A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor α coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA

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One class of the nuclear receptor AF-2 coactivator complexes contains the SRC-1/TIF2 family, CBP/p300 and an RNA coactivator, SRA. We identified a subfamily of RNA-binding DEAD-box proteins (p72/p68) as a human estrogen receptor α (hERα) coactivator in the complex containing these factors, p72/p68 interacted with both the AD2 of any SRC-1/TIF2 family protein and the hERα A/B domain, but not with any other nuclear receptor tested. p72/p68, TIF2 (SRC-1) and SRA were co-immunoprecipitated with estrogen-bound hERα in MCF7 cells and in partially purified complexes associated with hERα from HeLa nuclear extracts. Estrogen induced co-localization of p72 with hERα and TIF2 in the nucleus. The presence of p72/p68 potentiated the estrogen-induced expression of the endogenous pS2 gene in MCF7 cells. In a transient expression assay, a combination of p72/p68 with SRA and one TIF2 brought an ultimate synergism to the estrogen-induced transactivation of hERα. These findings indicate that p72/p68 acts as an ER subtype-selective coactivator through ERα AF-1 by associating with the coactivator complex to bind its AF-2 through direct binding with SRA and the SRC-1/TIF2 family proteins.

Keywords: coactivator/DEAD-box/estrogen receptor α/ N-terminal activation domain

Introduction

The biological actions of steroid hormones and related lipophilic ligands are mediated by transcriptional control of target genes by their nuclear receptors. Nuclear receptors form a steroid–thyroid hormone receptor superfamily, acting as a ligand-inducible transcription factor (Beato et al., 1995; Mangelsdorf et al., 1995; Chambon, 1996). Based on similarities in structure and function, the nuclear receptors can be divided into functional domains designated domains A–F (E). The DNA-binding domain is located in the well-conserved middle region (C domain) of the receptor. The less conserved C-terminal E/F domain serves as the ligand-binding domain. The N-terminal A/B domain and the C-terminal E/F domain are required for the ligand-induced transactivation functions of nuclear receptors. The autonomous activation function (AF-1) in the A/B domain is constitutively active on its own, while the autonomous activation function (AF-2) in the ligand-binding E/F domain is dependent on ligand binding (Lees et al., 1989). Moreover, the activities of both AF-1 and AF-2 are cell type and promoter content specific (Tora et al., 1989).

The liganded nuclear receptor forms a large complex to initiate transcription. This complex is believed to contain basic transcription machinery and coactivator complexes (Freedman, 1999; Glass and Rosenfeld, 2000). Two classes of nuclear receptor coactivator complexes for AF-2 have so far been identified to associate in a ligand-dependent manner, and activate the transactivation function (AF-2) in the C-terminal E/F domain. The better described of the two AF-2 coactivator complexes is supposed to contain CBP/p300, the steroid receptor coactivator (SRC-1/TIF2) family and an RNA coactivator, SRA, along with other unknown components (Onate et al., 1995; Vögel et al., 1996; Anzick et al., 1997; Torchia et al., 1997; Lanz et al., 1999). One of the molecular mechanisms underlying the coactivator complex’s actions is supposed to modulate chromatin structure, in terms of its intrinsic histone acetyltransferase (HAT) activity, which is indeed found in several complex components such as CBP/p300 and the SRC-1/TIF2 family proteins (Freedman, 1999; Glass and Rosenfeld, 2000). The other complex without HAT activity is the recently reported DRIP/TRAP complex, composed of at least 12 proteins (Gu et al., 1999; Rachez et al., 1999). However, it is still unclear whether these AF-2 coactivator complexes directly interact with the N-terminal transactivation function (AF-1) of nuclear receptors through a known component, or an unknown factor in the complexes.

