Geldanamycin, a Heat Shock Protein 90-Binding Benzoquinone Ansamycin, Inhibits Steroid-Dependent Translocation of the Glucocorticoid Receptor from the Cytoplasm to the Nucleus†

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Received March 20, 1997⊗

ABSTRACT: When they are translated, steroid receptors are assembled into a multiprotein complex containing hsp90, p23, an immunophilin, and often some hsp70. Some of the receptors, such as that for progesterone, have nuclear localization signals that are functional in the absence of hormone, and they move into the nucleus where they exist in the same multiprotein heterocomplex with hsp90. Other receptors, such as the glucocorticoid receptor, are localized predominantly in the cytoplasm in the absence of hormone and move into the nucleus in a hormone-dependent fashion. We have previously proposed that hsp90 and the immunophilin play a role in receptor trafficking [Pratt, W. B. (1993) *J. Biol. Chem. 268*, 21455−21458]. In this work, we show that treatment of L cells with geldanamycin, a benzoquinone ansamycin that binds to hsp90 and disrupts its function, impedes dexamethasone-dependent trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. Because geldanamycin treatment of hormone-free cells causes a rapid loss of steroid binding activity, receptors were prebound with dexamethasone by incubating cells with hormone at 0 °C prior to shifting the temperature to 37 °C for 20 min to permit receptor transformation and translocation in the presence or absence of geldanamycin. Geldanamycin does not cause steroid to dissociate from prebound receptors, and it does not inhibit hormone-mediated receptor transformation assayed by conversion to the DNA-binding state. However, as reported previously for the progesterone receptor, geldanamycin blocks assembly of the glucocorticoid receptor·hsp90 heterocomplex at an intermediate state of assembly where the receptor is bound to hsp70 and p60, both of which are required components in the assembly mechanism. Our observations support the proposal that dynamic association of receptors with hsp90 is required for receptor translocation from the cytoplasm to the nucleus.

As ligand-regulated transcription factors, the steroid receptors must move through the cytoplasm, traverse the nuclear pores, and subsequently move within the nucleus to their sites of action. Their nuclear localization is mediated by nuclear localization signal (NLS) sequences in the receptors themselves (Picard & Yamamoto, 1987), and bidirectional shuttling of receptors into and out of the nucleus occurs constantly [Guichon-Mantel et. al., 1991; Chandran & DeFranco, 1992; Madan & DeFranco, 1993; Dauvois et al., 1993; for review, see DeFranco et al. (1995)]. Under steady-state conditions in hormone-free cells, two patterns of shuttling are seen, depending upon the receptor involved. The progesterone receptor (PR),[1] for example, is predominantly localized in the nucleus (Perrot-Applanat et al., 1985, 1986), whereas the glucocorticoid receptor (GR) is predominantly localized in the cytoplasm of most cells (Picard & Yamamoto, 1987; Qi et al., 1989). Probably, the GR is cytoplasmic because the NLS in the untransformed GR is not readily accessible to the NLS-binding protein, and when it becomes accessible upon transformation (Urda et al., 1989; Scherrer et al., 1993), nuclear translocation occurs.

Regardless of whether the unliganded receptors are predominantly cytoplasmic or nuclear, they exist in multiprotein heterocomplexes that contain hsp90 and an immunophilin, such as FKBP52 (also called hsp56) or CyP-40 (Owens-Grillo et al., 1995). In 1992, we proposed that the receptors travel through the cytoplasm to the nucleus in this heterocomplex form, with hsp90 and the immunophilin acting as a protein transport unit or *transportosome* (Pratt, 1992, 1993). This model of receptor movement was supported by experiments of Kang et al. (1994) in which hsp90 was targeted to the nucleus by fusion to the nucleoplasmin NLS, and it was shown that coexpression of the hsp90-NLS and cytoplasmic receptor mutants devoid of an NLS resulted in complete nuclear localization of the receptors. The interpretation of this observation was that the receptors undergo "piggyback" transport with the hsp90-NLS (Kang et al., 1994). It should be emphasized, however, that receptor·hsp90 complexes are in a dynamic state, in that they are constantly dissociating and being reformed under physiological conditions in the cell (Smith, 1993); thus, the proposed piggyback movement might involve chaperone-mediated association of the receptor with several hsp90-NLS molecules.

† This investigation was supported by National Institutes of Health Grant CA28010. M.J.C. is a trainee under Pharmacological Sciences Training Program Grant GM07767 from the NIH.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: hsp, heat shock protein; GR, glucocorticoid receptor; PR, progesterone receptor; FKBP, FK506 binding protein; GA, geldanamycin; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulfonic acid; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TA, triamcinolone acetonide.

S0006-2960(97)00648-X CCC: $14.00

In the heterocomplex model, it is proposed that the immunophilin is the component responsible for the targeting of receptor movement (Pratt et al., 1993; Czar et al., 1995; Owens-Grillo et al., 1996). The immunophilin FKBP52 contains a sequence of eight amino acids [EDLTDDED, rabbit (Lebeau et al., 1992)] with six negatively charged residues that is located in a short hinge segment linking the first and second globular domains predicted by Callebaut et al. (1992). The sequence is retained in human and mouse FKBP52 (Peattie et al., 1992; Schmitt et al., 1993), and it is electrostatically complementary to the receptor NLSs [e.g., the NL1 sequence RKTKKKIK of rat GR (Picard & Yamamoto, 1987)]. Consistent with the notion that the immunophilin may play a role in GR movement, we recently reported that intracellular injection of an antibody directed against this conserved negative sequence of FKBP52 impeded dexamethasone-mediated cytoplasmic−nuclear translocation of the receptor (Czar et al., 1995).

Twelve years ago, Raaka et al. (1985) reported that treatment of intact cells with molybdate retards steroid-induced conversion of receptors from 9S (hsp90-bound) to 4S (hsp90-free). Yang and DeFranco (1996) recently introduced sodium molybdate into cells with a liposome-mediated delivery system and found that molybdate both stabilized GR·hsp90 complexes *in vivo* and inhibited hormone-dependent nuclear import of the GR. The molybdate treatment trapped both the GR and the PR in the cytoplasm of cells chronically exposed to hormone, suggesting that the receptors can export from nuclei but cannot be reimported into nuclei in the presence of molybdate. Because molybdate stabilization caused receptors to accumulate in the cytoplasm, Yang and DeFranco (1996) proposed that association of receptors with hsp90 must be dynamic to permit the access of macromolecular components that participate in the delivery of receptors to their nuclear sites of action. This observation is consistant with Munck's model of the receptor cycle, where reassociation of receptors with hsp90 in the cytoplasm is required for recycling into the nucleus (Orti et al., 1992; Hu et al., 1994), but it suggests an expansion of the model to require a dynamic process of receptor·hsp90 complex assembly−disassembly for nuclear translocation.

