Direct Evidence That the Glucocorticoid Receptor Binds to hsp90 at or near the Termination of Receptor Translation in Vitro*

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We have translated the rat glucocorticoid receptor in both reticulocyte lysate and in wheat germ extract. Receptor synthesized in the reticulocyte lysate is immunoabsorbed by the 8D3 monoclonal antibody directed against the 90-kDa heat shock protein (hsp90) and it has a normal ability to bind glucocorticoid in a high affinity manner. Although the wheat germ extract synthesizes the full length receptor, the receptor is not immunoabsorbed by 8D3 and we cannot demonstrate high affinity steroid binding. Receptor synthesized by the reticulocyte lysate can be immunoabsorbed by antibody directed against hsp90 as soon as the translation product is full length, suggesting that the receptor becomes associated with hsp90 late during translation or immediately at the termination of translation. When newly synthesized receptor is bound with steroid and incubated at 25°C, it is converted to a form that binds to DNA. This study provides direct evidence that association of hsp90 with the glucocorticoid receptor is a very early event and that the newly formed heteromeric receptor–hsp90 complex is fully competent to undergo transformation.

The steroid binding activity of glucocorticoid receptors (GR)1 in cytosol preparations is very labile, but the receptors can be stabilized in their steroid binding state by molybdate and some other group 6A transition metal oxyanions (1, 2). In addition to stabilizing the receptor in its steroid binding conformation, these metal anions inhibit the transformation of the receptor to its DNA binding state (see Ref. 3 for review of molybdate effects). The effects of molybdate on receptor function correlate well with the ability of molybdate to stabilize the association of the GR with the 90-kDa heat shock protein (4–6). The core heteromeric receptor unit that

is stabilized by molybdate contains two molecules of hsp90 per molecule of glucocorticoid binding protein (7–10). There is good reason to think that molybdate exerts its effects on the receptor by interacting with the binding site for an ubiquitous, endogenous metal anion that stabilizes the association of the GR with hsp90 and produces the same effects on GR function as molybdate (11, 12). Both molybdate and hsp90 appear to interact with the steroid binding domain of the receptor (13, 14).

Recently, we have reported that hydrogen peroxide, through its ability to promote disulfide bond formation, produces all of the effects on the GR that are produced by molybdate (15, 16). Since both peroxide and the transition metal oxyanions stabilize the association of hsp90 with unliganded receptors and since conditions that disrupt the heteromeric complex inactivate the steroid binding capacity of cytosols, we postulated that interaction of hsp90 with the GR might be required to generate and/or stabilize a competent steroid binding conformation of the receptor (16). This proposal was supported by the observations that the glucocorticoid binding capacity of immunopurified receptors correlates with the relative amount of hsp90 that is present and that immunopurified, hsp90-free GR does not bind steroid (17).

One of the major problems that has hampered the study of the receptor-hsp90 interaction is that no one has yet been able to reassociate hsp90 with any steroid receptor. It has also not been possible to reassociate hsp90 with the avian viral transforming protein pp60\565. The failure to reassociate hsp90 with the receptor has made it impossible to study directly any role of hsp90 in generating the steroid binding conformation of the GR. Glucocorticoid receptors that are translated in vitro by a reticulocyte lysate system are able to bind steroid (e.g. Refs. 18 and 19), and in this paper, we show that GR synthesized by the reticulocyte lysate in vitro becomes associated with hsp90 during or immediately at the termination of translation. Although GR synthesized in the rabbit reticulocyte lysate binds glucocorticoid with high affinity, GR synthesized in wheat germ lysate, where there is no identifiable hsp90, does not bind steroid. While this work was in progress, Denis and Gustafsson (20) reported that rat glucocorticoid receptor translated in reticulocyte lysate has a sedimentation coefficient of 9 S and can be transformed to the DNA binding state by heating the lysate at 25°C in the presence of steroid.

**EXPERIMENTAL PROCEDURES**

*Materials

\( L-[\beta^35]S\)Methionine (1100 Ci/mmol), \( ^{125}I\) labeled conjugate of goat anti-mouse IgG (9.3 mCi/mg), [6,7-\( ^3\)H]triamcinolone acetonide (42.5

\[1^3\]

