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Direct Stoichiometric Evidence That the Untransformed M_r 300 000, 9S, Glucocorticoid Receptor Is a Core Unit Derived from a Larger Heteromeric Complex†

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ABSTRACT: We have used three methods to measure the stoichiometry of the glucocorticoid receptor and the 90-kDa heat shock protein (hsp90) in L-cell glucocorticoid receptor complexes that were purified by immunoadsorption to protein A–Sepharose with an anti-receptor monoclonal antibody, followed by a minimal washing procedure that permits retention of receptor-associated protein. In two of the methods, receptor was quantitated by radioligand binding, and receptor-specific hsp90 was quantitated against a standard curve of purified hsp90, either on Coomassie blue stained SDS gels by laser densitometry or on Western blots by quantitative immunoblotting with ^{125}I-labeled counterantibody. The stoichiometry values obtained by densitometry and immunoblotting are 7 and 6 mol of hsp90/mol of receptor, respectively. In a third method, which detects total receptor protein rather than just steroid-bound receptor, the ratio of hsp90 to receptor was determined by immunopurifying receptor complexes from [^{35}S]methionine-labeled L cells, and the amount of ^{35}S incorporated into receptor and hsp90 was corrected for the established methionine content of the respective proteins. In complexes from L cells which are labeled to steady state (48 h), the ratio of hsp90 to GR is 4:1. When immunoadsorbed receptor complexes are washed extensively with 0.5 M NaCl and 0.4% Triton X-100 in the presence of molybdate, the ratio of hsp90 to GR is 2:1. In addition to hsp90, preparations of [^{35}S]methionine-labeled untransformed receptor complex also contain a 55-kDa protein that resolves into several isoelectric forms on two-dimensional gel electrophoresis. These observations lead to the conclusion that the untransformed L-cell glucocorticoid receptor exists in cytosol in a much larger heteromeric complex than considered to date. We propose that the 9S receptor form that is commonly observed by density gradient centrifugation, and by gel filtration chromatography, must be a "core unit" containing two hsp90 and one GR which is derived from this larger structure.

The untransformed[1] glucocorticoid receptor (GR)[2] can exist in cytosolic preparations as an 8–9S (M_r 300 000) heteromeric complex consisting of a single molecule of the steroid-binding protein (Gehring & Arndt, 1985; Okret et al., 1985) and two molecules (Mendel & Orti, 1988) of a non-steroid-binding phosphoprotein (Joab et al., 1985; Sullivan et al., 1985; Housley et al., 1985; Mendel et al., 1986) which has been identified as the 90-kDa heat shock protein (hsp90) (Sanchez et al., 1985; Schuh et al., 1985; Catelli et al., 1985; Denis et al., 1987). There is strong evidence that this heteromeric untransformed receptor complex is derived from the physiologically inactive form of the receptor that is turned on by the hormone in intact cells: (1) Mendel et al. (1986) have shown that hsp90 is associated with GR obtained from hormone-free cells but not from cells that were exposed to hormone at 37 °C, a condition that ensures transformation; (2) Rexin et al.

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[1] The term transformation will be used throughout this paper to describe the process whereby the receptor is converted from a non-DNA-binding to a DNA-binding form.

[2] Abbreviations: GR, glucocorticoid receptor; TA, triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; hsp90, 90-kDa heat shock protein.

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(1988) have cross-linked the GR to hsp90 in intact cells under conditions where the receptor is untransformed; (3) Pratt et al. (1988) have demonstrated a direct correlation between the constitutive versus hormone-inducible properties of GRs produced from modified receptor cDNAs and their respective recovery from transfected hormone-free cells as the transformed 4S, dissociated receptor protein or as untransformed 9S, hsp90-containing complexes.

These observations, combined with the direct observation that glucocorticoids promote the dissociation of GR from hsp90 in cytosol with concomitant generation of the DNA-binding state (Sanchez et al., 1985, 1987; Denis et al., 1988b), have provided strong support for the notion that a GR–hsp90 complex represents a biologically meaningful inactive state of the receptor. To understand how this complex is altered during the initial events of signal transduction, we must ultimately define its structure. Some information regarding stoichiometry of the GR and hsp90 components is available from direct studies. Denis et al. (1988a) have determined that hsp90 that has been dissociated from the untransformed GR behaves as a dimer, an observation consistent with the demonstration that purified hsp90 is dimeric (Ianotti et al., 1988). Mendel and Orti (1988) have used a [^{35}S]methionine labeling technique to directly demonstrate that there are two molecules of hsp90 per molecule of steroid-binding protein in immunoadsorbed untransformed GR complexes. The question arises, however, as to whether this GR–hsp90 unit exists as a free entity in cytosol and in the intact cell or whether it is a "core unit" that has been separated from a much larger complex as a result of the manipulations required for its isolation and analysis (e.g., density gradient centrifugation, molecular sieve chromatography, and immunoadsorption followed by washing). This is a special concern in light of the fact that hsp90 (Sanchez et al., 1988; Redmond et al., 1989) has been found to colocalize with cytoskeleton by indirect immunofluorescence in intact cells.

In this paper, we examine the stoichiometry of hsp90 to GR in untransformed receptor complexes immunoadsorbed from L-cell cytosol. We have recently shown that these immunopurified untransformed complexes are able to bind steroid and that there is a good correlation between the presence of hsp90 in the complex and steroid-binding capacity (Bresnick et al., 1989). In this system, the immunoadsorbed receptor has undergone a minimal washing procedure to preserve as much of the structure of the native receptor complex as possible while at the same time eliminating hsp90 that is present in a manner that is nonspecific for the presence of the receptor. Our results show that this complex contains several molecules of hsp90 and that a core unit containing one molecule of GR and two molecules of hsp90 can be derived from this larger complex by more stringent washing with salt and detergent in the presence of molybdate. In addition, we show the presence of a 55-kDa protein with several isoelectric forms that is present in the larger complex obtained after the minimal washing procedure.

