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Aluminum Fluoride Inhibition of Glucocorticoid Receptor Inactivation and Transformation†

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ABSTRACT: Fluoride, in the presence of aluminum ions, reversibly inhibits the temperature-mediated inactivation of unoccupied glucocorticoid receptors in cytosol preparations from mouse L cells. The effect is concentration-dependent, with virtually complete stabilization of specific glucocorticoid-binding capacity at 2 mM fluoride and 100 μM aluminum. These concentrations of aluminum and fluoride are ineffective when used separately. Aluminum fluoride also stabilizes receptors toward inactivation by gel filtration and ammonium sulfate precipitation. Aluminum fluoride prevents temperature-dependent transformation of steroid-receptor complexes to the DNA-binding state. Aluminum fluoride does not inhibit calf intestine alkaline phosphatase, and unoccupied receptors inactivated by this enzyme in the presence of aluminum fluoride can be completely reactivated by dithiothreitol. The effects of aluminum fluoride are due to stabilization of the complex between the glucocorticoid receptor and the 90-kDa mammalian heat-shock protein *hsp*90, which suggests that aluminum fluoride interacts directly with the receptor. Endogenous thermal inactivation of receptors in cytosol is not accompanied by receptor dephosphorylation. However, inactivation is correlated with dissociation of *hsp*90 from the unoccupied receptor. These results support the proposal that *hsp*90 is required for the receptor to bind steroid and dissociation of *hsp*90 is sufficient to inactivate the unoccupied receptor.

The untransformed[1] mouse glucocorticoid receptor (GR)[2] in cytosol preparations exists as an 8S–9S heteromeric complex containing the steroid-binding phosphoprotein and a 90-kDa non-steroid-binding phosphoprotein (Housley & Pratt, 1983; Housley et al., 1985; Mendel et al., 1986; Sanchez et al., 1987a). The steroid-binding phosphoprotein contains 783 amino acids with a predicted mass of 86 kDa (Danielsen et al., 1986), although it exhibits anomalous electrophoretic mobility on SDS–polyacrylamide gels with an apparent mass of ~100 kDa (Housley et al., 1985; Mendel et al., 1986). The 90-kDa protein has been identified as the mammalian heat-shock protein *hsp*90 (Sanchez et al., 1985). *hsp*90, or a similar 90-kDa protein, is also found as a common component of untransformed progesterone, estrogen, androgen, mineralocorticoid, and aryl hydrocarbon receptors (Joab et al., 1984; Schuh et al., 1985; Catelli et al., 1985; Perdew, 1988; Denis et al., 1988; Rafestin-Oblin et al., 1989). Transformation of the steroid-bound GR to the DNA-binding state is accompanied by dissociation of *hsp*90 from the GR (Sanchez et al., 1985; Mendel et al., 1986), concomitant with a reduction in the size of the GR from an apparent mass of ~300 kDa to an apparent mass of ~100 kDa (Holbrook et al., 1983; Vedeckis, 1983).

The possibility that receptors are dephosphorylated during transformation has been inferred from the ability of phosphatase inhibitors to prevent steroid receptor transformation. The group VIA transition metal oxyanion molybdate is an effective inhibitor of transformation (Toft & Nishigori, 1979; Leach et al., 1979), and molybdate has been widely used in the purification of untransformed steroid receptors. Although F^- was initially reported to be ineffective at inhibiting transformation of steroid receptors (Leach et al., 1979; Nishigori & Toft, 1980), more recent studies suggest that F^- can partially inhibit transformation of the steroid-bound GR induced by dialysis of AtT-20 cell cytosol (Reker et al., 1987). On the basis of this and other indirect evidence obtained in both intact cells and cytosols, several groups have suggested that transformation may require dephosphorylation of the GR or some other cytosol component (Munck & Leung, 1977; Sando et al., 1979; Barnett et al., 1980; Reker et al., 1987). However, the effects of molybdate are not mediated by phosphatase inhibition but rather are due to a direct interaction with steroid

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[1] The term "transformation" is used to describe the process whereby the steroid-bound receptor is converted to a form that binds to nuclei, DNA–cellulose, etc. The terms "inactivation" and "reactivation" are used to describe the loss and restoration of specific steroid-binding capacity, respectively.

[2] Abbreviations and trivial names: GR, glucocorticoid receptor; *hsp*90, 90-kDa heat-shock protein; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DEAE, diethylaminoethyl; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate.

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receptors [reviewed in Dahmer et al. (1984)], and it is now evident that the GR is not dephosphorylated during ligand-dependent transformation in vitro or in intact cells (Tienrungroj et al., 1987; Mendel et al., 1987). Additionally, a recent report demonstrates that in intact murine WEHI-7 cells the GR undergoes an agonist-dependent increase in phosphate content that is blocked by a glucocorticoid antagonist (Orti et al., 1989).

The possibility that receptor phosphate is required for maintaining the ligand-binding activity of steroid receptors has also been suggested by several observations. The steroid-binding capacity of the unoccupied GR in cytosol is labile, and receptor inactivation can be promoted by a variety of treatments including heat and the addition of exogenous protein phosphatases [reviewed in Housley et al. (1984)]. Endogenous thermal inactivation of the GR can be partially inhibited by the phosphatase inhibitors F^-, glucose 1-phosphate, and inorganic orthophosphate, and molybdate completely inhibits inactivation (Nielsen et al., 1977b). F^- stabilizes the unoccupied progesterone receptor in chick oviduct cytosol (Grody et al., 1980), and F^- prevents inactivation of the calf uterine estrogen receptor by a nuclear enzyme (Auricchio et al., 1981). These stabilizing effects are similar to the properties of an endogenous small, heat-stable factor present in cytosol that inhibits receptor inactivation and transformation (Cake et al., 1976; Bailly et al., 1977; Sando et al., 1977; Granberg & Ballard, 1977; Leach et al., 1982). Recently, this factor has been extensively purified from rat liver cytosol, and it has been proposed to be a metal anion (Meshinchi et al., 1988) or an unusual ether aminophosphoglyceride (Bodine & Litwack, 1988a,b). It is not known if receptor phosphate groups are required for the interaction between the GR and the endogenous factor.

