Evidence that the Hormone Binding Domain of Steroid Receptors Confers Hormonal Control on Chimeric Proteins by Determining Their Hormone-Regulated Binding to Heat-Shock Protein 90[†]

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ABSTRACT: Previously, it has been shown that the hormone binding domain of the glucocorticoid receptor acts as a transferable regulatory cassette that can confer hormonal control onto chimeric proteins [Picard, D., Salser, S. J., & Yamamoto, K. R. (1988) *Cell 54*, 1073–1080]. The hormone binding domain of the glucocorticoid receptor contains its site of interaction with the 90-kDa heat-shock protein, hsp90 [Dalman, F. C., Scherrer, L. C., Taylor, L. P., Akil, H., & Pratt, W. B. (1991) *J. Biol. Chem. 266*, 3482–3490]. We have now transfected COS cells with cDNAs for fusion proteins containing β-galactosidase and portions of the glucocorticoid receptor, and we demonstrate a correlation between hormone regulation of fusion protein localization and binding of the fusion proteins to hsp90. The hormone binding domain (residues 540–795) of the rat glucocorticoid receptor is sufficient for conferring hormone regulation onto a fusion protein and for intracellular binding of a fusion protein to hsp90. The hormone binding domain of the rat glucocorticoid or the human estrogen receptor is also sufficient to permit reticulocyte lysate-mediated refolding of a fusion protein into association with hsp90. Consistent with the results of fusion protein localization in intact cells, binding of a fusion protein to hsp90 blocks binding of an antibody directed against the NL1 nuclear localization signal of the glucocorticoid receptor. These observations argue strongly that the hormone binding domain of the glucocorticoid receptor confers hormonal control on fusion proteins by conferring hormone-regulated binding to hsp90.

Steroid receptors are direct signal transduction systems in which the receptor both receives the signal input by binding the hormone and then, in its hormone-activated form, binds to receptor-specific enhancer sequences in the genome, where it alters the transcription rates of specific genes [see Yamamoto (1985) for review]. The receptor comprises separate domains that are responsible for signal reception and subsequent DNA binding [see Evans (1988) for review]. Receptor truncation studies demonstrated that deletion of the hormone binding domain (HBD)[1] in the COOH terminus yields glucocorticoid receptors that are constitutive activators of transcription (Godowski et al., 1987; Hollenberg et al., 1987; Danielsen et al., 1987). Thus, it became clear rather early that the hormone binding domain performs three functions: (1) it represses the transcriptional activating activity of the receptor, (2) it binds hormone, and (3) it determines hormone-regulated derepression of receptor function.

The DNA binding domain can be moved as a cassette from one receptor to another, with its function being brought under control of another hormone binding domain (Green & Chambon, 1987; Kumar et al., 1987). Remarkably, the DNA binding domain of the bacterial LexA repressor exchanged for the DNA binding domain of the glucocorticoid receptor resulted in a hybrid protein that activated transcription from a promoter linked to the *lex* operator in a hormone-dependent manner (Godowski et al., 1988).

In addition to controlling transcriptional activating activity, the HBD of the glucocorticoid receptor controls its nuclear localization. By studying the cellular localization of fusion proteins of β-galactosidase and various portions of the rat glucocorticoid receptor, Picard and Yamamoto (1987) defined two distinct nuclear localization signals, NL1 and NL2. NL1 is located just to the COOH-terminal side of the DNA binding domain and acts constitutively when fused to β-galactosidase. NL2 is located within the HBD and is fully hormone-dependent, conferring on β-galactosidase a cytoplasmic or nuclear localization depending upon the absence or presence of hormone. The β-galactosidase activity of the fusion proteins is not hormone regulated (Picard et al., 1990).

The regulatory function of the HBD of the GR is remarkably versatile. Even when its position is moved from the COOH terminus to the NH2 terminus of the receptor, it still confers hormone response (Picard et al., 1988). A surprising and

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[1] Abbreviations: hsp70 and hsp90, 70- and 90-kDa heat-shock proteins; GR, PR, and ER, glucocorticoid, progesterone, and estrogen receptors; HBD, hormone binding domain; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanosulfonic acid.

unexpected observation was that the activity of an unrelated protein, the adenovirus E1A gene product, became glucocorticoid-dependent when fused to the HBD of the GR (Picard et al., 1988). Similarly, fusion of the HBD of the estrogen receptor to the Myc oncoprotein rendered cellular transformation estrogen-dependent (Eilers et al., 1989). It has been speculated that the ability of the HBD to confer hormone regulation on structurally different proteins is conferred via its association with the 90-kDa heat-shock protein, hsp90 (Picard et al., 1988).