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Ci/mmol), and [6,7-3H]dexamethasone-21-mesylate (48.9 Ci/mmol) were obtained from Du Pont-New England Nuclear. Sodium molybdate, radiodinated dexamethasone, nonimmune mouse IgG, TES, Tris, Heps, protein A-Sepharose CL-4B, bovine serum albumin, and goat anti-mouse IgM were from Sigma. Immobilon P membranes were from Millipore Corp. Rabbit reticuloocyte lysate, wheat germ lysate, and Ribobond core system transcription kit were from Promega. BuGR2 monoclonal antibody prepared against the rat glucocorticoid receptor (21) was kindly provided by Dr. Robert Harrison III (Dept. of Medicine, Univ. of Arkansas, Little Rock), and the AC88 monoclonal antibody against the 90-kDa heat shock protein (22) was kindly provided by Dr. David Toft (Dept. of Biochemistry and Molecular Biology, Mayo Medical School, Rochester, MN). The SD3 monoclonal antibody (IgM) was originally prepared against partially purified Ah receptor (the dioxin receptor) and subsequently found to be specific for hsp90 (29). The EC1 monoclonal antibody, which was originally prepared against partially purified rabbit progesterone receptor and subsequently found to react with a 59-kDa receptor-associated protein (24), was kindly provided by Dr. Lee Faber (Depts. of Obstetrics/ Gynecology and Physiology, Medical College of Ohio, Toledo, OH). Plasmid T3.1118 containing the gene for the rat glucocorticoid receptor was kindly provided by Dr. Keith Yamamoto (Dept. of Biochemistry and Biophysics, University of California, San Francisco).

Methods

In Vivo Transcription and Translation—Transcription reactions (100 ml) contained 2 ml of PvuII-linearized plasmid T3.1118, 2.5 ml ATP, CTP, GTP, and TTP, 10 ml DTT, 1 unit/ml RNase A, 20 units SP6 polymerase in the buffer provided by Promega. For 2 h at 37 °C, the mixture was extracted with phenol, followed by precipitation of nucleic acids with ethanol. The nucleic acid pellet was dissolved in 10–30 ml of diethyl pyrocarbonate-treated water.

Translation reactions (50 ml) contained 35 ml of rabbit reticuloocyte lysate or wheat germ lysate, 1 ml amino acids mix (minus methionine), 2 ml of RNA substrate in H2O, and 2 ml of [35S]methionine. Samples were incubated at 30 °C for times up to 1 h, and reactions were stopped by boiling in ice or in one experiment by incubation with 50 ml pyrogenic acid. For experiments in which hormone binding was measured and when translation products were analyzed by immunoblotting, 1 ml radiodinated methionine was used instead of the [35S]methionine.

Preparation of L Cell Cytosol—L292 murine fibroblasts were grown in monolayer culture in modified Eagle’s medium supplemented with calf serum. Cells in log phase were harvested by scraping into Earle’s saline and centrifuged at 800 × g for 5 min. Following a wash by resuspension into Earle’s saline and centrifugation, cells were resuspended in 1.5 volumes of 10 mM Heps, 1 mM EDTA, 10 mM molybdate, pH 7.35 at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged at 100,000 × g for 1 h, and after removal of the floating lipid layer the supernatant (referred to as lysate or cytosol) was frozen.

Incubation with Antibodies and Adsorption to Protein A-Sepharose—BuGR2, AC88, and EC1 antibodies were prebound to 30 ml of protein A-Sepharose pellets in 0.1 ml of TEG buffer for 2 h at 4 °C with rotation and pellets were then washed once with TEG buffer to remove unbound antibody. The SD3 monoclonal antibody against hsp90 (30 ng of IgM) was adsorbed to 30 ml of protein A-Sepharose that had been prebound with goat IgG directed against mouse IgM. Translation reactions (25 ml) were diluted to 100 ml with cold TEG buffer containing 20 mM molybdate and were added to protein A-Sepharose pellets with prebound antibodies. After rotation for 3 h at 4 °C, Sepharose pellets were washed three times with 1-ml aliquots of TEG buffer containing 10 mM molybdate. Samples were then boiled in SDS sample buffer and proteins were resolved by SDS-PAGE.

Gel Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed in 1.5- or 3%-w/v slab gels according to the procedure of Laemmli (5). Slab gels were cooled to 2 °C during electrophoresis. All samples were extracted from protein A-Sepharose by boiling in SDS sample buffer containing 10% β-mercaptoethanol. M, standards were: myosin, M, = 205,000; β-galactosidase, M, = 116,000; phosphorylase b, M, = 97,000; bovine serum albumin, M, = 66,000; ovalbumin, M, = 45,000; and carbonic anhydrase, M, = 29,000.

