Evidence That the 90-kDa Phosphoprotein Associated with the Untransformed L-cell Glucocorticoid Receptor Is a Murine Heat Shock Protein*

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Two phosphoproteins are adsorbed to protein-A-Sepharose when cytosol from 32P-labeled L-cells is incubated with a monocular antibody against the glucocorticoid receptor: one is a 98–100-kDa phosphoprotein that contains the steroid-binding site and the other is a 90-kDa nonsteroid-binding phosphoprotein that is associated with the untransformed, molybdate-stabilized receptor (Housley, P. R., Sanchez, E. R., Westphal, H. M., Beato, M., and Pratt, W. B. (1985) J. Biol. Chem. 260, in press). In this paper we show that the 90-kDa receptor-associated phosphoprotein is an abundant cytosolic protein that reacts with a monoclonal antibody that recognizes the 90-kDa phosphoprotein that binds steroid receptors in the chicken oviduct. The 90-kDa protein immunoadsorbed from L-cell cytosol with this antibody reacts on Western blots with rabbit antiserum prepared against the 89-kDa chicken protein that binds steroid receptors in the chicken oviduct. The 90-kDa protein immunoadsorbed from L-cell cytosol with this antibody reacts on Western blots with steroid receptor-associated phosphoprotein in L-cell cytosol.

EXPERIMENTAL PROCEDURES

Materials

[32P]Orthophosphate (carrier-free) was from Amersham Corp.; normal nonimmune mouse IgG, nonradioactive triamcinolone acetonide, dithiothreitol, TES, protein A-Sepharose CL-4B, and molecular weight marker proteins were from Sigma; and sodium molybdate (dibasic salt) was from Baker Chemical Co.

Methods

Cell Culture, Labeling Conditions, and Cytosol Preparation—L929 mouse fibroblasts were grown in monolayer culture in Joklik medium supplemented with 10% calf serum at 37 °C. For 32P-labeling experiments, cells in log phase of growth were washed with phosphate-free medium containing 10% dialyzed calf serum and incubated in this medium containing [32P]orthophosphate (20 μCi/ml) at 37 °C for 18 h. All subsequent steps were done at 0–4 °C. Cells were harvested by scraping into Earle’s balanced saline and centrifuged at 600 × g for 10 min. Following a wash by resuspension in Earle’s saline and centrifugation, cells were suspended in 1.5 volumes of 10 mM Hepes, 0.4 mM EDTA, pH 7.35, at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged first at 27,000 × g for 30 min, dithiothreitol (2 mM) and sodium molybdate (10 mM) were added, and the mixture was centrifuged at 105,000 × g for 1 h. After removal of the floating lipid layer, the supernatant fluid (referred to as cytosol) was either immediately used or stored at −70 °C.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed in 7% acrylamide slab gels according to Laemmli (7). Gels were cooled to 4 °C during electrophoresis. All samples were extracted

We have reported the molybdate-stabilized cytosol prepared from L-929 mouse fibroblasts contains two phosphoproteins (a 90-kDa1 and a 98-kDa) that copurify on affinity chromatography in a manner consistent with the predicted behavior of the glucocorticoid receptor (1). Both of these phosphoproteins are adsorbed to protein-A-Sepharose after incubation of L-cell cytosol with a monoclonal antibody against the glucocorticoid receptor (2). The 98-kDa phosphoprotein has been shown by covalent affinity labeling with [3H]dexamethasone 21-mesyate to contain the steroid binding site. The 90-kDa phosphoprotein is a structurally different protein that does not bind steroid but is associated with the untransformed, molybdate-stabilized L-cell glucocorticoid receptor (2).

Two laboratories have reported that molybdate-stabilized progesterone receptors are eluted from steroid affinity columns in association with a 90-kDa phosphoprotein that does not bind progesterone (3–6). Sullivan et al. (4) and Joab et al. (6) have prepared monoclonal antibodies against the 90-kDa nonsteroid-binding phosphoprotein that is associated with the chick oviduct progesterone receptor in the untransformed S S complex. These monoclonal antibodies against the 90-kDa protein also interact with molybdate-stabilized glucocorticoid, estrogen, and androgen receptors in chick oviduct cytosol.