Experimental Procedures

Materials

[6,7-^{3}H]Triamcinolone acetonide (42.5 Ci/mmol), L-[^{35}S]methionine, and ^{125}I conjugate of goat anti-mouse IgG (9.3 mCi/mg) were from New England Nuclear (Boston, MA). Sodium molybdate, radioinert dexamethasone, nonimmune mouse IgG, TES, Tris, HEPES, protein A–Sepharose CL-4B, bovine serum albumin, Tween 20, and goat anti-mouse IgG–horseradish peroxidase conjugate were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose paper was from Bio-Rad (Richmond, CA), and Immobilon P membranes were from Millipore Corp. (Bedford, MA). BuGR2 monoclonal antibody prepared against the rat glucocorticoid receptor (Gametchu & Harrison, 1984) was kindly provided by Dr. Robert Harrison, and the AC88 monoclonal antibody against the 90-kDa heat shock protein (Riehl et al., 1985) was kindly provided by Dr. David Toft.

Methods

Cell Source and Fractionation. L929 murine fibroblasts were grown in monolayer culture in modified Eagle's medium supplemented with 10% calf serum at 37 °C. Cells were harvested by scraping into Earle's saline and centrifuged at 600*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 HEPES, 1 mM EDTA, and 10 mM molybdate, pH 7.35, at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged at 100000*g* for 1 h, and, after removal of the floating lipid layer, the supernatant (referred to as cytosol) was immunoadsorbed and washed as described below right after preparation. For ^{35}S-labeling experiments, confluent L cells were split 1 to 5 in fresh medium containing nonradioactive methionine, 10% undiluted calf serum, and [^{35}S]methionine at 5 μCi/mL and grown for 24–72 h at 37 °C.

Incubation with Antibodies and Adsorption to Protein A–Sepharose. Aliquots of freshly prepared cytosol were mixed with 0.1 volume of TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, and 50 mM NaCl, pH 7.6 at 4 °C]. BuGR anti-receptor antibody was added at 3% of the final volume. The mixture was incubated for 2 h at 0 °C, and then each sample was added to a protein A–Sepharose pellet (10 μL of pellet per 0.1 mL of L-cell cytosol). Samples were mixed by rotation for 2.5 h at 4 °C, and protein A–Sepharose pellets were washed 6 times by resuspension in 1-mL aliquots of TEG buffer or TEG buffer containing 10 mM molybdate (TEGM buffer), vortexing for 5 s, and centrifugation. This is the minimal wash procedure that was used to eliminate non-receptor-specific hsp90 from immune pellets. In some experiments where noted, this pellet was submitted to additional severe washing with TEGM buffer containing 0.5 M NaCl and 0.4% Triton X-100.

Gel Electrophoresis and Immunoblotting. SDS–polyacrylamide gel electrophoresis was performed in 7% slab gels according to Laemmli (1970) as described previously (Bresnick et al., 1989). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Immunoadsorbed GR complexes were dissolved by incubating them for 2 h in 9.5 M urea, 2% (w/v) NP-40, 5% ampholines (comprised of 4% pH range 5–8 and 1% pH range 3–10), and 5% β-mercaptoethanol.

Immunoblotting was carried out by transferring proteins from acrylamide slab gels to nitrocellulose paper or to Immobilon P membranes, followed by overnight incubation with 1% BuGR antibody against the glucocorticoid receptor or 0.4% AC88 antibody against hsp90. Immunoblotted proteins were detected either by reaction with goat anti-mouse peroxidase-conjugated IgG or by reaction with ^{125}I-conjugated goat anti-mouse IgG followed by rabbit anti-goat peroxidase-conjugated IgG. To quantitate the relative amounts of receptor and hsp90, the peroxidase-visualized receptor and hsp90 bands were excised and counted for radioactivity, as described by Howe and Hershey (1981) and modified by Dalman et al. (1988). During the course of this work, we found the Immobilon P membranes to be far superior to nitrocellulose for

retention of blotted proteins, in particular hsp90.

Purification of Hsp90 from L Cells. Hsp90 was purified by a modification of the method of Welch and Feramisco (1982). 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. Hsp90 was detected by Coomassie blue staining and immunoblotting with the AC88 antibody probe. Fractions containing hsp90 were pooled and concentrated by adsorption with poly(ethylene glycol). The concentrated material was diluted with an equal volume of 20 mM K_2HPO_4/1 mM EDTA, pH 7.5, it was chromatographed on a 2 × 8 cm hydroxylapatite column (which was equilibrated in the same K_2HPO_4 buffer), and proteins were eluted with a 200-mL gradient of 0–0.4 M K_2HPO_4. Fractions containing hsp90 were pooled, concentrated by poly(ethylene glycol) adsorption, and frozen at −70 °C. The final protein concentration determined by amino acid analysis was 565 μg/mL.

Measurement of Receptor and Hsp90 Stoichiometry. (*A*) *Method 1.* The amount of receptor was measured by binding [^{3}H]triamcinolone acetonide, and receptor-specific hsp90 was assayed by densitometry of Coomassie blue stained protein after resolution by SDS–polyacrylamide gel electrophoresis. L-cell cytosol was incubated with 50 nM [^{3}H]TA (a concentration in excess of that required to occupy all steroid-binding sites) with or without 50 μM radioinert dexamethasone (in the presence of 10 mM molybdate and 10 mM DTT) for 2 h at 0 °C. Receptors were immunoadsorbed to protein A–Sepharose with BuGR or nonimmune mouse IgG, and the Sepharose pellets were washed 6 times with TEGM buffer. This washing procedure does not result in any dissociation of steroid from the immunoadsorbed receptor. Pellets were boiled in 0.3 mL of SDS–sample buffer, and duplicate 14-μL aliquots were counted for radioactivity. This steroid-binding value was used to calculate the amount of receptor in the immune pellet. The remaining 272 μL and increasing amounts of purified hsp90 were resolved by SDS–PAGE. Proteins were visualized by Coomassie staining, and receptor-specific hsp90 was quantitated by linear regression of the values obtained by laser densitometric scan of the purified hsp90. A molecular weight of 83 300 for hsp90 (Welch & Feramisco, 1982) was used to convert the protein measurement by amino acid analysis to picomoles of hsp90.