Immunoblotting was carried out by transferring proteins from polyacrylamide slab gels to Immobilon P membrane, followed by overnight incubation with 1% BuGR antibody against the glucocorticoid receptor or 0.3% AC88 antibody against hsp90. Immunoblotted proteins were detected by reaction with 125I-conjugated goat anti-mouse IgG, followed by autoradiography.

Steroid Binding—Translation reactions prepared with nonradioactive methionine were diluted 1:1 with Heps buffer containing 40 mM sodium molybdate and incubated for 4 h at 0 °C with either 100 nM [3H]dexamethasone mesylate or 25 nM [3H]triamcinolone acetonide in the presence or absence of either 50 or 25 μM radiodinated dexamethasone, respectively. Receptors bound with [3H]dexamethasone-21-mesylate were resolved by SDS-PAGE. In the case of [3H]triamcinolone acetonide-bound receptors, free steroid was removed with charcoal and specific binding was determined by subtracting the nonspecific value obtained in the presence of nonradioactive dexamethasone from the total binding obtained in the presence of vehicle. In the rabbit reticuloocyte lysate, nonspecific binding of [3H]triamcinolone acetonide is 15–20% of the total binding, and in the wheat germ lysate, all of the binding observed at 25 nM [3H]triamcinolone acetonide is nonspecific. Twenty-five nM triamcinolone acetonide is well beyond the concentration required to occupy all receptor sites having a normal dissociation constant for this steroid in the range of 2 nM (26).

Receptor Transformation and DNA Binding—Reticuloocyte lysate containing [35S]methionine-labeled translation products was diluted with an equal volume of 10 mM Heps buffer, pH 7.4, with 10 mM DTT and 10% glycerol, with or without 40 mM sodium molybdate, and incubated 2 h on ice with 100 nM triamcinolone acetonide. This mixture and receptor sterones was diluted with 2.5 volumes of Heps buffer with or without molybdate and incubated 25 min at 25 °C to transform receptors. An equal volume of 12.5% DNA-cellulose was added and the mixture was rotated at 4 °C for 45 min. After washing the DNA-cellulose pellet three times, DNA-bound proteins were eluted by boiling in 2 × SDS sample buffer.

Purification of [3H]hsp90 from L Cells—[3H]hsp90 was purified by a modification of the method of Welch and Feramisco (27). L cell cytosol (20 ml at 10.7 mg of protein/ml) was chromatographed on a 2 × 20-cm DEAE column equilibrated in 10 mM Tris, 0.1 mM EDTA, pH 7.1, and proteins were eluted with a 200-ml gradient of 0–0.4 M KCl.

Samples of eluted fractions were resolved by SDS-PAGE and hsp90 was detected by immunoblotting with the AC88 antibody. Fractions containing hsp90 were pooled, concentrated by adsorption with polyethylene glycol. The concentrated material was diluted with an equal volume of 20 mM K2HPO4, 1 mM EDTA, pH 7.5, and was chromatographed on a 2 × 8-cm hydroxylapatite column (which was equilibrated in the same K2HPO4 buffer), and proteins were eluted with a 200-ml gradient of 0–0.4 M K2HPO4. Fractions containing hsp90 were pooled, concentrated by polyethylene glycol adsorption, and frozen in aliquots at −70 °C. Prior to addition to the translation reactions, the hsp90 preparation was diluted in Heps buffer and concentrated by Centricon centrifugal devices to remove salt. hsp90 was prepared in this manner is about 98% pure.2 The protein concentration of the hsp90 preparation was determined by amino acid analysis.

RESULTS

Properties of GR Synthesized by Reticuloocyte and Wheat Germ Lysates—Fig. 1 shows the [35S]methionine-labeled proteins synthesized by rabbit reticuloocyte and wheat germ lysates after addition of Brome mosaic viral RNA or GR RNA. With 60 min of translation, both systems produce the full length rat GR. As with a large number of other mRNAs, the wheat lysate is less efficient than the reticuloocyte lysate at synthesizing both the viral proteins and the GR. For this reason, twice as much GR RNA and two to three times as much wheat germ lysate was added to subsequent incubations as to those with reticuloocyte lysate. In the wheat germ system, the specific activity of the [35S]methionine is not diluted by endogenous methionine, but according to the manufacturer, the reticuloocyte lysate contains about 5 μM endogenous methionine; thus, the difference in translation efficiency is greater than that indicated by the [35S]methionine autoradiograms presented in this paper.

