occur by this mechanism. Moreover, a strand realignment step occurring during SSA can lead to the formation of apparent gene conversion products, with two copies of the repeated sequences, unassociated with crossing-over.

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Interaction of the Immunosuppressant Deoxyspergualin with a Member of the Hsp70 Family of Heat Shock Proteins

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Deoxyspergualin (DSG) is a potent immunosuppressant whose mechanism of action remains unknown. To elucidate its mechanism of action, an intracellular DSG binding protein was identified. DSG has now been shown to bind specifically to Hsc70, the constitutive or cognate member of the heat shock protein 70 (Hsp70) protein family. The members of the Hsp70 family of heat shock proteins are important for many cellular processes, including immune responses, and this finding suggests that heat shock proteins may represent a class of immunosuppressant binding proteins, or immunophilins, distinct from the previously identified *cis-trans* proline isomerases. DSG may provide a tool for understanding the function of heat shock proteins in immunological processes.

Deoxyspergualin (DSG) is a synthetic analog of spergualin, a natural product isolated from *Bacillus laterosporus* that possesses potent immunosuppressive activity (*1*–*6*). In many models of T cell–dependent immune responses, such as antibody production, delayed-type hypersensitivity, and allograft rejection, DSG exerts potent immunosuppressive effects (*2*, *3*, *7*). The mechanism of action of DSG is believed to be different from that of the immunosuppressants cyclosporin A and FK506. Unlike cyclosporin A and FK506, DSG does not alter the amount of interleukin-2 (IL-2) produced in response to T cell activation (*2*, *5*), and the time course of inhibition of the mixed lymphocyte response for DSG differs from that for cyclosporin A (*5*, *7*, *8*). Furthermore, in contrast to cyclosporin A, the effect of DSG on the generation of secondary cytotoxic T lymphocytes cannot be reversed with exogenous IL-2 (*5*). In an attempt to elucidate the mechanism of action of DSG, we have isolated a 70-kD intracellular DSG binding protein and identified this protein as a member of the Hsp70 family of heat shock proteins. This finding provides evidence for a class of immunophilins distinct from the previously identified *cis-trans* proline isomerases (*9*–*13*) that bind cyclosporin A and FK506.

Jurkat cell (human T cell) lysates were subjected to chromatography on a methoxy DSG-Sepharose column (Fig. 1). Methoxy DSG (Me-DSG) was used because it is more stable to hydrolysis than DSG but still possesses similar immunosuppressive activity (*7*, *14*). After application of the Jurkat cell lysate, the column was washed with a low- to high-salt gradient. On the subsequent application of 5 mM DSG, one major protein with an apparent molecular mass of 70 kD was specifically eluted. Only a small amount of the 70-kD protein was eluted from the column by the polyamines putrescine and spermidine, which have limited structural similarity to DSG and are not immunosuppressive (Fig. 2). Furthermore, the 70-kD protein did not bind to resin that did not contain DSG and was retained on the affinity column in the presence of 2 M NaCl or 0.5% Nonidet P-40, which suggests that the protein and affinity matrix interact with high affinity. In addition to Jurkat cells, we have identified the 70-kD protein in THP-1 cells (human monocytic), calf spleen cells, and thymus cells, as well as nonhematopoietic HeLa cells.

To identify the 70-kD protein, we subjected the affinity-purified protein to trypsin digestion, and the resulting peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) (*15*). Several fractions from the HPLC-purified tryptic digest were chosen for amino acid sequencing. Although some fractions contained more than one peptide, we could unambiguously identify the sequences of six peptides (*16*). Each of the sequences of the DSG binding protein matched identically the sequence of the human constitutive heat shock cognate 70 (Hsc70) heat shock protein (*15*, *16*). Although there is an 81% sequence identity between the inducible Hsp70 heat shock protein and Hsc70, there are six amino acid differences between the peptide sequences of the DSG binding protein and inducible Hsp70 (*16*). These data identify the DSG binding protein as the constitutive or cognate member of the Hsp70 heat shock protein family.

To confirm the sequencing results, we also determined whether monoclonal antibodies to Hsp70 recognized the affinity-purified 70-kD protein (Fig. 3). Three different monoclonal antibodies, 7.10, 3a3, and N27F3-4, that recognize different epitopes found on both the constitutive and inducible Hsp70 proteins (*17*) each bound

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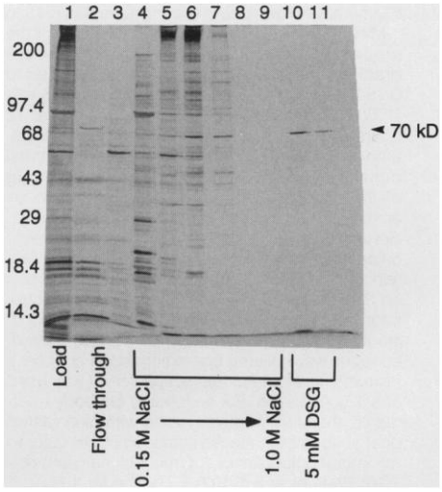

Fig. 1. SDS–12.5% polyacrylamide gel of protein fractions eluted from the Me-DSG affinity column (*36*). Lane 1, Jurkat cell lysate; lane 2, flow-through fraction; lanes 3 to 9, NaCl gradient (0.15 to 1 M) fractions; lanes 10 and 11, 5 mM DSG eluate fractions. Aliquots (20 μl) of every third 7-ml fraction were loaded on the gel. The gel was silver stained with the Gelcode system (Pierce). The position of molecular mass standards (in kilodaltons) and the 70-kD DSG binding protein are indicated on the left and right, respectively.