(*B*) *Method 2.* The amount of receptor in immunoadsorbed GR complexes was assayed as described above, and the amount of receptor-specific hsp90 was measured by quantitative immunoblotting using purified hsp90 as a standard. After resolution of the remaining 272-μL aliquot by SDS–PAGE, proteins were immunoblotted with AC88 and ^{125}I-conjugated and peroxidase-conjugated antibodies as described above. Hsp90 bands were cut out and counted for radioactivity. The amount of receptor-specific hsp90 was determined from a standard curve prepared from purified hsp90 that was run on the same gel.

(*C*) *Method 3.* The amounts of receptor and receptor-specific hsp90 were determined by labeling L cells for 48 h with [^{35}S]methionine. Receptors were immunoadsorbed to protein A–Sepharose with BuGR or with mouse IgG, and pellets were washed 6 times with TEGM buffer. Cytosol prepared from one T175 flask of ^{35}S-labeled L cells was used for each condition (approximately 150 μL of cytosol). The immunoadsorbed proteins were resolved by SDS–PAGE, gel lanes were sliced into 1.5-mm slices, and slices were digested in 30% hydrogen peroxide by incubating for 5 h at 90 °C.

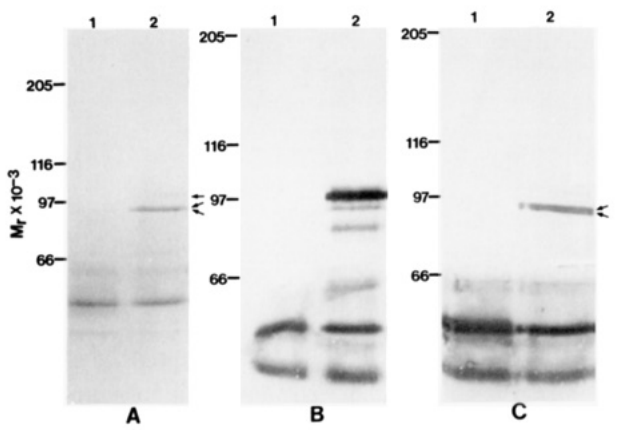

FIGURE 1: Coomassie blue stain and Western blot analysis of immunoadsorbed glucocorticoid receptor complexes. Aliquots of L-cell cytosol (panels A and C, 600 μL; panel B, 150 μL) were immunoadsorbed to protein A–Sepharose with nonimmune mouse IgG (lane 1) or with BuGR (lane 2). The Sepharose pellets were washed 6 times with 1-mL aliquots of TEGM buffer, they were boiled in SDS–sample buffer, and the eluted proteins were resolved by SDS–PAGE. Proteins were visualized by Coomassie blue staining (panel A) or by immunoblotting with the BuGR (panel B) and AC88 (panel C) antibody probes for receptor and hsp90, respectively. The immunoblots were developed by the peroxidase method. The prominent bands present in both nonimmune and BuGR samples are immunoglobulin. The arrows indicate the hsp90 doublet bands in lane 2 of panels A and C and the faint Coomassie blue stained receptor band migrating just above the 97-kDa marker in lane 2 of panel A.

Digested samples were counted for radioactivity, and a background radioactivity value was obtained by averaging the values for the three slices before the receptor peak and the three slices after the hsp90 peak as indicated on the gel profile presented in the inset in Figure 4. This background value was subtracted from both receptor and hsp90 values to obtain the amount of radioactivity in the receptor and hsp90. The sums of the specific receptor or hsp90 values were corrected for the methionine content of GR and hsp90 as described by Mendel and Orti (1988). The hsp90:GR ratio was multiplied by 1.33 assuming 18 methionines for hsp90 (Moore et al., 1987) and 24 methionines for receptor (Danielson et al., 1986). The results are expressed as the moles of hsp90 per mole of GR.

Results and Discussion

Stoichiometry of Hsp90 and GR in Immunoadsorbed Receptor Complexes after the Minimal Washing Protocol. Figure 1 shows both Coomassie blue stained and Western-blotted gels of unliganded glucocorticoid receptors that have been immunoadsorbed to protein A–Sepharose and washed 6 times with TEGM buffer as reported previously (Bresnick et al., 1989). Lane 1 in panels A and C shows that this minimal washing procedure eliminates hsp90 from samples immunoadsorbed with nonimmune IgG. When the immunoadsorbed GR complexes are resolved by SDS–PAGE and stained with Coomassie blue (lane 2 in panel A), two immune-specific bands are detected, a weakly stained band migrating just above the 97-kDa marker and a more intensely stained doublet band migrating just below the marker at 90 kDa. The more intensely stained 90-kDa doublet is developed relatively weakly on immunoblotting with the AC88 anti-hsp90 monoclonal antibody (lane 2 in panel C). In contrast, the weakly staining 100-kDa band is developed intensely by immunoblotting with the BuGR2 anti-GR monoclonal antibody (lane 2 in panel B). The increased staining intensity of the hsp90 band versus that of the 100-kDa receptor is also observed after silver stain (data not shown). The different intensities of the immunoblot signals reflect different intrinsic reactivities of the primary antibodies.

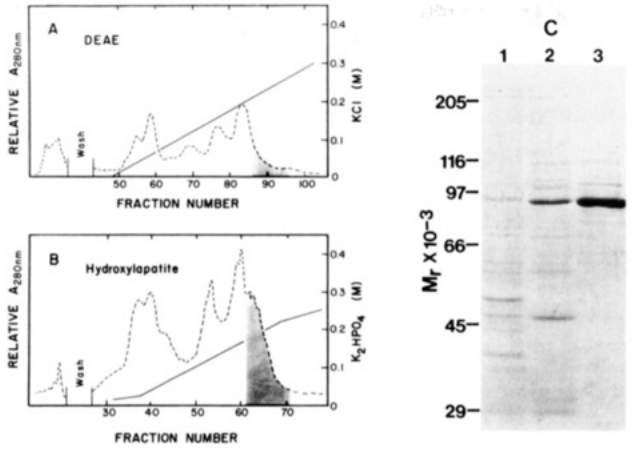

FIGURE 2: Purification of hsp90 from L cells. L-cell cytosol was chromatographed on a DEAE column (panel A), and proteins were eluted with a 200-mL KCl gradient (solid line). Fractions containing hsp90 (indicated by the shaded area) were pooled, concentrated, and chromatographed on a hydroxylapatite column (panel B). Panel C: Coomassie blue stain of (lane 1) 12 μg of L-cell cytosol protein, (lane 2) 12 μg of DEAE-purified protein, and (lane 3) 12 μg of DEAE/hydroxylapatite-purified protein from the shaded area in panel B.

