Animal and Plant Cell Lysates Share a Conserved Chaperone System That Assembles the Glucocorticoid Receptor into a Functional Heterocomplex with hsp90[†]

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Received May 24, 1995; Revised Manuscript Received October 27, 1995[⊗]

ABSTRACT: The hormone-binding domain of the glucocorticoid receptor must be bound to heat shock protein (hsp) 90 for it to have a high-affinity steroid-binding conformation. Cell-free assembly of a glucocorticoid receptor−hsp90 heterocomplex is brought about in reticulocyte lysate by a preformed protein-folding complex containing hsp90, hsp70, and other proteins [Hutchison, K. A., Dittmar, K. D., & Pratt, W. B. (1994) *J. Biol. Chem. 269*, 27894−27899]. In this "foldosome" system, hsp70 is required for assembly of the receptor−hsp90 complex and concomitant activation of steroid-binding activity [Hutchison, K. A., Dittmar, K. D., Czar, M. J., & Pratt, W. B. (1994) *J. Biol. Chem. 269*, 22157−22161]. All previous experiments involving cell-free assembly of both receptor−hsp90 and protein kinase−hsp90 heterocomplexes have been carried out with the protein-folding system in rabbit reticulocyte lysate. In this work, we show that concentrated lysates of receptor-free mouse (L cells) and insect (Sf9) cells and also a plant (wheat germ) lysate fold the immunopurified glucocorticoid receptor into a functional (i.e., steroid binding) heterocomplex with hsp90. Receptor heterocomplex formation in animal lysates and in the plant lysate are not identical in that the dynamics of complex assembly are different, but both systems produce a functional complex that binds steroid. Also, in contrast to animal and insect complexes, receptor−plant hsp90 complexes are not stabilized by molybdate. When added to the other lysate, purified plant and animal hsp90s show partial complementarity, in that a receptor−hsp90 complex is formed but the receptor is not converted to the steroid-binding conformation. When added to rabbit reticulocyte lysate that has been depleted of endogenous hsp70, purified wheat germ and mouse hsp70's are equally active in promoting both assembly of receptor−hsp90 heterocomplexes and conversion of receptor to the steroid-binding conformation. Thus, hsp70 from the plant kingdom has conserved the ability to interact functionally with chaperone proteins of the animal kingdom to cooperate in protein folding as evidenced by formation of a functional receptor−hsp90 heterocomplex.

When immunoadsorbed, unliganded steroid receptors are incubated with rabbit reticulocyte lysate, they are folded into a heterocomplex with rabbit hsp90[1] (Smith et al., 1990; Scherrer et al., 1990). To be in a conformation that binds steroid in a high-affinity manner, the hormone-binding domain of the glucocorticoid receptor (GR) must be bound to hsp90 (Bresnick et al., 1989), and the protein-folding system in reticulocyte lysate converts the GR from a non-steroid-binding to a steroid-binding state (Scherrer et al., 1990). There is a direct relationship between the extent of hsp90 association with the GR and the number of specific binding sites that are formed by reticulocyte lysate (Hutchison et al., 1992b), and generation of steroid-binding activity is blocked by peptides that block GR association with hsp90 (Dalman et al., 1991). Formation of receptor−hsp90 complexes is an ATP/Mg^{2+}-dependent process (Hutchison et al., 1992b; Smith et al., 1992) that requires the presence of a monovalent cation, such as K^+ or NH_4^+ (Hutchison et al., 1992b). Both assembly of the GR−hsp90 complex and concomitant conversion of the GR to the steroid-binding conformation are dependent upon the presence of hsp70 (Hutchison et al., 1994b). It is thought that, in addition to hsp70, at least two other proteins are required for hsp90 to bind to the hormone-binding domain of the receptor [for review of models for receptor heterocomplex assembly, see Pratt, (1993) and Smith and Toft (1993)].

In hypotonic lysates of mammalian cells, hsp90 and hsp70 exist together in multiprotein complexes that contain a couple of immunophilins, the p50 protein that was originally identified as part of the $pp60^{src}$−hsp90 complex, a 23 kDa protein, and a 60 kDa stress-related protein (Sanchez et al., 1990; Perdew & Whitelaw, 1991; Whitelaw et al., 1991; Smith et al., 1993). In addition to hsp70, p23 is required to form the receptor−hsp90 heterocomplex (Johnson & Toft, 1994). An analogous hsp-containing multiprotein complex has recently been described in yeast (Chang & Lindquist, 1994). The multiprotein complex isolated from reticulocyte lysate with an antibody to hsp90 acts as a self-sufficient

[†] This investigation was supported by National Institutes of Health Grant DK31573 (to W.B.P.) and by a grant from the National Sciences and Engineering Research Council of Canada (to P.K.). L.F.S. is a trainee under PSTP Training Grant GM07767.

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[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1996.

[1] Abbreviations: hsp, heat shock protein; GR, glucocorticoid receptor; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

0006-2960/96/0435-554$12.00/0

protein-folding structure, or "foldosome", in that it contains everything that is required to assemble the glucocorticoid receptor into a functional (i.e., steroid binding) heterocomplex with hsp90 (Hutchison et al., 1994a).

The reticulocyte lysate hsp heterocomplex appears to have a broad protein-folding activity in that it can form stable hsp90 complexes with some protein kinases (e.g., pp60^{src}, v-Raf) (Hutchison et al., 1992a,c; Stancato et al., 1993) and it can refold thermally denatured firefly luciferase to the active conformation (Schumacher et al., 1994). To date, all of the cell-free protein-folding experiments with the hsp heterocomplex have utilized rabbit reticulocyte lysate, and efforts to use cytosols prepared from other cell sources have failed. But, given the abundant, ubiquitous, and conserved nature of the proteins involved and the likelihood of a much broader protein-folding function than indicated by the most intensively studied steroid receptor model, it is reasonable to presume that the folding activity should itself be ubiquitous.

