CELL REGULATION, Vol. 1, 291–299, February 1990

Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors

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The glucocorticoid receptor accumulates in nuclei only in the presence of bound hormone, whereas the estrogen receptor has been reported to be constitutively nuclear. To investigate this distinction, we compared the nuclear localization domains of the two receptors and the capacity of their respective hormone-binding regions to regulate nuclear localization activity. As with the glucocorticoid receptor, we showed that the human estrogen receptor contained a nuclear localization signal between the DNA-binding and hormone-binding regions (amino acids 256–303); however, in contrast to the glucocorticoid receptor, the estrogen receptor lacked a second nuclear localization domain within the hormone-binding region. Moreover, the hormone-binding domain of the unliganded estrogen receptor failed to regulate nuclear localization signals, although it efficiently regulated other receptor functions. We conclude that the two receptors employ a common mechanism for signal transduction involving a novel "inactivation" function, but that they differ in their control of nuclear localization. Thus, despite the strong relatedness of the estrogen and glucocorticoid receptors in structure and activity, certain differences in their properties could have important functional implications.

Introduction

The receptors for steroid hormones are members of a superfamily of intracellular proteins that associate with cognate ligands, and in response to hormone binding, regulate the transcription of specific genes. The receptors share common organizational features, including a DNA-binding domain comprised of two zinc fingers, and at the C-terminus, a large hormone-binding segment (for review, see Evans, 1988; Green and Chambon, 1988; Godowski and Picard, 1989). In addition, we showed previously that the glucocorticoid receptor contains two independent nuclear localization signals, one (NL1) between the DNA- and hormone-binding regions, and the other (NL2) overlapping the hormone-binding region (Picard and Yamamoto, 1987). In the absence of hormone, these signals are nonfunctional within the context of the glucocorticoid "aporeceptor" (apo-GR); thus, apo-GR is a cytoplasmic protein. Indeed, we showed that apo-GR contains a "protein inactivation" activity within the hormone-binding domain which inhibits other receptor functions (DNA binding, and probably transcriptional enhancement and repression), as well as the activities of nonreceptor proteins to which the hormone-binding domain is fused; importantly, this inactivation is fully reversed upon hormone addition, thus suggesting a general scheme for signal transduction (Picard *et al.,* 1988).

Previous studies with estrogen receptor have suggested that it is associated with the nucleus not only in the presence of bound estrogens, but also in the absence of ligand (apo-ER) (King and Greene, 1984; Welshons *et al.,* 1984, 1985). This difference between apo-GR and apo-ER is somewhat unexpected in view of the strong functional similarities and evolutionary relatedness of the two proteins. It therefore seemed conceivable that these two receptors might contain two distinct types of nuclear localization activities or might employ fundamentally different signal transduction mechanisms. To investigate these possibilities, we reexamined the intracellular location of apo-ER, mapped the nuclear localization signal of the human estrogen receptor, and compared it to the nuclear localization signals of the glucocorticoid receptor; furthermore, we tested the putative "inactivation domain" of apo-ER and its effect upon nuclear localization activity.

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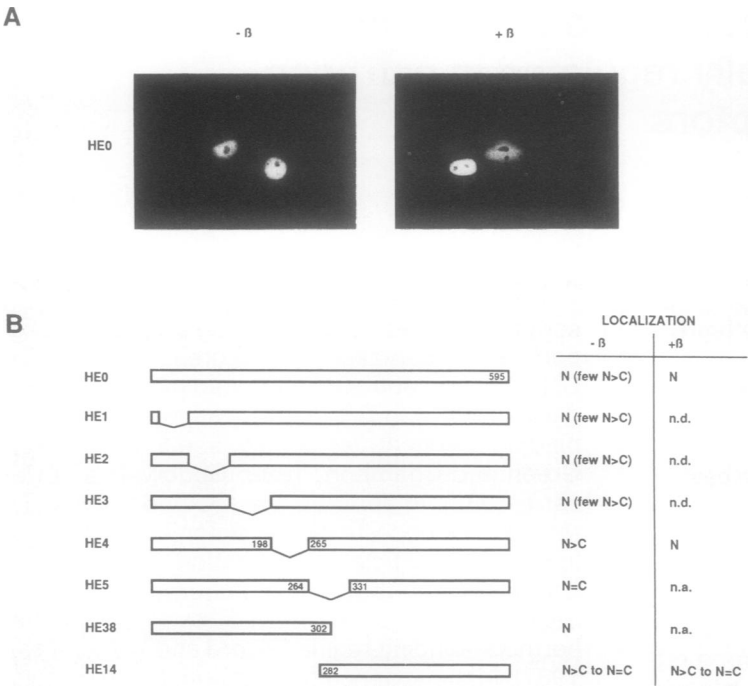

