Purification of the Endogenous Glucocorticoid Receptor Stabilizing Factor†

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ABSTRACT: A ubiquitous, low molecular weight, heat-stable component of cytosol stabilizes the glucocorticoid receptor in its untransformed state in association with hsp90. This heat-stable factor mimics molybdate in its effects on receptor function, and it has the heat stability, charge, and chelation properties of a metal oxyanion [Meshinchi, S., Grippo, J. F., Sanchez, E. R., Bresnick, E. H., & Pratt, W. B. (1988) *J. Biol. Chem. 263*, 16809–16817]. In this paper, we describe the further purification of the endogenous factor from rat liver cytosol by anion-exchange HPLC (Ion-110) after prepurification by molecular sieving, cation absorption, and charcoal absorption. Elution of the factor with an isocratic gradient of ammonium bicarbonate results in recovery of all of the bioactivity in a single peak which coelutes with inorganic phosphate and contains all of the endogenous molybdenum. The bioactivity can be separated from inorganic phosphate by chromatography of the partially purified endogenous factor on a metal-chelating column of Chelex-100. The chelating procedure results in complete loss of bioactivity with recovery of 98% of the inorganic phosphate in both the column drop-through and a subsequent 1 M NaCl wash. The factor preparation purified through the Ion-110 HPLC step inhibits temperature-mediated dissociation of the immunopurified glucocorticoid receptor–hsp90 complex, but it is considerably more effective at stabilizing the unpurified receptor–hsp90 complex in a Chelex-treated cytosol system that has been depleted of metal components. These observations support the proposal that an endogenous metal can stabilize the binding of hsp90 to the receptor but it is likely that other cytosolic components that are not present in the immunopurified complex must contribute to the stability of the soluble protein–protein complex in cytosol.

Cytosols prepared from a variety of sources contain a low molecular weight factor that stabilizes the glucocorticoid receptor–90-kDa heat shock protein (hsp90)[1] complex, preventing its transformation to the DNA binding state (Leach et al., 1982; Meshinchi et al., 1988). Separation of the glucocorticoid receptor from the low molecular weight components of cytosol promotes rapid, temperature-dependent loss of steroid binding capacity of the unliganded receptor (Leach et al., 1982) and enhances the transformation of the steroid-bound receptor to the DNA binding state (Leach et al., 1982; Cake et al., 1976; Goidl et al., 1977). The stabilizing factor is a small molecular weight, heat-stable compound that is strongly anionic (Leach et al., 1982; Meshinchi et al., 1988; Bailly et al., 1977). This endogenous heat-stable compound also stabilizes cytosolic estrogen, progestin, and androgen receptors (Bailly et al., 1977; Sato et al., 1980), suggesting that it may perform a general role in regulating steroid receptor function in vitro.

The first evidence for the existence of an endogenous low molecular weight inhibitor of steroid receptor transformation was reported by the laboratories of Litwack (Cake et al., 1976; Goidl et al., 1977) and Milgrom (Bailly et al., 1977) about 15 years ago, and Litwack and his colleagues have called this endogenous activity "modulator" (Goidl et al., 1977; Bodine & Litwack, 1990a). As summarized by Bodine and Litwack (1990a) in a recent and balanced review, two possibilities have emerged regarding the molecular composition of the stabilizing factor. One line of evidence has led to the identification of a bioactive phosphoglyceride (Bodine & Litwack, 1988a,b, 1990b), and a second line of evidence ascribes the activity to a metal anion (Meshinchi et al., 1988).

In 1988, Bodine and Litwack (1988a,b) achieved a large-scale purification of an inhibitor of glucocorticoid receptor transformation. The inhibiting activity was purified from rat liver cytosol that had been preheated at 37 °C. Extensive purification was achieved by a three-step procedure, including gel filtration, anion-exchange chromatography on Dowex 1, and preparative silica HPLC. Physical analysis of the purified product by infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometry indicated that the factor is an ether aminophosphoglyceride (an aminophospholipid). Because the factor could not be inactivated by any of a variety of enzymes that degrade aminophospholipids and because the activity of the factor could not be replaced with any of a variety of phospholipids, it was suggested that the phospholipid was of novel structure (Bodine & Litwack, 1988b). This factor (modulator) has subsequently been separated into two isoforms that interact synergistically in stabilizing the unoccupied, untransformed receptor (Bodine & Litwack, 1990b). The modulator preparation is devoid of molybdenum and other

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[1] Abbreviations: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; 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; SRIXE, synchrotron radiation induced X-ray emission.