The best characterized coactivators, SRC-1 (NCaA-1), TIF2 (GRIP1/NCaA-2) and AIB1 (pCIP/ACTR), form a p160 nuclear receptor coactivator family. Molecular dissection of SRC-1 and TIF2 (Onate et al., 1995; Vögel et al., 1996; Anzick et al., 1997; Torchia et al., 1997) has shown that both of these coactivators harbor two autonomous activation domains (AD1 and AD2) and a nuclear receptor interaction domain (NID) with three LXXLL motifs (Kalkhoven et al., 1998; Vögel et al., 1998). The NID and AD1 domains are located in the middle of the coactivators and, more recently, CBP/p300 (Kamei et al., 1996) was shown to interact directly with AD1 to mediate its transcriptional activity (Kalkhoven et al., 1998; Vögel et al., 1998). However, the property of the transcriptional mediator of AD2 mapped to the C-terminal domains with ~150 amino acid residues still remains unclear.
Therefore, the present study was undertaken to identify a mediator of the AD2 domains of the SRC-1/TIF2 proteins by a yeast two-hybrid screening of a HeLa cell cDNA library with the AIB1/AD2 domain as bait. The identified p72 is an RNA-binding DEAD-box protein (Lamm et al., 1996), and was found to form a subfamily with p68, which we previously identified as a human estrogen receptor α (hERα) AF-1 coactivator (Endoh et al., 1999). p72/p68 interacted directly with both the AD2 of any p160 member and the hERα/A/B domain, but not with hERβ or any other nuclear receptor tested. p72/p68, together with TIF2 and SRA, was co-immunoprecipitated with hERα in MCF7 cells only when estrogen (E2) was bound, and all these factors were detected in partially purified endogenous complexes associated with E2-bound hERα from HeLa nuclear extracts, indicating that p72/p68 bridges between the N-terminal hERα AF-1 and the coactivator complex bound to its AF-2 domain. In agreement with these findings, E2-dependent co-localization of p72/p68 with hERα and TIF2 was observed in the nucleus. The presence of p72/p68 potentiated the E2-induced expression of the endogenous pS2 gene. A transient expression assay showed that a combination of p72/p68 and SRA with TIF2 brings an ultimate synergism to the ligand-induced transactivation of hERα by far-western blotting. Three proteins of ~120, 72 and 68 kDa from MCF7 nuclear extracts were found to associate with the phosphorylated hERα from HeLa nuclear extracts, indicating that p72/p68 bridges between the N-terminal hERα AF-1 and the coactivator complex bound to its AF-2 domain. In agreement with these findings, E2-dependent co-localization of p72/p68 with hERα and TIF2 was observed in the nucleus. The presence of p72/p68 potentiated the E2-induced expression of the endogenous pS2 gene. A transient expression assay showed that a combination of p72/p68 and SRA with TIF2 brings an ultimate synergism to the ligand-induced transactivation of hERα through its AF-1. Thus, together with the direct SRA binding of p72/p68, the present study showed that a subfamily of the RNA-binding DEAD-box proteins, p72/p68, acts as an ERα coactivator to potentiate AF-1 by associating with the coactivator complex bound to its AF-2 domain through direct binding to SRA and the SRC-1/TIF2 family proteins.

Results
Identification of a subfamily of RNA-binding DEAD-box proteins (p72/p68) interacting with the SRC-1/TIF2 family proteins and hERα, but not hERβ

We first characterized functional domains of AIB1 (pCIP/ACTR) (Anzick et al., 1997; Chen et al., 1997), and found that this factor possesses AD1 and AD2 at locations similar to those of SRC-1 and TIF2 (Figure 1A) as reported in the other two 160 kDa members. p300 potently enhanced AIB1 AD1, but not AD2 (data not shown). We further observed that transcriptional squelching in the AD2s takes place among these three coactivators (data not shown), indicating the presence of a common factor directly associating with each of the SRC-1/TIF2 family proteins through AD2. In order to clone this common factor, we performed a yeast two-hybrid screening (Takeyama et al., 1999) of a HeLa cell cDNA library with the AIB1 AD2 domain as bait under a screening condition where an intrinsic activity of the AD2 in yeast was suppressed in the presence of 100 mM 3-amino-1,2,4-triazone. One of several candidate clones encodes a partial sequence of a reported protein (p72) (Lamm et al., 1996) containing a typical DEAD-box RNA-binding motif, which is supposed to retain an RNA helicase activity by direct RNA binding (Figure 1A) (Lamm et al., 1996; Eisen and Lucchesi, 1998; Hamm and Lamond, 1998). Expression of the p72 gene with two transcripts was observed in many tissues by northern blotting (Figure 1B). With the cloned full-length p72 cDNA, we confirmed the p72 interaction with the AIB1 AD2 domain in a yeast two-hybrid assay, and the p72 interaction was also seen with the AD2 domains of both SRC-1 and TIF2, as expected from their sequence similarity in the AD2 domains (data not shown). Furthermore, in a glutathione S-transferase (GST) pull-down assay, we observed physical interactions of p72 with the AD2 domain (Figure 1C) and the full-length protein (data not shown), but not with the AD1 domain (data not shown), in each of the three coactivators (Takeyama et al., 1999; Yanagisawa et al., 1999). The interactions of p72 with AD2s were also detected in COS-1 cells with a mammalian two-hybrid system, while p300 interacted with AD1s (Kalkhoven et al., 1998; Vogel et al., 1998), but not with the AD2s (data not shown).