Figure 1. **Subcellular localization of the full-length estrogen receptor and of mutant derivatives.** (A) Immunofluorescence micrographs of wild-type estrogen receptor (plasmid HE0) expressed in CV-1 cells in the absence ($-\beta$) or presence ($+\beta$) of 0.1 μM β-estradiol. (B) Summary of immunofluorescence analyses. Estrogen-receptor segments included in each derivative are represented by open boxes. For clarity in aligning the diagrams, deleted receptor sequences are represented by fine lines connecting the fused segments. Amino acid positions at fusion junctions or end points of deletion mutants are indicated within open boxes. The tabulated data summarize the results from a total of >100 positive cells for each assay condition. N and C, nuclear and cytoplasmic fluorescence, respectively; N > C, predominantly nuclear fluorescence accompanied by some cytoplasmic staining; N (few N > C), vast majority of cells are N, while a few cells are N > C; N = C, equal cytoplasmic and nuclear staining. n.d., not done; n.a., not applicable (derivatives are defective for hormone binding).

Results

Estrogen aporeceptor is nuclear

The unliganded estrogen receptor (apo-ER) appears to be a nuclear protein, according to immunocytochemical analyses of human breast tumors and of tissues from ovariectomized animals (King and Greene, 1984) and of tissue culture cells (King and Greene, 1984; Welshons *et al.,* 1984, 1985). In contrast, our studies with the unliganded glucocorticoid receptor (apo-GR) suggested that it was a cytoplasmic protein and that nuclear localization was a hormone-dependent process (Picard and Yamamoto, 1987). Although we also employed cultured cells, our experiments differed in several technical aspects from the previous studies of apo-ER in cultured cells. For example, we used culture medium lacking phenol red, a compound that appears to have weak agonist activity both for the estrogen receptor (Berthois *et al.,* 1986) and for the glucocorticoid receptor (Picard and Yamamoto, 1987). We therefore began the present work by reexamining the subcellular localization of the intact human estrogen receptor under the same experimental conditions that we had employed for the glucocorticoid receptor. Thus, monkey CV-1 cells, which lack endogenous estrogen receptors, were transfected with appropriate expression vectors and subcellular localization of various receptor derivatives was assessed by indirect immunofluorescence. To minimize spurious agonist activities in the absence of added hormone, cultures were generally maintained in growth medium lacking phenol red and supplemented with charcoal-treated fetal calf serum; during experiments, cultures were incubated either in this same medium, or in medium lacking serum altogether (Picard and Yamamoto, 1987); similar results were obtained under both conditions (data not shown). As shown in Figure 1A, apo-ER (construct HE0; Green *et al.,* 1986) indeed resides predominantly in the nucleus, in contrast to the cytoplasmic localization of apo-GR in this same assay (Picard and Yamamoto, 1987).

Nuclear localization signal of the estrogen receptor

For preliminary mapping of the estrogen receptor nuclear localization signal(s) we used the immunofluorescence assay to monitor the intracellular distribution of a series of receptor deletion mutants (Kumar *et al.,* 1986, 1987). Deletions within the N-terminal 264 amino acids (aa) had little or no effect (Figure 1B; derivatives HE1–HE4), whereas deletion of aa 265–330 (mutant HE5) abolished nuclear accumulation. Instead, HE5 was distributed throughout the cell, perhaps reflecting the relatively free diffusion through the nuclear pores of proteins of <70 kDa (Paine *et al.,* 1975; Bonner, 1975; Lang *et al.,* 1986). These results suggest that a nu-

clear localization signal may reside, at least in part, within the deleted region. In addition, derivative HE38, which contains aa 1–302, is strongly nuclear, implying that a nuclear localization signal resides somewhere in this portion of the receptor. Finally, derivative HE14, which encompasses the C-terminal half of the receptor (aa 282–595) including the steroid-binding domain, showed only a weak preference for nuclear accumulation either in the absence or presence of hormone. Thus, our results are consistent with the view that a signal resides N-terminal to aa 302 and overlaps aa 264–331.

