The ATPase Domain of hsp70 Possesses a Unique Binding Specificity for 3′-Sulfogalactolipids*

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The region(s) of hsp70 critical for sulfogalactolipid (SGL) recognition has been defined through deletion analysis and site-directed mutagenesis. Truncated polymerase chain reaction products of hsp70 generated N-terminal fragments of 43, 35, 29, and 22 kDa. The C terminus substrate-binding domain (28 kDa) was also expressed. The N-terminal ATPase domain (rP43) shared the binding specificity of hsp70, because only sulfogalactosyl ceramide and sulfogalactosyl glycerolipid were recognized by both TLC overlay and RELISA. The C-terminal domain showed no binding. SGL binding of rP29 and rP22 was severely reduced. The loss of SGL binding for rP25 by RELISA but not TLC overlay was considered as a function of receptor presentation. The truncation of rP43 to rP35 demonstrates that residues 318–387 (the base of the ATP binding cleft) are critical for high affinity SGL binding. Mutagenesis showed that Arg342 and Phe198 are crucial for this process. SGL binding, mediated by these conserved residues within the ATPase domain of hsp70, implies that this binding specificity is evolutionarily conserved.

Heat shock proteins of the 70-kDa family (hsp70) have traditionally been described as intracellular chaperones that facilitate protein folding (1), degradation (2), translocation across membranes (3), and disassembly of protein oligomers (4). These functions are driven by ATPase activity contained within the N-terminal domain of all hsp70 family members (5). Hsp70s have also been described on the surface of bacteria (6–9), male germ cells (10), and carcinoma cell lines (11, 12). The absence of extracellular ATP, however, likely renders the hsp70 chaperone function inoperative. Exogenous hsp70 has recently been shown to elicit a cytokine response after binding to the plasma membrane of monocytes (13) and to bind to the surface of antigen-presenting cells and undergo receptor-mediated endocytosis (14), consistent with a cell surface “receptor” for hsp70.

We have previously described a novel function of hsp70 family members as cell surface-associated, SGL-specific adhesins. Anti-hsp70 antibodies prevent the attachment of mycoplasma (15), acid-stressed Helicobacter pylori (16), and temperature-stressed Hemophilus influenzae (17) to SGC.1 This SGL binding specificity was found to be shared by the bovine brain hsp70, recombinant mycoplasma hsp70s (15), and the recombinant testis-specific hsc70 (18).

We have recently extended this survey to demonstrate that recombinant hsp70 family members from Chlamydia trachomatis (6), H. pylori (19), H. influenzae (17), Escherichia coli (20), and an hsp70-related extracellular domain from the egg receptor of the sea urchin, Strongylocentrotus purpuratus (21), all possess the same restricted “lectin” binding specificity for SGC and SGG in vitro.2 Further we found that heterogeneity within the lipid moiety of SGC can differentially modulate binding by prokaryote, as compared with eukaryote, hsp70s, which may reflect their different in vivo adhesin functions.

Sulfogalactolipids are found in a variety of tissues and blood cells. SGC is the major sulfoglycolipid of the kidney (23), brain, gastrointestinal tract (24, 25), and endometrium (26). SGG (with or without SGC) is the major glycolipid of mammalian male germ cells (27) and has, together with an SGG-binding protein (28–30) subsequently identified as the testes-specific hsc70 (18), been implicated in sperm/egg binding (31, 32). SGC alone is found in the male germ cells of lower vertebrates (33) and in red and white blood cells (34). Low levels of SGG are found in the mammalian brain (35), where SGC and SGG synthesis are associated with myelination (36).

The correlation between SGL localization throughout the body, the tissue tropism of the bacterial pathogens, and the data suggesting that surface-associated hsp70 family members function as SGL-specific adhesins, indicate that hsp70-mediated SGL binding plays a physiological role in, at least, bacterial-host and germ cell binding.

To investigate the molecular basis of SGL recognition by hsp70, we have used the recombinant murine testes-specific hsp70.2 gene product, rP70, as our model system (18). The generation of recombinant truncated products of rP70 and site-directed mutagenesis identified a minimal region within the highly conserved N-terminal ATPase domain, critical for SGL binding. The localization of the SGL-binding site is considered within the context of both the chaperone and adhesin functions of hsp70s.

