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Selectivity of the Molecular Chaperone-Specific Immunosuppressive Agent 15-Deoxyspergualin

MODULATION OF HSC70 ATPASE ACTIVITY WITHOUT COMPROMISING DNAJ CHAPERONE INTERACTIONS

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ABSTRACT. The immunosuppressive and cytostatic agent 15-deoxyspergualin (DSG) binds to the Hsc70 class of molecular chaperones with a K_D = 4 μM. Because Hsc70s represent a diverse group of cellular effectors and because Hsc70 function frequently requires a DnaJ molecular chaperone, the specificity of DSG for different Hsc70s and the ability of DSG to block the productive interaction between an Hsc70 and its DnaJ partner were examined. DSG stimulated the ATPase activity of a mammalian and yeast cytosolic Hsc70 from 20 to 40%, but was unable to elicit such a response in a homologous Hsc70, Binding Protein (BiP), that resides in the lumen of the endoplasmic reticulum. In addition, the DnaJ-stimulated Hsc70 ATPase activity and the DnaJ-mediated release of an unfolded polypeptide from an Hsc70 were unaffected by DSG. These results indicate that Hsc70s exhibit substrate selectivity for DSG and that DSG does not compromise Hsc70 functions that require DnaJs. Thus, the immunosuppressive and cytostatic effects of DSG may be specific for a subset of cellular Hsc70s and confined to DnaJ-independent Hsc70-mediated activities. BIOCHEM PHARMACOL **57**;8:877–880, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 15-deoxyspergualin; molecular chaperone; Hsp70; BiP; DnaJ

DSG† is a synthetic analogue of the antibiotic spergualin and has been shown to be a potent anti-tumor (cytostatic) and immunosuppressive agent [1, 2]. DSG successfully prevents rejection in kidney transplant recipients, most likely because it interferes with macrophage, cytolytic T cell, and B cell function [2].

To elucidate the molecular mechanism of DSG action, Nadler and co-workers [3] synthesized a DSG-affinity matrix and found that cellular Hsc70 was retained on the column; subsequent studies confirmed that DSG binds to Hsc70 and determined that the K_D for this interaction is 4 μM [4]. Hsc70s are the constitutive cellular analogues of the stress-inducible Hsp70 molecular chaperones, 70-kDa heat shock proteins that bind and release polypeptide substrates concomitant with ATP binding and hydrolysis [5, 6]. Hsc70s play a vital role in protein biogenesis [5], are required for steroid hormone receptor action [7], and facilitate nuclear transport [8, 9]. Because the steady-state ATPase activity of Hsc70s may be enhanced 2-fold by specific polypeptides [6], it is not surprising that DSG stimulates the ATPase activity of an Hsc70 by ~60% since the structure of DSG somewhat resembles a tripeptide [4]. It is generally assumed that the ability of Hsc70s to engineer such a wide variety of processes arises from their capacity to modulate the activity of a spectrum of cellular polypeptides.

In most cases, however, Hsc70s do not act on polypeptide substrates alone but must cooperate with another molecular chaperone in the cell, a unique DnaJ family member [5]. DnaJs (Hsp40s) catalyze the steady-state ATPase activity of Hsc70s up to 13-fold [10] and, because they also may bind unique polypeptides, DnaJs can target substrates to the Hsc70. Indeed, DnaJ homologues are also required for protein biogenesis [5] and steroid hormone action [7]; however, it is unknown whether DnaJs, like Hsc70s, are required for nuclear transport.

While it is thought that DSG acts through Hsc70s to exert profound effects on cell growth and the immune system [1, 2], the question as to which of the many Hsc70-catalyzed processes are targeted by DSG remains unsolved. Nevertheless, it has been proposed that DSG affects nuclear import since DSG (1) inhibits the heat shock-inducible increase of nuclear Hsp70 in L1210 cells, and (2) compromises the NF-κB-induced expression of κ Ig light chains in pre-B cells, an event known to require the import of NF-κB into the nucleus [11]. Whether these *in vivo* effects of DSG are mediated by Hsc70 is unclear, particularly since DSG also binds to Hsp90 with a K_D of 5

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† *Abbreviations*: DSG, 15-deoxyspergualin; ER, endoplasmic reticulum; CMLA, carboxymethyl-lactalbumin; BiP, Binding Protein; Hsp70, heat shock protein, molecular weight 70,000; Hsc70, Hsp70 cognate protein; and Hsp40, heat shock protein, molecular weight 40,000.

