Energy-Dependent Conversion of Transformed Cytosolic Glucocorticoid Receptors from Soluble to Particulate-Bound Form†

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ABSTRACT: We have recently published that soluble cytosolic glucocorticoid receptors are converted to a particulate form when they are incubated at 37 °C in a tubulin-polymerizing buffer [Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C., & Welsh, M. J. (1989) *Cancer Res. (Suppl.)* *49*, 2222s–2229s]. In this work, we further define this phenomenon and demonstrate that the L-cell glucocorticoid receptors are binding to a protein particulate composed largely of cytoskeletal proteins. Incubation of L-cell cytosol with glutamate at 37 °C converts the glucorticoid receptor to a form that pellets when cytosol is centrifuged at 150000*g*. The particulate material formed in a temperature-dependent and glutamate-dependent manner contains a large amount of tubulin, actin, and vimentin, but it is not the product of a cold-labile, colchicine-sensitive polymerization process. Very few cytosolic proteins are present in this complex, but the glucocorticoid receptor is tightly bound to it. Binding of the receptor to the cytoskeletal complex occurs after receptor transformation and is at least partially energy-dependent. Examination of the behavior of β-galactosidase receptor fusion proteins and the nt[i] glucocorticoid receptor demonstrates that residues 445 to the COOH-terminus of the receptor (DNA-binding and hormone-binding domains) contain the features required for binding to the cytoskeletal complex. Although it is the transformed receptor that associates tightly with the complex, DNA-binding activity is not required for association with the cytoskeletal particulate. The temperature-dependent, energy-dependent, and glutamate-dependent conversion of cytosolic receptors to a particulate form may provide a useful system in which to study a unique protein–protein interaction of the transformed receptor.

Steroid receptors are recovered from hormone-free cells in the cytosolic fraction as ~9S complexes [for a review, see Sherman (1984)]. The 9S form of the receptors is stabilized by the transition-metal oxyanions molybdate, vanadate, and tungstate [for a review, see Dahmer et al. (1984)]. The classically described 9S form of the receptor is known to be a heterocomplex in which the receptor is bound to the 90-kDa heat shock protein (hsp90)[1] [for a review, see Pratt (1990)]. When unliganded steroid receptors are immunoadsorbed under the gentlest conditions designed to maintain as much of the structure of the native heteroprotein complex as possible, several other proteins have been found to coimmunoadsorb with the receptor (Kost et al., 1989; Smith et al., 1990; Bresnick et al., 1990), suggesting that the receptors are bound to a multimolecular structure.

In their 9S form, the receptors do not have DNA-binding activity and are considered to be untransformed.[2] Cross-linking studies suggest a core unit of two molecules of hsp90 and one molecule of hsp56 per molecule of steroid-binding protein in the untransformed glucocorticoid receptor (GR) complex (Rexin et al., 1991; Alexis et al., 1992). This untransformed heteromolecular state of the receptor is considered soluble in that it remains in the supernatant fraction when cytosol is centrifuged at 150000*g*.

When cytosols are heated, the glucocorticoid receptor dissociates from hsp90, with the rate of dissociation being much faster if the GR is bound by steroid (Sanchez et al., 1987). The soluble heterocomplex is also dissociated by salt or increased pH. Dissociation of the GR from hsp90 is accompanied by simultaneous transformation of the receptor to the DNA-binding state (Sanchez et al., 1987; Meshinchi et al., 1990). The transformed GR behaves as a monomer migrating at 4 S on sucrose gradients [for a review of receptor transformation, see Pratt (1987)].

Although the 4S receptor can be regarded as derived from the hormone-activated form of the receptor in the cell, it is unreasonable to assume that in intact cells the hormone promotes receptor dissociation from a multimolecular structure, leaving a soluble entity that proceeds in a random way to the precise sites where its effects on transcriptional activation occur. Rather, it seems inescapable that the hormone-transformed receptor must enter into one or more new protein–protein interactions that precisely direct its movement to its final site of action. It is entirely possible that elements of the system involved in this trafficking of the transformed receptor are present in the cytosol fraction after cell rupture and that conditions may be achieved under which these protein–protein interactions can be directly or indirectly detected.

In some cases, very high molecular weight complexes have been found after receptor transformation in cytosol. For example, Nelson et al. (1989) reported that estrogen receptors in mouse uterine cytosol exposed to conditions usually used for receptor transformation (25 °C, 60 min) enter into a large complex that elutes between dextran blue 2000 and thyroglobulin (M_r 669 000) on size-exclusion HPLC. Because high molecular weight complexes did not form when cytosol

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[1] Abbreviations: CHO, Chinese hamster ovary; hsp90, 90-kDa heat shock protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

[2] 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.

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components were concentrated or precipitated, Nelson et al. (1989) concluded that they did not result from generalized aggregation and they suggested that through transformation the estrogen receptors may have acquired the capacity to engage in intermolecular associations with other proteins that are meaningful with respect to further events in hormone action.