MATERIALS AND METHODS

The murine hsp70.2 gene was cloned, and its recombinant gene product, rP70, was expressed and purified as described previously (18).

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‡ The abbreviations used are: SGC, 3′-sulfogalactosyl ceramide; SGL, sulfogalactolipid; SGG, 3′-sulfogalactosylglycerolipid; MES, 2-(N-morpholino)ethanesulfonic acid; TBS, Tris-buffered saline; PIBM, polyisobutylmethacrylate; GC, galactosylceramide; GM₃, monosialylgangliotetraosyl ceramide; G₀, sulfogalactosylceramide; G₁, globotetraosyl ceramide; G₂, globotriaosylceramide (Forssman antigen); RELISA, receptor enzyme-linked immunosorbent assay.

2 Mamelak, D., Mylvaganam, M., Whetstone, H., Hartmann, E., Lennarz, W., Wyrick, P., Raulston, J., Han, H., Hoffman, P., and Lingwood, C. (2001) Biochemistry, in press.
The cloning and purification of the hsp70 family member from *C. trachomatis* was recently described. *E. coli* XL-1 Blue supercompetent cells, DpnI, and Pfu polymerase, all restriction enzymes, ligases, and respective buffers were purchased from Amersham Pharmacia Biotech. DNA and protein standards were from Life Technologies, Inc. and Bio-Rad, respectively. TA cloning and pTrc expression vectors were purchased from Invitrogen Corp. (San Diego, CA). QuikChange™ site-directed mutagenesis kit was purchased from Stratagene. A cobalt affinity column was purchased from CLONTECH. The Minicycler™ was purchased from Fisher. Mutagenic primers with restriction enzyme sites for the C terminus of the ATPase domain and further C-terminally truncated products. In addition, the segment was designed to overlap with the C terminus of the ATPase domain. Oligonucleotide primers with restriction enzyme sites for EcoRI and HindIII synthetically engineered into the upstream and downstream primers, respectively, were employed in the amplification reaction. Polymerase chain reaction was performed using the recombinant expression vector pDMX1.9 harboring the hsp70.2 gene as the template (18). The Minicycler™ (Fisher) was programmed with the following cycling parameters: a hot start at 94 °C for 10 min, followed by an initial cycle of denaturing for 5 min at 94 °C, followed by annealing for 2 min at 58 °C, and then extending for 2 min at 72 °C. The 2nd to 29th cycles involved denaturing for 5 min at 94 °C, followed by annealing for 1 min at 58 °C, and then extending for 2 min at 72 °C. The 30th and final cycle is similar to the middle cycles except that the final extension time at 72 °C was 10 min. Pfu was employed as the polymerase for these reactions. Taq polymerase was added to the amplified reaction after polymerase chain reaction for 10 min at 72 °C with no extension time.

**TABLE I**

| Mutagenic primers | Amino acid mutation |
|-------------------|---------------------|
| 5″ACAACGCCTAGTACGGACTCGGTGTCGTGCT3′ | N171P |
| 5″GCGGACGAAAGCCTGCTATCAGCCTGGGCGGACC3′ | F198A |
| 5″CCTGGTGGGGCTCAACGCCATCTCTGATCCAGAG3′ | R342A |

The histidine tag adds 3000 kDa to the molecular mass.

**FIG. 1.** SDS-polyacrylamide gel electrophoresis of rP70, deletion constructs, and site-specific mutants. Coomassie Blue stain (A) and Western blot (B) using rabbit polyclonal anti-rP70 antiserum. C-term, C terminus.
pTrc-35, pTrc-29, pTrc-22, and pTrc-Cterm. Derivatives of E. coli DH5α harboring these vectors were selected on LB agar containing 100 µg ml⁻¹ ampicillin. Restriction analysis of recovered plasmid DNA confirmed the presence of recombinant vectors with appropriate size inserts.

All lots of overnight cultures of E. coli strain DH5α harboring the recombinant expression vectors were diluted 1:1000 into LB supplemented with 100 µg ml⁻¹ ampicillin and grown (37 °C, 250 rpm) to an 860 of 0.6. Expression of the truncated hsp70.2 gene products was induced upon the addition of isopropylthiogalactoside (1 mM). After a 5-h induction growth period, cultures were harvested (10,000 rpm, 5 min), and pellets were lysed with Buffer A (8 M urea, 100 mM NaCl, 10 mM Tris-HCl, 50 mM NaH₂PO₄, pH 8). The supernatant was applied to a cobalt affinity column (CLONTECH) and washed three times with Buffer B (8 M urea, 20 mM MES, 100 mM NaCl, 10 mM NaH₂PO₄, pH 6). All protein samples were dialyzed against 10 mM Tris, pH 7.4.