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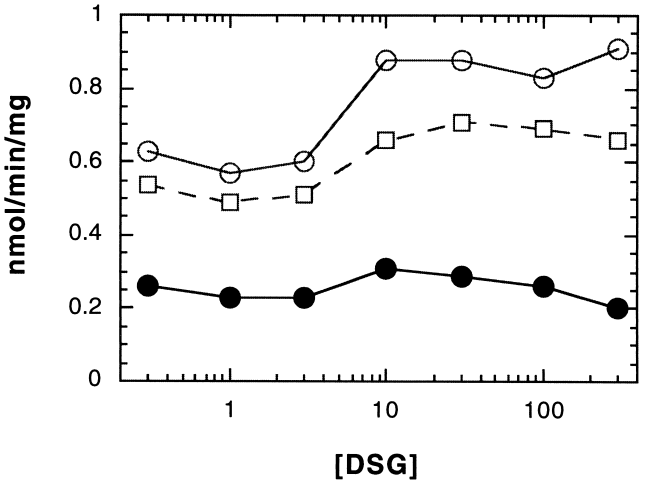

FIG. 1. Activation of ATPase activity of Hsc70s by DSG. Measurements of Hsc70 ATPase activity were performed as described in Materials and Methods using either Ssa1p (○), bovine Hsc70 (□), or yeast BiP (●) at the indicated concentrations of DSG (in micromolar). The resulting specific activities of nanomoles of ATP hydrolyzed per minute per milligram of protein shown are the means from three independent determinations. Representations of SD fit within the displayed symbols.

μM [4] and because Hsp90s are known to facilitate hormone action [7].

To further elucidate the molecular mechanism of DSG, the effects of the drug on Hsc70 function were examined directly using established *in vitro* assays. It was first observed that DSG exhibits selectivity, as the ATPase activities of two cytoplasmic Hsc70s were enhanced by DSG whereas the activity of an ER Hsc70 was refractory to DSG. In addition, the effects of DSG on two Hsc70 activities mediated by a DnaJ were examined. DSG failed to block either DnaJ-catalyzed activity, indicating that the cellular action of DSG on Hsc70 is independent of DnaJ.

MATERIALS AND METHODS

DSG was obtained from Nippon Kayaku American Inc. and was resuspended to a final concentration of 10 mg/mL in double-distilled water immediately prior to use. Mammalian Hsc70 was purchased from StressGen. Ssa1p, BiP, and Ydj1p were purified as previously described [12, 13]. Steady-state ATPase measurements and reduced CMLA release assays were conducted as published [14, 15]. Briefly, [α-^{32}P]ATP was incubated with the indicated purified protein(s) in reaction buffer at 30°, and a 2-μL aliquot of the reaction was spotted onto a thin-layer chromatography plate after 30 min. The chromatogram was resolved in a lithium acetate/formic acid mixture, and the amount of liberated [α-^{32}P]ADP was measured by PhosphorImager analysis. CMLA was radiolabeled with Na^{125}I and incubated with the indicated protein(s) for 60 min at 30° before the Hsc70-bound and free forms of CMLA were resolved by native polyacrylamide gel electrophoresis.

RESULTS

DSG has been shown to stimulate the steady-state ATPase activity of bovine cytosolic Hsc70 by ~40% [4]. To determine the range of DSG substrate selectivity, increasing concentrations of DSG were incubated with the bovine cytosolic Hsc70, with a yeast cytosolic Hsc70 (Ssa1p), and with a yeast ER luminal Hsc70, BiP, and specific ATPase activities were determined as described [14, 15]. As shown in Fig. 1, both the bovine and yeast cytosolic Hsc70s exhibited DSG concentration-dependent increases in ATPase activities; in contrast, the ATP hydrolytic activity of BiP was largely unaffected by DSG. Half-maximal levels of stimulation ($K_{0.5}$) of ~5 μM were obtained for bovine Hsc70 and Ssa1p from these data, which agree well with the $K_{0.5}$ of 3 μM determined previously for bovine Hsc70 [4]. In these particular experiments, DSG enhanced the ATPase activities of Hsc70 and Ssa1p by 22 and 42%, respectively.

The yeast cytosolic DnaJ homolog, Ydj1p, significantly stimulates the ATPase activity of the yeast Hsc70, Ssa1p [13–15]. To determine whether Ydj1p also enhances the ATPase activity of mammalian Hsc70, steady-state ATPase activities of Hsc70 were measured in the presence of increasing concentrations of Ydj1p. As shown in Fig. 2, mammalian Hsc70 exhibited an ~7-fold increase in ATP hydrolysis in the presence of the yeast DnaJ homolog, and a $K_{0.5}$ of ~1.3 μM (0.8 μg of Ydj1p) was obtained. The molar ratio of Ydj1p:Hsc70 (1.3:1.0) required for half-maximal stimulation was nearly identical to the ratio of Ydj1p:Ssa1p required for half-maximal stimulation of the ATPase activity of Ssa1p (data not shown).

