Estrogen Receptor Accessory Proteins: Effects on Receptor–DNA Interactions

Carolyn Church Landel,1 Peter J. Kushner,2 and Geoffrey L. Greene1,3

1Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois; 2Metabolic Research Unit, University of California, San Francisco, California; 3Ben May Institute, University of Chicago, Chicago, Illinois

Despite a wealth of information about the structure and composition of steroid receptors and their functional domains, little is known about the role of accessory proteins as mediators of receptor activity. To better define the role of such proteins in estrogen receptor (ER) function, we have used immunofluorescent, steroid affinity, and site-specific DNA-affinity chromatography to identify and characterize proteins that associate with human ER (hER) in extracts from MCF-7 cells and hER-expressing CHO (CHO-ER) cells. In addition to the expected 66-kDa hER, a 70-kDa protein was obtained and subsequently identified as a member of the heat shock protein family (hsp70). A 55-kDa protein, detected by all three approaches, was identified as a member of the protein disulfide isomerase family (PDI). Two proteins that were preferentially retained by an ER-specific DNA affinity column (p48 and p45) remain unidentified. Maximal interaction of purified hER with the vitellogenin A2 estrogen response element (ERE) occurred in the presence of all four associated proteins isolated by DNA-affinity chromatography. The increased stability of this complex was due primarily to an increase in the association rate of hER with ERE. Thus, accessory proteins may be required for optimal interaction of ER with EREs. — Environ Health Perspect 103(Suppl 7):23-28 (1995)

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Introduction

The cloning and molecular analysis of the known steroid receptors has led to the definition of common functional domains by which they interact with responsive genes in hormone sensitive tissues (1–4). As a consequence of these interactions, DNA synthesis is altered as well as the synthesis of specific RNAs and proteins involved in cell proliferation, differentiation, and physiologic function and development. One member of this family of transcription factors is the estrogen receptor (ER), which mediates estrogenic responses in diverse tissues including the brain, mammary gland, tissues of the reproductive tract, and cancers derived from some of these tissues (5).

All of the steroid receptors including ER are activated by one or more ligands and bind with high affinity and specificity to short cis-acting DNA sequences called hormone response elements (HREs). Interaction of steroid-receptor complexes with responsive genes in vivo can result in either induction or suppression of transcription, depending upon the target gene and the tissue (1, 6, 7). The molecular mechanisms by which either pathway occurs are still obscure although it is generally accepted that, for transcriptional activation, receptor–DNA complexes recruit or facilitate the recruitment of other transcription factors that comprise a functional transcription complex (3, 8). This process involves protein–protein interactions between receptor and other factors, which may be either general [e.g., transcription factor IIIB (TFIIB)] (9), tissue specific (certain cofactors) (10), or receptor specific [e.g., for N-terminal domain of progestosterone receptor (PR) B isoform] (11). Some of these interactions may result in the formation of DNA loops (12) to accommodate long stretches of DNA between promoters and HREs or possibly by altering the local chromatin organization (13, 14) to permit access of other transcription factors. DNA bending may also be involved (15, 16). It has also been suggested that nonhistone protein acceptor sites (17, 18) that are part of the nuclear matrix play a key role in receptor action, possibly by directing receptor to a target gene. Although such sites have been described, they have not yet been linked in an obligatory manner to a functional transcription complex in vivo. Obviously, all or any combination of these processes could occur.

