Quantitation of the Interaction of the Immunosuppressant Deoxyspergualin and Analogs with Hsc70 and Hsp90

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ABSTRACT: Deoxyspergualin (DSG), a spermidinyl, α-hydroxyglycyl, 7-guanidinoheptanoyl peptidomimetic, shows immunosuppressive activity. In confirmation of a recent report that immobilized methoxyDSG selectively retains the heat shock protein Hsc70, we report here quantitative binding of DSG and analogs to both Hsc70 and the 90-kDa heat shock protein Hsp90. We have utilized affinity capillary electrophoresis to obtain K_d values for DSG and analogs, and stimulation of the ATPase activity of Hsc70 to obtain K_m values for DSG, that are comparable and corroborative. K_d values are 4 μM for DSG binding to Hsc70 and 5 μM for DSG binding to Hsp90. Two active analogs, methoxy- and glycylDSG, bind with similar affinities. Glyoxylylspermidine and des(aminopropyl)DSG, two inactive metabolites, have much reduced affinity for Hsc70 and Hsp90. These data validate binding of these novel immunosuppressant agents to these molecular chaperones, at concentrations in the range of pharmacologically active doses, and indicate that further characterization of Hsc70 and/or Hsp90 as potential targets for DSG is warranted.

15-Deoxyspergualin (DSG)[1] (Muindi et al., 1991; Umezawa et al., 1981), an analog of the bacterial metabolite spergualin, has potent immunosuppressive effects in a number of T-cell-dependent assays. DSG, structurally a mimic of a linear peptide, is composed of spermidine, α-hydroxyglycine, and 7-guanidinoheptanoic acid (Figure 1). It is very different structurally from both the cyclic undecapeptide cyclosporin A (CsA) and the cyclic macrolide FK506, which exhibit potent immunosuppressive activity by blockade of IL-2 production. While CsA and FK506 enter T-cells, bind to separate immunophilins (cyclophilin and FKBP-12), and then converge to inhibit the protein serine phosphatase activity of calcineurin (Schreiber, 1992), deoxyspergualin clearly acts at a distinct point in the T-cell activation pathway (Tepper, 1993; Nadler et al., 1992). Each of the immunophilins has catalytic peptidyl prolyl isomerase activity (PPIase) thought to be involved in protein folding. It is believed that CsA and FK506 bind to the active site of their respective PPIase and are presented as specific bound conformers to calcineurin (Liu et al., 1991).

Deoxyspergualin may exert its immunosuppressive activity by binding to a separate component of the cellular folding machinery, on the basis of the recent observation that an immobilized methoxyDSG derivative selectively retained a cytoplasmic, constitutive form of a 70-kDa heat shock protein, Hsc70 (Nadler et al., 1992). Hsp70's are molecular chaperones distributed ubiquitously and found in every cell compartment where protein folding, sorting, translocation, and refolding occurs (Rothman, 1989). To characterize the interaction of DSG with Hsc70, we have now quantitated the strength and specificity of the interaction of DSG and several spermidine-containing analogs with pure Hsc70 both by affinity capillary electrophoresis, to obtain K_d values directly, and by stimulation of the ATPase activity of Hsp70's, to obtain K_m values as positive effectors. A second major class of cytosolic molecular chaperones is the 90-kDa class, Hsp90, most notably involved, with Hsc70, in complexation with steroid receptors for estrogens, androgens, progesterones, glucocorticoids, and the toxin dioxin (Pratt, 1990). We show here by affinity electrophoresis that pure human Hsp90 and Hsc70 have equivalent affinities for DSG (K_d = 5 and 4 μM, respectively), which are therapeutically relevant concentrations (Muindi, 1991). We have also analyzed some DSG analogs and metabolites for Hsp binding. Among the DSG analogs tested, there appears to be a correlation between binding to heat shock proteins and immunosuppressive activity.

MATERIALS AND METHODS

Materials. DSG and all DSG analogs were provided by Bristol Myers Squibb. Trypanothione was purchased from the Bachem Company. N^1-Glutathionylspermidine was a generous gift of Dr. Alan Fairlamb of the London School of Hygiene and Tropical Medicine. All references to bovine, mouse, or cytosolic *Trypanosoma cruzi* Hsp70 refer to the constitutive form of this family, Hsc70. Human Hsp90 was purchased from StressGen (Vancouver, BC, Canada). Bovine Hsc70 was purified and donated by the laboratories of Bristol Myers Squibb. *T. cruzi* Hsc70 and *T. cruzi* Mtp70 were provided in a GST fusion overexpression system from the laboratories of Dr. David Engman of Northwestern University Medical School. Murine Hsc70 was donated by Dr. Stuart Calderwood of the Dana Farber Cancer Institute. The peptides KFERQ and HWDFAWPW were generous gifts of the laboratories of Dr. Alfred Goldberg of Harvard Medical School and of Dr. Sylvia Blond-Elguindi of the laboratories of Dr. Mary Jane Gething of the University of Texas Southwestern Medical School, respectively. CsA was given to the Walsh laboratories by Sandoz Company. Dr. George

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[1] Abbreviations: Hsp90, heat shock protein of the 90-kDa class; ACE, affinity capillary electrophoresis; Hsp70, heat shock protein of the 70-kDa class; FITC, fluoroisothiocyanate; SR, steroid receptor; ER, endoplasmic reticulum; BiP, ER form of Hsc70; SDS, sodium dodecyl sulfate; SRp, steroid receptor peptide of 33-aa length corresponding to residues 631–622 of murine glucocorticoid receptor; Hsp59, heat shock protein of the 59-kDa class; Hsc70, Hsc70 class protein, constitutively expressed; DSG, deoxyspergualin; DAS, des(aminopropyl); Mtp70, mitochondrial heat shock protein of the 70-kDa class.

