Hsp70s Contain a Specific Sulfogalactolipid Binding Site. Differential Aglycone Influence on Sulfogalactosyl Ceramide Binding by Recombinant Prokaryotic and Eukaryotic Hsp70 Family Members†

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ABSTRACT: Specific 3′-sulfogalactolipid [SGL-sulfogalactosyl ceramide (SGCer) and sulfogalactosylglycerolipid (SGG)] binding is compared for hsp70s cloned from *Helicobacter pylori*, *Haemophilus influenzae*, *Chlamydia trachomatis* serovar E, *Escherichia coli*, murine male germ cells, and the hsp70-like extracellular domain within the sperm receptor from *Strongylocentrotus purpuratus*. This lectin activity, conserved among the different hsp70 family members, is modulated by the SGL aglycone. This is shown by differential binding to both SGC fatty acid homologues and 3′-sulfogalactolipid neoglycoproteins generated by coupling bovine serum albumin (BSA) and glycosyl ceramide acids synthesized by oxidation of the double bond of sphingosine. Eukaryotic hsp70s preferentially bound the SGCer fatty acid homologues SG^{24}Cer, SG^{18}Cer, and $SG^{20:OH}$Cer, while prokaryotic hsp70s bound $SG^{18:1}$Cer and $SG^{20:OH}$Cer. Eukaryotic hsp70s bound SGCer−BSA and SG^{24}Cer−BSA conjugates where the latter is the main constituent in SGCer−BSA, while prokaryotic hsp70s bound $SG^{20:OH}$Cer−BSA. None of the hsp70s bound sulfogalactosyl sphingosine (SGSph) or SGSph−BSA, further demonstrating the important role of the aglycone. Although the primary SGL recognition domain of all hsp70s is conserved, we propose that aglycone organization differentially influences the interaction with the sub-site. Heterogeneous SGCer aglycone isoforms in cells and the differential in vitro binding of eukaryotic and prokaryotic hsp70s may relate to their different adhesin roles in vivo as mediators of germ cell and bacterial/host interactions, respectively.

The function of hsp70s[1] as chaperones in normal cell physiology and their intracellular modulation of the adverse effects of environmental stress are widely studied and well characterized (*2*, *3*). Their role as mediators of intercellular adhesion, both under normal and under stress conditions, is less well appreciated. Cell-surface hsp70s have been reported in a variety of prokaryotic (*4*−*8*) and eukaryotic cell systems (*9*−*11*). Their function at the cell surface, however, has remained tenuous. We have shown that the recombinant testis-specific hsc70 (*10*), bovine brain hsc70, and the hsp70 from mycoplasma specifically bind the sulfated galactolipids, SGC and SGG (*12*). In addition, monoclonal anti-hsp70 antibodies inhibited the binding of acid-stressed *Helicobacter pylori* (*6*), temperature-stressed *Heamophilus influenzae* (*7*),

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[1] Abbreviations: Natural glycosphingolipids (GSLs) terminate with "Cer" (for ceramide), and deacyl- or lyso-GSLs (dGSLs) terminate with "Sph" (for sphingosine), for example, sulfogalactosyl ceramide (SGCer) and lyso-SGCer (SGSph). Other GSLs: glucosyl ceramide (GlcCer), galactosyl ceramide (GalCer), lactosyl ceramide (LacCer), globotriaosyl ceramide (Gb_3Cer), globotetraosyl ceramide (Gb_4Cer), gangliotetraosyl ceramide (Gg_4Cer), and monosialylganglioside (GM1). Sulfogalactolipids (SGL), sulfogalactosylglycerolipid (SGG), and cholesterol sulfate (CS). Acyl chains on ceramide specified as: SG^{18}Cer, $SG^{18:1}$Cer, SG^{24}Cer, $SG^{24:1}$Cer, $SG^{20:OH}$Cer where 18, 18:1, 24, 24:1, and 20:OH correspond to stearic acid, oleic acid (*cis*-9), lignoceric acid, nervonic acid (*cis*-15), and 2-hydroxyeicosinoic acid, respectively. All abbreviations following the center dot indicate modifications to the ceramide or sphingosine; for example, acids derived from the oxidation of SG^{18}Cer will be SG^{18}Cer·^{C}COOH, where the superscript "C" denotes ceramide acid (*1*). Similarly in BSA conjugates, for example, (SG^{18}Cer·^{C}CONH)$_n$BSA and (SGSph·NHCO)$_n$BSA are from SG^{18}Cer·^{C}COOH and SGSph, respectively. Proteins: heat shock protein (hsp), bovine serum albumin (BSA). Reagents: 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), isopropyl thiogalactopyranoside (IPTG), polyisobutyl methacrylate (PIBM), ampicillin (Amp), Tris-buffered saline (TBS), phosphate-buffered saline (PBS), goat anti-rabbit immunoglobulin (GAR), dichloromethane (DCM), *tert*-butyl alcohol (^{t}BuOH), isopropyl alcohol (^{i}PrOH), 1,2-dichloroethane (DCE), pyridine (Py), diethyl ether (Et_2O), methanol (MeOH), ethanol (EtOH), triethylamine (Et_3N), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), 1-hydroxy-7-azabenzotriazole (HOAT). Others: mass spectra or mass spectroscopy (MS), electrospray-MS (ESMS), matrix-assisted laser desorbed ionization time-of-flight-MS (MALDIToF-MS), high-performance thin-layer chromatography (HPTLC).

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and cultured *Mycoplasma hominis* (*12*) to SGCer and SGG, in vitro.

SGLs are found in the outer leaflet of the outer membrane of epithelial cells. SGG [also termed seminolipid (*13*)] is the major glycolipid of mammalian male germ cells (*14*−*16*). High levels of SGCer are found in the kidney, brain (*17*), gastric mucosa (*18*), and endometrium [where it has been implicated as a receptor for zygote implantation (*19*)].

The intracellular pathogen *Chlamyida trachomatis* (serovars D−K), like mycoplasma, is a reproductive tract pathogen capable of causing endometriosis, sterility, and ectopic pregnancy (*20*). Its hsp70 homologue has been reported to be immunoaccessible on native elementary bodies (EB) (*21*), and antibodies against hsp70 were able to neutralize chlamydial infectivity in vitro (*21*). The outer membrane-associated hsp70 from *Chlamydia trachomatis* serovar E has been implicated in attachment of infectious EB to endometrial epithelial cells (*4*), though the receptor is currently unknown.

In addition to being a receptor for bacterial pathogens, SGG has also been implicated in the mechanism of sperm/egg binding (*22*). SLIP1, a germ cell plasma membrane protein that specifically binds SGLs (*23*), shares many biochemical and immunological properties with hsp70s (*12*) and is likely the testis-specific hsc70 protein (*10*, *24*, *25*). Immunofluorescent studies have identified hsp70 family members on the surface of mammalian sperm (*9*, *10*), co-localized with SGG (*10*, *26*). Furthermore, SLIP1 is a potent inhibitor of sperm/egg binding in vitro (*27*), while anti-SLIP1 inhibited fertilization in vivo (*28*). The role of hsp70s in mediating sperm/egg interactions is further supported by the observations that GST45A, a recombinant egg receptor for sperm from the sea urchin *Strogylocentrotus purpuratus*, is related to the hsp70 family and a potent inhibitor of sperm/egg binding (*29*).

The tissue localization of SGL throughout the body corresponds with the tropism of the pathogenic organisms and eukaryotic cells addressed in this study. Thus, hsp70-mediated SGL recognition may be an important mechanism mediating both bacterial/host and sperm/egg binding. The ceramide fatty acid composition of GSLs can modulate GSL−protein interactions (*30*). Studies with globotriaosyl ceramide and verotoxins demonstrated that aglycone modulation could be particularly important in eliciting binding selectivity within a homologous group of proteins (*31*−*34*). Since the lipid-free sugar of SGCer is not bound by hsp70 (*10*), the effects of SGCer fatty acid homologues and neoglycoprotein conjugates on hsp70 binding were investigated.