Among the family of proteins containing the DEAD-box motif, p72 is highly related only to the p68 protein (Figure 1A), which we have identified recently as a coactivator acting specifically for the activation function (AF-1) in the N-terminal A/B domain of hERα by directly binding to the A/B domain (Endoh et al., 1999). In fact, like p72, p68 interacted with the AD2s (Figure 1C). The p68 interaction with the hERα A/B domain is potentiated by the phosphorylation of the Ser118 residue in the A/B domain by mitogen-activated protein (MAP) kinase (Endoh et al., 1999). With the hERα A/B domain phosphorylated by MAP kinase in vitro, we searched for endogenous interactants from the MCF7 nuclear extracts by far-western blotting. Three proteins of ~120, 72 and 68 kDa from MCF7 nuclear extracts were found to associate with the phosphorylated hERα: AD2s (Figure 1D), raising the possibility that the detected interactants are p72 and p68 DEAD-box motif proteins, although p120 remains to be identified. We then examined the direct interactions of p72 with the A/B and D/E/F domains of hERα and hERβ in a GST pull-down assay. Similarly to p68, p72 interacted with the hERα A/B domain, but showed no interaction with the hERα D/E/F domain, nor any domain of hERβ (Figure 1C). Such a physical interaction with p72 was not detected in the tested nuclear receptors such as VDR, RARα, MR and PR (data not shown). Note that human helicase Q1 (Q1) (Seki et al., 1994), a typical DEAD-box protein with little homology to p68 and p72 in the alignment except for the DEAD-box motif, showed no interaction with any nuclear receptor tested (Figure 1C). These findings suggest that, like p68, p72 serves as an hERα coactivator through its AF-1 by associating with the AF-2 coactivator complex through direct binding to the SRC-1/TIF2 family proteins.

Estrogen-induced co-localization of p72 with hERα and TIF2 in the nucleus

Since the AF-2 coactivator complex containing the SRC-1/TIF2 family proteins is recruited to the nuclear receptor only when the ligand-binding D/E/F domain (D/E/F) is occupied by cognate ligand (Cavaillies et al., 1994; Shiau, 1998; Freedman, 1999; Glass and Rosenfeld, 2000), we tested the idea that p72 is present, physically associating with the SRC-1/TIF2 family proteins, in the same natural coactivator complex in MCF7 cells, by an immunoprecipitation of hERα following western blotting with antibodies for p72 and TIF2 (Figure 2A). p72 was co-immunoprecipitated with E2-bound hERα along with TIF2.
Co-immunoprecipitation of p72 with E2-bound hERα and TIF2 was also observed when these factors were overexpressed, whereas a TIF2 deletion mutant lacking the AD2 domain (TIF2ΔAD2) suppressed the co-immunoprecipitation of p72 by hERα (Figure 6, lane 6). Similar results were observed for p68, while Q1 was not co-immunoprecipitated with E2-bound hERα (data not shown). These findings were supported further by detection of p72 in a partially purified hERα-associated complex(es) (Figure 2B). A nuclear complex(es) associated with E2-bound hERα was purified from the HeLa nuclear extracts with a GST–hERα(D/E/F) fusion protein as a probe, and further fractionated on a glycerol gradient.

