion proteins or GST alone. Inputs are 20% of the labeled protein used. (C) Coupled GST pull-down/phosphorylation assays performed with GST fused to point-mutated hERα domains. Experiments used 10 μg of CHO-K1 WCE and *in vitro* translated hERα or COUP-TFI domains. Quantification of the radioactivity incorporation is shown as in Figure 4.

and LBD-mediated enhancement of hERα activity (Figure 6A). Western blots using an antibody specifically targeting the phosphorylated S118 were conducted to test the *in vivo* relevance of the phosphorylation processes (Figure 6B). In transfected HepG2 cells (AF-1-permissive), the amounts of hERα phosphorylated at S118 are increased after E2 treatment, as expected (Kato *et al.*, 1995). Co-expressing COUP-TFI also induces this process, but in a ligand-independent manner. This COUP-TFI-mediated effect needs the formation of the hERα–COUP-TFI complex. Indeed, the COUP-TFI C141S mutant, which cannot interact with hERα (Figure 2), is unable to increase *in vivo* the S118 phosphorylation above the level found for ER in the absence of COUP-TFI. Treatment with PD098059 abolishes this COUP-TFI-mediated effect, as does S118 point mutation. In AF-2-permissive HeLa cells, the hERα S118 is not phosphorylated, and COUP-TFI has no effect. The results obtained using the PD098059 inhibitor indicated that the MAPKs are involved, either directly or indirectly, in the COUP-TFI-mediated processes that affect hERα phosphorylation and activity. This was analyzed further using a purified constitutively active ERK2/p42^{MAPK} (Sigma) instead of WCE in the pull-down/*in vitro* phosphorylation assay. Phosphorylation of the hERα N-terminal region by ERK2 is not enhanced after interaction with any hERα domains in the presence of estradiol (Figure 6C). In contrast, COUP-TFI 153–423 and 57–153 regions increase the phosphorylation state of the hERα B and BD domains by 6- or 14-fold, respectively, in a ligand-independent manner. Together with previous results indicating a requirement for an AF-1-permissive cell context, these data demonstrate that a phosphorylation process involving MAPK pathways is the key for the cell-specific enhancement of hERα transcriptional activity by COUP-TFI.

hERα hyperphosphorylation induced by COUP-TFI involves an enhanced recruitment of MAPK

The connection between the enhanced phosphorylation state and an increased targeting of ERK2 to hERα through interaction with COUP-TFI subsequently was tested. As illustrated in Figure 7A, a His-tagged ERK2 protein binds to hERα and COUP-TFI in a ligand-independent manner. This interaction requires the *in vitro* phosphorylation of the bacterially expressed ERK2 by CHO-K1 WCE. In contrast, phosphorylation by HeLa WCE allows a 10- to 20-fold less efficient association of ERK2 with both NRs, although ERK2 has been phosphorylated to the same extent by both WCEs (Figure 7A, right). Co-immunoprecipitation experiments (Figure 7B) demonstrate this enhanced recruitment *in vivo*. COUP-TFI does not enhance the level of phosphorylated ERK2, as revealed by western blot using a specific antibody against the phosphorylated form of ERK2. COUP-TFI increases the amounts of activated ERK2 present in the hERα immunoprecipitated complex from HepG2 but not HeLa cells. This requires the physical interaction between the two NRs, since the COUP-TFI C141S mutant does not allow this increase in ERK2 recruitment. Altogether, these data demonstrate that the molecular basis of the positive hERα–COUP-TFI interplay is an enhanced recruitment of ERK2.