We now report specific SGL binding by recombinant DnaK homologues from *H. pylori*, *H. influenzae*, and *C. trachomatis* serovar E, the recombinant hsp70-related sperm receptor from the sea urchin *S. purpuratus*, as well as *E. coli* DnaK, the intracellular hsp70 family member most widely studied for chaperone function. The selective recognition by these recombinant hsp70 family members of different SGCer glycoconjugates is discussed as a function of the interfacial organization around the SGL glycosidic linkage (i.e., sugar/hydrocarbon interface). An intramolecular H-bonding model is proposed to explain this effect.

MATERIALS AND METHODS

Solvents: DCM, ^{t}BuOH, ^{i}PrOH, DCE, Py, Et_2O, MeOH, and $CHCl_3$ were purchased from either Caledon (Georgetown, Ontario) or Aldrich (Milwaukee, WI), and EtOH was from Commercial Alcohols Inc. (Brampton, Ontario). Reagents: Stearic acid ($C_{18}H_{32}O_2$), oleic acid ($C_{18}H_{30}O_2$), lignoceric acid ($C_{24}H_{48}O_2$), nervonic acid ($C_{24}H_{46}O_2$), 2-hydroxyeicosanoic acid ($C_{20}H_{40}O_3$), EDAC, and *N*-hydroxysuccinimide (NHS) were from Fluka (Oakville, Ontario). Chromatographic materials: Silica gel 60 (40−63 μm or 230−400 mesh) and aluminum-backed nanosilica HPTLC-plates (Alugram NanoSIL GI UV_{254}, Macherey & Nagel) were supplied by Caledon; microtiter 96-well plates were from Diamed (Mississauga, Ontario); Centricon-30 centrifugal concentrators were purchased from Amicon. Proteins: Goat anti-rabbit IgG horseradish peroxidase conjugate was from BIO-RAD (Hercules, CA); BSA (99%, essentially fatty acid free) was purchased from Sigma and further purified on a hydroxyapatite column. Glycolipids: LacCer, Gb_3Cer, and Gb_4Cer were purified from human kidney (*35*); GM_1 and SGG were purified from bovine brain and testes (*36*), respectively; GalCer, GlcCer, and SGCer (bovine brain) were purchased from Sigma (St. Louis, MO); Gg_4Cer was prepared by acid hydrolysis of GM_1 with 1 M acetic acid at 80 °C for 1 h (*37*). Deacylated SGCer was prepared by saponification (*38*).

Hsp70 Family Members and Corresponding Antisera. Escherichia coli strains MC4100 (Δdnak, sidB1) harboring plasmids pUHE21-dnaK[1−385] and pUHE21-dnaK, encoding the N-terminal ATPase domain of DnaK and full-length DnaK, respectively, were kindly provided by B. Bukau (*39*). The generation of GST45A, the extracellular domain (amino acids 96−586) of the egg receptor for sperm from the sea urchin, *Strogylocentrotus purpuratus*, and corresponding antisera have been described previously (*29*). The expression of the DnaK homologue from *Haemophilus influeznae* as an N-terminal $(His)_6$-tagged fusion protein will be described in a separate report (Hartmann, E., Lingwood, C., and Reidl, J. *Infect. Immun.* (in press).

A monospecific polyclonal antiserum (anti-2) was raised against an N-terminal region of the *C. trachomatis* serovar E DnaK homologue (residues 154−171) containing a predicted surface-exposed domain and B cell epitope. A similar antibody (anti-1) was raised against a domain (residues 351−368) similar to the *S. purpuratus* sperm receptor (*40*).

Preparation of Anti-Hsp70 Antisera. Purified GST-Hsp70 (100 μg) from *H. pylori* (GST70K) was emulsified with Freund's complete adjuvant and injected subcutaneously into New Zealand White rabbits. A secondary (booster) inoculum, using 100 μg of purified GST70K emulsified with Freund's incomplete adjuvant, was administered 10 days later and repeated after a further month. The following week, the rabbit was bled from the marginal ear vein, and sera were collected and tested for optimal anti-GST70K titer by western blot. In addition to the hsp70 from *H. pylori*, this antiserum was reactive with hsp70 from *H. influenzae* and *E. coli* DnaK. Anti-rP70 specific antiserum was similarly prepared (*10*).

Cloning and Expression of Hsp70 Family Members. The *Helicobacter pylori* hsp70 gene (*41*) was amplified by PCR from strain Hp500 with primer pairs HSP70BHIF (5′GAG-

GATCCATGGGAAAAGTTATTGGA3′) and HSP70ERIR (5′CTGAATTCCCGCATCAATCCACATCGTC3′). Primer HSP70BHIF was designed to produce an in-frame translational fusion with the 3′ region of glutathione-*S*-transferase by restriction of the amplicon with *Bam*HI and *Eco*RI, and ligation into the *Bam*HI−*Eco*RI site of pGEX-2T. The GST-hsp70 protein was overexpressed in *E. coli* DH5α and purified by affinity chromatography following the manufacturer's instructions (AmershamPharmacia Biotech, QC).

The gene encoding the DnaK homologue from *Chlamydia trachomatis* serovar E was amplified by PCR from the plasmid, pPBW58 (*42*). The oligonucleotide primers were designed to the upstream (5′CAGGATCCATGAGCGAAAAAAGAAAGTCT3′) and downstream (5′CAGGTACCCTATAGAATACTCAAGCTTCG3′) regions of the coding frame with synthetically engineered 5′ *Bam*HI and *Kpn*I restriction enzyme sites, respectively. The amplified product was subcloned into pCRII cloning vector (InVitrogen, Carlsbad, CA). Recombinant plasmids were recovered from transformed *E. coli* DH5α and digested with *Bam*HI and *Kpn*I, and the insert was ligated into the expression vector, pTRCB (InVitrogen) The *C. trachomatis* DnaK homologue was expressed as a recombinant N-terminal $(His)_6$-tagged fusion protein and purified from *E. coli* via cobalt affinity chromatography, as described by the manufacturer (Clonetech, CA).

Preparation of Whole Cell Lysate. Overnight cultures of *E. coli* strains MC4100 harboring plasmid-encoded DnaK and the DnaK ATPase domain were subcultured (1:500) into fresh medium, grown (37 °C, 250 rpm) to mid-log phase, and induced with 1 mM IPTG for 5 h. The cells were harvested by centrifugation at 10 000 rpm for 10 min. After vortexing the cells with Buffer A (50 mM NaH_2PO_4, 10 mM Tris, 100 mM NaCl, 0.75 μg/mL lysozyme, pH 8), they were placed on ice for 30 min, followed by two 30 s sonication bursts. The supernatant was collected after centrifugation at 12 000 rpm for 20 min.

Electrophoresis and Western Blotting. Protein samples were separated on 12% sodium dodecyl sulfate−polyacrylamide gels (*43*) and detected by staining with Coomassie blue. For western blotting, the hsp70 family members from *E. coli*, *H. influenzae*, and *H. pylori* 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-GST70K (1:1000 in blocking solution), overnight at 4 °C. The secondary antibody, peroxidase-conjugated GAR (diluted 1:2000 in 50 mM TBS), was incubated with the nitrocellulose for 2 h at room temperature. Bound antibody was visualized with chloro-1-naphthol (*23*).