Fractions containing complexes of ~600 kDa were analyzed by western blotting (Lanz et al., 1999). p72 was detected in the fractions containing SRC-1 (Figure 2B). Furthermore, p72 was also detected in the TIF2 complex recruited to E2-bound hERα when tagged hERα and p72 with TIF2 were overexpressed in COS-1 cells in the presence of E2. The complex recruited to E2-bound hERα was purified sequentially with two affinity columns for Flag-hERα and His-p72. Both p72 and TIF2 were detected in this complex (Figure 2C). Moreover, TIF2 was co-immunoprecipitated with p72 even when hERα was not associated in the absence of E2 (Figure 2D). These findings suggest that p72 is present in the complex containing the SRC1/TIF2 family proteins. Together with the fact that p68 serves as an hERα AF-1 coactivator, we
Fig. 2. Detection of p72 in the hERα-associated endogenous coactivator complex containing the SRC-1/TIF2 family proteins and SRA.

(A) Co-immunoprecipitation of E2-bound hERα with p72 and TIF2 in MCF7 cells. p72 in a natural complex associated with E2-bound hERα was detected by immunoprecipitation with an antibody to hERα followed by western blotting using anti-TIF2, anti-p72 or anti-Q1 antibody. The MCF7 cells incubated with E2 (10⁻⁸ M) were lysed in TNE buffer and immunoprecipitated with the monoclonal anti-hERα antibody or a non-specific IgG, then the immunoprecipitate was immunoblotted (Yanagisawa et al., 1999). Note that in the absence of E2, p72 was not co-immunoprecipitated with hERα (data not shown). SRA was detected using the SRA-specific primers (Lanz et al., 1999), but the GAPDH transcript was not detected with the specific primers by RT–PCR amplification of total RNA purified from the immunoprecipitate. (B) Detection of p72 in a natural complex associated with E2-bound hERα. Nuclear extracts of HeLa cells were passed over immobilized GST–ERα LBD in the presence of E2 (10⁻⁸ M). The nuclear complexes associated with E2-bound hERα were then fractionated on a glycerol gradient (Rachez et al., 1998), and each fraction was immunoblotted with the anti-TIF2, anti-p72 or anti-Q1 antibody. SRA, but not the GAPDH transcript, was detected by RT–PCR using specific sets of primers (Lanz et al., 1999). The positions of marker proteins are shown above the fraction numbers. (C) p72 and TIF2 in the same complex are recruited to E2-bound hERα. To test whether p72 and TIF2 are present in the complex recruited to E2-bound hERα, Flag-tagged hERα, His-tagged p72 and TIF2 were overexpressed in COS-1 cells and their presence confirmed by western blotting using either the anti-Flag, anti-His or anti-TIF2 antibody. The complex associated with E2-bound hERα was analyzed to detect p72 and TIF2 by purification from the cell extracts with anti-Flag M2 resin and Ni resin, following by western blotting using the anti-Flag, anti-His or anti-TIF2 antibody. (D) TIF2 was co-immunoprecipitated with p72 even when hERα was not associated in the absence of E2.
suppose that p72/p68 associates with not all of the coactivator complex containing CBP/p300 and the SRC-1/TIF2 family proteins.

We then investigated the localizations of p72, hERα and TIF2 in living cells. hERα, which is detected as red by immunostaining with a monoclonal antibody for the hERα A/B domain, localized in the nucleus irrespective of ligand binding (Figure 3B, D and G), while ubiquitous expression of green fluorescent protein (GFP; data not shown) and cytosolic expression of GFP fusion protein [extra-cellular signal-responsive MAP kinase (ERK2)] as seen (Figure 3A). The p72 fusion with GFP was found in the nucleus (green), but the expression pattern did not overlap with that of E2-unbound hERα (Figure 3E). However, a 4 h treatment with E2 (10 nM) produced co-localization of p72 and hERα (Figure 3H, in yellow). Furthermore, co-localization of p72 with TIF2 (Figure 3K) appeared to be enhanced by the presence of E2-bound hERα (Figure 3N). These findings suggest that the complex containing p72 and SRC-1/TIF2 family proteins is recruited to E2-bound hERα for transactivation.