From the experiment shown in Fig. 2, one can obtain a general impression of the relative amount of rat GR synthesized in the reticuloocyte lysate. In this experiment, the rat GR was immunoadsorbed from the lysate, resolved by SDS-
with no added RNA; translation of PvuII-linearized plasmid pT3.1118 was as described under "Methods." Translation was carried out in the presence of $^{35}$S methionine and total lysates were resolved by SDS-PAGE and autoradiography. Samples applied to the gel were: lane 1, rabbit lysate with no RNA; lane 2, rabbit lysate with Brome mosaic viral RNA; lane 3, rabbit lysate with rat GR RNA; lane 4, wheat lysate with no RNA; lane 5, wheat lysate with Brome mosaic viral RNA; lane 6, wheat lysate with rat GR RNA.

Fig. 2. Immunoreactivity of GR translated in the rabbit reticulocyte lysate. Unlabeled translation products were immunoabsorbed with nonimmune IgG (lanes 1 and 3) or BuGR (lanes 2 and 4). Proteins were resolved by SDS-PAGE, transferred to an Immobilon membrane, and the blot was developed by reacting with BuGR, followed by $^{125}$I-conjugated goat anti-mouse IgG. Proteins were visualized by autoradiography. Samples applied to the gel were: lane 1, lysate with no RNA immunoadsorbed with nonimmune IgG; lane 2, lysate with no RNA immunoadsorbed with BuGR; lane 3, lysate with GR RNA immunoadsorbed with nonimmune IgG; lane 4, lysate with GR RNA immunoadsorbed with BuGR; lane 5, 10 µl of L cell cytosol boiled directly in SDS sample buffer and added directly to the gel.

PAGE, and immunoblotted with the BuGR anti-GR antibody, followed by $^{125}$I-conjugated anti-mouse IgG. The autoradiogram shows the in vitro translated rat GR in lane 4 and the GR contained in 10 µl of mouse L cell cytosol in lane 5. For unknown reasons, the cytosolic mouse GR routinely migrates at 98–100 kDa, whereas the rat GR migrates at 94 kDa (see Fig. 5 in Ref. 16), despite the fact that the rat GR has a slightly longer amino acid chain. Fig. 2 shows that the in vitro translated rat GR migrates in a similar more rapid fashion with respect to the cytosolic mouse GR. Our standard L cell cytosol contains about 10 pmol of GR/ml. By excising and counting the radioactivity in the receptor bands in lanes 4 and 5, it was estimated that the lysate reaction synthesized an amount of rat GR equivalent to that present in 0.64 µl of L cell cytosol or 180 fmol of rat GR/ml of lysate (assuming equivalent reaction of the BuGR antibody with the rat and mouse GR).

The experiment of Fig. 3 examines the steroid binding capacity of the GR translated in both systems. Fig. 3A shows the $^{35}$S methionine-labeled receptor and Fig. 3B shows the translation products after site specific affinity labeling with $^{3}$$H$[dexamethasone 21-mesylate. The $^{35}$S methionine-labeled bands (the full length product only) and the $^{3}$$H$ dexamethasone 21-mesylate-labeled band were excised and counted. The wheat germ lysate synthesized 87% as much full

![Fig. 1. In vitro translation of the rat glucocorticoid receptor in rabbit reticulocyte and wheat germ lysates. Transcription of PvuII-linearized plasmid pT3.1118 was as described under "Methods." Translation was carried out in the presence of $^{35}$S methionine and total lysates were resolved by SDS-PAGE and autoradiography. Samples applied to the gel were: lane 1, rabbit lysate with no added RNA; lane 2, rabbit lysate with Brome mosaic viral RNA; lane 3, rabbit lysate with rat GR RNA; lane 4, wheat lysate with no RNA; lane 5, wheat lysate with Brome mosaic viral RNA; lane 6, wheat lysate with rat GR RNA.](image)

![Fig. 2. Immunoreactivity of GR translated in the rabbit reticulocyte lysate. Unlabeled translation products were immunoabsorbed with nonimmune IgG (lanes 1 and 3) or BuGR (lanes 2 and 4). Proteins were resolved by SDS-PAGE, transferred to an Immobilon membrane, and the blot was developed by reacting with BuGR, followed by $^{125}$I-conjugated goat anti-mouse IgG. Proteins were visualized by autoradiography. Samples applied to the gel were: lane 1, lysate with no RNA immunoadsorbed with nonimmune IgG; lane 2, lysate with no RNA immunoadsorbed with BuGR; lane 3, lysate with GR RNA immunoadsorbed with nonimmune IgG; lane 4, lysate with GR RNA immunoadsorbed with BuGR; lane 5, 10 µl of L cell cytosol boiled directly in SDS sample buffer and added directly to the gel.](image)