Generation of Site-specific Mutants—The rP70 mutants F198A and R342A were generated by the QuikChange™ site-directed mutagenesis kit (Stratagene) using mutagenic oligonucleotides encoding alanine substitutions for phenylalanine 198 and arginine 342. The steps were performed according to the manufacturer’s instructions. In addition, a mutagenic oligonucleotide encoding a proline substitution for asparagine 171 was designed complementary to the template DNA, and mutagenesis was performed by the method described by Kunkel et al. (39). Isolated plasmid DNA harboring the F198A and R342A mutations was confirmed by sequencing with oligonucleotide primers specific for the 5’ and 3’ ends of the N-terminal ATPase domain. The N171P mutation was confirmed by sequencing plasmid DNA with a primer designed 90 base pairs upstream from the proline substitution using the fmo® DNA cycle sequencing system (Promega). Expression and purification of site-specific mutants was performed as described above.

Electrophoresis and Western Blotting—Protein samples were separated on 12% sodium dodecyl sulfate polyacrylamide gels (40). Separated proteins were detected by staining with Coomassie Blue. For Western blotting, separated proteins were transferred to nitrocellulose and blocked with 5% milk powder, 0.05% Tween 20 in 50 mM Tris, pH 7.4, for 0.5–1 h at room temperature. Western blots were probed with the primary antibody anti-rP70 (1:1000 in blocking solution) overnight at 4 °C. The secondary antibody, peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted 1:2000 in 50 mM TBS), was incubated with the nitrocellulose for 2 h at room temperature. Bound antibody was visualized by developing with chloro-1-naphthol (28).

Thin Layer Chromatography Overlay—Glycolipids (5 µg) were separated on thin layer chromatography plates using a solvent system of chloroform:methanol:RCl (65:25:4 v/v). For dose-response assays 1–10 fmol of SGC/SGG was used. The plates were dried, and the reference plate was treated with orcinol to reveal the positions of the (glyco)lipids. All other plates were soaked in a solution of 0.5% PIBM and hexane for 5 min with gentle agitation, dried, and then immersed again for 3 min (41). After drying, the plates were sprayed with blocking buffer (1% bovine serum albumin in 50 mM TBS) and incubated facedown for 1 h. The blocking buffer was removed, and protein (5 µg/ml in blocking buffer) was added to the plates. After a 2-h incubation, the plates were washed with phosphate-buffered saline four times prior to adding anti-rP70 (1:1000 in blocking buffer) for 1 h. The plates were washed as above and incubated with goat anti-rabbit immunoglobulin secondary anti-sera (1: 2000 in 1.5% bovine serum albumin, 50 mM TBS) for 1 h. After a final wash, plates were turned faceup, and bound protein was visualized by developing with chloro-1-naphthol. All steps were performed at room temperature. Developed plates were scanned, and the density of the signal resulting from protein-SGL binding was quantified using NIH Image.

**FIG. 2.** TLC overlay demonstrating sulfogalactolipid binding of rP70 and deletion constructs. Glycolipid (5 µg) standards were separated on each plate as indicated in the orcinol stain. Binding to SGC and SGG was restricted to rP70, rP43, and rP35 and greatly reduced for the 29- and 22-kDa species. Weak binding of rP35, rP29, and rP22 can be observed to GC. No binding (to any glycolipid) is observed by the recombinant C-terminal substrate-binding domain. C-term, C terminus.