To map the nuclear localization activity of the estrogen receptor in greater detail, we fused portions of the receptor coding sequence to the *Escherichia coli* β-galactosidase gene (Figure 2); β-galactosidase itself is predominantly cytoplasmic when expressed in mammalian cells (Picard and Yamamoto, 1987). Thus, we sought to detect a segment of the estrogen receptor that would elicit nuclear localization of the fusion gene product. As β-galactosidase is a large tetrameric protein, this scheme reduces the relative size differences in the various products; the β-galactosidase-receptor fusion proteins that we tested were all in the 120–170 kDa (monomer) range.

We first tested fusion protein Z.HE0 (see Figure 2B), which contains all but the first 120 aa of the estrogen receptor fused to β-galactosidase. As expected, Z.HE0 accumulates efficiently in nuclei, but unlike the receptor alone, nuclear localization occurred efficiently only in the presence of β-estradiol. Thus, a nuclear localization function within the estrogen receptor between aa 121 and 595 can indeed be observed for the Z.HE0 protein, but nuclear localization of this chimeric species, more than twice the mass of the intact estrogen receptor, appears to comprise a more stringent test of the localization signal. According to this view, an "inactivation activity" of apo-ER might reduce but not fully inactivate a nuclear localization signal within the receptor, and the residual activity remaining would suffice for nuclear accumulation of apo-ER, but not of apo-Z.HE0. A similar increase in the "stringency" of hormonal control of nuclear localization was observed in fusions of the glucocorticoid receptor to β-ga-

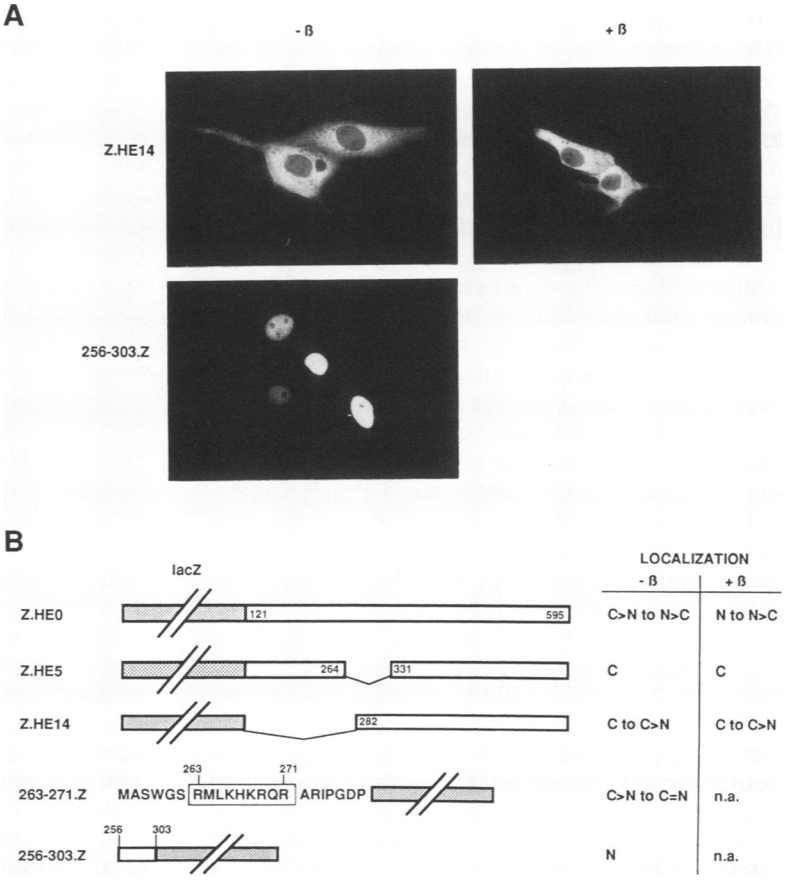

***Figure 2.* Subcellular localization of β-galactosidase-receptor fusion derivatives.** (A) Immunofluorescence micrographs of recombinant proteins Z.HE14 and 256-303.Z expressed in CV-1 cells in the absence (−β) or presence (+β) of β-estradiol. (B) Summary of immunofluorescence analyses. β-galactosidase (lacZ) and estrogen-receptor sequences are represented by stippled boxes (β-galactosidase sequences are not drawn to scale) and open boxes, respectively. Numbers within or above open boxes indicate relevant amino acid positions of estrogen-receptor moieties. For diagrammatic alignment of derivatives Z.HE0, Z.HE5, and Z.HE14, deleted receptor sequences (relative to Z.HE0) are represented by fine lines connecting the fused segments. For derivative 263–271.Z, the amino acids encoded by linker sequences are indicated N- and C-terminal to the estrogen-receptor segment (amino acid sequence in open box). Receptor sequence included in derivative 256-303.Z is presented in Figure 4. Assessment of the fluorescence staining was as described in the legend to Figure 1.

lactosidase (Picard and Yamamoto, 1987); analogous context effects on the efficacies of nuclear localization signals have been described in other systems as well (Roberts *et al.,* 1987; Nelson and Silver, 1989).