SYNTHESIS OF SGCER GLYCONJUGATES

Synthesis of $SG^{18}Cer$, $SG^{18:1}Cer$, $SG^{24}Cer$, $SG^{24:1}Cer$, and $SG^{20:OH}Cer$. Fatty acid (2 mg, approximately 2 equiv), HOAT (30 μL of a 0.2 M solution in 5:1 CH_3CN/Et_3N, 6 μmol), and solid EDAC (2−3 mg, 10−15 μmol) were added to a solution of SGSph (2 mg, 4 μmol) in 5:1 CH_3CN/Et_3N (2 mL), and the mixture was stirred at 60 °C for 3 h and monitored by TLC ($CHCl_3$/MeOH/0.88% KCl, 80:20:2). Upon completion, the reaction mixture was dried under a stream of N_2, and the crude product was dissolved ($CHCl_3$/MeOH, 98:2) and loaded onto a silica column (0.5 × 10 cm of silica gel in $CHCl_3$/MeOH, 98:2). HOAT and excess fatty acid were eluted with $CH_3COOH/CHCl_3$ (4:1). Product was eluted with $CHCl_3/MeOH/H_2O$, 80:20:2 (*6*, 4 mL fractions were collected). The estimated yield by TLC was >90%.

Mass Spectroscopy. All samples were dissolved in aqueous methanol (9:1, $MeOH/H_2O$) containing 0.1% NaCl to give a final concentration of 1 mg/mL. Electrospray mass spectra were acquired on an API III Plus triple quadrupole mass spectrometer (PE Sciex, Thornhill, Canada) fitted with an articulated pneumatically assisted nebulization probe. Negative-ESMS (M−H) confirmed the identity of each SGCer isoform: $SG^{24}Cer$, 890.6; $SG^{24:1}Cer$, 888.8; $SG^{20:OH}Cer$, 850.6; $SG^{18}Cer$, 806.6; $SG^{18:1}Cer$, 804.6.

Synthesis of Ceramide Acids from SGCer, $SG^{24}Cer$, and $SG^{20:OH}Cer$. Peracetylated SGCer (300−500 mg dissolved in $^tBuOH/H_2O$, 1:1) was oxidized under neutral conditions at 38 °C for 4 h (*1*, *44*). Prior to coupling, the acetate groups were deprotected with $Et_3N/MeOH/H_2O$, dried, dissolved in $CHCl_3/MeOH/H_2O$ (80:20:2), and loaded onto a silica column (a 9 in. Pasteur pipet plugged with glass wool, packed with 0.5 cm of Celite at the bottom and 0.5 cm of silica, and equilibrated with $CHCl_3$/MeOH, 98:2). The acetate ions were washed with 5 mL of $CHCl_3/MeOH/H_2O$, 80:20:2, and then 5 mL of MeOH to elute the ceramide acid. ESMS of $SG^{20:OH}Cer{\cdot}^{C}COOH$: 688.4 (M+H); 710.2 (M+Na); 732.2 (M−H+2Na); 730.0 $(M(OAc)_1+H)$; 752.2 $(M(OAc)_1+Na)$; 774.2 $(M(OAc)_1-H+2Na)$; 796.2 $(M(OAc)_1-2H+3Na)$. ESMS of $SG^{24}Cer{\cdot}^{C}COOH$: 728.4 (M+H); 750.2 (M+Na); 772.2 ((M−H+2Na); 720 $(M(OAc)_1+H)$; 792.4 $(M(OAc)_1+Na)$; 814.2 $(M(OAc)_1-H+2Na)$; 836.4 $(M(OAc)_1-2H+3Na)$. Note: Ceramide acid samples contain small amounts of partially deprotected monoacetate species [for example, $SG^{24}Cer(OAc)_1{\cdot}^{C}COOH$ denoted as $M(OAc)_1$]. During ESMS analysis, $M(OAc)_1$ species give ions (M+H and M+Na) that are approximately 100 times more stable than the fully deprotected ceramide acid (*44*).

Synthesis of SGCer−BSA Glycoconjugates. Deprotected ceramide acids were coupled to globulin-free BSA as described (*1*). Here, a ratio of BSA to ceramide acid of 2:1(w/w) was employed. Products were purified using the Centricon filtration system. During the separation of coupling reagents from the BSA-conjugates, a mixture of 5:1 (v/v) 40 mM phosphate buffer and EtOH was used in the first two washing cycles, followed by two cycles of 40 mM phosphate buffer. Since the coupling reagents EDAC and NHS were reactive in the BCA-based protein assay (Pierce, Rockford, IL), the final wash was checked by BCA assay to ensure complete removal of coupling reagents. The number of glycosyl units per BSA was determined using MALDI-TOF spectroscopy (summarized in Table 2) as previously described (*1*). MALDI-TOF was recorded on a VOYAGER-ELITE spectrometer (sinapinic acid matrix, linear mode, delayed extraction) using standard conditions.

SGCer−BSA Interface Models. A 3D model of $Gal^{OH}Cer$ was built using standard parameters in the CS CHEM 3D program (Cambrigesoft.Com). The simplified model contained a C-3 fatty acid chain and a sphingosine chain truncated at carbon-6. Appropriate restrictions were imposed such that the intramolecular hydrogen bonds observed in the X-ray structure were maintained. Then the 3′-OH was replaced with a sulfate moiety and minimized using MM2

parameters. This model (not shown) was used to build the structures shown. The interface models of $SG^{20:OH}$Cer−BSA were created by substituting the double bond with an amide bond and followed by energy minimization with minimum perturbation to H-bonded structures. Additional H-bonded structures were manually imposed and minimized.

LIGAND BINDING

Thin-Layer Chromatography (TLC) Overlay. Glycolipids (5 μg) were chromatographed on plates in a solvent system of $CHCl_3$/MeOH/0.88% KCl, 65:25:4. Orcinol staining of one plate revealed the relative migratory distances of the (glyco)lipids. Nontreated plates were either coated with 0.5% PIBM (in $CHCl_3$ and hexane) or untreated and blocked with 1% BSA in 50 mM TBS for 1 h. Purified protein (5 μg/mL) or whole cell *E. coli* MC4100 extract (10 μL/mL) in 1% BSA in 50 mM TBS was incubated with the plates for 2.5 h. The plates were then washed 4× with PBS and incubated with primary antisera (1:1000 in 1% BSA in 50 mM TBS) for 1 h. Anti-GST70K antisera were employed as the primary antisera in the detection of the DnaK homologues from *E. coli*, *H. pylori, and H. influenzae*. TLC overlays involving the *C. trachomatis* hsp70 and GST45A were treated with separate antibodies raised against these proteins. After washing as above, the plates were incubated for 1 h with secondary antisera (goat anti-rabbit 1:2000 in 1.5% BSA in 50 mM TBS). The plates were washed 4× with PBS, before developing with chloro-1-naphthol. All steps were performed at room temperature.

Receptor ELISA (Relisa) Binding. Aliquots (50 μL) of 0.5-dilutions of lipid in ethanol were applied to the wells of a microtiter plate and allowed to dry overnight at room temperature. For BSA-conjugates, 50 μL of 0.5-dilutions in PBS was similarly applied and adsorbed overnight at 4 °C. The highest concentration per well was 100 ng for SGCer/SGG, 200 ng for the SGCer fatty acid homologues, and 150 ng for the SGCer−BSA conjugates. Blocking solution (100 μL of 1% BSA, 10 mM histidine in 50 mM TBS) was applied followed by washing (2×) with 200 μL of wash buffer (0.2% BSA, 1 mM histidine in 50 mM TBS). Protein (50 ng), in 100 μL aliquots of the wash buffer, was added to the wells for 2 h. After washing 3× with 0.2% BSA/50 mM TBS, primary antisera (1:1000 in 0.2% BSA/50 mM TBS) were added for 1 h. To avoid possible cross-reactivity with the anti-2 peptide (40% sequence similarity with BSA), the anti-1 antiserum was employed for *C. trachomatis* hsp70 detection. EDAC-treated BSA (0.02%) was added to the *C. trachomatis* hsp70-specific antisera to further reduce background binding. The wells were washed as above and incubated with secondary antisera (GAR 1:2000 in 1.5% BSA in 50 mM TBS) for 1 h. The wells were washed again and rinsed quickly with 50 mM TBS. The microtiter plates were developed in the dark for 20 min with 100 μL of freshly made ABTS solution (0.5 mg/mL ABTS in citrate−phosphate buffer, pH 4, 3 μL of hydrogen peroxide/10 mL) and read at 405 nm. All steps were carried out at room temperature.