![Fig. 3. Steroid binding activity of GR translated in rabbit reticulocyte and wheat germ lysates. A, translation was carried out in the presence of $^{35}$S methionine, and receptors were immunoadsorbed with BuGR, and resolved by SDS-PAGE and autoradiography. Lane 1, rabbit reticulocyte lysate with GR RNA; lane 2, wheat germ lysate with GR RNA. B, unlabeled translation products (50 µ) were made 10 mM with respect to molybdate, incubated with 100 nM $^{3}$$H$ dexamethasone 21-mesylate in the presence or absence of competing dexamethasone, immunoadsorbed with BuGR, and resolved by SDS-PAGE and autoradiography (developed for 2 days). Lane 1, rabbit lysate with GR RNA labeled with $^{3}$$H$ dexamethasone 21-mesylate alone; lane 2, rabbit lysate with GR RNA labeled with $^{3}$$H$ dexamethasone 21-mesylate in the presence of competing dexamethasone; lane 3, wheat lysate with GR RNA labeled with $^{3}$$H$ dexamethasone 21-mesylate in the presence of competing dexamethasone; lane 4, wheat lysate with GR RNA labeled with $^{3}$$H$ dexamethasone 21-mesylate in the presence of competing dexamethasone. C, unlabeled translation products from reactions performed with or without GR RNA were diluted with an equal volume of Hepes buffer containing 20 mM molybdate and 20 mM DTT and incubated with 25 nM $^{3}$$H$ triamcinolone acetonide (7A) in the presence or absence of competing dexamethasone. Steroid binding was assayed by charcoal absorption and values are expressed as counts/min of specifically bound $^{3}$$H$ triamcinolone acetonide. D, aliquots of unlabeled reticulocyte lysate translation products were incubated with various concentrations of $^{3}$$H$ triamcinolone acetonide in the presence (○) or absence (●) of competing radioinert dexamethasone and steroid binding was assayed by charcoal absorption.](image)
length GR as rabbit reticulocyte lysate by \[^{[35}S\]methionine labeling but there was no comparable labeling of the wheat germ translation product with \[^{[3}H\]dexamethasone 21-mesy late. It should be mentioned that in some experiments we can see trace labeling of the wheat germ translation product. If the gel of Fig. 3B is developed for 2 weeks, for example, on direct examination of the autoradiogram a faint band is visible at M,, 94,000 in lane 3 but not in lane 4.

Consistent with the \[^{[3}H\]dexamethasone 21-mesylate labeling, only GR synthesized by the reticulocyte lysate bound \[^{[3}H\]triamcinolone acetonide as determined by charcoal absorption assay (Fig. 3C). In this particular synthesis, there were 2.7 pmol of \[^{[3}H\]triamcinolone acetonide bound specifically per ml of reticulocyte lysate. This estimate of the concentration of synthesized rat GR is roughly an order of magnitude higher than the estimate made from radioactive immunoblotting in the experiment of Fig. 2. It should be noted, however, that the amount of GR synthesized from one experiment to the next varies over about a 5-fold range, both on the basis of \[^{[35}S\]methionine labeling and of \[^{[3}H\]steroid binding.

Fig. 3D shows the concentration dependence of \[^{[3}H\]triamcinolone acetonide binding to the reticulocyte lysate translation product. The apparent \(K_d\) from one half saturation is about 3 nM, which is a normal binding affinity for this ligand (26).

GR Translated in the Reticulocyte Lysate Is Bound to hsp90—The reticulocyte lysate contains a 90-kDa protein that reacts with the AC88 monoclonal antibody against hsp90 on immunoblotting, but the wheat germ lysate does not (data not shown). The amount of hsp90 in the reticulocyte lysate can be estimated by the method shown in Fig. 4. In this method, aliquots of reticulocyte lysate or L cell cytosol were resolved on SDS-PAGE along with standardized amounts of purified hsp90. The relative amount of hsp90 in each lane was determined by quantitative immunoblotting with AC88 followed by \[^{[3]}I\]anti-mouse IgG. After excision of the bands and counting, the amount of hsp90 is determined from a standard curve as shown in the figure. From two such experiments, we determined that the reticulocyte lysate contains about 0.15 mg of hsp90/ml of lysate, or about 1.6 \(\mu\)g of hsp90/mg protein. This is comparable to the concentration of hsp90 in our standard L cell cytosol, which we calculate to be 0.09 mg of hsp90/ml or 4.5 \(\mu\)g/mg protein (L cell cytosol has a lower protein content than reticulocyte lysate). Thus, L cell cytosol contains roughly 1 \(\mu\)m hsp90, with the ratio of hsp90 to GR being approximately 100:1. The reticulocyte lysate contains approximately 2 \(\mu\)m hsp90, and because of the relatively low and variable amounts of GR produced from one experiment to another, the hsp90 to GR ratio ranges from about 300:1 to more than 1500:1.