In contrast to molybdate, which permits receptor heterocomplex assembly but impedes GR·hsp90 dissociation, geldanamycin permits (and even may facilitate) heterocomplex dissociation but blocks reassembly. Geldanamycin is a benzoquinone ansamycin antibiotic that was found to revert transformation induced in cultured cells by oncogenic tyrosine kinases, such as pp60^{v-src} (Uehara et al., 1988; Whitesell et al., 1992). Both the Src and the Raf-1 protein kinases are assembled into heterocomplexes with hsp90 by the same reticulocyte lysate system that forms receptor·hsp90 heterocomplexes (Hutchison et al., 1992a; Stancato et al., 1993). It has been shown that geldanamycin does not inhibit the kinase activity of the proteins (June et al., 1990; Whitesell et al., 1992), but it binds in a pharmacologically specific manner to hsp90 (Whitesell et al., 1994). The action of geldanamycin on hsp90 apparently inhibits formation of the Src·hsp90 heteroprotein complex by reticulocyte lysate (Whitesell et al., 1994) and prevents newly synthesized Raf-1 from associating with hsp90 in MCF7 cells (Schulte et al., 1995). Geldanamycin destabilization of Raf-1·hsp90 complexes is accompanied by a marked decrease in the half-life of the Raf-1 protein (Schulte et al., 1995). The mechanism of the geldanamycin effect is not completely understood, but cell-free experiments have shown that it prevents the association of the p23 component of the heterocomplex assembly system with hsp90 (Johnson & Toft, 1995). Smith et al. (1995) demonstrated that PR heterocomplex assembly in both reticulocyte lysate and intact cells is blocked at an intermediate stage of assembly where the hormone binding domain is not properly folded and it cannot bind steroid. Similar observations have been made by Whitesell and Cook (1996) for the GR.

Because geldanamycin does not inhibit hsp90 dissociation from receptors but blocks heterocomplex reassembly, it should be a useful tool for determining whether the dynamic association of steroid-bound receptors with hsp90 is required for their trafficking through the cytoplasm to the nucleus. In this work, we show that geldanamycin does not affect steroid-dependent transformation of the GR, but geldanamycin treatment of L cells containing glucocorticoid-bound receptors impedes their trafficking to the nucleus as measured by indirect immunofluorescence with anti-receptor antibody and by a shift of specifically bound [^{3}H]triamcinolone acetonide from the cytosolic to the nuclear fraction. Because geldanamycin treatment of hormone-free cells causes a rapid loss of steroid binding activity, we have prebound the receptors by incubating cells with steroid at 0 °C prior to shifting the temperature to 37 °C to permit receptor transformation and translocation to the nucleus. As reported for the PR (Smith et al., 1995), geldanamycin causes the accumulation of GR heterocomplexes at an intermediate stage of assembly where they are bound to both hsp70 and p60. Our observations support the proposal that dynamic association of steroid-bound receptors with hsp90 is required for their trafficking to the nucleus.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Triamcinolone acetonide (35.4 Ci/mmol) and ^{125}I-conjugated goat anti-mouse IgG were obtained from DuPont NEN. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). BuGR anti-glucocorticoid receptor IgG for Western blotting was from Affinity BioReagents (Golden, CO). BuGR ascites for immunofluorescence and the L-M(TK$^-$) subline of mouse L929 fibroblasts (L cells) were kindly provided by D. DeFranco (University of Pittsburgh, Pittsburgh, PA). The DS14F5 anti-p60 monoclonal mouse IgG (Smith et al., 1993) was a gift from D. Smith (University of Nebraska, Omaha, NE). Nonimmune mouse IgG, protein A−Sepharose, anti-mouse IgG−horseradish peroxidase conjugate, DNA−cellulose, and FITC−dextran (average M_r of 70.1 kDa) were from Sigma (St. Louis, MO). The AC88 monoclonal IgG against hsp90 and the N27F3-4 anti-72/73-kDa heat shock protein monoclonal IgG (anti-hsp70) were from StressGen (Victoria, BC). Rhodamine-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA). Hybridoma cells producing FiGR monoclonal IgG against the GR (Bodwell et al., 1991) were generously provided by J. Bodwell (Dartmouth Medical School). Purified FiGR antibody was used to immunoadsorb the GR, and for this purpose, it was covalently linked to Actigel-ALD (activated aldehyde agarose) affinity support for protein immobilization from Sterogene Biochemicals (San Gabriel, CA). Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute.

Cell Culture and Fractionation. L929 mouse fibroblasts (L cells) and the L-M(TK$^-$) subline of L929 fibroblasts were grown in a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% iron-supplemented calf serum. Cells were harvested by scraping into Earle's balanced saline, suspended in 1.5 volumes of HE buffer [10 mM HEPES (pH 7.4) and 1 mM EDTA] or HE buffer with 20 mM sodium molybdate, and ruptured by Dounce homogenization. Cell homogenates were centrifuged for 1 h at 100000*g*, and the supernatant from this centrifugation is referred to as the "cytosol".

L-M(TK$^-$) cells were prepared for immunofluorescence by lifting from flasks using 0.05% trypsin/0.5 mM EDTA in calcium- and magnesium-free Hanks buffered saline. The cells were plated onto 11 × 22 mm glass coverslips (10/(100 mm dish)] in DMEM supplemented with 10% iron-supplemented calf serum. One day prior to nuclear translocation experiments, coverslips containing L-M(TK$^-$) cells were washed extensively with and then grown in phenol red-free DMEM supplemented with 10% charcoal-stripped, delipidated bovine calf serum in individual 35 mm Petri dishes.

Immunofluorescence. L-M(TK$^-$) cells were prepared for immunofluorescent staining of the GR by simultaneous fixation and permeabilization by immersion in methanol at −20 °C for at least 10 min, as described previously (Czar et al., 1995). The cells were then rinsed twice with phosphate-buffered saline (PBS) at room temperature, and the coverslips were inverted onto a 30 μL drop of BuGR (1:60 ascites) for 45 min at room temperature in a humid chamber. After two 10 min washes in PBS, the coverslips with attached cells were inverted onto a 30 μL drop of rhodamine-conjugated donkey anti-mouse IgG (1:60). After 30 min, the cells were washed twice for 10 min with PBS and mounted on microscope slides using *p*-phenylenediamine mounting medium as described (Welsh, 1983). Cells on coverslips were photographed using T-max 400 film. Photos were taken on a Leitz Aristoplan epiillumination fluorescence microscope fitted with a Leitz Vario-Orthomat camera (Leitz, Rockeigh, NJ). For each experiment, an exposure time was determined and all fields were photographed manually for the same exposure time.

Assay for Steroid Binding Capacity. Cytosolic steroid binding capacity was assayed by diluting aliquots (45 μL) of cytosol treated as described in the figure legends with an equal volume of HE buffer plus 20 mM sodium molybdate and incubating them overnight on ice with 50 nM [^{3}H]-triamcinolone acetonide in the presence or absence of a 1000-fold excess of competing cold dexamethasone. Each incubation mixture was then mixed with 0.15 mL of a suspension of dextran-coated charcoal [1% charcoal (w/v) and 0.2% dextran (w/v) in 10 mM HEPES at pH 7.3] for 10 min at 0 °C. The radioactivity in the charcoal supernatant was assayed, and the specific binding was determined by subtracting the radioactivity obtained in the presence of cold dexamethasone from that obtained in its absence.

For the whole cell binding experiments of Table 1, cells were grown to confluence in 35 mm dishes in phenol red-free DMEM containing 10% charcoal-stripped, delipidated serum. The cells were incubated on ice for 30 min with 0.5 mL of phenol red-free DMEM containing 50 nM [^{3}H]-triamcinolone acetonide with or without a 1000-fold excess of cold dexamethasone, and with the cells still on ice, 10 μM geldanamycin or 0.1% DMSO vehicle was added to the medium and the incubation continued for an additional 30 min at 0 °C. All cultures were then incubated for an additional 20 min at 37 °C, and the cells were washed three times for 5 min with PBS containing 1% bovine serum albumin. Bound steroid was extracted from the cells by incubating them for 30 min on ice with 0.5 mL of 100% ethanol. The ethanol was transferred to scintillation vials and was counted. The specifically bound steroid counts were determined by subtracting the counts per minute obtained under conditions with cold dexamethasone from the counts per minute obtained under conditions without.

