3′Sulfogalactolipid Binding Specifically Inhibits Hsp70 ATPase Activity in Vitro†

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ABSTRACT: A method for the generation of soluble glycosphingolipid derivatives that retain the receptor activity of the parent (BBRC 257:391−394, Carb Res 335:91−100) was used to investigate the consequence of 3′sulfogalactolipid (SGL) specific binding within the N-terminal ATPase-containing domain of Hsc70. Sulfogalactosyl ceramide (SGC) was deacylated, and the resulting sulfogalactosylsphingosine coupled to an α-adamantane or a norbornane rigid hydrophobic frame. The resulting conjugate preferentially partitioned into water, as opposed to organic solvent. In the range of 100−300 μM, these conjugates inhibited the specific binding of bovine brain Hsc70 to immobilized SGLs. A similar dose-related inhibition of bovine brain Hsc70 ATPase activity was seen between 100 and 300 μM adamantylSGC (adaSGC). Adamantyl conjugates of glycolipids not bound by Hsp70s had no effect. Kinetic analysis indicated that adaSGC was a noncompetitive inhibitor of Hsc70 ATPase activity, a special case of mixed inhibition since the K_m values were not statistically different, 0.89 ± 0.024 μM to 0.93 ± 0.038 μM, but the V_{max} decreased from 0.20 ± 0.012 pmol min^{-1} μg^{-1} to 0.15 ± 0.016 pmol min^{-1} μg^{-1}. A reproducible 5 min lag was observed prior to ATPase inhibition that could be eliminated by preincubation of adaSGC with Hsc70 or by adding the cochaperone Hdj-1. The dependence of ATPase inhibition on the rate of hydrolysis indicates that adaSGC binding occurs at a specific stage of the ATPase cycle. These studies identify a new mechanism for the regulation of Hsp70 ATPase activity.

Members of the HSP70 family are intracellular chaperones under both normal and stress conditions in all cells (*1*−*4*). Hsp70s are comprised of two functionally coupled domains, the N-terminal ATPase domain and the C-terminal protein binding domain. Binding and hydrolysis of ATP in the N-terminal domain has been linked to a conformational change in the C-terminal protein binding domain leading to tight binding of hydrophobic exposed segments of proteins undergoing Hsp70-assisted folding (*5*). The N-terminal ATPase activity is central to Hsp70 chaperone activity (*6*) and is regulated by several cochaperones. Cochaperones such as DnaJ (*7*, *8*) (Hdj-1 in eukaryotes) and Hip (*9*) are positive regulators of the ATPase cycle and function by maintaining the ADP conformation of Hsp70, which has a high affinity for protein substrates. DnaJ stimulates the hydrolysis of ATP forming the Hsp70−ADP conformation (*10*), and Hip binds to and maintains this conformation (*11*). The activity of Hip can be competed with the nucleotide exchange factor Bag-1 (*12*), a negative regulator of the Hsp70 ATPase cycle that promotes the exchange of ADP for ATP leading to the release of protein bound substrates. The C-terminal Hsp70-interacting protein (CHIP) is another negative regulator of the ATPase cycle that inhibits Hdj-1 stimulation of the ATPase activity of Hsp70 (*13*).

The majority of Hsp70s are cytosolic, but in eukaryotes additional Hsp70s are found in the ER, nucleus, mitochondria, and lysosomes (*14*−*16*). Hsp70 chaperone activity functions as quality control in the folding, translocation, degradation, and cellular sorting of newly synthesized or unfolded proteins. Small subpopulations of Hsp70s have been located at the plasma membrane (*17*−*19*), and these surface located Hsp70s can elicit cellular immune responses (*20*). We have previously shown eukaryotic and prokaryotic cell surface associated Hsp70s can function as adhesins by specific binding to the 3′sulfogalactolipids (SGL)[1], sulfogalactosylceramide (SGC), and sulfogalactoglycerolipid (SGG). Our initial studies were concerned with the SGG binding of a 68 kDa testicular cell surface protein (*21*), later identified as the testes-specific cognate Hsc70 (P70) (*22*). Mycoplasma