To determine if the difference in staining intensity between the bands reflects a considerable stoichiometric excess of hsp90 over GR in the immunoadsorbed complex, we used three methods to measure stoichiometry. Two of the methods require the use of a standard curve prepared with purified hsp90. Figure 2 shows the purification of hsp90 from cytosol of unstressed L cells by sequential chromatography on DEAE and hydroxylapatite according to the method of Welch and Feramisco (1982). In the experiment shown in the top panel of Figure 3, the GR was quantitated by radioligand binding, and hsp90 was quantitated by laser densitometry using the purified hsp90 as a standard. In this experiment, the stoichiometry was 5 mol of hsp90/mol of GR, and the mean stoichiometry from three such experiments is 7.2 × 1.4 (SE) mol of hsp90/mol of GR. In the method shown in the bottom panel of Figure 3, the relative amount of immunoreactive hsp90 is determined by reacting blots containing immunoadsorbed complexes and standard amounts of the purified hsp90 with ^{125}I-anti-mouse IgG and directly counting the excised hsp90 bands as previously described (Bresnick et al., 1989). Receptor-specific hsp90 is calculated by subtracting the value obtained for hsp90 immunoadsorbed with nonimmune mouse IgG from that obtained in samples immunoadsorbed with an equivalent amount of anti-receptor antibody. The nonimmune hsp90 value does not exceed 15% of the hsp90 value obtained in the BuGR immunoadsorbate (data not shown). In this experiment, the stoichiometry is 7 mol of hsp90/mol of GR, and the mean stoichiometry from four such experiments is 6.2 ± 1.0 (SE) mol of hsp90/mol of GR.

In the methods used for estimating stoichiometry in Figure 3, it is assumed that all of the receptors in cytosol are capable of binding steroid. If a significant proportion of the receptors cannot bind steroid, then the hsp90 to GR ratios would be inappropriately large. To directly assay the relative amounts of hsp90 and GR protein, we used the [^{35}S]methionine biosynthetic labeling technique of Mendel and Orti (1988). L cells were cultured in medium containing [^{35}S]methionine, and at various times, cells were removed, the untransformed receptors were immunoadsorbed with the BuGR anti-GR antibody, and the GR and receptor-associated hsp90 were resolved by SDS–polyacrylamide gel electrophoresis. The inset to Figure 4 shows the distribution of radioactivity in gel slices

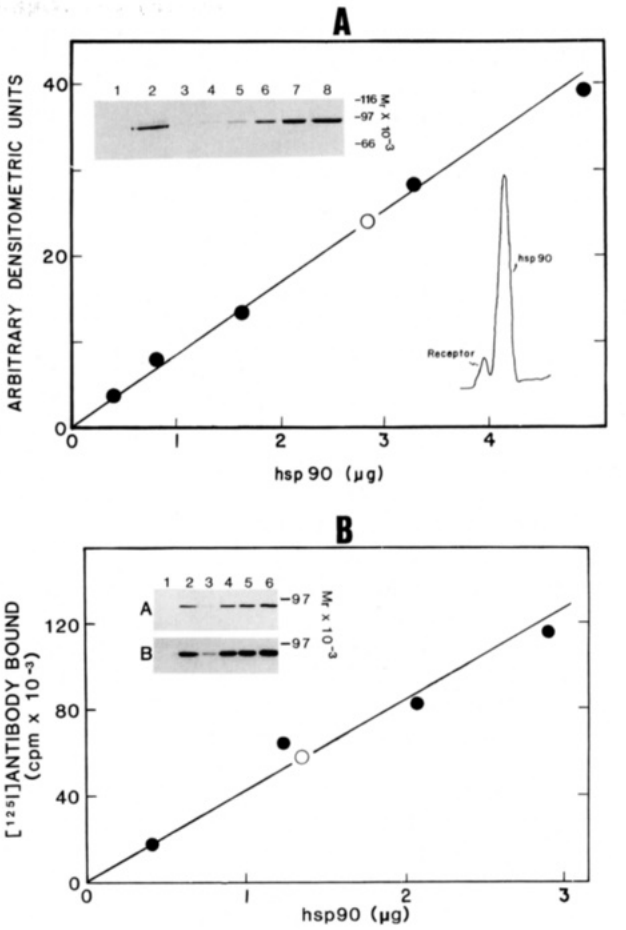

FIGURE 3: Measurement of receptor and hsp90 stoichiometry in immunoadsorbed molybdate-stabilized glucocorticoid receptor complexes after minimal washing. Panel A: Steroid–receptor complexes were formed by incubating molybdate and DTT-containing L-cell cytosol with 50 nM [^{3}H]triamcinolone acetonide (TA) with or without 50 μM radioinert dexamethasone for 2 h at 0 °C. Cytosol was immunoadsorbed to protein A–Sepharose with BuGR or nonimmune mouse IgG, and the Sepharose pellets were washed 6 times with TEGMD buffer and boiled in SDS–sample buffer. Replicate aliquots of the extract were counted for ^{3}H, and the values (which were corrected for nonspecifically bound radioactivity) were used to calculate the amount of GR. The remaining aliquot of extract and increasing amounts of purified hsp90 were resolved by SDS–PAGE, and proteins were visualized by staining with Coomassie blue. The amount of GR-specific hsp90 and the standard amounts of purified hsp90 were quantitated by laser densitometry, and the values are expressed as arbitrary densitometric units. The closed circles show a representative standard curve obtained by scanning the bands of purified hsp90 shown in lanes 3–8 in the inset at the upper left, and the open circle shows the GR-specific hsp90 value determined from lane 2 in the inset. Samples applied to the gel were as follows: lane 1, cytosol adsorbed with nonimmune mouse IgG; lane 2, cytosol adsorbed with BuGR; lanes 3–8, 0.2, 0.4, 0.8, 1.6, 3.3, and 4.8 μg of purified hsp90. The lower inset shows a densitometric scan of GR and hsp90 in lane 2. Panel B: The amount of GR was calculated in an identical manner as in panel A. The amount of GR-specific hsp90 was measured by quantitative immunoblotting with ^{125}I-labeled counterantibody. The closed circles show the standard curve obtained by excising and counting the hsp90 bands, and the open circle shows the GR-specific hsp90 value determined from lane 2. The inset shows the peroxidase stain (row A) and the ^{125}I autoradiogram (row B) of the same blot. Samples applied to the gel were as follows: lane 1, cytosol adsorbed with nonimmune mouse IgG; lane 2, cytosol adsorbed with BuGR; lanes 3–6, 0.4, 1.2, 2.0, and 2.8 μg of purified hsp90.