Although it is widely believed that an allosteric alteration of receptor structure occurs following hormone binding, which exposes the DNA-binding domain, the nature of this change is still not understood. The participation of other proteins, both before and after hormonal activation, has been the subject of much investigation (19). At least three members of the heat shock protein family have been identified as putative accessory proteins by virtue of their association with several receptors in vitro. One of these, hsp90, has been implicated in the in vitro stabilization of the inactive form of receptors for glucocorticoids (GR) (20, 21), progestins (PR) (22), and estrogens (ER) (23). In support of the
hypothesis of an essential role for hsp90, recent experiments in which expression of the hsp90 gene was conditionally regulated in *Saccharomyces cerevisiae* demonstrated that reduced levels of hsp90 severely compromised GR transcriptional activity (24). ER activity was less affected in this model. In contrast, members of the thyroid (25) and retinoid (26) receptor family do not appear to associate with hsp90, but rather are synthesized in an active form that is able to bind to HRES in vivo in the absence of ligand. hsp56, which was recently identified as an immunophilin, also appears to be part of the unactivated complex of several steroid receptors (27) and may (28) or may not (29) be able to modify the transcriptional activity of some receptors (e.g., PR or GR) in response to immunosuppressants such as FK506. It has also been suggested that p59 (p56) may be the nuclear localization signal-binding protein (27). Another heat shock protein, hsp70, has been shown to bind to both PR and GR in the absence of hormone but, unlike hsp90, this association appears to be maintained, at least in part, in the activated receptor complex after hormone treatment (30). A recent study using baculovirus-overexpressed human GR in *Spodoptera frugiperda* cells suggested that hsp70 is associated with the GR-glucocorticoid response element (GRE) complex (31). However, Onate et al. (32) observed that hsp70 was not present or involved in specific recognition of a progesterone response element (PRE) by PR. Clearly, the role of hsp70 in ER, PR, and GR function remains unresolved.

An additional class of receptor-associated proteins are those that interact with the activated receptor complex. Thus, a 95-kDa nuclear accessory factor (NAF) has been reported to be essential for maximal binding of the vitamin-D receptor (VDR) to the vitamin-D response element from the human osteocalcin promoter (33). Similarly, a 65-kDa factor termed triiodothyronine receptor- auxiliary protein (TRAP), which exhibits limited independent DNA binding, has been shown to enhance thyroid hormone receptor (TR) binding to DNA (34). More recently, both retinoid x receptor α and β (RxRα and RxRβ) have been shown to function as NAF- (35) or TRAP-like proteins (36) by forming heterodimers with retinoic acid receptors (RAR), VDR, and TR. Such heteromers can have both positive and negative transcriptional activity (2). In addition, one or more members of a heteromeric complex may interact with mixed DNA elements or half sites in a responsive gene (37). In regard to other interactions with activated receptors, the nonhistone high-mobility-group chromatin protein (HMGG-1) has been shown to substitute for an unidentified factor present in partially purified progesterone receptor fractions that is responsible for promoting PR-DNA binding (38). Also, an unidentified single strand DNA-binding protein has been implicated as being necessary for high affinity binding of ER to the vitellogenin A2 estrogen response element (ERE) (39). In addition, a 110-kDa receptor accessory factor (RAF) has been observed to potentiate the DNA-binding activity of the estrogen receptor (AR) in vitro (40). This protein was recently identified as insulin-degrading enzyme, and it is not clear what, if any, role it plays in AR action.

The nature of agonist–receptor versus antagonist–receptor interaction and the resulting altered transcriptional activity are also still poorly understood at present. It is very likely that an altered conformation of receptor occurs in the presence of an agonist (41), which could affect receptor stability (42), DNA binding (43,44), interaction with other transcription factors such as AP1 (45,46), phosphorylation (47), or interaction with hsp90 or hsp70. It has been proposed for ER that partial agonists restrict TAF-2 (transcriptional activation function) activity but not TAF-1 activity and that the promoter and cell context is therefore crucial for agonist versus antagonist activity (48–50). For both ER (51) and PR (52,53), the pattern of phosphorylation appears to be essentially the same in the presence of either agonist or partial agonists, suggesting that altered phosphorylation may not reflect agonist versus antagonist activity. For estrogen receptor it has been proposed that higher order protein–protein interactions differ when DNA-bound ER is associated with estradiol versus 4-hydroxytamoxifen (54). It has also been suggested that some agonists, like the "pure" estrogen agonist-ICl-164 (or the closely related ICI-182,780), may interfere with dimerization (55) or promote receptor degradation (42). It has recently been observed that the carboxyterminal tail of at least several steroid receptors may be a critical determinant of receptor response to ligand and that antagonism may result from a failure of antagonists to interact with a 30-amino acid domain at the carboxy terminus (56,57). Obviously, there are still a number of key dynamic and molecular aspects of ligand-mediated receptor activity that are not resolved at this time.