Induction of the endogenous pS2 gene by E2 is potentiated by p72/p68

To investigate this possibility, we studied the action of p72 in the E2-induced expression of an endogenous target gene for E2-bound ER, pS2, in MCF7 cells. In untreated cells, the overexpression of p72 itself had no effect on pS2 gene expression (Figure 4 at 0 h). Cells treated with E2 (for either 4 or 8 h) resulted in a significant induction of the pS2 gene without affecting expression of a non-target gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 4). This induction by E2 was clearly potentiated by overexpressing p72, but not Q1 (Figure 4). These inductions by E2 were completely abrogated by a transcriptional inhibitor, actinomycin D, while the GAPDH mRNA levels remained unchanged irrespective of p72 overexpression (data not shown). Furthermore, when this 4 h induction was aborted by drug treatment for another 4 h, overexpression of p72 modulated the decay of pS2 mRNA (data not shown), indicating that p72 has no effect on post-transcriptional events such as RNA splicing and mRNA degradation. A similar action was also seen for p68 (data not shown). Given these observations, it is likely that the p72-mediated potentiation of ER-mediated endogenous gene induction by E2 takes place at a transcriptional level.

Synergistic actions of p72/p68 with SRA by direct binding in the ligand-induced transactivation function of hERα

We explored the molecular mechanisms of p72/p68-mediated potentiation in the E2-induced transactivation...
function of hERα. During the characterization of p72 as an hERα coactivator, Lanz et al. (1999) discovered that SRA acts as an RNA coactivator in the nuclear receptor coactivator complex containing the SRC-1/TIF2 family proteins and CBP/p300 (Kamei et al., 1996; Lanz et al., 1999) to potentiate ligand-induced transactivation functions of steroid hormone receptors including ERα through their AF-1s. Together with the fact that both p72 and p68 harbor a DEAD-box RNA-binding motif, we determined whether p72 acts together with SRA by direct association in the same coactivator complex. An RNA gel-shift assay (Peng et al., 1998) showed that p72 clearly binds labeled SRA (Figure 5A, lane 2), and this binding is completely abolished in the presence of an excess of non-labeled SRA (Figure 5A, lane 5). Furthermore, only deletion mutants containing the DEAD-box motif retained the SRA-binding activity (Figure 5B), while a mutant lacking a well-conserved motif VIII (Figure 5B), which is essential for RNA binding, showed no SRA-binding activity (Figure 5A, lane 3). Neither a mutation to abolish ATP binding [p72 (mATP BD); Figure 5A, lane 4] nor an ATP addition (data not shown) affected the SRA binding. However, the RNA-binding specificity of p72 appeared very low, at least in vitro, since SRA binding to p72 was competed even with antisense SRA (Figure 5A, lane 6) or other RNAs tested (data not shown), and p72 bound to all RNAs tested although their affinities were different (data not shown). Together with the fact that SRA binds to Q1 (Figure 5A, lane 7), it is likely that some DEAD-box proteins strongly interact physically with SRA in vitro.

We then attempted to delineate the functional domains for the interactions with the hERα A/B domain, the SRC-1/TIF2 family of proteins and SRA, and the transactivation function (Figure 5B). With the TIF2 AD2 domain as a probe, the TIF2 interaction region in p72 was mapped to the C-terminal domain by GST pull-down assay (Figure 5B). In accordance with the physical TIF2 interaction, truncation of the C-terminal domain [p72(m1)–p72(m3)] resulted in loss of potentiation by p72 of the TIF2-mediated transactivation by E2-bound hERα (Figure 5B, right panel). Deletion mutants retaining only the AD2 interaction region [p72(m5), p72(m6)] acted in a dominant-negative way, possibly competing with endogenous p72 for the TIF2 interaction (Figure 5B). Note that this region is not conserved among the DEAD-box family proteins except for p68 (Hamm and Lamond, 1998; Luking et al., 1998), and is also required for interaction with the hERα A/B domain. We then investigated whether SRA binding reflects the p72 coactivator action by introducing a deletion in the putative RNA-binding motif. A deletion mutation [p72(ΔVIII)] in the RNA-binding motif (region VIII in Figure 5B) (Gross and Shuman, 1998), which caused a loss of RNA-binding activity (Figure 5A, lane 3), resulted in a significant reduction in the potentiation by p72, suggesting that direct SRA binding is required for the p72 coactivator activity.
The p72-binding site was mapped to the core AF-1 region of hERα (Figure 5C) (Kobayashi et al., 2000), in agreement with previous results for p68 (Endoh et al., 1999). The lack of any significant sequence homology between this region (56–127 amino acids) and any hERβ region is supposed to cause selectivity between hERα and hERβ in the p72/p68 interaction.