Fusion product Z.HE5 is similar to Z.HE0, but carries a deletion of receptor aa 265–330 (Figure 2B); consistent with the results obtained with the simple deletion derivative HE5 (see Figure 1B), Z.HE5 failed to localize to nuclei. Similarly, derivative Z.HE14 (Figure 2) failed to accumulate in nuclei, supporting the view that the steroid-binding domain of the estrogen receptor, unlike that of the glucocorticoid receptor, lacks a nuclear localization signal.

Many nuclear localization signals correspond to short stretches of basic amino acids (for a review see Goodson and Silver, 1989). Therefore, the results obtained with HE5, HE38, and HE14 (and their β-galactosidase fusion derivatives), together with our knowledge of the position and sequence of the glucocorticoid receptor signal NL1, prompted us to test whether aa 263–271 (RMLKHKRQR) might confer nuclear localization activity. However, this segment (with flanking linker sequences; derivative 263–271.Z, Figure 2B) failed to direct β-galactosidase into nuclei. On the other hand, a 48aa fragment encompassing that region, aa 256–303, indeed mediated efficient nuclear localization of a β-galactosidase fusion protein (derivative 256–303.Z, Figure 2). This portion of the receptor appears to include three basic stretches, aa 256–260, 266–271, and 299–303 (see Figure 4). A comparison of estrogen receptor sequences from chicken (Krust *et al.,* 1986), human (Green *et al.,* 1986; Greene *et al.,* 1986), mouse (White *et al.,* 1987), and rat (Koike *et al.,* 1987) reveals that overall this region is one of the least conserved within receptor. Interestingly, the first two basic stretches have been perfectly conserved with only one amino acid change in the chicken sequence and the third stretch has been conserved at least in charge, whereas the other sequences in this region have diverged considerably. None of the basic stretches is itself sufficient as a localization signal, according to results obtained with HE5, 263–271.Z, and Z.HE14. It remains to be shown directly that these basic sequences actually correspond to the signal; note, however, that derivative HE4, which contains the two most C-terminal basic sequences, retains at least partial nuclear localization activity (Figure 1B).

apo-ER carries an inactivation domain that fails to inhibit nuclear localization signals

In studies of the glucocorticoid receptor, we described an "inactivation function" within the steroid-binding region of apo-GR, which disables all known receptor activities other than steroid-binding region of apo-GR, which disables all known receptor activities other than fused (Picard *et al.,* 1988; Eilers *et al.,* 1989). In contrast, the estrogen receptor contains a nuclear localization signal that is constitutively active, thus confirming and extending previous reports of others. As the transcriptional regulatory activity of the estrogen receptor is fully hormone dependent, at least one receptor function other than nuclear localization must be under hormonal control, thus providing hormone-mediated signal transduction. Indeed, Kumar and Chambon (1988) have shown that the DNA-binding activity of the estrogen receptor (HE0) is hormone dependent, whereas that of a deletion mutant (HE38), which lacks the steroid-binding domain, is constitutive.

Three simple models could explain the differences observed between the estrogen and glucocorticoid receptors: first, they might employ entirely different mechanisms for signal transduction; second, apo-ER might contain a special type of nuclear localization signal that is not affected by the inactivation function; third, the apo-ER inactivation domain, unlike that of apo-GR, might fail to disable nuclear localization signals. To investigate these possibilities, we constructed glucocorticoid receptor-estrogen receptor chimeras. Thus, GR.ER fuses a constitutive derivative of the glucocorticoid receptor (N525, which lacks its own steroid-binding domain) to the ligand-binding region of the estrogen receptor (Figure 3A). The glucocorticoid receptor nuclear localization signal NL1, present in GR.ER, is normally inactivated in intact apo-GR. However, GR.ER was constitutively nuclear (Figure 3A), suggesting that the estrogen-binding domain was unable to inactivate NL1. A similar chimera lacking NL1, GR(ΔNL1).ER, failed to accumulate in nuclei, confirming that NL1 mediates the nuclear localization of GR.ER.