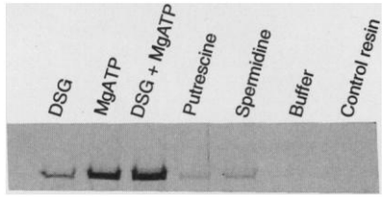

Fig. 2. Elution of Hsc70 from the Me-DSG affinity resin by 5 mM DSG, 5 mM MgATP, 5 mM MgATP plus 5 mM DSG, 5 mM putrescine, 5 mM spermidine, or buffer alone. Control resin represents activated CH-Sepharose not coupled to DSG, with 5 mM DSG used as the eluent. Only the 70-kD region of the gel is shown; no other proteins were eluted (*36*).

to the DSG binding protein on immunoblots. A third antibody, which recognizes only the inducible Hsp72 protein and not Hsc70, did not interact with the DSG binding protein (*18*). These results suggest that the DSG binding protein is Hsc70. However, because the inducible Hsp70 is present in cells in much smaller amounts than Hsc70, we cannot rule out the possibility that this form may also bind to the Me-DSG affinity resin.

One of the known functions of heat shock proteins is to bind a wide array of peptides and proteins (*19–22*), the release of which requires the binding and hydrolysis of adenosine triphosphate (ATP) by the heat shock protein (*21, 22*). ATP appeared to elute Hsc70 more effectively from the Me-DSG affinity column than DSG, and DSG and ATP combined eluted more Hsc70 than either compound alone (Fig. 2). These data suggest that DSG may bind to the heat shock protein in a manner similar to that of peptides or that an ATP-induced conformational change of Hsc70 is sufficient to release the protein from the affinity resin. Hence, this result provides additional functional evidence that the DSG binding protein is a heat shock protein. Providing further evidence that DSG binds Hsc70 in solution, prior incubation of bovine Hsc70 with 10 mM DSG (a concentration similar to that of Me-DSG on the Sepharose resin) inhibited the binding of the protein to the affinity resin (*23*).

Our data show that the Hsc70 constitutive heat shock protein binds the immunosuppressant DSG. Heat shock proteins appear to have several functions (*22, 24–26*) that, in addition to the binding and ATP-dependent release of a wide variety of peptides and proteins (*20, 21, 27*), include the facilitation of the folding and unfolding of proteins (*20, 24*) and the shuttling of proteins and peptides between various cellular organelles (*19, 24, 25*). Our data and other studies also suggest that Hsc70 in particular plays a role in immune responses (*28, 29*). Immunoregulatory functions in which heat shock proteins appear to participate include the binding and stabilization of immunoglobulin heavy chains before the binding of light chains (*30*) and antigen processing and presentation with major histocompatibility complex class II molecules (*28, 31*). This latter function involves a protein termed PBP 72/74, which appears to be a constitutive member of the Hsp70 family (*28*).

Our results showing the binding of the immunosuppressant DSG to a member of the Hsp70 family suggest that heat shock proteins may represent a new class of immunophilins. Cyclophilin, the cyclosporin A binding protein, FK506 binding protein, and heat shock proteins all participate in the folding of proteins, which suggests that a common mechanism of action of immunosuppressants may include binding to protein "foldases." Alternatively, Hsc70 may act as a carrier, or "molecular chaperone," to translocate DSG to the nucleus or other organelles where the drug may elicit its immunosuppressive effects. Recently, Yem *et al.* (*32*) and Tai *et al.* (*33*) have demonstrated that a protein termed p59, a putative heat shock protein (*34*), interacts with the immunosuppressant FK506. This protein is found in a complex with the glucocorticoid receptor, as well as members of the Hsp90 and Hsp70 family of heat shock proteins (*35*). Modulating glucocorticoid receptor activity by means of the binding of immunosuppressants to the associated heat shock proteins may also be a mechanism of immunosuppression.