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DSG and ANALOGS:

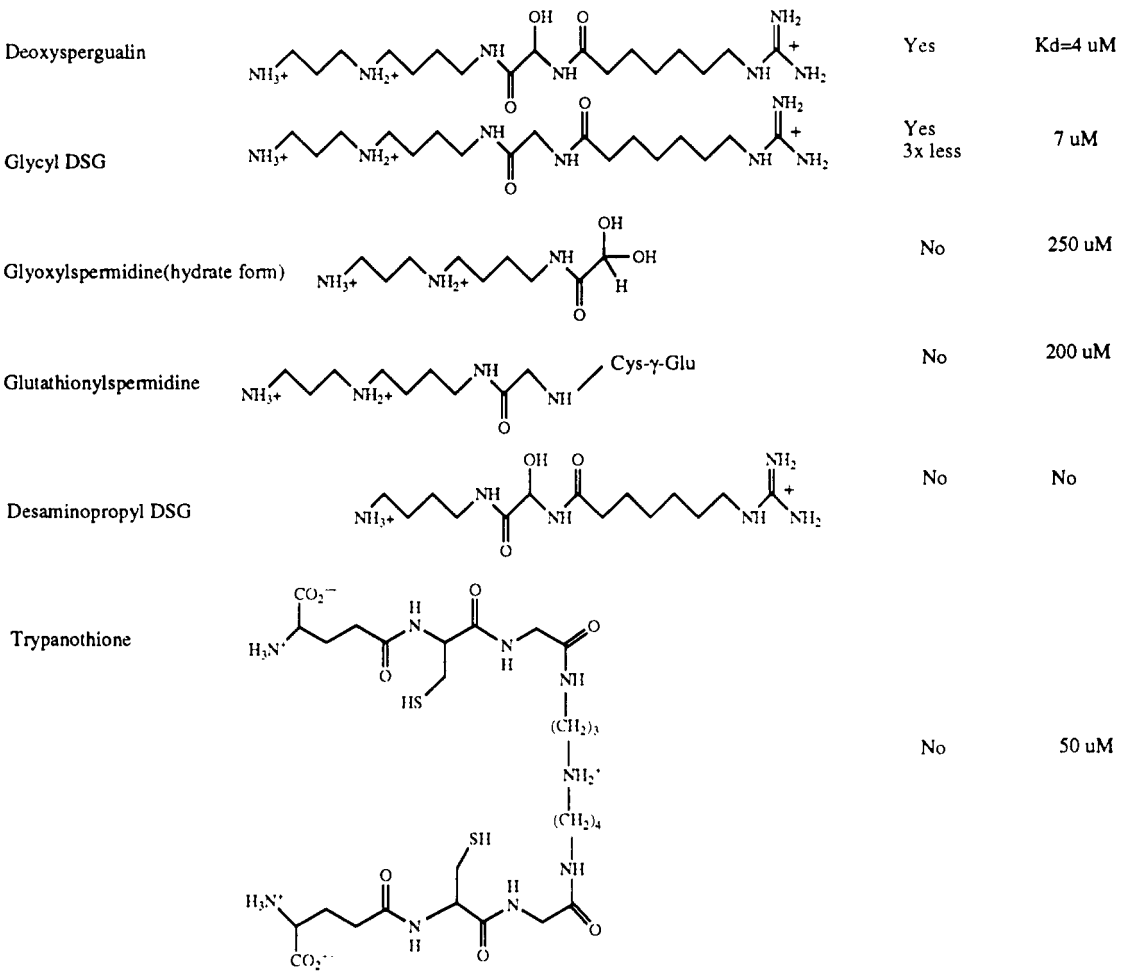

FIGURE 1: DSG and its structural analogs with a comparison of binding to Hsc70 and immunosuppression activity. K_d was determined by capillary zone electrophoresis (see Materials and Methods), and immunosuppression activity was assayed according to Tepper et al. (1991).

Whitesides and his laboratory members in the Harvard University Chemistry Department allowed the use of the ISCO Model 3140 capillary zone electrophoresis unit. *Trypanosoma cruzi* Hsc70 vs Mtp70 sequence analysis was performed by Dr. Steven Nadler using the MacVector sequence analysis package. All other reagents and chemicals were of the highest grade commercially available.