At a recent meeting, we reported that conditions that permit cell-free polymerization of tubulin convert the glucocorticoid receptor from a solbule to particulate-bound form in L-cell cytosol (Pratt et al., 1989). Because the steroid receptors are classically regarded as soluble entities in cytosols, we have pursued this observation further. In this paper, we show that incubation of cytosol at 37 °C in the presence of glutamate causes the formation of large heteromeric complexes containing several cytoskeletal proteins, including tubulin, vimentin, and actin. The glucocorticoid receptor is tightly bound to this cytoskeletal aggregate. Binding to the cytoskeletal protein complex occurs after receptor transformation, is at least partially energy-dependent, and requires only the COOH-terminal half of the receptor without requiring DNA-binding activity. We present this particulate binding as a heretofore undescribed physical behavior of the glucocorticoid receptor with the possibility that it could reflect protein–protein interactions relevant to those that occur after receptor transformation in the cell.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Dexamethasone 21-mesylate (44.7 Ci/mmol) and ^{125}I-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from DuPont–New England Nuclear. DNA–cellulose (double-stranded from calf thymus), nonimmune mouse IgG, goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugate, TES, Hepes, protein A–Sepharose Cl-4B, and monoclonal antibodies against rat brain β-tubulin, porcine vimentin, and β-galactosidase were from Sigma. Immobilon P membranes were from Millipore Corp., and the anti-actin monoclonal antibody was from Chemicon. The rabbit anti-hsp70 serum prepared against the C-terminal 21 amino acid sequence from human hsp70 by Erhart et al. (1988) was kindly provided by Dr. Ettore Appella (National Cancer Institute). The four COOH-terminal amino acids of human hsp70 and hsp90 are identical, and the antiserum recognizes both proteins (Erhart et al., 1988). The BuGR2 monoclonal antibody against the GR (Gametchu & Harrison, 1984) was kindly provided by Drs. William Hendry and Robert Harrison (University of Arkansas for Medical Science). The plasmid (pSV2Wrec) containing the cDNA for the wild-type mouse glucocorticoid receptor (Danielsen et al., 1986) was provided by Dr. Gordon Ringold (Syntex Research, Palo Alto, CA), and plasmids containing cDNAs for β-galactosidase (Z) and β-galactosidase fused to residues 4–795 (Z.4C) or 4–445 (Z.4–445) of the rat glucocorticoid receptor were prepared as described by Picard and Yamamoto (1987) and provided by Dr. Keith Yamamoto (Department of Biochemistry and Biophysics, University of California, San Francisco). The nt^i (*n*uclear *t*ransfer *i*n-creased) variant of the S49 mouse lymphoma cell line (S49.1A.55R) was originally selected by Sibley and Tomkins (1974) and was provided by Dr. Keith Yamamoto. NB cells are clone-transfected CHO cells expressing mouse glucocorticoid receptor that binds hormone but is unable to bind DNA due to a point mutation in the DNA-binding domain in which Arg^{484} has been changed to His (Hirst et al., 1990). The NB cells were provided by Dr. Gordon Ringold.

Cell Culture and Fractionation. L929 mouse fibroblasts (L cells) were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum. NB cells were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 40 μg/mL proline and 10% iron-supplemented calf serum. The NB cells were originally transfected with a plasmid containing the cDNA for dihydrofolate reductase and a plasmid containing mutant mouse glucocorticoid receptor cDNA, and then selected for amplification by growth in methotrexate. To maintain overexpression, the NB cells are maintained in 10 μM methotrexate. S49 nt^i cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate in a 5% CO_2 atmosphere at 37 °C. Cells were harvested in late-log-phase growth by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500*g*. The washed cells were resuspended in 1.5 volumes of Hepes buffer (10 mM Hepes and 1 mM EDTA, pH 7.4) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100000*g*. The 100000*g* supernatant is the cytosol, and it was used fresh (i.e., without freezing or storage).

Receptor Pelleting Assay. Cytosol (50 μL) containing unbound receptors (or steroid-bound receptor where noted) was mixed with an equal volume of Hepes buffer (10 mM Hepes and 1 mM EDTA, pH 7.4), glutamate buffer (Hepes buffer containing 100 mM monosodium glutamate, pH 7.4), or microtubule stabilizing buffer (100 mM glutamate, 20 mM sodium phosphate, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM GTP, pH 6.8) as noted in the figure legends. The mixtures were incubated for 20 min at 37 °C (or for the time and temperatures noted in the figure legends) and then centrifuged for 10 min at 150000*g* in a Beckman Airfuge at room temperature using a rotor with a 30° angle at 30 psi. The supernatants were removed and mixed with SDS sample buffer. The tightly-packed pellets were rinsed by adding 100 μL of distilled water to the tube and removing the water without disturbing the pellet. After being rinsed, the pellet was suspended in SDS sample buffer, and both supernatant and pellet samples were heated at 100 °C for 2 min.

Gel Electrophoresis and Immunoblotting. SDS–PAGE was performed in 7% slab gels essentially as described by Dalman et al. (1988). Gels were cooled to 4 °C during electrophoresis. Molecular weight standards were the following: myosin, M_r 205 000; β-galactosidase, M_r 116 000; phosphorylase B, M_r 97 000; bovine serum albumin, M_r 66 000; egg albumin, M_r 45 000; carbonic anhydrase, M_r 29 000. Immunoblotting was carried out by transferring proteins to an immobilon P membrane and incubating with 1% BuGR antibody against the glucocorticoid receptor or monoclonal antibody against β-tubulin, followed by a second incubation with peroxidase-conjugated goat anti-mouse IgG. In some experiments, the relative amount of receptor and tubulin in various samples was quantitated by incubating also with ^{125}I-labeled goat anti-mouse IgG and then excising receptor bands visualized by peroxidase staining and counting for ^{125}I as described previously (Bresnick et al., 1989).

Immunoadsorption. In one experiment, the glucocorticoid receptor was immunoadsorbed with monoclonal antibodies to cytoskeletal proteins. Aliquots (200 μL) of L-cell cytosol that had been mixed with an equal volume of glutamate buffer and incubated at 37 °C were cooled and added to 25 μL of protein A–Sepharose pellets prebound with 15 μL of nonimmune mouse IgG (1 mg/mL) or a comparable amount of monoclonal IgG antibody to β-tubulin, vimentin, or actin. The mixture

was rocked at 4 °C for 90 min, the immunopellets were centrifuged in a microfuge, and the pellets were washed 3 times with 1 mL of 10 mM Hepes, 1 mM EDTA, and 10% (w/v) glycerol. The pellets were boiled in SDS sample buffer, and the receptor was resolved by SDS–PAGE and Western blotting with the BuGR antibody as probe.