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metals (Bodine & Litwack, 1990b).

For many years, our laboratory has been interested in the ability of boiled rat liver cytosol to enhance and stabilize the glucocorticoid binding activity of unliganded receptors (Sando et al., 1977) and to inhibit the transformation of liganded receptors (Leach et al., 1982). In 1988, we demonstrated (Meshinchi et al., 1988) that the receptor stabilizing factor in boiled liver cytosol has a low molecular weight (approximately 340), its bioactivity is eliminated by adsorption to a metal-chelating resin, and its activity is completely stable to heating at 340 °C for 1 h. These findings led us to propose that the stabilizing factor is a metal component of cytosol that normally stabilizes the glucocorticoid receptor complex (Meshinchi et al., 1988).

Both the highly purified factor isolated by the Litwack group and the partially purified factor we have studied have been noted (Meshinchi et al., 1988; Bodine & Litwack, 1988b, 1990a) to produce the same effects on the cytosolic glucocorticoid receptor as reported for molybdate, vanadate, and tungstate (Leach et al., 1979; Dahmer et al., 1984). Although both the factor we are studying and the factor isolated by the Litwack laboratory have the same effects on glucocorticoid receptor function and structure, they are clearly different in physical composition. In contrast to our factor, the purified modulator does not bind to Chelex-100 and is destroyed at 220 °C (Bodine & Litwack, 1990a,b). Thus, there appear to be two distinct classes of endogenous molecules that can interact with and stabilize steroid receptors that are associated with hsp90.

In this study, we present additional purification of the endogenous heat-stable factor by high-pressure liquid chromatography on a column (Ion-110) with high resolving properties for the separation of metal anions. All of the bioactivity is recovered in a single peak eluting with inorganic phosphate. The bioactive HPLC peak contains all of the endogenous molybdenum that was present in the preparation of factor loaded onto the column. The bioactivity in the factor preparation can be separated from inorganic phosphate by its absorption to a metal-chelating resin. These observations reinforce the proposal (Meshinchi et al., 1990) that an endogenous metal anion component of cytosol stabilizes the complex between the receptor and hsp90. However, the HPLC-purified factor is not as effective at stabilizing the purified glucocorticoid receptor–hsp90 complex as it is at stabilizing the unpurified complex in cytosol, suggesting that other cytosolic components also contribute to stabilizing the protein–protein interaction.

Experimental Procedures

Materials

[6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol) and ^{125}I-conjugated goat anti-mouse IgG were obtained from New England Nuclear (Boston, MA). Radioinert dexamethasone, rabbit anti-goat IgG–horseradish peroxidase conjugate, sodium molybdate, ammonium bicarbonate, Hepes buffer, protein A–Sepharose, and marker proteins were from Sigma Chemical Co. (St. Louis, MO). Chemicals for electrophoresis and Chelex-100 resin were from Bio-Rad Laboratories (Richmond, CA). Phosphocellulose P-11 cation-exchange resin was obtained from Whatman (Hillsboro, OR). Immobilon-P transfer membrane (0.45 μm) was from Millipore (Bedford, MA). The Ion-110 anion-exchange HPLC column was from Rainin Instruments (Woburn, MA). The BuGR2 anti-glucocorticoid receptor monoclonal antibody (Gametchu & Harrison, 1984) was kindly provided by Dr. R. W. Harrison (The University of Arkansas for Medical Sciences), and the AC88 monoclonal antibody (Riehl et al., 1985) against the 90-kDa heat shock protein was kindly provided by Dr. David Toft (Mayo Medical School).

Methods

Cell Source and Fractionation. Rat liver cytosol was prepared from 100–200-g male Sprague-Dawley rats which had been adrenalectomized and maintained on 0.9% saline for 1 day prior to sacrifice. Livers were removed immediately upon death, placed in ice-cold Earle's balanced salt solution, and homogenized in a Waring blender in 1.5 volumes of 10 mM Hepes buffer, pH 7.4, per gram of wet weight, followed by Dounce homogenization. The tissue homogenate was centrifuged for 20 min at 27000*g*, and the top half of the resulting supernatant was centrifuged at 100000*g* for 2 h.

L-Cell cytosol was prepared from L929 murine fibroblasts which were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37 °C. Cells were harvested by scraping and washed in Earle's balanced saline. The cell pellet was suspended in 1.5 volumes of 10 mM Hepes/0.1 mM EDTA, pH 7.4 at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged at 100000*g*, and the supernatant was used immediately.

