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The aglycone of sulfogalactolipids can alter the sulfate ester substitution position required for hsc70 recognition

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Abstract

3′-Sulfogalactolipids(SGLs), sulfogalactosyl ceramide (SGC), and sulfogalactoglycerolipid (SGG) bind to the N-terminal ATPase-containing domain of members of the heat shock protein 70 family. We have probed this binding specificity using a series of synthetic positional sulfated or phosphorylated glycolipid analogues, containing either a long-chain bisalkyl hydrocarbon–2-(tetradecyl)hexadecane (B30) or C_{18} ceramide (SGC_{18}) backbone. By TLC overlay and receptor ELISA, recombinant hsc70 bound ceramide-based glycoconjugates having 3′- or 4′-sulfogalactose glycone moieties and the 4′-sulfogalactose positional isomer conjugated to B30. Hsc70 binding was significantly decreased to the 3′-sulfogalactose conjugated to the long-chain branched alkane. 3′-Sulfoglucose conjugated to B30 was not bound, nor were similarly conjugated di-, tri-, and tetra-sulfated or phosphorylated galactolipids. These results highlight the importance of the position, rather than the number of sulfate esters within the galactose ring. This binding selectivity was shared by the sea urchin hsp70-related sperm receptor. A 3′-SGC-based soluble inhibitor, in which the acyl chain was replaced with an adamantyl group, inhibited binding of hsc70 to both 3′- and 4′-SGC species with an IC_{50} of 50 and 75 μM, respectively, indicating a shared sulfogalactose binding site. These studies demonstrate the highly specific nature of hsc70/SGL binding and show, for the first time, that the lipid aglycone can alter the substitution position requirement for glycolipid recognition.

Keywords: Sulfatide; Cerebroside sulfate; Sulfogalactose isomers; Soluble glycolipid analog; Stress protein

Abbreviations: SGC, 3′-sulfogalactosylceramide; SGG, 3′-sulfogalactosylglycerolipid; SGL, sulfogalactolipid; Lyso-SGC, deacylated SGC; GC, galactosylceramide; SLIP1, sulfoglycolipid immobilized protein 1; ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); IPTG, isopropyl 1-thiogalactoside; GAR, goat anti-rabbit; hsp, heat shock protein; TLC, thin-layer chromatography; HOAT, [1-Hydroxy-7-azabenzotriazole; EDAC, [1-ethyl-3-(3-dimethylamino-propyl)carbodiimide; RELISA, receptor enzyme linked immunosorbant assay; GAR-HRP, goat anti-rabbit conjugated horseradish peroxidase; BSA, bovine serum albumin; TBS, Tris buffered saline; PIBM, polyisobutylmethacrylate; RT, room temperature; s, second (s); Amp, ampicillin; H_2O^S, 0.8% KCl solution.

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1. Introduction

Cell-surface glycolipids can mediate eukaryotic cell adhesion[1–3] and serve as host surface receptors for a variety of microbial pathogens[4,5] We have described a novel function of cell surface or outer membrane-associated hsp70 family members as 3′-SGL (SGC/SGG)-specific adhesins[6,7] with the potential of mediating bacterial–host and sperm–egg binding.[6,8–12] Anti-hsp70 antiserum prevents the stress-induced SGL binding of *Helicobacter pylori*[9] and *Haemophilus influenzae*[8,13] and the SGL binding of mycoplasmas.[6] SLIP1,[11] the SGL binding protein isolated from rat testes and a member of the testes specific hsp70 family,[14] as well as the recombinant sea urchin egg receptor from *Strogylocentrus purpuratus*, which possesses 50% homology with the hsc70 family[12,15] and binds SGL,[7] are potent inhibitors of sperm–egg binding.

Sulfogalactolipids are found in a variety of tissues and blood cells. SGC (3′-sulfogalactosylceramide) is the major sulfoglycolipid of the kidney,[16] brain, gastrointestinal tract,[17] where it has been proposed as a receptor for *H. pylori*,[18] and endometrium.[19] SGC is found in the male germ cells of vertebrates[20] and in red and white blood cells.[21] SGG (3′-sulfogalactosylglycerolipid) (with or without SGC), the major sulfoglycolipid of mammalian male germ cells,[22] is crucial for spermatogenesis[23] and has been strongly implicated in sperm–egg binding[11,24,25]. Low levels of SGG are found in the mammalian brain[26] where SGC and SGG synthesis are associated with myelination.[27]

Given the tropism of certain bacterial infections to epithelia of high SGL content, and our recent observations that many (probably all) hsp70 family members specifically bind SGL[7] we propose that cell surface hsp70-SGL recognition is a novel mediator of cellular adhesion.