Enzyme Assay for ATPase Activity. To assay the hydrolysis of ATP by the heat shock proteins, release of inorganic phosphate was determined using a malachite green assay (Geladopoulos et al., 1991). Enzyme (Hsc70) was diluted with buffer (50 mM Hepes and 2 mM Mg^{2+}, pH 7.2), and ATP was added to 2 mM concentration. Typically the total reaction volume was 100 μL after addition of 10 μL of enzyme and the reaction mixture was incubated at 25 °C for 10–20 min. The assay was quenched with 800 μL of malachite green reagent and 100 μL of sodium citrate, and after 10 min at 25 °C the absorbance at 630 nm was determined. Concentrations of drug, peptide, or protein used to test enhancement of Hsc70 ATPase activity varied from 2 to 1000 μM.

Immunosuppression Studies (in Vivo). To determine whether DSG or its analogs were active with respect to immunosuppressive ability, in vivo delayed type hypersensitivity assays were performed according to Meade (1992), and KLH (keyhole limpet hemocyanin) antibody models were constructed according to Tepper et al. (1991).

Determination of Molecular Weights and Protein/Peptide Concentration. The molecular weights for all proteins analyzed were determined by SDS–PAGE with standards of myosin heavy chain (200-kDa), phosphorylase B (97-kDa), bovine serum albumin (68-kDa), ovalbumin (43-kDa), and carbonic anhydrase (29-kDa). All protein and peptide concentrations were determined by the Bradford method according to Bio-Rad.

Kinetic Analysis. Michaelis constants (K_m) and maximal initial velocities (V_{max}) for ATP and peptides were determined by the malachite green assay. Initial velocity measurements were made at five substrate concentrations. Kinetic constants were calculated from Lineweaver-Burk plots.

Capillary Zone Electrophoresis. An ISCO Model 3140 automated capillary zone electrophoresis unit was used with an uncoated fused silica capillary from Polymicrotechnologies Inc. The capillary was 70 cm long and 50 μm in diameter, and the OD at 200 nm was measured at 45 cm down the length of the capillary with a sensitivity of 0.005 absorbance units. All runs were performed at 25 °C and 30 kV, using Tris (0.03 M)/glycine (0.2 M) buffer at pH 8.3. Eight nanoliters of sample (the receptor) was injected for each run by vacuum; concentrations were 0.6 μM (human Hsp90 and mouse or bovine Hsc70). All samples which contained binary complexes

Table 1: Summary of Binding Data Obtained from Capillary Zone Electrophoresis and from ATPase Studies

Hsp	substrate	K_d (μM)	K_m (μM)	x-fold stimulation of ATPase
Hsc70 (mouse)	deoxyspergualin	4.0	3.0	2.0
Hsc70 (mouse)	glutathionylspermidine	200.0	150.0	1.4
Hsc70 (mouse)	trypanothione	50.0	60.0	1.6
Hsc70 (bovine)	glycylDSG	7.0	12.0	1.8
	glyoxylylspermidine	250.0	900.0	1.2
	DAP-DSG	0	0	0
	methoxyDSG	5.0	4.0	2.0
Hsc70 (*T. cruzi*)	deoxyspergualin	0.4	0.6	1.4
	glutathionylspermidine	100.0	95.0	1.2
	trypanothione	30.0	40.0	1.4
Mtp70 (*T. cruzi*)	deoxyspergualin	0	0	0
	glutathionylspermidine	0	0	0
	trypanothione	0	0	0
DnaK (*E. coli*)	deoxyspergualin	0	0	0
Hsp90 (human)	deoxyspergualin	5.0		
	glutathionylspermidine	230.0		
	trypanothione	100.0		
	glycylDSG	20.0		
	glyoxylylspermidine	130.0		
	spermidine	970.0		
	DAP-DSG	0		
	methoxyDSG	10.0		

(Hsc70–DSG, Hsp90–DSG) consisted of bovine Hsc70 (0.6 μM), DSG (10 μM), and human Hsp90 (0.6 μM) and were incubated for 5 min in the Tris/glycine buffer at 25 °C before they were run in a Tris/glycine-buffer-equilibrated capillary. When receptor was injected into buffer containing ligand at different concentrations, time shifts were measured and Scatchard plot analyses were obtained (Avila et al., 1992). Each run was carried out at room temperature and repeated five times to ensure that the time shift for a particular concentration of ligand and receptor was reproducible. Mesityl oxide was used as the neutral marker to allow calibration of the mobilities of Hsp's and their complexes. The mesityl oxide peak varied in intensity because different concentrations were used for each run, but the concentration of Hsp's was constant. Scatchard analysis was used on electropherograms (run under the same conditions) that are not shown. Peak shape variabilities from run to run occurred in the Hsp90–DSG electropherogram.

Elution of Hsc70 from a MethoxyDSG Affinity Column with Synthetic Peptide. A methoxyDSG affinity column was run using Jurkat cell lysates as previously described (Nadler et al., 1992). After the affinity column was washed, the protein was eluted first with 5 mM of a synthetic peptide with the sequence KRQIYTDLEMNRLGK followed by a combination of 5 mM DSG and 5 mM ATP. Five 3-mL fractions were collected for each eluent and then analyzed on a 12.5% SDS–polyacrylamide gel.