To examine whether DSG would compromise the ability of Ydj1p to stimulate the ATPase activity of Ssa1p or Hsc70, reactions containing a final concentration of 300 μM DSG were preincubated for 30 min at 4° with either Ssa1p or Hsc70 before Ydj1p and ATP were added (Fig. 3).

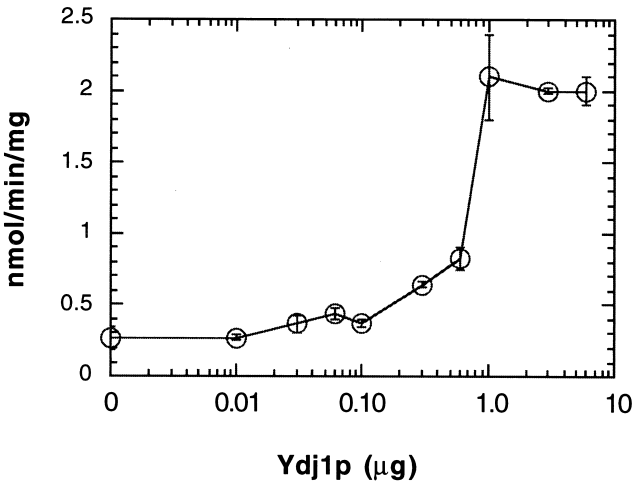

FIG. 2. Ydj1p activation of the ATPase activity of bovine cytosolic Hsc70. ATPase activity measurements were performed as in Fig. 1 supplemented with the indicated amounts of Ydj1p. Data represent the means ± SD from three independent determinations.

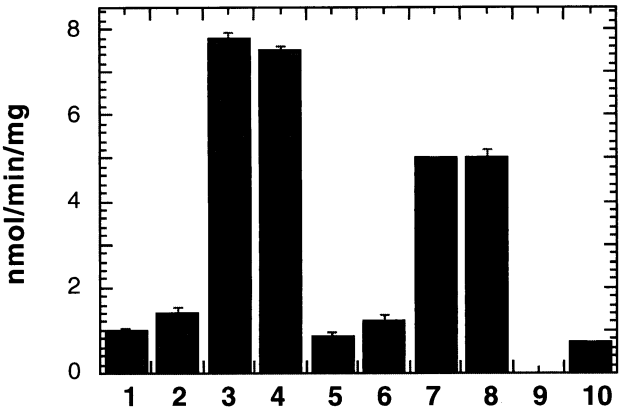

FIG. 3. Effect of DSG on the Ydj1p-stimulated ATPase activity of Ssa1p and Hsc70. ATPase activity measurements were performed as in Fig. 1 under the following conditions: lanes 1–4, Ssa1p; lanes 5–8, bovine Hsc70. Lanes 2, 4, 6, and 8 were supplemented with DSG to a final concentration of 300 μM, and lanes 3, 4, 7, and 8, were supplemented with 3 μg of Ydj1p. Lanes 9 and 10 depict ATPase activities of 300 μM DSG and 3 μg Ydj1p, respectively, in the absence of Ssa1p or Hsc70. Data represent the mean specific activities ± SD from three independent determinations.

DSG increased the ATPase activities of Ssa1p and Hsc70 by 38 and 47%, respectively (compare lanes 2 to 1 and 6 to 5), while Ydj1p stimulated their activities by 8- and 5.9-fold, respectively (compare lanes 3 to 1 and 7 to 5). It is important to note that DSG was present at an ~300-fold molar excess relative to Hsc70, Ssa1p, and Ydj1p, and that neither DSG nor Ydj1p alone had appreciable levels of ATPase activity (lanes 9 and 10). As shown in Fig. 3, the level of Ydj1p-induced stimulation was unaffected by DSG (compare lanes 4 to 3 and 8 to 7), indicating that the drug does not prevent the productive interaction between the DnaJ homolog and either Hsc70 or Ssa1p.