In hormone-free cells and cytosols prepared from hormone-free cells, steroid receptors are associated with hsp90 [see Pratt (1987, 1990) for reviews]. Several studies of hsp90 binding by receptors deleted for various regions have established that HBD is necessary for interaction of GR or PR with hsp90 (Pratt et al., 1988; Howard et al., 1991; Dalman et al., 1991; Cadepond et al., 1991; Schowalter et al., 1991). This conclusion is supported by the demonstration that hsp90 remains bound to the HBD when receptor in the heterocomplex is cleaved by protease (Denis et al., 1988; Chakraborti & Simons, 1991). However, neither the deletion nor the cleavage approach can determine if the HBD is *sufficient* for formation of a complex with hsp90, and it has not been determined if any of the hormone-regulated fusion proteins bind hsp90. In this work we examine the hsp90 binding of several fusion proteins containing β-galactosidase and portions of the rat glucocorticoid receptor and compare hsp90 binding to previously published cellular localization (Picard & Yamamoto, 1987) for these fusion proteins. We show that only those β-gal–GR chimeras whose localization is under hormonal control are bound to hsp90. By extension of this example, our observations support the notion that the hormone binding domains of steroid receptors regulate the function of other proteins through hormone-regulated binding of chimeras to hsp90.

EXPERIMENTAL PROCEDURES

Materials

Rabbit reticulocyte lysate was from Promega Inc. (Madison, WI). [^{3}H]Dexamethasone 21-mesylate and ^{125}I-labeled goat anti-mouse and anti-rabbit IgGs were from DuPont/NEN (Boston, MA). Monoclonal anti-β-galactosidase antibody was obtained from Sigma (St. Louis, MO). Rabbit antiserum against both hsp70 and hsp90 (Erhart et al., 1988) was a generous gift from Dr. Ettore Appella (National Cancer Institute). The BuGR2 (Gametchu & Harrison, 1984) monoclonal antibody against the GR was kindly provided by Dr. Robert W. Harrison III (University of Arkansas for Medical Science). AP64 rabbit antiserum against a peptide containing the glucocorticoid receptor nuclear localization sequence was described previously (Urda et al., 1989). The epitope of AP64 has been shown to lie in a segment between amino acids 506 and 514 of the rat GR, which covers most of the SV40 large T-antigen nuclear translocation sequence (Miyashita and Simons, in preparation). Wild-type mouse glucocorticoid receptor plasmid pSV2Wrec (Danielsen et al., 1986) was kindly provided by Dr. Gordon Ringold (Syntex Research, Palo Alto, CA). Rat glucocorticoid receptor–β-galactosidase fusion protein plasmids were described previously (Picard & Yamamoto, 1987). The plasmid containing cDNA for a fusion protein of β-galactosidase and residues 282–595 of the human estrogen receptor has been described by Picard et al. (1990).

Methods

Cell Culture and Transfection. COS-1 cells were maintained in DMEM supplemented with 10% charcoal-stripped fetal calf serum under 5% CO_2 in air. Cells were split into 150-mm tissue culture dishes (2 × 10^5 cells/dish), and the following day, cells were transfected as follows. Each dish was washed two times with 25 mL of phosphate-buffered saline (PBS) and then incubated with 5 mL of DNA cocktail (25 μg of plasmid and 1 mg/mL DEAE-dextran in PBS) for 1 h at 37 °C. Next, 20 mL of DMEM containing 80 μM chloroquine was added to each dish and incubation was continued for 5 h. Cells were then shocked with 10% DMSO in DMEM for 2 min and incubated with 25 mL of fresh medium for 60 h. Cytosol was prepared by Dounce homogenization of the washed, transfected cells in 1.5 volumes of HEPES buffer (10 mM HEPES and 1 mM EDTA, pH 7.4) containing 20 mM sodium molybdate, followed by centrifugation at 100000g for 1 h.