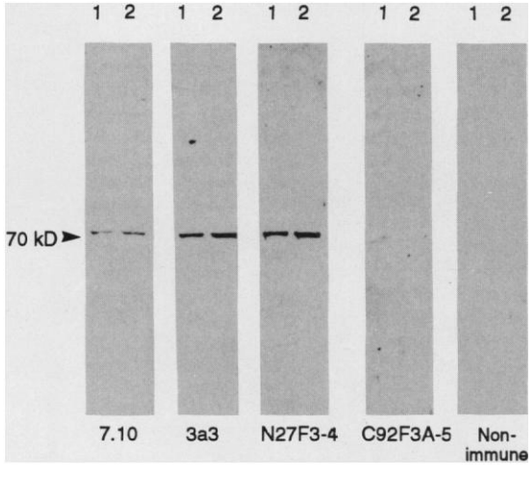

Fig. 3. Immunoblot analysis of the DSG binding protein (*37*). Bovine brain Hsc70 (lanes 1) or affinity-purified DSG binding protein from Jurkat cells (lanes 2) were immunoblotted with the 7.10 rat monoclonal antibody to Hsp70 (Affinity BioReagents, Neshanic Station, New Jersey), the 3a3 mouse monoclonal antibody to Hsp70 (Affinity BioReagents), the N27F3-4 mouse monoclonal antibody to Hsp70 (StressGen, Victoria, Canada), the C92F3A-5 mouse monoclonal antibody to Hsp72 (heat-inducible form) (StressGen), or nonimmune serum.

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15. To determine the amino acid sequence of the DSG binding protein, we prepared tryptic peptides. For the trypsin digestion, 200 μg of DSG binding protein was reduced with dithiothreitol (DTT) and carboxamidomethylated with iodoacetamide. The protein was then digested for 18 hours at 37°C with tosyl-lysine–chloromethyl ke-

tone–trypsin. The resulting peptides were separated on a C_{18} reversed-phase HPLC column (Waters) with a water-acetonitrile gradient. Approximately 1 nmol of each peptide was submitted to amino acid sequencing on an Applied Biosystems sequencer. The following six DSG binding protein peptide sequences were determined: DSG(362–384), SINPDEAVA*YGAAVQAAILS*GDK; DSG(273–290), TLSSSTQ*ASI*EIDSLY*EG; DSG(37–47), TTPSYVAFTDT; DSG(525–531), YKAEDEK*; DSG(265–269), TACER; and DSG(448–451), AMTK. The numbers correspond to the amino acid sequence for the human constitutive heat shock protein Hsc70 (*16*). An asterisk to the right of an amino acid indicates a difference between the Hsc70 sequence and that of the human inducible Hsp70 protein sequence. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

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36. Me-DSG was prepared as described (*2*) and coupled to activated CH-Sepharose (Pharmacia/LKB). We coupled Me-DSG via the spermidine primary amino group because at least one analog modified at this position has been shown to be an active immunosuppressant (K. Maeda, paper presented at the Third International Conference on Drug Research in Immunologic and Infectious Diseases, Washington, DC, 27 June to 1 July 1992). Jurkat cells (1×10^9) were washed twice with phosphate-buffered saline and then suspended in affinity buffer [10 mM sodium phosphate (pH 7.5), 0.1 mM EDTA, 0.15 M NaCl, 0.1 mM dithiothreitol, pepstatin (1 μg/ml), and 0.1 mM phenylmethylsulfonyl fluoride]. The cells were then lysed by sonication and centrifuged at 40,000*g* for 90 min. All subsequent steps were performed at 4°C. The supernatant was applied to the affinity column (2.5 cm by 3.0 cm) at a flow rate of 0.25 ml/min and then washed with 3-column volumes of affinity buffer at a flow rate of 3.0 ml/min, followed by a linear gradient of 0.15 to 1 M NaCl in affinity buffer. The total volume of the gradient was ~20 column volumes. After the gradient, the NaCl concentration was reduced to 0.15 M, and bound protein was eluted with 5 mM DSG. The elution of DSG was performed over 12 hours because protein dissociated from the column at a slow rate. From 1×10^9 Jurkat cells (total protein ≃ 48 mg), we were able to purify ~60 μg of the DSG binding protein, as determined by amino acid analysis.
37. Bovine brain Hsc70 (200 ng) (StressGen Biotechnologies) and affinity-purified DSG binding protein (200 ng) were subjected to electrophoresis on SDS–12.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose. Monoclonal antibodies specific for various Hsp70 epitopes were diluted according to the supplier's instructions. Nonimmune mouse serum was used at a 1:500 dilution. For secondary antibody treatments, the blots were incubated with biotinylated goat antibodies to mouse immunoglobulin G (or to rat immunoglobulin G for monoclonal antibody 7.10) (Pierce) at a 1:10,000 dilution for 1 hour at room temperature. After extensive washing, blots were incubated for 45 min with avidin conjugated with horseradish peroxidase (Pierce) at a 1:1000 dilution. The washed blots were then developed in a solution that contained 3,3′-diaminobenzidine tetrahydrochloride (0.25 mg/ml) and 0.01% hydrogen peroxide.
38. We thank J. Brown and K. E. Hellström for their support of this project; K. Willlams and K. Stone of the Yale University Protein Chemistry Facility and E. Kozlowski for performing the amino acid sequencing; and P. Kiener, I. Takenaka, K. Webster, J. Boice, and L. Hightower for helpful comments.

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