The interaction between an Hsc70 protein and a DnaJ protein can also be assessed by observing whether DnaJ and ATP catalyze the release of an unfolded polypeptide from the Hsc70 [15]. One polypeptide commonly used is reduced CMLA, a chemically unfolded protein that can be radiolabeled and resolved by native acrylamide gel electrophoresis from Hsc70-bound CMLA [15]. Thus, reactions were assembled to determine the ability of DSG to inhibit (1) the formation of an Ssa1p–CMLA complex, and (2) the Ydj1p-stimulated release of CMLA from this complex. As shown in Fig. 4, CMLA bound to Ssa1p in either the presence (lane 3) or absence (lane 2) of DSG. While a 22% reduction in the amount of Hsc70-bound CMLA was noted in the presence of DSG, these experiments were conducted such that the molar ratio of DSG to CMLA was ~3700:1. Therefore, in these experiments, DSG did not interfere significantly with the peptide binding site of Hsc70, as noted previously [11]. DSG also failed to block the Ydj1p-catalyzed release of CMLA from Hsc70 (compare lanes 4 and 5), even though it was present at an ~200-fold excess relative to Ydj1p. Together, these results indicate that DnaJ-catalyzed Hsc70 activities are unaffected by DSG.

DISCUSSION

DSG did not prevent the ability of DnaJ chaperones to modulate two Hsc70 activities: stimulation of ATPase activity and polypeptide release. This result is profound, since many Hsc70-mediated processes absolutely require DnaJ association [5], as underscored by the fact that

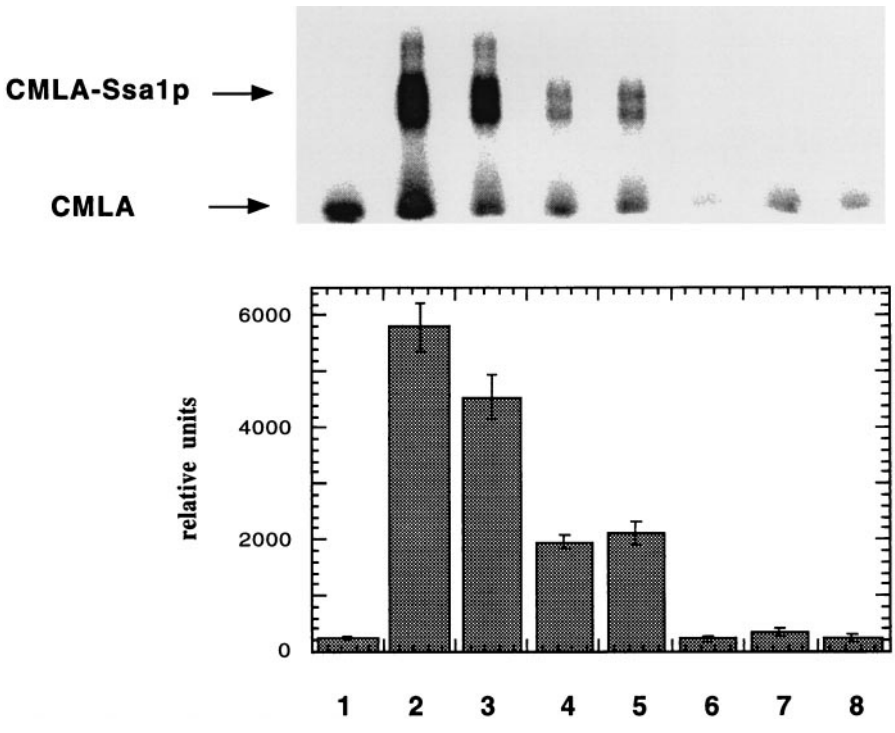

FIG. 4. DSG effect on Ydj1p-mediated release of CMLA from Ssa1p. CMLA binding and release reactions were assembled as described in Materials and Methods. The top half of the figure depicts an autoradiogram from one set of reactions, and the bottom half represents the averaged values from two experiments ± range. Note that a doublet of Ssa1p-bound CMLA is apparent in the top half of the figure, most likely representing monomeric and dimeric Ssa1p species. For simplicity, much of the free, unbound CMLA has been deleted from the figure. All lanes contained CMLA, and lanes 2–5 contained Ssa1p in the absence of any other factors (lane 2), in the presence of 300 μM DSG (lane 3), in the presence of 2 μg Ydj1p (lane 4), and in the presence of both DSG and Ydj1p (lane 5). Control reactions included CMLA alone (lane 1), Ydj1p alone (lane 6), Ydj1p and DSG (lane 7), and DSG alone (lane 8).