We then tested whether GR.ER confers transcriptional regulation mediated by a glucocorticoid response element (GRE), and whether its activity is estrogen dependent despite its constitutive accumulation in nuclei. Thus, we cotransfected the GR.ER expression vector together with a GRE-CAT reporter plasmid, G_{46}TCO, into CV-1 cells. As expected, enhance-

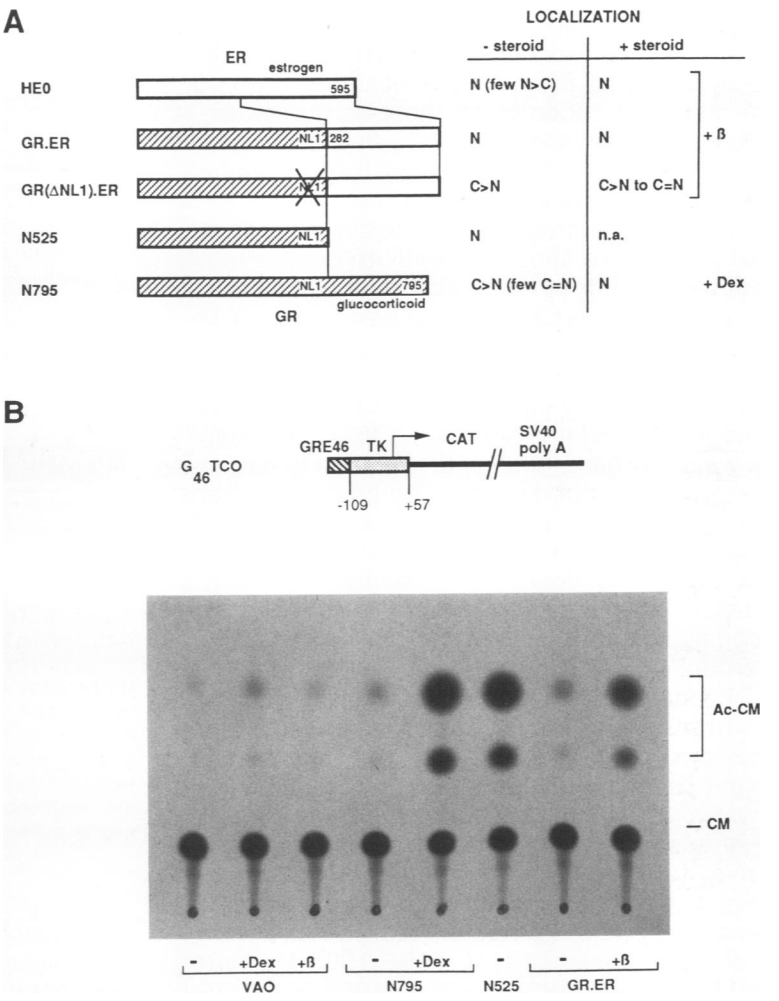

Figure 3. **The steroid-binding domain of the estrogen receptor contains an inactivation function that does not regulate nuclear localization signals.** (A) Subcellular localization of chimeric receptor derivatives expressed in CV-1 cells in the absence or presence of appropriate steroids (β, 0.1 μM β-estradiol; Dex, 0.1 μM dexamethasone). Estrogen and glucocorticoid receptor sequences are represented by open and hatched boxes, respectively. The position of the NL1 nuclear localization signal of the glucocorticoid receptor is indicated. The deletion of NL1 in derivative GR(ΔNL1).ER is represented by an overlaid X. Data for N795 (wild-type glucocorticoid receptor) and N525 (glucocorticoid-receptor derivative containing aa 1-525) are from Picard and Yamamoto (1987). Assessment of the fluorescence staining was as described in the legend to Figure 1. n.a., not applicable. (B) CAT assay of a cotransfection experiment in CV-1 cells. Reporter plasmid G_{46}TCO contains a synthetic 46 bp GRE (GRE46; ⊡) linked to the thymidine kinase promoter (TK; □) at position −109, driving the CAT gene; the arrow indicates the transcription start site and direction. G_{46}TCO was cotransfected with the indicated plasmids. VAO, expression vector lacking receptor sequences. Quantitation of two independent CAT assays yields the following values: N795 +Dex, 100% (94-fold induction); N525, GR.ER −, and GR.ER +β are 90%, 2%, and 28% of N795 +Dex, respectively.

ment of CAT enzyme expression (Figure 3B) by the intact glucocorticoid receptor (N795) was observed only in the presence of the glucocorticoid dexamethasone, and the truncated derivative N525 was constitutively active. However, N525 activity was abolished upon fusion to the steroid-binding domain of the estrogen receptor in derivative GR.ER, and this inactivation was at least partially relieved by addition of β-estradiol.