RESULTS

Expression and Homology of Different Family Member Hsp70s. The recombinant hsp70 products were separated by SDS−PAGE (Figure 1). Each recombinant hsp70 was detected as a single species. Transfer of the *H. influenzae*, *H. pylori* hsp70 homologues as well as the whole cell lysate of *E. coli* MC4100 to nitrocellulose and probing with anti-GST70K antisera demonstrated a titer of 1:1000 reacted optimally with all products to show a single immunoreactive species in each case.

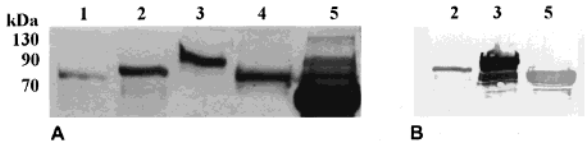

FIGURE 1: SDS−PAGE of recombinant hsp70s used in this study. Panel A: Coomassie blue stain. Panel B: Western blot profile of hsp70 family members cross-reactive with anti-GST70K. Lanes: 1, *C. trachomatis*; 2, *H. pylori* (GST70K); 3, *H. influenzae* hsp70 (reactivity is also observed to a minor C-terminal breakdown product of *H. influenzae* hsp70 and *E. coli* DnaK); 4, *S. purpuratus* (GST45A); 5, lysate of *E. coli* pUHE21-dnaK.

The hsp70s implicated in mediating SGL recognition are listed in Table 1. Entire amino acid sequences and a portion representing the highly conserved N-terminal ATPase domain (amino acids 1−400) were compared to the corresponding sequences of the murine testis-specific P70. The hsp70s of *H. pylori* and *C. trachomatis* are 63% and 64% homologous with the murine testis-specific cognate hsc70, P70. The extracellular domain of the sea urchin egg receptor for sperm, which was reclassified as a member of the hsp110 family (*45*), still possesses 50% homology with the highly conserved N-terminal ATPase domain of P70.

Ligand Binding

Recombinant Hsp70 Homologues Possess Binding Specificity for Sulfogalactolipids. All recombinant members of the hsp70 family from both prokaryotic and eukaryotic organisms employed in this study showed a binding specificity for both SGCer and SGG by TLC overlay (Figure 2). None of the recombinant hsp70s bound any other sulfated or sialylated (glyco)lipid. Furthermore, no binding was observed to galactosyLCeramide, the nonsulfated precursor of SGCer. The hsp70s from *H. pylori* and *H. influenzae* showed increased binding to SGCer/SGG without PIBM pretreatment whereas the binding of that from *C. trachomatis* significantly decreased. Other hsp70s were unaffected (not shown).

Glycolipid RELISA

(A) SGCer Fatty Acid Homologues. The hsp70 homologues from prokaryotic and eukaryotic species showed different binding selectivity to the SGCer fatty acid homologues (Figure 3 and summarized in Table 3). The hsp70s from pathogenic bacteria, *H. influenzae*, *H. pylori*, and *C. trachomatis*, bound strongest to $SG^{18:1}$Cer. The DnaK homologues from *H. influenzae* and *H. pylori* bound less to $SG^{20:OH}$Cer and very weakly, if at all, to SG^{18}Cer, SG^{24}Cer, and $SG^{24:1}$Cer. The *C. trachomatis* hsp70 family member bound strongest to $SG^{18:1}$Cer, but unlike the hsp70s from the other two pathogens, exhibited moderate binding to SG^{24}-Cer, $SG^{20:OH}$Cer, SG^{18}Cer, and $SG^{24:1}$Cer. The eukaryotic hsp70s, rP70 and GST-45A, both implicated in fertilization (*27*−*29*), preferentially bound the saturated fatty acids, SG^{24}-Cer and SG^{18}Cer, and the hydroxylated fatty acid, $SG^{20:OH}$-

Table 1: Summary of Hsp70 Family Members Possessing Sulfoglycolipid Binding Specificity

source of hsp70 family member	GenBank accession no.	% similarity to murine testis-specific hsc70[a]	% similarity to N-terminal amino acids 1−400	binding SGCer/SGG	ref
murine testis-specific (rP70)	M20567	100	100	+/+	(*10*)
bovine brain (hsc70)	U02892	93	94	+/+	(*12*)
Strogylocentrotus purpuratus (GST45A)	U72874	47	49	+/+	this work
Escherichia coli DnaK	D10765	61	64	+/+	this work
Helicobacter pylori (GST70K)	E64533	47	63	+/+	(*41*)/this work
Heamophilus influenzae	P43736	66	62	+/+	(*7*)
Mycoplasma hyopneumoniae	S73733	55	54	+/+	(*70*), (*12*)
Chlamydia trachomatis	L22180	67	64	+/+	this work

[a] Amino acid sequence comparisons of hsp70 family members relative to the murine testis-specific hsc70 were performed using SeqWeb (Wisconsin Package Version 10).

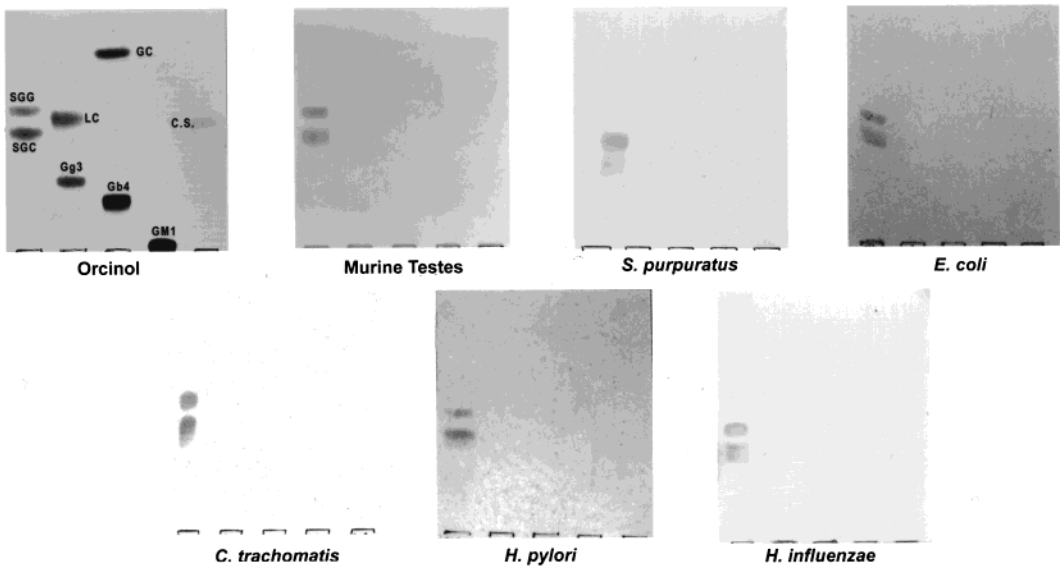

FIGURE 2: Glycolipid binding specificity of many recombinant hsp70s by TLC overlay. The indicated glycolipids (5 μg) were separated by TLC. One plate (upper left panel) was treated with orcinol for chemical staining. All other plates were dried (treated with PIBM, if required for optimum binding) and blocked with BSA as described under Materials and Methods. The plates were incubated with 5 μg/mL of the hsp70 from the indicated organism (listed below each panel) at room temperature for 2.5 h. Whole cell *E. coli* MC4100 (10 μL/mL) extract cured of its plasmid was employed as a negative control. PIBM was present for binding by the hsp70s from murine testes, *S. purpuratus C. trachomatis*, and *E. coli*, but not from *H. pylori* and *H. influenzae*. The hsp70s from murine testes and *S. purpuratus* bound equally well in the absence of PIBM (not shown). The plates were washed, and bound hsp70 was visualized immunologically (*10*).