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[1] Abbreviations: SGC, sulfogalactosylceramide; SGG, sulfogalactosylglycerolipid; SGL, sulfogalactolipid; SGS, sulfogalactosylsphingosine, deacylated SGC; GalC, galactosylceramide; adaSGC, *N*-adamantyl SGS, adamantyl SGC; Hsp, heat shock protein; Hsc, constitutively expressed cognate heat shock protein; P70, testes specific cognate Hsc70; rP70, recombinant P70; TLC, thin-layer chromatography; HOAT, 1-hydroxy-7-azabenzotriazole; EDAC, 1-ethyl-3(3-dimethylamino-propyl) carbodiimide; RELISA, receptor enzyme linked immunosorbent assay; BSA, bovine serum albumin; TBS, Tris buffered saline; PIBM, polyisobutyl methacrylate; Gb_3, globotriaosylceramide; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid; $CHCl_3$, chloroform; MeOH, methanol; CH_2Cl_2, dichloromethane; HCl, hydrochloric acid; CH_3CN, acetonitrile; EtOH, ethanol; NH_3, ammonia; H_3PO_4, phosphoric acid; Et_3N, triethylamine; Et_2O, ethyl ether; KOH, potassium hydroxide; NaCl, sodium chloride; CH_3COONa, sodium acetate; DTT, dithiothreitol; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); CMLA, bovine carboxymethyl α-lactalbumin.

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SGL binding in vitro (*23*, *24*) was prevented by pretreatment with anti-Hsp70 antibodies and recombinant mycoplasma Hsp70 bound SGL (*25*). Brief stress treatment of *Helicobacter pylori* (*26*, *27*) or *Hemophilus influenzae* (*28*) induced SGL binding, inhibited by anti-Hsp70. Subsequently, cell surface associated SGL-binding Hsp70s have been identified on *H. influenzae* after but not prior to heat shock (*29*).

Recombinant Hsp70s from the prokaryotes *Escherichia coli*, *Chlamydia trachomatis*, *H. pylori*, mycoplasma, and *H. influenzae* and the eukaryotic recombinant Hsp70s, P70, Hsc70, and BiP specifically bind to SGL (*30*). Truncation analysis localized the SGL binding site to the highly conserved N-terminal ATPase domain of Hsp70s (*31*). Sequence homology in this domain is 60−70% indicating that SGL recognition may be an important conserved function among Hsp70s.

Hsp70/SGL binding, though dependent on the glycolipid carbohydrate moiety (*32*), is not inhibited by the lipid-free 3′sulfogalactose (*22*). The lipid moiety differentially affects the SGL binding of different Hsp70s (*30*, *32*). In this respect, the binding is similar to that we have described for verotoxin and its glycolipid receptor, globotriaosyl ceramide (Gb_3) (*33*−*35*). We developed a novel procedure to generate a water-soluble analogue of Gb_3 that, unlike the lipid-free Gb_3 oligosaccharide, retained high affinity verotoxin binding (*36*). We have now used this procedure to develop similar water-soluble analogues of SGC (*32*) and investigated their ability to inhibit Hsc70/SGL binding. Amino acids important for SGL binding were identified within the ATPase cleft (*31*). Therefore, we investigated the effect of these soluble SGC analogues on Hsc70 ATPase activity.

EXPERIMENTAL PROCEDURES

Proteins and Antisera. Bovine brain Hsc70 (90% Hsc70 and 10% Hsp70) (SPP-750), recombinant human Hdj-1 (>90% pure) (SPP-400), and rat anti-Hsc70 monoclonal antibody (SPA-815) were purchased from StressGen Biotechnologies Corp. (Victoria, BC). Horseradish peroxidase conjugated rabbit anti-rat IgG (A5795), CMLA, and BSA were purchased from Sigma (St. Louis, MO).