RESULTS

Quantitation of Binding of Deoxyspergualin and Analogs to Hsc70 and Hsp90. Given the recent finding that the heat shock protein Hsc70 was selectively retained by an immobilized methoxyDSG column (Nadler et al., 1992), we have determined the K_d for interaction of DSG with Hsc70 in solution. To this end, we have used either purified mouse Hsc70 or bovine Hsc70 and the technique of affinity capillary electrophoresis to assess binding of DSG, DSG analogs, and DSG metabolic fragments (see Table 1 and Figure 1 for summary). Briefly, the mobility of Hsc70 in an electric field is altered upon binding of a ligand to form a complex with different

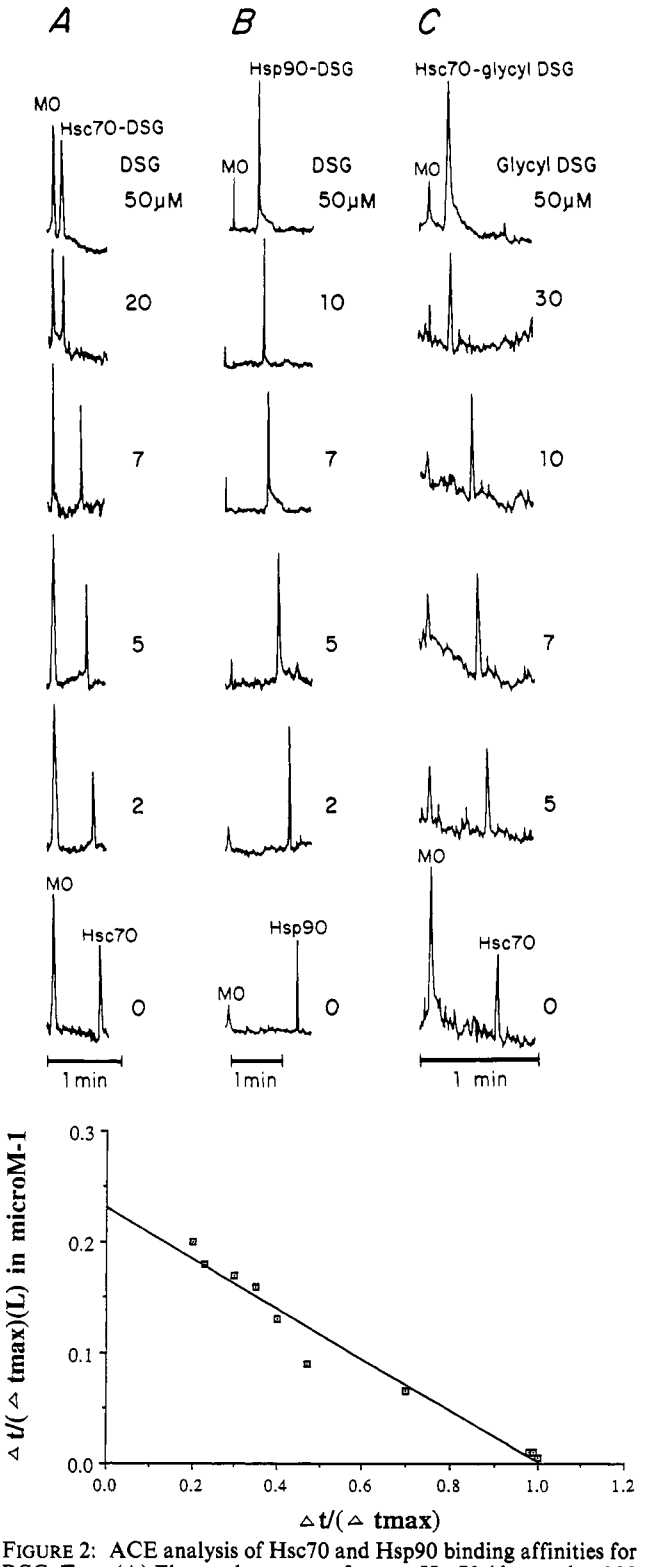

FIGURE 2: ACE analysis of Hsc70 and Hsp90 binding affinities for DSG. Top: (A) Electropherogram of mouse Hsc70 (detected at 200 nm) as increasing concentrations of the positively charged DSG were incubated in the capillary (from 0 to 50 μM DSG). The relative mobility shifts compared to a neutral marker, mesityl oxide, allow one to calibrate mobilities of Hsp's and their complexes. (B) Electropherogram of human Hsp90 as various concentrations of DSG (0–50 μM) were perfused throughout the capillary. (C) Same as (A), except glycylDSG was used. Bottom: Scatchard analysis of the data in (A) above.

mass and charge from those of free, unbound Hsc70 (Avila et al., 1993). In the experiment demonstrated in Figure 2A (top), mouse Hsc70 at 0.6 μM was injected into a capillary column equilibrated with different fixed levels of DSG from