It is notable that we chose the junction sequences for construction of GR.ER somewhat arbitrarily, rather than attempting to recombine at regions of homology between the two receptors. Thus, the finding that GR.ER is fully hormone dependent supports the view that the hormone-binding domain of apo-ER, like apo-GR, carries an inactivation function that operates on many activities without strict regard to protein structure. In addition, the failure of the estrogen receptor inactivation function to disable nuclear localization signals from either receptor, in contrast to the strong inactivation of nuclear localization by the glucocorticoid receptor inactivation domain, suggests that it is the inactivation functions of the two receptors, and not their nuclear localization signals, that differ in strength or in their ability to affect nuclear localization signals in particular.

Discussion

We have confirmed and extended previous reports (King and Greene, 1984; Welshons *et al.*, 1984, 1985) that apo-ER is a nuclear protein. In particular, we have mapped the nuclear-localization activity to a single segment of 48aa between the DNA-binding and steroid-binding domains. Moreover, our studies suggest that the hormone-binding domain of apo-ER, like that of apo-GR, "inactivates" proteins with which it is associated in a hormone-reversible manner

(Figure 4). Indeed, we have recently demonstrated this movable inactivation function directly by fusing the hormone-binding region of the estrogen receptor to the c-*myc* protooncogene and showing that transformation activity of the fusion protein becomes fully hormone dependent (Eilers *et al.*, 1989).

The human estrogen-receptor clones used in this study were shown recently to carry a mutation in the hormone-binding region (*gly* to *val* at aa 400). Whereas the wild-type receptor is exquisitely sensitive to spurious agonist activities, the mutation decreases receptor stability and ligand affinity but does not otherwise affect receptor behavior (Tora *et al.*, 1989), including, as we show here, nuclear localization in particular. We have not excluded the possibility that the wild type estrogen-binding domain carries a second constitutive nuclear localization signal; such a finding would only strengthen our conclusion that nuclear localization is differentially regulated in the estrogen and glucocorticoid receptors.

The mechanism of hormone-regulable protein inactivation is unknown, but we have speculated previously that the heat shock protein hsp90 might be involved (Picard *et al.*, 1988; Yamamoto *et al.*, 1988). An abundant protein even in unstressed cells (for review, see Lindquist and Craig, 1988), hsp90 has been reported to bind several aporeceptors of the nuclear receptor gene family (see, e.g., Catelli *et al.*, 1985; Schuh *et al.*, 1985; Sanchez *et al.*, 1985, 1987; Howard and Distelhorst, 1988; Perdew, 1988; Rafestin-Oblin *et al.*, 1989) apparently by associating with the hormone-binding domain (Gehring and Arndt, 1985; Denis *et al.*, 1988a; Pratt *et al.*, 1988); importantly, these complexes are disrupted upon hormone binding. In addition, studies of the glucocorticoid receptor imply that the hsp90-apo-GR interaction may also be essential for establishment of a competent hormone-binding domain (Bresnick *et al.*, 1989). We have suggested that hsp90 might inactivate other functions either by actively altering the conformation of the bound polypeptide (by analogy with the putative effects of certain heat shock and related proteins, such as hsp70 [Rothman, 1989]), or by passive interference with essential dimerization or oligomerization activities.

Although the inactivation function appears to operate upon a broad range of protein structures, its effects are not unlimited. Thus, our experiments reveal that apo-ER fails to inactivate either its own nuclear localization signal or that from the glucocorticoid receptor. Moreover, fusions of the apo-GR inactivation domain to β-galactosidase and several other enzymes have not produced significant reductions in enzyme activity (D. Picard, unpublished observations). On the other hand, it is also clear that regulable inactivation is distinct from the exquisite structural constraints of classical allosteric regulation, and as such, suggests a novel and efficient mechanism for the evolution of this class of receptors, and of the gene networks under their control (Picard *et al.*, 1988; Yamamoto *et al.*, 1988).