Solvents. $CHCl_3$, MeOH, CH_2Cl_2, HCl, CH_3CN, and Et_2O were purchased from Caledon (Georgetown, ON) or Aldrich (Milwaukee, WI), and EtOH was purchased from Commercial Alcohols Inc. (Brampton, ON). NH_3, H_3PO_4, and Et_3N were obtained from Sigma (St. Louis, MO).

Reagents. NaCl and KOH were purchased from Fisher Scientific Company (Fair Lawn, NJ), and activated charcoal was obtained from BDH Chemicals (Toronto, ON). EDAC, CH_3COONa, ABTS, and PIBM where purchased from Sigma (St. Louis, MO). Adamantaneacetic acid and norbornaneacetic acid were purchased from Aldrich (Milwaukee, WI), and HOAT was purchased from Fluka (Oakville, ON).

Chromatography. Silica gel 60 (40−63 μM or 230−400 mesh) and aluminum-backed nanosilica plates (Alugram NanoSIL G UV_{254}, Macherey-Nagel) were purchased from Caledon (Georgetown, ON), and reverse-phase C-18 cartridges were purchased from Waters (Mississauga, ON).

Glycolipids. Purified SGC and GalC were purchased from Sigma, or SGC was purified from bovine brain extract (*37*) (Sigma, Cat #B1014), which contains 10% SGC, 30% GalC, 30% sphingomyelin, and some gangliosides. SGG was purified from bovine testes (*38*). Evergreen microtiter plates were purchased from DiaMed Lab Supplies Inc. (Mississauga, ON).

Protein Expression and Purification. Recombinant wild-type Hsc70 was expressed in *E. coli* BL21 (DE3) using the plasmid pRSET-Hsc70 (*39*) and purified to >95% homogeneity (*40*, *41*). Cultures were grown in Luria−Bertani broth at 37 °C until the optical density of 0.6 at 600 nm and induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Cells were harvested by centrifugation (5000 rpm for 10 min) and lysed by incubation with lysozyme (0.3 mg/mL) at 4 °C for 45 min in 20 mM Tris-HCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride at pH 8.0 followed by sonication. Cell debris was removed by centrifugation for 30 min at 20 000 rpm and for 15 min at 17 000 rpm. The supernatant was loaded onto a Q-Sepharose (Amhersham-Pharmacia Biotech AB, Uppsala, Sweden) column, and Hsc70 was eluted with a linear gradient of 0.0−1.0 M KCl. Hsc70 was dialyzed overnight against 20 mM HEPES (pH 7.0), 25 mM KCl, and 10 mM EDTA. After the addition of $Mg(CH_3COO)_2$ to a final concentration of 25 mM, recombinant Hsc70 was purified using ATP-agarose (Sigma). Hsc70 was dialyzed against 25 mM Bis-Tris (pH 6.3) and loaded onto a MonoP isoelectric focusing column followed by gel filtration using Superdex-75 with 20 mM MOPS (pH 7.0), 150 mM KCl, and 3 mM $MgCl_2$ (Amhersham-Pharmacia Biotech AB, Uppsala, Sweden). Recombinant Hsc70 was concentrated to 4−6 mg/mL, aliquoted, and stored at −70 °C.

Synthesis of AdaSGC. SGS was prepared from SGC according to Koshy and Boggs (*42*). α-Adamantaneacetic acid (or 2-norbornaneacetic acid) (1.3 mg, approximately 2 equiv), HOAT (30 μL of 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 SGS (2 mg, 4 μmol) in 5:1 CH_3CN/Et_3N (2 mL) and stirred at 60 °C for 3 h. The progress of the reaction was monitored by TLC ($CHCl_3$/MeOH/0.88% KCl; 65:25:4), and upon completion the reaction mixture was dried under a stream of N_2. The crude product was dissolved in $CHCl_3$/MeOH (98:2) and loaded on to a silica column (0.5 × 10 cm of silica gel $CHCl_3$/MeOH; 98:2). HOAT and excess adamantane acetic acid were eluted with CH_3COOH/$CHCl_3$ (4:1). Product was eluted with $CHCl_3$/MeOH/H_2O; 80:20:2 (6 and 4 mL fractions were collected). The estimated yield by TLC was 70% (*32*).