In 1979, this laboratory reported that glucocorticoid receptors that had been inactivated by incubating L cell cytosol at 25 °C could be reactivated to the steroid-binding form in an ATP-dependent manner (Sando et al., 1979). This inactivation of glucocorticoid-binding activity in L cell cytosol is now known to be due to dissociation of hsp90 from the receptor (Bresnick et al., 1989), and it follows that this previously reported ATP-dependent reactivation might have reflected re-formation of the GR−hsp90 heterocomplex. Thus, in this work, we show that a concentrated L cell cytosol assembles the glucocorticoid receptor into a heterocomplex with hsp90, and that concentrated cytosols prepared from cultured monkey (COS-7) and human (HeLa) cells are nearly as active at receptor folding as L cell cytosol. We also show that concentrated lysates of insect (Sf9) and plant (wheat germ) cells are efficient at forming functional GR heterocomplexes. These results suggest that the hsp chaperone activity is ubiquitous among eukaryotes. We have purified two components of the heterocomplex, hsp90 and hsp70, from wheat germ and from rabbit reticulocytes and have asked whether the function of these proteins is conserved in mammalian and plant folding systems, respectively. The plant hsp70 interacts with the other components of the rabbit reticulocyte folding system to yield functional receptor complexes with rabbit hsp90. Thus, the ability of hsp70 to interact with the other components of the folding system has been preserved since the divergence of the plant and animal kingdoms.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol) and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from Dupont NEN. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Wheat germ lysate was purchased from Promega, and the wheat germ used as a source for purification of hsp90 was from ICN Biomedicals (Aurora, OH). Protein A−Sepharose, ATP−agarose (coupled at the C8 position of the purine ring), (carboxymethyl)cellulose, and goat anti-mouse and anti-rabbit IgG−horseradish peroxidase conjugates were from Sigma. The BuGR2 monoclonal IgG antibody against the GR was from Affinity Bioreagents. The rabbit antiserum against hsp70 and hsp90 was a generous gift from Dr. Ettore Appella (National Cancer Institute). The rabbit R2 antiserum against plant hsp90 was raised against a fusion protein comprising a portion of *Pharbitis nil* (Japanese morning glory) hsp90 fused to the trpE protein of *E. coli*, and a detailed description of the antibody will be published elsewhere (P. Krishna, M. Sacco, and R. F. Felsheim, submitted manuscript). The L929 E8.2 cell line lacking the glucocorticoid receptor (Housley & Forsthoefel, 1989) was kindly provided by Dr. Paul Housley (University of South Carolina).

Cell Culture and Fractionation. L929 mouse fibroblasts (L cells) and the E8.2 L cell subline which does not express the gene for the glucocorticoid receptor were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. L cells were harvested by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500*g*. The washed cells were suspended in 1.5 volumes of HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100000*g*, with the supernatant from this step being the "cytosol" from which the GR was immunoadsorbed.

Receptor Immunoadsorption. Prior to immunoadsorption, antibody was prebound to protein A−Sepharose pellets by incubating 40 μL of a 20% slurry of protein A−Sepharose for 1 h at 4 °C with 40 μL of BuGR at a concentration of 10 μg/mL and 150 μL of TEG buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, and 10% glycerol, pH 7.6), followed by centrifugation and washing with TEG. Glucocorticoid receptors were immunoadsorbed from 400 μL aliquots of L cell cytosol by rotation for 2 h at 4 °C with 8 μL of protein A−Sepharose prebound with the BuGR antibody. Prior to incubation with reticulocyte and other lysates, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet an additional 2 h at 4 °C with 0.5 M NaCl followed by one wash with 1 mL of TEG and a second wash with HEPES.

Glucocorticoid Receptor Heterocomplex Reconstitution. Reconstitution of GR−hsp90 complexes was performed essentially as described by Scherrer et al. (1990). Immune pellets (8 μL of protein A−Sepharose) containing GR stripped of hsp90 were incubated with 50 μL of untreated rabbit reticulocyte lysate, E8.2 L cell cytosol, Sf9 cytosol, or wheat germ lysate. Cytosols used for reconstitution were prepared by rupturing E8.2 cells or Sf9 cells in 1 volume of 10 mM HEPES, pH 7.4, and centrifugation at 100000*g*. Dithiothreitol was added to each incubation to a final concentration of 5 mM, and 5 μL of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM $MgCl_2$, and 100 units/mL creatine phosphokinase) was added to all incubations to yield a final assay volume of 56 μL. The assay mixtures were incubated for 20 min at 30 °C with resuspension of the pellets by shaking the tubes every 5 min. At the end of the incubation, one-fourth of the suspension was removed for assay of steroid binding, and the remainder was used for Western blotting of receptor and associated proteins. The portion of the immunopellet used for steroid-binding assay was washed 1 time with 1 mL of iced TEGM (TEG buffer plus 20 mM sodium molybdate) while the portion used for Western blotting was washed 4 times with 1 mL of TEGM.

Assay of Steroid Binding Capacity. Immune pellets to be assayed for steroid binding were incubated overnight in 100 μL of TEGM buffer plus 5 mM dithiothreitol and 100 nM [^{3}H]triamcinolone acetonide. All samples were then washed

3 times with 1 mL of TEG buffer and counted by liquid scintillation spectrometry. Because one-fourth of an immunopellet prepared originally from 400 μL of L cell cytosol was used to assay steroid-binding activity, the binding values are presented as cpm [^{3}H]triamcinolone acetonide bound per BuGR immunopellet prepared from 100 μL of L cell cytosol. As noted previously (Hutchison et al., 1992b), 100 μL of L cell cytosol contains about 60 000 cpm of [^{3}H]triamcinolone acetonide binding capacity and with 10% BuGR we immunoadsorb about 50% of the glucocorticoid receptor. Thus, ~30 000 cpm represents 100% of receptors reactivated to the steroid-binding form, and in this paper, the extent of receptor reactivation with whole reticulocyte lysate varies from 50 to 100%.

Western Blotting of Receptor and Associated Proteins. For assay of GR and associated proteins, immune pellets were boiled in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved on 7% SDS−polyacrylamide gels as described previously (Hutchison et al., 1992b). Proteins were then transferred to Immobilon-P membranes and probed with 2 μg/mL BuGR2 monoclonal antibody for the glucocorticoid receptor, 0.05% rabbit antiserum for hsp70 and hsp90, or 0.2% R2 rabbit antiserum for plant hsp90. The immunoblots were then incubated a second time with the appropriate ^{125}I-labeled or horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands. In most cases, with appropriate cutting of the immunoblot, the GR, hsp90, and hsp70 can be assayed on a single immunotransfer.