### Results and Discussion

As discussed above, a wealth of data implicates the interaction of ER with other cytoplasmic and nuclear proteins, both before and after activation by hormone or specific binding to DNA response elements in target genes. An objective that has evolved from our studies on the DNA-binding properties of the human estrogen receptor (hER), as well as from the over expression and purification of hER from mammalian cells, is the determination of the role of receptor-associated proteins in DNA binding, transcriptional activity, or cytoplasmic-nuclear transport of ER. In addition to the association of several heat shock proteins with unactivated receptors described above, other proteins have been implicated in the specific recognition of DNA response elements on target genes by receptors (39) and as mediators of transcriptional activity (9,58). It is also clear that the TAF-1 and TAF-2 functions of ER are not identical and that their respective roles in transcriptional activation/repression are therefore almost certainly mediated by different intermediary factors. These factors may be cell- and promoter-specific as well as limiting, as suggested for PR TAF-1 (58), or general, as suggested for TAF-2 (TFF1B) (9). However, it is still unclear how many different factors participate or how steroid receptors influence the formation or stability of transcription initiation or elongation complexes.

To isolate, identify, and characterize both cytoplasmic and nuclear ER-associated accessory proteins, we used immunoadfinity (H222-Sep, steroid affinity (E-Sep), and site-specific DNA (B-ERE) affinity chromatography to identify proteins that co-purify with hER in extracts from MCF-7 human breast cancer cells and from CHO cells stably transfected with hER (CHO-ER cells) (Figure 1) (59,60). Analysis of eluted proteins by SDS-PAGE revealed similar, but not identical, patterns of associated proteins for each of these techniques (Table 1). In each case, the expected 66-kDa hER was observed in silver-stained gels as well as on Western blots. In addition to hER, a 70-kDa band was retained by all three methods. Western blot analysis identified this species as hsp70. Treatment of CHO-ER extract in *vitro* with ATP reduced the amount of ER-bound hsp70, similar to the result reported by Smith and Toft for chick PR treated with ATP (19,61).

An additional species that co-purified with hER by all three methods was a 55-kDa protein (Table 1) that we have recently identified by N-terminal amino
acid sequencing as protein disulfide isomerase (PDI) (59, 62), or thyroid hormone binding protein (p55) (63). It is not yet clear what role PDI may have in ER action. However, this ubiquitous and abundant protein is essential for yeast viability (64), and a recent report suggests that one important function of PDI is to catalyze disulfide bond formation and rearrangements within kettically trapped, structured folding intermediates (65). Interestingly, deletion of the catalytic domain of PDI is not lethal in yeast (66), suggesting that the essential role of PDI lies in a different function. One possibility is that PDI may serve as a chaperone in the cytoplasmic/nuclear transport of proteins since a subpopulation of p55 has been localized to the nuclear membrane of human A431 and rat GH3 cells (67).

Two additional protein bands, which migrate at 45 and 48 kDa (p45 and p48), were observed in eluates containing hER purified on B-ERE-agarose (Table 1). Although these two bands are also present to a lesser extent in immunoaffinity eluates, their intensity is significantly enhanced when ER is bound to ERE, suggesting that their association with hER is promoted or stabilized by ER/ERE interaction. One of these species may be the 45-kDa single strand DNA-binding protein identified earlier by Mukherjee and Chambon (39), as mentioned above. Although most of our studies were carried out with CHO-ER cells, a virtually identical pattern of ER-associated proteins was observed by SDS-PAGE with affinity-purified whole cell extracts from MCF-7 breast cancer cells. Because the CHO-ER cells express very high levels of hER (2–3 × 10^6 molecules/cell) (68), it is a good model system for studies of ER-associated proteins.