0 to 50 μM, spanning the K_d. As the ligand (DSG) concentration is raised from 0 to subsaturating levels, there is an induced mobility shift relative to the unchanged migration of an uncharged standard, mesityl oxide. Maximal mobility shift occurs at saturation, 50 μM DSG. The K_d for the DSG–Hsc70 complex is obtained from Scatchard analysis by plotting $\Delta t/(\Delta t_{max})$ (L) (ligand concentration) vs Δt (the change in the mobility of the peak)/Δt_{max} to obtain a slope of –Kb (binding constant) which is the reciprocal of K_d (dissociation constant). By obtaining linear fits of the data, the Scatchard analysis reflects a monovalent ligand binding site. Since the x-intercept is 1, DSG and Hsc70 are in a 1:1 stoichiometry (Figure 2, bottom). DSG has a K_d value of 4 μM for binding to Hsc70 (data not shown). Similar results were obtained for glycylDSG, as seen in Figure 2C (top). We have also analyzed the affinity of pure human Hsp90 for DSG as a ligand for comparison with Hsc70. DSG clearly binds to human Hsp90 (Figure 2B, top) and by Scatchard analysis yields a K_d of 5 μM, indicating very similar affinities of Hsp90 and Hsc70 for this immunosuppressive agent.

To probe specificity of Hsc70 and Hsp90 for the specific structural components of DSG, we tested the affinity by affinity capillary electrophoresis (ACE) of five analogs and/or fragments of DSG (Figure 1) as well as spermidine. Removal of the hydroxy group from the α-hydroxyglycyl unit gives glycylDSG. As noted in Table 1, glycylDSG has 3-fold higher affinity for Hsc70 (K_d = 7 μM) than for Hsp90 (K_d = 20 μM) but is similar to DSG in its ability to bind to Hsc70 (K_d = 4 μM). Glyoxylylspermidine and des(aminopropyl)DSG (DAP-DSG), which represent human metabolic degradation fragments (Muindi et al., 1991) of DSG, had much reduced affinities for Hsc70 (the K_d of glyoxylylspermidine for Hsc70 is 250 μM, and the K_d between DAP-DSG and Hsc70 was undetectable by affinity capillary electrophoresis methods at concentrations up to 1 mM) (Table 1).

To analyze the selectivity of DSG for other 70-kDa heat shock family members, we assessed by ACE the ability of DSG to form a complex with Hsp70's from the causative agent of Chagas disease, the protozoan parasite *Trypanosoma cruzi*, and with the Hsc70 prokaryotic representative, DnaK. *Trypanosoma cruzi* has both cytosolic and mitochondrial forms of Hsc70 (Markell et al., 1981). DSG has a high affinity for the *T. cruzi* cytoplasmic Hsc70 (K_d = 0.4 μM) and no detectable affinity for mitochondrial Hsp70 from this parasite; nor does DnaK bind DSG detectably (Table 1). Mtp70 and DnaK were determined to be active by their ability to bind and hydrolyze ATP and by their affinity for peptides from hemaglutinin and rhodanese (data not shown).

Effects of DSG and Analogs on ATPase Activities of Hsc70. Hsc70 (k_{cat} = 0.04–1.0 min^{-1}) family members have catalytic ATPase activity that can be stimulated by peptide or protein ligands (Rothman, 1989; Nadeau et al., 1992). It was reported that ATP induced release of Hsc70 from the methoxyDSG affinity column, resulting in elution of more Hsc70 than with the equivalent amount of DSG. In an in vitro assay, addition of DSG results in a 1.6-fold stimulation of bovine Hsc70 ATPase (Figure 3) (also detected with mouse Hsc70; data not shown), allowing a K_m determination of DSG as effector of 3 μM. This K_m correlates well with the K_d from the ACE experiments and provides independent validation of the binding constant. The DSG analogs were also assayed for their effect on ATPase activity as noted in Table 1. GlycylDSG, spermidine, glyoxylylspermidine, and des(aminopropyl)DSG all had increased K_m's for Hsc70 when compared to DSG. DSG had no effect on DnaK and *T. cruzi* mitochondrial Hsp70

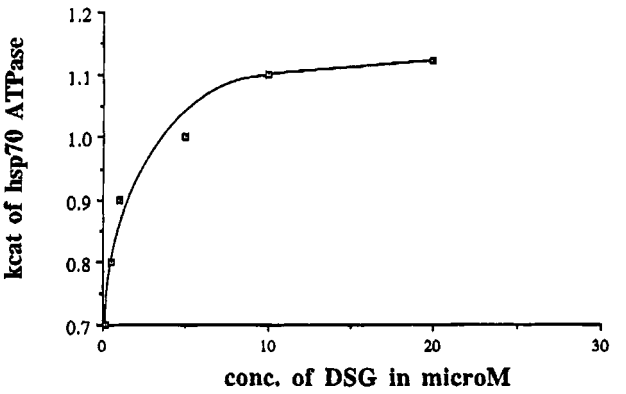

FIGURE 3: ATPase analysis of bovine Hsc70 with DSG as effector. Increasing concentrations from 0 to 20 μM DSG were incubated with Hsc70 and tested using the malachite green assay (see Materials and Methods) for DSG's ability to enhance Hsc70 ATPase (baseline k_{cat} of 0.7 min^{-1}).