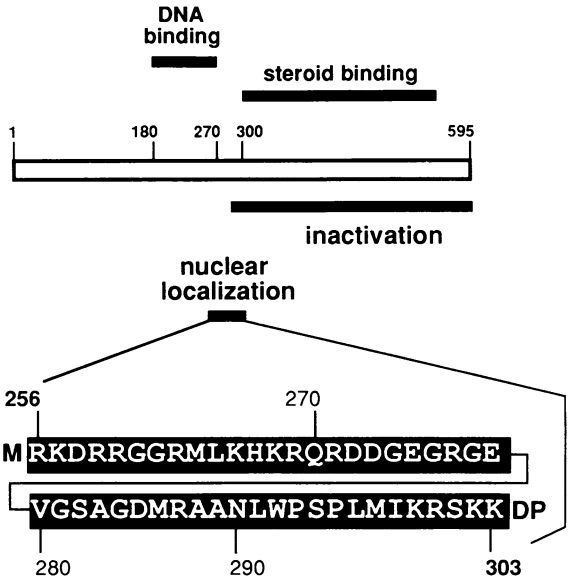

***Figure 4.* The nuclear localization and inactivation domains of the human estrogen receptor.** The 595 aa receptor is depicted by the open box. For orientation, solid bars above the receptor indicate the positions of the DNA and steroid-binding domains (Kumar *et al.*, 1986; 1987). Below the receptor diagram are shown the positions of the inactivation domain, and the position and sequence of the nuclear localization signal (aa 256–303); amino acids shown in black are additional residues present in the β-galactosidase-receptor fusion protein 256–303.Z.

We propose that four well-studied receptors (glucocorticoid, estrogen, progesterone, and thyroid hormone) may be viewed as prototypes of three functional classes distinguished by differential efficacies of inactivation. A receptor of the first class, defined by apo-GR, is cytoplasmic and requires hormone binding for virtually all of its actions–nuclear translocation (Picard and Yamamoto, 1987), specific DNA binding (Denis *et al.*, 1988b) and likely for transcriptional regulatory activity. A second class, typified by the estrogen and progesterone receptors, is characterized by aporeceptors that are competent for nuclear translocation (see also Perrot-Ap-

planat, 1985, 1986; Guiochon-Mantel *et al.*, 1989), but whose binding to cognate DNA sequences (Bagchi *et al.*, 1988) and subsequent transcriptional effects remain strongly hormone dependent. The third class, exemplified by the unliganded thyroid receptor (apo-TR), is localized to the nucleus, binds with high affinities to specific DNA sequences and represses transcription from a TRE-associated promoter; thus, hormone binding serves only to relieve this repression and perhaps to stimulate expression modestly from the same promoter (for discussion see Damm *et al.*, 1989).

It is interesting to speculate that the progenitor of the nuclear receptor family may have utilized a more conventional allosteric-type mechanism for signal transduction. By this view, the acquisition of the regulable inactivation function, presumably coinciding with acquiring the capacity for ligand-regulated binding of hsp90, rendered signal transduction relatively independent of structure, thus allowing diversification of the general mechanism and of this particular gene family. Indeed, the hormone-binding domains of different members of the gene family appear to have acquired other distinct functions during evolution, such as nuclear localization activity in the case of the NL2 signal of the glucocorticoid receptor (Picard and Yamamoto, 1987). In this context, it is intriguing that in the case of the thyroid receptor, ligand binding appears to control only an aspect of its transcriptional regulatory activity. Conceivably, this relatively restricted domain of regulation may reflect use of a more classical allosteric-like signal transduction mechanism, rather than the more global protein inactivation device. It is particularly notable in this regard that Dalman *et al.* (1990) find that apo-TR does not form a complex with hsp90.

Finally, the observation that apo-TR is a functional species raises the interesting possibility that apo-GR and apo-ER might also function within their respective compartments as cytoplasmic and non-DNA bound nuclear proteins. In principle, these aporeceptors could exert regulatory effects, perhaps being relieved by hormone binding, that are independent of DNA binding and transcription. Such nontranscriptional effects of steroids have been inferred (Szego, 1974), but have not been investigated at the molecular level. In any case, it is apparent from our present findings that functional subclasses within the nuclear receptor superfamily could impart extensive diversity on the signal transduction properties of this closely related group of regulatory factors.

Materials and methods

Plasmids

Plasmids HE0 (Green *et al.*, 1986), HE1, HE2, HE3, HE4, HE5, and HE14 (Kumar *et al.*, 1986), HE38 (Kumar *et al.*, 1987), N795 (Picard and Yamamoto, 1987), and G_{46}TCO (pUC18 derivative of plasmid 5'MTV46 [Sakai *et al.*, 1988]) have been described.