Protein Purification. hsp90 was purified from rabbit brain cytosol and hsp70 from mouse L cell cytosol exactly as described by Hutchison et al. (1994b). The same procedure was used for purifying the plant hsp90 and hsp70 from 30 g of wheat germ, except that wheat germ lysate was dropped through a column (2 × 20 cm) of (carboxymethyl)cellulose prior to adsorption of the dropthrough fraction to DEAE-cellulose.

RESULTS

Mouse and Insect Cell Lysates Form Functional GR−hsp90 Complexes. Figure 1 (lane 5) shows the reconstitution of the GR−hsp90 heterocomplex by cytosol prepared from the E8.2 subline of the L cell. The E8.2 L cells are unique among glucocorticoid-resistant cell lines in that they do not express the gene for the receptor (Housley & Forstboefel, 1989). In our previous work reconstituting the glucocorticoid receptor heterocomplex with rabbit reticulocyte lysate, we have noted that the reconstituting activity declines markedly with as little as 1:1 dilution of the lysate. Thus, we have prepared a very concentrated L cell cytosol by mixing 1 volume of packed cells with only 1 volume of buffer prior to cell homogenization. In Figure 1, immunopurified L cell GR that had been stripped of associated proteins (lane 1) was incubated with rabbit reticulocyte lysate or concentrated E8.2 L cell cytosol. The receptor and receptor-associated hsp90 are shown in the Western blot and the steroid-binding activity in the bar graph. The reticulocyte lysate (lane 3) and the E8.2 L cell cytosol (lane 5) both reactivate the immunoadsorbed receptors. As the concentrated cytosol from cultured mouse cells was active at receptor heterocomplex assembly, we asked if similar cytosols prepared from other cultured mammalian cells were active. We found that freshly prepared, concentrated cytosols from monkey (COS-7) cells and human (HeLa) cells were nearly as active at receptor reactivation as the mouse E8.2 cytosol and rabbit reticulocyte lysate (data not shown).

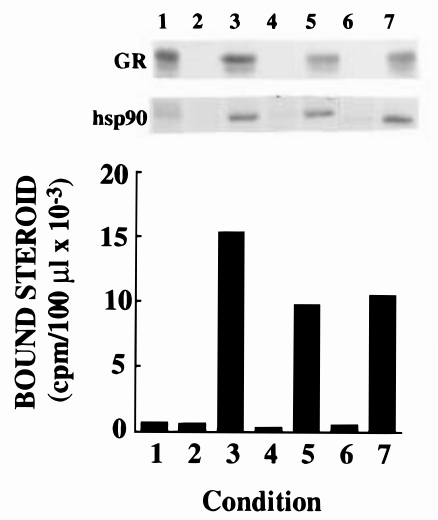

FIGURE 1: Cytosols from mouse and insect (Sf9) cells assemble the receptor heterocomplex. Glucocorticoid receptors were immunoadsorbed from aliquots of L cell cytosol to protein A−Sepharose prebound with BuGR or nonimmune IgG. Receptor-associated proteins were stripped from the indicated immunopellets by incubation with 0.5 M NaCl, and stripped immunopellets were incubated with reticulocyte lysate or E8.2 L cell cytosol or Sf9 cell cytosol as described under Experimental Procedures. Receptor and hsp90 were assayed in each sample by Western blotting, and a portion of the immunopellet was incubated with [^{3}H]triamcinolone acetonide to determine steroid-binding activity. Lane 1, stripped receptor; lanes 2 and 3, stripped nonimmune or immune pellet, respectively, incubated with reticulocyte lysate; lanes 4 and 5, stripped pellets incubated with E8.2 cytosol; lanes 6 and 7, stripped pellets incubated with Sf9 cytosol.

Previously, it was shown that glucocorticoid receptors that are overexpressed in Sf9 (*Spodoptera frugiperda*) insect cells using a baculovirus expression vector are in the heterocomplex form and bind steroid (Srinivasan & Thompson, 1990). Here, we show that concentrated Sf9 cytosol (Figure 1, lane 7) reactivates immunoadsorbed receptors to the same extent as mouse E8.2 cytosol (lane 5). It is clear that Sf9 cytosol forms a complex with the insect hsp90, which migrates somewhat faster than the rabbit and mouse hsp90s, and that the resulting complex between the mouse receptor and insect hsp90 binds steroid. In contrast to the insect cells, we were not able to prepare a lysate of yeast cells that was active at receptor heterocomplex assembly.

Assembly of Functional GR−hsp90 Complexes by Wheat Germ Lysate. In a previous study in which we translated the full-length glucocorticoid receptor in reticulocyte lysate, we found that the reticulocyte-translated receptor was bound to hsp90, bound steroid with high affinity, and did not have DNA-binding activity until it was transformed (Dalman et al., 1989). In contrast, receptor translated in wheat germ lysate had only trace steroid-binding activity, and it did not require transformation in order to bind to DNA (Dalman et al., 1989, 1990). Thus, it seemed that the plant lysate was not producing a functional receptor−hsp90 complex, but that it could not be directly tested at that time due to lack of an antibody against plant hsp90. Two developments allow us to return to the question of whether a plant lysate can form a receptor−hsp90 heterocomplex and whether a plant chaperone system can fold the hormone-binding domain to the steroid-binding conformation. First, the availability of the R2 antiserum, which reacts with plant but not animal

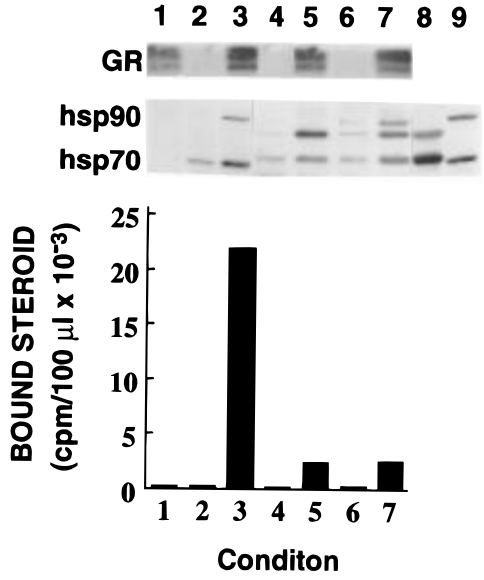

FIGURE 2: Wheat germ lysate assembles the receptor heterocomplex. Immunoadsorbed stripped receptors were incubated with reticulocyte lysate or wheat germ lysate or with wheat germ lysate plus 120 μg of purified rabbit hsp90, and samples were assayed for steroid-binding activity and immunoblotted for receptor and associated proteins. The hsp70 and the hsp90 region of the Western blot was probed with anti-hsp70/hsp90 serum to detect rabbit hsp90 and hsp70 and wheat hsp70, and it was also probed with the R2 antibody to detect wheat hsp90. Lane 1, stripped receptor; lanes 2 and 3, stripped nonimmune or immune pellet, respectively, incubated with reticulocyte lysate; lanes 4 and 5, stripped nonimmune and immune pellets incubated with wheat germ lysate; lanes 6 and 7, nonimmune and immune pellets incubated with wheat germ lysate and purified rabbit hsp90; lane 8, 10 μL of wheat germ lysate; lane 9, 10 μL of rabbit reticulocyte lysate.