Preparation of Filtered Rat Liver Cytosol for Assay of the Bioactivity of the Stabilizing Factor. The concentration of low molecular weight components in rat liver cytosol was substantially reduced by dilution and filtration as previously described by Leach et al. (1982). Rat liver cytosol (10 mL) was diluted 1:1 with 10 mM Hepes buffer and filtered to 5 mL on an Amicon YM10 filter that excludes molecules with a molecular weight greater than approximately 10000. After filtration, the large molecular weight excluded material was washed by addition of 10 mL of Hepes buffer and refiltration to the original cytosol volume. Thus, the concentration of receptor is the same as in whole cytosol, but the concentration of small molecular weight components that pass through the filter has been reduced to roughly one-sixth that of whole cytosol. As previously described (Leach et al., 1982), steroid-bound receptors in filtered cytosol are rapidly transformed to the DNA binding form on incubation at 20 °C, and this transformation is inhibited by the heat-stable factor.

Prepurification of the Stabilizing Factor from Rat Liver. The stabilizing factor was prepared by boiling rat liver cytosol, centrifuging out the denatured material, and passing the supernatant through sequential columns of Sephadex G-50 and G-10 as described previously (Meshinchi et al., 1988). The preparation was then concentrated 20-fold by lyophilization and passed through a column (2.5 cm × 10 cm) of phosphocellulose P-11 to remove the cationic components, and the phosphocellulose drop-through was concentrated back to the original volume loaded on the column (i.e., 20-fold concentrated with respect to boiled cytosol). The concentration of factor is expressed in relative units [defined in Meshinchi et al. (1988)], with 1 relative unit of factor being the concentration of factor normally present in boiled cytosol. An aliquot (500 μL) of the P-11-treated factor was added to a 30-μL pellet of dextran-coated charcoal and centrifuged after 10 min at 4 °C to remove the charcoal.

High-Performance Liquid Chromatography. An analytical Ion-110 HPLC column was equilibrated with 500 mL of 100 mM ammonium bicarbonate. An aliquot (50 μL) of 20-fold-concentrated charcoal-treated factor prepurified through the phosphocellulose P-11 step was injected into the column and eluted at 1 mL/min with an isocratic gradient of 100 mM

ammonium bicarbonate. The column eluent was monitored at 254 nm, and 1-mL fractions were collected. Each fraction was assayed for its phosphate content by the method of Ames (1966) and for its ability to prevent receptor transformation in the filtered rat liver cytosol assay.

Assay for Factor Bioactivity. Individual fractions eluted from the HPLC column were lyophilized and resuspended in water several times to remove the ammonium bicarbonate. Each fraction was assayed for factor bioactivity as follows: 120 μL of filtered rat liver cytosol containing [^{3}H]triamcinolone acetonide bound receptors was added to each tube containing lyophilized material, the final volume was adjusted to 240 μL with 10 mM Hepes and DTT at a final concentration of 10 mM, each sample was incubated for 60 min at 20 °C, and the amount of receptor transformation was measured by binding to DNA–cellulose. To assay DNA binding, 0.1-mL aliquots of incubation mix were incubated for 45 min with 0.1 mL of a 12.5% suspension of DNA–cellulose, and the pellets were washed and assayed for radioactivity. This assay is described in detail in Meshinchi et al. (1988).

Metal Analysis. Aliquots of samples applied to the Ion-110 column and aliquots of a pool composed of all elution fractions containing inorganic phosphate were assayed for molybdenum, vanadium, and tungsten by using synchroton radiation induced X-ray emission (SRIXE). The work was carried out at the X26 beam line of the Brookhaven National Synchrotron Light Source. SRIXE was chosen for these analyses since it is a method which can be used with small specimens and has excellent detection limits. The details of the X26 experimental arrangement have been described by Jones et al. (1990). In the present work, samples were prepared by deposition of 50-μL volumes on 5 mg/cm^2 filter paper. A sensitivity curve was determined by measurement of National Institute of Standards and Technology standard reference materials 3171 and 3172 (Gordon et al., 1990). Values for the minimum detection limits were calculated from the spectra for the two elements. A detection limit of 4 fg was found for Mo. Values for vanadium and tungsten were about 10 times that value because of the proximity of peaks from fluorescent Cr and Zn X-rays and larger incoherent scattering backgrounds. The system calibration was checked by measurement of a 10 μM sodium molybdate specimen. Agreement between the SRIXE determination and the nominal value was within 5%.