Receptor Immunoadsorption and Heterocomplex Reconstitution. Aliquots (400 μL) of each cytosol were immunoadsorbed to protein A–Sepharose with either nonimmune IgG or monoclonal anti-β-galactosidase (or BuGR, for wild-type GR). The immune pellets were washed four times with 1 mL of TEGM buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 20 mM molybdate, and 50 mM NaCl, pH 7.6) and analyzed by SDS–PAGE and Western blotting for wild-type receptor (BuGR primary antibody) or fusion protein (anti-β-galactosidase primary antibody) and hsp90 as described previously (Hutchison et al., 1992). The immunoblots were incubated with the appropriate ^{125}I-labeled counterantibody to visualize the immunoreactive bands.

In the experiment using the AP64 anti-NL1 antiserum, cytosol was prepared from transfected cells with and without 20 mM molybdate. The cytosol without molybdate was incubated for 3 h with 500 mM NaCl to transform any fusion protein–hsp90 complex. Aliquots of cytosol (500 μL) were then incubated for 3 h with 50 μL of AP64 of preimmune rabbit serum and bound to protein A–Sepharose. The pellets were washed 3 times with 1 mL of TEGM buffer and analyzed for the presence of fusion protein by SDS–PAGE and Western blotting.

Reconstitution of heterocomplexes with hsp90 was carried out essentially as described previously (Hutchinson et al., 1992). Immunoadsorbed fusion proteins were stripped of associated hsp90 by incubating the immunopellets with TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, and 50 mM NaCl, pH 7.6) plus 500 mM NaCl for 2 h on ice. The immunopellets were washed three times with 1 mL of TEG buffer and then once with 1 mL of 10 mM HEPES, pH 7.4. The washed pellets were mixed with 100 μL of rabbit reticulocyte lysate, incubated for 20 min at 30 °C, and washed four times with 1 mL of TEGM buffer. Fusion protein-associated hsp90 was analyzed by Western blotting. In one experiment, the Z.540C fusion protein was affinity-labeled with [^{3}H]dexamethasone 21-mesylate by incubating the transfectant cytosol with 100 nM ligand for 6 h on ice.

RESULTS

Association of β-Galactosidase–GR Fusion Proteins with hsp90. COS-1 cells were transfected with plasmids containing cDNAs for the wild-type mouse GR, β-galactosidase, or β-galactosidase–rat GR fusion protein. The expressed proteins were immunoadsorbed to protein A–Sepharose and both the expressed protein (Figure 1) and its associated hsp90 (Figure
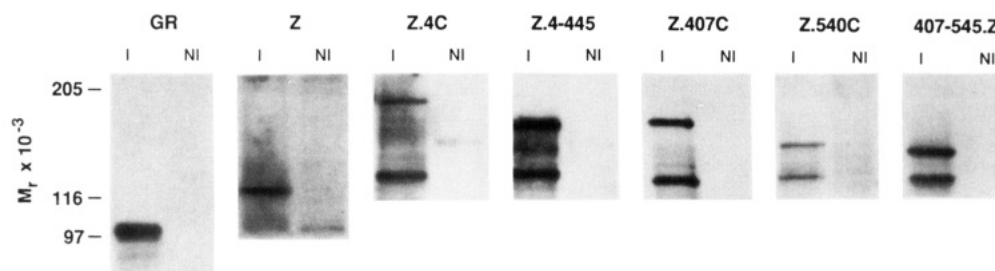
FIGURE 1: Expression of β-galactosidase–glucocorticoid receptor fusion proteins. COS-1 cells were transfected with one of seven plasmids containing the coding sequence for either wild-type mouse glucocorticoid receptor (GR) or β-galactosidase (Z) or β-galactosidase fused to rat GR residues 4–795 (Z.4C), 4–445 (Z.4-445), 407–795 (Z.407C), 540–795 (Z.540C), or 407–545 (407-545.Z). Cytosols were prepared from the transfected cells and the expressed protein was immunoadsorbed to protein A–Sepharose with nonimmune IgG or with BuGR (for the wild-type receptor in the panel at the far left) or monoclonal anti-β-galactosidase antibody (all other panels). The pellets were washed four times with 1 mL of TEGM buffer and analyzed by SDS–PAGE and Western blotting for the presence of expressed wild-type receptor, β-galactosidase, or β-galactosidase–receptor fusion protein. The figure shows Western blots of protein immunoadsorbed with nonimmune (NI) or immune (I) antibodies. The Western blots were probed with BuGR for the GR (far left panel) or anti-β-galactosidase for Z and fusion proteins (all other panels). The M_r standards were myosin, M_r = 205 000; β-galactosidase, M_r = 116 000; and phosphorylase b, M_r = 97 000. Z.407C and 407-545.Z were run on a gel with narrower lanes and the photographs were enlarged by 50% to match the width of the other bands and aligned through the lower bands; thus, the fusion protein (upper band) in these lanes appears at a slightly higher M_r than its actual migration.
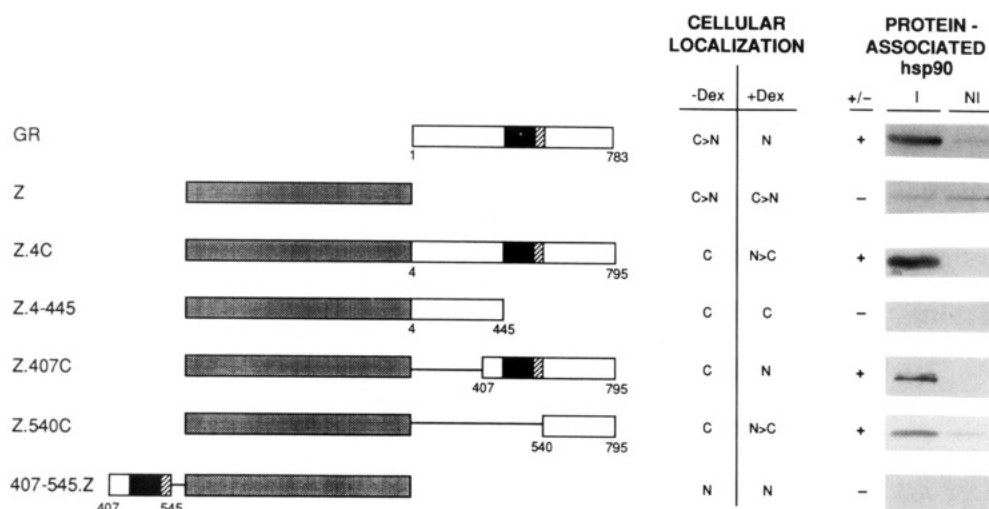