The effects of F^- on certain biological systems have been shown to be due to the presence of Al^{3+}. The activation of the regulatory GTP-binding protein of adenyl cyclase by F^- requires Al^{3+} (Sternweis & Gilman, 1982), and the involvement of GTP-binding proteins has been demonstrated or implicated in other metabolic systems that are affected by aluminum fluoride [for recent reviews, see Casey and Gilman (1988), Macdonald and Martin (1988), and Freissmuth et al. (1989)]. The inhibition of microsomal glucose-6-phosphatase (EC 3.1.3.9) by F^- is due to a complex between Al^{3+} and F^- (Lange et al., 1986), and it is possible that a coordinate complex between Al^{3+} and F^- can account for other observed F^- effects. In this paper, the effects of F^- on the GR have been reexamined, and evidence is presented to show that aluminum fluoride is a potent inhibitor of both receptor inactivation and transformation. These effects are not due to inhibition of receptor dephosphorylation but rather are due to stabilization of the GR–*hsp*90 complex. The properties of aluminum fluoride resemble those of the endogenous heat-stable factor and molybdate, which suggests that all three interact with the same site on the GR. A preliminary account of portions of this work has appeared in abstract form (Housley & Laks, 1988).

Experimental Procedures

Materials. [6,7-^{3}H]Triamcinolone acetonide (42.5 Ci/mmol) and ^{125}I-labeled goat anti-mouse IgG (7.2 μCi/μg) were obtained from New England Nuclear (Boston, MA). [^{32}P]-Orthophosphate (carrier free), Hepes (ultrapure), and electrophoresis reagents were from ICN (Irvine, CA). Immobilon P membranes were from Millipore (Bedford, MA). DEAE-Sephacel and Sephadex G-50 were from Pharmacia-LKB (Piscataway, NJ). Sodium fluoride and aluminum chloride (analytical reagent grade) were from Mallinckrodt (Paris, KY). Sodium molybdate and routine chemicals were from Fisher (Atlanta, GA). Radioinert dexamethasone, nonimmune mouse IgG, protein A–Sepharose CL-4B, calf intestine alkaline phosphatase (type VII-NT, 1060 units/mg), and rabbit anti-goat IgG–peroxidase conjugate were from Sigma Chemical Co. (St. Louis, MO). The BuGR2 anti-receptor monoclonal antibody is derived from one of eight antibody-producing hybridomas previously described (Gametchu & Harrison, 1984) and was kindly provided by Drs. W. J. Hendry and R. W. Harrison. The AC88 monoclonal antibody against the 90-kDa heat-shock protein *hsp*90 (Riehl et al., 1985) was kindly provided by Dr. D. O. Toft.

Cell Culture, ^{32}P Labeling, and Cytosol Preparation. Mouse L929 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Hyclone) at 37 °C. For ^{32}P-labeling experiments, cells in the log phase of growth were washed with phosphate-free medium containing 10% dialyzed calf serum and incubated in this medium containing [^{32}P]orthophosphate (20 μCi/mL) for 18 h. All subsequent steps were done at 0–4 °C. Cells were harvested by scraping into Earle's balanced saline and centrifuged at 600*g* for 10 min. Following a wash by resuspension in Earle's saline and centrifugation, cells were suspended in 1.5 volumes of 10 mM Hepes–0.4 mM EDTA, pH 7.35 at 4 °C, and ruptured by Dounce homogenization. The homogenate was centrifuged at 27000*g* for 30 min, followed by 105000*g* for 1 h. The resulting supernatant fluid (cytosol) was either used immediately or stored frozen at −70 °C.

Incubation Conditions, Steroid-Binding Assay, and DNA-Binding Assay. For inactivation studies, cytosol was present at 50% of the final reaction volume unless otherwise specified. As fluoride solutions can leach aluminum from glass (Lange et al., 1986), all experiments were performed in plastic tubes. Specific glucocorticoid-binding capacity was assayed with 50 nM [^{3}H]triamcinolone acetonide as previously described (Housley et al., 1982). For transformation experiments, cytosol was incubated with 50 nM [^{3}H]triamcinolone acetonide plus vehicle or 50 μM radioinert dexamethasone for 4 h on ice to occupy GR binding sites. Steroid-bound receptors were transformed by heating for 1 h at 25 °C with the indicated additions. To assay DNA binding, duplicate 0.2-mL aliquots of each sample were added to 0.1 mL of a 25% suspension of DNA–cellulose and mixed by rotation at 4 °C for 45 min. The pellets were washed and assayed for radioactivity as previously described (Leach et al., 1979). Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate as described (Leach et al., 1983).