These observations support a general model in which a variety of untransformed steroid receptors are associated with a nonsteroid-binding 90-kDa phosphoprotein. In this communication, we will present data showing that the 90-kDa receptor-associated phosphoprotein in L-cell cytosol is the 90-kDa murine heat shock protein.
from protein A-Sepharose by boiling in SDS sample buffer containing 10% β-mercaptoethanol. Molecular weight standards were: myosin, M, = 205,000; β-galactosidase, M, = 116,000; phosphorylase b, M, = 97,000; bovine serum albumin, M, = 66,000; and ovalbumin, M, = 45,000.

**Incubation with Antibodies and Adsorption to Protein A-Sepharose**

The monoclonal anti-receptor antibody (GR49) used in this work is derived from one of seven anti-receptor antibody-producing hybridomas selected from 102 fusions by Westphal et al. (8). The original antigen was the partially purified, transformed form of the glucocorticoid receptor from rat liver. GR49 is an IgG antibody that cross-reacts extensively (90%) with the mouse glucocorticoid receptor (8). The preparation of GR49 used in these experiments was precipitated from ascitic fluid with ammonium sulfate at 40% of saturation, redissolved in phosphate-buffered saline containing 0.1% NaN₃ at a concentration of 10 mg protein/ml, and subsequently diluted 10-fold in TEG buffer (10 mM TES, 50 mM NaCl, 10% glycerol, 4 mM EDTA, pH 7.6, at 0–4 °C) and frozen at −70 °C. The AC88 monoclonal antibody against the 90-kDa phosphoprotein was prepared against the 88-kDa protein purified from the water mold *Achlya ambisexualis* as described by Riehl et al. (9) and has been shown to react with the 90-kDa phosphoprotein from chick oviduct. The preparation and properties of the 4F3 monoclonal antibody against the chicken oviduct 90-kDa phosphoprotein have been described by Sullivan et al. (4). The preparation and properties of the rabbit antisera against gel-purified 89-kDa chicken heat shock protein has been described by Kelley and Schlesinger (10). This antibody is referred to throughout the text as anti-hsp89. The rabbit antisera against glucocorticoid receptor purified from L-cells will be described in another report from this laboratory.

The following protocol was used in all of the antibody experiments. Aliquots of 100 to 300 μl of either 32P-labeled or unlabeled L-cell cytosol were incubated for 2–4 h at 0 °C with 50 mM nonradioactive dexamethasone to form steroid-bound receptor complexes and mixed with an equal volume of TEG buffer containing 20 mM dithiothreitol and 20 mM sodium molybdate. Monoclonal antibody (all preparations contained 1 mg of protein/ml) or nonimmune mouse IgG (at the same IgG concentration as that of the monoclonal preparation) or rabbit antisera against the mouse glucocorticoid receptor was added to the cytosol at the indicated per cent of final volume, and the mixtures were incubated for 15 to 16 h on ice. Each mixture was added to 50 μl bed volume of protein A-Sepharose pre-equilibrated in TEG buffer and mixed by rotation at 4 °C for 2 h. Protein A-Sepharose was then pelleted by centrifugation, the supernatant was removed, and the pellet was washed 3 times by suspension in 1 ml of TEG buffer containing 20 mM sodium molybdate and 20 mM dithiothreitol. The pellet was washed sequentially with 3 x 1-ml volumes of molybdate-containing TEG buffer with 0.4 M NaCl, buffer with 0.4 M NaCl and 0.2% Triton X-100, and finally with a 10 mM Tris buffer, pH 6.8, containing 10% (w/v) glycerol, 20 mM sodium molybdate and 20 mM dithiothreitol. It is important to note that sodium molybdate is present in all buffers, including wash buffers, except as noted in the legend to Fig. 3. The washed pellet was suspended in 4% SDS sample buffer boiled for 4 min, and the eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis.

**Immunoblotting—** Immunoblotting was carried out by transferring proteins from 7.5% acrylamide slab gels to nitrocellulose paper, overnight incubation with the antibody preparation, and subsequent reaction with peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies as previously described (2).