Assay for Stabilization of the Immunopurified L-Cell Glucocorticoid Receptor–hsp90 Complex. Aliquots (500 μL) of cytosol containing untransformed and unliganded receptors were mixed with an equal volume of TEG buffer (10 mM TES, 4 mM EDTA, 10% glycerol, and 50 mM NaCl, pH 7.6 at 4 °C), and BuGR anti-receptor antibody was added at 4% of the final volume. The mixture was incubated for 2 h at 0 °C, and each sample was added to a pellet of protein A–Sepharose (10-μL pellet per 0.1 mL of L-cell cytosol). Samples were mixed by rotation for 3 h at 4 °C, and the protein A–Sepharose pellets were washed 3 times by resuspension in 1-mL aliquots of TEG buffer. The immunopurified receptor–hsp90 complex was then incubated at 0 or 25 °C for 2 h in the presence of 100 μL of buffer to permit dissociation of hsp90 as described by Bresnick et al. (1989). At the end of the incubation, protein A–Sepharose pellets from buffer controls and from samples containing molybdate or HPLC-purified factor were then washed an additional 3 times with TEG buffer containing 20 mM sodium molybdate. One-fifth of each sample was used to assay the glucocorticoid receptor and the remaining four-fifths to assay for receptor-associated hsp90 by SDS–PAGE and immunoblotting.

Assay for Stabilization of the Glucocorticoid Receptor–hsp90 Complex in Chelex-Treated L-Cell Cytosol. Endogenous metals were removed from L-cell cytosol containing unliganded and untransformed glucocorticoid receptors by rapidly passing the cytosol through a bed of Chelex-100 resin as previously described (Meshinchi et al., 1990). The Chelex-treated, metal-depleted cytosol was incubated at 20 °C with buffer or the indicated concentration of purified stabilizing factor. At various times, 400-μL aliquots were removed and mixed with an equal volume of TEG buffer and 4% BuGR anti-receptor antibody. Immunoadsorbed proteins were adsorbed to protein A–Sepharose, washed, and assayed for receptor and hsp90 by SDS–PAGE and immunoblotting.

Western Blot Assay for Receptor and Receptor-Associated hsp90. SDS–polyacrylamide gel electrophoresis was performed in 7% slab gels as previously described (Bresnick et al., 1989). Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon P transfer membranes, followed by overnight incubation with 1% BuGR2 monoclonal antibody to detect the glucocorticoid receptor or with 0.4% AC88 antibody to detect hsp90. Immobilon membranes were subsequently probed with horseradish peroxidase conjugated goat anti-mouse IgG to detect hsp90. The hsp90 bands were then visualized either by the peroxidase method or by incubating with ^{125}I-labeled anti-mouse IgG as previously described (Bresnick et al., 1988).

Results

Purification of the Stabilizing Factor by Anion-Exchange HPLC. In previous work (Meshinchi et al., 1988), we have published a four-step purification of the receptor-stabilizing factor. Rat liver cytosol is first boiled and centrifuged to remove denatured protein. The supernatant is then passed through a column of Sephadex G-50 to eliminate all of the large components excluded by that matrix. All of the material smaller than the Sephadex G-50 macromolecular peak is passed through a long column of Sephadex G-10, and the material lying between the excluded volume and the conductivity peak determined by the smallest salts is pooled to yield heat-stable molecules with a molecular weight range of approximately 100–1000. There is no loss of total receptor-stabilizing bioactivity units through these steps. This preparation is then passed through a column of phosphocellulose P-11 to remove cations, and the cation-free drop-through material now has about 130% of the bioactivity of the original boiled cytosol (Meshinchi et al., 1988).

This phosphocellulose drop-through can be extracted with charcoal, with the removal of about 95% of the remaining UV absorbance (254 nm) and no loss of bioactivity. To this point, the endogenous factor has been separated from protein and most organic material, and to separate the factor from other anions, the charcoal-extracted material (50 μL of a 20-fold-concentrated phosphocellulose P-11 drop-through) was further resolved on an Ion-110 anion-exchange HPLC column. As documented in the manufacturer's brochure, HPLC with the Ion-110 matrix is particularly useful for separating metal anions, and the factor bioactivity (inhibition of receptor transformation to the DNA binding state in the filtered liver cytosol assay) elutes from Ion-110 in the same fractions as inorganic phosphate. This Ion-110 procedure separates the bioactivity from most of the residual UV absorbance.