**FIG. 3.** Glycolipid binding specificity of rP70 and the N- and C-terminal domains. Both rP70 (A) and rP43 (C) specifically bound SGC and SGG but not the other glycolipids tested. B, heparin (1 mM) preincubated with rP70 did not inhibit binding to SGC/SGG. D, the 28-kDa C-terminal substrate-binding domain fragment of rP70 showed no binding to any species tested. Each glycolipid was used at a concentration of 100 ng/well. (Glycolipids: 1, SGC; 2, SGG; 3, Gg; 4,Gb2; 5, lactosyl ceramide; 6, G M1; 7, GC; 8, cholesterol sulfate.)
Microtitre Plate Binding Assays—Stock solutions of all lipids were prepared in ethanol. Lipids at specified concentrations were applied, in 50-μl aliquots, to the wells of microtitre plates and allowed to dry overnight at room temperature. The wells were blocked with blocking reagent (200 μl/well 2% bovine serum albumin in 50 mM TBS, pH 7.4), containing 10 mM histidine) for 1 h. Histidine was included in the blocking and washing buffers as a means of preventing any nonspecific interactions between the His<sub>6</sub>-tagged proteins and the wells of the microtitre plate. It has been our experience that His<sub>6</sub>-tagged proteins can bind certain plastics, which is dramatically reduced by the addition of (A) SGC (filled symbols) and (B) SGG (open symbols). Symbols:  ● rP70; ○ rP43; □ rP35; ▲ rP29; X rP22.

Fig. 4. Comparison of the SGL binding of rP70 and its truncated derivatives by RELISA. Binding of protein products to increasing doses of (A) SGC (filled symbols) and (B) SGG (open symbols). Symbols:  ● rP70; ○ rP43; □ rP35; ▲ rP29; X rP22.

The highly conserved N-terminal 43-kDa fragment of the hsp70.2 gene product bound the sulfogalactolipids, SGC, and SGG by either TLC overlay (Fig. 2) or RELISA (Fig. 3). Furthermore, the recombinant N-terminal domain product, rP43, maintained the SGL binding specificity of rP70 (18), because no binding was detected to other negatively charged glycolipids (O<sub>M</sub>), sulfated lipids (cholesterol sulfate), the desulfated derivative of SGC (GC), or ganglioseries (Gg<sub>3</sub>) or globoseries (Gb<sub>1</sub>) neutral glycolipids (Figs. 2 and 3). Coincubation with heparin did not affect the ability of rP70 to bind SGC/SGG (Fig. 3). The C-terminal 28-kDa fragment (substrate-binding domain) of rP70 showed no binding to SGC, SGG, or any other glycolipid tested (Figs. 2 and 3). These results demonstrate that the SGL binding epitope of rP70 is distinct from the heparin-binding domain (42) and is localized only within the highly conserved N-terminal ATPase domain of hsp70.

Minimum SGL-binding Domain of hsp70 T<br>L<br>C<br>O<br>L<br>l<br>l<br>y<br>E<br>v<br>n<br>a<br>t<br>i<br>y<br>

The ability of rP70 and its truncated derivatives to bind increasing concentrations of SGC and SGG was also compared by RELISA (Fig. 3). The recombinant N-terminal domain product, rP43, did not affect the ability of rP70 to bind SGC/SGG (Fig. 3). The C-terminal 28-kDa fragment (substrate-binding domain) of rP70 showed no binding to SGC, SGG, or any other glycolipid tested (Figs. 2 and 3). These results demonstrate that the SGL binding epitope of rP70 is distinct from the heparin-binding domain (42) and is localized only within the highly conserved N-terminal ATPase domain of hsp70.

Results

Generation, Expression, and Purification of Truncated hsp70.2 Gene Products and Site-specific Mutants

The polymerase chain reaction was successfully employed to amplify the N-terminal ATPase coding region of hsp70.2 and C-terminal truncated segments using specifically designed oligonucleotide primers listed in Table I. All amplified products were cloned, expressed, and purified (Fig. 1). Sequence analysis confirmed the base pair substitutions TTT→GCT, CGC→GCC, and AAT→CCT, yielding the rP70 site-specific mutants F198A, R342A, and N171P, respectively. The apparent molecular weights of each truncated and site-directed mutant protein were determined by SDS-polyacrylamide gel electrophoresis. Transfer of the purified protein products to nitrocellulose and probing with anti-rP70 anti-sera demonstrated equal reactivity with all products, showing a single major immunoreactive species in each case (Fig. 1).