Incubation of Intact Cells with [^{3}H]TA Followed by Cell Fractionation. For assay of nuclear and cytosolic receptors bound with [^{3}H]triamcinolone acetonide in intact cells (Figure 7), we used a modification of a previously reported method (Tienrungroj et al., 1987). L cells were harvested by scraping into fresh serum-containing culture medium and suspended at a cytocrit of 0.1 mL of packed cells per milliliter of suspension. [^{3}H]Triamcinolone acetonide was added at a final concentration of 50 nM in the presence or absence of a 200-fold excess of unlabeled dexamethasone, and the suspensions were incubated exactly according to the protocol of Figure 5. At the end of the incubations, cells were washed three times with Earle's balanced saline, resuspended in 1 mL of HE, and ruptured by Dounce homogenization. The homogenate was centrifuged at 3500*g* for 20 min to yield a crude nuclear pellet and a cytosolic fraction. The nuclear pellet was washed once by resuspension in 1 mL of 1 mM HEPES (pH 7.6), 1 mM EGTA, and 3 mM $MgCl_2$ with 0.3 M sucrose and repeat centrifugation. The pellet from sucrose centrifugation was suspended in 300 μL of water and 100 μL of 10% SDS in TEG buffer [10 mM TES (pH 7.6), 50 mM NaCl, 4 mM EDTA, and 10% glycerol], boiled, and suspended. The nuclear and cytosolic fractions were then assayed by scintillation counting. For each condition in each experiment, samples with and without competing dexamethasone were performed in triplicate. Specifically bound steroid counts were determined by subtracting the counts per minute obtained under conditions with nonradioactive dexamethasone from the counts per minute obtained under conditions without.

Immunoadsorption. The GR was immunoadsorbed from replicate (250−300 μL) aliquots of L cell cytosol by rotation for 2 h at 4 °C with 8 μL of Actigel-ALD precoupled to 80 μL of FiGR ascites suspended in 300 μL of TEGM (TEG buffer with 20 mM sodium molybdate). After immunoadsorption of GR from cytosol, the immune pellet was washed three times by suspension in 1 mL of TEGM buffer and centrifugation.

Gel Electrophoresis and Immunoblotting. For assay of GR, hsp90, hsp70, and p60, immunopellets were boiled in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved on 7 or 8% SDS−polyacrylamide gels as previously described (Bresnick et al., 1989). Proteins were then transferred to Immobilon-P membranes and probed with 2 μg/mL BuGR2 for the GR, 1 μg/mL AC88 for hsp90, 1 μg/mL N27F3-4 for hsp70, or 0.1% DS14F5 for p60. The immunoblots were then incubated a second time with ^{125}I-labeled goat anti-mouse IgG followed by horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands. In some experiments, the bands visualized by the peroxidase reaction were excised from the membrane and quantitated by γ counting as described previously (Meshinchi et al., 1990).

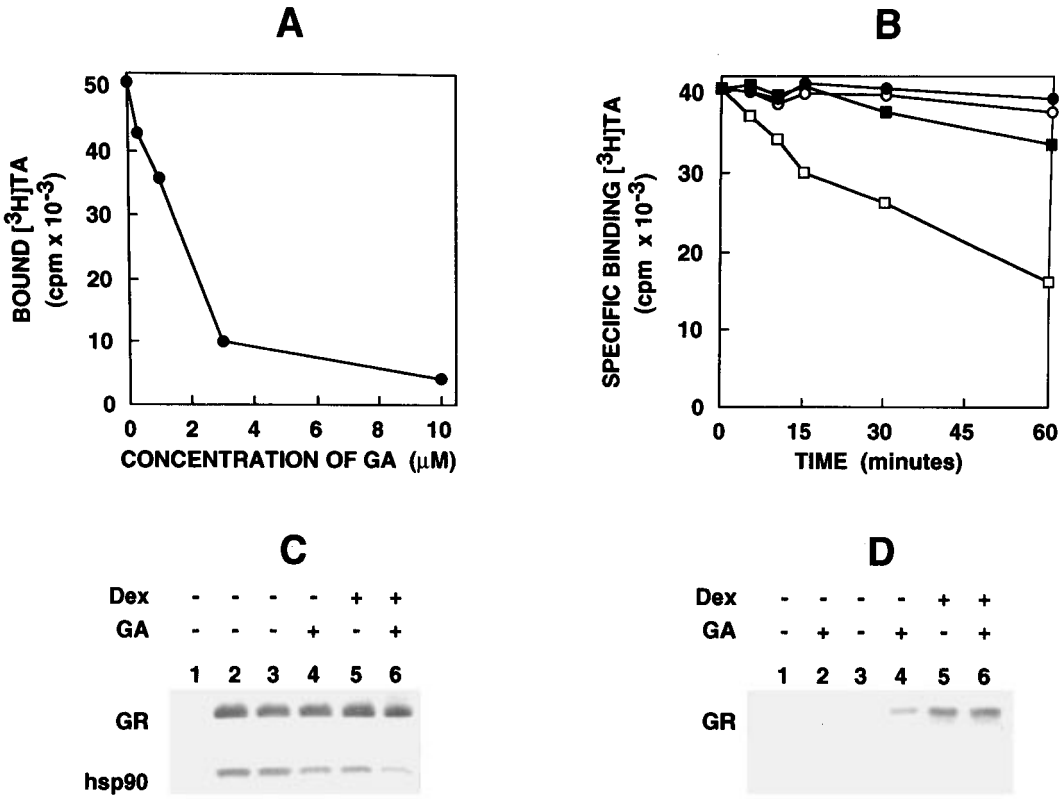

FIGURE 1: GR·hsp90 complexes formed in the absence of geldanamycin can be transformed in the presence of geldanamycin. (A) Geldanamycin inhibition of receptor reactivation to the steroid binding state by reticulocyte lysate. Replicate samples of hsp90-free GR were incubated with reticulocyte lysate and geldanamycin at the indicated concentrations, and steroid binding was assayed as described in Experimental Procedures. (B) Temperature-dependent inactivation of steroid binding activity of cytosolic GR by (10 μM) geldanamycin. Portions of molybdate-free L cell cytosol were incubated in the presence or absence of geldanamycin at 0 or 20 °C, and at various times, aliquots were removed and steroid binding capacity was assayed. Each point is the average of duplicate samples: cytosol incubated at 0 °C with (○) or without (●) geldanamycin and incubation at 20 °C with (□) or without (■) geldanamycin. (C) Steroid-dependent dissociation of the GR·hsp90 complex occurs in the presence of geldanamycin. Aliquots of cytosol (two of which contained GR prebound with 1 μM dexamethasone) were incubated for 1.5 h at 0 or 20 °C with or without 10 μM geldanamycin; GR was immunoadsorbed with BuGR, and the immunopellets were washed. GR and GR-associated hsp90 were detected by Western blotting: lanes 1 and 2, samples kept at 0 °C immunoadsorbed with nonimmune IgG or BuGR, respectively; lanes 3 and 4, samples with hormone-free receptors incubated at 20 °C without (lane 3) or with (lane 4) geldanamycin; and lanes 5 and 6, samples with dexamethasone-bound receptors incubated at 20 °C without (lane 5) or with (lane 6) geldanamycin. (D) Geldanamycin does not affect steroid-dependent GR transformation to the DNA−binding state. Aliquots of cytosol containing steroid-bound or unbound receptors were incubated for 1.5 h at 0 or 20 °C, and binding to DNA−cellulose was assayed as described in Experimental Procedures. DNA-bound GR was detected by Western blot: lanes 1 and 2, cytosol with unliganded GR incubated at 0 °C without (lane 1) or with (lane 2) geldanamycin; lanes 3 and 4, unliganded GR incubated at 20 °C without (lane 3) or with (lane 4) geldanamycin; and lanes 5 and 6, dexamethasone-bound GR incubated at 20 °C without (lane 5) or with (lane 6) geldanamycin.