prepared from the region of the gel containing GR and hsp90. The hsp90 to GR ratios from several experiments are plotted in the main part of Figure 4. The ratio reaches a steady state by 48 h of [^{35}S]methionine labeling, and receptor complexes from cells which are labeled for 48 and 72 h have a mean

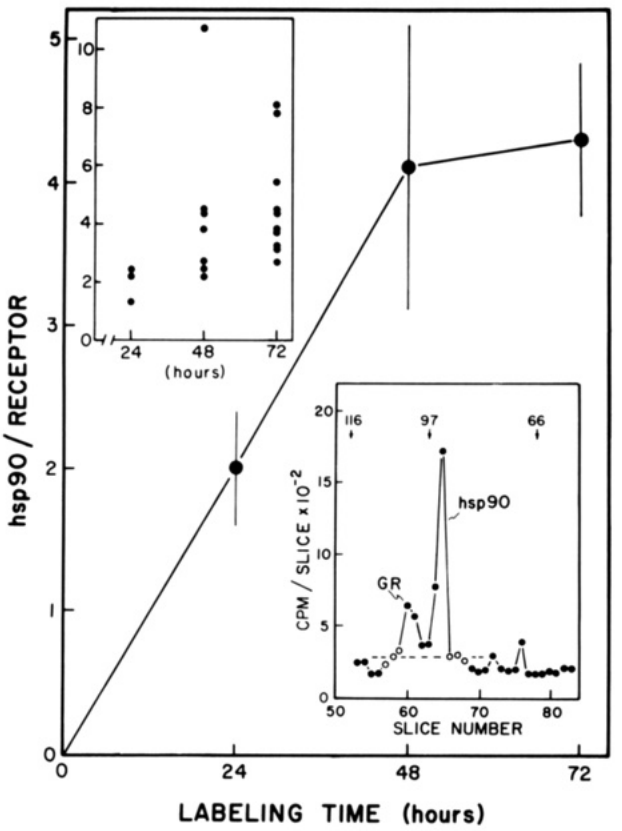

FIGURE 4: Measurement of receptor and hsp90 stoichiometry by biosynthetic labeling. L cells were labeled with [^{35}S]methionine for 24, 48, or 72 h, and GR complexes were immunoadsorbed to protein A–Sepharose with BuGR. The Sepharose pellets were washed 6 times with TEGM buffer, and the eluted proteins were resolved by SDS–PAGE (6% gel). Gel lanes were sliced and counted for radioactivity. Stoichiometry values were calculated as described under Experimental Procedures and are expressed as moles of hsp90 per mole of GR in the immune complex (mean ± SE: 24 h, n = 3; 48 h, n = 8; 72 h, n = 10). The upper inset is a scatter plot of all the data. The lower inset shows a representative gel profile of ^{35}S-labeled proteins. The open circles show the values which were averaged to yield a nonspecific value (dotted line). The stoichiometry in this experiment is 4 mol of hsp90/mol of GR.

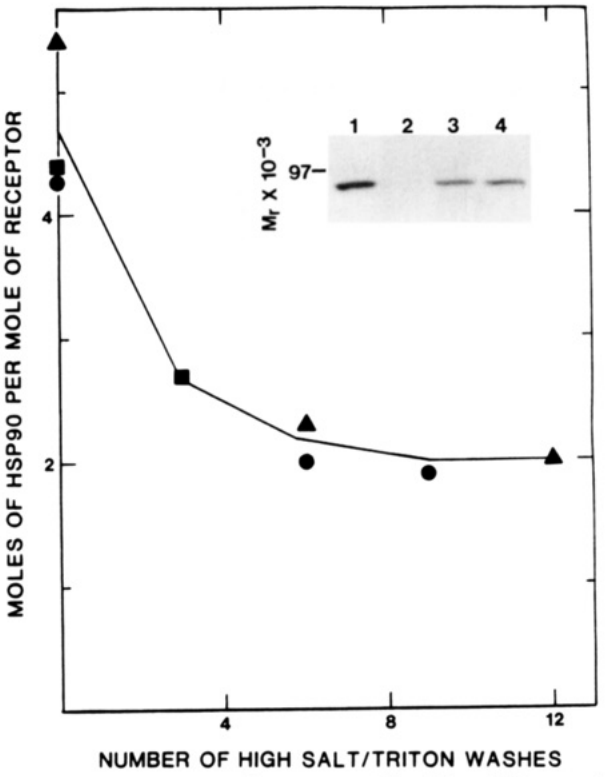

FIGURE 5: Measurement of receptor and hsp90 stoichiometry in immunoadsorbed glucocorticoid receptor complexes after stringent washing. L cells were labeled with [^{35}S]methionine for 72 h, and GR complexes were immunoadsorbed to protein A–Sepharose with BuGR. The Sepharose pellets were washed 5 times with TEG buffer, followed by 0, 3, 6, 9, or 12 times with TEGM buffer containing 0.5 M NaCl and 0.4% Triton X-100 (TEGMNT), and the eluted proteins were resolved by SDS–PAGE (6% gel). Gels lanes were sliced and counted for radioactivity. Stoichiometry values were calculated as described in the legend to Figure 4 and are expressed as moles of hsp90 per mole of GR in the immune complex. The results were pooled from three separate experiments which are indicated by the different symbols. The inset shows an autoradiogram of an immunoblot that was developed with AC88 followed by ^{125}I counterantibody. Samples applied to the gel were as follows: lane 1, cytosol adsorbed with BuGR and washed 5 times with TEG buffer; lane 2, cytosol adsorbed with BuGR and washed 5 times with TEG buffer, followed by 3 times with TEGNT buffer; lane 3, identical with lane 2 except molybdate was included in the TEGNT buffer; lane 4, identical with lane 2 except molybdate and DTT were included in the TEGNT buffer.