Immunoadsorption, Gel Electrophoresis, and Immunoblotting. For immunoadsorption, aliquots of each sample were mixed with an equal volume of TEG buffer (10 mM TES, 20 mM sodium molybdate, 10% glycerol, 50 mM NaCl, 4 mM EDTA, pH 7.6 at 4 °C). The BuGR2 antibody (as hybridoma culture fluid) or nonimmune mouse IgG (at the same concentration as the BuGR2 antibody) was added at 5% of final volume, and the samples were incubated on ice for 12–16 h. Protein A–Sepharose (50-μL packed volume) in TEG buffer was added, and the samples were mixed by continuous rotation at 4 °C for 3 h. The protein A–Sepharose was then pelleted by centrifugation and washed five times by suspension in 1 mL of TEG buffer containing 0.4 M NaCl and 0.2% Triton X-100, followed by three washes with TEG buffer. All washes were done at 0–4 °C. The washed pellet was suspended in 2× SDS sample buffer and then heated at 100 °C for 4 min,

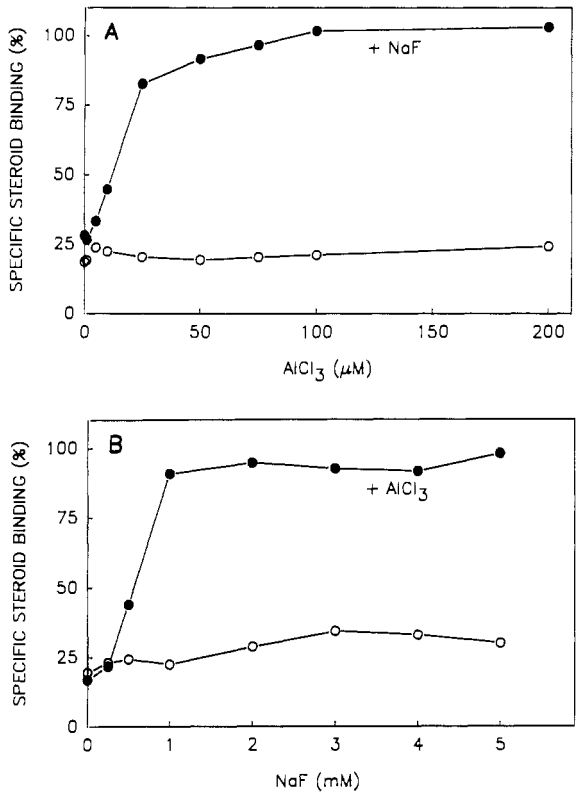

FIGURE 1: Effect of aluminum fluoride concentration on the thermal inactivation of receptor binding capacity. Aliquots of L cell cytosol containing aluminum chloride, sodium fluoride, or both were incubated at 25 °C for 1 h and assayed for remaining specific steroid-binding capacity as described under Experimental Procedures. (Panel A) Aluminum chloride at the indicated final concentration was present in samples with (●) or without (○) 2 mM sodium fluoride. (Panel B) Sodium fluoride at the indicated concentration was present in samples with (●) or without (○) 100 μM aluminum chloride.

and the eluted proteins were resolved by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Proteins were electrophoretically transferred to Immobilon P membranes, and the remaining protein-binding sites on the membrane were blocked by incubation with Western buffer (20 mM Tris, 0.5% bovine serum albumin, 0.5% Tween 20, 180 mM NaCl, pH 7.5 at 25 °C). To detect the GR and *hsp*90, blots were incubated with 1% BuGR2 or 10 μg/mL AC88, respectively, in Western buffer at 4 °C overnight. Immune complexes were detected by incubating the membranes with ^{125}I-labeled goat anti-mouse IgG (0.25 μg/cm^2) followed by peroxidase-conjugated anti-goat IgG. After peroxidase staining, the bound radioactivity was visualized by autoradiography. To quantify the amounts of GR and *hsp*90, the stained bands were excised from the blot and assayed for ^{125}I by γ crystal scintillation spectrometry as described (Howe & Hershey, 1981). After background values for nonimmune controls have been subtracted, this method is linear for samples containing from 0.05 to 10 pmol of GR. The amount of receptor-associated *hsp*90 in each sample was estimated by normalizing ^{125}I radioactivity bound to the *hsp*90 band to the ^{125}I radioactivity bound to the GR band and is reported as the ratio *hsp*90:GR. To determine the phosphate content of receptors after inactivation, samples containing ^{32}P-labeled cytosol were processed as above, and the GR bands were excised and counted for ^{125}I and ^{32}P as previously described (Tienrungroj et al., 1987; Dalman et al., 1988).

Column Chromatography. Gel filtration of cytosol samples was performed at 4 °C on columns of Sephadex G-50 (1.5 × 12 cm) equilibrated in 10 mM Hepes, pH 7.35, with or without 100 μM $AlCl_3$ plus 2 mM NaF. Protein concentrations in the eluates were estimated as described (Bradford, 1976) with bovine serum albumin as the standard. For ion exchange chromatography, columns of DEAE-Sephacel (1 × 8 cm) were equilibrated in 10 mM Hepes, pH 7.0 at 4 °C, with the indicated additions. Steroid-bound receptors were eluted from the column with a gradient (180 mL) of 0–0.5 M NaCl in column buffer. Aliquots of each 2-mL fraction were assayed for [^{3}H]triamcinolone acetonide bound receptors by liquid scintillation spectrometry. The NaCl concentration in column fractions was determined by conductivity measurements referenced to a standard curve.

