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Identification of a Binding Site on Hsc70 for the Immunosuppressant 15-Deoxyspergualin

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Hsc70, the constitutive form of the heat shock protein 70 family of proteins, is involved in a number of biological activities which include protein folding and molecular chaperoning. Previously, we had shown that the immunosuppressant 15-deoxyspergualin (DSG) specifically interacted with Hsc70, as well as the Hsp90 family of proteins. Although the exact binding site on Hsc70 for protein substrates is unknown, a recent study shows that the extreme C-terminal four amino acids ^{647}EEVD^{650} play a role in regulating ATPase activity, substrate binding, and interaction with HDJ-1. These four amino acids are also found at the C-terminus of Hsp90 and may be involved in similar functions. In this study, we show that DSG binds specifically to this EEVD regulatory domain. Binding of DSG to Hsc70 did not affect its ability to bind peptides. These results suggest that in addition to the ATP binding domain, there are two additional substrate binding domains on Hsc70. DSG should provide a tool for understanding the role of the EEVD motif in biological processes.

Deoxyspergualin is an immunosuppressant which has shown efficacy in numerous animal models of transplant rejection and autoimmune disease. *In vitro*, DSG has potent effects on B-cell differentiation (1,2), monocyte function (3) and T-cell responses (4). Although the exact molecular mechanism of action of DSG is unknown, a recent study suggests DSG inhibits the nuclear localization of the transcription factor NF-kappa B (1). Previously, in an attempt to understand the mechanism of action of DSG we showed, using affinity chromatography, that DSG specifically interacted with Hsc70, the constitutive member of the heat shock protein 70 family of proteins (5,6). Subsequently, using capillary zone electrophoresis, we showed that DSG also interacted with Hsp90 (7). The affinity constants of DSG for Hsc70 from various species, and Hsp90 ranged from 0.4 to 5.0 micromolar (7), which is well within the range which DSG reaches in peripheral blood lymphocytes after six hours (8). Hsc70 is composed of at least two domains, a 45 kilodalton (kD) ATP binding domain (9), an 18 kD peptide binding domain (10), and a 10 kD domain involved in clathrin uncoating (11). In order to gain further insight into how DSG might affect Hsc70 function, we have identified a specfic DSG binding site on Hsc70, and provide data showing that DSG does not affect peptide binding.

MATERIALS AND METHODS

Enzymes and reagents. Bovine Hsc70 was kindly supplied by Dr. Larry Hightower (Univ. of Connecticut). Carbon-14 labeled 15-deoxyspergualin was prepared via the acid catalyzed condensation of 7-(^{14}C-guanidino)heptanoic acid hydrochloride and glyoxyoylspermidine dihydrochloride. The identity and purity of the final radioactive product was established by ion-pair reversed phase HPLC. The radiochemical purity of the sample was 97% and the specific activity was 41.3 microcuries/milligram. The procedure is described in more detail in reference 12.

Chymotrypsin digestion of Hsc70. Bovine Hsc70 was digested with a 1:25 (wt:wt) ratio of chymotrypsin for 3 hours at 37°C. The reaction was quenched with 0.2mM phenylmethyl sulfonyl fluoride. Affinity chromatography was performed essentially as described previously (5). Either undigested Hsc70 or the chymotryptic digest was loaded, using a batch technique, on the methoxy-DSG affinity column (the amount of the 45kD fragment loaded on the resin was approximately 5-fold less then that of the undigested protein due to a low yield from the chymotryptic digest). The resin was washed three times (only the washes of the 45kD domain are shown) with cold phosphate-buffered saline. The resin was then boiled in SDS sample buffer, electrophoresed on a 12% SDS-polyacrylamide gel and stained with Coomassie blue.