Fig. 6. *In vivo* enhancement of hER α activity by COUP-TFI involves the MAPK pathway. (A) hER α was expressed alone or in combination with COUP-TFI constructs in CHO-K1 cells, which were treated subsequently with 10 μ M of the ERK inhibitor PD098059. Normalized luciferase activities are shown as the mean $\pm$ SEM. (B) WCEs from HepG2 or HeLa transfected cells were subjected to western blots using the antibodies indicated on the left. (C) A 25 ng aliquot of purified MAPK was used in the GST pull-down/*in vitro* phosphorylation assays using 250 or 150 ng of the GST-hER α B or BD fusion proteins, respectively, with either the DF or CD domains of hER α or COUP-TFI. 32 P incorporation was measured and expressed as in Figure 4.

Discussion

Although COUP-TFI was depicted originally as a repressor for other NR functions, there is growing

Fig. 7. Interaction with COUP-TFI enhances ERK2/p42^{MAPK} recruitment by hER α . **(A)** *In vitro* pull-down experiments were performed using 2 μ l of the *in vitro* labeled proteins indicated and 500 ng of the His-tagged ERK2 previously phosphorylated by 10 or 20 μ g of CHO-K1 or HeLa WCE, respectively. Controls (C) were run using ion resin previously incubated with bacterial extract expressing no fusion proteins. Inputs (I) are 25% of the proteins used. A radioactive *in vitro* phosphorylation assay is shown on the right, revealing similar activation of ERK2 by the two WCEs. **(B)** The H222 antibody was used to co-immunoprecipitate the hER α -COUP-TFI-ERK2 complex in cells. A rat pre-immune serum (PreI) was used as a control. The presence of each component of the complex and activated ERK2 was checked before and after precipitation.

evidence of cross-talk between COUP-TFI and hER α , some of which results in an increased hER α activity (Ktistaki and Talianidis, 1997; Lazennec *et al.*, 1997). This study was therefore initiated to elucidate the molecular basis underlying the positive transcriptional interplay between COUP-TFI and hER α .

Regions of COUP-TFI required (i) to physically associate with hER α and (ii) to enhance the hER α activity are multiple but identical (DBD and C-terminal regions). This implies that the hER α –COUP-TFI interaction is required for the transcriptional cooperation. Within this ‘heteromer’, the DBD–DBD surface is of importance since the

COUP-TFI C141S mutant neither provides a proper hER α interaction surface, nor permits the transcriptional interplay. Moreover, the isolated COUP-TFI DBD is sufficient to enhance hER α activity. The COUP-TFI LBD strengthens the contacts with hER α and the transcriptional cooperation. As in the case of some nuclear receptors or hER α homodimers (He *et al.*, 1999; Métivier *et al.*, 2001), the N-terminal region of hER α interacts with COUP-TFI, and vice versa. These observations strongly support a tight association between COUP-TFI and hER α . This confirms a conclusion previously reached by Klinge (1999), using other approaches. It is recalled that in the cell, the two proteins are, of course, entire. Given that the C141S mutation disrupts the other interaction surfaces, we propose that the formation of the complex would be directed first by the DBD–DBD interface. The other interaction surfaces, which could each act separately, stabilize the complex. This sequence of events is of interest when compared with the way in which NR homodimers are formed on DNA: there, dimerization is initiated by the LBD–LBD interface, and stabilized further by allosterical interactions due to the DNA and to the use of the DBD–DBD interface (Lefstin and Yamamoto, 1998). When considered in conjunction with these results, the fact that specific DNA-binding sequences for both NRs dissociate the hER α –COUP-TFI complex is likely to imply that the formation of the hER α –COUP-TFI complex occurs in solution. Actions leading to the transcriptional cooperation between the two NRs are thus likely to occur downstream of the necessary formation of this ‘heteromer’.