GR Heterocomplex Reconstitution. Reconstitution of GR·hsp90 heterocomplexes was performed essentially as described by Scherrer et al. (1990). Receptors immunoadsorbed from 200 μL of L cell cytosol were stripped of associated hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 0.5 M NaCl followed by one wash with 1 mL of TEG and a second wash with 1 mL of 10 mM HEPES buffer at pH 7.4. The stripped BuGR immune pellets (8 μL of protein A−Sepharose) were mixed with 50 μL of rabbit reticulocyte lysate, 1 μL of dithiothreitol (final concentration of 5 mM), and 5 μL of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM MgOAc, and 100 units/mL creatine phosphokinase). The assay mixtures were incubated for 20 min at 30 °C with resuspension of the pellets by shaking the tubes every 5 min. At the end of the incubation, the immunopellet was washed once with 1 mL of iced TEGM. Steroid binding capacity was assayed by incubating the washed pellets overnight at 0 °C in 100 μL of TEGM buffer plus 1 mM dithiothreitol with 50 nM [^{3}H]triamcinolone acetonide (Hutchison et al., 1992b). Samples were then washed three times with 1 mL of TEG buffer and counted by liquid scintillation spectrometry.

GR Binding to DNA−Cellulose. Cytosol was first cleared of any transformed GR by preincubation of 700 μL of cytosol with a 100 μL pellet of DNA−cellulose for 1 h. Then, replicate 100 μL aliquots of cytosol (DNA−cellulose supernatant) were or were not incubated with 1 μM dexamethasone for 2 h at 0 °C. Geldanamycin (10 μM) was added to some aliquots, and the cytosol was incubated for 1.5 h at 0 or 20 °C. Each cytosol sample was rotated for 1 h at 4 °C with 200 μL of a 12.5% suspension of DNA−cellulose, and the DNA-bound GR in the washed pellets was then resolved by SDS−PAGE and Western blotting.

RESULTS

Geldanamycin Permits Transformation of the Native GR Heterocomplex in Vitro. When immunoadsorbed, hsp90-free GR is incubated with rabbit reticulocyte lysate, the receptor is assembled into a complex with hsp90, and its hormone binding activity is restored (Scherrer et al., 1990; Hutchison et al., 1992b). Figure 1A shows the concentration-

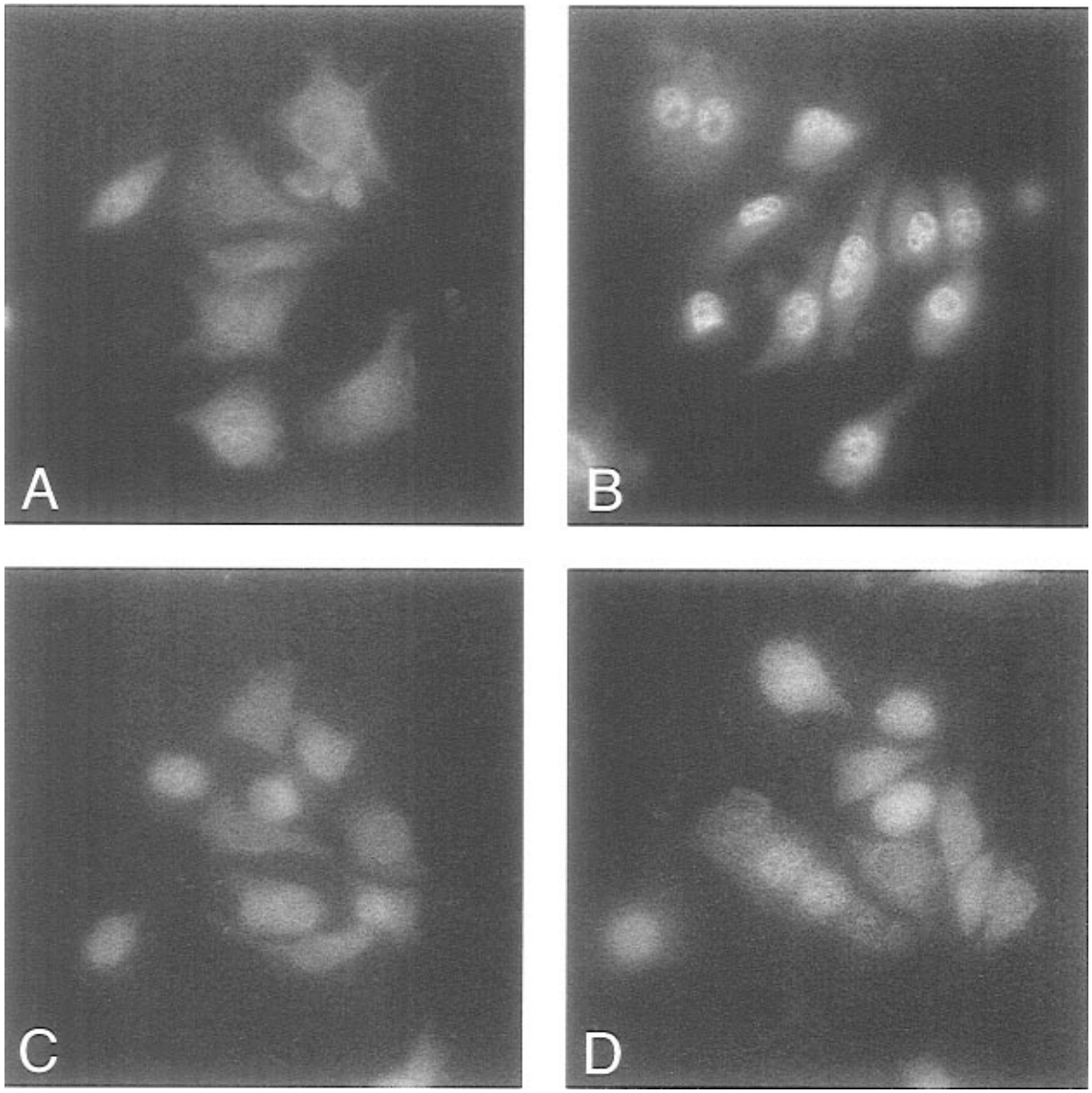

FIGURE 2: Geldanamycin does not itself cause GR translocation. Cells were pretreated for 60 min at 37 °C with 10 μM geldanamycin or with 0.1% DMSO vehicle; 1 μM dexamethasone or 0.1% ethanol vehicle was then added, and incubations were continued for an additional 10 min at 37 °C. Cells were then fixed in cold methanol, and receptors were detected by immunofluorescence after incubating with BuGR followed by rhodamine-conjugated anti-mouse IgG. Each field of cells was photographed identically: (A) cells treated with vehicle alone, (B) cells treated with dexamethasone alone, (C) cells treated with geldanamycin alone, and (D) cells pretreated with geldanamycin and then dexamethasone.