FIGURE 2: Association of hsp90 with β-galactosidase–glucocorticoid receptor fusion proteins. The same immunopellets shown in Figure 1 were Western blotted for the presence of hsp90 using the rabbit antiserum of Erhart et al. (1987). The gray bars in the diagram indicate β-galactosidase, and the solid black and hatched bars represent the DNA binding domain and hinge region, respectively, of the glucocorticoid receptor. The first column shows the relative nuclear (N) and cytoplasmic (C) localization of the receptor in the presence or absence of dexamethasone (Dex) detected by indirect immunofluorescence; these results are taken from Picard and Yamamoto (1987). The right-hand column shows Western blots of hsp90 present in immune (I) and nonimmune (NI) pellets, and the plus or minus sign denotes the presence or absence of immune-specific hsp90.

2) were visualized by Western blotting. Two bands that blotted with the anti-β-galactosidase antibody were observed in each of the fusion protein transfectants. The lower band migrates at about 125 kDa (just above the β-galactosidase shown in the second panel). The upper band in each case represents the full-length fusion protein, and the lower band is either a proteolytic product or an artifact of the plasmid construction.

As summarized in Figure 2, three fusion proteins containing the hormone binding domain, Z.4C, Z.407C, and Z.540C, were immunoadsorbed in association with hsp90, as was the wild-type mouse receptor. Picard and Yamamoto (1987) showed that all of these fusion proteins undergo a hormone-dependent shift from a cytoplasmic to nuclear localization in intact cells. In contrast, we found that fusion proteins Z and Z.4-445, which remain predominantly cytoplasmic and do not shift location with hormone (Picard & Yamamoto, 1987), were not associated with hsp90, nor was the constitutively nuclear fusion 407-545.Z. In these experiments, transfections were harvested and analyzed pairwise: for each hsp90-negative species tested, an hsp90-associated derivative was analyzed as the other member of the pair to provide a positive control.

Figure 2 also shows that hsp90 did not associate with the unidentified $\sim$125-kDa anti-β-galactosidase-reacting protein, which was detected in all fusion protein cytosols.