Table I: Reversibility of Aluminum Fluoride Stabilization and Recovery of Receptor Binding Activity after Gel Filtration[a]

sample	incubation at 25 °C (min)	specific binding capacity (cpm/mg of protein)
control cytosol	0	94 600
	30	68 100
	60	28 300
cytosol plus aluminum fluoride	30	95 500
	60	91 700
column 1 fraction		
control	0	33 100
	30	300
plus aluminum fluoride	0	49 400
	30	37 500
column 2 fraction	0	92 700
	30	73 100

[a] L cell cytosol (at 98% of the final volume) was incubated in the presence of buffer or 100 μM aluminum chloride plus 2 mM sodium fluoride at 25 °C for 30 min, chilled on ice, and assayed for specific binding capacity. Aliquots (1.0 mL) of the sample containing aluminum fluoride were chromatographed on Sephadex G-50 columns at 4 °C in buffer alone to remove aluminum fluoride (column 1) or in buffer containing aluminum fluoride (column 2). The macromolecular fraction from column 1 was divided into two portions, and aluminum chloride (100 μM) and sodium fluoride (2 mM) were added to one portion. Each sample was assayed for specific binding before and after a 30-min incubation at 25 °C. The macromolecular fraction from column 2 was also assayed for specific binding capacity before and after a 30-min incubation at 25 °C. The cytosols remaining from the original incubation were returned to 25 °C and assayed for remaining specific binding capacity after an additional 30 min. Values are reported as cpm/mg of protein to correct for dilution after the gel filtration step.

RESULTS

Aluminum Fluoride Inhibition of GR Inactivation. When L cell cytosol is incubated at 25 °C, the glucocorticoid-binding capacity of the GR is inactivated with a half-life of 25 min. When either NaF (1–5 mM) or $AlCl_3$ (1–200 μM) is present, there is no effect on the rate of inactivation. However, when both NaF and $AlCl_3$ are present, there is virtually complete stabilization of the GR. As shown in Figure 1, this synergistic effect is concentration-dependent. The maximum stabilization is observed at 2 mM NaF and 100 μM $AlCl_3$, and these concentrations were used for subsequent experiments. As shown in Table I, the inhibition of receptor inactivation by aluminum fluoride is reversible.

Unoccupied receptors are also inactivated at 0–4 °C by gel filtration and by precipitation with ammonium sulfate, and sodium molybdate inhibits inactivation under these conditions (Leach et al., 1979). The presence of aluminum fluoride in the elution buffer also inhibits inactivation of GR steroid-binding capacity during gel filtration (Table I). Appreciable receptor binding activity can be recovered from cytosol after ammonium sulfate precipitation in the presence of aluminum fluoride, as shown in Table II. When [^{3}H]triamcinolone acetonide bound receptors are precipitated with ammonium sulfate under the conditions listed in Table II, there are no

Table II: Effect of Aluminum Fluoride on Recovery of Receptor Binding Activity after Precipitation with Ammonium Sulfate[a]

condition	specific binding capacity (cpm/0.1 mL) expt 1	expt 2
control cytosol	77 500	86 300
cytosol precipitated and redissolved in		
buffer alone	4 500	9 400
buffer plus fluoride	8 700	10 200
buffer plus aluminum	5 300	13 700
buffer plus aluminum fluoride	21 700	58 600
buffer plus molybdate	59 700	80 300

[a] Aliquots (0.5 mL) of L cell cytosol containing sodium fluoride (2 mM), aluminum chloride (100 μM), both aluminum chloride (100 μM) and sodium fluoride (2 mM), or sodium molybdate (10 mM) were fractionally precipitated with ammonium sulfate at 55% of saturation. Precipitated proteins were redissolved in the original volume of buffer containing the same respective additions, and specific binding capacity was assayed for each sample. All procedures were done at 0–4 °C.

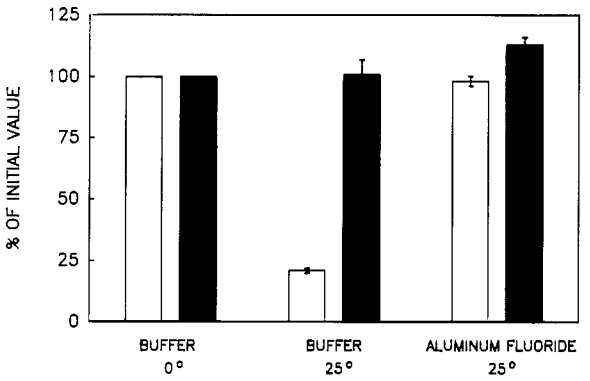

FIGURE 2: Thermal inactivation of unoccupied glucocorticoid receptors is not accompanied by receptor dephosphorylation. Aliquots (0.6 mL) of cytosol prepared from ^{32}P-labeled L cells were mixed with an equal volume of 10 mM Hepes buffer or buffer containing 100 μM aluminum chloride plus 2 mM sodium fluoride and incubated at 0 or 25 °C for 1 h as indicated. The GR in each sample was analyzed by immunoadsorption and quantitative immunoblotting as described under Experimental Procedures. The amount of phosphate per receptor ($^{32}P/^{125}I$) is expressed as the percent of the initial value (solid bars, mean ± SE, n = 3) for three independent preparations of ^{32}P-labeled cytosol. Aliquots of unlabeled cytosol were incubated under identical conditions and assayed for remaining specific binding capacity. Steroid-binding capacity is expressed as the percent of the initial value (open bars, mean ± SE, n = 3).

differences in the amounts of steroid-bound GR recovered in the precipitates (data not shown), which indicates that the effects of aluminum fluoride and molybdate are not due to an increased amount of receptor protein being precipitated.