Cer. Moderate binding was observed to the monounsaturated homologues $SG^{18:1}Cer$ and $SG^{24:1}Cer$. Both rP70 and GST-45A showed saturation binding to $SG^{18}Cer$ and $SG^{18:1}Cer$ at 100 ng, while rP70 reached saturation binding to $SG^{24}Cer$ at similar concentrations. The binding of hsp70s from *H. influenzae* and *H. pylori* saturated at 50 ng of $SG^{20:OH}Cer$, while that of *C. trachomatis* reached saturation at 100 ng of $SG^{18}Cer$ and $SG^{24}Cer$.

(B) SGC−BSA Glycoconjugates. The SGCer−BSA glycoconjugates were characterized by MALDI-TOF mass spectroscopy, and the weight of glycose unit per conjugate was determined (Table 2). Dilutions of SGCer−BSA conjugates were standardized based on glycose content and applied in the microtiter binding assay. The hsp70s from both eukaryotic and prokaryotic organisms bound the nonhydroxylated SGCer−BSA glycoconjugates preferentially (Figure 4 and summarized in Table 3). Interestingly, rP70 exhibited a preference for bovine brain SGCer coupled to BSA as compared to $SG^{24}Cer$−BSA, which was bound preferentially by all other hsp70s (Table 3). Nevertheless, rP70 exhibited saturation binding to both SGCer−BSA and $SG^{24}Cer$−BSA glycoconjugates. Neither eukaryotic hsp70 bound the $SG^{20:OH}Cer$−BSA glycoconjugate. Conversely, this glycoconjugate (and its glycolipid equivalent) was bound by all the prokaryotic hsp70s. No hsp70 bound SGSph−BSA (or SGSph).

DISCUSSION

The results presented in this study demonstrate that constitutively expressed and stress-inducible hsp70 homologues from prokaryotic and eukaryotic species specifically bind glycolipids containing a 3′-sulfogalactose moiety, i.e., SGCer and SGG. None of the hsp70s bound the nonsulfated galactose moiety, as in GalCer, or the sulfate group of cholesterol sulfate or the negatively charged sialic acid moiety of GM1. SGL recognition is not related to the C-terminal hydrophobic peptide-binding site of hsp70s, since it is contained within the N-terminus (*46*) and negatively charged, rather than more hydrophobic neutral glycolipids being recognized.

The present binding assays support our earlier proposal for the stress-inducible mechanisms of hsp70-mediated SGL recognition of *H. pylori* (*6, 41, 47*) and *H. influenzae* (*7*). SGL-specific recognition by the hsp70 homologue from

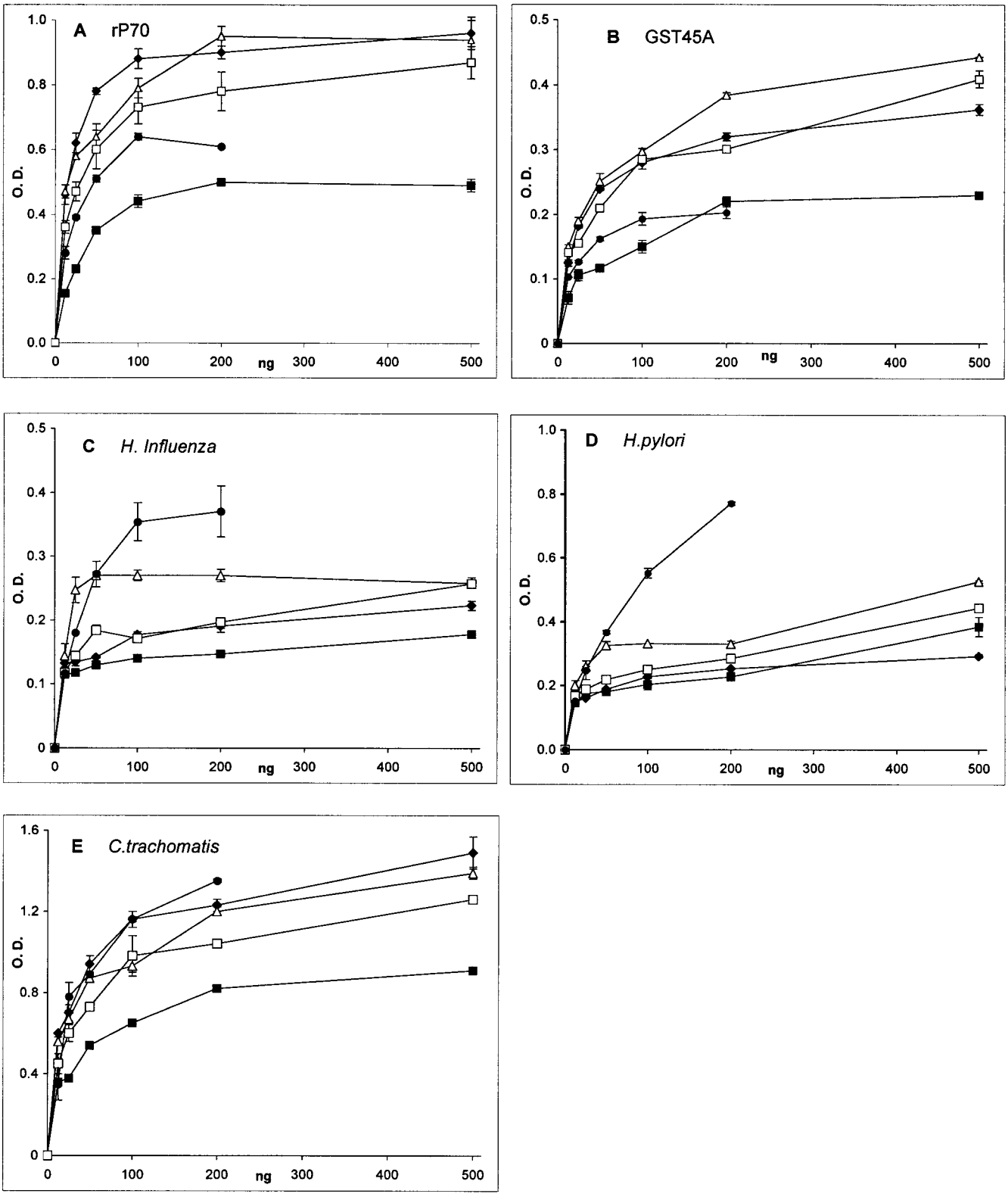

FIGURE 3: Relisa binding of hsp70 family members to SGC fatty acid homologues. (A) rP70; (B) GST45A; (C) *H. influenzae* hsp70; (D) *H. pylori* hsp70; (E) *C. trachomatis* hsp70. The plotted values are averages of each point assayed in triplicate. (◆) $SG^{24}Cer$; (■) $SG^{24:1}Cer$; (△) $SG^{20:OH}Cer$; (□) $SG^{18}Cer$; (●) $SG^{18:1}Cer$.