Evidence that hsp90 Blocks or Alters NL1. The NL1 nuclear localization signal appears sufficient for constitutive nuclear localization of 407-545.Z, but when the hormone binding domain is present (as in Z.4C and Z.407C), then NL1 function is abrogated and the fusion protein remains cytoplasmic in the absence of hormone (Picard & Yamamoto, 1987). The experiments of Figure 3 show that 407-545.Z is immunoadsorbed by an NL1-specific antiserum (Urda et al., 1989), regardless of whether it is treated with molybdate or salt, conditions that stabilize or dissociate, respectively, receptor–hsp90 complexes (Sanchez et al., 1987). In contrast, when both the hormone binding domain and NL1 are present, as in Z.407C, the molybdate-stabilized fusion protein was not immunoadsorbed (lane 2), while the NL1 epitope was readily detected after salt treatment (lane 3). These data are consistent with the notion that binding of hsp90 to the hormone binding domain of the glucocorticoid receptor either blocks antibody access to NL1 or alters the folding of this region
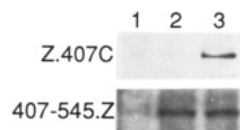
FIGURE 3: A fusion protein containing the hormone binding domain does not react with an antibody against NL1 until the receptor–hsp90 complex is dissociated with salt. Cytosol from cells transfected with Z.407C or with 407-545.Z was incubated at low ionic strength or brought to 500 mM NaCl. The fusion proteins were immunoadsorbed to protein A–Sepharose with AP64 (anti-NL1) antiserum or preimmune serum, and the pellets were analyzed for the presence of fusion protein by SDS–PAGE and Western blotting with BuGR. Lane 1, molybdate-containing cytosol immunoadsorbed with preimmune serum; lane 2, molybdate-containing cytosol immunoadsorbed with AP64; lane 3, molybdate-free cytosol incubated with NaCl and immunoadsorbed with AP64.
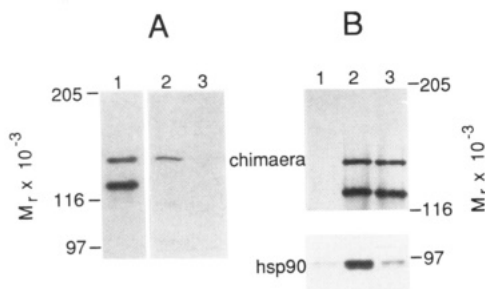

FIGURE 4: Evidence that a fusion protein containing only the hormone binding domain of the GR is functional in cytosol. (A) Steroid binding: Aliquots (200 μL) of cytosol from COS-1 cells transfected with Z.540C were incubated for 6 h with 100 nM [³H]dexamethasone 21-mesylate in the presence (lane 3) or absence (lane 2) of 10 μM nonradioactive dexamethasone. Proteins were resolved by SDS–PAGE and visualized by autoradiography. Lane 1 presents a Western blot to show that only the upper anti-β-galactosidase-reactive band (~140 kDa) has steroid binding activity. (B) Dissociation of hsp90: Aliquots of Z.540C transfectant cytosol were incubated with vehicle or 10⁻⁷ M dexamethasone for 3 h on ice and then incubated at 25 °C for 50 min. The fusion protein was adsorbed to protein A–Sepharose with anti-β-galactosidase, the immunopellets were washed four times with 1 mL of TEGM buffer, and protein-associated hsp90 was assayed by Western blotting. Lane 1, unbound fusion protein immunoadsorbed with nonimmune IgG; lane 2, unbound fusion protein immunoadsorbed with anti-β-galactosidase; lane 3, dexamethasone-bound fusion protein immunoadsorbed with anti-β-galactosidase.

such that NL1 is not in the appropriate epitope conformation. By either mechanism, the NL1 would be inactive as a nuclear targeting signal, determining a predominantly cytoplasmic localization for the fusion protein.