The significance of these interactions has led us to investigate the structural features of both hsp70 and SGL required for this recognition event. Sulfatide is specifically bound by several proteins[28–30] possessing a consensus SGL-binding motif.[31,32] Although this motif is lacking in hsp70s, binding studies with deletion constructs and site-directed mutants of the recombinant testes-specific hsc70.2 gene product, have demonstrated that F198 and R342, localized within the highly conserved N-terminal ATPase domain are crucial for SGL recognition.[33] Previous studies from our group have demonstrated the importance of the lipid moiety in conferring glycolipid receptor activity.[34–36] Hsp70–SGL binding is not inhibited by the lipid-free 3′-sulfogalactose,[14] demonstrating that SGL receptor function is modulated by the lipid moiety.[37,38] Moreover, differences in SGC fatty acid composition can markedly affect hsp70 recognition,[7] a selectivity which may reflect their proposed *in-vivo* functions, further indicating the importance of the aglycone in sulfatide–hsp70 binding.

In studies on the positional requirement of the sulfate ester for hsp70–SGL binding using a series of synthetic sulfated and phosphorylated glycolipid analogues, we have found that not only is the sulfate and its substitution position crucial, but this restriction can be modulated by the lipid moiety.

2. Experimental

Materials and methods.—All glycolipids were run/spotted on high-performance TLC plates from Machery–Nagel (Germany). PIBM, ABTS, IPTG and chloro-1-naphthol were all purchased from Sigma (St. Louis, MO). Ninety-six well microtitre plates were purchased from Diamed (Mississauga, ON). The purification of recombinant testes specific hsc70 (a cognate member of the hsp70 family, used as our model hsp70),[14] GST45A, the sea urchin sperm receptor hsp,[12] and corresponding antisera have been described.[7,14,33] The synthesis of the mono and multimeric phosphorylated and sulfated positional glycolipid analogues coupled to 2-(tetradecyl)hexadecyl-β-D-galactopyranoside has been described previously.[39,40] Bovine brain SGC was purchased from Sigma (St. Louis, MO), while SGG was purified from testes.[41] Deacylated SGC was prepared by saponification.[42]

Thin-layer chromatography (TLC) overlay and inhibition assays.—Glycolipids (5 μg) were chromatographed on TLC plates using a solvent system of 65:25:4 (v/v) $CHCl_3$–MeOH–H_2O^S. The plates were dried and the reference plate was treated with orcinol to reveal the positions of the glycolipids. All other plates were immersed in a solution of 0.5% PIBM.[43] After soaking for 5 min with gentle agitation, the plates were blown dry and immediately soaked again for 3 min.[44] After drying, the plates were sprayed with 1% BSA in 50 mM TBS and incubated face down in the same buffer for 1 h. Purified protein (5 μg/mL) in 1% BSA/50mM TBS was incubated with the plates for 2.5 h. For inhibition, hsc70 (68 nM) was pre-incubated with increasing concentrations of SGS-Nada (0, 10, 100, 1000 μM) for 1–2 h in 50 mM TBS, pH 7.4. Mixtures (1 mL) of hsc70 and the compounds were added to separate plates. The plates were then washed 4× with PBS and incubated with primary anti-sera (1/1000 in 1% BSA/50mM TBS) for 1 h. After washing as above, the plates were incubated for 1 h with secondary anti-sera (GAR 1/2000 in 1.5% BSA/50mM TBS). The plates were washed 4× with PBS, before developing with chloro-1-naphthol. All steps were performed at RT unless otherwise stated.

Glycolipid receptor (RELISA) binding and inhibition assays.—Stock solutions of all lipids were prepared in EtOH. Lipids (100 ng) were applied, in 50 μL aliquots, to the wells of microtitre plates and allowed to dry overnight at RT. No lipids were recovered in subsequent washing steps and we have therefore assumed that each species is adsorbed equally. The wells were blocked for 1 h with buffer A (2% BSA, 10 mM histidine in 50 mM TBS). After washing the wells twice with 200 μL of wash buffer (0.2% BSA, 1 mM histidine in 50 mM TBS), 50 ng of protein, in 100 μL aliquots of the wash buffer, were added to the wells in triplicate for 2 h. For inhibition, increasing concentrations of SGS-Nada, (0–1 mM) were pre-incubated (2 h) with hsc70 (6.8 nM) prior to applying to the wells of the microtitre plates. The wells were then washed 3× with buffer B (0.2% w/v) BSA/50 mM TBS) and then incubated with primary anti-sera (1/200 in buffer B) for 1 h. The wells were washed as above and the secondary anti-sera (GAR-HRP 1/2000 in buffer C (1.5% BSA/50 mM TBS)) was added. After 1 h, the wells were washed again as above, then rinsed quickly with 50 mM TBS. The wells were developed in the dark for 30 min with 100 μL of freshly made ABTS solution (0.5 mg/mL) in citrate–phosphate buffer (pH 4) containing H_2O_2 (mixture of 3 μL of 30% H_2O_2 and 10 mL buffer) and then read at 405 nm. The plates were sealed with parafilm for each incubation step (except developing) to prevent evaporation. All steps were carried out at RT.