Because the absolute amount of GR synthesized in the 25–50 \(\mu\)l of reticulocyte lysates is so small, we cannot employ our usual technique of detecting receptor-associated hsp90, which involves immunoadsorbing receptors with the BuGR anti-receptor antibody and then detecting the immune-specific presence of hsp90 by immunoblotting using AC88 as a probe for hsp90 (5, 6, 17). Thus, we used the method shown in Fig. 5, which takes advantage of the strong \[^{[35}S\]methionine signal produced by the limited number of GR molecules that are present. As shown in \(\lambda\)ne 6 of Fig. 5, the GR can be immunoadsorbed with the 8D3 monoclonal (IGM) and absorption is immune-specific as suggested by the protein A-Sepharose-bound anti-IgM control of \(\lambda\)ane 5. In contrast, the EC1 monoclonal antibody against the 59-kDa receptor-associated protein does not immunoadsorb the newly synthesized receptor.

In the experiment of Fig. 6, the amount of protein A-Sepharose-bound 8D3 anti-hsp90 antibody was tripled to permit immunoadsorption of most of the hsp90 in the reticulocyte lysate. The relative amount of hsp90 (Fig. 6A) recovered in the pellet and supernatant after immunoadsorption was determined by immunoblotting samples and probing them with the AC88 antibody against hsp90 followed by \[^{[3]}I\]conjugated goat anti-mouse IgG and peroxidase-conjugated rabbit anti-goat IgG. The peroxidase-stained hsp90 bands were excised and counted for \[^{[3]}I\]radioactivity. The inset shows an autoradiogram of the gel: lane 1, L cell cytosol; lane 2, rabbit reticulocyte lysate; lanes 3–7, 0.08, 0.16, 0.32, 0.63, and 0.95 \(\mu\)g of purified hsp90. The solid circles in the main figure represent the radioactivity from the purified hsp90 standards and the triangle and the square represent the radioactivity from L cell and reticulocyte hsp90, respectively, plotted on the standard curve.

**Fig. 4. Quantitation of the hsp90 concentration in rabbit reticulocyte lysate and L cell cytosol.** Aliquots of L cell cytosol (7.5 \(\mu\)l) and rabbit reticulocyte lysate (5 \(\mu\)l) were resolved by SDS-PAGE on a gel that also contained standardized amounts of purified L cell hsp90. The proteins were transferred to an Immobilon P membrane and the blot was probed with the AC88 antibody, followed by \[^{[3]}I\]conjugated anti-mouse IgG and peroxidase-conjugated rabbit anti-goat IgG. The peroxidase-stained hsp90 bands were excised and counted for \[^{[3]}I\]radioactivity. The inset shows an autoradiogram of the gel: lane 1, L cell cytosol; lane 2, rabbit reticulocyte lysate; lanes 3–7, 0.08, 0.16, 0.32, 0.63, and 0.95 \(\mu\)g of purified hsp90. The solid circles in the main figure represent the radioactivity from the purified hsp90 standards and the triangle and the square represent the radioactivity from L cell and reticulocyte hsp90, respectively, plotted on the standard curve.

Sepharose-bound 8D3 anti-hsp90 antibody was tripled to permit immunoadsorption of most of the hsp90 in the reticulocyte lysate. The relative amount of hsp90 (Fig. 6A) recovered in the pellet and supernatant after immunoadsorption was determined by immunoblotting samples and probing them with the AC88 antibody against hsp90 followed by \[^{[3]}I\]conjugated goat anti-mouse IgG. The relative amount of GR in each sample was determined after excising and counting the complete translation product labeled with \[^{[35}S\]methionine (Fig. 6B). When this larger amount of antibody was used, we immunoadsorbed 65% of the hsp90 and 51% of the \[^{[35}S\]methionine-labeled full length translation product. This suggests that nearly all of the newly transformed GR may be bound to hsp90 and would be immunoadsorbed if all of the hsp90 could be immunoadsorbed.

**Evidence That Binding to hsp90 Occurs Very Late in the Translation Process**—To determine if the GR was undergoing cotranslational association with hsp90, we performed time course experiments like that of Fig. 7. As shown in Fig. 7A, full length receptor produced after 20 or 30 min of translation is coadsorbed in an immune-specific manner by the 8D3 monoclonal antibody against hsp90. In some experiments, some full length receptor has been synthesized by 10 min and this nascent receptor is immunoadsorbed by 8D3 (see inset, Fig. 7A), suggesting that the GR binds to hsp90 either late during translation or as soon as translation is complete.