To determine the recovery of factor bioactivity, inorganic phosphate, and the relevant transition metals from the Ion-110 HPLC procedure, 4 × 50 μL aliquots were resolved, and the fractions containing inorganic phosphate in each column run were pooled and lyophilized. The ammonium bicarbonate was

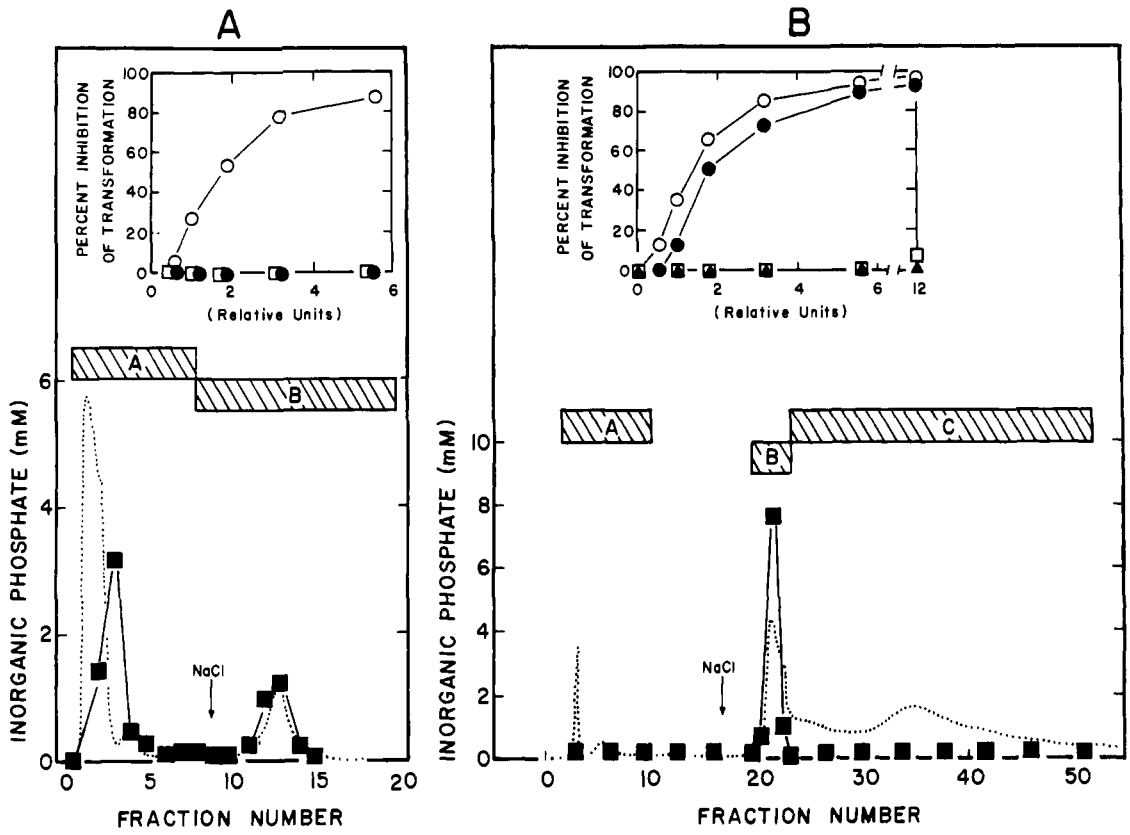

FIGURE 1: Chelex chromatography of the factor preparation. (Panel A) Chromatography on untreated Chelex-100. A column (1.5 × 10 cm) of Chelex-100 that had been adjusted to pH 7.2 in Hepes buffer ws eluted extensively with distilled water, 0.6 mL of an 8-fold-concentrated solution of the factor purified through the Sephadex G-10 step was applied, and the column was washed with distilled water. After elution of the drop-through material, determined by A_{280nm} (dotted line), the column was eluted with 1 M NaCl as indicated by the arrow. Inorganic phosphate (■) was assayed in each tube. The fractions indicated by the bars labeled A and B were pooled and lyophilized to dryness. Pool B was desalted by passage through a column of Sephadex G-10, lyophilized and reconstituted in 10 mM Hepes buffer. The inset presents the activity of pool A (□), pool B (●), and control untreated factor (○) in inhibiting receptor transformation in the filtered liver cytosol assay. (Panel B) Chromatography on copper-saturated Chelex-100. Chelex resin (pH 7.2) was saturated with copper by washing it batchwise several times with 0.25 M copper chloride. A column (1.5 × 10 cm) was prepared with the copper-chelated resin, which is deep blue in color, and the column was then washed extensively with distilled water. When unchelated copper had been removed (determined from the conductivity of the eluate), 0.6 mL of 8-fold-concentrated factor was applied, and the column was eluted first with distilled water and then with 1 M NaCl as in panel A. The fractions indicated by the bars were pooled, pools B and C were desalted, and all fractions were reconstituted in 10 mM Hepes buffer. The inset presents the activity of pools A (□), B (●), and C (▲) and untreated factor (○) in inhibiting receptor transformation in the filtered liver cytosol assay.