mutations preventing such interactions compromise protein transport, lead to cell death, inviability under stress conditions, and in one case have been shown to abrogate cellular transformation and tumorigenesis [14, 16, 17]. Thus, either (1) the immunosuppressive and cytostatic effects of DSG modulate an Hsc70 activity that does not depend on DnaJ action, or (2) the *in vivo* target of DSG may be another molecule. In support of the first hypothesis, DSG alters cellular processes that require nuclear transport [11], and a role for a DnaJ protein in nuclear transport has not been observed. In support of the second hypothesis, DSG binds to another heat shock protein family, the Hsp90s, with relatively high affinity (K_D = 5 μM) [4]. Hsp90 chaperones are required for a number of cellular processes, including steroid hormone receptor activation [7]. Hsp90 has also been found to present tumor antigens to the immune system; Hsp90 derived from analogous tumor cells prevents metastasis and slows the growth of preexisting tumors when administered to mice [18]. Thus, the ability of DSG to compromise immune system function [1, 2] may derive from its affinity for Hsp90. Future work will be directed toward this hypothesis.

DSG was shown to exhibit specificity for a subset of cellular Hsc70s (Fig. 1). While the ATPase activity of two homologous Hsc70s was enhanced by DSG, DSG had no effect on the activity of a third Hsc70. The amino acid identity between bovine Hsc70 and Ssa1p is ~86%, and the identity between the Hsc70 and BiP is ~66% [19]; furthermore, the cytosolic Hsc70s execute cellular functions distinct from those performed by BiP [5]. Regardless of the many interesting questions raised by this and other work, DSG is the first drug known to differentiate between Hsc70 family members, a result that may aid future studies on the action of unique molecular chaperones both *in vivo* and *in vitro*.

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References

1. Gores PF, Deoxyspergualin: Clinical experience. *Transplant Proc* **28**: 871–872, 1996.
2. Hughes SE and Gruber SA, New immunosuppressive drugs in organ transplantation. *J Clin Pharmacol* **36**: 1081–1092, 1996.
3. Nadler SG, Tepper MA, Schacter B and Mazzucco CE, Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. *Science* **258**: 484–486, 1992.
4. Nadeau K, Nadler SG, Saulnier M, Tepper MA and Walsh CT, Quantitation of the interaction of the immunosuppressant deoxyspergualin and analogs with Hsc70 and Hsp90. *Biochemistry* **33**: 2561–2567, 1994.
5. Hartl FU, Molecular chaperones in cellular protein folding. *Nature* **381**: 571–579, 1996.
6. Flynn GC, Chappell TC and Rothman JE, Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* **245**: 385–390, 1989.
7. Pratt WB and Tofts DO, Steroid receptor interactions with heat shock proteins and immunophilin chaperones. *Endocr Rev* **18**: 306–360, 1997.
8. Shi Y and Thomas JO, The transport of proteins into the nucleus requires the 70-kDa heat shock protein or its cytosolic cognate. *Mol Cell Biol* **12**: 2186–2192, 1992.
9. Yang J and DeFranco DB, Differential roles of the heat shock protein 70 in the *in vitro* nuclear import of glucocorticoid receptor and simian virus 40 large tumor antigen. *Mol Cell Biol* **14**: 5088–5090, 1994.
10. McCarty JS, Buchberger A, Reinstein J and Bukau B, The role of ATP in the functional cycle of the DnaK chaperone system. *J Mol Biol* **249**: 126–137, 1995.
11. Nadler SG, Eversole ACB, Tepper MA and Cleaveland JS, Elucidating the mechanism of action of the immunosuppressant 15-deoxyspergualin. *Ther Drug Monit* **17**: 700–703, 1995.
12. Brodsky JL, Hamamoto S, Feldheim D and Schekman R, Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic Hsc70. *J Cell Biol* **120**: 95–102, 1993.
13. Caplan AJ, Cyr DM and Douglas MG, YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. *Cell* **71**: 1143–1155, 1992.
14. Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y, Whyte P, Rundell K, Brodsky JL and Pipas JM, The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J-domain. *Mol Cell Biol* **17**: 4761–4773, 1997.
15. Cyr DM, Lu X and Douglas MG, Regulation of hsp70 function by a eucaryotic DnaJ homolog. *J Biol Chem* **267**: 20927–20931, 1992.
16. Wall D, Zylicz M and Georgopoulos C, The conserved G/F motif of the DnaJ chaperone is necessary for the activation of the substrate binding properties of the DnaK chaperone. *J Biol Chem* **270**: 2139–2144, 1995.
17. Feldheim D, Rothblatt J and Schekman R, Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation. *Mol Cell Biol* **12**: 3288–3296, 1992.
18. Tamura Y, Peng P, Liu K, Daou M and Srivastava PK, Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* **278**: 117–120, 1997.
19. Boorstein WR, Ziegelhoffer T and Craig EA, Molecular evolution of the hsp70 multigene family. *J Mol Evol* **38**: 1–17, 1994.