Chlamydia trachomatis serovar E suggests that SGCer/SGG may function as a cellular receptor for *C. trachomatis* colonization. The function of hsp70s as mediators of bacterial/host colonization through SGL binding is further supported by the observation that in situ, the tissues colonized by these pathogens are rich in SGLs. SGL binding by *E. coli* DnaK demonstrated that this novel recognition function is conserved among strictly intracellular hsp70 chaperones as well.

SGL binding by the recombinant extracellular sea urchin sperm/egg binding domain, GST45A (*29*), and the surface-associated murine testes-specific hsc70 gene product supports

Table 2: Characterization of BSA Conjugates Using MALDI-TOF Mass Spectroscopy

type of BSA conjugate	BSA-conjugate[a] (kDa)	hexose unit[b] (Da)	hexose mass/ BSA[d] (kDa)	hexose units/ BSA (n)[e]
$(SGC^{C}CONH)_n$BSA	76.8	624[c]	4.3	7 ± 7
$(SG^{24}C^{C}CONH)_n$BSA	79.4	710	6.9	10 ± 4
$(SG^{20:OH}CCCONH)_n$BSA	81.0	669	8.5	13 ± 5
$(SGSNHCO)_n$BSA	85.0	523	12.5	24 ± 14
$(GalSNHCO)_n$BSA	78.8	443	6.3	14 ± 5

[a] Corresponds to the mean mass. [b] Molecular mass of glycose monomers less 18, for loss of water during coupling. [c] Bovine SGCer consists mainly of SG^{24}Cer and $SG^{24:1}$Cer, and the weight of the oxidized glycosyl ceramide acid was calculated using a weighted ratio of 0.68 and 0.32, respectively. [d] Calculated by subtracting the mass of BSA-control (72.5 kDa) obtained by treating BSA with only EDAC and NHS. [e] The range was calculated using the equation: $({}^{A}n_{1/2} - {}^{B}n_{1/2})/m$, where ${}^{A}n_{1/2}$, ${}^{B}n_{1/2}$, and m correspond to the half-peak heights of the BSA-conjugate and the BSA-control and the mass of glycose monomers given in column 3, respectively.

and extends the role of hsp70-mediated SGL recognition we have proposed in sperm/egg binding (*27, 28*). GST45A demonstrates 50% homology with the testis-specific hsc70, P70, and binds specifically SGCer/SGG. Furthermore, purified GST45A was demonstrated to be a powerful inhibitor of sperm/egg binding (*29*). Thus, hsp70-mediated SGL recognition may be an important mechanism mediating bacterial/host interaction, as well as a general, non-species-specific mechanism mediating male/female gamete interaction.

Hsp70s constitute one of the most highly conserved protein families in nature, yet members have been shown to differ greatly in localized intracellular function(s), e.g., facilitators of protein folding (*2*) and membrane translocation (*48*). Nevertheless, as with the conserved catalytic ATPase domain, the amino acid sequence similarities among the hsp70s studied here suggest that all share a conserved primary recognition domain for the 3′-sulfogalactolipid glycone. With the exception of *E. coli* DnaK, the different hsp70s from prokaryotic and eukaryotic species described in this study have been implicated in different SGL-binding events, i.e., bacterial/host and sperm/egg binding, respectively. Although it is unknown whether specific parameters exist for SGCer−hsp70 interaction, earlier studies have demonstrated that heterogeneity in the ceramide fatty acid chain length, hydroxylation, and unsaturation of GSLs can influence ligand recognition (*30*). For example, binding of verotoxin (VT) family members, VT1 and VT2c, to Gb_3Cer is differentially modulated by fatty acid chain length and unsaturation (*32*). Since heterogeneous species (i.e., having different acyl chains) of SGCer are found in tissues throughout the body (*49*), we speculate that the aglycone moiety of the 3′-sulfogalactolipid may influence specific hsp70 binding events.

SGCer homologues with C18, C18:1, C20:OH, C24, and C24:1 were synthesized, where the latter two are the main constituents of commercially available bovine brain SGCer (*44*). Hsp70s from prokaryotic organisms preferentially bound $SG^{18:1}$Cer and $SG^{20:OH}$Cer fatty acid homologues, while the eukaryotic hsp70s preferred SG^{24}Cer, SG^{18}Cer, and $SG^{20:OH}$Cer.

Packing of the hydrocarbon chains in a membrane bilayer structure may influence the organization (supramolecular and solvation) of the SGL *interface*—the region sandwiched between the sugar and hydrocarbon domains, which includes the serine-like moiety of GSLs (*44*). Major changes of *H. pylori* and *H. influenzae* hsp70/SGL binding were observed upon minor modification of the fatty acid SG^{18}Cer to $SG^{18:1}$Cer (Figure 3). We attribute these changes to the fluid nature of the C18:1 (*cis*-9) in comparison to denser packing of the saturated analogue. It is reasonable to suggest that similar packing restraints are applicable to SGCer homologues adsorbed to an ELISA plate. Supercomputer simulation of biological membranes depicts a significant level of organized water dipoles around the interface region (*50*). Therefore, water molecules could participate in hydrogen bond formation within the interface, yielding an organized solvated structure. We propose that the interface of SG^{18}Cer is more densely packed and therefore is less solvated. However, $SG^{18:1}$Cer will have greater flexibility and thus greater solvation at the interface. Such flexibility and solvation effect is likely to be less in the case of $SG^{24:1}$Cer and SG^{24}Cer, since the double bond of the C24:1 homologue is positioned further away (*cis*-15) and the packing anomalies generated around C15 (as opposed to C9 in $SG^{18:1}$Cer) would be less effectively translated to the interface.

The fact that SGG (which lacks H-bond donors) is bound by hsp70s as well as SGCer implies that the H-bond acceptor groups of the glycerolipid are sufficient to allow appropriate packing for efficient hsp70 recognition. This, in turn, implies the involvement for water-mediated intramolecular organization.

Conserved amino acid sequences among the different hsp70s contribute to a conserved 3′-sulfogalactose glycone recognition domain (*46*). However, the region(s) surrounding the binding domain, i.e., the sub site, could be variable (*51*). We speculate that hsp70s which preferentially bind the SGCer glyconjugates with a more solvated interface (i.e., $SG^{18:1}$Cer) have charged amino acids in their subsite. We observed that in the TLC binding assay, the hsp70s from *H. pylori* and *H. influenzae* preferentially bound SGL in the absence of PIBM. The hydrophobic nature of PIBM, when coated onto a glycolipid-bearing TLC plate, decreases the hydrophilicity, particularly at the (glyco)lipid/silica surface. Ligands that have polar amino acids in their subsite(s) and require water to stabilize subsite interaction(s) with their 'lectins' will not bind as well in the presence of PIBM. We infer that the subsites of the hsp70 homologues from *H. pylori* and *H. influenzae* have solvated charged residues, and, as a result, binding to SGCer/SGG is reduced in the presence of PIBM. In contrast, the hsp70 homologue from *C. trachomatis* bound preferentially in the presence of PIBM, suggesting that hydrophobic residues may be present in its subsite. In comparison to *H. pylori* and *H. influenzae* hsp70s, increased binding to the less hydrophilic species, SG^{18}Cer and SG^{24}Cer, should thus occur in the presence of such a