The complex containing p72/p68, TIF2 and SRA is recruited to liganded hERα
We next tested whether SRA is indeed included in the complex containing E2-bound hERα, p72 and the SRC-1/TIF2 family proteins by immunoprecipitation. RT–PCR analysis with SRA-specific primers (Lanz et al., 1999) showed the presence of SRA in the immunoprecipitate of E2-bound hERα, along with p72 and TIF2 (Figure 2A). Unlike in the RNA gel-shift assay, antisense SRA (Figure 6, lanes 11 and 12) and an RNA containing the 3′-untranslated region AUUUA motif (data not shown) (Malter, 1989) were not associated with p72 by an immunoprecipitation assay when these factors were over-expressed. Moreover, in contrast to the in vitro SRA binding to p72, the ATP binding to p72 appeared to be essential for the in vivo integration of SRA into this complex (compare lane 5 with lane 8 in Figure 6). As expected, when the co-immunoprecipitation of p72 and E2-bound hERα was abrogated by overexpression of the TIF2ΔAD2 mutant, SRA could not be detected (Figure 6, lane 6). Similar results were observed in p68, whereas SRA was not detected in the immunoprecipitate of Q1 (data not shown). Thus, it is likely that p72/p68 is integrated with SRA in the coactivator complex containing the SRC-1/TIF2 family proteins, at least when this complex is recruited by E2-bound hERα, and that the SRA-specific integration of p72/p68 in the nuclear complex requires ATP.

p72/p68 acts as an hERα coactivator through AF-1
We then studied the combined effects of p72/p68, SRA and TIF2 on the E2-induced transactivation function of hERα (Kato et al., 1995b). These three factors caused an ultimate potentiation of the hERα transactivation function (Figure 7D, lanes 13 and 17). The actions of SRA, p72 and p68 appeared to be mediated through AF-1 (Figure 7B), but not AF-2 (Figure 7C), as estimated from the activities of the AF-1 and AF-2 hERα deletion mutants (Figure 7A). Interestingly, such actions of SRA and p72/p68 were not detected in hERβ (Figure 7B and C). It should also be noted that this ultimate potentiation by the three factors was abrogated by a deletion mutation [p72/(ΔVIII)] with loss of the SRA-binding activity (Figure 7E, lane 14), and a point mutation in ATP binding [p72 (mATP BD)] (Figure 5B). In agreement with these findings in the transient expression assay, this p72/(ΔVIII) mutant failed to potentiate the E2-induced expression of the endogenous p52 gene in MCF7 cells (Figure 4). Similar findings with either SRC-1 or AIB1 instead of TIF2 were also seen (data not shown). Thus, it is likely that SRA binding is indispensable for p72/p68 to function as an hERα coactivator.

Discussion
p72/p68 is a novel class of hERα coactivator
In the present study, we report that a subfamily of the RNA-binding DEAD-box proteins, p72/p68, acts as a novel class of ERα coactivator through AF-1 potentiation. p72/p68 interacted directly with SRA and the hERα A/B domain, but not with hERβ or any other receptor tested. p72/p68 physically and functionally interacted with the SRC-1/TIF2 family proteins through the CBP/p300-independent AD2 domains of all three members of the family (Kalkhoven et al., 1998; Vögel et al., 1998). p72/p68 was co-immunoprecipitated with E2-bound hERα, TIF2 and SRA. In such p72/p68 interactions, no functional difference among the SRC-1/TIF2 proteins was detected. In this respect, the function of the recently identified methyltransferase (CARM1) associating with AD2s appears distinct from that of p72/p68, since CARM1 was shown to be effective in physical interaction and transactivation only for SRC-1 and GRIP1 (TIF2), but not for ACTR (AIB1) (Chen et al., 1999). Co-localization of p72/p68 with liganded hERα and TIF2 was observed in the nucleus. p72/p68 acted synergistically with SRA and TIF2 in the ligand-induced transactivation function of hERα through its AF-1. Moreover, the presence of p72/p68 potentiated the E2-induced expression of the endogenous p72 gene, an E2 target gene. Thus, from these observations, we propose that p72/p68 bridges between the ERα A/B domain and the coactivator complex bound to the E/F domain through direct binding to SRA and SRC-1/TIF2 family proteins.