Reconstitution of a Fusion Protein–hsp90 Heterocomplex. The unliganded glucocorticoid receptor may be unique among steroid receptors in its strong dependence on hsp90 association in order to achieve a high-affinity steroid binding conformation (Bresnick et al., 1989; Scherrer et al., 1990). Binding of steroid to the cytosolic receptor then promotes temperature-dependent dissociation of hsp90 (Sanchez et al., 1987; Meshinchi et al., 1990). If these cell-free observations are related to hormonal control of the fusion protein, then the hormone binding domain should be sufficient to confer both high-affinity hormone binding and steroid-promoted hsp90 dissociation. A β-galactosidase fusion protein containing only the hormone binding domain of GR (Z.540C) bound the site-specific glucocorticoid affinity-labeling ligand [³H]dexamethasone 21-mesylate (Figure 4A), and 0.1 μM dexamethasone promoted hsp90 dissociation (Figure 4B), as described previously for the full-length receptor (Sanchez et al., 1987; Meshinchi et al., 1990).

It has been shown that incubation of immunoadsorbed, hormone-free, wild-type mouse GR with rabbit reticulocyte
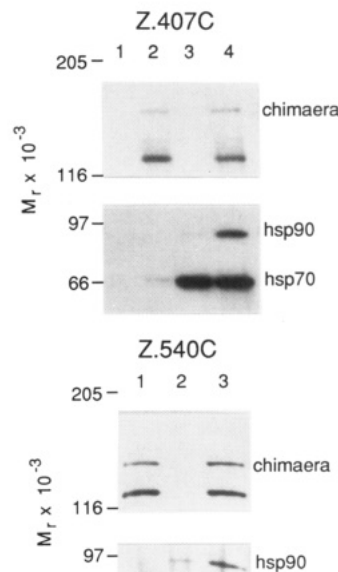

FIGURE 5: Reconstitution of fusion proteins into a heterocomplex with hsp90. Fusion protein from Z.407C (top panel) or Z.540C (bottom panel) transfectant cytosol was immunoadsorbed to protein A–Sepharose with anti-β-galactosidase. Associated hsp90 was stripped by incubating the immunopellets with 0.5 M NaCl on ice for 2 h. Receptor-bound hsp70 is not affected by the stripping conditions (data not shown). An aliquot of the stripped immunopellet was incubated with reticulocyte lysate for 20 min at 30 °C and washed four times with TEGM buffer. The pellets were analyzed by SDS–PAGE and Western blotting for fusion protein and fusion protein-associated hsp90. Top panel: lane 1, sample immunoadsorbed with nonimmune IgG and stripped; lane 2, immunoadsorbed with anti-β-galactosidase and stripped; lane 3, nonimmune, stripped, and incubated with reticulocyte lysate; lane 4, immunoadsorbed with anti-β-galactosidase, stripped, and incubated with reticulocyte lysate. Bottom panel: lane 1, fusion protein immunoadsorbed with anti-β-galactosidase and stripped; lane 2, nonimmune, stripped, and incubated with reticulocyte lysate; lane 3, immunoadsorbed with anti-β-galactosidase, stripped, and incubated with reticulocyte lysate.

lysate results in formation of a mouse GR–rabbit hsp90 heterocomplex (Hutchison et al., 1992; Scherrer, 1990). If the GR hormone binding domain is sufficient to confer a hormone response onto a fusion protein and binding to hsp90 is intrinsic to that process, then the hormone binding domain alone might be sufficient to reconstitute the fusion protein into a complex with hsp90 in vitro. Figure 5 demonstrates reconstitution of both Z.407C and Z.540C into heterocomplexes with hsp90.