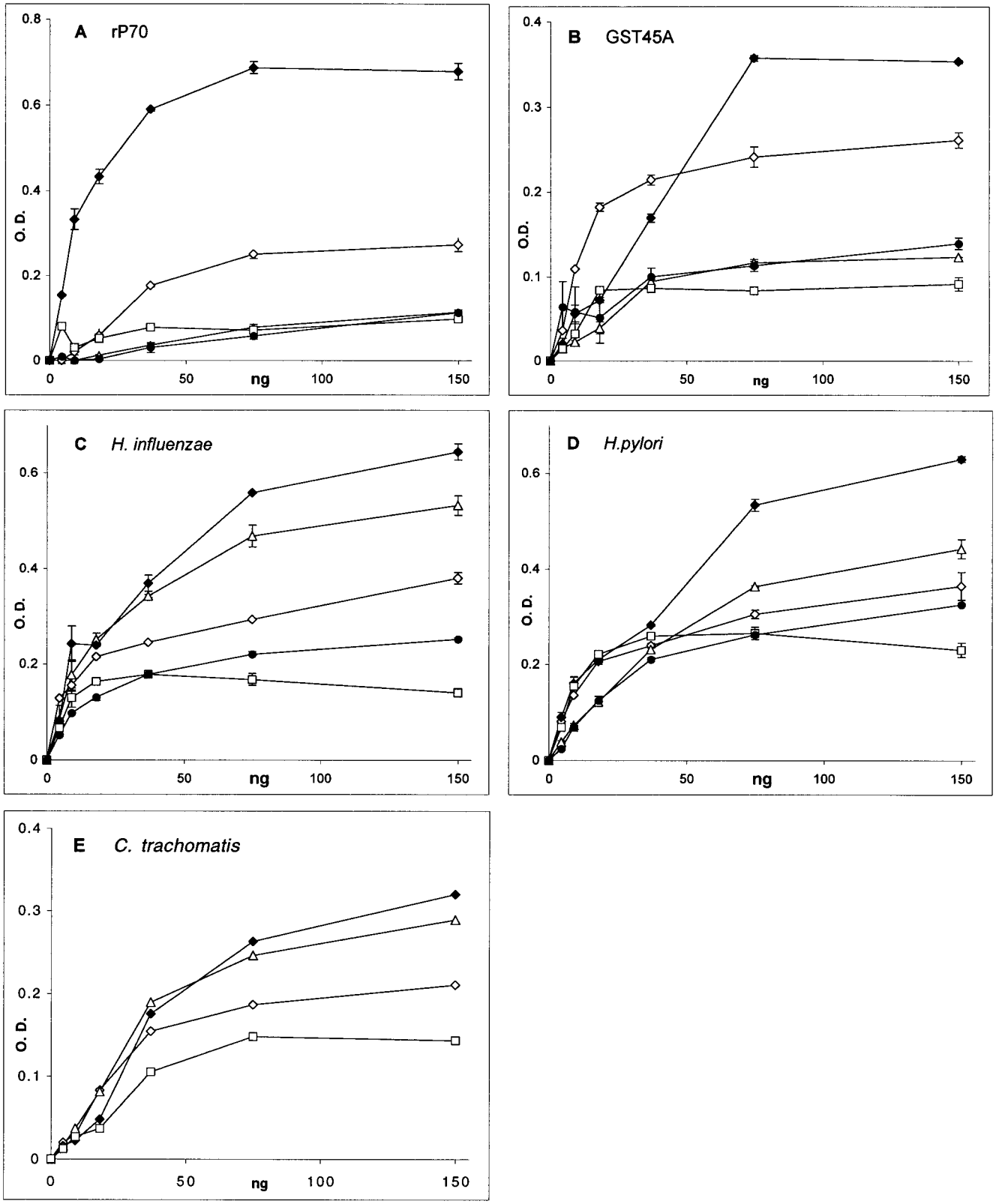

FIGURE 4: Relisa binding of hsp70 family members to SGC−BSA glycoconjugates. (A) rP70; (B) GST45A; (C) *H. influenzae* hsp70; (D) *H. pylori* hsp70; (E) *C. trachomatis* hsp70. The plotted values are averages of each point assayed in triplicate, except for panel E (in duplicate). BSA conjugated to (◇) SGCer, (◆) $SG^{24}Cer$, (△) $SG^{OH}Cer$, (□) SGSph, and (●) GalSph.

hydrophobic subsite, and, indeed, the *C. trachomatis* hsp70 did bind these species more effectively.

To further dissect the fatty acid modulation of hsp70/SGCer binding, we investigated the effect of presenting the 3′-sulfogalactolipid on a protein aglycone scaffold. The sphingosine double bond of natural SGCer and fatty acid homologues was oxidized and coupled to BSA (*1*). Also SGSph was coupled directly to BSA via the sphingosine amine. In SGCer−BSA glycoconjugates, the organization of the glycose might not correlate to the membrane bilayer

Table 3: Index Values Assigned to Selective Binding Specificities of Hsp70 Family Members[a]

			fatty acid homologues (% maximum)									
		hsp70 homologues	saturated		unsaturated		hydroxylated	BSA-conjugates (% maximum)				
origin	functions	source	$SG^{18}Cer$	$SG^{24}Cer$	$SG^{18:1}Cer$	$SG^{24:1}Cer$	$SG^{20:OH}Cer$	SGCer[b]	$SG^{24}Cer$	$SG^{20:OH}Cer$	SGSph	GalSph
eukaryotic	fertilization	murine testes	82	100	60	44	100	100	40	15	0	15
		sea urchin egg receptor	76	76	48	48	100	74	100	36	0	22
prokaryotic	host−cell interactions	*H. pylori*	12	12	100	12	30	54	100	68	18	52
		H. influenzae	32	32	100	12	62	54	100	78	16	30
		C. trachomatis	68	46	100	46	86	30	100	78	0	ND[c]

[a] To allow quantitative comparison between the selective binding of hsp70s to the SGCer glycoconjugates, we assigned index values to the readings measured at A_{405nm} (Figures 3 and 4). The index values are a relative measure of the saturation binding. For each protein, a maximum value of 100% was assigned to the SGCer glycoconjugate most bound by that protein. All other values were similarly calculated and expressed as a percentage of this maximum. Any calculated values less than or equal to values for GalSph−BSA should be considered background. BCA protein assay confirmed no loss of BSA-glycoconjugates from wells during washing. [b] Bovine brain SGCer contains 23% $SG^{24}Cer$ and 48% $SG^{24:1}Cer$ and other fatty acid species (*44*). Upon oxidation and coupling, a number of different conjugates are produced. [c] ND: not determined.