Truncated hsp70.2 Gene Products

Restriction of the Sulfatide-specific Binding Site within the Major N-terminal hsp70 Domain

The highly conserved N-terminal 43-kDa fragment of the hsp70.2 gene product bound the sulfogalactolipids, SGC, and SGG by either TLC overlay (Fig. 2) or RELISA (Fig. 3). Furthermore, the recombinant N-terminal domain product, rP43, maintained the SGL binding specificity of rP70 (18), because no binding was detected to other negatively charged glycolipids (O<sub>M</sub>), sulfated lipids (cholesterol sulfate), the desulfated derivative of SGC (GC), or ganglioseries (Gg<sub>3</sub>) or globoseries (Gb<sub>1</sub>) neutral glycolipids (Figs. 2 and 3). Coincubation with heparin did not affect the ability of rP70 to bind SGC/SGG (Fig. 3). The C-terminal 28-kDa fragment (substrate-binding domain) of rP70 showed no binding to SGC, SGG, or any other glycolipid tested (Figs. 2 and 3). These results demonstrate that the SGL binding epitope of rP70 is distinct from the heparin-binding domain (42) and is localized only within the highly conserved N-terminal ATPase domain of hsp70.

Minimum SGL-binding Domain of hsp70 TLC Overlay—The recombinant protein products rP35, rP29, and rP22 further truncated from the rP43 C terminus were compared for binding by TLC overlay relative to the intact protein, rP70 (Fig. 2). The binding of rP35 to SGG was similar, even enhanced, compared with rP70 and rP43. rP70, rP43, and rP35 bound SGG in preference to SGC. Binding of SGG by either TLC overlay (Fig. 2) or RELISA (Fig. 3). For rP29 and the smallest recombinant product, rP22, was severely reduced (Fig. 2). SGC binding was greatly diminished for the rP29 and eliminated for the 22-kDa recombinant fragment. The truncated derivatives rP35, rP29, and rP22 were found to bind very weakly to GC but not to any other lipid tested.

Glycolipid RELISA Binding—The ability of rP70 and its truncated derivatives to bind increasing concentrations of SGC and SGG was also compared by RELISA (Fig. 4). Dose-dependent binding to SGC (Fig. 4A) or SGG (Fig. 4B) was seen for rP70 and rP43. Similar binding was observed for rP70 and rP43 to both SGC/SGG. The binding of rP35, rP29, and rP22 to both SGC and SGG was not above background over the entire lipid concentration range (Fig. 4, A and B).

hsp70.2 Site-specific Mutants

TLC Overlay

The site-specific mutants of rP70 retained reactivity with the anti-hsp70 antiserum (Fig. 1). The SGL binding of N171P was not reduced relative to wild type (Fig. 5A). However, N171P

3 D. Mamelaok, unpublished observations.
bound SGC and SGG in a manner similar to the hsp70 from *C. trachomatis*, for which (unlike rP70) binding was dependent on PIBM pretreatment (Fig. 5A). The binding of F198A and R342A was significantly reduced (Fig. 5B), particularly for SGC. SGC/SGG binding relative to wild type was quantitated by digital image analysis and expressed relative to the binding of rP70 to 10 μg of SGC (100%). LC, lactosyl ceramide; C.S., cholesterol sulfate; C. trach, *Chlamydia trachomatis*.

Glycolipid RELISA

rP70 showed a similar dose response for binding SGC and SGG by RELISA (Fig. 6). No binding was detected to GC. The recombinant C-terminal substrate-binding domain showed no binding to increasing concentrations of SGC/SGG or GC. Whereas the N171P mutant showed efficacy of SGL binding equivalent to wild type, the F198A and R342A mutants showed defective SGL binding by RELISA. At 200 ng of SGC/SGG, binding was reduced by 62 and 56%, respectively, for R342A and 37 and 28%, respectively, for F198A relative to rP70. The SGC/SGG binding affinity, as reflected by the initial slope of the binding curve, was reduced by 25 and 46%, respectively, for F198A and 53 and 81%, respectively, for R342A. The SGC concentration required to reach the saturation binding of rP70 (200 ng) was calculated to be increased 7-fold for F198A and 12-fold for R342A mutants.