Cell Transfection. COS-1 cells were maintained in monolayer culture in DMEM containing 10% fetal calf serum and supplemented with 4 mM glutamine, 10 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 100 μg/mL kanamycin sulfate. Cells were plated into 150 cm^2 dishes at 5×10^6 cells/dish, and the following day they were washed 2 times with 25 mL of Dulbecco's PBS and then incubated with 5 mL of DNA cocktail (5 μg/mL supercoiled plasmid DNA and 500 μg/mL DEAE-dextran in PBS) for 30 min at 37 °C. Next, 20 mL/dish of 80 μM chloroquine in serum-free DMEM was added, and the cells were incubated for 2.5 h at 37 °C. The medium was removed, and the cells were shocked with 10% DMSO (v/v) in DMEM for 10 min at room temperature. The DMSO solution was removed, 25 mL of DMEM containing 10% fetal calf serum was added, and the cells were incubated at 37 °C in 5% CO_2. After 72 h, cells were harvested by scraping, and cytosol was prepared as described for L cells.

DNA-Binding Assay. To measure receptor binding to DNA, 200-μL aliquots of incubation mix containing steroid-bound receptors were incubated for 1 h at 4 °C with a 200-μL suspension of 12.5% (v/v) DNA–cellulose containing 20 mM sodium molybdate. The DNA–cellulose pellets were washed 3 times with 1 mL of Hepes buffer containing 10 mM molybdate and assayed for radioactivity of bound steroid or solubilized with SDS sample buffer and analyzed by quantitative Western blotting with ^{125}I-labeled antibody as described (Bresnick et al., 1989).

RESULTS

Conditions Required for Converting Receptor from Soluble to Particulate Form. We reported previously (Pratt et al., 1989) that dilution of L-cell cytosol (prepared in Hepes buffer) with an equal volume of microtuble stabilizing buffer permitted temperature-dependent conversion of the receptor from a soluble to a particulate-bound form. The microtubule stabilizing buffer was prepared according to conditions published by Asnes and Wilson (1979), and it contains monosodium glutamate, sodium phosphate, EGTA, $MgCl_2$, and GTP at pH 6.8. The experiment of Figure 1 was performed to determine the minimum buffer conditions required to pellet the receptor. Aliquots of cytosol were diluted with an equal volume of solutions containing various components of the microtuble stabilizing buffer (SB), and after incubation at 37 °C, the relative amounts of glucocorticoid receptor and tubulin in supernatant and particulate form were assayed. Conditions 3 and 4 in Figure 1 demonstrate that the receptor is still converted from soluble to particulate-bound form when the stabilizing buffer is prepared at pH 7.4 and when EGTA, GTP, and $MgCl_2$ are eliminated. It is clear from conditions 5 and 6 that, of the two remaining constituents, glutamate and phosphate, glutamate is the important component for receptor pelleting. Although less than half of the tubulin is pelleted under any of the conditions, there is a rough correlation between conditions that favor pelleting of receptor and those that promote pelleting of tubulin. In the rest of the experiments in this paper, we use the Hepes buffer containing only glutamate at pH 7.4 to promote conversion of the receptor to particulate–bound form.

At 37 °C, we never see more than 50% of the tubulin converted to the particulate form, but with a 90-min incubation

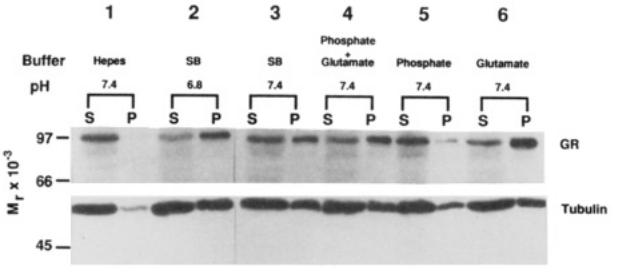

FIGURE 1: Glutamate alone is sufficient to promote conversion of the receptor from solbule to particulate form. Aliquots (50 μL) of cytosol were diluted with an equal volume of the buffers indicated below, the mixtures were incubated at 37 °C for 20 min, and they were then centrifuged at 150000*g*. The receptor and the tubulin in the pellet and supernatant were resolved by SDS–PAGE and Western blotting. The buffers used for each condition are as follows: (1) Hepes (10 mM Hepes and 1 mM EDTA) at pH 7.4; (2) SB, complete microtubule stabilizing buffer (100 mM sodium glutamate, 20 mM sodium phosphate, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM GTP) adjusted to pH 6.8; (3) stabilizing buffer at pH 7.4; (4) Hepes with 20 mM sodium phosphate and 100 mM glutamate; (5) Hepes with phosphate alone; (6) Hepes with glutamate alone.

in the presence of glutamate alone, we recover 70–90% of the receptor in the pellet (data not shown). Although glutamate is well-known to stabilize tubulin and to enhance the self-association of tubulin into microtubules (Wilson, 1970; Arakawa & Timascheff, 1984), neither the conversion of receptor nor the conversion of tubulin from soluble to particulate form in this L-cell cytosol system reflects classical tubulin polymerization, as observed, for example, when brain tubulin is purified by multiple cycles of polymerization (Asnes & Wilson, 1979). Even at a concentration of 200 μM, colchicine does not affect pelleting of either the receptor or tubulin from L-cell cytosol, whereas it completely blocks polymerization of purified bovine brain tubulin in parallel assays (data not shown). Nocodazole does not affect the pelleting either. Cycling microtubules depolymerize when the temperature is lowered to 4 °C, but pelleting of tubulin or receptor from L-cell cytosol that has been incubated with the stabilizing buffer at 37 °C is not reduced by cooling in ice before centrifugation and the proteins are not resolubilized if the centrifuged pellet is resuspended in cold buffer (data not shown). These observations, combined with the lack of a requirement for added GTP and Mg^{2+}, raise the possibility that a general protein aggregate is being formed in a glutamate-dependent manner.