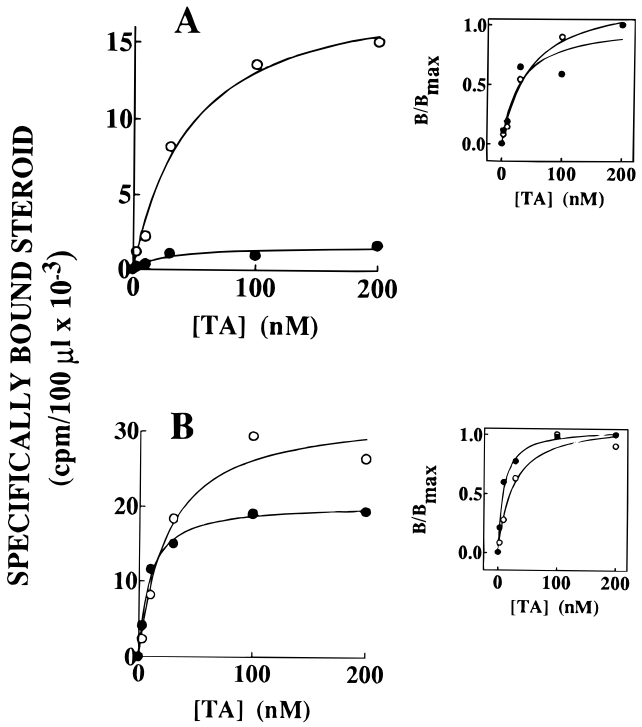

FIGURE 3: Binding of radiolabeled steroid to receptors during incubation with wheat germ lysate detects receptor heterocomplexes that are not detected by incubation of washed, reconstituted immunopellets. Immunoadsorbed stripped receptors were incubated with wheat germ lysate (A) or reticulocyte lysate (B). In samples identified by open circles, [^{3}H]triamcinolone acetonide was present during the 20 min incubation with lysate at 30 °C. In the samples identified by closed circles, the washed reconstituted immunopellets were suspended in buffer for 16 h at 4 °C with [^{3}H]triamcinolone acetonide, as in the previous figures. Values for nonspecific binding determined by binding to nonimmune pellets were only 1−2% of total binding and have been subtracted. The abscissa represents the concentration of [^{3}H]triamcinolone acetonide, [TA]. The insets to the right of each panel show the data plotted as B/B_{max} to demonstrate that the binding curves are similar under the two conditions. The lines were generated by a least-squares fit to a single-site binding isotherm.

hsp90, permits direct detection of any heterocomplexes formed between receptor and the plant hsp90. Second, in our heterocomplex reconstitution system, we are assaying steroid binding with at least 10 times more receptor in the immunopellet than we had in incubations where we translated the receptor *in vitro*. Thus, if the wheat germ lysate reconstitutes only low levels of steroid-binding activity, we have a higher likelihood of detection.

In the experiment of Figure 2, immunoadsorbed receptors were incubated with either reticulocyte lysate or wheat germ lysate, and the receptor-associated hsp90 was detected by Western blotting, using antiserum against hsp70/hsp90 to detect rabbit and wheat hsp70 and rabbit hsp90, and the R2 antiserum to detect wheat hsp90. The antiserum against hsp70/hsp90 has a weak reactivity with the plant hsp90, but the reactivity is not sufficient to detect the amount of protein that is bound to the receptor in the reconstitution assay. As shown in Figure 2, incubation of stripped receptors (lane 1) with wheat germ lysate yielded a complex with wheat germ hsp90 (lane 5), which migrates somewhat faster than the rabbit hsp90 (cf. lanes 8 and 9). As shown by the bar graph, receptors reactivated with rabbit reticulocyte lysate had approximately 10 times the steroid-binding activity of receptors reactivated with wheat germ lysate (cf. lanes 3 and 5).

There are several possible explanations for the low steroid-binding activity of receptors incubated with wheat germ lysate. One possibility is that the receptor−plant hsp90 complex has a much lower steroid-binding affinity than the receptor−rabbit hsp90 complex. A second possibility is that both receptor−wheat hsp90 complexes and receptor−rabbit hsp90 complexes bind steroid with similar affinities, but that only ∼10% of the receptors incubated with wheat germ lysate are bound to hsp90 versus those incubated with reticulocyte lysate. Smith (1993) has shown that the assembly of receptor−hsp90 complexes in reticulocyte lysate is a dynamic process in which complex assembly and disassembly proceed simultaneously. It is possible that during the 30 °C incubation in wheat germ lysate there is a relatively higher rate of disassembly versus assembly relative to reticulocyte lysate such that only ∼10% of the receptors incubated in wheat germ lysate are in complex with hsp90 when steroid binding is assayed. If this is the case, then it might be possible to trap the steroid-binding state by having [^{3}H]triamcinolone acetonide present during the 20 min incubation with the wheat germ lysate at 30 °C. hsp90 is required for steroid binding, but after steroid is bound, hsp90 is not necessary for maintenance of binding. In the experiments shown in Figure 3, stripped receptors were incubated with wheat germ lysate (Figure 3A) or reticulocyte lysate (Figure 3B) in the presence of various concentrations of [^{3}H]triamcinolone acetonide which were present during the 30 °C incubation with lysate (open circles). Identical immunopellets were reconstituted and incubated with steroid only after lysate removal and washing (closed circles) as in the previous experiments.