eliminated by multiple lyophilization, and the final material was redissolved in water to yield 200 μL of 20-fold-concentrated factor. Both the material loaded onto the column and the material in the pooled eluate peaks (concentrated back to the original volume) were assayed for bioactivity. The recovery of bioactivity was complete. Each 50-μL aliquot of column load contained 12.5 μmol of inorganic phosphate, and the recovery of inorganic phosphate in the pooled peak material was 103%. The remainder of the pooled eluate was used to assay for molybdenum by SRIXE. Neither vanadium nor tungsten was detectable in the phosphocellulose P-11 drop-through that was applied to the column. However, the material loaded onto the Ion-110 column contained an average 5.7 ± 1.4 (SE) μM molybdenum, and the eluate contained 5.4 ± 1.7 (SE) μM molybdenum. Thus, essentially all of the molybdenum in the factor preparation behaves as an anion that is eluted with both the inorganic phosphate and the bioactivity. As the factor preparation is 20-fold-concentrated, the concentration of molybdenum in unconcentrated material would be about 0.27 μM, which is close to the molybdenum concentration (0.18 μM) previously determined in the crude factor preparation (Sephadex G-10 step) by atomic absorption (Meshinchi et al., 1988).

Separation of Bioactivity from Inorganic Phosphate by Chromatography on Chelex-100. Because the endogenous factor behaves as a small heat-stable anion, it is difficult to determine the degree of purification we have achieved. By the time the material has been eluted from the Ion-110 column, there is no protein. Also, there is no absorbance peak at 254 nm when the pooled bioactive fractions eluted from Ion-110 are rerun on the same column. Thus, to use either protein or units of OD λ_{254} as a reference for purification would be inappropriate.

In contrast to OD λ_{254}, the bioactivity copurifies with inorganic phosphate throughout all of the purification steps. As inorganic phosphate should not have the chelation properties of a metal, we sought to use chromatography on Chelex-100 as a means of separating bioactivity from inorganic phosphate. Passage of the factor preparation through Chelex-100 eliminates both bioactivity and the endogenous molybdenum (Meshinchi et al., 1988). In the experiment of Figure 1A, factor that was purified through the Sephadex G-10 step was applied to a column of Chelex-100, and both the drop-through fractions and a subsequent 1 M NaCl elution were assayed for both inorganic phosphate and bioactivity. Two-thirds of the phosphate is present in the drop-through fraction, and 98% of the inorganic phosphate applied to the column was recovered in pools A (drop-through) and B (NaCl peak) defined by the bars in Figure 1A. Neither of these pools contains any factor bioactivity (see inset, Figure 1A).

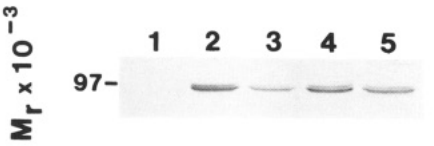

FIGURE 2: Purified stabilizing factor inhibits temperature-mediated dissociation of hsp90 from the immunopurified receptor–hsp90 complex. Aliquots of L-cell cytosol were immunoadsorbed to protein A–Sepharose either with the BuGR monoclonal anti-receptor antibody (lanes 2–5) or with nonimmune IgG (lane 1). The washed, immunoadsorbed pellets were incubated with either buffer, or 10 mM molybdate, or 2 relative units of the factor purified through the anion-exchange HPLC step, and incubated at 0 or at 25 °C for 2 h. Samples were then washed by suspension and centrifugation, and receptor-associated hsp90 was assayed by SDS–polyacrylamide gel electrophoresis and immunoblotting with rabbit antiserum against hsp90. Lane 1, immunoadsorbed with nonimmune mouse IgG; lane 2, the immunopurified receptor–hsp90 complex was incubated at 0 °C with buffer; lane 3, the complex was incubated at 25 °C with buffer, lane 4, the complex was incubated at 25 °C with molybdate; lane 5, the complex was incubated at 25 °C with the purified factor.