Crosslinking of ^{14}C-DSG to Hsc70. ^{14}C-DSG was crosslinked to bovine Hsc70 using the following procedure. Approximately 100 micrograms of Hsc70 was incubated with ^{14}C-DSG in 25mM Hepes, pH7.6 for 30 minutes at 22°C at a final DSG and Hsc70 concentration

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Abbreviations used: Hsc70, 73kDa heat shock cognate protein; DSG, 15-deoxyspergualin; Hsp90, heat shock protein 90; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; FITC, fluorescein isothiocyanate; LA, reduced carboxymethylated lactalbumin.

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of 500 micromolar and 3 mg/ml, respectively. EDC was then added to a concentration of 1.0 millimolar and incubated an additional 90 minutes at 22°C. After crosslinking, the samples were gel filtered through Bio-Spin-6 columns (BioRad Co) to remove unincorporated ^{14}C-DSG. For competition studies, either 1mM ATP or 5mM DSG were added to the protein for 15 minutes prior to starting the crosslinking procedure.

Purification and analysis of ^{14}C-DSG labeled cyanogen bromide peptides. Cyanogen bromide cleavage was performed as described in reference 13. CNBr peptides of ^{14}C-DSG labeled Hsc70 were chromatographed on a gel permeation Bio-Sil TSK-250 column (7.5 × 600mm). Peptides generated from 40 micrograms of Hsc70 were loaded on the column. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) in water containing 40% acetonitrile at 0.2ml/min at 30°C. 0.5 ml. fractions were collected. The following proteins were used as markers: carbonic anhydrase (Mr 31,600), lactalbumin (Mr 14,800), cytochrome C (Mr 12,300) and insulin (Mr 5,700). Repurification of peptide 2 was performed by C-18 reversed phase HPLC on a Vydac C-18 (1 × 150mm) column. Elution was achieved with a linear 40 minute gradient of 5.0% acetonitrile to 65% acetonitrile in 0.1% TFA in water. The column was operated at a flow rate of 100 microliters/minute at 40°C. Mass analysis of the peptides was recorded on a Finnigan-MAT TSQ-7000 (San Jose, CA) triple quadropole mass spectrometer equipped with an electrospray ion source and a C-18 microcapillary HPLC column (0.1 × 150mm).

Binding of [^{3}H]-peptide to bovine Hsc70. A cytochrome C peptide with the sequence IFAGIKKKAERADLIAYLKQATAK was synthesized and radiolabeled with [^{3}H]-sodium borohydride and formaldehyde. For the binding assay, two micrograms of bovine Hsc70 was incubated with varying concentrations of labeled peptide for 30 minutes at 37°C in a buffer consisting of 0.15M NaCl, 0.1mM EDTA, 50mM Tris-Cl pH7.5. The samples were then loaded on a G-50 spin column and centrifuged to remove free peptide from the Hsc70-peptide complex. The total volume of each reaction was 50 microliters. For the competition studies, the inhibitor was preincubated for 30 minutes at 37°C with the Hsc70 prior to adding the radiolabeled peptide.

RESULTS

DSG does not bind to the 45kD ATP binding domain. The 45 kD ATP binding domain on Hsc70 can be generated from the holoenzyme by chymotrypsin cleavage (9). This domain has been shown to be functional with respect to its ability to bind ATP. Using a methoxy-DSG affinity resin, we determined whether the 45 kD N-terminal domain could bind DSG. Figure 1 shows that whereas the undigested Hsc70 binds well to the affinity resin, the 45 kD fragment was unable to bind. This indicates that the DSG binding site does not reside within the 45 kD N-terminal ATP binding domain.

EDC crosslinking of [^{14}C]-DSG to Hsc70. To specifically identify the DSG binding site on Hsc70, we synthesized radiolabeled [14C]-DSG (12) and utilized the primary amino group on the spermidine moiety of DSG for chemical crosslinking to Hsc70. Using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), we were able to covalently crosslink [14C]-DSG to purified bovine Hsc70. The crosslinking most likely occurs between the DSG primary amino group and the alpha-carboxy or a carboxylate containing amino acid, such as glutamic or aspartic acid, on Hsc70 (14). We determined that there were approximately 0.96 ± 0.27 (mean±S.D.) moles of DSG bound per mole of Hsc70 (mean of three experiments). Unlabeled DSG (5mM) effectively competed with [14C]-DSG crosslinking to the protein, reducing the amount of [14C]-DSG crosslinked to 0.14 moles DSG/mole Hsc70 (85% inhibition). Incubation of the crosslinking reaction with 1mM ATP caused a 50% decrease in the amount of [14C]-DSG incorporated on Hsc70. Since DSG does not appear to bind to the ATP binding domain, the inhibition caused by ATP is probably due to conformational changes of Hsc70 which modify the DSG binding site.