Synthesis of soluble glycolipid analogues.—Deacyl-SGC[42] or deacyl-GC (0.5 mg) were dissolved in 5:1 CH_3CN–Et_3N (1 mg/mL) and adamantyl acetic acid (1:1 by weight), HOAT (1:1 by weight) and solid EDAC (1 mg) were added in the given order. The reaction mixture was heated at 50 °C and monitored by TLC (65:25:4 $CHCl_3$–MeOH–H_2O^S) every 2 h. Once all the deacyl-SGC was consumed, 2 mL of NH_3 (2 M solution in EtOH) was added and dried under a stream of nitrogen. Crude product was dissolved (1 mL of 98:2 $CHCl_3$–MeOH) and loaded on to a neutral alumina column (5 × 1 cm, 98:2 $CHCl_3$–MeOH) and washed (90:15:1 $CHCl_3$–MeOH–0.88% KCl) until all the HOAT was removed. The column was eluted with MeOH, dried, and adamantyl-SGC purified on a silica column.

3. Results

The glycone and aglycone moieties that constitute the glycolipids employed in this study are depicted in Fig. 1. The glycolipids are based on native 3′-sulfogalactosyl ceramide and differ in the position, number, and type of anionic ester and/or aglycone moiety. Bovine brain SGC ($I^3SO_3GalCer$) is comprised of a 2:1 mixture of saturated–unsaturated acyl chains (C_{24}), with the unsaturation occurring at C_{19}.[45] This differs from the synthetic SGC analogues, $I^3SO_3Gal^{18}Cer$ and $I^4SO_3GalCer$, whose ceramide fatty acid chains (C_{18}) are saturated. All other SGL derivatives have a hydrocarbon backbone consisting of two alkyl chains:B30.

Thin-layer chromatography overlay.—Hsc70 bound specifically SGG, SGC and the synthetic SGLs, $I^3SO_3GalCer$, $I^4SO_3GalB30$ and $I^4SO_3GalCer$ (Fig. 2). Very weak binding to $I^3SO_3GalB30$, and no binding to lyso-SGC was also observed. With the exception of $I^3SO_3GalB30$, GST45A exhibited similar SGL binding selectivity as observed for hsc70 (Fig. 2). Neither hsc70 nor GST-45A bound any of the multi-sulfated or multi-phosphorylated analogues (not shown).

Glycolipid RELISA.—Binding of hsc70 to the phospho- and sulfo-glycolipids was also assayed by RELISA (Fig. 3). Rank order of binding was $I^4SO_3Gal^{18}Cer > I^3SO_3Gal^{18}Cer > SGC = SGG > I^4SO_3GalB30 > I^{3,4}SO_3GalB30 > I^3SO_3GalB30$. Consistent with the TLC overlay, no binding was observed to any of the multi-phosphorylated compounds, nor to the 1-deoxynorjirimycin derivatives, *N*-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-glucitol and *N*-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-galactitol.

Dose-dependent hsc70 binding to, $I^3SO_3GalCer$, $I^4SO_3GalB30$ and $I^4SO_3GalCer$ was seen (Fig. 4), with saturation only occurring for $I^3SO_3GalCer$ and $I^4SO_3GalCer$. Very weak dose-dependent binding was seen for $I^3SO_3GalB30$.

The RELISA receptor function of each glycoconjugate for hsc70 was assessed relative to $I^4SO_3GalCer$, (Table 1).