Of significance is the finding that only AF-1-permissive cells (i.e. cells in which hER α activity is driven mainly by this AF) allow the increase of hER α activity by COUP-TFI. This cell dependency gave the first clue about a specific mechanism that allows COUP-TFI to enhance hER α AF-1 activity. Co-immunoprecipitation assays demonstrated that the formation of the hER α –COUP-TFI complex alone is not this key process. Phosphorylation pathways are known to influence AF-1 (Ali *et al.*, 1993; Le Goff *et al.*, 1994). We thus hypothesized a role for the phosphorylation pathways, which allow COUP-TFI to target hER α AF-1. Mutation of the phosphorylation target site S118 impairs the positive effect of COUP-TFI on hER α activity. This COUP-TFI-mediated enhancement of hER α S118 phosphorylation occurs only in hER α AF-1-permissive cells, and requires COUP-TFI LBD and DBD. MAPK pathways can lead to hER α S118 phosphorylation (Kato *et al.*, 1995). We also show that ERK2/p42^{MAPK} is involved in the COUP-TFI-induced phosphorylation of the hER α S118. *In vivo* assays demonstrate that COUP-TFI enhances the interaction between hER α and ERK2 in AF-1- but not AF-2-permissive cells. Thus, the stabilized recruitment of MAPK within a complex involving hER α and COUP-TFI is the basis for the cell-specific effect of COUP-TFI on hER α AF-1.

COUP-TFI but not hER α enhances the S118 phosphorylation through DBD–DBD interactions. Moreover, ERK2 is not involved in the S118 phosphorylation that takes place following the hER α N-terminal region ligand-dependent association with the C-terminal part of the protein (Métivier *et al.*, 2001). This illustrates the

specificity of COUP-TFI in the hER α S118 modification. Both COUP-TFI DBD and LBD were able to enhance the phosphorylation of hER α S118. This suggests that the interactions between these two regions of COUP-TFI and hER α induce specific and identical conformational changes within the hER α N-terminal region. Such structural adaptations through dimerization and/or DNA binding were demonstrated for the hER α , glucocorticoid (GR, NR3C1) and progesterone receptors (Kumar *et al.*, 1999; Bain *et al.*, 2000; Greenfield *et al.*, 2001). Furthermore, limited proteolysis experiments evidenced some differences in the overall three-dimensional structure of the hER α –COUP-TFI complex, compared with a hER α homodimer (not shown).

The mechanism reported here is important to integrate the actions of E2 in the whole organism. As is well known, E2 exhibits pleiotropic and opposite effects depending upon the tissues, leading to a cell-specific modulation of the transcription of a repertoire of genes (Nilsson *et al.*, 2001). Cell-specific controls of the expression of the ER and its isoforms, due to a differential use of the multiple promoters of the hER α gene together with alternative splicing, are one of the elements that contribute to explaining these pleiotropic effects (Koř *et al.*, 2001). In addition, the ER activity in itself can be regulated in a cell-specific manner, for instance through the preferential use of AF-1 over AF-2, to activate the transcription of target genes. The cell-specific transcriptional interplay between ER and other NRs such as COUP-TFI is another way to diversify estrogenic responses. Indeed, the two NRs coexist in certain cell types, such as uterine epithelial cells, ovaries, neonatal reproductive tract, kidney, prostate and liver (Pereira *et al.*, 1995; Shigeta *et al.*, 1996; Qiu *et al.*, 1997; Chu *et al.*, 1998). The relative levels of COUP-TFI might be decisive in discriminating between the positive and negative actions of COUP-TFI on ER functions. These opposite actions are known to depend on two variables: promoter and cell context (Ktistaki and Talianidis, 1997; Klinge, 1999). The repressive actions of COUP-TFI are thought to be due to competition for either the occupancy of DNA-binding sites or for a common co-regulator (Cooney *et al.*, 1993). In the same cell, one way for COUP-TFI to discriminate between opposite actions on other NR activities relies on their relative affinity for a shared response element (Sugiyama *et al.*, 2000). We demonstrate here that the additional element of cell-dependent interplay with kinase pathways plays a role in orienting the balance between repression and activation of hER α activity by COUP-TFs.