The amino acids Phe198 and Arg342 are conserved among all the hsp70 family members that we have shown to possess SGL binding activity (Fig. 7A). The hsp70 from *C. trachomatis* is the only one to possess a proline rather than an asparagine at position 171. The position of the mutations made and the 8-kDa sequence required for SGL binding relative to bound ADP from the crystal structure of the N terminus of the clathrin-uncoating ATPase (43) are shown in Fig. 7B. Arg342 and Phe198 are close in space and adjacent to the bound ADP, Arg342 is 5 Å from the adenosine ring, and Phe198 is ~11 Å from Arg342. The 8-kDa sequence forms three α-helices across the bottom of the ATPase cleft. Asn171 is also at the base of this cleft but on the other side of the molecule and connects strands Iα/Ib and IIA/IIB forming the cleft. Replacement with proline might well alter the angle of this cleft.

**Fig. 5.** TLC overlay of rP70 and site-specific mutants N171P, F198A, and R342A. A, binding of N171P is similar to *C. trachomatis* hsp70, because binding to SGC/SGG is now decreased in the absence of PIBM. B, dose response. (i) Orcinol staining and (ii) wild-type rP70, (iii) F198A, and (iv) R342A binding. The binding intensities of each protein to SGC and SGG were quantitated by image analysis and expressed relative to the binding of rP70 to 10 μg of SGC (100%). LC, lactosyl ceramide; C.S., cholesterol sulfate; C. trach, *Chlamydia trachomatis*. 

rP70 showed a similar dose response for binding SGC and SGG by RELISA (Fig. 6). No binding was detected to GC. The recombinant C-terminal substrate-binding domain showed no binding to increasing concentrations of SGC/SGG or GC. Whereas the N171P mutant showed efficacy of SGL binding equivalent to wild type, the F198A and R342A mutants showed defective SGL binding by RELISA. At 200 ng of SGC/SGG, binding was reduced by 62 and 56%, respectively, for R342A and 37 and 28%, respectively, for F198A relative to rP70. The SGC/SGG binding affinity, as reflected by the initial slope of the binding curve, was reduced by 25 and 46%, respectively, for F198A and 53 and 81%, respectively, for R342A. The SGC concentration required to reach the saturation binding of rP70 (200 ng) was calculated to be increased 7-fold for F198A and 12-fold for R342A mutants.

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DISCUSSION

hsp70 family members have recently been described to possess a novel adhesion function to SGL (15–17). The present study describes a two-stage approach to map the sulfogalactolipid-binding site of rP70. The generation of deletion constructs identified an 8-kDa sequence at the C terminus of the 44-kDa N-terminal ATPase domain of rP70, critical for SGL binding. Site-directed mutagenesis within this fragment identified asparagine 342 to be critical, primarily for SGC binding (as monitored by TLC overlay), whereas the mutation F198A significantly reduced binding to both SGC and SGG equally. In contrast, mutagenesis of asparagine 171 to proline did not affect the efficacy of SGL binding.

In a previous study, we found that an N-terminal breakdown product of rP70 bound to an SGC column, whereas a C-terminal fragment did not (18). To confirm and further define the SGL-binding site, we monitored the effect of progressive C-terminal deletion within the ATPase domain (amino acids 1–381) on SGL binding. Both TLC overlay and RELISA showed that the SGL-binding site is contained within the N-terminal 44-kDa ATPase domain. Despite the propensity to bind hydrophobic species, the C-terminal fragment (amino acids 381–633), containing the substrate-binding domain, showed no glycolipid binding. hsp70 has no sequence similarity to the SGC-binding domain present in the human immunodeficiency virus coat protein, gp120, laminin, thrombospondin, and other extracellular matrix proteins (44–46). The localization of the SGL-binding site distinguishes it from that of heparin (LIGRR, residues 74–78) (42), consistent with our finding that heparin had no effect on SGL binding.

By TLC overlay, equivalent SGL binding was seen for rP70, rP43, and rP35. Binding was significantly reduced for rP29, particularly to SGC, and even further reduced for rP22. This differential binding is not a result of reduced anti-hsp70 reactivity, because the truncated species showed equivalent immunoreactivity.

In comparison to TLC overlay, the glycolipid RELISA provides a closer functional mimic of the eukaryotic cell membrane lipid. SGL binding was lost for the rP29 and rP22 truncated species. However, in contrast to TLC overlay, RELISA showed that the rP35 species had lost SGL binding. Thus rP35 bound SGL by TLC overlay but not by RELISA. This indicates that major elements of the high affinity SGL-binding site are contained within an 8-kDa domain, 35 kDa from the N terminus. This sequence defines three short α-helices at the bottom of the ATPase-containing cleft (43). Remaining elements or a secondary subsite of the primary recognition domain (48) might be within the 13-kDa fragment between rP35 and rP22.