Inactivation of GR Does Not Result from Receptor Dephosphorylation. Several observations have led to the proposal that receptor phosphate groups may be required to maintain the active, steroid-binding state of the GR, and it has been suggested that inactivation of steroid-binding activity may involve, at least in part, dephosphorylation of the unoccupied GR or some other component by an endogenous phosphatase (Nielsen et al., 1977b; Sando et al., 1979). Fluoride complexed to aluminum inhibits glucose-6-phosphatase (Lange et al., 1986), and it is possible that the stabilizing effect of aluminum fluoride on the GR is due to phosphatase inhibition. This possibility was examined directly by submitting cytosol from ^{32}P-labeled L cells to conditions that inactivate steroid-binding capacity. As shown in Figure 2, incubation of unoccupied cytosolic receptors at 25 °C does not result in detectable loss of receptor phosphate, whether or not aluminum fluoride is present. Thus, aluminum fluoride is not stabilizing the GR indirectly as a result of inhibiting an endogenous receptor phosphatase.

Table III: Inhibition of Steroid–Receptor Complex Transformation by Aluminum Fluoride[a]

condition	specific binding to DNA–cellulose (cpm/0.1 mL)
unheated control cytosol	6 200
additions to steroid-bound cytosol	
before 25 °C incubation	
sodium chloride	45 700
sodium fluoride	42 700
aluminum chloride	47 700
aluminum fluoride	8 300
molybdate	2 500
additions to steroid-bound cytosol	
after 25 °C incubation	
sodium chloride	43 500
sodium fluoride	37 700
aluminum chloride	41 200
aluminum fluoride	39 800
molybdate	41 600

[a] L cell cytosol was incubated for 4 h at 0 °C with 50 nM [^3H]-triamcinolone acetonide to occupy GR binding sites and divided into several aliquots. Sodium chloride (10 mM), sodium fluoride (2 mM), aluminum chloride (100 μM), aluminum chloride (100 μM) plus sodium fluoride (2 mM), or sodium molybdate (10 mM) was added either before or after incubation at 25 °C for 1 h to transform steroid-bound receptors to the DNA-binding state. Duplicate aliquots were removed and assayed for steroid binding and binding to DNA–cellulose as described under Experimental Procedures. The total specific binding capacity of cytosol was 95 600 cpm/0.1 mL.

Aluminum Fluoride Inhibition of GR Transformation. As molybdate has been shown to be an effective inhibitor of both GR inactivation and transformation to the DNA-binding state and as aluminum fluoride also inhibits the inactivation of unoccupied receptors, it seemed possible that aluminum fluoride might also inhibit temperature-mediated transformation of the steroid-bound GR. The effect of aluminum fluoride on transformation was examined, and the results are listed in Table III. Neither Al^{3+} nor F^- alone has an effect on transformation, but the combination is a potent inhibitor of the process. This inhibition is not due to an effect on the ability of transformed receptors to bind to DNA but rather is due to direct inhibition of the transformation reaction (Table III).

The steroid-bound GR is also transformed to the DNA-binding state by chromatography on DEAE ion exchange columns (Parchman & Litwack, 1977; Sakaue & Thompson, 1977; Munck & Foley, 1980), and if molybdate is present during elution of the column, this conversion is prevented (Dahmer et al., 1981). Like molybdate, aluminum fluoride prevents transformation on DEAE columns. As shown in Table IV, when [^3H]triamcinolone acetonide bound receptors are adsorbed to DEAE-Sephacel and eluted with a gradient of NaCl, the majority of the GR elutes at low salt concentrations. If aluminum fluoride is present in the column buffer, the GR elutes at higher salt concentrations. The GR peak eluting in the low-salt region has previously been shown to contain transformed, DNA-binding receptors, and the peak eluting in the high-salt region contains untransformed, non-DNA-binding receptors (Dahmer et al., 1981). The untransformed GR is more tightly bound to the matrix through the additional interactions of receptor-associated *hsp*90 (Sanchez et al., 1987b).

Stabilization of GR–hsp90 Complex by Aluminum Fluoride. As transformation of the GR has been shown to be accompanied by dissociation of *hsp*90 from the receptor (Sanchez et al., 1985, 1987b; Mendel et al., 1986), it seemed

Table IV: Effect of Aluminum Fluoride on Steroid–Receptor Complexes during Chromatography on DEAE-Sephacel[a]

condition	% receptors eluted in low salt (140–165 mM)	% receptors eluted in high salt (240–280 mM)
untransformed receptors eluted in		
buffer alone	93	7
buffer plus aluminum fluoride	9	91
buffer plus molybdate	2	98
transformed receptors eluted in		
buffer alone	94	6
buffer plus aluminum fluoride	92	8

[a] Aliquots (2.0 mL) of L cell cytosol containing untransformed or temperature-transformed [^{3}H]triamcinolone acetonide bound receptors were submitted to ion exchange chromatography on DEAE-Sephacel columns at 4 °C. The column buffer contained no additions, aluminum chloride (100 μM) plus sodium fluoride (2 mM), or sodium molybdate (10 mM) as indicated. Steroid-bound receptors were eluted with a gradient of sodium chloride in column buffer and analyzed as described under Experimental Procedures. The sodium chloride concentrations at which each receptor peak was eluted are shown in parentheses. The receptors eluting in the low-salt region and in the high-salt region of the gradient are expressed as a percentage of the total receptors eluting in both regions.