Our results also give rise to another question: what are the mechanisms that restrict ERK2 interaction with COUP-TFI and hER α only to certain cells? A specific conformation or alteration within the hER α and COUP-TFI proteins in AF-2-permissive cells due to variations in post-translational modifications might prevent the recruitment of the kinase. Conversely, a specific conformation adopted by the hER α –COUP-TFI complex in AF-1-permissive cells would also be responsible for ERK2 recruitment. Similar amounts of activated ERK2 were present in the *in vitro* phosphorylation assays (Figures 4 and 5). This implies that in the WCEs from AF-2-permissive cells, other proteins prevent the recruitment of ERK2 to the hER α –COUP-TFI complex.

We observed in the *in vitro* assays that the profile of endogenous phosphorylated proteins was different for each WCE (Figure 4). Principally, two bands were specific to HepG2 WCEs: one at 90 kDa, presumably the pp90rsk (Joel *et al.*, 1998), and one at 140 kDa. Conversely, other proteins (85 and 100 kDa) were visualized specifically using HeLa WCEs. When the identity of these proteins is known, their potential role in the interrelationships between COUP-TFI and hER α might be tested. It might be plausible that the designation of cells as AF-1 or AF-2 permissive might be due to the expression of these proteins. Another explanation for the cell-specific recruitment of ERK2 could be that pathways leading to an activation of hER α by ERK2 are impaired in AF-2-permissive cells.

It should be noted that a similar phosphorylation-mediated mechanism was described recently, in which NR interactions with TF_{II}H were linked to targeting to the cdk7 kinase (Chen *et al.*, 2000). A prolonged phosphorylation state correlated with an increased activity of the STAT5 transcription factor was also observed after physical interaction with GR (Wyszomierski *et al.*, 1999). Modulation of a given NR phosphorylation state by physical interaction with another NR could then be a general process involved in the complex network of pathways orchestrated by these factors. What would be the functional consequence of these events? Upon phosphorylation in the absence of ligand, one could propose that hER α interaction with co-repressors might be reduced, making the receptor more accessible for co-activators. The phosphorylation of specific residues in PPAR (NR3C3), SF-1 and ER β modulates the recruitment of p160 co-activators (Shao *et al.*, 1998; Hammer *et al.*, 1999; Tremblay *et al.*, 1999). Phosphorylation of hER α S118 by MAPK enhances its physical interaction with the AF-1 co-activator, p68 RNA helicase (Endoh *et al.*, 1999). One can then propose that COUP-TFI modulates the interaction of p68 RNA helicase with hER α . COUP-TFI also interacts with the CBP-p300 complex (Bailey *et al.*, 1998), which is a co-activator for hER α AF-1 that is known to bind SRC-1 and the p68 RNA helicase (Yao *et al.*, 1996; Endoh *et al.*, 1999; Kobayashi *et al.*, 2000). Upon interaction with hER α , COUP-TFI might thus also recruit large protein complexes to modulate hER α actions.

In conclusion, this study of the interplay between COUP-TFI and hER α shows that these NRs are physically interacting, in a ligand-independent manner. This results in an enhancement of hER α transcriptional activity in AF-1-permissive cells, by a mechanism that leads to an increased binding of ERK2. These data add to our understanding of NRs and signal transduction cross-talk, which allow new mechanisms to be envisaged that contribute to an explanation of the pleiotropic actions of E2.

Materials and methods

Host strains

We used *Escherichia coli* DH5 α , *E. coli* BL21 (DE3) pLysS (Invitrogen Corp., Carlsbad, CA) or *E. coli* TG1 bacteria strains, and Y190 yeast strain (Clontech Laboratories, Inc., Palo Alto, CA). Two-hybrid clones were obtained, selected and analyzed in three independent experiments, as described previously (Métivier *et al.*, 2001).

Antibodies

The rabbit polyclonal anti-GFP, anti-Gal4DBD, anti-GAPDH, anti-p42/p44, H184 (directed against the N-terminal part of the hER α) and goat polyclonal anti-COUP-TFI were purchased from TEBU (Le-Perray en Yvelines, France); and the anti-phosphorylated ERK2 from NEB (9106; Frankfurt, Germany). The anti-S118P antibody was a gift from Dr S. Ali (Chen *et al.*, 2000), and the rat monoclonal H222 (directed against the C-terminal part of the hER α) was from Dr B.S. Katzenellenbogen.