dependent inhibition of receptor reactivation by geldanamycin. A concentration of 10 μM (5 μg/mL) geldanamycin is sufficient for essentially complete inhibition, and this concentration will be used for all of the experiments in this work. In the experiment of Figure 1B, geldanamycin was added to L cell cytosol containing native GR·hsp90 complexes, and after various times of incubation at 20 °C, aliquots of cytosol were removed and incubated with [^{3}H]-triamcinolone acetonide. Geldanamycin causes a temperature-dependent loss of steroid binding capacity. Because the GR must be bound to hsp90 to have a high-affinity steroid binding site (Bresnick et al., 1989; Hutchison et al., 1992b), the loss of steroid binding capacity may reflect the ability of geldanamycin to promote dissociation of the GR·hsp90 complex. In Figure 1C, aliquots of cytosol containing hormone-free or hormone-bound receptors were incubated for 1.5 h with geldanamycin at 20 °C, and the GR was immunoadsorbed and assayed for associated hsp90 by Western blotting. Geldanamycin causes the loss of about one-third of the receptor-bound hsp90 (cf. lanes 3 and 4). Steroid also causes the loss of hsp90, regardless of whether geldanamycin is present (lane 6) or absent (lane 5). As a second assay for receptor transformation, we determined DNA binding activity. As shown in Figure 1D, steroid promotes conversion of receptors to the DNA binding form in the presence (lane 6) or absence of geldanamycin (lane 5). Also, consistent with its ability to cause a modest dissociation of hsp90 (Figure 1C), geldanamycin alone at 20 °C causes a modest increase in the DNA-binding activity (cf. lanes 3 and 4).

Geldanamycin Effect on GR in Intact Cells. We have previously shown that GR transfer to the nucleus (assessed by immunofluorescence) is complete about 10 min after addition of dexamethasone to L cells [L-M(TK$^-$)] at 37 °C (Czar et al., 1995). Figure 2 shows the nuclear transfer of the GR caused by dexamethasone in the absence of geldanamycin (cf. Figure 2A,B). Treatment of cells for 1 h with geldanamycin does not itself cause GR translocation (Figure

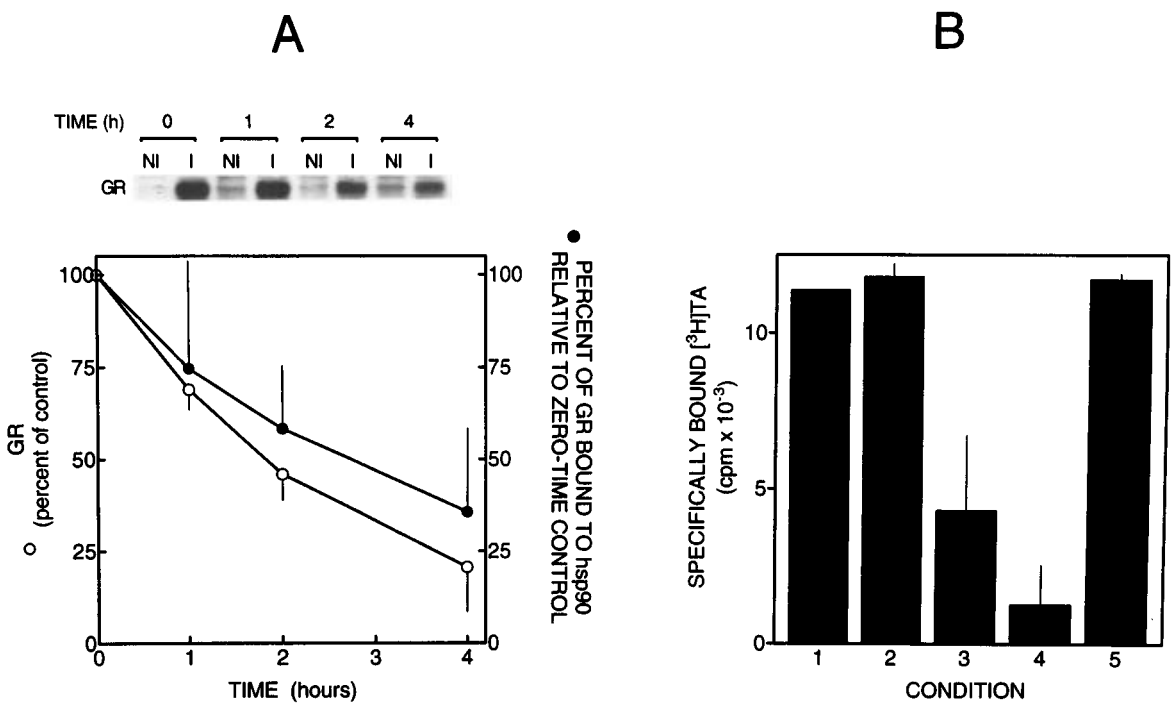

FIGURE 3: (A) Effect of geldanamycin treatment of intact cells on the amount of GR, GR-associated hsp90, and steroid binding capacity. Cytosols were prepared (in buffer containing 20 mM sodium molybdate) from L cells treated at 37 °C for 1, 2, or 4 h with 10 μM geldanamycin or for 4 h with vehicle. This 4 h vehicle treatment is used as the zero-time control for geldanamycin treatment because vehicle does not affect the amount of GR or GR-associated hsp90. Aliquots (300 μL) were immunoadsorbed with nonimmune IgG (NI) or FiGR antibody against the GR (I); the immune pellets were washed three times, and proteins were resolved by SDS−PAGE and Western blotting for GR and hsp90. The Western blots were probed with ^{125}I-labeled counterantibody, and the bands were excised and counted to determine the relative amount of GR in each immunopellet and the hsp90 to GR ratio relative to control, which is set at 100%. The graph presents the means ± SEM of three experiments, and the immunoblot above the graph shows the GR autoradiogram from one experiment. (B) Geldanamycin treatment of intact cells causes a temperature-dependent elimination of steroid binding capacity. Cells were incubated at 37 °C or on ice with 10 μM geldanamycin, after which cells were incubated with [^{3}H]triamcinolone acetonide, washed, and extracted with ethanol for determination of whole cell specific steroid binding activity as described in Experimental Procedures. The values are the means from two experiments with the range indicated by the vertical line in each bar. Conditions are as follows: 1, untreated cells; 2, cells incubated for 30 min at 37 °C with 0.1% DMSO; 3, cells incubated for 10 min at 37 °C with geldanamycin; 4, cells incubated for 30 min at 37 °C with geldanamycin; and 5, cells incubated for 30 min on ice with geldanamycin.

2C), and the GR does not translocate when dexamethasone is added to geldanamycin-pretreated cells (Figure 2D).