likely that the effects of aluminum fluoride on the GR are mediated by stabilizing the association of *hsp*90 with the receptor. Accordingly, the amounts of receptor-associated *hsp*90 after inactivation and transformation were determined. As shown in Figure 3A, *hsp*90 dissociates from the unoccupied GR during thermal inactivation, and this dissociation is prevented by aluminum fluoride and molybdate. The amounts of receptor-associated *hsp*90 were measured by quantitative immunoblotting with ^{125}I-labeled goat anti-mouse IgG and normalized to the amount of GR in each sample. The ratios of *hsp*90:GR radioactivity for the inactivated samples in Figure 3A (lanes 2–4) indicate that 70–80% of the receptor-associated *hsp*90 has been lost under conditions that inactivate 80% of the steroid-binding capacity, whereas aluminum fluoride and molybdate completely prevent this loss (Figure 3A, lanes 5 and 6). Thus, there is a good correlation between dissociation of *hsp*90 from the unoccupied receptor and inactivation of steroid-binding capacity. As shown by the experiment in Figure 3B, temperature-mediated transformation of the steroid-bound GR is accompanied by dissociation of *hsp*90 from the receptor, and both aluminum fluoride and molybdate prevent this dissociation. The ratios of *hsp*90:GR radioactivity for the transformed samples in Figure 3B (lanes 2–4) indicate that incubation of steroid–receptor complexes at 25 °C results in a loss of 90% of receptor-associated *hsp*90, although only 50% of these receptors are in the DNA-binding form.

Aluminum Fluoride Permits Reactivation of Phosphatase-Inactivated Receptors. It has been known for some time that when exogenous purified calf intestine alkaline phosphatase is added to unoccupied receptors in cytosol, the steroid-binding capacity is inactivated in an enzyme-dependent manner (Nielsen et al., 1977a). This enzyme is not inhibited by molybdate, and phosphatase-mediated inactivation of the GR occurs in the presence of molybdate (Leach et al., 1983). We have shown that phosphatase-inactivated receptors can be reactivated by reduction with DTT, provided molybdate is present during the incubation with the enzyme (Housley et al., 1982). When unoccupied ^{32}P-labeled receptors are incubated with alkaline phosphatase in the presence of molybdate, the rate of receptor dephosphorylation is similar to the rate of receptor inactivation under these conditions (unpublished experiments). These results provided indirect evidence that receptor phosphate groups might be required to maintain the steroid-binding conformation of the GR and suggested that molybdate was stabilizing the receptor directly. The experiments with aluminum fluoride suggested that it might also stabilize the GR under these conditions, provided alkaline phosphatase is not inhibited. Neither Al^{3+} (up to 1 mM) nor F^- (up to 100 mM), nor the combination, inhibits the activity of calf intestine alkaline phosphatase assayed with *p*-nitrophenyl phosphate. As shown in Table V, if aluminum fluoride is present during the incubation of unoccupied receptors with alkaline phosphatase, receptors can be completely reactivated by the addition of DTT. This result suggests that aluminum fluoride, like molybdate, maintains the inactivated receptor in a form that can be reactivated by sulfhydryl reduction. This form is composed of a complex between the GR and *hsp*90. The data in Table V suggest that if *hsp*90 remains associated with the oxidized, nonbinding GR, steroid-binding capacity can be reactivated by the addition of DTT. Thus, receptor-associated *hsp*90 is necessary, but not sufficient, for steroid-binding activity.

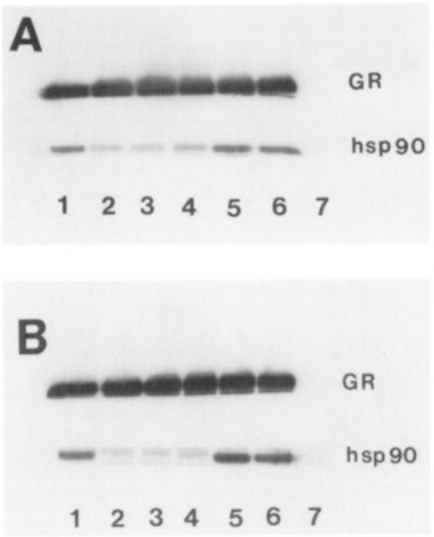

FIGURE 3: Aluminum fluoride prevents dissociation of *hsp*90 from the glucocorticoid receptor during inactivation and transformation. Aliquots of L cell cytosol containing unoccupied receptors (panel A) or [^{3}H]triamcinolone acetonide bound receptors (panel B) were incubated at 25 °C for 1 h with the following additions: (lanes 1 and 7) unheated control cytosol; (lane 2) no additions; (lane 3) 100 μM aluminum chloride; (lane 4) 2 mM sodium fluoride; (lane 5) 100 μM aluminum chloride plus 2 mM sodium fluoride; (lane 6) 10 mM sodium molybdate. Samples were assayed for DNA-binding activity and/or steroid-binding capacity, and an aliquot (0.5 mL) of the remainder was immunoadsorbed to protein A–Sepharose with the BuGR2 antibody (lanes 1–6) or nonimmune mouse IgG (lane 7). Half of each immunoadsorbed sample was used to determine the amount of GR with the $BuGR_2$ antibody, and the other half was used to determine the amount of receptor-associated *hsp*90 with the AC88 antibody by the quantitative immunoblot procedure described under Experimental Procedures. The autoradiogram shown was obtained after incubation of each blot with ^{125}I-labeled anti-mouse IgG and peroxidase staining. Each band was excised from the blot and assayed for ^{125}I to quantify the amount of GR and *hsp*90 in each sample, as discussed in the text.