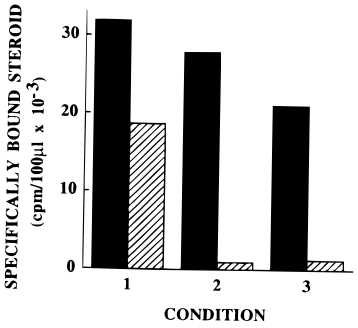

FIGURE 4: Evidence that only a small fraction of receptors are in the heterocomplex form at any time during incubation with wheat germ lysate. Stripped receptors were incubated with reticulocyte lysate (solid bars) or wheat germ lysate (hatched bars), and steroid binding was determined under three conditions with 100 nM [^{3}H]-triamcinolone acetonide. Condition 1, [^{3}H]steroid was present only during the 20 min incubation with lysate at 30 °C. Condition 2, receptors were incubated with lysate at 30 °C; the incubation mixture was placed in ice and then incubated with [^{3}H]steroid for 16 h at 0 °C. Condition 3, washed reconstituted immunopellets were suspended in buffer with [^{3}H]steroid for 16 h at 0 °C. The stripped receptor sample bound 2200 cpm/100 μL, and this value has been subtracted from the binding values in the figure.

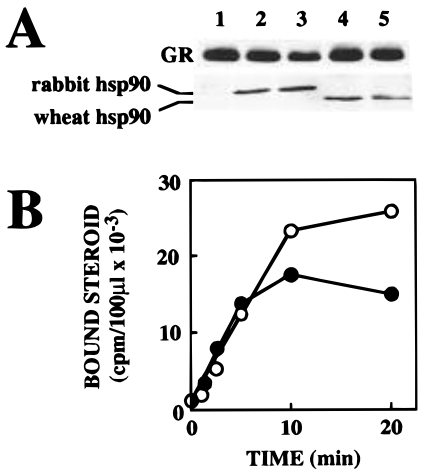

FIGURE 5: Evidence that the rates of GR−hsp90 complex assembly are similar in wheat germ and reticulocyte lysates. (A) Stability of GR−hsp90 complexes at 0 °C. Immunoadsorbed stripped receptors were incubated with reticulocyte lysate or wheat germ lysate. After being washed, the immunopellets were suspended in 100 μL of HE buffer and incubated on ice for 16 h. After a second washing, receptor and receptor-associated hsp90 were assayed by SDS−PAGE and Western blotting. Lane 1, stripped GR; lane 2, GR−rabbit hsp90 complex; lane 3, GR−rabbit hsp90 complex incubated on ice for 16 h; lane 4, GR−wheat hsp90 complex; lane 5, GR−wheat hsp90 complex incubated for 16 h. (B) Rate of generation of steroid-binding activity by rabbit reticulocyte and wheat germ lysates. Immunoadsorbed stripped receptors were incubated at 30 °C with rabbit reticulocyte lysate (open circles) or wheat germ lysate (closed circles) in the presence of 100 nM [^{3}H]triamcinolone acetonide, and at the indicated times, the amount of steroid bound to the immunopellet was assayed.

It is clear from Figure 3A that having the steroid present during the reconstitution with wheat germ lysate yields much more steroid binding than when receptors are exposed to steroid only after heterocomplex reconstitution. In the case of the rabbit reticulocyte reconstitution system, the difference between the two methods of steroid binding is much less. It also seems clear that the receptor−plant hsp90 complex has an apparent affinity for [^{3}H]triamcinolone acetonide similar to that of the receptor−rabbit hsp90 complex. It should be noted that the curves described by the open circles do not represent equilibrium binding but merely the amount of steroid bound during the 20 min incubation with lysate. However, the curves described by the closed circles do represent equilibrium binding at 0 °C, and as shown in the insets to each panel, the curves for the lysates are similar when plotted as the fraction bound versus steroid concentration. The apparent K_D's for the solid and open circles in Figure 3A were both 29 nM, and in Figure 3B, they were 9 and 21 nM, respectively. In the experiment of Figure 3, the wheat germ lysate yielded about half the steroid-binding activity of the reticulocyte lysate when the ligand was present during the reconstitution. Over the course of several experiments, we have found that the wheat germ lysate generates an average of 65% of the steroid-binding capacity that is generated by reticulocyte lysate, and that fraction is not increased by increasing the [^{3}H]trimcinolone acetonide concentration above 200 nM.

The experiment of Figure 3A suggests that the wheat germ lysate makes many more receptor−hsp90 complexes than is apparent if receptors are exposed to steroid only at the end of the incubation with lysate. As suggested above, this could reflect a different ratio of rates of heterocomplex assembly and disassembly, such that the actual fraction of receptors in heterocomplex form at any time is low with respect to reticulocyte lysate. Alternatively, the fraction of receptors in heterocomplex with plant hsp90 during the incubation may be high, but the plant hsp90 dissociates from the receptor when the lysate is removed and the immunopellet is washed prior to incubation with [^{3}H]steroid. In the experiment of Figure 4, stripped receptors were incubated with 100 nM [^{3}H]triamcinolone acetonide during the 20 min incubation with wheat germ lysate at 30 °C (condition 1), or, following reconstitution at 30 °C, the mixture of receptors and lysate was immediately placed in ice and [^{3}H]steroid was added (condition 2), or receptors were incubated with [^{3}H]steroid only after removal of lysate and washing the immunopellets (condition 3). It is clear from Figure 4 that the cooled wheat germ reconstitution mixture from which nothing has been removed has a very low steroid-binding capacity compared to the similarly treated reticulocyte lysate reconstitution mixture (condition 2). This observation, coupled with the fact that we do not see any difference in the inherent stability of receptor−wheat hsp90 and receptor−rabbit hsp90 complexes in the washed immunopellets (Figure 5A), is consistent with the notion that a small fraction of the receptors being incubated with wheat germ lysate is associated with hsp90 and that the presence of [^{3}H]steroid during the incubation with lysate (condition 1) "traps" the steroid-binding conformation. To use steroid binding as an accurate reporter of GR−hsp90 complex assembly, we assume that the rate of steroid binding is fast relative to the rates of complex assembly and disassembly.