Composition of the Cytoskeletal Protein Aggregate. The experiments of Figure 2 were performed to determine if incubation of L-cell cytosol in the presence of glutamate causes all cytosolic proteins to aggregate in a nonspecific manner or if selected cytosolic proteins are involved. As shown in the panel on the left in Figure 2, most of the cytosolic proteins are not converted to particulate form. Four bands lying in the range of M_r 45 000–55 000 constitute the great majority of the Coomassie blue-stained material in the pellet. The band just above the 45-kDa marker was identified as actin, the major component of microfilaments, and the most heavily stained band at 55 kDa is tubulin. The light Coomassie blue-stained band just above tubulin was identified as the intermediate filament protein vimentin. The band just above the 29-kDa marker and the three lighter staining bands migrating at about M_r 80 000 have not been identified.

Two abundant heat shock proteins that are known to associate with steroid receptors are not present in the pellet formed on incubation with glutamate (Figure 2, right panel). Hsp90 is associated with the untransformed state of steroid

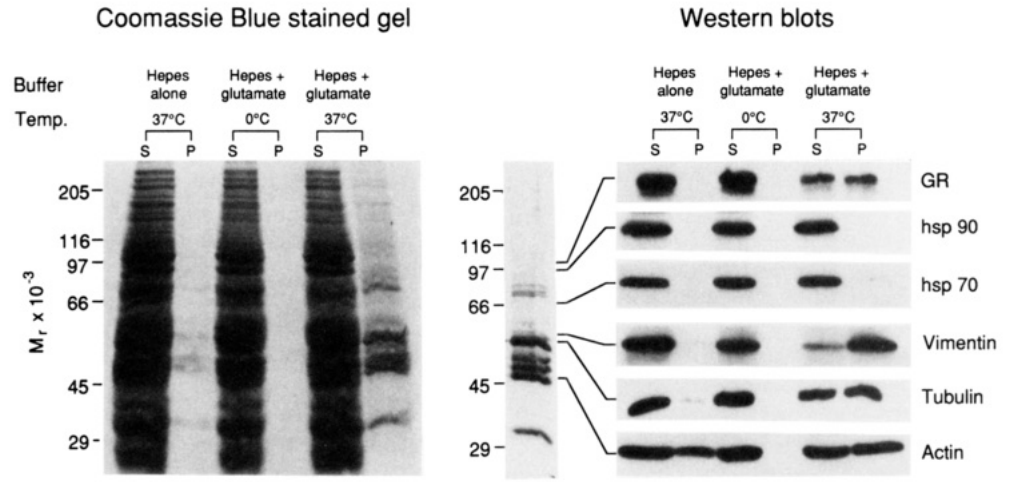

FIGURE 2: Cytoskeletal proteins are major components of the particulate material. Aliquots of L-cell cytosol mixed with Hepes buffer or glutamate buffer were incubated for 50 min at 0 or 37 °C as indicated above the lanes. After centrifugation, the proteins in the supernatant (S) and pellet (P) derived from a 50-μL aliquot of incubation mix were resolved by SDS–PAGE. On the left is shown a Coomassie blue stain of the gel. The right-hand panel summarizes data from three identical experiments where the proteins were transferred to Immobilon P membranes, the membranes were cut in half, and each half was blotted with an antibody against one of the proteins indicated on the right. The gel lane shown in the right panel is a Coomassie blue stain of the pellet proteins obtained from a 37 °C incubation with glutamate, with the lines indicating the position of the individual Western-blotted proteins on the stained gel.

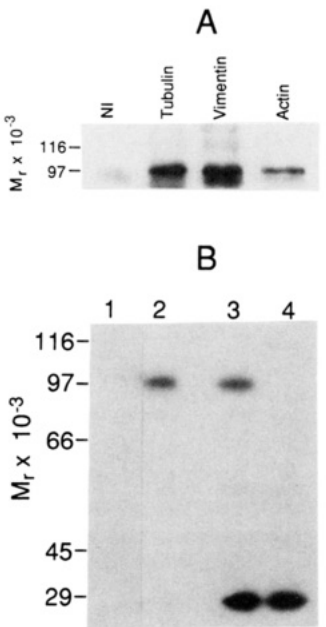

FIGURE 3: Immunoadsorption of the glucocorticoid receptor with monoclonal antibodies to cytoskeletal proteins. (A) Cytosol mixed with glutamate buffer was incubated for 1 h at 37 °C, and 200-μL aliquots were immunoadsorbed at 0–4 °C to protein A–Sepharose prebound with antibody. The protein A–Sepharose-bound proteins were pelleted by centrifugation for 30 s in a microfuge at 12000*g*, and the pellets were washed 3 times as described under Experimental Procedures. The immunoadsorbed receptor was resolved by SDS–PAGE and immunoblotting with BuGR as the probe antibody. From left to right, the lanes represent (NI) absorption with nonimmune mouse IgG and absorption with a monoclonal antibody against β-tubulin, vimentin, and actin, respectively. (B) Lanes 1 and 2; 60-μL aliquots of L-cell cytosol containing [^{3}H]dexamethasone mesylate-bound receptors were incubated for 25 min at 37 °C with glutamate, then diluted to 600 μL with Hepes–glutamate buffer, and immunoadsorbed with nonimmune IgG (lane 1) or with the monoclonal anti-β-tubulin antibody (lane 2). Lanes 3 and 4, 30 μL of the same [^{3}H]dexamethasone mesylate-bound cytosol (lane 3) or an aliquot bound in the presence of competing nonradioactive dexamethasone (lane 4) was applied to the gel and electrophoresed. Shown is an autoradiogram of the gel.