p72/p68 acts as an hERα activator through AF-1 potentiation
As expected from the high homology with p68 acting as an hERα AF-1 coactivator (Endoh et al., 1999), p72 directly associated with the hERα A/B domain to potentiate the ligand-induced transactivation of hERα, but not hERβ. We have reported previously that interaction of p68 with the hERα A/B domain is potentiated by phosphorylation of the Ser118 residue in the hERα A/B domain by MAP kinase, which is activated by growth factor signaling (Kato et al., 1995a; Endoh et al., 1999). Similarly to p68, p72 exhibited higher affinity for the MAP kinase-phosphorylated hERα A/B domain (data not shown). Furthermore, p72 was as potent as p68 for the ligand-induced transactivation of the full-length hERα bound to 4-hydroxytamoxifen, an ERα AF-1 agonist/AF-2 antagonist (Figure 7D, lanes 21–25). Thus, these findings indicate that p72 and p68 are functionally identical as an hERα coactivator to potentiate the AF-1 in a MAP kinase-mediated phosphorylation-dependent manner.

p72/p68 directly binds an RNA coactivator, SRA
Although many RNA-binding DEAD-box proteins exist (Lamm et al., 1996; Eisen and Lucchesi, 1998; Hamm et al., 1998), only p72/p68 are highly related in terms of homologies of their entire amino acid sequences, and of interaction domains for the hERα A/B domain and the AD2 of the p160 members. The coactivator activity of p72/p68 was only partially impaired by mutations in the ATP-binding domain to abolish putative RNA helicase activity, whereas lack of
RNA-binding activity in p72/p68 by deletion of motif VIII abrogated the synergistic action with SRA and the p160 members in the ligand-induced transactivation of hERα. Thus, it is likely that p72/p68 serves as an acceptor protein to integrate SRA into the coactivator complex in the nucleus, although p72/p68 binding to RNA does not appear to be RNA sequence specific, at least in vitro. Moreover, this SRA-specific interaction with p72 in vivo appears to require a proper conformational change of p72 and/or SRA induced by ATP hydrolysis. In this respect, it will be of interest to identify other SRA-binding proteins associating with the A/B domains of other steroid hormone receptors in the coactivator complex containing the SRC-1/TIF2 family proteins and CBP/p300, since SRA was reported to be potent for the AF-1 functions of other steroid hormone receptors (Lanz et al., 1999).

**Unknown tissue-specific component(s) in the reported coactivator complexes?**

The ratio of AF-1 to AF-2 activity in ERα has been shown to be cell type specific (Tora et al., 1989; Kobayashi et al., 2000), and modulated by growth factor signaling. Such cell type specificity in the transactivation function of ERα is unlikely to be elicited by only the limited numbers of reported components in the coactivator complexes (Freedman, 1999; Glass and Rosenfeld, 2000). As recent reports revealed that the coactivator complexes for distinct classes of transcription factors share several common components (Björklund et al., 1999), it is likely that one...
class of the nuclear receptor coactivator complexes contains CBP/p300 and p160 family proteins as common components, and uncommon ones such as p72/p68 and SRA, since the actions of these latter factors depend on receptor types. It is therefore more likely that such uncommon components exert cell type and receptor type specificities by forming a particular set of the coactivator complex subtypes with the common components. Based on ubiquitous expression patterns of the p72/p68 genes in various tissues, unknown factor(s) may be associated with p72/p68 to potentiate the hER\textsubscript{a} AF-1 activity in a cell type-specific manner. Alternatively, it is a possibility that a novel coactivator complex specifically interacting with the ER\textsubscript{a} AF-1 contributes to the cell type-specific activities of the ER\textsubscript{a} AFs. In this respect, a PPAR\gamma AF-1 coactivator, PGC-2 (Castillo et al., 1999), is a good example of a factor that may be a component of the reported coactivator complex or of an unknown coactivator complex. In order to delineate further the physiological role of p72/p68 as an hER\textsubscript{a} coactivator in the ligand-induced transactivation function of hER\textsubscript{a}, purification and identification of the endogenous complex containing p72/p68, SRA and CBP/p300 along with, hopefully, cell type-specific components are necessary.