Thin-Layer Chromatography Overlay Assay of SGL Binding. The binding of bovine brain Hsc70 to SGL was determined by using the TLC overlay assay described (*22*, *43*). Glycolipids (5 μg) were separated on TLC plates ($CHCl_3$/MeOH/0.88% KCl; 65:25:4) and dried, and one plate was stained with oricinol. The remaining plates were treated with 0.5% (v/v) PIBM in hexane. The dried plates were sprayed with 1% (w/v) BSA in 50 mM TBS and incubated upside down in the same BSA buffer for 1 h. Hsc70 (5 μg/mL) in BSA buffer was then incubated with the plates for 2 h. The plates were then washed with PBS (4×) and incubated with rat anti-Hsc70 monoclonal antibody (1 mg/mL 1:500 in BSA buffer) for 1 h. After washing as above, the plates were incubated for 1 h with horseradish peroxidase conjugated rabbit anti-rat IgG (4 mg/mL 1:2000 in BSA buffer).

The plates were washed again with PBS (4×) before developing with 4-chloro-1-naphthol. For inhibition studies, SGC was separated using TLC, and bands were cut out after detection with iodine. The plate sections were incubated with bovine brain Hsc70 preincubated with increasing concentrations of adaSGC for 1 h. Binding was visualized as above. All steps were performed at room temperature.

Receptor ELISA (RELISA) Binding. Hsc70/SGL binding was quantitated using the RELISA (*30*) with the following modifications: SGC (50 μL in EtOH) was dried overnight at room temperature in Evergreen microtiter plate wells. The plates were then blocked with 180 μL of 2% (w/v) BSA in 50 mM TBS followed by washing (2×) with 0.2% (w/v) BSA in 50 mM TBS (wash buffer). Hsc70 (50 ng/100 μL) in wash buffer was added to the wells and incubated for 2 h. After washing (3×) with wash buffer, 180 μL/well rat anti-Hsc70 monoclonal antibody (1 mg/mL, 1:750 in 0.2% (w/v) BSA in 50 mM TBS) was added and incubated for 1 h. The plates were washed as above and then incubated with 180 μL/well horseradish peroxidase conjugated rabbit anti-rat IgG (4 mg/mL, 1:2000 in 0.2% (w/v) BSA in 50 mM TBS) for 1 h. The wells were washed again with wash buffer and then once quickly with 50 mM TBS. The microtiter plates were developed in the dark with 100 μL/well freshly prepared ABTS solution (0.5 mg/mL ABTA in citrate−0.1 M phosphate buffer (pH 4.3), 1 μL hydrogen peroxide/10 mL), and the optical density was determined at 405 nm. All steps were performed at room temperature. For inhibition, increasing concentrations of adaSGC were mixed with the Hsc70 1 h prior to either binding assay.

ATPase Assay. ATP hydrolysis by Hsc70 was determined by measuring the release of $^{32}P_i$ from [γ^{32}-P]ATP according to the procedure of Sadis and Hightower (*44*) with the following modifications. Soluble glycosphingolipid (GSL) derivatives (i.e., adaSGC) were dried under N_2 in an Eppendorf tube, and 5 μM ATP, 0.1 μM CMLA, and 2 μCi [γ^{32}-P]ATP (3000 Ci/mM) in buffer A (20 mM Hepes-KOH (pH 7.0), 25 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $Mg(OAc)_2$, and 1 mM DTT) was added to the tube on ice and then vortexed vigorously. To start the reaction, 0.1 μM Hsc70 was then added to the reaction tube. For experiments requiring the DnaJ cochaperone, Hdj-1, 0.8 μM was added. When adaSGC (or other soluble glycosphingolipid analogues) and Hsc70 were preincubated, adaSGC was dried in an Eppendorf tube, and half the volume of buffer A required was added containing 0.1 μM Hsc70 and 0.1 μM CMLA. The reaction mixture was vortexed and then incubated at room temperature for 1 h. The [γ^{32}-P]ATP and ATP were then added in an equal volume of buffer A to start the reaction. The *Prism* 3.0 program (Graphpad, San Diego, CA) was used for curve fitting to estimate the K_m and V_{max} of bovine brain Hsc70. The significant difference between the kinetic constants was determined using an independent samples t-test with $P < 0.05$.