Inhibition of hsc70-SGL binding.—TLC overlay binding of hsc70 to SGC and SGG was completely inhibited by 100 μM adamantylSGC while 1 mM of the nonsulfated derivative, adamantylGC had no inhibitory effect (Fig. 5(A)). The IC50s for

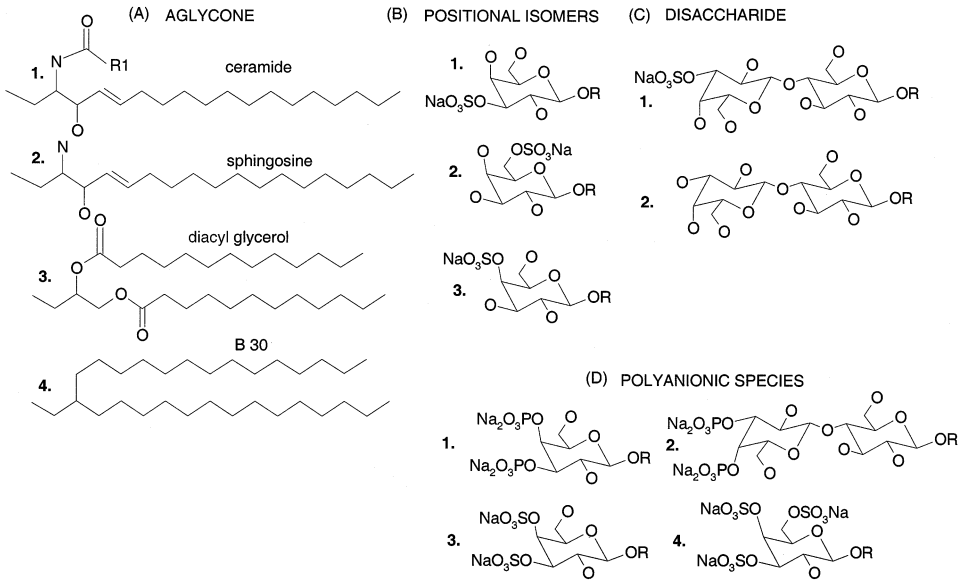

Fig. 1. Examples of aglycones and positional sulfate isomers used in current study.

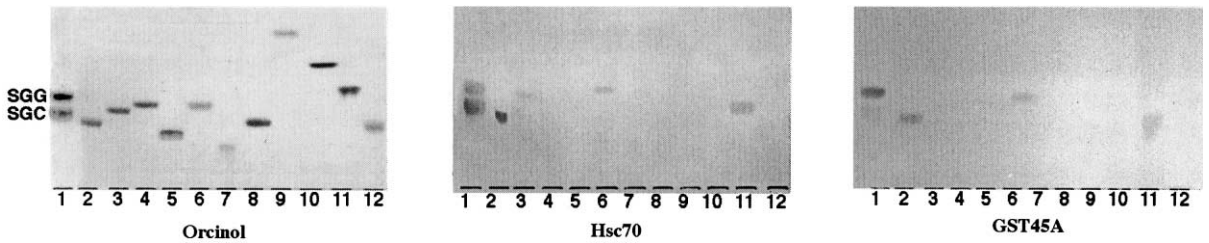

Fig. 2. Binding specificity of hsc70 and GST45A to SGC derivatives by TLC overlay. (a). Orcinol stain indicating the position of the SGC derivatives. (b) Hsc70 binds SGC/SGG, $I^3SO_3Gal^{18}Cer$, $I^4SO_3GalB30$ and $I^4SO_3Gal^{18}Cer$. Weak binding can also be observed to $I^3SO_3GalB30$. (c) GST45A binds specifically SGC, SGG, $I^3SO_3Gal^{18}Cer$, $I^4SO_3GalB30$ and $I^4SO_3GalCer$. Lanes: (1) SGC/SGG, (2) $I^3SO_3Gal^{18}Cer$, (3) $I^3SO_3GalB30$ (4) $I^3SO_3GluB30$ (5) $II^4SO_3LacB30$ (6) $I^4SO_3GalB30$ (7) $I^{3,4}SO_3GalB30$ (8) $I^6SO_3GalB30$ (9) GalB30 (10) LacB30 (11) $I^4SO_3Gal^{18}Cer$ (12) $I^3SO_3GalSph$.