The low level of GR−hsp90 complex present in wheat germ lysate *versus* reticulocyte lysate could reflect either a relatively low rate of complex assembly or a relatively high rate of complex disassembly during the 30 °C incubation with the wheat lysate. We can measure the rate of GR−hsp90 complex assembly indirectly by examining the initial rate of accumulation of steroid-bound GR when stripped receptors are incubated with lysate at 30 °C in the presence of [^{3}H]triamcinolone acetonide. As soon as a GR−hsp90 complex is formed, it is bound by steroid; thus, the rate of accumulation of steroid−receptor complexes approximates the rate of assembly of GR−hsp90 complexes. As shown in Figure 5B, the rates of accumulation of steroid−receptor

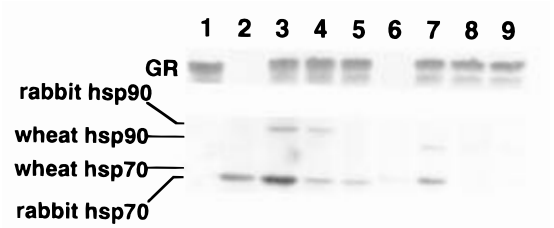

FIGURE 6: Molybdate does not stabilize the complex of receptor with plant hsp90. Immunoadsorbed stripped receptors were incubated with rabbit reticulocyte lysate or wheat germ lysate. After removal of the lysate, the immunopellets were washed once with buffer at 0 °C and suspended in 250 μL of TEG with or without 20 mM molybdate as indicated. Samples were then incubated 1 h at 0 or 30 °C, washed twice with TEGM buffer, and assayed for receptor, hsp90, and hsp70 by Western blotting. Lane 1, stripped receptor; lane 2, nonimmune pellet incubated with reticulocyte lysate; lanes 3−5, stripped receptor incubated with reticulocyte lysate and then washed and incubated at 0 °C without molybdate (lane 3), at 30 °C with molybdate (lane 4), or at 30 °C without molybdate (lane 5); lane 6, nonimmune pellet incubated with wheat germ lysate; lanes 7−9, stripped receptor incubated with wheat germ lysate and then washed and incubated at 0 °C without molybdate (lane 7), at 30 °C with molybdate (lane 8), or at 30 °C without molybdate (lane 9).

complexes are similar for wheat germ and reticulocyte lysates.

We have reported previously (Hutchison et al., 1992c; Stancato et al., 1993) that heterocomplexes formed in reticulocyte lysate between hsp90 and either the glucocorticoid receptor or the tyrosine kinases (pp60$^{v\text{-}src}$ and v-Raf) are stabilized by molybdate. It was surprising to find that addition of molybdate to the wheat germ lysate during heterocomplex reconstitution and the presence of molybdate in washing buffers after reconstitution did not affect either the amount of hsp90 recovered in the immunopellet or the steroid-binding activity of the immunopellet (data not shown). As shown in Figure 6, when receptor−rabbit hsp90 complexes are heated at 30 °C, hsp90 dissociates (cf. lanes 3 and 5) and the dissociation is inhibited by 20 mM molybdate (lane 4). However, dissociation of receptor−wheat hsp90 complexes (cf. lanes 7 and 9) is not inhibited by molybdate (lane 8).

Effect of Purified Wheat and Rabbit hsp90 When Added to the Other Lysate. To determine if the plant hsp90 would function in the rabbit lysate and *vice versa*, hsp90 was purified from wheat germ and from rabbit brain and added to the rabbit or wheat lysate, respectively. It should be mentioned that we do not, as yet, have a method by which we can deplete either lysate of its endogenous hsp90. Thus, the purified protein was added to whole lysate. As shown in Figure 2, addition of rabbit hsp90 to the wheat germ lysate results in assembly of receptor heterocomplexes with both rabbit hsp90 (upper hsp90 band in lane 7) and wheat hsp90 (lower hsp90 band in lane 7). Stripped receptors incubated with either wheat or rabbit hsp90 in the absence of lysate do not bind hsp90 (data not shown). Importantly, the low steroid-binding activity generated with the wheat germ lysate is not increased by the presence of rabbit hsp90 (cf. lanes 5 and 7 in bar graph). It should be noted that different antibodies were used to probe for rabbit and wheat hsp90 and, thus, the relative amounts of animal and plant hsp90 cannot be assessed.

As shown in Figure 7, when purified wheat hsp90 is added to the rabbit reticulocyte lysate, there is a concentration-dependent decline in the amount of rabbit hsp90 that becomes

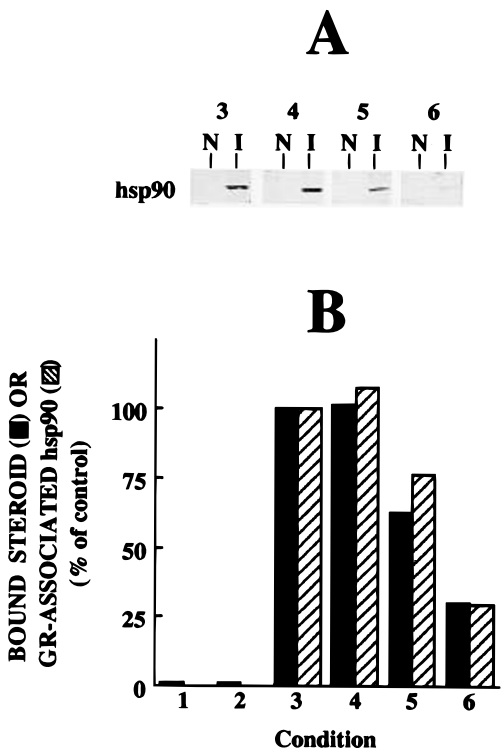

FIGURE 7: Purified plant hsp90 added to rabbit reticulocyte lysate inhibits formation of a receptor−rabbit hsp90 complex. Aliquots (50 μL) of rabbit reticulocyte lysate were preincubated 30 min at 30 °C with the indicated amounts of purified wheat germ hsp90, and the mixture was incubated 20 min at 30 °C with immunoadsorbed stripped receptors. After being washed with TEGM buffer, immunopellets were assayed for steroid binding and receptor-associated rabbit hsp90. The Western blot in panel A shows the rabbit hsp90 in nonimmune (N) and immune (I) (i.e., BuGR) pellets of conditions 3−6. In this experiment, the blot was incubated with both horseradish peroxidase-conjugated and ^{125}I-conjugated anti-rabbit IgGs. The peroxidase reaction was developed for the immunoblots shown, and the peroxidase-stained bands were cut out and counted in a gamma counter to determine the relative amount of ^{125}I-labeled counterantibody radioactivity in each hsp90 band. The bar graph in panel B shows the steroid binding (solid bars) and the amount of GR-associated hsp90 (hatched bars) in each condition plotted as a percent of that achieved with reticulocyte lysate alone (i.e., condition 3). Condition 1, stripped receptor; condition 2, stripped nonimmune pellet treated with reticulocyte lysate; condition 3, stripped receptor incubated with reticulocyte lysate without plant hsp90; conditions 4−6, stripped receptor incubated with reticulocyte lysate supplemented with 6 μg (lane 4), 30 μg (lane 5), or 120 μg (lane 6) of purified wheat germ hsp90.