RESULTS

Synthesis of AdamantylSGC, NorbornaneSGC. SGC was deacylated, and the amine generated was coupled to the carboxyl group of either 2-adamantane or 2-norbornane acetic acid using EDAC/HOAT activation (Figure 1A). Adamantyl-GC and adamantylGb_3 were similarly prepared (*31*). The structures of adamantylSGC and norbornaneSGC were confirmed by electrospray mass spectrometry. AdamantylSGC (Figure 1B) shows parent ions at 716 (M − H) (negative mode), and norbornaneSGC (Figure 1C) shows parent ions at 678 (M + H), 700 (M + Na), and 722 (M + 2Na − H) (positive mode), respectively.

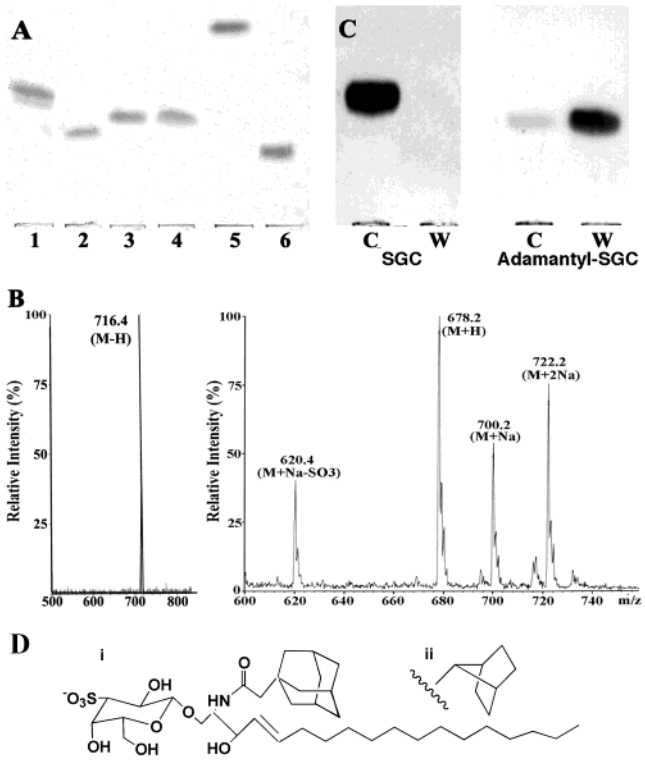


FIGURE 1: Characteristics of glycolipid derivatives. (A) TLC separation of glycolipids and derivatives visualized with orcinol. Lane 1, SGC; 2, SGS; 3, adaSGC; 4, norbornaneSGC; 5, norbornaneGC; and 6, adamantylGb_3. (B) Electrospray mass spectroscopy of adamantylSGC and norbornaneSGC; assignments of (i) negative mode spectrum of adaSGC and (ii) positive mode spectrum of norbornaneSGC. (C) Solubility of adaSGC. TLC separation of equivalent aliquots of SGC or adaSGC partitioned between chloroform (C) and water (W). (D) (i) Structure of adamantylSGC and (ii) structure of norbornaneSGC.

Properties of adaSGC. AdaSGC was compared with SGC for relative partitioning between water and chloroform (Figure 1C). SGC partitioned completely into the organic phase, while >90% of the adaSGC was recovered in the aqueous phase. Thus, adaSGC is essentially water soluble.