Plasmids

Oligonucleotides (Eurogentec, Seraing, Belgium) had the following sequences: EREc, GATCCAGGTCACAGTGACCT; DR1, TCGAGGTCAGAGGTCACGA; and AP-1, TCGACGCTTGATGACTCAGCCGGAA. The *SmaI-SmaI*, *SmaI-EcoRI*, *SmaI-XmnI*, *XmnI-EcoRI* and *EcoRI-EcoRI* fragments of the pECE/COUP-TFI or pECE/COUP-TFI C141S vectors (Lazennec *et al.*, 1997) were cloned into pACT2 (Clontech) or pGEX2T (Pharmacia, Buckinghamshire, UK) vectors. COUP-TFI 84/423 and COUP-TFI 153-423/SV40NLS were obtained by PCR, and the latter was cloned in the pEGFP-C3 vector (Clontech). The Gal4DBD fusions with wild-type and deleted COUP-TFI were constructed by excising the corresponding inserts from pACT2 constructs, and ligation into pAS2-1 (Clontech) or pCDNA-3 (Invitrogen). The pCDNA/COUP-TFI and pCDNA/COUP-TFI C141S, and 0.2 basic, chicken Vg and ERE-TK reporter plasmids are described (Lazennec *et al.*, 1997; Flouriot *et al.*, 2000). The hER α /Gal4DBD, hER α /Gal4AD, hER α AB/Gal4DBD, hER α CF/Gal4DBD and hER α DF/Gal4DBD fusions were expressed by pAS2-1 or pACT2 constructs. hER α _{S118A} and hER α _{S118E} included in plasmid pSG5 were provided by Professor P. Chambon, while pCMV-5/hER α , pCMV-5/hER α _{S167A}, pCMV-5/hER α _{S154A} and pCMV-5/hER α _{S104A,S106A} are from Dr B.S. Katzenellenbogen. The GST-hER α B, GST-hER α BD and point mutant versions were expressed using pGEX2T constructs. pRSET/hERK2 was a gift from Dr A. Nebreda.

Cell culture

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Quentin Fallavier, France) supplemented with 5% fetal calf serum (FCS; Sigma) at 37°C and 5% CO₂. Transient transfections were performed as previously (Métivier *et al.*, 2001) in 6-well plates (HeLa, HepG2, MDA-MB231, PC3, TsuPR1 and DU145 cell lines) or 24-well plates (COS-7, P19 and CHO-K1). Cells were treated for 36 h in DMEM-F12/2.5% charcoal-stripped FCS media supplemented with 10⁻⁸ M E2 or ethanol. MCF-7 cells were only treated for 6 h. Treatment with 10 μ M of the inhibitor PD098059 (Sigma) or dimethylsulfoxide (DMSO) as a control was performed 4 h before harvesting the cells (Alessi *et al.*, 1995). Luciferase activities were normalized by β -galactosidase activity, and expressed as the mean $\pm$ SEM from reporter activity fold induction obtained in four independent transfections.