Fusions involving β-galactosidase (Z), the glucocorticoid receptor (GR), or the estrogen receptor (HE or ER) are denoted by a decimal point between the fusion partners. The expression vector, denoted VAO, for all β-galactosidase fusion proteins and for GR.ER, GR(ΔNL1).ER, N795, and N525 (identical to derivative N525 in Godowski *et al.* [1987] except for the expression vector) was as described in Picard and Yamamoto (1987). For all β-galactosidase fusion proteins except 256–303.Z, 5'-untranslated sequences and the first three codons were from the herpes simplex virus thymidine kinase gene.

Recombinants Z.HE0 and Z.HE5 were constructed by inserting a *Pst*I-*Bam*HI fragment from HE0 and HE5, respectively, into recombinant Z (Picard and Yamamoto, 1987) using the polylinker from pUR291 (Rüther and Müller-Hill, 1983) to achieve in-frame fusion. For recombinant Z.HE14, a *Bam*HI-*Sac*I fragment from HE14 was cloned into recombinant Z using the polylinker from pUR290 (Rüther and Müller-Hill, 1983) for in-frame fusion.

Recombinant 263–271.Z was obtained by substituting an oligonucleotide encoding aa 263–271 from the human estrogen receptor for the *Bam*HI-*Sac*I fragment encompassing glucocorticoid receptor sequences in construct 407–545.Z (Picard and Yamamoto, 1987). The relevant segment was verified by sequencing.

To construct recombinant 256–303.Z sequences encoding estrogen receptor aa 256–303 were fused in-frame to β-galactosidase sequences in plasmid Z as a *Xba*I-*Bam*HI fragment; the estrogen-receptor fragment was obtained by the polymerase chain reaction (Mullis and Faloona, 1987) with Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) according to the manufacturer's instructions using the following two oligonucleotides: 5'GTCTAGACCATGCGAAAAGACCGAAGA3' (contains the initiation codon for translation), 5'CGGATCCTTCTTAGAGCGTTTG3'.

To construct recombinant GR.ER, glucocorticoid-receptor sequences encoding aa 1–524 were fused to estrogen-receptor sequences by joining a *Bgl* II site from an appropriate *Bal* 31 deletion mutant (P.J. Godowski, unpublished observations) to the *Bam*HI site in HE14. GR(ΔNL1).ER is identical to GR.ER except for the introduction of the LS10 receptor mutation (Godowski *et al.*, 1988).

Cell culture and transfection

Monkey CV-1 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) lacking phenol red and supplemented with 5% fetal calf serum. The fetal calf serum was treated with 20 mg/ml acid-washed charcoal (Norit A, Baker) for 90 min at 4°C and refiltered. Cells were transfected by the calcium phosphate coprecipitation technique as described (Picard and Yamamoto, 1987; Picard *et al.*, 1988); β-estradiol and dexamethasone were added to 0.1 μM for 24 h.

Immunofluorescence and CAT assays

Indirect immunofluorescence was carried out as described (Picard *et al.*, 1988). The mouse monoclonal antibody against β-galactosidase (Promega) was diluted 1:1000. Recombinants GR.ER and GR(ΔNL1).ER were analyzed with the mouse monoclonal antibody 250 against rat glucocorticoid

receptor (Okret *et al.*, 1984) at a dilution of 1:2500. For the analysis of derivatives HE0, HE1, HE2, HE4, HE5, and HE14, the rat monoclonal antibody H222 against human estrogen receptor (Abbott Laboratories, Abbott Park, IL) was used as indicated by the manufacturer. Derivative HE38, which lacks the H222 epitope (Kumar *et al.*, 1986), was analyzed with the rat monoclonal antibody H226 against human estrogen receptor (Abbott Laboratories) at ~50 μg/ml.

CAT assays were performed according to Gorman *et al.* (1982). Within a given experiment, the same amount of protein was used for all samples.

Acknowledgments

We thank Rick Dalman and Bill Pratt for communication of unpublished results, Katharina Strub for help with sequencing, Jeff Miner, Marty Privalsky, and Katharina Strub for critical reading of the manuscript. We also acknowledge Kathleen Mulherin for preparation of the manuscript and Bonnie Maler for help with the figures. This research was supported by a grant from the National Science Foundation; postdoctoral support (D. Picard) was from the Swiss National Foundation and from a Leukemia Society of America Special Fellowship.

Received: December 1, 1989.
Revised and accepted: January 17, 1990.

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