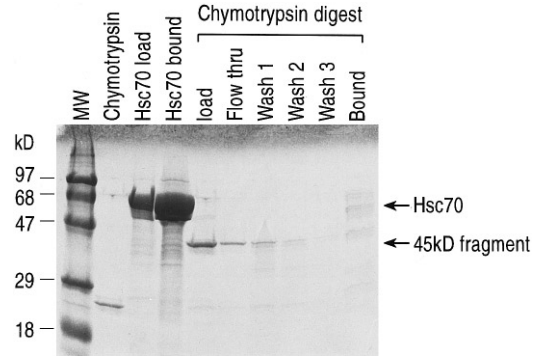

FIG. 1. Methoxy-DSG chromatography of the 45kD ATP binding domain of Hsc70. 12% SDS polyacrylamide gel of Hsc70 and Hsc70 chymotryptic digest before and after chromatography on a methoxy-DSG affinity resin. The proteins were detected by Coomassie blue staining.

Identification of the DSG binding site. The [C14]-labeled Hsc70 was then submitted to cyanogen bromide (CNBr) cleavage followed by HPLC gel permeation chromatography. Two major peaks of radioactivity, peak 1 and peak 2, were obtained which corresponded to molecular weights of approximately 12.6 kD and 3.5 kD (figure 2a). Since peak 2 contained a major portion of the radioactivity, and appeared to be the peak with the highest specific radioactivity, it was analyzed first by chromatography on a reversed-phase HPLC column. Figure 2b shows the chromatogram from the C-18 reversed-phase chromatography of peak 2. Only fractions a and b contained detectable radioactivity (these peaks accounted for 76% of the radioactivity loaded on the column) and were therefore pooled for amino acid sequencing.

Amino acid sequencing of the pool of peaks a and b revealed only one sequence. This peptide had the sequence PGGFPGGGAPPSGGASSGPTIEEV(D)* (*this amino acid was tentatively identified) which corresponds to amino acids 626-650 of bovine Hsc70 (15). Based on the reactivity of EDC (14), the only amino acids which could be labeled with [14C]-DSG are the three C-terminal carboxyl containing amino acids Glu-647, Glu-648 and Asp-650. Mass spectrometry confirmed that one molecule of DSG was covalently cou-

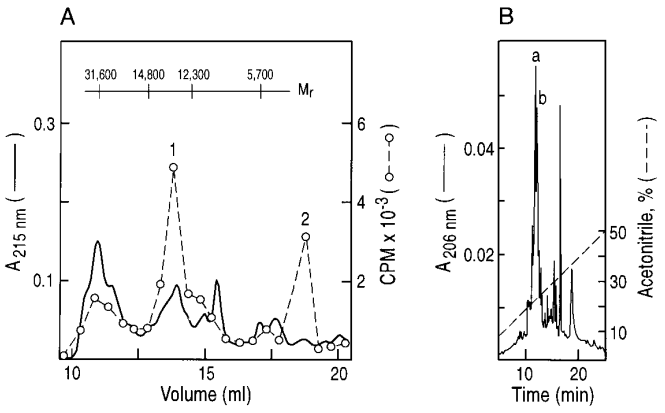

FIG. 2. HPLC chromatography of [14C]-DSG labeled Hsc70 cyanogen bromide peptides. Panel A, Gel permeation chromatography of CNBr fragments purified as described in Materials and Methods. Panel B, Purification of peak 2 from panel A on a C-18 reverse phase column.