To examine possible differential effects of estrogen agonists and antagonists in situ on the interaction of hER with the associated proteins listed in Table 1, cells were metabolically labeled with 35S-methionine prior to treatment with estradiol (E2), ICI-182,780 (ICI-182), or 4-hydroxytamoxifen (OH-Tam). hER was then isolated on B-ERE-agarose or H222-Sepharose. None of the tested ligands (E2, ICI-182, OH-Tam) had any effect on the stoichiometry of protein association with hER purified by adsorption to B-ERE. The same 45-, 48-, and 66-kDa (hER) bands that were observed by silver stain were seen in the autoradiogram except for 35S-hsp70, which was absent due to the low turnover rate of hsp70 (69). However, on Western blots, hsp70 was readily observed, as were hER (66 kDa) and PDI (p55). Like the other three associated proteins (Table 1), the hsp70/hER stoichiometry was constant for each in situ treatment.

In contrast to the B-ERE chromatography results, when total hER complexes were isolated by immunoabsorption (H222-Sepharose), a significant reduction in the amount of associated hsp70 was observed following treatment of CHO-ER cells in situ with either estradiol or the partial antagonist OH-Tam (Table 1), whereas dissociation of hsp70 did not occur in the absence of ligand or when cells were treated with ICI-182, a complete estrogen antagonist. In contrast to other published reports on the effect of ICI-164 (an analog of ICI-182) on ER stability in mouse uterus (42), no significant loss of ER was observed in extracts of CHO-ER cells treated with ICI-182. The hsp70/ICI-182 results suggest that hsp70 may be required for high affinity ER/ERE binding and that a subpopulation of ER that is competent for DNA binding remains associated with hsp70. It has been reported that treatment of chick PR with hormone in vitro partially disrupts its interaction with hsp70 (70). However, Onate et al. (32) subsequently observed that hsp70 was not present or involved in specific recognition of a progestrone response element (PRE) by PR. Therefore, ER and PR may function differently with respect to hsp70 interaction. As mentioned earlier, a recent study suggests that hsp70 is associated with the GR-GRE complex (31). Thus, it will be especially important to determine the role of hsp70 in ER transcriptional activity or in the stabilization of ER/ERE interactions.

Recent data (59) indicate that some or all of the ER-associated proteins discussed above can influence the affinity or rate of ER-DNA complex formation. Two approaches were used in conjunction with gel retardation analyses to address this question: removal of ER-associated components and reconstitution experiments. Purification schemes are outlined in Figure 1. When analyzed by gel retardation (Table 1), maximal binding of hER to the vitellogenin 32P-PERE (27 bp of natural vitellogenin A2 gene sequence) occurred in the presence of all four hER-associated proteins (hsp70, p55, p48, p45) that were isolated by B-ERE chromatography (Figure 1, scheme C). This interaction is at least as good as the interaction between unpurified ER (CHO-ER nuclear extract) and the vitellogenin ERE. Notably, the B-ERE eluate gives rise to two hER/ERE complexes. Subsequent removal of the p45 and p48 proteins by fractionation of the B-ERE eluate on estradiol-Sepharose (E-Seph) in the presence of 0.7 M NaCl afforded hsp70/hER-p55 (55, 66, 70) complex that bound to 32P-PERE with significantly reduced affinity (Table 1). The same hsp70-hER-p55 complex obtained by a single step purification of CHO-ER whole cell extract (WCE) on E-Seph (Figure 1, scheme B), behaved similarly in gel shift experiments (Table 1). Proteins in the B-ERE/E-Seph

### Table 1. Summary and properties of ER-associated proteins isolated by several chromatographic techniques (Figure 1).

| Source of hER | Protein present, kDa | Relative DNA binding | Rate of association |
|---------------|----------------------|----------------------|---------------------|
| CHO-ER nuclear extract | Total nuclear proteins | +++ | NA |
| B-ERE | 70, 66, 55, 48, 45 | +++ | +++ |
| B-ERE → E-Seph EL | 70, 66, 55 | ++ | ++ |
| B-ERE → E-Seph NA | 48, 45 | - | - |
| E-Seph | 70, 66, 55 | ++ | ++ |
| E-Seph/ATP | 66, 55 | + | + |