ATPase activity, but caused a small but measurable 1.4-fold stimulation of the cytosolic *T. cruzi* Hsc70 ATPase activity, yielding a K_m of 0.6 μM. Although we have found that glutathionylspermidine and trypanothione are not immunosuppressive (data not shown), since these compounds have some structural similarity to DSG (Markell et al., 1981), and given that DSG affects *T. cruzi* Hsc70, we assessed the recognition of glutathionylspermidine and trypanothione by Hsc70 and Hsp90 by ACE assays. As seen in Table 1, these compounds bind more weakly than DSG to Hsc70 and Hsp90.

It was also observed that increased amounts of DSG (greater than 200 μM) decrease ATPase activity of Hsc70 to baseline (k_{cat} = 0.7 min^{-1}). At these high concentrations of DSG used in capillary zone electrophoresis, binding to Hsc70 and Hsp90 was still detected (data not shown); therefore, the decrease in ATPase activity observed with higher concentrations of DSG may be due to allosteric effects or a second binding site.

Experiments to assess effector roles for DSG and analogs in ATPase activity of human Hsp90 (Nadeau et al., 1993) were inconclusive due to variable levels of ATPase, which ranged from undetectable to high levels in different preparations of the Hsp90. At present we are concerned that ATPase activity variably associated with higher eukaryotic Hsp90's could be due to small, variable amounts of contaminant enzymes.

Effect of CsA on DSG Binding and ATPase Activity of Hsc70. Given the recent findings of Moss et al. (1992) that fluorescently tagged CsA derivatives bound to Hsc70 and actin, we performed ATPase assays and ACE studies of bovine Hsc70 with CsA. No shift was detected by ACE in efforts to obtain K_d measurements for CsA binding to Hsc70 (data not shown). CsA at up to 1 mM concentrations also did not affect the ATPase activity of Hsc70. When CsA at 10 μM and DSG at 10 μM were incubated together with Hsc70, a 2-fold increase in ATPase activity comparable to that of DSG alone was still detected (data not shown). From ACE studies, CsA (as a competitor ligand) did not affect the shift in Hsc70 caused by DSG in experiments where both CsA and DSG were present at 10 μM concentrations throughout the capillary when Hsc70 was injected (data not shown).

Effect of DSG on Peptide and Protein Interactions. In order to determine the effects of DSG on protein–protein interactions, we injected the preformed binary complex of Hsp90 (0.6 μM) and Hsc70 (0.6 μM) into a capillary perfused with 30 μM DSG and electrophoresed the complex for 6 min at 30 kV. Under these conditions an Hsp90–Hsc70–DSG complex was obtained (trace 2, Figure 4). By comparison

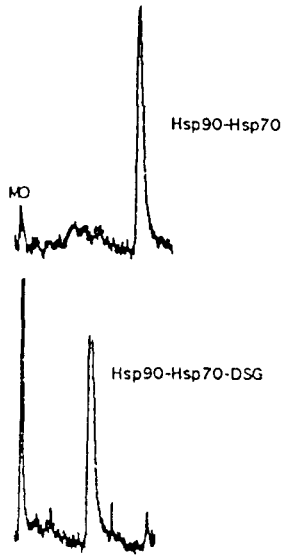

FIGURE 4: Capillary zone electrophoresis electropherogams of Hsp90 (0.6 μM) and Hsc70 (0.6 μM) injected together into a capillary perfused with Tris-glycine buffer at pH 8.3 (trace 1, top) and of Hsp90 (0.6 μM) and Hsc70 (0.6 μM) injected together into a capillary perfused with DSG at 50 μM in Tris-glycine buffer (trace 2, bottom).

with the mobility of the DSG–Hsc70 binary complex (Figure 2A), the DSG–Hsp90 complex (Figure 2B), and the Hsp90–Hsc70 complex (trace 1, Figure 4), it is clear that DSG elicits a shift of the preformed Hsc70–Hsp90 complex to a new mobility position. The K_d value for the DSG-induced shift by Scatchard analysis was 5 μM, similar to the values of DSG binding to either Hsp alone. We tentatively conclude that DSG can bind to Hsc70 at a site distinct from Hsp90's site and/or to Hsp90 at a site distinct from Hsc70's binding site, and the unique mobility profile of trace 2 of Figure 4 suggests a ternary DSG–Hsc70–Hsp90 complex. Although we cannot rule out a DSG–Hsc70–Hsp90–DSG complex, it is unlikely since the mobility shift corresponds to that predicted for a ternary complex. These initial findings show the power of ACE to detect higher order complexes and raise the issue of the extent to which DSG, peptides, and proteins bind to distinct binding sites of Hsc70 or Hsp90 molecular chaperones.