When aliquots of cytosol from geldanamycin-treated cells are immunoblotted, there is a marked loss of GR without any change in hsp90 (data not shown). The loss in receptor is reflected in a similar decrease in the amount of immunoadsorbed GR shown in Figure 3A. Whitesell and Cook (1996) showed previously that geldanamycin treatment of Hela cells produced a decrease in the GR protein half-life and provided evidence that the loss is due to proteasome-mediated digestion of the receptor. Figure 3A also shows that a decreasing fraction of the immunoadsorbed receptors are bound to hsp90. This response is similar to the disruption of the Raf-1·hsp90 complex and the decreased Raf-1 half-life reported upon geldanamycin treatment of MCF7 cells (Schulte et al., 1995) and the destabilization of the p185^{erbB2}·GRP94 complex and the decreased p185^{erbB2} half-life reported upon geldanamycin treatment of SKBr3 human breast carcinoma cells (Chavany et al., 1996). The half-time for GR disappearence from geldanamycin-treated L cells is about 2 h, and this effect cannot account for the failure of steroid to promote nuclear translocation after 1 h of geldanamycin treatment (Figure 2D) when cells still have about 70% of their cytosolic GR (Figure 3A).

However, as shown in Figure 3B, geldanamycin treatment of cells causes a rapid loss of glucocorticoid binding activity, with most of the receptors being inactivated within 10 min. Thus, the failure of dexamethasone to cause nuclear translocation of the GR in the experiment of Figure 2 could reflect the failure of steroid to bind to the receptor. As Smith et al. (1995) have shown for the PR in COS cells, the GR recovered from L cells treated with geldanamycin is arrested at an intermediate state of heterocomplex assembly. This is shown in Figure 4, where it can be seen that the GR from cells treated with geldanamycin for 20 min is bound to large amounts of hsp70 and p60, both of which are present in receptor complexes at an intermediate stage of heterocomplex assembly (Smith, 1993).

Geldanamycin Inhibits Nuclear Translocation of Dexamethasone-Prebound Receptors. In the experiment of Figure 5, cells were preincubated at 0 °C with dexamethasone to form steroid−receptor complexes. When the temperature was raised to 37 °C for 20 min, the receptors underwent a dexamethasone-dependent shift to the nucleus (cf. panels B and A of Figure 5). The presence of geldanamycin during the incubation at 37 °C inhibited receptor translocation (cf. panels B and D of Figure 5). The relative nuclear immunofluorescence in 50 cells per condition per experiment was scored on a scale of 0−4, and the results of five experiments like that of Figure 5 are summarized in Table 1. Although in this experimental protocol geldanamycin did not block nuclear translocation of the GR, it markedly inhibited it.

It should be noted that the effect of geldanamycin on steroid binding activity in intact cells is temperature-dependent, and geldanamycin treatment of cells at 0 °C does not affect the ability of GR to bind steroid (Figure 3B). Thus,

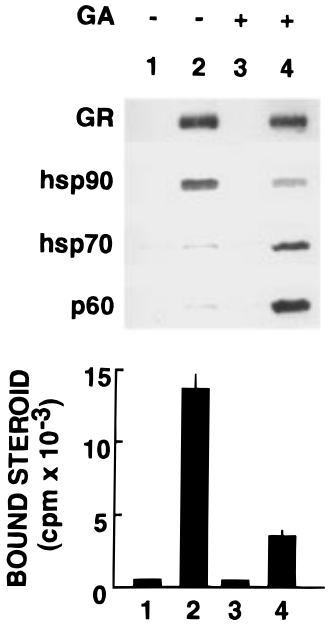

FIGURE 4: Effect of geldanamycin treatment of intact cells on GR-associated proteins. L cells were treated for 20 min at 37 °C with 10 μM geldanamycin or vehicle. Cytosols were prepared and immunoadsorbed with nonimmune IgG or with FiGR antibody against the GR. The immune pellets were split; one portion of each was washed three times, and proteins were resolved by SDS−PAGE and Western blotting for GR, hsp90, hsp70, and p60. The other portion of each immunopellet was incubated with 50 nM [^{3}H]-triamcinolone acetonide to determine steroid binding activity (bar graph). Lanes 1 and 2 are nonimmune and FiGR pellets, respectively, from vehicle-treated cells; lanes 3 and 4 are nonimmune and FiGR pellets, respectively, from geldanamycin-treated cells.

in experiments like that of Figure 5, a 30 min equilibration time with geldanamycin at 0 °C was allowed before the incubation temperature was raised to 37 °C. Assuming complete equilibration of geldanamycin, dexamethasone-dependent GR nuclear transfer and geldanamycin inhibition of heterocomplex assembly should both start when the temperature is shifted to 37 °C, and the degree of inhibition would reflect the relative rates of receptor transfer *versus* geldanamycin inhibition of heterocomplex assembly. It is important to note that geldanamycin does not cause a loss of steroid from the preformed steroid−receptor complexes during the 20 min shift to 37 °C (Table 1).

In Figure 6, cytosols were prepared from cells treated as in the protocol of Figure 5 and GR heterocomplexes were immunoadsorbed. It can be seen that dexamethasone treatment causes a loss of the GR from the cytosolic fraction that is inhibited but not blocked by geldanamycin. Receptors isolated from cells treated with geldanamycin are associated with large amounts of hsp70, again suggesting that they have entered a new cycle of heterocomplex assembly and become blocked at an intermediate stage in the process.

The effect of geldanamycin on nuclear translocation of [^{3}H]steroid-bound receptors is presented in Figure 7A. In these experiments, cells were suspended in growth medium, receptors were prebound with [^{3}H]triamcinolone acetonide at 0 °C, and the cells were then incubated for 20 min at 37 °C to permit receptor translocation. This is the same protocol that was used for the immunofluorescence experiments of Figure 5 and Table 1. In cells kept at 0 °C, ∼85% of the [^{3}H]steroid-bound GR is recovered in the cytosolic fraction, but after 20 min at 37 °C, >90% of the receptor is present in the nuclear fraction (Figure 7A, open bars). In the presence of geldanamycin, half of the [^{3}H]steroid-bound receptors are recovered in the cytosol and half in the nuclear fraction after incubation at 37 °C (Figure 7A, solid bars). This method provides a different and more quantitative assessment of the geldanamycin inhibition of GR translocation that is visualized by immunofluorescence in Figure 5.

In the event that steroid-transformed receptors cycle back out of the nucleus very rapidly, then geldanamycin inhibition of nuclear translocation could be due to geldanamycin blocking the association of hsp90 with hsp90-free receptors exported from the nucleus. These receptors would not be able to bind steroid and recycle to the nucleus. That this is not the case is supported by the experiment of Figure 7B, where cells that were incubated at 37 °C for 20 min to permit GR translocation were incubated an additional 20 min in the presence of geldanamycin. Although the antibiotic caused a small (∼16%) loss of the total specific binding consistent with the loss of the cytosolic GR shown in Figure 3A, the ratio of nuclear to cytoplasmic receptor was similar to the control. This argues strongly against an effect of geldanamycin solely on GR that has come out of the nucleus and is being recycled back.

DISCUSSION

Smith et al. (1995) have shown that geldanamycin is a useful reagent for studying steroid receptor heterocomplex assembly both *in vitro* in rabbit reticulocyte lyate and in intact cells, where in both cases PR heterocomplex assembly was arrested at a step where receptors are bound to the normally transient intermediate assembly components hsp70 and p60. If the effect of geldanamycin is such that receptors can be transformed (i.e., dissociated from hsp90 by biochemical criteria) but that their reassembly into mature heterocomplexes is blocked, then one can test the notion that dynamic assembly of receptor·hsp90 heterocomplexes is required for their trafficking. Because the untransformed GR is cytoplasmic, trafficking can be assayed as nuclear translocation.