**RESULTS AND DISCUSSION**

As shown in lane 2 of Fig. 1, immunoadsorption of 32P-labeled, molybdate-stabilized L-cell cytosol with a monoclonal antibody (GR49) specific for the 98-kDa steroid-binding phosphoprotein results in the immunoadsorption of both a 98-kDa phosphoprotein and a 90-kDa phosphoprotein. If the same 32P-labeled cytosol is incubated with the AC88 monoclonal antibody against the 90-kDa protein (*lanes 3 and 6*), a large amount of 32P-labeled 90-kDa protein is immunoadsorbed. The AC88 monoclonal antibody was raised against an abundant 88-kDa protein of *A. ambisexualis*, a water mold that has steroidal receptors which are stabilized in an 8 S complex by molybdate (11). This AC88 monoclonal antibody reacts with the 90-kDa phosphoprotein that is associated with the chick oviduct progesterone receptor, and its reactive site is directed against an epitope that is conserved in 90-kDa proteins in a variety of avian, rodent, and human cells. It is clear from examining the Coomassie Blue stain (not shown) of the gel used for the autoradiogram of Fig. 1 that the 90-kDa protein is present in very large amounts in L-cell cytosol. In contrast to the AC88 monoclonal antibody, the 4F3 monoclonal antibody, which was raised against the steroid receptor-associated 90-kDa phosphoprotein of chick oviduct, shows only a very low level of reactivity against the 90-kDa protein in L-cell cytosol.

We now know several facts about the 90-kDa glucocorticoid receptor-associated protein of L-cells: 1) it is an abundant cytosolic protein, 2) it is phosphorylated on serine moieties (1, 3) it has regions that are highly conserved, and 4) it is present in a wide variety of cell types. Taken together, these observations describe very well the 90-kDa proteins in a variety of avian, rodent, and human cells. It is clear from examining the Coomassie Blue stain (not shown) of the gel used for the autoradiogram of Fig. 1 that the 90-kDa protein is present in very large amounts in L-cell cytosol. In contrast to the AC88 monoclonal antibody, the 4F3 monoclonal antibody, which was raised against the steroid receptor-associated 90-kDa phosphoprotein of chick oviduct, shows only a very low level of reactivity against the 90-kDa protein in L-cell cytosol.

Kelley and Schlesinger (10) have raised a rabbit antisera against gel-purified chicken hsp89 and demonstrated that it...
reacts with proteins of similar mobilities in human, rodent, frog, and Drosophila cells. We show in Fig. 2 (lane 1) that the antibody reacts with the abundant L-cell cytosol phosphoprotein that is immunoabsorbed by the AC88 monoclonal. The experiment of Fig. 2 (lanes 2 and 4) also shows that immunoabsorption of molybdate-stabilized L-cell cytosol with anti-receptor antibody results in the immune-specific adsorption of a 90-kDa protein that is recognized by the anti-hsp89 antibody on Western blot. As the 90-kDa receptor-associated phosphoprotein has several properties in common with the 90-kDa heat shock protein and reacts with antisera prepared against the heat shock protein, we propose that hsp90 is a component of the untransformed, molybdate-stabilized glucocorticoid-receptor complex in L-cell cytosol. We have tried to immunoabsorb untransformed glucocorticoid receptors bound with [3H]triamcinolone acetonide using the anti-hsp89 antisera and protein A-Sepharose. So far, we have only adsorbed about 5% of the specifically bound [3H]triamcinolone acetonide using this approach. This very low level of immunoabsorption probably reflects the fact that there is a huge excess of hsp90 in the cytosol that is not bound to the glucocorticoid receptor and we are only causing the immunoabsorption of a portion of the total hsp90. Also, anti-hsp89 was raised against denatured protein and it may react very poorly with native structures in a complex in murine cells.

Several laboratories have demonstrated that transformation of glucocorticoid-receptor complexes is accompanied by a reduction in their size from an apparent, Mr, of about 320,000 to about 100,000, leading to the proposal that transformation involves dissociation of the receptor either from itself or from nonsteroid-binding components (13–15). Both transformation to the DNA-binding state and the reduction in molecular size are prevented by molybdate (16–18). It is clear from the results of the experiment shown in Fig. 3 that molybdate must be present to recover the complex between the 90-kDa protein and the receptor. If molybdate is not present to preserve the complex during the reaction with antibodies and washing of the protein A-Sepharose pellet, the 90-kDa protein is lost. The sample shown in lane 5 of Fig. 3 was incubated at 25 °C for 1 h in the absence of molybdate to permit receptor transformation and then molybdate was present during the rest of the procedures. By comparing lanes 4 and 5 in the Western blot shown in part A of the figure, it is clear that most of the 90-kDa protein dissociates from the receptor during temperature-mediated transformation, whereas molybdate stabilizes the complex. In part B of Fig. 3, the Western blot has been reacted with the GR49 antibody to demonstrate that the glucocorticoid receptor is present in all samples.