Association of hsp90 with the Estrogen Receptor Hormone Binding Domain. Chambraud et al. (1990) reported that a fragment (HE14) of the human estrogen receptor containing the hormone binding domain (amino acids 282 to the COOH terminus) is recovered in transfected COS-7 cell cytosol only in the 4–5S form, implying that it is not sufficient for hsp90 binding. In the experiment of Figure 6A, we transfected COS-1 cells with a plasmid coding for the Z.HE14 fusion protein (Picard et al., 1990). When the fusion protein was immunoadsorbed with anti-β-galactosidase, we failed to detect fusion protein-associated hsp90 (lane 2), but incubation with reticulocyte lysate resulted in hsp90 association (cf. lanes 4 and 5). Given the possibility that the native fusion protein–hsp90 complex might be somewhat unstable under conditions of cytosol preparation and immunoadsorption, we repeated the cytosol preparation in molybdate-containing buffer and proceeded rapidly to the immunoadsorption step. Under these conditions, we were able to detect hsp90 associated with Z.HE14 (Figure 6B).
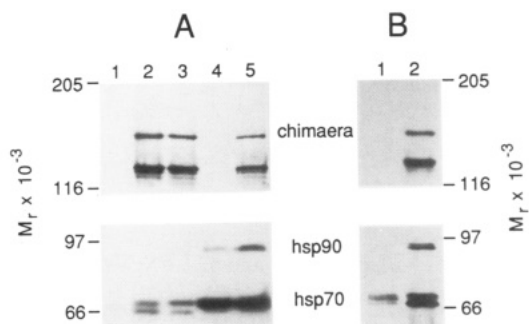
FIGURE 6: Association of hsp90 with the estrogen receptor hormone binding domain. Cytosol was isolated from cells transfected with Z.HE14, and the fusion protein was immunoadsorbed from 500-μL aliquots with nonimmune IgG or anti-β-galactosidase antibody. The immunoadsorbed pellets were stripped with salt and incubated with reticulocyte lysate as described in the legend to Figure 5. Samples were analyzed by SDS–PAGE and Western blotting for the presence of fusion protein, hsp90, and hsp70. Lane 1, nonimmune sample, unstripped; lane 2, anti-β-galactosidase-adsorbed sample, unstripped; lane 3, anti-β-galactosidase-adsorbed sample, stripped; lane 4, nonimmune sample, stripped and incubated with reticulocyte lysate; lane 5, anti-β-galactosidase-adsorbed sample, stripped and incubated with reticulocyte lysate. (B) Molybdate-containing cytosol was prepared and immunoadsorbed as rapidly as possible to preserve the native Z.HE14-hsp90 heterocomplex. Lane 1, nonimmune; lane 2, anti-β-galactosidase.

Hsp70 has been reported to be coimmunoadsorbed with two receptors that reside in the nuclei of hormone-free cells—progesterone receptors and mouse GR overexpressed in CHO cells (Kost et al., 1989; Smith et al., 1990; Sanchez et al., 1990). Estrogen receptors were the first receptors reported to be located in the nuclei of hormone-free cells (King & Greene, 1984; Welshons et al., 1984). Interestingly, when the native Z.HE14 fusion protein complex was immunoadsorbed from cytosol, we detected bound hsp70 (cf. lanes 1 and 2 in panels A and B of Figure 6) in addition to hsp90, implying that the hormone binding domain of the estrogen receptor may be sufficient for hsp70 binding in the cell. In contrast to the Z.HE14 fusion protein, we have seen either no hsp70 at all (the usual case) or only a trace immune-specific hsp70 signal with the transfected wild-type GR and the β-gal–GR fusion proteins: Figure 5 shows a trace amount of hsp70 coimmunoadsorbed with Z.407C (cf. lanes 1 and 2). This is consistent with previous reports where hsp70 was not recovered at all or was recovered only in trace amounts with native GR complexes immunoadsorbed from cytosols prepared from L929, WEHI-7, HTC, and HeLa cells (Rexin et al., 1991; Sanchez et al., 1990; Alexis et al., 1992). As noted in studies of progesterone (Smith et al., 1992) and glucocorticoid (Hutchison et al., 1992) receptor heterocomplex reconstitution, hsp70 seems to be rather "sticky", as large amounts are recovered in both immune and nonimmune pellets incubated with reticulocyte lysate [Figure 5 (top), lanes 3 and 4, and Figure 6A, lanes 4 and 5]. The high nonspecific background likely prevents detection of any specific hsp70 binding to the small amount of fusion protein.

DISCUSSION

The data presented in this paper support a model in which the hormone binding domain of the glucocorticoid receptor confers hormonal control onto the cellular localization of β-galactosidase–receptor fusion proteins by conferring hormone-regulated binding to hsp90. Of the several β-galactosidase–GR fusion proteins examined in Figure 2, only those whose cellular localization is under hormonal control are bound to hsp90. In Figure 3, we show that the presence of the HBD

prevents reaction with an antibody to NL1 under conditions where the fusion protein is bound to hsp90 but not under conditions where it is dissociated from hsp90. This is consistent with the notion (Urda et al., 1989) that either hsp90 itself or an hsp90-associated protein in the heterocomplex blocks the NL1 site so that it is not accessible to the antibody, or that the NL1 site is conformationally altered by hsp90 such that it is not a functional epitope. The HBD of the GR clearly contains all of the features required for reconstituting the fusion protein into a complex with hsp90 (Figure 5).