receptors, including the glucocorticoid receptor from L cells [for a review, see Pratt (1990)]. During the 37 °C incubation, the unliganded glucocorticoid receptor is transformed and hsp90 dissociates from the receptor within a few minutes (data not shown). It has recently been reported that hsp70 is associated with avian and human progesterone receptors (Kost et al., 1989; Smith et al., 1990) and also with mouse glucocorticoid receptors overexpressed in CHO cells (Sanchez et al., 1990). Hsp70 is not associated with glucocorticoid receptors recovered from L cells (Sanchez et al., 1990), and, like hsp90, it is not present in the pellet when L-cell cytosol is incubated with glutamate. These two heat shock proteins serve as controls to demonstrate that all abundant cytosolic proteins are not being trapped in a large nonspecific protein aggregate. Also, a 29-kDa protein that binds [^{3}H]dexamethasone mesylate nonspecifically (see Figure 3B) is not pelleted on incubation with glutamate (data not shown). Although all of the proteins in the pellet have not been identified, three major cytoskeletal proteins (tubulin, actin, and vimentin) account for at least half of the Coomassie blue-stained material that pellets.

Sedimentation of the Receptor–Cytoskeletal Protein Aggregate. Although we do not know the size of the cytoskeletal protein complex, we know that a rather high centrifugal force (>30000*g*) is required to pellet it. One could argue that the cytoskeletal proteins are forming reticular arrays that are nonspecifically trapping the receptors via weak forces as they are centrifuged out of solution and compacted into the pellet. The data of Figure 3A, however, suggest that the receptors are tightly associated with the complexes before they are pelleted. In this experiment, cytosol was incubated at 37 °C with glutamate and then incubated on ice with protein A–Sepharose that was prebound with nonimmune IgG or with monoclonal antibodies directed against β-tubulin, vimentin, or actin. The protein A–Sepharose beads were then pelleted by centrifugation for 30 s in a microfuge at 12000*g*, a speed at which receptor that is associated with free complex (i.e., complex that is not immunoadsorbed) is not pelleted. As shown in Figure 3A, all three antibodies cause immune-specific coadsorption of the receptor. None of the antibodies reacts directly with the receptor on Western blot, and coimmunoadsorption of the receptor by the antibodies suggests that the receptor may be associated with complexes containing the three proteins.

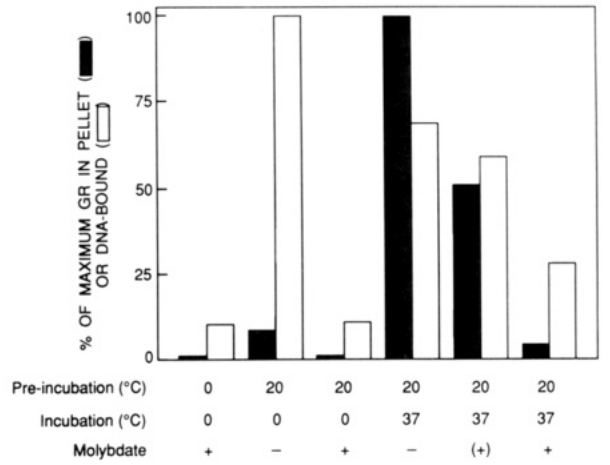

FIGURE 4: Effect of molybdate on the ability of pretransformed receptors to pellet on subsequent incubation at 37 °C. L-cell cytosol containing steroid-bound receptors was mixed with glutamate buffer, and portions were incubation for 50 min at 0 or 20 °C with or without 50 mM sodium molybdate. After this preincubation, molybdate was added to one sample that had been preincubated at 20 °C without molybdate, and all samples were incubated for an additional 25 min at either 0 or 37 °C as indicated. At the end of the second incubation, all samples were assayed for receptor binding to DNA and receptor pelleting by the ^{125}I-Western blot procedure. Results are presented as a percent of the maximum receptor (GR) recovery in the pellet (solid bars) or bound to DNA–cellulose (open bars). + without parentheses represents molybdate present throughout both incubations; (+) is molybdate present only during the 37 °C incubation.

The experiment of Figure 3B was performed to show that a large amount of receptor is immunoadsorbed from cytosol when a large amount of tubulin is immunoadsorbed. Receptors were bound with [^{3}H]dexamethasone mesylate, cytosol was incubated at 37 °C with glutamate, and lanes 1 and 2 show the amount of receptor immunoadsorbed from 60 μL of cytosol by nonimmune IgG or anti-β-tubulin monoclonal antibody, respectively. Lane 3 shows the total amount of receptor in a 30-μL aliquot of cytosol. The dark band at about 29 kDa is an L-cell protein that is heavily labeled by [^{3}H]dexamethasone mesylate in a nonspecific manner (cf. lanes 3 and 4) and is not coimmunoadsorbed by the anti-tubulin antibody. In this experiment, it was determined by quantitative Western blotting with anti-tubulin antibody (not shown) that 43% of the total tubulin was immunoadsorbed and by densitometry of the autoradiogram in Figure 3B that 38% of the receptor was immunoadsorbed. Since each of the immune pellets in Figure 3A,B was washed 3 times with a relatively large volume of buffer, it would seem that the receptor is bound to the cytoskeletal complex rather tightly.