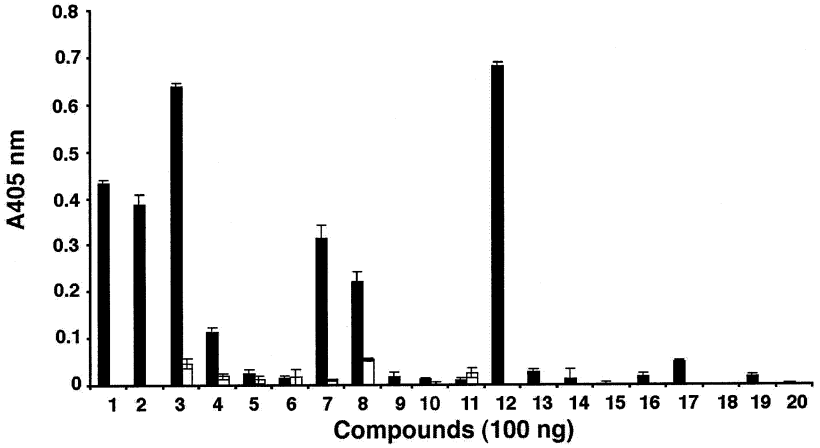

Fig. 3. RELISA of hsc70 and SGC derivatives. Binding of hsc70 (closed bars) to SCG, SGG, $I^3SO_3GalC^{18}Cer$, $I^4SO_3GalB30$ and $I^4SO_3GalCer$ and $I^{3,4}SO_3GalB30$. No binding was observed by the recombinant C-terminal substrate binding domain (28 kDa) of hsc70 (open bars). Lanes: (1) SGC, (2) SGG, (3) $I^3SO_3Gal^{18}Cer$, (4) $I^3SO_3GalB30$, (5) $I^3SO_3GluB30$, (6) $II^4SO_3LacB30$, (7) $I^4SO_3GalB30$, (8) $I^{3,4}SO_3GalB30$, (9) $I^6SO_3GalB30$, (10) GalB30, (11) LacB30, (12) $I^4SO_3GalCer$, (13) I^3SO_3Gal Sph, (14) *N*-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-glucitol, (15) *N*-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-galactitol, (16) $I^{2,3,4,6}SO_3GalB30$, (17) $I^{3,4,6}SO_3GalB30$, (18) $II^{3,4}PO_3LacB30$, (19) $I^{2,3,4,6}PO_3GalB30$, (20) $I^{3,4}PO_3GalB30$.

adamantylSGC inhibition of hsc70/SGL binding were determined by quantitative RELISA to be 50 μM for SGC and SGG and 75 μM for $I^4SO_3GalB30$ and $I^4SO_3GalCer$ (Fig. 5(B)).

4. Discussion

SGL recognition by both prokaryotic and eukaryotic members of the hsp70 family is dependent upon the 3′-sulfogalactose moiety linked to either ceramide or glycerol. The free 3′-sulfogalactose glycone is not recognized by hsc70,[14] while alterations in the ceramide fatty acid significantly altered the binding affinities of hsp70s for SGC.[7] The present study elaborates further on the structural features of SGL that determine the specificity of recognition by hsp70.

To determine whether the specificity is dependent upon the substitution position of the sulfate, binding of hsc70 to 3′-, 4′- and 6′-sulfogalactose-containing SGL was examined. The relative role of charge-dependent binding was evaluated using glycolipids derived from polysulfo- or polyphospho-galactosyl sugars. To elaborate on the role of aglycone modulation and receptor function, sulfogalactosyl sugars were conjugated to branched or linear hydrocarbon chains and their binding was compared to natural SGLs containing either ceramide or glycerol lipids (Fig. 1). Binding assays were performed on both charged-silica (TLC overlay) and hydrophobic-surfaces (microtiter plate RELISA), as the different solid-state properties of these surfaces might influence the binding specificity or affinity for adsorbed glycolipids.

Hsc70 and GST45A exhibited similar glycolipid-binding specificities when assayed by TLC overlay. These observations are consistent with our earlier finding that eukaryotic

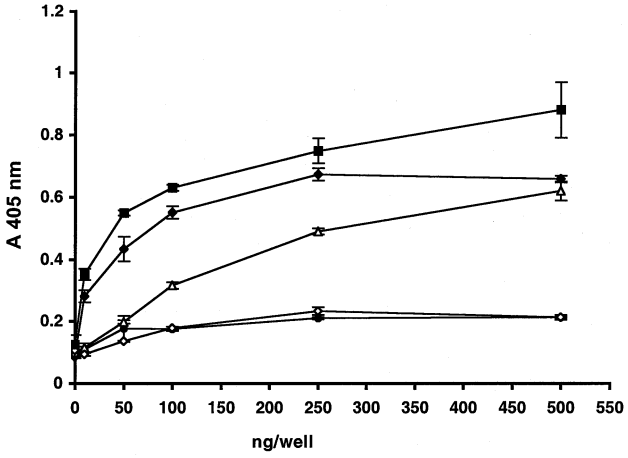

Fig. 4. Dose–response binding of hsc70 to SGLs by RELISA. At low concentrations, hsc70 preferentially binds the ceramide-based glycolipids, $I^4SO_3GalCer$, $I^3SO_3Gal^{18}Cer$. Saturation binding was achieved at approximately 100 ng of each. Only at higher concentrations did hsc70 exhibit saturation binding to $I^4SO_3GalB30$. Very weak saturable binding was observed to $I^3SO_3GalB30$. Open squares, $I^4SO_3GalCer$; circles, $I^3SO_3GalB30$; triangles, $I^4SO_3GalB30$; diamonds, $I^3SO_3GalCer$.