Discussion

The experiments shown in Figure 1 and in Tables I–IV demonstrate that aluminum fluoride inhibits inactivation of the unoccupied GR and also prevents transformation of the steroid-bound GR to the DNA-binding state. These effects are mediated by stabilizing the association between *hsp*90 and the GR (Figure 3), an activity which is also exhibited by the purified endogenous heat-stable factor (Meshinchi et al., 1988). Additionally, aluminum fluoride stabilizes the oxidized nonbinding form of the receptor by maintaining the GR–*hsp*90 complex, and steroid-binding capacity can be recovered by reduction with DTT (Table V). The properties of aluminum

Table V: Aluminum Fluoride Permits Reactivation of Receptor Binding Activity after Incubation with Alkaline Phosphatase[a]

additions	specific binding capacity (cpm/0.1 mL) A (-DTT)	specific binding capacity (cpm/0.1 mL) B (+DTT)	receptor-associated *hsp*90 (*hsp*90:GR) A (-DTT)	receptor-associated *hsp*90 (*hsp*90:GR) B (+DTT)
none	18300	23900	0.012	0.013
alkaline phosphatase	100	2100	0.012	0.012
aluminum fluoride	90900	84700	0.119	ND
alkaline phosphatase plus aluminum fluoride	4900	77600	0.107	0.108
molybdate	90300	89700	0.111	ND
alkaline phosphatase plus molybdate	4800	85900	0.109	0.107

[a] L cell cytosol was incubated at 25 °C for 1 h with the additions listed and then chilled on ice. Aliquots were removed to assay for specific binding capacity and for receptor-associated *hsp*90 by immunoadsorption and quantitative immunoblotting as described in the legend to Figure 3 (A). To the remainder of each sample, dithiothreitol (DTT) was added to a final concentration of 10 mM, and aliquots were assayed for specific binding capacity and for receptor-associated *hsp*90 (B). The concentrations of the additions were as follows: calf intestine alkaline phosphatase, 100 μg/mL; aluminum fluoride, 100 μM aluminum chloride plus 2 mM sodium fluoride; sodium molybdate, 10 μM. The initial specific binding capacity was 85600 cpm/0.1 mL of cytosol. For unheated control cytosol, the ratio of *hsp*90-bound ^{125}I to GR-bound ^{125}I (*hsp*90:GR) was 0.079. ND, not determined.

fluoride thus resemble those of the group VIA transition metal oxyanion molybdate and of the endogenous heat-stable factor previously described (Meshinchi et al., 1988; Bodine & Litwack, 1988a,b), and it is proposed that aluminum fluoride interacts with the GR at the same site as molybdate and the heat-stable factor.

Several years ago high concentrations of F^- (25–100 mM) were reported to partially stabilize the GR (Nielsen et al., 1977b,c) and the progesterone receptor (Grody et al., 1980). More recently, 50 mM F^- was reported to partially inhibit GR transformation in vitro (Reker et al., 1987). Commercial preparations of NaF are commonly contaminated with aluminum fluosilicates (*Merck Index*, 1983), and these high concentrations of F^- might contain sufficient aluminum to form the active aluminum fluoride complex. Additionally, aqueous solutions of F^- can leach Al^{3+} from glassware (Lange et al., 1986), and Al^{3+} is also present in blood and tissues (Hammond & Beliles, 1980; Yokel & Melograna, 1983). Thus the unsuspected presence of Al^{3+} could have contributed to the previously reported effects of F^- on steroid receptors.

The stoichiometry of the complex between Al^{3+} and F^- that stabilizes the GR is unknown. F^- can combine with Al^{3+} to form six complexes, ranging from AlF_1^{2+} to AlF_6^{3-} (Brossel & Orring, 1943). The active species that interacts with GTP-binding proteins has usually been assumed to be AlF_4^- [e.g., see Sternweis and Gilman (1982) and Bigay et al. (1985)]. At the concentrations used in the experiments in this paper, the formation constants of aluminum fluoride complexes predict that the active species might be AlF_3 or AlF_4^- (Brossel & Orring, 1943; Goldstein, 1964). However, Al^{3+} also has a strong affinity for phosphate, and recent papers have questioned the existence of AlF_4^- in aqueous solutions containing phosphorylated compounds (Martin, 1988; Jackson, 1988). Regardless of the coordinate structure of the active species, the present results suggest that the effects of Al^{3+} on the GR are dependent on the presence of F^-.