In several experiments where geldanamycin was added to cytosol containing native GR·hsp90 heterocomplexes, we show that geldanamycin does not inhibit steroid-dependent receptor transformation assayed by GR·hsp90 dissociation or acquisition of DNA binding activity (Figure 1). Indeed, geldanamycin itself appears to have a modest destabilizing effect in L cell cytosol at 20 °C (Figure 1B−D). To have heterocomplex assembly activity, L cell cytosol must be more concentrated than the cytosols we have prepared here and it must be supplemented with an ATP-regenerating system (Stancato et al., 1996). As there is no heterocomplex assembly occurring in the L cell cytosol used for our experiments, the effect of geldanamycin shown in Figure 1 suggests it may bind to hsp90 in complex with the receptor and at least partially destabilize preformed, native GR·hsp90 complexes.

The fact that geldanamycin treatment of intact cells leads to both a loss of cytosolic GR (Figure 3A) and a loss of steroid binding capacity (Figure 3B) places some constraints on how we can ask whether geldanamycin affects GR translocation. Because the loss of receptor that occurs with geldanamycin treatment occurs relatively slowly (half-time of ∼2 h, Figure 3A) with respect to the normal rate of steroid-dependent GR translocation (half-time of ∼5 min;

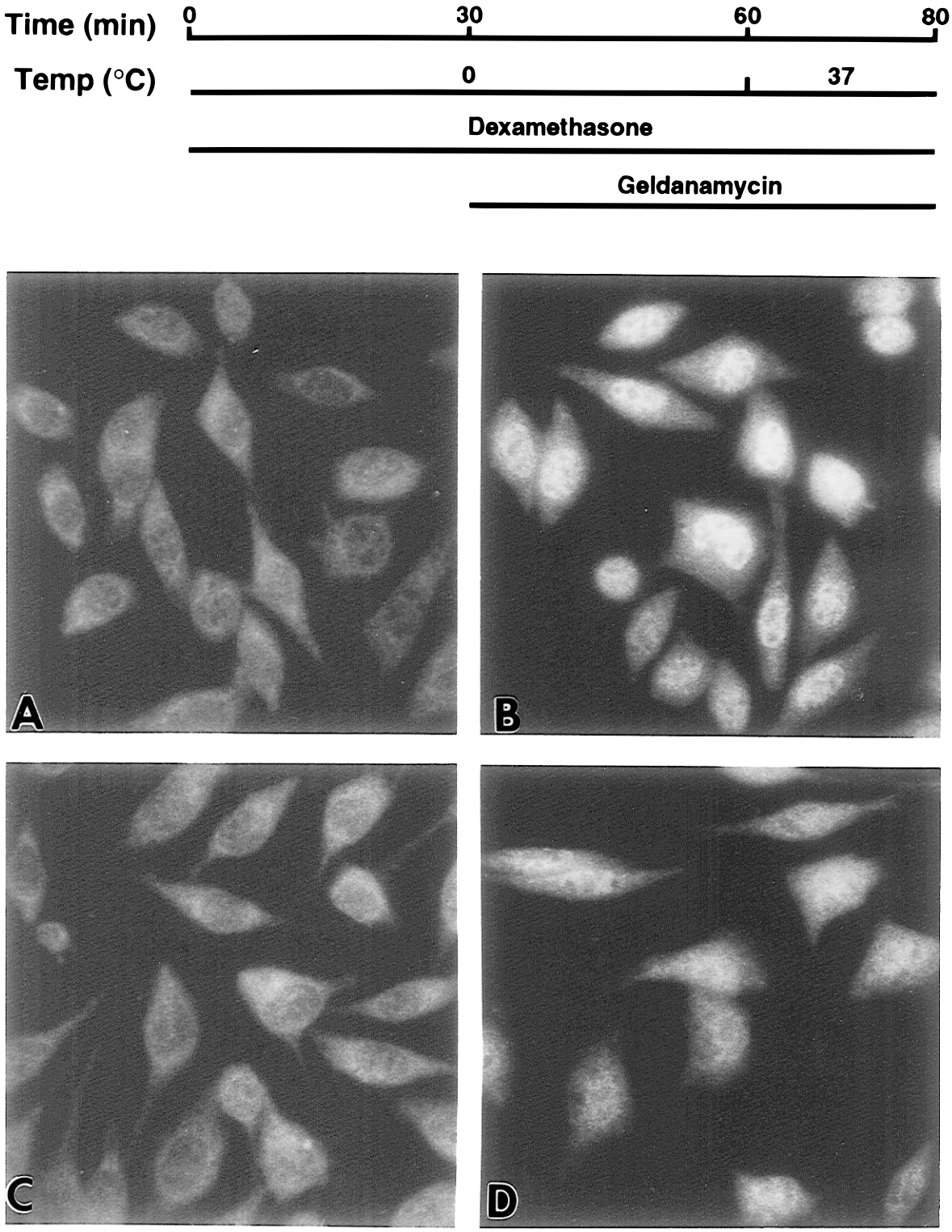

FIGURE 5: Geldanamycin inhibits dexamethasone-induced translocation of the GR to the nucleus. Dexamethasone (1 μM) or vehicle (0.1% ethanol) was added to cells that had been precooled for 10 min on ice. Cells were maintained on ice for 1 h, with 10 μM geldanamycin or vehicle (0.1% DMSO) being added at 30 min. At the end of the 1 h preincubation at 0 °C, cells were shifted to 37 °C for 20 min to allow the steroid-bound receptors to translocate to the nucleus. After fixation in cold methanol, cells were immunostained with BuGR, followed by rhodamine-conjugated anti-mouse IgG. Each field of cells was photographed identically: (A) cells treated with vehicle, (B) cells treated with dexamethasone alone, (C) cells treated with geldanamycin alone, and (D) cells treated with dexamethasone and geldanamycin.

Czar et al., 1995), we can assay nuclear translocation after 20 min of a shift to 37 °C without a significant loss of receptor. However, the inactivation of steroid binding activity by geldanamycin is rapid (Figure 3B). Thus, it is not possible to preincubate cells with geldanamycin and subsequently add dexamethasone to induce nuclear translocation as was done in the protocol of Figure 2. To avoid this problem in the protocol of Figure 5, we first bound the GR with steroid at 0 °C and then shifted the cells to 37 °C. The cells could be preequilibrated with geldanamycin at 0 °C because the effect of geldanamycin is temperature-dependent (Figure 3B). Using this experimental protocol, we were able to show that geldanamycin inhibits nuclear translocation of the GR (Figures 5 and 7A and Table 1).

This inhibition of receptor trafficking appears to reflect the geldanamycin blockade of proper GR·hsp90 heterocom-

Table 1: Geldanamycin Inhibits Nuclear Translocation of Steroid-Bound GR without Causing Loss of Bound Steroid

condition	nuclear translocation score[a]	specific steroid binding[b] [(cpm/(35 mm dish)]
ethanol/DMSO	1.48 ± 0.17	
Dex/DMSO	3.72 ± 0.09	9600 ± 1050
ethanol/GA	1.62 ± 0.31	
Dex/GA	2.50 ± 0.11	11200 ± 1850

[a] Cells were treated with dexamethasone (Dex) and geldanamycin (GA) according to the protocol of Figure 5 and prepared for immunofluorescence. The nuclear receptor was scored for each condition using a score of 4 for nuclear fluorescence much greater than cytoplasmic, fluorescence, 3 for nuclear fluorescence greater than cytoplasmic fluorescence, 2 for nuclear fluorescence equal to cytoplasmic fluorescence, 1 for nuclear fluorescence less than cytoplasmic fluorescence, and 0 for nuclear fluorescence much less than cytoplasmic fluorescence. The scores represent the mean ± SEM from five experiments in which at least 50 cells per condition per experiment were scored in randomly chosen fields. The translocation scores in dexamethasone-treated cells exposed to DMSO vehicle were different from those exposed to geldanamycin at $p < 0.001$. [b] Cells in 35 mm dishes were treated according to the protocol of Figure 5 except that 50 nM [^{3}H]triamcinolone acetonide was substituted for dexamethasone, and whole cell specific steroid binding was assayed as described in Experimental Procedures. The values from five experiments are expressed as the specific counts per minute of [^{3}H]TA binding per dish ± SEM.