Table 1
Glycolipids functioning as hsc70 receptors in solid phase binding assays

Compound	Structures (based on Fig. 1)	Hsc70/glycolipid binding	
		TLC	RELISA (%)
I^3SO_3GalCeramide	A1B1, R = $(CH_2)_{22}CH_3$	++	63
I^3SO_3Gal1-*O*-alkyl-2-*O*-acylglycerol	A3B1	+++	56
$I^3SO_3Gal^{18}$Ceramide	A1B1, R = $(CH_2)_{16}CH_3$	++	94
I^3SO_3GalB30	A4B1	−	17
I^3SO_3GlcB30	ns	−	4
II^4SO_3LacB30	A4C1	−	3
I^4SO_3GalB30	A4B3	+	46
$I^{3,4}$ SO_3GalB30	A4D3	−	32
I^6SO_3GalB30	A4B2	−	3
$I^4SO_3Gal^{18}$Ceramide	A1B3	++	100
GalB30	ns	−	2
LacB30	A4C2	−	2
$I^{3,4,6}SO_3$GalB30	A4D4	−	7
$I^{2,3,4,6}SO_3$GalB30	ns	−	3
$I^{3,4}PO_3$GalB30	A4D1	−	0.6
$I^{2,3,4,6}PO_3$GalB30	ns	−	3
II ^{3,4} PO_3LacB30	A4D2	−	0
I^3SO_3GalSph	A2B1	−	4
N-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-glucitol	ns	−	2
N-decyl-1,5-dideoxy-1,5-imino-3-sulfo-D-galactitol	ns	−	0.3

All compounds employed in the solid-phase binding assays are listed above. Their chemical structures are based on the glycone and aglycone moieties depicted in Fig. 1. The superscript before SO_3 denotes position of the sulfate group; the superscript before Ceramide denotes the chain length of the *N*-acetyl group. Rank order of binding of hsc70 to the compounds, by TLC is summarized by weakest (−) to strongest (+++). Binding by RELISA is summarized as percentage of maximum binding of hsc70 to $I^4SO_3Gal^{18}$Cer.

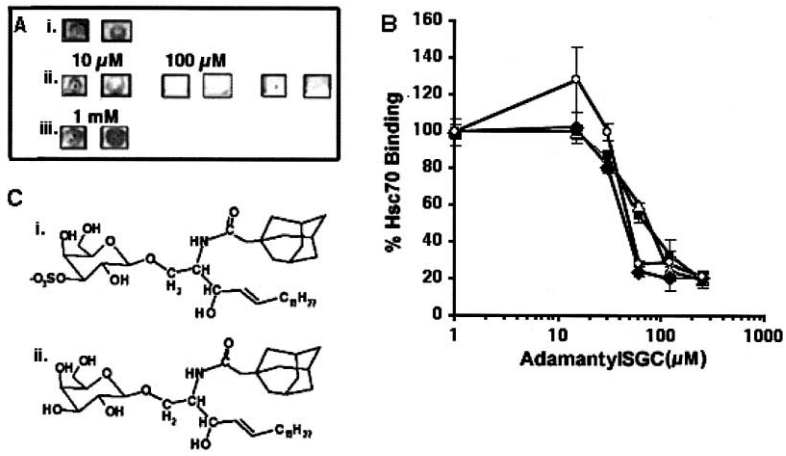

Fig. 5. Inhibition of hsc70 binding to SGC/SGG. (A) TLC overlay. TLC plates were prepared by spotting 5 μg of SGC/SGG in an area of 1 cm^2 and overlaying with hsc70 pre-treated with; (i) no inhibitor; (ii) adamantylSGC and (iii) adamantylGC (1 mM). Complete inhibition of hsc70 binding to SGC/SGG was observed with 100 μM adamantylSGC. (B) By RELISA, 50 and 75 μM of adamantlySGC were shown to be the effective IC_{50}s of hsc70 binding to the 3′- and 4′-sulfogalactosyl ceramides, respectively. Symbols ◆, SGC; ○, SGG; ■, I^4SO_3GalCer; △, I^4SO_3GalB30. (C) Structures of (i) adamantylSGC and (ii) nonsulfated derivative, adamantylGC.