Evidence That the Untransformed Receptor Does Not Bind to the Cytoskeletal Complex. In a series of kinetic experiments, we demonstrate that unliganded receptors in cytosol heated at 37 °C are maximally transformed to the DNA-binding state within 2 min and formation of the cytoskeletal complex (as measured by tubulin conversion to a particulate form) is complete within 5 min, yet pelleting of the receptor requires 60 min or more to reach maximum (data not shown). Although this demonstrates that the receptor transforms to the DNA-binding state before it binds to the cytoskeletal complex, it does not indicate whether or not the untransformed receptor can bind to the cytoskeleton. Molybdate stabilizes steroid receptor–hsp90 complexes and inhibits their transformation to the DNA-binding state (Pratt, 1987). At 37 °C, molybdate inhibition of receptor transformation is partial, whereas at 25 °C, it is virtually complete. Because the temperature dependency of receptor transformation is different

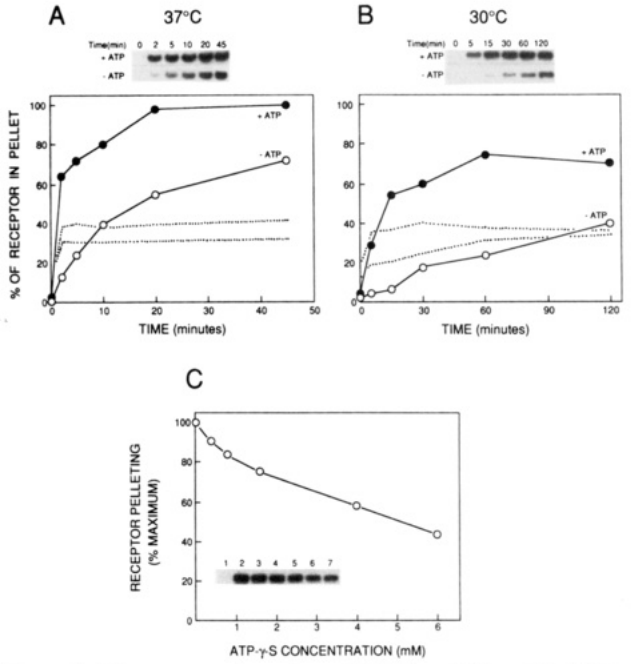

FIGURE 5: Time courses of receptor and tubulin pelleting with addition of an ATP-regenerating system and inhibition of receptor pelleting by ATPγS. (A and B) One hundred microliter aliquots of a 1:1 mixture of L-cell cytosol and glutamate buffer were mixed with 10 μL of an ATP-regenerating system [250 mM creatine phosphate, 50 mM ATP, 20 mM $MgCl_2$, 100 units/mL type I creatine phosphokinase, 5% (v/v) glycerol, and 100 mM Hepes, pH 7.4] or 10 μL of ATP-regenerating system buffer [20 mM $MgCl_2$, 5% (v/v) glycerol, and 100 mM Hepes, pH 7.4]. The samples were incubated at 37 or 30 °C for the indicated times and then centrifuged. Receptor and tubulin were resolved by SDS–PAGE and Western blotting. The insets show autoradiograms of the ^{125}I-labeled bands of receptor on the Western blot. These bands were cut out and counted for radioactivity, and the data are presented as the percent of total receptor that was recovered in the pellet fraction at 37 °C (panel A) or 30 °C (panel B) in the presence (●) or absence (○) of the ATP-regenerating system. The dotted lines show the time course of tubulin pelleting in the same samples, with the upper line being tubulin pelleting in the presence of the ATP-regenerating system and the lower line in the absence of the system. (C) Various concentrations of ATPγS were added to aliquots of a 1:1 mixture of L-cell cytosol and glutamate buffer, the mixtures were incubated for 20 min at 37 °C, and receptor pelleting was quantitated as described above. The graph shows receptor pelleting as a percentage of the total pelleting obtained in the absence of the inhibitor. The inset shows a Western blot of receptor pelleting data: 0 °C incubation with no ATPγS (lane 1); 37 °C incubation with 0, 0.4, 0.8, 1.6, 4 or 6 mM ATPγS (lanes 2–7, respectively).

from that of receptor pelleting, we can ask whether molybdate has less of an effect on pelleting when pretransformed receptors are incubated at 37 °C than when untransformed receptors are incubated at 37 °C. In the experiment of Figure 4, cytosol containing steroid-bound receptors was preincubated at 20 °C for 50 min to allow the majority of the receptors to transform. As shown by the second pair of bars from the left in Figure 4, receptors preincubated at 20 °C in the absence of molybdate were transformed, but only a small portion of the receptors were converted to particulate-bound form. A subsequent incubation at 37 °C resulted in a lot of receptor pelleting, but if molybdate was present throughout both incubations, the pelleting was markedly inhibited (cf. the fourth pair and the last pair of bars in Figure 4). If, however, receptors were pretransformed at 20 °C in the absence of molybdate and molybdate was present only during the subsequent incubation at 37 °C (fifth pair of bars), then the degree of inhibition is much less. This observation is consistent with the proposal that the untransformed receptor either does not

bind to the cytoskeletal complex or binds weakly with respect to the transformed receptor.

Binding of the Receptor to the Cytoskeletal Complex Is an Energy-Dependent Process. In our original experiments characterizing the receptor pelleting system, we found that addition of ATP had little effect (data not shown). It was not until all of the other experiments presented in this paper had been performed that we asked whether addition of an ATP-regenerating system would alter the kinetics of receptor pelleting. As shown in Figure 5, addition of an ATP-regenerating system cuases more rapid receptor pelleting at both 30 and 37 °C. As shown by the dotted lines in Figure 5, the pelleting of tubulin is not markedly affected by the presence of the energy-regenerating system. This leads us to suggest that it is not the formation of the cytoskeletal complex that requires energy but rather there is a step (or steps) involved in the binding of the transformed receptor to the complex that has a partial dependence upon the presence of the energy-generating system. The rate of transformation itself is not enhanced by the presence of the energy-regenerating system (data not shown), and transformation proceeds more rapidly than even the ATP-enhanced pelleting of the receptor. As shown in panel C of Figure 5, addition of ATPγS to the L-cell cytosol produces partial inhibition of receptor pelleting in a concentration-dependent manner.