In this work, we have presented evidence that, in its untransformed state in molybdate-stabilized L-cell cytosol, the glucocorticoid receptor exists in a complex with the 90-kDa murine heat shock protein. When the receptor is transformed, it dissociates from hsp90 and binds to DNA. Both this dissociation and transformation to the DNA-binding state are inhibited by molybdate which acts to stabilize the receptor-hsp90 complex. It has been shown that the phosphoproteins mediating transformation of cells infected with Rous (pp60vsrc), Fujinami (pp140vsrc), and Y73 (pp94vsrc) avian sarcoma viruses also associate transiently with hsp90 in infected cells (19–21). It will be interesting to see if molybdate stabilizes complexes between hsp90 and viral transforming proteins in the same manner that it stabilizes hsp90-steroid receptor complexes. If so, this might suggest that molybdate and some of the other group 6A transition metal oxyanions have the general prop-

FIG. 2. Demonstration that the 90-kDa protein, immunoabsorbed from molybdate-stabilized cytosol in a receptor-specific manner, reacts with antibody prepared against the 89-kDa chicken heat shock protein (aHS89). Aliquot (100–200 μl) of L-cell cytosol were diluted with TEG buffer containing molybdate, incubated with the indicated antibodies, and processed for immunoblotting as described under “Experimental Procedures.” The nitrocellulose immunoblots were reacted with 4% aHS89 serum. Lane 1, 100 μl of cytosol adsorbed with 2% AC88. Lane 2, 200 μl of cytosol adsorbed with 5% GR49. Lane 3, 200 μl of cytosol adsorbed with 5% nonimmune mouse IgG (NI). Lane 4, 200 μl of cytosol adsorbed with 10% polyclonal rabbit antireceptor serum (PC). Lane 5, 200 μl of cytosol adsorbed with 10% preimmune rabbit serum (PI).

FIG. 3. Relationship of 90-kDa protein to molybdate-stabilized and temperature-transformed states of the L-cell receptor. Aliquot (200 μl) of L-cell cytosol containing 20 mM dithiothreitol but no molybdate were preincubated at 0 °C with 200 μM dexamethasone to form steroid-receptor complexes. Each aliquot was then incubated for 1 h at either 0 or 25 °C in the presence or absence of 20 mM sodium molybdate as indicated. All samples were then incubated with 10% antireceptor serum, adsorbed to protein A-Sepharose, and the pellets were washed essentially as described under “Experimental Procedures.” In samples 1 and 4, molybdate was present during all procedures, including the washing of the protein A-Sepharose pellet. In samples 2 and 3, no molybdate was present during any procedure. In sample 5 cytosol containing steroid-bound receptors was incubated at 25 °C for 1 h in the absence of molybdate, then molybdate was added and molybdate was present during incubation with antisera and all subsequent procedures. After the protein A-Sepharose pellets were washed, the proteins were extracted with SDS sample buffer and analyzed by SDS-gel electrophoresis and immunoblotting as described under “Experimental Procedures.” Panel A, all lanes on the nitrocellulose paper were reacted with 4% AC88 monoclonal antibody, developed by the peroxidase method, and the wet immunoblot was photographed. Panel B, the same immunoblot was then washed and reacted with 10% GR49 hybridoma fluid and developed again. Thus, panel A shows only the 90-kDa nonsteroid-binding protein and panel B shows both the 90-kDa protein and the steroid-binding proteins in the same immunoblot. Lane 1, steroid-receptor complexes incubated at 25 °C in the presence of molybdate; lane 2, incubated at 0 °C in the absence of molybdate; lane 3, incubated at 25 °C in the absence of molybdate; lane 4, incubated at 25 °C in the presence of molybdate; lane 5, incubated for 1 h at 25 °C in the absence of molybdate, then immunoabsorbed and washed in the presence of molybdate.
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property of stabilizing associations between hsp90 and other proteins.

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