and prokaryotic hsp70s share a specific sulfogalactolipid binding domain.[7] By both TLC and RELISA, hsc70 bound the 4′ positional isomer of SGC (non physiological) with affinity comparable to (if not greater than) natural 3′-SGC (Figs. 2 and 3, Table 1). Site

directed mutational analyses of hsc70 showed that Arg342, located in the ATP-binding domain, could be involved in SGC binding.[33] Thus, we propose that the sulfate group of either *cis*-3′-hydroxy-4′-sulfate or *cis*-3′-sulfate-4′-hydroxy moieties could interact with this Arg residue while the hydroxyl group could act as a hydrogen bond acceptor with another residue. Assuming the relative organization of these residues is perpendicular to the axis defined by the *cis*-sulfate-hydroxyl moiety, switching positions of the sulfate and hydroxyl units should minimally perturb the interaction. Binding is poorly accommodated by the 3′,4′-disulfate, showing the importance of the sulfate-adjacent hydroxyl group.

The effect of the aglycone on the presentation and receptor function of the 3′- and 4′-sulfogalactose moieties was tested with glycolipids containing either branched or linear hydrocarbon backbones that lack the serine-like or glycerol-like moieties (i.e. potential H-bond donors/acceptors) found in the interface (that is, adjacent to the glycosidic linkage) of natural SGC and SGG, respectively. Using the X-ray structure of galactosylceramide[46] as a model, we have proposed a possible role of interface organization in glycone presentation.[7] Hydroxyl and amide functional groups could form intra- and inter-molecular hydrogen bonds and lead to specific organizations around the interface. An organized interface in turn, could restrict the solvation and number of possible glycone conformations around the glycosidic linkage and also, interactions with amino acid residues adjacent to the sulfogalactosyl binding domain of hsc70 that is, subsite assistance.[47] Thus, the presentation of the sulfogalactosyl moiety for favourable receptor-recognition is a function of the sum of (*i*) low energy glycone conformation(s), (*ii*) orientation around the glycosidic linkage, (*iii*) the specific inter- and intra-molecular hydrogen-bond networks, and (*iv*) the solvation associated with the interface.

By RELISA, hsc70 exhibited strongest binding to $I^4SO_3Gal^{18}Cer$ (100%) and $I^3SO_3Gal^{18}Cer$ (94%), whereas binding to the B30-based glycolipids, $I^4SO_3GalB30$ and $I^3SO_3GalB30$, was significantly lower at 46 and 17%, respectively (Fig. 3 and Table 1). The reduced hsc70 binding affinity for $I^4SO_3GalB30$ relative to $I^4SO_3GalCer$ was more profound for $I^3SO_3GalB30$, as compared to $I^3SO_3GalCer$. In the dose response (Fig. 4) $I^3SO_3GalB30$ binding was barely above background. Thus the 3′-sulfate is selectively disfavoured in the context of the B30 lipid. Glycolipid-dose dependent RELISA gave similar binding curves for ceramide glycolipids ($I^4SO_3GalCer$, $I^3SO_3GalCer$) but a significantly different curve for the B30-hydrocarbon $I^4SO_3GalB30$ conjugate. Ceramide glycolipids showed rapid increase in binding with concentration and reached 80% saturation binding with 50 ng of lipids. However, for $I^4SO_3GalB30$, 80% binding was reached only at concentrations greater than 250 ng.

Although the monophosphorylated species were not tested, the finding that the $I^{3,4}PO_3GalB30$ shows no binding as compared to $I^{3,4}SO_3GalB30$ (Fig. 3) indicates that phosphate cannot substitute for sulfate.

Reduced binding of B30-hydrocarbon glycoconjugates is attributed to the lack of interface organization, resulting in unfavourable glycone presentation for recognition.[33,45] The serine-like and glycerol-like moieties of SGC and SGG could form extended water-mediated hydrogen-bonding networks which could lead to interface organization for favourable presentation of the glycone, even at low lipid concentrations, as observed for the SGC species (Fig. 4). However, when such H-bond mediated stabilising factors are lacking, as with $I^4SO_3GalB30$, packing for favourable glycone presentation is only achieved at higher lipid densities (Fig. 4), indicating that hydrocarbon- and ceramide-based glycones can confer similar receptor binding capacity. Lipid packing effects could also contribute to the lower (than expected) binding of $I^3SO_3GalB30$ glycoconjugate.