pled to this peptide since the calculated mass of this peptide with one molecule of DSG is $(M + H)^+ = 2567.8$, and the EDC crosslinked peptide had an observed $(M + H)^+ = 2567.6$. This is in agreement with our earlier finding that only one molecule of DSG is covalently incorporated per molecule of Hsc70. An additional piece of data confirming the specificity of the interaction between DSG and Hsc70 was evidenced by the finding that the reagent disuccinimidyl suberate (DSS) was unable to crosslink [14C]-DSG to Hsc70 (data not shown). This is most likely due to the fact that for covalent crosslinking, DSS requires a primary amino group on the protein which is not present in the primary sequence in the vicinity of the EEVD binding site. We also found when we crosslinked ^{14}C-spermidine to Hsc70 that this non-immunosuppressive polyamine did not interact with the EEVD domain but was bound to other sites within the protein (data not shown).

Peak 1 (Figure 2a) was further analyzed by gel electrophoresis, electroblotting, and amino acid sequencing. This peak was found to contain a minimum of 2-3 peptides. One sequence corresponded to amino acids 550-650, and represents a larger CNBr fragment containing amino acids 626-650. This DSG modified peptide had a calculated molecular weight of 11,155, that was in good agreement with the Mr=12,600 determined by SDS-PAGE and a specific activity similar to peptide 626-650 of peak 2. We also found that peak 1 contained a peptide corresponding to amino acids 128-237. Clearly, however, based on the total and specific radioactivity, the major site of specific labeling was found in peak 2 (Figure 2a).

DSG does not compete for peptide binding. As seen in Figure 3a, we analyzed the binding of a radiolabeled peptide to Hsc70. This peptide had an affinity for Hsc70 of approximately 5 micromolar. Figure 3b shows that the unlabeled peptide effectively competes for the binding of the radiolabeled peptide to Hsc70. In contrast, DSG was unable to compete for binding of the cytochrome C peptide to the protein. Given that we had previously shown that DSG has an affinity of 500nM-5μM for Hsc70 (7), and that the peptide bound with an affinity of approximately 5μM, we would have expected that DSG should have competed for binding of the peptide in the low micromolar range. Since DSG did not compete for peptide binding, this suggests that peptides (at least the cytochrome C peptide) bind to a different site than DSG on Hsc70.

DISCUSSION

This represents the first report identifying a specific drug binding site on Hsc70. Despite the wealth of studies on Hsc70, except for the localization of the peptide binding domain to the C-terminal 18 kD fragment (10), a more defined binding site for substrates on Hsc70 has not been determined. Recently, however, Freeman, et.al. (16), have identified a regulatory motif which consists of the extreme C-terminal four amino acids EEVD, corresponding to those which we identified in

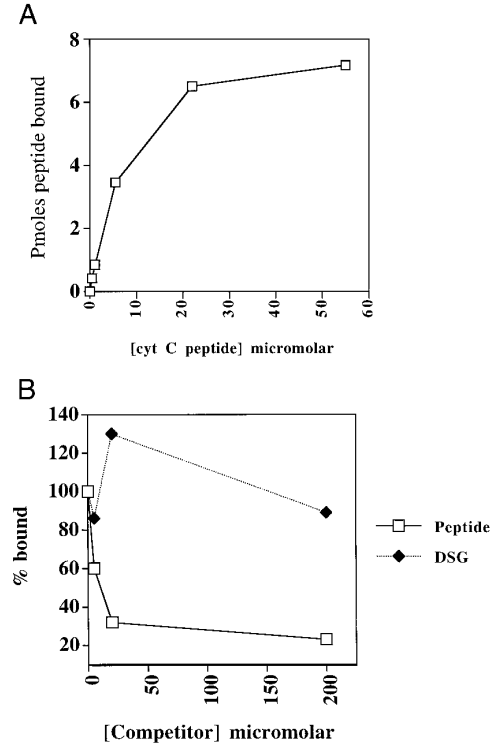

FIG. 3. Binding of peptides to bovine Hsc70. (A) [^{3}H]-cytochrome C peptide was titrated with bovine Hsc70 and separated on a Sephadex G-50 as described in the Materials and Methods. (B) Competition between DSG and [^{3}H]-cytochrome C peptide binding to Hsc70. Various concentrations of DSG were preincubated with Hsc70 followed by the addition of 8 micromolar of labeled peptide. The complex was separated as described above.