**Materials and methods**

**Yeast two-hybrid screening**

The AD2 domain of AIB1 (amino acids 1135–1421) was cloned in-frame with the GAL4-DNA-binding domain (DBD) in pGBT9 (Clontech). A HE\textsubscript{a} cDNA library (Clontech) was co-transfected with the pGBT9-AD2 bait plasmid in Y153 yeast cells. Yeast two-hybrid screening was performed as described (Clontech Matchmaker Two-Hybrid Protocol), followed by a β-galactosidase assay (Yanagi et al., 1999).

**Northern analysis**

Multiple tissue northern blots were obtained from Clontech. p72 full-length cDNA was radiolabeled by the random primer method as a probe. Pre-hybridizations and hybridizations were carried out as described previously (Takeyama et al., 1997). A human GAPDH probe from Clontech was used for the control experiments.

**GST pull-down assay**

GST-fused proteins were expressed in Escherichia coli and purified on glutathione–Sepharose beads (Pharmacia). The beads were incubated with \textsuperscript{35}S-methionine-labeled proteins. Bound proteins were eluted and analyzed by SDS–PAGE. Proteins were translated in vitro in the presence

**Immunoprecipitation**

After washing the MCF7 cells twice with ice-cold phosphate-buffered saline (PBS), the collected cells were resuspended in 1 ml of ice-cold lysis buffer [10 mM Tris–HCl pH 4.7, 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) NP–40] and incubated on ice for 30 min, then centrifuged again for 5 min at 500 g. The sedimented nuclear fraction was resuspended in TNE buffer (10 mM Tris–HCl pH 7.5, 1% NP–40, 0.15 M NaCl, 1 mM EDTA) and incubated for 30 min on ice. After centrifugation, the supernatant was collected and used as whole-cell extracts of MCF7 cells for immunoprecipitation of anti-hER α/β antibody (anti-ER α/β–4, Neo Markers) following western blotting of anti-hER α/β antibody, anti-TIF2 antibody (GRIP1 M 343; Santa Cruz Biotechnology) or anti-p72 monoclonal antibody raised against an N-terminal region (amino acids 35–55). In Figure 3B, the whole-cell extracts of COS-1 cells transfected with Flag-hER α, His-TIF2, His-TIF2AADD2, Mpc-p72 or Mpc-p72 (mATP BD) were used for immunoprecipitation with anti-Flag antibody, following western blotting of either anti-Flag, anti-His or anti-Myc antibody (Figure 3B) (Yanagisawa et al., 1999).

**Fractionation of an hERα-associated complex by glycerol density gradient**

Immobilized GST–ERα LBD fusion protein was pre-incubated for 1 h at 4°C in GST binding buffer [20 mM Tris–HCl pH 7.9, 180 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT)] containing bovine serum albumin (BSA; 1 mg/ml) and E2 (1 nM). The bead-immobilized proteins were then incubated at 4°C for 6–10 h with HeLa nuclear extracts in the presence of 10 mM E2. After washing with GST wash buffer (GST binding buffer containing 0.1% NP-40) three times, the beads were washed further with a GST wash buffer containing 0.2% N-lauryl sarcosine (Sarkosyl; Sigma). The complexes bound to E2-bound hERα were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris–HCl pH 7.9, 10 mM NaCl, 3 mM MgCl2, 0.5% glycerol). Protein standards were vitamin B12 (1.3 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), γ-globulin (158 kDa) and thyroglobulin (667 kDa) (Rachez et al., 1998).

**Phosphorylation of the hERα A/B domain in vitro**

A much more efficient phosphorylation of the Ser118 residue in the hERα A/B domain by MAP kinase (anti-MAP-Ab–4, Neo Markers) following western blotting of anti-hER α/β antibody, anti-TIF2 antibody (GRIP1 M 343; Santa Cruz Biotechnology) or anti-p72 monoclonal antibody raised against an N-terminal region (amino acids 35–55). In Figure 3B, the whole-cell extracts of COS-1 cells transfected with Flag-hER α, His-TIF2, His-TIF2AADD2, Mpc-p72 or Mpc-p72 (mATP BD) were used for immunoprecipitation with anti-Flag antibody, following western blotting of either anti-Flag, anti-His or anti-Myc antibody (Figure 3B) (Yanagisawa et al., 1999).

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