To elute chelated metals from Chelex-100, either the resin must be exchanged with a strongly chelated metal, such as copper, or it must be eluted with acid. Unfortunately, both the metals used for displacement and the eluting acid remaining after multiple lyophilizations destroy the system we use to assay receptor-stabilizing activity. Thus, we have not been able to demonstrate recovery of bioactivity from the Chelex column. As shown in Figure 1B, however, the chelating sites on the column can be tied up with a metal, and this prevents elimination of bioactivity from the factor preparation. In this case, the column matrix was saturated by exposing it to copper chloride and then washed extensively to eliminate free metal. When cytosol is passed through this copper-chelated column, all of the phosphate and all of the bioactivity are weakly adsorbed and are recovered in a sharp peak (pool B in Figure 1B) immediately after the start of elution with 1 M NaCl. The data provided in Figure 1 suggest that the bioactivity is eliminated by Chelex-100 because the bioactive factor has the chelating properties of a metal, and when the chelation sites are occupied with Cu^{2+}, the column then acts as an anion-exchange matrix, yielding weak retention of both phosphate and bioactivity.

Activity of the Factor on Purified versus Cytosolic Receptor Complexes. We have reported that glucocorticoid receptor–hsp90 complexes that have been immunopurified from L-cell cytosol are stabilized by molybdate, by peroxide (Bresnick et al., 1989), and by the crude (Sephadex G-10 step) factor preparation (Meshinchi et al., 1988). The ability of the HPLC-purified factor (at a concentration of 2 relative units) to stabilize the immunopurified L-cell receptor–hsp90 complex is compared to that of molybdate (10 μM) in Figure 2. In this experiment, untransformed receptors were immunoadsorbed to protein A–Sepharose using the BuGR monoclonal antibody; the pellet was washed and heated to permit temperature-mediated dissociation of hsp90 as described by Bresnick et al. (1989). The proteins in the immunopellet were resolved by SDS–PAGE, and the hsp90 remaining with the immunoadsorbed receptor was detected by immunoblotting. As shown in Figure 2, incubation at 25 °C removes most but not all of the hsp90 (lane 3), and the complex is stabilized by molybdate (lane 4) and partially stabilized by the purified factor (lane 5).

The fact that the most purified form of the factor can stabilize a purified form of the receptor–hsp90 complex suggests that the factor interacts directly with the receptor or hsp90 or both. However, it is important to note that we

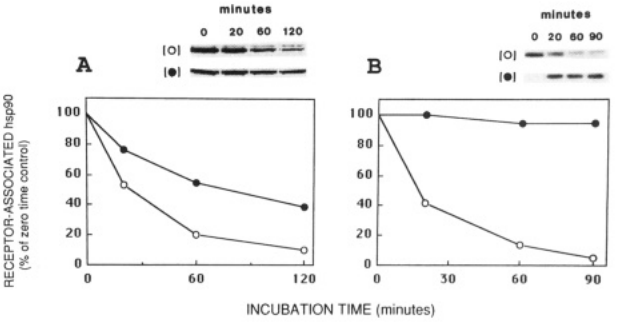

FIGURE 3: Factor is less effective at stabilizing the immunopurified receptor–hsp90 complex than the receptor–hsp90 complex in cytosol. (Panel A) Stabilization of the immunopurified receptor–hsp90 complex. Untransformed, unliganded receptors in 4 mL of L-cell cytosol were immunoadsorbed to protein A–Sepharose with the BuGR anti-receptor antibody; the immune pellet was washed and then heated at 20 °C in the presence of buffer alone (O) or 1 relative unit of HPLC-purified factor (●). At various times, the amount of hsp90 remaining in the immune pellet was quantitated by ^{125}I Western immunoblot analysis using the AC88 monoclonal anti-hsp90 antibody as the probing agent. The autoradiogram of the hsp90 is shown above the graph, and these bands were excised and counted for ^{125}I radioactivity to yield the values shown in the graph. (Panel B) Stabilization of receptor–hsp90 complexes in Chelex-treated cytosol. Chelex-treated L-cell cytosol was incubated at 20 °C in the presence of buffer (O) or 1 relative unit of factor (●). At various times, 400-μL aliquots were removed, and receptors were immunoadsorbed to protein A–Sepharose with the BuGR antibody. Receptor-associated hsp90 was assayed and quantitated as described above.