Protein cell extracts and analysis

Yeast WCEs were obtained as previously described (Petit *et al.*, 1999). Other WCEs were obtained by thawing-freezing cycles of cells in WCE buffer [20 mM HEPES pH 7.9, 400 mM KCl, 2 mM dithiothreitol (DTT) and 20% glycerol]. These conditions were adequate for a maximal ERK2 activation under basal conditions, as checked by western blotting. Analysis of protein expression was performed using the ECL kit (Amersham Pharmacia, Piscataway, NJ), with 50 μ g of yeast WCEs or 150 μ g of HepG2 or HeLa WCE precipitated by 30% (NH₄)₂SO₄. For immunoprecipitations, 800 μ g of WCE in LSB (50 mM Tris pH 7.9, 3 mM MgCl₂, 0.1 M KCl, 1 mM DTT, 0.1% NP-40 and 20% glycerol) were cleared for 2 h with 4 μ g of rat or goat pre-immune IgG (Sigma), and 50 μ l of a protein G-Sepharose beads slurry (Sigma). Supernatants were incubated with 2 μ g of H222 or anti-COUP-TFI antibody chemically cross-linked to 50 μ l of protein G-Sepharose beads for 6 h at 4°C. After three washes in LSB buffer, retained IgG-protein complexes were denatured before SDS-PAGE, and blotted on a PVDF membrane. Blots were incubated overnight with primary antibodies, washed and incubated for 1 h with corresponding horseradish peroxidase-conjugated antibodies (TEBU).

Pull-down and *in vitro* phosphorylation assays

In vitro translation of proteins using the TNT kit (Promega), ligand treatment, purification of proteins and GST pull-down assays were performed as described previously (Métivier *et al.*, 2001). His₆-ERK2 was purified on Probond beads (Invitrogen). After purification, the protein was phosphorylated by incubation with 10 or 20 μ g of CHO-K1 or HeLa

WCE, respectively, in phosphorylation buffer (PB; 40 mM Tris-HCl pH 7.4, 50 mM MgCl₂, 5 mM DTT, 250 µM ATP and phosphatase inhibitors: 1 µM okadaic acid and 200 µM orthovanadate). Similar ERK2 activation was checked by performing the *in vitro* phosphorylation with [γ -³²P]ATP and evaluation of the His₆-ERK2 protein labeling. After binding to *in vitro* labeled proteins, the beads were washed twice in HWB (20 mM Na₂HPO₄ pH 7.8, 300 mM NaCl, 0.04% Tween-20 and 10 µg/ml concentrations of the protease inhibitors leupeptin, pepstatin and aprotinin), and proteins were eluted in 0.5 ml of HEB (250 mM imidazole, 20 mM Na₂HPO₄ pH 6.0, 500 mM NaCl and protease inhibitors), precipitated with trichloroacetic acid (TCA) before fractionation by SDS-PAGE. In oligonucleotide competitions, increasing amounts of double-stranded DNA (25–200 ng) were added. A pull-down experiment coupled to an *in vitro* phosphorylation assay was designed to evaluate the impact of hER α -COUP-TFI association on the phosphorylation of the hER α N-terminal region. For this, 250 ng of GST-hER α B or 150 ng of the GST-hER α BD fusion proteins were incubated in binding buffer [50 mM NaCl, 50 mM Tris pH 8, 0.02% bovine serum albumin (BSA), 0.02% Tween-20, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors] for 3 h with equal amounts of *in vitro* translated proteins. These amounts were determined by taking into account the number of methionines and ³⁵S incorporation. Beads were washed four times in WB300 (50 mM Tris pH 8, 300 mM NaCl, 1 mM PMSF, 0.05% Tween-20 and protease inhibitors) and resuspended in binding buffer, before incubation at 30°C for 30 min with 10 µCi of [γ -³²P]ATP in PB with 10 or 20 µg of WCEs. Ten, 25 or 50 ng of activated purified ERK2/p42^{MAPK} (double mutant constitutively active; Sigma M3172) were also used instead of the WCE. Proteins bound to the beads were washed twice in WB300, denatured and fractionated by SDS-PAGE. After Coomassie Blue staining and drying, gels were exposed for autoradiography with Kodak Biomax films (Rochester, NY). Amounts of incorporated ³²P in phosphorylation assays were quantified using a phosphorimager (Packard). Experiments were carried out at least twice, and were reproducible. The phosphorylations observed in these experiments were due specifically to kinases present in the WCE, and not to those contained in rabbit reticulocyte lysate (Foulkes *et al.*, 1983).

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