bound to receptors and a similar decrease in the amount of steroid-binding activity that is generated. The concentration of endogenous rabbit hsp90 in the reticulocyte lysate is ~2 μM (Dalman et al., 1989), and under the condition of lane 6, the ratio of wheat hsp90 to rabbit hsp90 is about 7 to 1. Addition of 120 μg of purified rabbit hsp90 to the reticulocyte lysate has no effect on steroid-binding activity, suggesting that the decline is specific to the presence of excess wheat hsp90 in the reconstitution system. Although not determined in this particular experiment, we can show that a small amount of plant hsp90 is bound to the receptor under conditions like lanes 5 and 6 where there is a significant decrease in receptor−rabbit hsp90 heterocomplex assembly.

Purified Wheat hsp70 Is Completely Functional in Rabbit Reticulocyte Lysate. We have previously shown that reticulocyte lysate can be depleted of its endogenous hsp70 by passing the lysate through a column of ATP−agarose (Hutchison et al., 1994b). hsp70 has a high avidity for

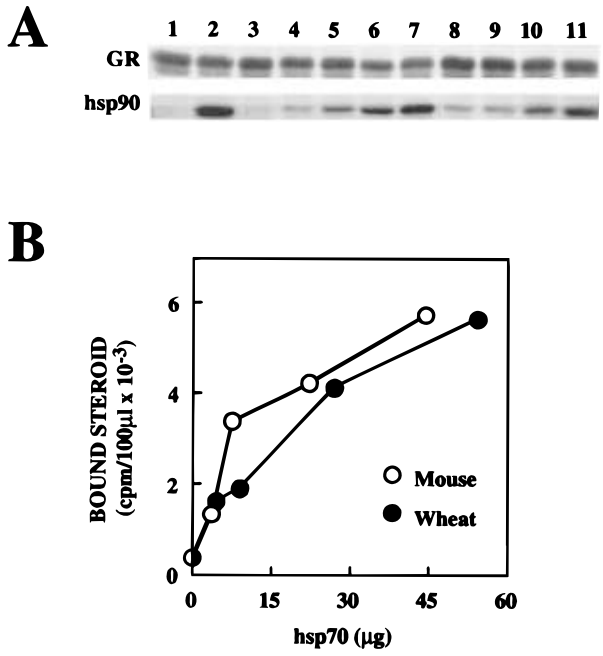

FIGURE 8: Purified wheat hsp70 is as potent as purified mouse hsp70 at reconstituting functional GR−hsp90 heterocomplex in hsp70-depleted rabbit reticulocyte lysate. Rabbit reticulocyte lysate was depleted of hsp70 by passage through a column of ATP−agarose exactly as described by Hutchison et al. (1994b). Immunoadsorbed stripped receptors were incubated 20 min at 30 °C with mock-extracted reticulocyte lysate (exposure to all conditions but ATP−agarose), hsp70-depleted lysate, or depleted lysate plus the indicated amounts of purified wheat or mouse hsp70. Samples were assayed for steroid-binding activity and for receptor and hsp90 by Western blotting. Panel A shows the Western blot. Lane 1, stripped receptor; lane 2, stripped receptor plus mock-depleted lysate; lane 3, stripped receptor plus depleted lysate; lanes 4−7, stripped receptor plus depleted lysate plus 4 μg (lane 4), 7.5 μg (lane 5), 22 μg (lane 6), or 44 μg (lane 7) of purified mouse hsp70; lanes 8−11, stripped receptor plus depleted lysate plus 4.5 μg (lane 8), 9 μg (lane 9), 27 μg (lane 10), or 54 μg (lane 11) of purified wheat hsp70. Panel B shows steroid binding with the various amounts of mouse (open circles) or wheat (closed circles) hsp70.

ATP−agarose and sticks to the matrix, while hsp90 is present in the dropthrough fraction, which serves as the hsp70-depleted lysate. As shown by the Western blot in Figure 8, hsp70-depleted lysate by itself (lane 3) does not form a heterocomplex between receptors and hsp90, but addition of either purified mouse hsp70 (lanes 4−7) or purified wheat hsp70 (lanes 8−11) permits heterocomplex assembly. As shown in the graph portion of Figure 8, purified wheat hsp70 (closed circles) is as effective as purified mouse hsp70 (open circles) at restoring receptor−hsp90 heterocomplex assembly activity. We cannot do the reverse experiment where mouse hsp70 is added to hsp70-depleted wheat germ lysate because even mock-extraction (i.e., exposure to all conditions but ATP−agarose) of wheat germ lysate destroys its heterocomplex reconstituting activity. We do not know why mock-extraction eliminates the activity of the wheat germ lysate, but this limitation means that we cannot say that plant and mammalian hsp70 are completely interchangeable.

DISCUSSION

Like reticulocyte lysate, concentrated L cell cytosol assembles the glucocorticoid receptor into a complex with hsp90 and returns the hormone-binding domain of the receptor to a steroid-binding conformation (Figure 1). In retrospect, it would seem that the heterocomplex assembly activity was first shown 15 years ago, when Sando et al. (1979) showed that heat-inactivated, unliganded glucocorticoid receptors in L cell cytosol were activated to the steroid-binding state by an ATP-dependent mechanism. Molybdate potentiated that ATP-dependent reactivation, an effect we now know is due to the metal's ability to stabilize the reassembled receptor−hsp90 complex. Because concentrated cytosols prepared from cultured insect (Figure 1), monkey (COS-7), and human (HeLa) cells also assemble functional receptor−hsp90 complexes, it would seem that this chaperone activity is broadly distributed (and possibly ubiquitous) throughout cells of the animal kingdom.