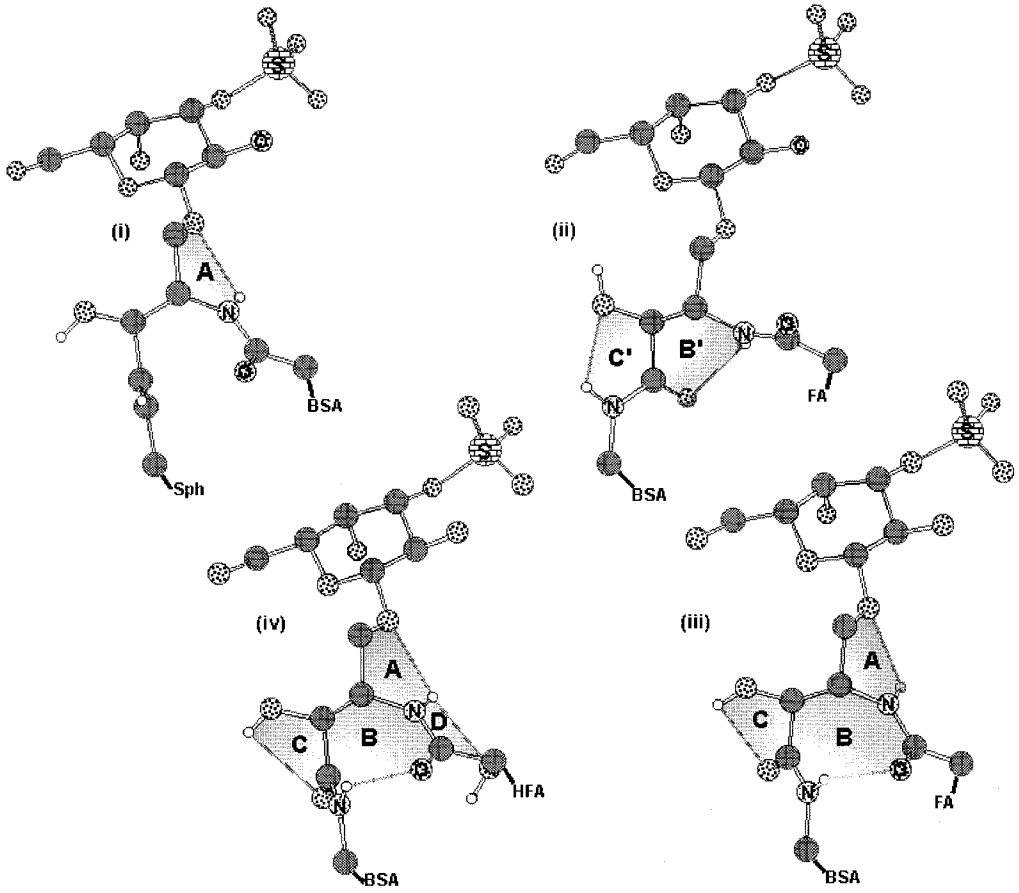

FIGURE 5: Possible structures of the interface of SGCer−BSA conjugates. Possibile intramolecular hydrogen bonds in the interface region. (i) SGSph−BSA; (ii and iii) $SG^{18/24}Cer$−BSA; (iv) $SG^{20:OH}Cer$−BSA. In the case of nonhydroxylated fatty acid containing BSA conjugates, two possibilities are presented to illustrate how the positioning of one amine linkage could give rise to different structural organizations. Shaded areas denoted by A, B, C, and D illustrate possible organizations around the interface. They result from related H-bonds and are defined by similar atoms. For example, in (iii), A and D result from the amide-H forming two H-bonds with the glycosidic oxygen and the oxygen of the hydroxylated fatty acid, respectively. The organization conferred by A and D is very close to what was observed in the crystal structure of $Gal^{OH}Cer$ (*57*). Also, area D in itself is not a stable arrangement, yet could be formed synergistically with areas A, B, and C so that the total organization of the interface is stabilized. Areas C′ and B′ are spatially related domains to C and B. We propose that lack of such intamolecular organization in (i) could be one of the factors preventing binding. Sp, sphingosine; HFA, hydroxylated fatty acid; FA, fatty acid (C24 or C18).

structure, but nevertheless could have different interfacial organizations (Figure 5). Unsaturated SGCer homologues were not coupled to BSA since oxidation of these species yields more than one type of glycosyl acid, thus complicating neoglycoprotein synthesis, characterization, and interpretation of any subsequent binding studies.

None of the hsp70s bound the SGSph−BSA glycoconjugate, despite the fact that this was the most highly substituted glycoconjugate (Table 2). Similarily, SGSph was not bound by TLC overlay (*52*). In SGSph−BSA conjugates, the amide linkage is formed between the amine of the sphingosine and the carboxyl groups of the aspartic/glutamic acids of BSA.

The interface of SGSph−BSA has fewer hydrogen bonds (Figure 5) and as a result has a low degree of interface organization. The absence of a strong hydrogen bond network allows numerous possibilities of sugar presentation, and the optimum for hsp70 recognition occurs at low frequency. A similar study involving the binding of the HIV coat protein gp120 to neoglycoproteins derived from GalSph (i.e., GalSph−BSA) also showed a linkage-sensitive modulation of binding (*1*).

An alternate explanation for the lack of SGSph and SGSph−BSA binding might be that the fatty acid amide of SGCer is directly involved in the binding of SGC by hsp70, particularly in light of the known fatty acid binding activities of hsp70s (*53, 54*). However, this is unlikely, since fatty acid binding invariably requires charge interaction with the free acid (*55, 56*) and other, synthetic SGCer analogues, lacking any fatty acid, are effectively bound by hsp70s (*52*).

Some of the binding profiles of neoglycoproteins were reversed from the profiles observed for fatty acid homologues (Figures 3 and 4). For example, rP70 $SG^{OH}Cer$ binding was significantly decreased in $SG^{OH}Cer$−BSA. In these conjugates, the amide linkages formed by coupling ceramide acids with lysine/arginine residues on BSA will be in the same location as the double bond of sphingosine. As depicted in Figure 5, the interface organization of $SG^{OH}Cer$−BSA could be different from $SG^{OH}Cer$. Some of the H-bonds proposed in this figure were based on the crystal structure of $Gal^{18:OH}Cer$ (*57*). In $SG^{OH}Cer$−BSA, the second amide linkage, introduced by oxidation/coupling, can augment the interface organization by forming additional hydrogen bonds (Figure 5, iii, iv, shaded areas B, C). The effects of hydroxylated fatty acids on other glycolipid receptor functions have been reported (*58, 59*) and an effect on carbohydrate conformation proposed (*60*). Our finding that the eukaryote binding to $SG^{20:OH}C$ was decreased following coupling to BSA suggests that more distal components of the sphingosine base may also play a role in the interface structure.

Structures in Figure 5 are not presented as final models but as an illustratory guide to suggest how the aglycone could influence the presentation of the sugar moiety. They indicate how substituting a double bond with an amide bond could organize the interface and how lack of hydroxylation in the fatty acid could be compensated by a hydroxyl group located on the sphingosine, giving rise to a different organization. These models suggest how different functional groups at different positions on the aglycone could give rise to different organizations around the sugar/lipid linkage. Data to define the interfacial GSL organization and its affect on glycone presentation for protein binding have been difficult to obtain (*61, 62*), due to the heterogeneity of the system, although NMR does support an effect of the fatty acid on packing (*61*). Our approach has been to substitute part of the aglycone with rigid hydrophobic globular frames to generate water-soluble GSL mimics (*63*) which retain receptor activity and thus likely, at least in part, the interfacial properties of the original GSL. Such a derivative of SGC is an effective inhibitor of hsp70−SGC/SGG binding (*52, 64*).

We propose that hsp70 family members have dual roles. When found within the cytoplasm and cytoplasmic organelles, hsp70 functions as a *housekeeping and stress-induced* chaperone. Alternatively, hsp70s localized to the cell membrane can function as SGL-specific adhesins. The observed differential binding of the hsp70 homologues from eukaryotic and prokaryotic species may be a function of their proposed in vivo adhesive roles, correlating with the lipid heterogeneity of SGCer found throughout the body (*49*). We have not determined whether the chaperone function of hsp70 family members is conserved by cell-surface-associated hsp70s, yet SGL-binding specificity is conserved among strictly intracellular hsp70 family members, such as bovine brain hsp70 (*12, 64*) and *E. coli* DnaK (this study). The relevance of SGL binding for intracellular hsp70 function has yet to be determined. Glycolipids are present in ER/Golgi/endosomes/lysosomes. Other ER chaperones have been found to have lectin activity (*65, 66*). SGL binding would result in a membrane-bound hsp70, a common mechanism for regulation of protein function (*67, 68*). However, unless there is a cytosolic SGL equivalent (*69*), cell-surface hsp70/SGL lectin function may be a fortuitous event used to advantage, perhaps initially in primitive gamete interaction.

Since surface-displayed hsp70s from both prokaryotic and eukaryotic organisms specifically bind SGLs, this evolutionary conserved lectin activity represents a new target for intervention in both gamete binding and bacterial−host interactions. The aglycone modulation of SGL recognition helps direct the rational design of soluble glycolipid mimics (*63*) to function as universal, and perhaps also selective, inhibitors of hsp70−SGL interactions.

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