TABLE I

Alignment of Extreme C-Terminal Amino Acids of Various Heat Shock Proteins

		Bind DSG?
Bovine Hsc70	^{643} G P T I E E V D ^{650}	Yes
Human Hsp90	^{724} T S R M E E V D ^{731}	Yes
Mtp70	^{649} Q Q G D Q Q K Q ^{656}	No
DnaK	^{628} V E E S D E D E ^{635}	No

Note. The numbering of amino acids is taken from references in (26–27). DSG binding was determined in reference (7).

this study. This domain appears to play a role in regulating the ATPase activity, and the ability of Hsc70 to interact with protein substrates, as well as, the human dnaJ homolog HDJ-1. It appears to modulate intramolecular interactions between the amino terminal ATP binding and carboxy terminal domains. Single point mutations of either the glutamic acid or aspartic acid residues reduced substrate binding by approximately 30%, whereas, double mutants had no binding activity. Since DSG binds to these same amino acids, it is possible that it modulates, to varying degrees, one or all of the functions attributed to this motif. In fact, we have previously shown that DSG stimulates the ATPase activity of Hsc70 (7). Figure 3 shows that DSG does not affect the ability of peptides to bind to Hsc70 which agrees with our earlier studies showing that DSG could not compete for Hsc70 binding to a peptide affinity column (7).

Our data support the notion that in addition to the ATP binding site, there are two additional substrate binding sites. One of these sites, perhaps the 18kD domain identified by Wang, et al. (10) may bind hydrophobic peptides (17-19), and the other site may bind more hydrophilic peptides as identified by Takenaka, et al. (20). It is this second putative site where we have shown DSG binds and is located in the very C-terminus of the protein.

As mentioned above, we previously showed that DSG bound to Hsp90, but did not bind to DnaK, or the mitochondrial Hsp70, Mtp70 (7). Interestingly, as seen in Table I, the EEVD motif is also found at the extreme C-terminus of Hsp90, whereas, it is not found in either DnaK or Mtp70. This suggests that DSG may bind to Hsp90 via this motif and implies that similar to Hsc70, the EEVD motif may act to regulate Hsp90 functions. Although it is still unclear as to whether Hsp90 possesses ATPase activity, it clearly has the ability to bind to YDJ1, the yeast homolog of HDJ1 (21). This interaction has been shown to regulate signal transduction pathways (21). It is possible that similar to Hsc70, the EEVD domain in Hsp90 regulates its interaction with YDJ1 and homologs, as well.

The question of how DSG modulates heat shock protein function *in vivo* has been difficult to answer. DSG does not affect steroid receptor function, or the expression of properly folded proteins (data not shown). Recent studies have shown that DSG inhibits the nuclear localization of at least one transcription factor, NF-kappa B (1). Since Hsc70 has been shown to be involved in transporting some, but not all proteins into the nucleus (22-24), we believe that DSG may be modulating, in an Hsc70 dependent process, the ability of NF-kappa B to translocate into the nucleus. In fact, Mandell and Feldherr (25) have shown that amino acids 611-650 are important for regulating the ability of Hsc70 to be transported into the nucleus. Perhaps the EEVD domain which is localized in this region regulates this process as well and is important for Hsc70 assisting in the transport of proteins from the cytosol to the nucleus. Finally, given the potent immunosuppressive activity of DSG, this report suggests that modulating the function of the EEVD motif in Hsc70 or Hsp90 may have immunosuppressive effects *in vivo*.

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