stoichiometry of 4.1 ± 1.0 and 4.3 ± 0.5 mol of hsp90/mol of GR, respectively. This value is about two-thirds that obtained with the other methods, which rely on the protein concentration of hsp90 standards determined by amino acid analysis. A small difference between the real and measured value of protein amount could account for the difference. Alternatively, this decrease in ratio could indicate that only about two-thirds of the receptors in cytosol are in an active steroid-binding conformation, despite the fact that steroid binding is being carried out under optimal conditions (i.e., plus DTT and molybdate) for maintaining the receptor in the steroid-binding state (Bresnick et al., 1988, 1989). It is clear from the scatter diagram of all the ratios presented in the upper inset of Figure 4 that there is a great deal of variation in ratios obtained from one experiment to another, with hsp90 to GR ratios obtained by the [^{35}S]methionine technique ranging from 2:1 to 11:1. In the lower inset of Figure 4, for example, there is 4.2 mol of hsp90/mol of GR, whereas in Figure 7A there is 8.1 mol of hsp90/mol of GR.

It should be noted here that we have attempted to use the [^{35}S]methionine biosynthetic labeling technique to measure the stoichiometry of GR complexes isolated by adsorption of L-cell receptors to a steroid affinity matrix (deoxycorticosterone–agarose). The high level of nonspecific protein binding to this matrix and the resulting high background counts (even after elution of bound proteins with glucocorticoid hormone) preclude the usefulness of this method for measuring the stoichiometry of minimally washed GR complexes (data not shown).

Stoichiometry of Molybdate-Stabilized Receptor Complexes after a Stringent Washing Protocol. The large variance in the ratios obtained with the minimal washing procedure would be expected if the GRs were associated with a large heteromolecular complex in cytosol that contains several molecules of hsp90 and if variable amounts of the complex were removed with each wash. It is known, however, that the molybdate-stabilized GR is still associated with hsp90 after stringent conditions of washing with salt and detergent (Sanchez et al., 1985, 1987). Figure 5 shows that, when minimally washed GR complexes are washed with buffer containing 0.5 M NaCl and 0.4% Triton X-100 in the presence of sodium molybdate, the hsp90:GR ratio decreases to 2:1 by six washes and it remains relatively constant thereafter. If GR complexes are not treated with molybdate, all of the hsp90 is eliminated by three washes with salt and detergent (see lane 2 in the inset in Figure 5). After washing immunoadsorbed receptors bound to a protein A–Sepharose column with

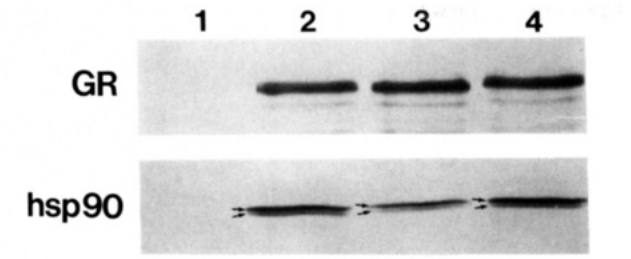

FIGURE 6: Immunoadsorbed glucocorticoid receptor complexes contain both forms of hsp90. Aliquots (0.6 mL) of cytosol were immunoadsorbed with nonimmune mouse IgG (lane 1) or BuGR (lanes 2–4), and the Sepharose pellets were washed 6 times with TEG buffer. Pellets were resuspended in 0.3 mL of HEPES buffer in the absence or presence of 10 mM molybdate and were incubated for 2 h at 0 or 25 °C. Pellets were washed once with TEGM buffer and boiled in SDS–sample buffer, and the eluted material was resolved by SDS–PAGE and analyzed for receptor (top panel) and hsp90 (bottom panel) by Western blotting and development with ^{125}I-conjugated IgG. Samples applied to the gel were the following: lane 1, cytosol adsorbed with nonimmune IgG; lane 2, cytosol adsorbed with BuGR and incubated at 0 °C; lane 3, cytosol adsorbed with BuGR and incubated at 25 °C; lane 4, cytosol adsorbed with BuGR and incubated at 25 °C in the presence of molybdate. The arrows point to the bands of the hsp90 doublet.

molybdate-containing buffer, Mendel and Orti (1988) also observed a 2:1 stoichiometry of hsp90 to GR.

As our original goal was to measure the hsp90 to GR stoichiometry in complexes washed in the mildest manner possible, we minimized the washing procedure to increase the probability of isolating a more native structure. Complexes isolated under these minimal washing conditions are relatively unstable and can be reduced to a 2:1 core unit with higher stringency washing under molybdate-stabilized conditions. We speculate that it is this 2:1 hsp90/GR unit that is observed as the 9S molybdate-stabilized form of the GR obtained on density gradient centrifugation.

Both the High and the Low Molecular Weight Forms of Hsp90 Are Present in the Receptor Complex. Murine hsp90 is present in cytosol as two separate gene products—a higher molecular weight form, hsp86, and a lower molecular weight form, hsp84 (Moore et al., 1987). In L-cell cytosol, the lower molecular weight form is somewhat more abundant (data not shown), but in the immunoadsorbed untransformed GR complex, either both forms are present in equivalent amounts (as shown in Figure 1) or, more often, the higher molecular weight form is more abundant as assessed by immunoblotting with the AC88 antibody. A typical experiment in which there appears to be a greater abundance of the high molecular weight form is shown in Figure 6. Mendel and Orti (1988) have reported that somewhat more of the receptor-associated hsp90 is present as the lower molecular weight form, but we have not found that to be the case in most of our experiments. As shown in lane 3 of Figure 6, when the immunoadsorbed complex is heated so that 50% of the hsp90 dissociates from the receptor, both forms are lost in roughly equivalent fashion.