In an assessment of the competition of a peptide ligand with DSG, the octapeptide HWDFAWPW, known to bind Hsp78 (BiP) with a K_m of 17 μM (Blond-Elguindi et al., 1993), caused a shift of the Hsc70–DSG complex but not of the Hsp90–DSG complex (Figure 5). This displacement could be caused either by peptides competing with the DSG binding site directly or by allosteric changes upon binding of the peptides to cause dissociation of the heat shock protein–DSG complex. Finally, another way to evaluate peptide binding was assessed by using the peptide KRQIYTDLEMNRLGK to elute Hsc70 from a methoxyDSG column (Figure 6). This peptide has previously been found by fluorescence binding studies to bind to Hsc70 with a K_d value of 770 μM (Rothman, 1989), but it was not able to elute Hsc70 from the methoxyDSG column under the following condition: Jurkat T-cell lysates were incubated with a methoxyDSG column as described in Nadler et al. (1992). After the column was washed extensively, 5 mM of the above 15-mer peptide failed to elute the bound Hsc70. Only 5 mM DSG and/or 5 mM ATP was successful in dissociating Hsc70 from the methoxyDSG resin. These results would suggest that this peptide binds at a site on Hsc70 different from the site for DSG.

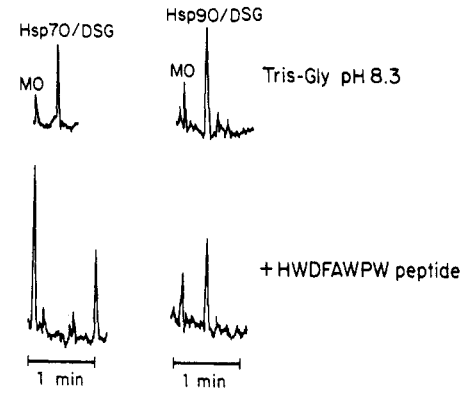

FIGURE 5: Displacement of DSG–Hsp90 or DSG–Hsp70 complexes by HWDFAWRW peptide. DSG (at 10 μM) was co-injected with Hsp70 or Hsp90 (0.6 μM), after a 5-min incubation, and the migration of the complex was monitored at 200-nm wavelength. When the HWDFAWRW peptide was perfused throughout the capillary (30 μM), only the Hsp70–DSG complex was affected as indicated by the mobility change of the Hsp70 complex.

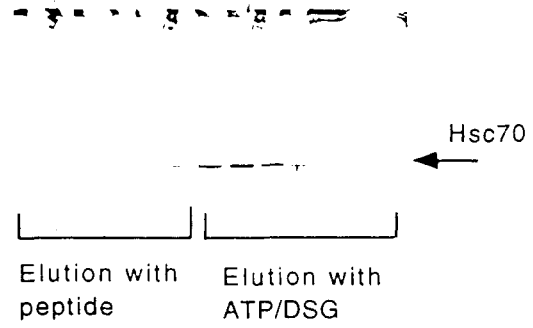

FIGURE 6: Elution of Hsc70 from a methoxyDSG affinity column. A methoxyDSG affinity column was run as described in Materials and Methods. Bound protein was eluted first with 5 mM synthetic peptide KRQIYTDLEMNRLGK, followed by 5 mM DSG plus 5 mM ATP. The gel was silver stained to detect Hsc70.

DISCUSSION

Deoxyspergualin shows promise as a clinically useful immunosuppressive drug in human transplant medicine, but to date its mechanism has been unclear. It acts on intracellular signaling events distinct from CsA and FK506, which bind to their respective immunophilins, CypA and FKBP-12, to convergently inhibit the protein serine phosphatase activity of calcineurin in T-cells and thereby block movement of the transcription factor NF-AT to the nucleus and its subsequent activation of early cytokine genes (Schreiber, 1992). Although the specific cell type that DSG acts upon is still unknown, preliminary evidence suggests that B-cell (Sterbenz & Tepper, 1993) and monocyte function (P. H. Hoeger, M. A. Tepper, and R. S. Geha, personal communication) may be affected by DSG. The recent demonstration that an immobilized methoxy derivative of DSG selectively retained Hsc70 from Jurkat T-cell extracts has impelled us to quantitatively determine the strength and specificity of the interaction both with respect to the DSG structure and with regard to various heat shock proteins.

K_d values for ligand binding to Hsc70 and Hsp90 were directly obtained by analysis of the mobility shifts of the heat shock proteins in capillary zone electrophoresis on complexation with DSG ligands. The running buffer contains different fixed concentrations of ligand so that it is dispersed throughout the capillary; hence, the migrating protein encounters an equivalent ligand concentration at each point. Fractional saturation of the protein with ligand causes a shift in the mobility of the protein due to changes in mass and/or charge.

Only one discrete peak is seen since the on/off rates of ligand and protein are faster than their migration through the column. The increments in mobility shift are related to fractional saturation, allowing for Scatchard analysis which yields K_d values. Affinity capillary electrophoresis was rapid, required only small amounts of ligands and receptors in unlabeled form, and was of general applicability for peptide–protein and protein–protein interactions (Avila et al., 1992). The K_d of 4 μM for DSG binding to mouse Hsc70 determined by ACE indicates an affinity consistant with its selective retention on the methoxyDSG column as previously reported (Nadler et al., 1992). While Hsc70 has a peptide-stimulated ATPase activity of 2–5-fold (Rothman, 1989), DSG stimulated ATPase was only 2-fold relative to unstimulated activity. However, this signal was sufficient to determine the K_m values for DSG activation of Hsc70 ATPase. The K_m value of 3 μM for DSG activation of Hsc70 ATPase activity is an independent validation of the affinity of Hsc70 for DSG and confirms the ACE data.