The hormone binding domain of the estrogen receptor apparently forms a less stable complex with hsp90. After careful examination of the sedimentation properties of a series of mutant human estrogen receptors produced in transfected COS-7 cells, Chambraud et al. (1990) concluded that a highly positively charged region in the DNA binding domain (residues 251–271) is necessary but not sufficient for the formation of the 8–9S form. However, the hormone binding domain of human ER encoded by HE14 (residues 282 to the COOH terminus) has been shown to confer hormonal control onto the transcriptional-activating activity of several proteins (Eilers et al., 1989; Umek et al., 1991; Burk & Klempnauer, 1991). If the conclusion of Chambraud et al. (1990) were valid, fusion proteins containing the HBD of the ER would not be bound to hsp90 and it would follow that conferral of hormonal control onto chimeric proteins could not be determined by hormone-regulated binding to hsp90. Our data of Figure 6 show that Z.HE14 can form a heterocomplex with hsp90. Thus, the region between amino acids 251 and 271 of the human estrogen receptor is not essential for forming a complex with hsp90. In the absence of this additional region, however, the complex may be less stable, perhaps not surviving procedures such as gradient centrifugation.

Picard et al. (1990) showed that the transcriptional activating activity of the GR is inactivated by the HBD of the ER in the chimera and that this inactivation is relieved by binding of β-estradiol. This inactivating function is common to both GR and ER hormone binding domains and can be conferred to other heterologous fusion proteins (Picard & Yamamoto, 1987; Picard et al., 1988; Eilers et al., 1989; Umek et al., 1991; Superti–Furga et al., 1991; Burk & Klempnauer, 1991). In this paper we have studied hsp90 binding in a series of fusion proteins originally constructed to study hormonal regulation of nuclear localization (Picard & Yamamoto, 1987); this activity provided a convenient assay for testing the relationship of hsp90 binding to hormone regulation (Figure 2). However, it is likely that general protein inactivation (rather than merely NL1 inactivation) is also by this same association of the hormone binding domain with hsp90 (Picard et al., 1988). Indeed, it is clear that the ER hormone binding domains binds hsp90 (Figure 6) and confers hormonal regulation onto fusion proteins (Eilers et al., 1989; Umek et al., 1991; Superti-Furga et al., 1991; Burk & Klempnauer, 1991) without regulating NL1 (Picard et al., 1990). Thus, the hsp90-mediated inactivation is not simply a function of intracellular localization.

Because the GR or ER hormone binding domain carries an inactivation function that operates on activities of structurally distinct proteins, it seems that a model of conferrable hormone regulation based on steric interference of receptor function by hsp90 alone is not adequate. Two models based on a role for hsp90 in receptor folding do accommodate a range of regulated structures and activities. In the model of Picard et al. (1988), hsp90 bound to the unliganded hormone binding domain causes the polypeptide as a whole to assume an "unfolded" confor-

mation that is reversed on hormone binding and transformation. A second model also assumes a role for hsp90 in determining the folding state of the hormone binding domain of the receptor (an assumption supported by several recent studies in which the hormone binding capacity of the GR is regenerated when the GR–hsp90 complex is reconstituted in vitro (Hutchison et al., 1992; Scherrer et al., 1990, 1992). This second model has been called a *docking* model [see Pratt (1990, 1992) for reviews] and it proposes that receptors or chimeric proteins bound to hsp90 are actually bound to a multiprotein structure to which they remain "docked" until they are released by steroid-mediated reversal of their unfolded conformation.

The principal difference between these two schemes is that Picard et al. (1988) assume that hsp90 determines an unfolded conformation in the region of the chimera that is regulated as well as in the hormone binding domain that is conferring the regulation. The inactivated protein is inactive because its active site is not properly folded. In the docking model (Pratt, 1990, 1992), hsp90 determines the conformation of only the hormone binding domain and the unfolded state does not have to be "propagated" to the rest of the molecule (although in some chimeras this may happen). Rather, the protein is biologically inactive until it is released from the hsp90-containing multiprotein structure and is transferred (or diffuses) to its ultimate site of action. In both models, regulation can be exerted in the nucleus, at the inside of the plasma membrane, or elsewhere in the cell. This opens up the possibility that hsp90 may regulate the activity of a variety of cellular processes through its role in protein folding.

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