In considering a core unit that contains two molecules of hsp90, the GR could be interacting directly with just one molecule of hsp90 which is present as a dimer or it could interact directly with both units of a dimer simultaneously. One problem in defining such a core unit is that, as yet, nobody has been able to determine whether the two forms of hsp90 normally interact with each other solely as heterodimers, solely as homodimers, or as both. Thus, it is not clear whether the GR could come into direct contact with a heterodimer and have different affinities for each component or whether it would only be complexed with homodimers. Our results permit us to rule out an exclusive interaction with either a high molecular weight or a low molecular weight homodimer, but all other options are still possible.

Other Components of the Immunoadsorbed Receptor Complex. To determine if the untransformed GR contains components other than hsp90, [^{35}S]methionine-labeled complexes were run on 6% and 12% gels to resolve potential high and low molecular weight components, respectively. It is clear from panels A and B of Figure 7 that there are no prominent immune-specific peaks of ^{35}S radioactivity with a molecular weight higher than the GR. Although no [^{35}S]methionine-labeled immune-specific bands are observed below the molecular weight 45K marker in the 12% gel shown in Figure 7 [an immune-specific protein band can be visualized at M_r 23 000 by Coomassie blue stain (not shown) but not by [^{35}S]methionine], there is a prominant peak of ^{35}S radioactivity at M_r 55 000 that we refer to as p55. The profiles in Figure 7 are representative in that the area under the p55 peak is greater in immune complexes prepared with BuGR than with

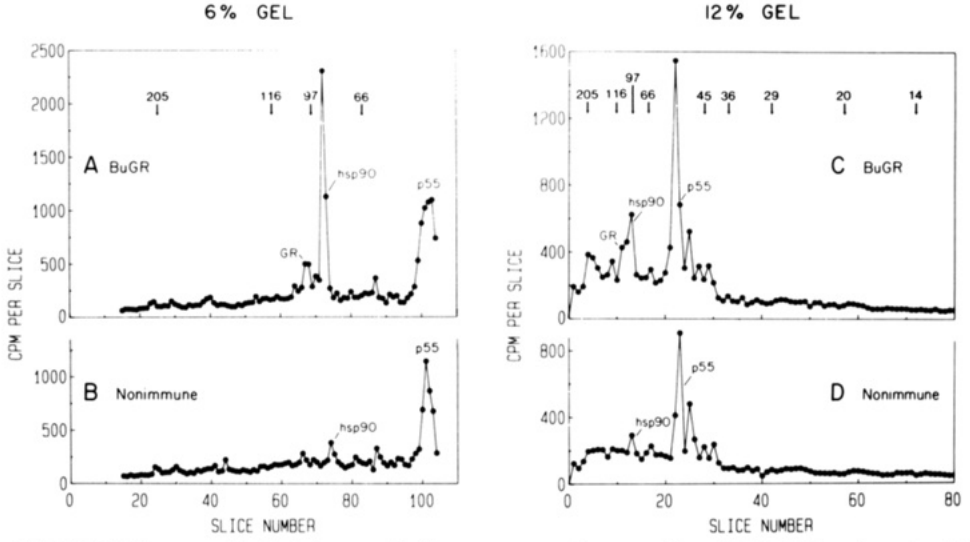

FIGURE 7: Analysis of ^{35}S-labeled immunoadsorbed glucocorticoid receptor complexes on 6% and 12% SDS–polyacrylamide gels. L cells were labeled with [^{35}S]methionine for 72 h, aliquots of ^{35}S-cytosol were immunoadsorbed to protein A–Sepharose with BuGR (panels A and C) or nonimmune mouse IgG (panels B and D), and the Sepharose pellets were washed 6 times with 1-mL aliquots of TEGM buffer. Pellets were boiled in SDS–sample buffer, and the eluted proteins were resolved on 6% (panels A and B) or 12% (panels C and D) gels. Gels were sliced, the slices were digested with peroxide, and the values are expressed as cpm per slice.