Evaluation of the binding of DSG to two Hsp70's from the parasitic protozoan *T. cruzi*, the causative agent of the fatal Chagas disease that afflicts 35 million people in Latin America, revealed at least a 5-fold higher affinity for the cytosolic *T. cruzi* Hsc70 than for the mammalian Hsc70, with a K_m of 0.6 μM, but no detectable affinity (at least 1000 times lower) for the mitochondrial Hsp70. Because *T. cruzi* and other trypanosomatids have high levels of spermidinyl glutathiones [monoglutathionyl and bis(glutathionyl) derivatives, the latter known as trypanothione] and these have structural similarity to DSG, we tested these as ligands for Hsc70's (Table 1 and Figure 1). In general they were 10–100-fold less potent than DSG for binding but were consistent in their inability to bind *T. cruzi* mitochondrial Hsc70. Since the mitochondrial Hsp70 has the highest homology to the prokaryotic Hsp70's (Lindquist & Craig, 1988) of which *Escherichia coli* DnaK is the best characterized, we tested DnaK for DSG binding. Pure DnaK also showed no mobility shift in ACE and no stimulation of ATPase activity by DSG. Homology analysis of the *T. cruzi* cytosolic and *T. cruzi* mitochondrial Hsp70's show that these Hsp70's differ substantially in sequence between residues 250 and 320 as well as in the approximately 100 C-terminal amino acids (S. Nadler, unpublished results), suggesting that this region may contain some of the binding determinants for DSG.

In cells, cytoplasmic Hsc70 is thought to bind many proteins transiently, either to assist nascent proteins to fold or to chaperone proteins in a loosely folded state to maintain them in a translocation-competent mode for transport into mitochondria, the endoplasmic reticulum, or the nucleus (Rothman, 1989; Gething & Sambrook, 1992). One of the better characterized partner proteins for Hsc70 is Hsp90 as part of a multiprotein complex (Hsp90–Hsp59–Hsc70) involved in binding to steroid receptors, for example, in cytoplasmic anchoring of the glucocorticoid receptors. Recent studies suggest that Hsp90–Hsp59–Hsc70 may be found in other complexes as well (Tai et al., 1993). As a preliminary evaluation of whether DSG might compete with Hsp90 for binding to Hsc70, we assessed separately the affinity of DSG for pure human Hsp90. By ACE, a K_d value of 5 μM establishes that Hsp90 has an affinity almost equivalent to that of Hsc70 for the immunosuppressant DSG. The question arises whether these low micromolar affinities of DSG for Hsc70 and Hsp90 are within the pharmacologically relevant range. It is estimated that in therapeutic doses serum concentrations of DSG reach 10–20 μM (Tepper, 1993), and DSG is transported into human lymphocytes to reach concentrations of approximately 500 μM (Cleaveland & Nadler, 1993). Hsp90 is a particularly abundant cytosolic protein, and its concentration may approach 2–10 μM, while that of Hsc70 can reach 5 μM (Hartl & Martin, 1992). Given K_d's of 4-5 μM, the DSG–Hsp complexes would be highly populated and DSG could compete effectively for other protein and peptide binding to Hsc70 and Hsp90 and thereby affect protein trafficking.

At this point it is unclear whether DSG binds to the same site as peptides on Hsc70 and Hsp90. On the basis of our studies, some peptides can compete for DSG binding to the heat shock proteins, whereas others cannot. This suggests that DSG may be mimicking the structure of certain peptides or that there are two binding sites on Hsc70. To support this latter hypothesis, we have seen that although low concentrations of DSG stimulate Hsc70 ATPase activity, high concentrations decrease the activity to basal levels (data not shown), suggesting that there may be a second allosteric site on Hsc70.

The results presented in this paper clearly document and quantitate the binding of DSG with both Hsc70 and Hsp90 proteins and show the utility of capillary electrophoresis for peptide and ligand interactions with proteins. It is unclear why our initial affinity chromatography studies (Nadler et al., 1992) did not show Hsp90 binding. One possibility may be that Hsp90 binds to the site on DSG which was attached to the affinity matrix. The DSG and DSG-like compounds in this study were also analyzed for in vivo immunosuppressive activity in mouse models of delayed-type hypersensitivity (DTH) and antibody production (Tepper et al., 1991). The results of this analysis showed comparable immunosuppressive activity for DSG and glycylDSG, and no activity for des-(aminopropyl)DSG or glyoxylylspermidine, possibly suggesting a correlation between immunosuppression and Hsp binding. However, it remains to be seen whether this correlation, from the limited number of structural analogs, will have any predictive value to link immunosuppressive activity with Hsp binding affinity to various isoforms of Hsc70 or Hsp90, either free or in various complexes with receptors.

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