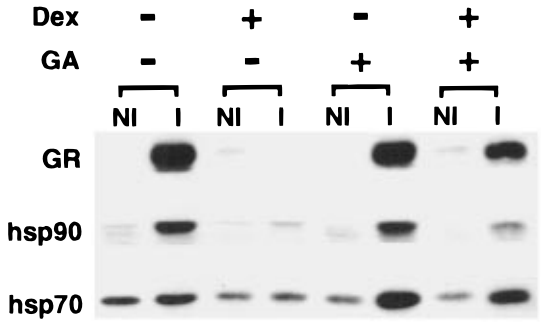

FIGURE 6: Geldanamycin treatment of intact cells inhibits nuclear translocation as assayed by the shift of GR from the cytosolic compartment. L cells were treated as in the experiment of Figure 5, and 300 μL aliquots were immunoadsorbed with nonimmune IgG (NI) or with FiGR antibody against the GR (I). The immune pellets were washed, and proteins were resolved by SDS−PAGE and Western blotted for GR, hsp90, and hsp70.

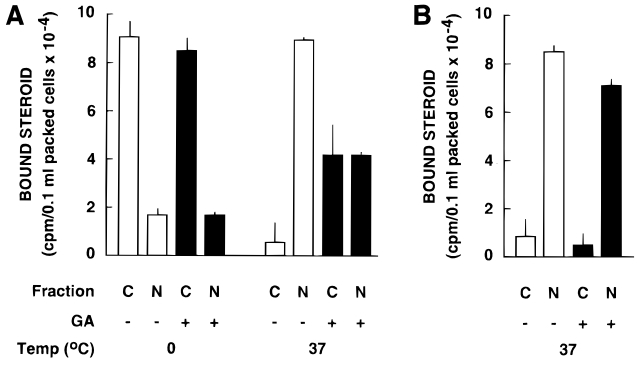

FIGURE 7: Geldanamycin treatment inhibits the shift of [^{3}H]steroid-bound receptors from the cytosolic to the nuclear fraction. (A) Geldanamycin inhibits the nuclear shift. Replicate suspensions of L cells in medium containing 50 nM [^{3}H]triamcinolone acetonide in the presence or absence of competing, nonradioactive dexamethasone were treated according to the protocol of Figure 5, except that half of the samples were incubated for 20 min at 37 °C to promote receptor translocation to the nucleus while the other half were maintained at 0 °C. Cytosolic (C) and nuclear (N) fractions were prepared and counted for [^{3}H]steroid as described in Experimental Procedures. The graph presents specific binding in the absence (open bars) or presence (solid bars) of geldanamycin. The data are the average values from three experiments expressed as the specific counts per minute of [^{3}H]triamcinolone acetonide bound per 0.1 mL of packed cells ± SEM. The nuclear values with geldanamycin at 37 °C are different from those without geldanamycin at $p < 0.003$. (B) Twenty minutes of geldanamycin treatment of cells after receptor translocation does not alter the nuclear: cytoplasmic ratio. L cells (0.1 mL packed volume) were suspended in 2 mL aliquots of medium containing 50 nM [^{3}H]triamcinolone acetonide in the presence or absence of competing dexamethasone, and the suspensions were incubated first for 1 h at 0 °C and then for 20 min at 37 °C to permit receptor translocation to the nucleus. Vehicle (open bars) or geldanamycin (solid bars) was then added, and the incubation was continued for an additional 20 min at 37 °C. The data are the average of values from two experiments, with the vertical lines representing the range of the two values.

plex assembly (Figures 4 and 6). In the protocol of Figures 5 and 7, the GR is in a complex with hsp90 when the cells containing steroid-bound receptors are shifted to 37 °C, and we have shown in Figure 1 that the steroid-bound receptor transforms normally *in vitro* when geldanamycin is present. Thus, the fact that geldanamycin inhibits subsequent nuclear transfer suggests that the transformed steroid−receptor complex requires additional cycles of heterocomplex assembly for translocation to occur.

This proposal deviates in a major way from the generally accepted model of steroid hormone action, which assumes that the cytoplasmic GR moves to the nucleus only after it has undergone steroid-dependent dissociation from hsp90. This general model is based on the observation that receptors that had undergone transformation *in vivo* or *in vitro* were no longer recovered as large (~9S) complexes that contain hsp90. It must be remembered, however, that the untransformed receptors are in extraordinarily tight association with hsp90, and very few proteins have been recovered in such stable complexes with this heat shock protein. Yet hsp90 is a highly abundant and ubiquitous essential protein that must have a general ability to chaperone many proteins. These predicted weak chaperone interactions with multiple cellular proteins have never been detected. Because the routine biochemical techniques we employ to detect hsp90 binding (e.g., coimmunoadsorption and cross-linking) have failed to detect hsp90 association with transformed receptors, it has become axiomatic that steroid-transformed receptors travel to the nucleus in the hsp90-free state. However, it may be that the conformational change that occurs in the hormone binding domain upon steroid binding to a receptor in the cell transforms the receptor's very tight interaction with hsp90 to a weak interaction typical of many proteins chaperoned by the hsp90 system.

It has been generally assumed that transformed receptors travel through the cytoplasm to the nucleus by diffusion. However, in a model where cyclic heterocomplex assembly is required for movement of the transformed receptor, it follows that there is some type of movement machinery. When we originally proposed the transportosome model (Pratt, 1992, 1993), it was assumed that receptors were bound to hsp90 while they moved. But Yang and DeFranco (1996) have suggested that dynamic association with hsp90 is required to permit the access of macromolecules that in essence link the receptor to a movement machinery. In our model, the immunophilin component of the receptor heterocomplex would provide the linkage to a movement machinery and target receptor movement to the nucleus (Pratt et al., 1993; Czar et al., 1995; Owens-Grillo et al., 1996).

In the same way that it seems intuitively reasonable that many proteins are likely to be chaperoned by hsp90, it seems

intuitively reasonable that many proteins might utilize for their own targeted movement the components of a movement machinery responsible for receptor delivery to the nucleus. Thus, we would suggest that dynamic association with hsp90 may be a general requirement for trafficking of many proteins. In that the general function of hsp90 in the biology of the cell has not been determined, it is possible that the suggested dynamic association with proteins during trafficking is its major function. It is interesting that the hsp90 homolog in bacteria is not essential, but hsp90 has been shown to be essential in yeast and *Drosophila* (Parsell & Lindquist, 1993). In comparison with prokaryotes, eukaryotic cells must move proteins in a precisely targeted way over great distances, and it is possible that a nonessential chaperone function of hsp90 in prokaryotes became essential to the solution of this trafficking problem in eukaryotes.

ACKNOWLEDGMENT

We thank Jack Bodwell, David Smith, and Don DeFranco for kindly providing antibodies used in this study.

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BI970648X