The truncated derivatives rP35, rP29, and rP22 show greater binding in the TLC overlay system than in the RELISA. In the TLC overlay, at least 50-fold more glycolipid is used (compared with the RELISA), with the hydrophilic sugar associated with the silica gel and the hydrocarbon chains exposed. Thus, the carbohydrate may be presented in a hydrophobic environment for ligand binding. Treatment with PIBM prior to overlay is proposed to reorient the sugar (and the lipid) to facilitate interaction with an exogenous ligand (49). Nevertheless, the carbohydrate presentation is less physiological (more hydrophobic because of the “exposed” hydrocarbon chains) than in the RELISA, wherein the glycolipid layer mimics, to a degree, the organization of a membrane bilayer. On this basis, we would infer that SGL binding by rP35 can occur at higher SGL concentrations and in the context of a hydrophobic environment, perhaps because of the exposure of an adjacent hydrophobic domain during the truncation of rP43 to rP35. Such an additional hydrophobic interaction may occur in the TLC overlay because the binding of rP35 is, if anything, greater than that of rP70 or rP43 when monitored by this means (Fig. 2). Although much reduced compared with rP40 and rP35, rP29 and rP22 showed residual SGL binding, but only by TLC overlay, suggesting that components (more hydrophobic?) of the SGL-binding site are contained in these fragments and can only bind at the higher SGL concentrations used in the TLC method (reduced affinity). The generation of a hydrophobic site in the deletion constructs may also explain the slight binding by rP35 and rP22. The truncated derivatives rP35, rP29, and rP22 may also explain the slight binding by rP35, rP29, and rP22 to GC (Fig. 2).

Any differential recognition of SGC and SGG could imply different but overlapping binding sites on the protein. Alternatively, the differential recognition could result from an effect of lipid moieties of SGC and SGG on the presentation of 3′-
sulfogalactose (50). We have recently shown that aglycone modulation of SGC influences binding by different hsp70 family members and that the lipid backbone of SGL strongly influences binding of hsp70 to different synthetic isoforms of SGL. The lipid moiety can modulate whether a glycolipid is recognized in either the TLC overlay or RELISA format (47). SGC and SGG differ only in the lipid species to which the galactose \(-\)sulfate is conjugated (ceramide versus glycerol). Thus, the hsp70 recognition epitope of SGG may be preferentially presented over that of SGC.

In the crystal structure of the sulfate-binding protein of Salmonella typhimurium, sulfate is bound in a solvent-free pocket and stabilized by hydrogen bonds donated by a specific amino acid sequence, GGS (52). The same GGS sequence is located in the 8-kDa fragment implicated in hsp70-SGL binding. Three site-specific mutations were made in this region to further define the SGL-binding site. Phe198 was selected because of the propensity of aromatic residues to stack against sugar rings in carbohydrate-binding sites (48). Arg342 was selected because of the appropriate charge coordination to bind sulfate. Arginine has also been implicated in stabilizing the binding of an E. coli adhesin to SGC (53). Asn171 was selected because it is the only residue in this region that clearly distinguishes the Chlamydia hsp70 (in which this residue is proline) from the other hsp70s (Fig. 7A). The Chlamydia hsp70 is the only hsp70 tested that requires PIBM for SGL binding by TLC overlay, suggesting that the “environment” around the SGL-binding site may be different for this hsp70. The N171P mutation did not alter the SGL binding specificity or efficacy of rP70 but rather rendered the mutant more like C. trachomatis hsp70, because SGL binding by TLC overlay was much reduced in the absence of PIBM. Thus, although Asn171 is not within the SGL-binding site, it is sufficiently close that mutation to proline affects SGL access.