We had hoped that we might be able to map the hsp90 binding site by determining if there was a critical length at which the translation product could be adsorbed by the 8D3 antibody. However, we are unable to absorb anything shorter
the full length product in an immune-specific manner. This is illustrated in Fig. 7B, where a ladder of translation products is shown.

**DISCUSSION**

In light of the observation that there is a correlation between the amount of hsp90 bound to the GR and its steroid binding activity (17), it is interesting that the full length GR synthesized in the wheat germ lysate does not bind more than trace amounts of steroid. A heat shock response similar to that observed in animal cells has been demonstrated in cultured plant cells (e.g. Ref. 28) and high M, heat shock proteins in the 80,000-94,000 range have been demonstrated (29). However, we have not been able to determine from the literature if plants, such as wheat, produce a protein that is in any way analogous to hsp90. Although there are a variety of potential explanations for our failure to demonstrate steroid binding activity on the part of the full length translation product synthesized in wheat germ lysate, absence of hsp90 or of an hsp90-like homologue could be an important factor. The trace amounts of specific [3H]dexamethasone 21-mesyate labeling of the wheat germ translation product that we have occasionally observed could represent binding to a low affinity state of the steroid binding site with consequent very low covalent labeling by [3H]dexamethasone 21-mesyate.

We have added purified mouse hsp90 to the wheat germ lysate to see if the translation product was converted to a form that could bind steroid and be immunoadsorbed with the 8D3 monoclonal antibody. The GR translated in the wheat germ lysate in the presence of purified hsp90 does not bind [3H]triamcinolone acetonide or more than trace amounts of [3H]dexamethasone 21-mesyate and it is not immunoadsorbed by 8D3. Thus, it appears that the wheat germ lysate lacks some property that permits the GR to become associated with the heat shock protein. We have added the same amount of hsp90 that we determined was normally present in the reticulocyte system (Fig. 4). As recently reported by Rose et al. (30), we find that addition of the purified hsp90 inhibits translation in the rabbit reticulocyte system, but addition of the same preparation of hsp90 to the wheat germ lysate does not inhibit GR synthesis. This may again suggest that the two systems are intrinsically different in their ability to respond to hsp90.

In another approach to directly determine if hsp90 is required for synthesis of a GR with a high affinity steroid binding site, we have tried to eliminate the endogenous hsp90 from the reticulocyte lysate prior to using it for the translation reaction. We have found that this approach is simply not possible, because any manipulations involved in immunoadsorption (even preadsorption with protein A-Sepharose without any antibody) result in a drastic reduction in translation activity.

Our inability to demonstrate immune-specific isolation of newly synthesizing GR shorter than the full length translation product could be explained in a couple of ways. It is possible that hsp90 binds to the GR prior to completion of translation but that only the full length translation product-hsp90 complex accrues in sufficient amount to allow detection with our methods. One could argue that the complex is not accessible to the 8D3 antibody until it has dissociated from the translation machinery, but we have terminated the translation by puromycin release and still cannot detect immune-specific absorption of incomplete translation products. Another possibility is that hsp90 binds only at the end of translation.

Rusconi and Yamamoto (31) have shown that deletion of the five carboxyl-terminal amino acids reduces the affinity of the GR for dexamethasone by two orders of magnitude. Car-
Fig. 7. hsp90 associates with the newly translated, full length glucocorticoid receptor. Reticulocyte translation reactions containing \[^{35}S\]methionine were stopped at 10, 20, or 30 min after initiation and the translation products were analyzed either directly or after immunoadsorption by 8D3. Lane 1, 10-min translation products immunoadsorbed by 8D3; lane 2, total 10-min translation products; lane 3, 20-min products immunoadsorbed by 8D3; lane 4, 20 min total; lane 5, 30-min products immunoadsorbed by 8D3; lane 6, 30 min total; lane 7, 30-min products immunoadsorbed by nonimmunne IgM. The inset in A is an autoradiogram from another experiment in which some full length translation product was synthesized by 10 min. Lane 1, product immunoadsorbed by nonimmune IgM; lane 2, product immunoadsorbed by 8D3; lane 3, total full length translation product. B shows a ladder of \[^{35}S\]methionine-labeled translation products built up during a 10-min reaction in lane 3 and after 20 min of synthesis in lane 4. Lane 1 contains the products of the 10-min translation reaction immunoadsorbed by nonimmune antibody and lane 2 by 8D3.