Direct evidence is lacking for a role of receptor phosphate in determining steroid-binding activity. In a recent report, it was shown that when the GR is purified free of other cytosolic components by immunoadsorption and subsequently inactivated by heating at 20 °C, there is no apparent loss of receptor phosphate (Bresnick et al., 1989). The experiments shown in Figure 2 extend this observation to whole cytosol and demonstrate that endogenous inactivation of the GR in cytosol during incubation at 25 °C is not due to receptor dephosphorylation. It has been established in several laboratories that sulfhydryl groups on the GR are essential for steroid binding, and NADPH-dependent thioredoxin reductase has been proposed to be responsible for maintaining the GR in a reduced, steroid-binding state through thiol-disulfide exchange with thioredoxin (Grippo et al., 1983, 1985). We have inferred from previous results (Housley et al., 1982) that dephosphorylation of unoccupied receptors promotes an oxidation of essential receptor sulfhydryl groups, and if molybdate is present, this oxidation is reversible. The endogenous heat-stable factor has also been reported to stabilize the phosphatase-inactivated GR such that steroid-binding can be reactivated by the addition of DTT (Bodine & Litwack, 1988b). The experiment in Table V demonstrates that aluminum fluoride is equally effective at stabilizing this nonbinding form of the GR and that there is a direct correlation between the presence of receptor-associated *hsp*90 in the nonbinding form and subsequent reactivation by DTT. While this paper was in preparation, an important study by Bresnick et al. (1989) was published that demonstrated that *hsp*90 is necessary for maintaining the steroid-binding conformation of the GR. Using immunoadsorbed receptors that were washed free of most other cytosolic components, they showed that the receptor was able to bind steroid only if it was still associated with *hsp*90. When immunoadsorbed GR complexes were heated at 20 °C, *hsp*90 dissociated from the receptor, and steroid-binding capacity was lost. The experiments shown in Figure 3A and Table V confirm this observation for receptors in whole cytosol, and they support the proposal that receptor-associated *hsp*90 is necessary but not sufficient for the steroid-binding conformation of the GR (Bresnick et al., 1989). It is not known if receptor phosphate groups are important in the normal interactions among the GR, the endogenous factor, and *hsp*90 that determine the steroid-binding conformation, but these results suggest that conditions which maintain the GR–*hsp*90 complex are sufficient to permit this active conformation to occur in a reducing environment. Direct evidence that receptor phosphate is required to maintain the GR in a substrate conformation for thioredoxin-mediated reduction will require site-directed mutagenesis of the GR cDNA at the sequences coding for the serine residues that serve as phosphorylation sites.

It is not clear why incubation of the unoccupied cytosolic receptor at 25 °C causes loss of steroid-binding capacity and dissociation of *hsp*90, whereas the unoccupied GR in whole cells at 37 °C is competent for ligand binding and remains associated with *hsp*90 in the absence of hormone (Rexin et al., 1988; Howard & Distelhorst, 1988). Some recent observations sugggest that one explanation for this discrepancy is the possibility that the structure of the GR complex in intact cells is highly organized and stable toward dissociation in the absence of ligand. It has been demonstrated that *hsp*90 is associated with tubulin in cytosol and the distribution of *hsp*90 in intact cells is similar to the pattern observed for microtubules by indirect immunofluorescence (Sanchez et al., 1988). This distribution of *hsp*90 and microtubules is similar to the pattern observed for the GR in intact cells by indirect immunofluorescence (Wikstrom et al., 1987). Thus, the unoccupied GR in intact cells may be associated with elements of the cy-

toskeleton, and this organization might contribute to a stabile heteromeric complex.

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Plasminogen Activator Activities of Equimolar Complexes of Streptokinase with Variant Recombinant Plasminogens†

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ABSTRACT: The steady-state kinetic characteristics of the amidolytic and plasminogen activator activities of equimolar streptokinase (SK)–human plasminogen (HPg) and SK–human plasmin (HPm) complexes have been determined, exploiting the generation and use of cleavage site resistant mutants of HPg to stabilize plasminogen within the complex. Whereas amidolytic kinetic constants for equimolar complexes of SK with the following proteins, viz., plasma HPm, insect (i) cell-expressed wild-type (wt) recombinant (r) HPm, R^{561}E-irHPg, and Chinese hamster ovary cell (c)-expressed R^{561}S-crHPg, are similar, it has been found that the various SK–HPg complexes are far better enzymes than SK–HPm complexes for activation of bovine plasminogen, a species of plasminogen that is resistant to activation by SK, alone. In addition, it is emphasized that as a result of mutating the cleavage site in plasminogen, it is possible to express this protein in mammalian cells, and thus provide it for use in complex with SK as a more efficient plasminogen activator than plasma plasminogen, which is rapidly converted to HPm within the SK complex. This finding has important implications in the assessment of thrombolytic therapeutic reagent employing SK–plasminogen and SK–plasmin complexes.

Human plasminogen (HPg)[1] is a plasma-derived zymogen of the fibrinolytic and fibrinogenolytic enzyme plasmin (HPm) and exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Wiman, 1973, 1977; Sottrup-Jensen et al., 1978; Malinowski et al., 1984; Forsgren et al., 1987; McLean et al., 1987), with Glu at its amino terminus ([Glu^1]Pg).[1] Activation of HPg results from cleavage of the Arg^{561}-Val^{562} peptide bond in the zymogen, producing the two-chain, disulfide-linked serine protease [Lys^{78}]Pm, which also lacks the amino-terminal 77 amino acids as a result of autolysis by HPm formed during the activation (Violand & Castellino, 1976). This activation is catalyzed by a variety of proteins, which include streptokinase (SK), urokinase (UK), and tissue plasminogen activator (tPA) [for a review, see Castellino (1983)]. Whereas the latter two proteins are enzymes that directly catalyze cleavage of the appropriate peptide bond in HPg, providing HPm, SK has no such inherent activity, and its plasminogen activator relies on its ability to form complexes with HPg and HPm utilizing the actual or latent

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[1] Abbreviations: HPg, any form of variant of human plasminogen; HPm, any form or variant of human plasmin; [Glu^1]Pg, native human plasminogen with Glu, residue 1, at the amino terminus; [Lys^{78}]Pg, proteolytically derived form of human plasminogen with Lys, residue 78, at the amino terminus; [Lys^{78}]Pm, human plasmin, which arises from [Glu^1]Pg by cleavage at the activation site Arg^{561}-Val^{562}, and also at Lys^{77}-Lys^{78}; BPg, bovine plasminogen; SK, streptokinase; tPA, tissue plasminogen activator; UK, urokinase; EACA, ϵ-aminocaproic acid; r, recombinant; wt, wild type; i, insect cell expressed; c, CHO cell expressed; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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