In 1990, Schena et al. (1991) demonstrated that the glucocorticoid receptor expressed in plant cells was capable of activating a reporter gene linked to glucocorticoid response elements, provided that the plant cells were treated with glucocorticoid. Thus, it would be predicted that plants have a similar system that assembles the receptor into a heterocomplex and that binding of the glucocorticoid receptor to plant hsp90 should determine a high-affinity steroid-binding conformation. The data of Figures 2−4 show that wheat germ lysate forms a receptor heterocomplex and that the complex with plant hsp90 is functional in the sense that the receptor is converted to the steroid-binding state.

There are differences between the wheat germ system and the rabbit reticulocyte systems. The wheat germ lysate appears to have a different ratio between the rates of receptor−hsp90 heterocomplex assembly and disassembly during the incubation with receptor at 30 °C such that only a small fraction of the receptor is associated with hsp90 at any time. As noted above, hsp90 must be bound to the GR for it to have steroid-binding activity, but once the steroid is bound, it remains bound to the receptor even though hsp90 dissociates. Thus, when the steroid is present during the incubation with wheat germ lysate (Figure 3A, Figure 4, condition 1, and Figure 5B), every time a receptor−hsp90 complex is assembled the steroid occupies the ligand-binding site. Generation of steroid binding under this incubation condition strongly suggests that a large fraction of the receptors must have been converted to the steroid-binding conformation during the incubation with wheat germ lysate (Figures 3A and 4). In condition 2 of Figure 4, steroid was not present during the incubation at 30 °C. Rather, at the end of the incubation at 30 °C, the sample was placed in ice and steroid was added in order to assay the amount of receptor−hsp90 complex that was present at that time. The extent of steroid binding shown in Figure 4, condition 2, indicates that only a small fraction of the receptors were present as receptor−hsp90 complexes. This suggests that, in wheat germ lysate at 30 °C, the rate of disassembly of complexes is rapid enough relative to the rate of assembly such that only a small proportion of the receptors are in complex with hsp90 at any time. In rabbit reticulocyte lysate, the rate of heterocomplex assembly versus disassembly is such that a higher proportion of receptors are in complex with hsp90 at any time, but here also the presence of steroid during the assay yields higher binding (Figure 3B). The data of Figure 5B strongly suggest that the initial rates of complex assembly in the two systems are similar. Because the complexes of GR with wheat and rabbit hsp90s seem to be equally stable at 0 °C, we suggest that the rate of complex disassembly during the 30 °C incubation with wheat germ lysate must be very rapid with respect to the rate of disassembly in reticulocyte lysate, accounting for a much

lower level of GR−wheat hsp90 complexes at any time.

In our experience, the complex with wheat hsp90 is unique in that molybdate appears to have no stabilizing effect (Figure 6). Because molybdate stabilizes hsp90 binding to both receptors and protein kinases (Hutchison et al., 1992a,c; Stancato et al., 1993), it is thought that the metal oxyanion interacts with the common hsp90 component of the complexes rather than with the protein that is bound to the heat shock protein (Hutchison et al., 1992c). Consistent with this interpretation, conformational changes have been demonstrated by alteration in the circular dichroism spectrum upon molybdate addition to purified hsp90 in solution (Csermely et al., 1993). If molybdate stabilizes receptor heterocomplexes through a direct interaction with hsp90, then it is possible that the metal oxyanion does not interact with the plant hsp90 or it does not induce the appropriate conformational change in the plant hsp90.

The purified wheat and rabbit hsp90s are not able to completely substitute for one another, but some activity in the other chaperone system is maintained. For example, rabbit hsp90 does not bind to the receptor by itself, but when added to wheat germ lysate, it can interact with the necessary plant factors such that some of it associates with the receptor (Figure 2). We have not been able to demonstrate, however, that the resulting complex is functional in terms of steroid-binding activity. A similar situation exists with purified wheat hsp90 added to reticulocyte lysate. We infer that the plant hsp90 interacts with rabbit factors because it inhibits the ability of the reticulocyte lysate to assemble a receptor−rabbit hsp90 complex (Figure 7) and also because a small amount of plant hsp90 associates with receptors when it is added to reticulocyte lysate (data not shown). However, as in the opposite situation, we are unable to demonstrate that any receptor−plant hsp90 complex formed in this way is functional. In previously published experiments where we incubated a washed immunoadsorbed hsp90 heterocomplex from rabbit reticulocyte lysate with stripped GR, we formed a GR−hsp90 complex that did not bind steroid unless other lysate factors were added (Hutchison et al., 1994a). In the experiments described here with the addition of purified hsp90, those factors required for a productive steroid-binding state are present in the lysate. Although our problem may reflect inherent differences between the wheat and rabbit hsp90s in terms of their abilities to react with factors in the other system, we think it most likely that our purification of hsp90 does not leave us with a completely active protein.

In contrast, the purification of hsp70 does leave us with an active protein, because purified wheat and mouse hsp70s are equally active in restoring the ability of hsp70-depleted reticulocyte lysate to form a functional complex between receptor and rabbit hsp90 (Figure 8). hsp70 possesses the ability to interact with and probably also to assist in the folding of hundreds of proteins. The fact that the plant hsp70 can interact productively with the mammalian multiprotein chaperone system attests to the very basic and essential function this ubiquitous and abundant hsp must perform in cells, be they animal cells or plant cells.

The fact that such diverse systems can form a receptor−hsp90 complex that returns the hormone-binding domain of the receptor to a steroid-binding conformation is consistent with a very basic and essential function for hsp90 in protein folding. It seems likely to us that the multiprotein complexes that have been isolated from animal and yeast cells (Sanchez et al., 1990; Perdew & Whitelaw, 1991; Whitelaw et al., 1991; Smith et al., 1993; Chang & Lindquist, 1994) will also be found in plant cells. The observations we present here also raise the possibility that receptors for plant hormones and growth factors may have brought their association with the hsp90 component of the chaperone complex under hormonal control, much as has happened with steroid and dioxin receptors in the animal kingdom (Pratt, 1993).

ACKNOWLEDGMENT

We thank Paul Housley for providing the mutant L cells without glucocorticoid receptors and Ettore Appella for providing antibody.

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BI9511649