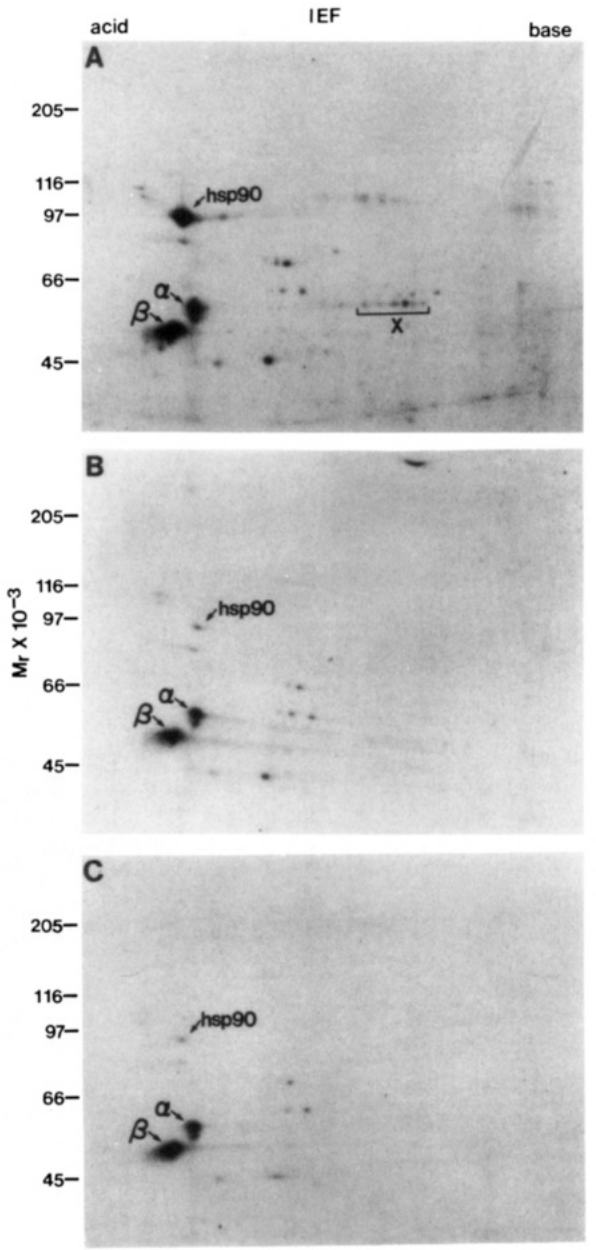

FIGURE 8: Two-dimensional SDS–polyacrylamide gel analysis of immunoadsorbed untransformed and transformed glucocorticoid receptor complexes. L cells were labeled with [^{35}S]methionine for 72 h, and aliquots of ^{35}S-cytosol were maintained at 0 °C (panels A and C, untransformed) or were incubated with 50 nM triamcinolone acetonide for 2.5 h at 0 °C, followed by an incubation for 1 h at 25 °C (panel B, transformed). Cytosol was immunoadsorbed to protein A–Sepharose with BuGR (panels A and B) or nonimmune mouse IgG (panel C), and the Sepharose pellets were washed 6 times with 1-mL aliquots of TEGM buffer. Pellets were incubated in sample buffer, and the eluted proteins were resolved by two-dimensional SDS–PAGE as described under Experimental Procedures. Gels were treated with "Amplify", and proteins were visualized by autoradiography.

nonimmune IgG, but nevertheless there is always a considerable amount of [^{35}S]methionine radioactivity in the p55 region of nonimmune samples. To resolve these proteins better, we have run two-dimensional gels of [^{35}S]methionine-labeled complexes after immunoadsorption of untransformed or transformed GR with BuGR or with nonimmune IgG. As shown in the experiment of Figure 8, the majority of the radioactivity in the p55 region is accounted for by α-tubulin and β-tubulin, with the identity of these spots having been confirmed by Western blotting with monoclonal antibodies directed against each tubulin form (data not shown). The transformed GR complex and the nonimmune complex contain almost no hsp90, and there are reduced amounts of tubulin. Because large amounts of tubulin bind to protein A–Sepharose in a nonspecific manner, it is impossible to determine by this method if any tubulin-containing cytoskeletal elements are present as a consequence of immunoadsorption of the GR.

The brackets marked by the X in panel A of Figure 8 point out several ^{35}S-labeled proteins that have the same molecular weight as the major α-tubulin species but migrate to a more basic region on isoelectric focusing. These basic p55 peak components are seen only with the untransformed GR and not with transformed GR (panel B) or nonimmune complexes (panel C). The behavior of the basic p55 components is very much like that of receptor-associated protein isoforms that are immunoadsorbed from human IM-9 cell cytosol by the EC1 monoclonal antibody (Sanchez, Faber, and Pratt, unpublished results). The EC1 monoclonal is an antibody prepared by Nakao et al. (1985) against a partially purified, molybdate-stabilized preparation of untransformed rabbit progesterone receptor. Tai et al. (1986) have shown that the EC1 monoclonal reacts with 9S progestin, estrogen, androgen, and glucocorticoid receptors but not with transformed receptors in rabbit uterine and liver cytosols. The protein recognized by the EC1 monoclonal antibody in human lymphocyte cytosol has the same molecular weight as α-tubulin, but like the basic p55 components bracketed in Figure 8A, the human EC1-reacting protein is resolved on two-dimensional SDS–PAGE into several isoforms with more basic p*I*'s than α-tubulin.

Rexin et al. (1988) have treated both mouse lymphoma cell extracts and intact cells with dimethyl suberimidate and then immunopurified the [^{3}H]dexamethasone mesylate labeled GR and resolved its cross-linked complexes by SDS–PAGE. Careful analysis of the size of the cross-linked products reveals complexes consistent with a GR linked to two molecules of a 90-kDa protein and one molecule of a 50-kDa protein and all subcombinations thereof (GR + p50, GR + p90, GR + p50 + p90, GR + 2 p90). Taken together, the direct studies presented in Figures 5 and 8, combined with the stoichiometry (Mendel & Orti, 1988), cross-linking (Rexin et al., 1988a,b), and EC1 immunoadsorption (Tai et al., 1986) observations of others strongly suggest that the core unit of the untransformed glucocorticoid receptor contains one molecule of steroid-binding protein, a dimer of hsp90, and one molecule of a 50–55-kDa protein. Interestingly, immunoadsorption of $pp60^{v\text{-}src}$ results in the coisolation of hsp90 and a p50 with unknown function (Brugge, 1986).

Our stoichiometry calculations with minimally washed complexes (Figures 3 and 4) show that the GR can be recovered from cytosol in association with multiple molecules of hsp90. We therefore propose that the core unit of untransformed GR is derived from some form of higher order structure, and on the basis of indirect immunofluorescence studies of hsp90 localization in intact cells (Sanchez et al., 1988; Redmond et al., 1989), we would speculate that this higher order structure may be derived from the cytoskeleton.

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Primary Structure of a Zinc Protease from *Bacillus mesentericus* Strain 76†

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ABSTRACT: The amino acid sequence of the neutral zinc protease from *Bacillus mesentericus* strain 76 (MCP 76) has been determined by using peptides derived from digests with trypsin, chymotrypsin, and cyanogen bromide and from cleavage with *o*-iodosobenzoic acid. The peptides were purified by means of gel filtration and reversed-phase high-performance liquid chromatography and analyzed by automatic sequencing. The protein contains 300 amino acid residues. It proved to be identical with the neutral protease deduced from the DNA precursor sequence of *Bacillus subtilis*. The residues for zinc and substrate binding are conserved, whereas the number of calcium binding sites is reduced compared to thermolysin. A classification of the neutral zinc protease is discussed.

The zinc protease isolated from *Bacillus mesentericus* strain 76 is a rennin-like enzyme first described by Emanouilov (1951). It is used in the cheese-making industry as a milk-clotting protease (MCP 76)[1] and belongs to the neutral metalloendopeptidases. These enzymes are inhibited by chealting agents but not by covalent inhibitors of cysteine or serine

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[1] Abbreviations: MCP 76, milk-clotting protease from *Bacillus mesentericus* strain 76 (neutral protease); HPLC, high-performance liquid chromatography.

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