NA, not available. *Reproduced with permission from Landel and Greene (60). †Three methods were used to isolate hER: site-specific DNA-affinity chromatography (B-ERE); estradiol-Sepharose affinity chromatography (E-Seph); and estradiol-Sepharose chromatography in the presence of ATP (E-Seph/ATP). A two-step purification using B-ERE followed by E-Seph was also used. The eluate (B-ERE→E-Seph EL) and nonadsorbed (B-ERE→E-Seph NA) are indicated above. ‡The identity of the proteins indicated in the table are: 70, hsp70; 66, hER; 55, PDI; 48 and 45 are unidentified.
nonadsorbed fraction (NA) (Figure 1, scheme C), which contained only the p45 and p48 species, did not interact with the 32P-labeled ERE in the absence of hER, at least under normal gel retardation conditions (Table 1). However, in the presence of a large excess of 32P-ERE, a weak gel-shift complex was observed, suggesting that at least one of the two proteins has some affinity for DNA, similar to the properties of the 45-kDa ER/ERE-binding protein reported by Mukherjee and Chambon (39). When CHO-ER WCE was treated with ATP prior to E-Seph chromatography (scheme A, Figure 1) to dissociate hsp70, the estradiol-eluted hER-p55 (55,66) complex displayed the weakest detectable interaction with the 32P-ERE probe (Table 1), suggesting that hsp70 may play an additional role in stabilizing or facilitating ER-ERE interaction.

To test whether proteins that were dissociated from hER could be added back to reconstitute a more active complex, the E-Seph/ATP eluate (hER-p55, scheme A, Figure 1) was first treated with an equimolar mixture of the p45 and p48 proteins (E-Seph NA, scheme C, Figure 1). As shown in Table 1, this combination enhanced the interaction of hER with 32P-ERE. If hsp70 was added separately to the ER-p55 complex, a similar enhancement of hER-ERE interaction was observed, except that in this case a second, more retarded complex was also seen, suggesting the formation of an hsp70-hER-p55 complex. Finally, if all three proteins (p45, p48, hsp70) were added to the ER-p55 complex, maximum hER-ERE formation was observed (Table 1), comparable to the original B-ERE purified material. In all experiments, a stoichiometric amount of each protein was added to a known, fixed amount of hER. Interestingly, a mutant ERE competitor (GGTCAnnnTGCAC), which has at least a 10-fold reduced affinity for fully complexed hER (54), competed significantly for the ER-p55-ERE complex, suggesting that one or more of the other associated proteins may contribute to ER-ERE specificity. Finally, when the rate of ER-DNA binding was assessed by timed gel-shift experiments, both hsp70 and the combined p45/p48 proteins contributed separately to a significant enhancement of the rate of association (Table 1).

Four conclusions can be drawn from the above results: a) maximal interaction of hER with vitellogenin ERE requires at least two of the four ER-associated proteins (45,48,55,70) obtained from the B-ERE column; b) a subpopulation of hER that is competent for DNA binding remains associated with hsp70, therefore, hsp70 may be required for high affinity ER/ERE interaction; c) it is possible to sequentially dissociate and reconstitute complexes that consist of hER and at least some associated proteins; and d) reconstitution studies indicate that a major contribution of these ER-associated proteins to hER-DNA interaction is to enhance the rate of association.

Based on our preliminary results, as well as the published data of others regarding receptor-associated proteins (19,27,71), we propose a model (Figure 2) in which unactivated p55-ER-hsp90/70 complex loses hsp90 following ligand binding; the resulting activated ER complex recruits or stabilizes the binding of two additional proteins (p45 and p48) when hER binds to ERE. The population of liganded hER that does not bind to ERE dissociates from hsp70. Other proteins (e.g., hsp56, TFIIB) may participate in one or more of these steps. This model is not meant to be complete. Future work will focus on the identification and further characterization of these and other ER-associated proteins, especially in regard to their ability to influence DNA binding or transcriptional activation by ER. Of particular interest is the mechanism(s) by which some estrogen antagonists (e.g., OH-tam) appear to be able to promote DNA binding but then either fail to activate gene expression or activate only a subset of genes, possibly by altered interaction of ER with accessory factors such as those described above or with other as yet unidentified intermediary factors.

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