The COOH-Terminal Half of the Receptor Is Required for Binding to the Cytoskeletal Protein Complex. The experiments of Figure 6 were performed to determine if a specific region of the receptor is required for pelleting. For this purpose, we used both cells containing mutant receptors and cells transiently transfected with plasmids prepared by Picard and Yamamoto (1987) in which the coding sequence for β-galactosidase is fused to subregions of the rat glucocorticoid receptor. The pelleting of two of these fusion proteins is shown in Figure 6. It is clear that neither β-galactosidase itself nor β-galactosidase fused to the NH_2-terminal half of the glucocorticoid receptor (Z.4–445) binds to the pellet whereas the fusion protein containing nearly the entire glucocorticoid receptor binds to the pellet [although pelleting is perhaps less effective than with the transfected receptor by itself (GR)]. Cytosol was also prepared from the nt^i variant of the S49 mouse lymphoma cell line which produces a 40-kDa receptor that contains only the DNA-binding and steroid-binding domains (Gehring & Hotz, 1983; Northrop et al., 1985). As shown in Figure 6, this truncated receptor pellets like the wild-type GR. Taken together, the behavior of the Z.4C, Z.4–445, and nt^i receptors shows that the COOH-terminal half of the receptor is both necessary and sufficient for pelleting.

Although the COOH-terminal half of the receptor is necessary for association with the cytoskeletal protein complex, DNA-binding activity is not required. This is shown with the NB mutant mouse glucocorticoid receptor in Figure 6. NB cells are CHO cells that overexpress a mouse glucocorticoid receptor that binds hormone but does not bind to DNA due to a point mutation at the base of the second zinc finger (Arg^{484} → His) (Hirst et al., 1990). We have previously shown that the NB receptor migrates on SDS–PAGE as several bands between 70 and 100 kDa as a result of either cleavage or alternate transcription start sites in the overexpressed state (Sanchez et al., 1990). As shown in Figure 6, the NB receptor pellets like the wild-type glucocorticoid receptor.

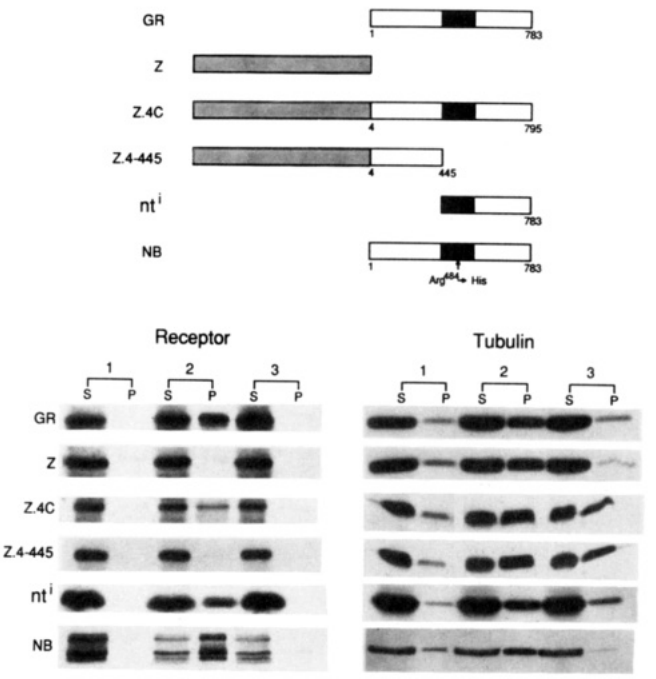

FIGURE 6: The COOH-terminal half of the receptor is required for pelleting. COS-1 cells were transfected with one of four plasmids containing the coding sequence for either the wild-type mouse glucocorticoid receptor (GR), β-galactosidase (Z), or β-galactosidase fused to residues 4–795 (Z.4C) or 4–445 (Z.4–445) of the rat glucocorticoid receptor. Cytosols prepared from the transfected COS cells and cytosols prepared from the nt^i variant of the S49 mouse lymphoma cell line or NB cells were mixed with an equal volume of Hepes or glutamate buffer, incubated at 0 or 37 °C for 25 min, and centrifuged at 150000*g*. Receptor and tubulin in supernatants (S) and pellets (P) were resolved by SDS–PAGE and Western blotting with monoclonal antibody against β-galactosidase or the receptor. The receptors in the nt^i cytosol were bound with [^{3}H]dexamethasone 21-mesylate prior to the incubation and were identified by autoradiography of the dried gel. The stippled bars indicate β-galactosidase, and the solid black bars represent the DNA-binding domain of the glucocorticoid receptor. The incubation conditions are as follows: (1) glutamate buffer at 0 °C; (2) glutamate buffer at 37 °C; (3) Hepes buffer at 37 °C.

DISCUSSION

In this work, we have described the temperature-dependent conversion of the cytosolic glucocorticoid receptor from a soluble to a particulate-bound form. This conversion requires only the presence of glutamate (Figure 1). The particulate that forms contains a considerable proportion of the cytosolic tubulin, actin, and vimentin (Figure 2), and for that reason, we refer to the particulate material as a cytoskeletal protein complex. In this work, we have monitored the formation of the complex by Western blotting for tubulin, although we could as readily have blotted for vimentin or actin. It is important to emphasize here that by using tubulin as an indicator we are not implying that formation of the cytoskeletal complex is related to polymerization of tubulin into classical microtuble structures. Formation of the complex is not affected by colchicine, nocodozole, or taxol under conditions where these agents are readily shown to affect polymerization of purified tubulin.