Fig. 8. Conversion of the translation product to the DNA binding form. \[^{35}S\]Methionine-labeled receptors in reticulocyte lysate translation reactions (25 \(\mu\)l) were bound with triamcinolone acetonide, incubated 45 min at 25 \(^\circ\)C in the presence (lanes 1–3) or absence (lanes 4–6) of molybdate, and binding to DNA-cellulose was assayed as described under "Methods." Lanes 1 and 4, total translation products; lanes 2 and 5, products bound to DNA-cellulose; lanes 3 and 6, supernatant after DNA-cellulose absorption.

Isted-Duke et al. (32) have demonstrated that the A ring of \[^{3}H\]triamcinolone acetonide forms a covalent adduct with Cys-754 on UV irradiation, making it clear that residues within 40 amino acids of the carboxyl terminus at Lys-795 are participating directly in the formation of the steroid binding pocket. Thus, the carboxyl terminus would seem to be critical for formation of a high affinity steroid binding conformation of the GR and it is possible that it is critical for association with hsp90.

After longer periods of translation (20 min or more), we can see the formation of degradation products (or possibly downstream initiation products) which can be immunoadsorbed with 8D3. The major band at \(M_r \approx 47,000\) in Fig. 6B, for example, is bound to hsp90, binds steroid (see faint band in Fig. 3B) and is transformed to the DNA binding state (Fig. 8). Mendel et al. (33) have shown that the rat GR can undergo considerable degradation and still be maintained in a molybdate-stabilized heteromeric complex, and Denis et al. (14) have cleared the receptor to the \(M_r \approx 27,000\) carboxyl-terminal fragment containing the steroid binding domain and have maintained the 9 S complex. When taken together with the results of experiments analyzing the physical state of receptors produced in cells transfected with GR cDNAs containing various domain deletions (13), these data strongly argue that the steroid binding domain of the GR contains the major sites of association with hsp90. We have recently shown that the hormone-free GR can be cleaved to the 27-kDa meroreceptor fragment with retention of both hsp90 binding and of high affinity steroid binding activity (17). Thus, it is not surprising that fragments smaller than the full length translation product can bind steroid and are associated with hsp90. But as far as we can tell at the moment, the GR must be essentially completely synthesized before there is detectable hsp90 binding, and it is likely that some hsp90-bound fragments are subsequently generated by proteolysis.

The observation that newly synthesized GR is associated with hsp90 is consistent with a model in which hsp90 is required for repression of receptor function (13). Thus, the GR would be in an inactive state in the cell from the time of its synthesis until it was subsequently exposed to hormone. It has been speculated that the binding of hsp90 to the GR may serve to stabilize the receptor in an unfolded state that is necessary for the high affinity steroid binding conformation (17, 34). We would speculate that the attachment of hsp90 to the GR at or near the termination of the receptor translation process may reflect some type of chaperone function of this ubiquitous, abundant, and conserved stress protein.

Association of hsp90 with a variety of proteins may play important general roles in maintaining protein stability and in protein attachment to systems responsible for intracellular protein movement and transmembrane passage. The association of hsp90 with newly synthesized pp60c-src may reflect such a primitive and necessary cellular function, facilitating its passage to the plasma membrane. One can envision, however, that during the process of evolution, the steroid receptor family and perhaps other transcription factors whose activity is regulated by small ligands, could have evolved a regulatory center which allows the subsequent dissociation of the protein-hsp90 complex to be directly controlled by the ligand as the primary event in signal transduction.

Such a model predicts that other as yet unidentified proteins that act in the nucleus and do not possess such a regulatory center may also be attached to hsp90 in a transient
manner between the time they are synthesized and the time they arrive at the nucleus, and that on arrival at the nuclear membrane or perhaps after passage through the nuclear pore system, hsp90 normally dissociates. In the case of steroid receptors, however, the receptors may remain attached to hsp90 in some kind of inactive “docking” mode until there is hormone-triggered release of hsp90. This could account for the fact that several of the steroid receptors that normally reside in the nucleus in the absence of hormone are recovered from hormone-free cells as 9 S, hsp90-containing complexes (see Ref. 3 for review). At any rate, the structures required for protein synthesis and protein movement and targeting in the cell must somehow be functionally connected. It is reasonable to propose that hsp90 plays some role in that general process and that steroid receptors have evolved a method of signal transduction in which their dissociation from this primitive and necessary component of the system is under hormonal control.

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