The packing organization(s) of the ceramide-based glycolipids, are not as influenced by minor variations in the glycone, such as the 3′ and 4′ positional isomers, as they are by the interfacial hydrogen-bond network. However, $I^3SO_3GalB30$ and $I^4SO_3GalB30$ positional isomers could alter the packing of B30-like hydrocarbon chains, as optimum presentation is

only achieved (for $I^4SO_3GalB30$) at higher packing densities. Compounded by the lack of interface organization, the $I^3SO_3GalB30$ and $I^4SO_3GalB30$ hydrocarbon glycolipids could proceed along different 'paths'[48] as packing density increases, modulated by the 3′-equatorial vs 4′-axial positioned sulfate. The path by which $I^3SO_3GalB30$ proceeds yields a poor presentation of the sulfogalactose moiety.

Hsc70 bound poorly to the disulfated compound, $I^{3,4}SO_3GalB30$ by RELISA (Fig. 3), whereas no binding was observed by TLC overlay (Fig. 2). This disparity could be attributed to differences in the adsorption isotherms of these compounds on silica versus plastic surfaces. Weak binding observed for $I^{3,4}SO_3GalB30$ by RELISA is, nevertheless, consistent with the requirement of *cis*-3-hydroxy-4-sulfo or *cis*-3-hydroxy-4-sulfo moiety, where a 3,4-disulfated species could be accommodated as well. The reduced affinity of the disulfated compound, relative to the monosulfated 3′- or 4′-sulfo species could be due to charge and/or steric repulsion effects and clearly demonstrates that SGL binding is not solely charge based.

$I^3SO_3GalSph$, whether adsorbed on a silica or plastic matrix did not bind hsc70. Although this might be attributed to the importance of the fatty acid in the overall organization of aglycone, the verotoxin/globotriaosylceramide interaction, also modulated by fatty acid composition,[35] bound strongly to deacyl Gb_3 adsorbed on a plastic matrix.[49] These distinct trends in aglycone-mediated receptor function are likely determined by the patterns in which the aglycones could be organized. For $I^3SO_3GalSph$, the charged amino function may interfere with the sulfate binding. However the lack of hsc70 binding was maintained even when coupled to BSA via this amine.[7] The inability of hsc70 to bind $II^4SO_3LacB30$ could be due to lack of aglycone mediated sub-site interaction(s); which are sterically prevented by the glucose residue located between the sulfogalactose and aglycone.

In the case of verotoxin-Gb_3 binding, an inhibitor designed by substituting the fatty acid of Gb_3 with a rigid adamantyl ring was soluble and gave an IC_{50} in the low micromolar range.[50] Using similar methodology, an adamantyl derivative of SGC was synthesized which exibited similar solubility properties as adamantylGb_3.[51] AdamantylSGC inhibited the binding of hsp70 to 3′- and 4′-sulfogalactosyl ceramide with an IC_{50} of 50 and 75 μM, respectively. The small difference in IC_{50}s could be attributed to a slightly higher affinity of hsp70 for the 4′-sulfogalactosyl species.

SGL recognition by hsp70s is highly specific given the strict requirements for either 3′- or 4′-sulfogalactose and that the desulfated derivatives GC and GG, nor other negatively charged (gangliosides) or sulfated (cholesterol sulfate) lipids are not recognized. Interestingly, a similar pattern of binding specificity is observed for GST45A, a recombinant domain of the sperm receptor from the sea urchin, *Strongylocentrus purpuratus*, possessing approximately 50% homology with hsp70[12] (Fig. 2). The lack of binding to $I^3SO_3GalB30$ by TLC could be due to differences in the SGL-binding subsites between the hsp70 family members.[33]

The mono- and poly-sulfated positional galactose isomers employed in this study have also been used to evaluate the sulfatide binding specificity of E and P selectins.[52] Selectin–sulfatide binding is entirely distinct from that of hsp70. Neither hsp70s nor selectins contain the sulfatide binding consensus sequence,[32] however, selectin binding is largely charge-dependent, with the polysulfated analogues showing highest activity while the lipid backbone plays only a minor role in the interaction.[52] However, these and other studies[53–55] involved milligram amounts of SGLs and their solubility comes into question. In contrast the polysulfated species are not bound by hsp70 and the aglycone plays a significant modulatory role. The generation of a soluble SGC mimetic[51] can effectively inhibit hsp70/SGL interactions. This is consistent with the concept of a specific SGL binding site within the hsp70 N-terminus[33] with specific coordination for sulfate binding and not an exclusive charge-mediated interaction.

The specificity of hsc70/SGL binding is contained within the N-terminal ATPase domain and involves F198 and R342.[33] This is the hinge region of ATPase domain[56] and SGL binding may therefore be dynamic, affected by

the conformation of the protein (i.e., in the ATPase cycle[57]).

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