consistently find that the factor is less effective at stabilizing the immunopurified complex than it is at stabilizing receptor–hsp90 complexes in cytosol. This is illustrated in Figure 3 where we compare the activity of 1 relative unit of HPLC-purified factor to stabilize the immunopurified L-cell receptor–hsp90 complex (Figure 3A) and the receptor–hsp90 complex in Chelex-treated L-cell cytosol (Figure 3B). We have recently shown that rapid passage of L-cell cytosol through a bed of Chelex-100 depletes the cytosol of endogenous metals and makes the glucocorticoid receptor unstable, thus markedly facilitating both its dissociation from hsp90 and its transformation to the DNA binding state (Meshinchi et al., 1990). As shown in Figure 3B, the factor is very effective at stabilizing the receptor–hsp90 complex in the Chelex-treated, metal-depleted cytosol, but it only partially stabilizes the immunopurified receptor complex (Figure 3A).

Discussion

The interaction of steroid receptors with hsp90 is an important determinant of their functional state, both in intact cells (Picard et al., 1990) and in cytosols prepared from hormone-free cells [see Pratt (1987, 1990) for a review]. The interaction between hsp90 and steroid receptors is a direct, noncovalent protein–protein interaction, and in the case of the glucocorticoid receptor, the heat shock protein is known to interact specifically with the hormone binding domain of the receptor (Pratt et al., 1988; Dalman et al., 1991). Binding of hsp90 to the glucocorticoid receptor is correlated with repression of DNA binding activity in vitro, and there is good evidence that hsp90 must be bound to the receptor for it to be in a high-affinity steroid binding conformation (Sanchez et al., 1987; Bresnick et al., 1989; Scherrer et al., 1990). Smith et al. (1990) have recently demonstrated that rabbit reticulocyte lysate can direct the association of hsp90 with the chicken progesterone receptor. Similar reconstitution of the glucocorticoid receptor–hsp90 complex is accompanied by functional restitution of both the high-affinity steroid binding conformation and the non-DNA binding state of the receptor

(Scherrer et al., 1990). The reconstitution of the receptor–hsp90 complex is both temperature-dependent and energy-dependent, and it is thought to require a protein unfolding activity of hsp70 to open up an hsp90 binding region on the receptor (Smith et al., 1990; Scherrer et al., 1990).

As the association of the glucocorticoid receptor with hsp90 is important for determining the functional state of the receptor in vitro, it will be important ultimately to define the nature of the protein–protein interaction and those cytosolic factors that contribute to the stability of the complex. As depletion of cytosolic metals by Chelex-100 destabilizes the receptor–hsp90 complex and re-addition of either transition-metal oxyanions (e.g., molybdate) or an endogenous anionic, heat-stable factor stabilizes the complex (Figure 3B) (Meshinchi et al., 1990), it is reasonable to consider that an endogenous metal anion is one of the important factors determining the stability of the protein–protein interaction in cytosols. The fact that the receptor-stabilizing activity can be resolved by HPLC on the Ion-110 matrix and the fact that the stabilizing activity is extracted by Chelex-100 in a manner that reflects the metal-chelating properties of that matrix (Figure 1) strongly support the proposal that the endogenous factor we have isolated is a metal anion.

Although the endogenous factor behaves on chromatography by charge and by size (Meshinchi et al., 1988) like inorganic phosphate, the bioactivity cannot be reproduced with phosphate (Meshinchi et al., 1988), and it can be separated from endogenous phosphate by chelation (Figure 1). It is of interest that the bioactivity copurifies on Ion-110 HPLC with endogenous molybdenum, because molybdate has a similar ability to stabilize the receptor–hsp90 complex and inhibit receptor transformation (Figure 2) (Meshinchi et al., 1988; Bodine & Litwack, 1988b, 1990a). We do not know, however, if the factor is an anionic form of molybdenum. If it is a form of molybdenum, it is not MoO_4^{2-}. This is inferred from the fact that 1 unit of factor contains only about 0.27 μM molybdenum and sodium molybdate must be added to purified receptor complexes or to cytosol in the 1–10 mM range to produce the degree of stabilization of receptor–hsp90 complex shown in Figures 2 and 3. However, molybdenum can assume a wide variety of oxo and thio anion forms in solution (Stiefel, 1977). Thus, it is possible that a bioactive anion form(s) of molybdenum that is responsible for the stabilizing activity of the factor preparation may be present in only trace amounts in commercial sodium molybdate or that the bioactive form might be formed in only trace amounts when sodium molybdate is added to cytosols for assay of receptor stabilization.

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