The cytoskeletal protein complex is formed in a glutamate-dependent manner. Zwitterionic compounds such as PIPES, MES, and glutamate have been widely used in microtuble assembly studies (Asnes & Wilson, 1979; Wilson, 1970; Arakawa & Timasheff, 1984; Hamel & Lin, 1981; Hamel et al., 1982; Foster & Rosenmeyer, 1986; and references cited therein). These compounds all stabilize microtuble structures, and we have found that MES substitutes for glutamate in our system (data not shown). Although the mechanism of stabilization is not well understood, Arakawa and Timasheff (1984) have noted there is a direct correlation between the

ability of compounds to induce preferential hydration of tubulin and their ability to stabilize assembled structures. It is clear from Figure 2 that only a limited number of proteins are present in the particulate, suggesting that a limited variety of protein–protein interactions are being stabilized by glutamate in this system. How the receptor binds to the cytoskeletal protein complex is unknown. Multiple proteins may be involved, and direct interaction of receptor with a cytoskeletal protein is not implied.

The association of receptor with particulate material that we have reported here may not be unique to the glucocorticoid receptor. Nelson et al. (1989) have reported that estrogen receptors in mouse uterine cytosol incubated at 25 °C enter into large complexes. Beyond determining that the complexes were likely composed of proteins, the components were not defined. This entry of mouse estrogen receptors into a high molecular weight complex in uterine cytosol may reflect an association of receptors with a cytoskeletal complex similar to the glutamate–dependent complex we have described for glucocorticoid receptors. Nelson et al. (1992) have recently transferred gel-resolved cytosolic proteins to an Immobilon membrane and then used radiolabeled, transformed estrogen receptor as a probe to detect interactions with two groupings of proteins in the region of the 66- and 45-kDa markers. In this respect, it should be made clear that although the type of complex we have shown in Figure 3 involves both the glucocorticoid receptor and cytoskeletal elements, the two may be bridged by noncytoskeletal proteins, which contain the receptor interaction sites.

Several observations suggest that receptor association with the cytoskeletal complex may involve specific protein–protein interactions. First, if a nonspecific event were occurring, one would expect a lot of cytosolic proteins to be converted from a soluble to a particulate-bound form, and as shown in Figure 2, that is not the case. Second, the rate of receptor pelleting is increased in the presence of an ATP-regenerating system (Figure 5), indicating that some step (or steps) in the process of receptor binding to the cytoskeletal complex is at least partially energy-dependent. Third, the fact that molybdate slows the rate of association with the complex (Figure 4) suggests that the hsp90-bound, untransformed receptor does not bind to the complex or binds only with low affinity such that it is not recovered with the pellet in our procedure. It is important to note that the formation of high molecular weight estrogen receptor complexes reported by Nelson et al. (1989) required the presence of ligand at 25 °C and was inhibited by molybdate, again suggesting that transformed receptors were preferentially or exclusively included in the complex.

It is possible that, after receptor transformation, a specific event occurs that is required for association with the cytoskeletal complex. Such a specific event could include the association of the receptor with another protein (or proteins) that mediates its binding to the complex. This would likely require a structure (or structures), such as a nuclear localization signal, located within a limited region of the receptor. Picard and Yamamoto (1987) have shown that nulcear localization signals lie just to the COOH-terminal side of the DNA-binding domain (NL1) and also within the hormone-binding domain of the glucocorticoid receptor. Nuclear localization signals of estrogen (Picard et al., 1990) and progesterone (Guichon-Mantel et al., 1989) receptors are located in the same general region as the NL1 signal of the glucocorticoid receptor. As shown in Figure 6, a structure or structures lying within the COOH-terminal half of the glucocorticoid receptor is/are both necessary and sufficient for association of the receptor with the cytoskeletal complex, and the NH_2-terminal half of the receptor (Figure 6, Z.4–445) does not confer any pellet-binding activity. Although it is the transformed state of the receptor that binds tightly to the cytoskeletal pellet, the fact that the NB mutant receptor binds to the pellet (Figure 6) shows that DNA-binding activity is not required.

It is of interest to note that β-galactosidase alone and β-galactosidase fused to the NH_2-terminal half of the receptor (Z.4–445) are localized to the cytoplasm in the absence or presence of glucocorticoid (Picard & Yamamoto, 1987) and do not bind to the cytoskeletal protein pellet (Figure 6) whereas β-galactosidase fused to the whole receptor (Z.4C) both undergoes a steroid-dependent shift from the cytoplasm to the nucleus (Picard & Yamamoto, 1987) and binds to the cytoskeletal pellet (Figure 6). The nt^i receptor binds tightly to the cell nucleus after stroid–mediated transformation (Gehring & Hotz, 1983) and also binds to the cytoskeletal protein pellet (Figure 6). Although nuclear localization signals are located within the region of the glucocorticoid receptor (Picard & Yamamoto, 1987) that is both necessary and sufficient for receptor pelleting, we do not know whether or not nuclear localization signals are involved in receptor binding to the cytoskeletal complex. We have added AP64 antibody directed against the NL1 segment of the glucocorticoid receptor (Urda et al., 1989) to the pelleting assay, but we did not see immune-specific of receptor pelleting (at very high concentrations, both nonimmune and AP64 antisera inhibit the process).

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Registry No. Glu, 56-86-0; ATP, 56-65-5.