Phe198 and Arg342 are highly conserved among most, if not all, hsp70 family members (Fig. 7A). Neither Arg342 nor Phe198 (nor Asn171) have been implicated in ATP binding or hydrolysis (54, 55). The decrease of SGL binding by F198A and R342A indicates that SGL docks into the pocket formed by the 8-kDa fragment immediately below the ATP-binding site (Fig. 7B). Arginine 342 would interact with the sulfate of SGC, which would be stabilized through stacking of galactose with the aromatic ring of phenylalanine 198. The 11 Å separating these residues is sufficient to accommodate the 3'\(-\)sulfogalactose moiety. The binding of both SGLs was compromised in both mutants, suggesting that the same site can be occupied by either SGL. The presentation of 3'\(-\)sulfogalactose on the glycerol backbone of SGG may be less favorable for interaction with arginine 342, as monitored by TLC. It is possible that the adjacent GGS sequence (Fig. 7A) plays a more significant role in SGG binding. However, it is apparent that the R342A mutation has a more significant effect on SGG (as compared with SGC) binding affinity, as monitored by RELISA (Fig. 6). Nevertheless, essentially the same site probably accommodates both SGC and SGG binding, because a soluble analogue of SGC

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Sulfatide Recognition by the ATPase Domain of hsp70

that we have made is that each truncated recombinant protein or, indeed, the site-specific mutants maintain the tertiary structure predicted from the crystal structure of the N terminus of hsc70 (43). Our finding that the nonconservative mutation N171P retains SGL binding strongly argues for the specific involvement of Arg342 and Phe398 in SGL recognition. Because the RELISA showed a significant decrease in SGL binding by truncating rP34 to rP35, the pre-
folded SGL docking site is probably within the tertiary structure representing this 8 kDa, at the base of the ATPase cleft (Fig. 7B). Alternatively, the 8-kDa loss may alter the “accessibility” of the SGL-binding site.

All hsp70 family members possess an ATPase function encoded within a highly conserved N-terminal domain (5). The catalytic hydrolysis of ATP is critical for hsp70 to facilitate protein folding, oligomerization, degradation, and membrane translocation (57). These chaperone functions of hsp70 are also regulated by the DnaJ (hsp40) co-chaperone (56) as well as by the nucleotide exchange factor GrpE (51). The location of the SGL-binding domain within the highly conserved N-terminal ATPase domain explains the conservation of SGL binding among members of the hsp70 family. SGL binding in this domain of hsp70 has the potential to influence chaperone function, either by affecting ATP binding/hydrolysis or the interaction of hsp70 with modulators of hsp70 function, e.g. DnaJ and GrpE, which also bind within this vicinity (20, 22). Although ATP has no effect on hsp70-SGL binding (data not shown), SGL binding to hsp70 could inhibit its ATPase activity in vitro.

Although the physiological relevance of SGL binding to intracellular hsp70s has yet to be established, our studies on bacterial surface hsp70s (15–17), together with the recent observation that exogenous hsp70 binds eukaryotic cells to effect a signaling cascade (13), clearly indicate that hsp70 possesses a novel “adhesin” function. Such an interaction could be mediated by the recognition process that we have described in this study.

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REFERENCES

1. Beckmann, R. P., Mizzen, L. E., and Welch, W. J. (1990) Science 248, 856–854
2. Chiang, H. L., Terlecky, S. R., Plant, C. P., and Dice, J. F. (1989) J. Cell. Biol. 108, 441–450
3. Chirico, W. J., Waters, M. G., and Blobel, G. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 10291–10294
4. DeLuca-Flaherty, F. C., McKay, D. B., Parham, P., and Hill, B. L. (1990) Cell 61, 851–861
5. Bukau, B., and Horwich, A. L. (1996) Annu. Rev. Cell Dev. Biol. 12, 75–109
6. Rau, J. E., Davis, C. H., Schmid, D. H., Morgan, M. W., and Wycko, B. P. (1995) J. Biol. Chem. 269, 23139–23147
7. Bukau, B., Reilly, P., McCarty, J., and Walker, G. C. (1993) J. Gen. Microbiol. 139, 95–99
8. Scorrano, A. Johnson, P., Laquerre, A., and Nelson, D. (1994) J. Bacteriol. 176, 6449–6456
9. Macelero, A., Tujulin, E., Hjalmarsson, K., and Norlander, L. (1998) Infect. Immun. 66, 5882–5888
10. Miller, D., Brough, S., and Al-Harbi, O. (1999) Hum. Reprod. 7, 637–645
11. Mulhoff, G., Botzler, C., Jenner, L., Schmidt, J., Ellwart, J., and Isells, R. (1997) J. Immunol. 158, 4341–4350

5 H. Whitestone, D. Melakel, and C. Lingwood, submitted for publication.