AdaSGC Inhibition of Hsc70/SGL Binding. The SGL binding specificity of bovine brain Hsc70, previously shown by RELISA (*25*), was confirmed by TLC overlay. Only SGC and SGG were recognized (Figure 2A, part ii). A linear dose response for SGC binding was observed for Hsc70 up to 100 ng of SGL by RELISA (Figure 2B). Preincubation of Hsc70 with adaSGC inhibited subsequent SGC binding by RELISA (Figure 2C) with an IC_{50} of ~80 μM. Similarly, adaSGC inhibited Hsc70/SGC binding as monitored by TLC overlay (Figure 2C).

AdaSGC Inhibition of Hsc70 ATPase. The ATPase activity of bovine brain Hsc70 was linear with enzyme concentrations of 0.05 and 0.2 μM at 5 μM ATP (not shown) up to 60 min and with ATP concentration up to 2.0 μM. Preincubation of the Hsc70 with SGC liposomes significantly inhibited

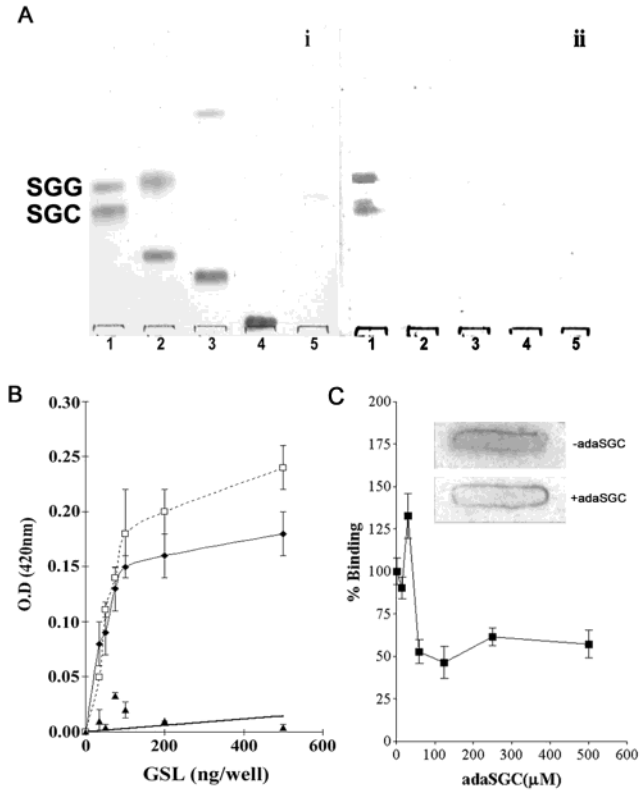


FIGURE 2: Glycolipid binding specificity of bovine brain Hsc70. The GSL binding specificity of bovine brain Hsc70 was monitored by (A) TLC overlay. Part i: glycolipids visualized with orcinol, (from the top) lanes 1, SGG, SGC; 2, LC, Gg_3; 3, GalC, Gb_4; 4, GM1; and 5, cholesterol sulfate. Part ii: bovine brain Hsc70 binding specifically to SGG/SGC. (B) RELISA -binding of brain Hsc70 to □ SGG; ◆ SGC; and ▲ GC. (C) AdaSGC inhibition of Hsc70 RELISA binding to SGC. Hsc70 binding to SGC by TLC overlay ±300 μM AdaSGC (insert). Concentrations >50 μM were inhibitory, and representative results are shown. Error bars represent the standard deviation of the mean for three independent experiments in triplicate.

Table 1: Kinetic Constants for the Hydrolysis of ATP by Recombinant Bovine Brain Hsc70 with and without 300 μM AdamantylSGC[a]

	control	adamantylSGC	*P* value
K_m (μM)	0.89 ± 0.024	0.93 ± 0.038	0.8927
V_{max} (pmol min^{-1} μg^{-1})	0.20 ± 0.012	0.15 ± 0.016	0.0032

[a] The kinetic constants were determined by fitting the equation $Y = V_{max} \times X/[K_m + X]$ to the initial linear velocities measured as a function of ATP concentration by nonlinear regression. The statistical difference between the kinetic constants was determined by an independent samples t-test with $P < 0.05$.

(300 μM—76%, 600 μM—89%, 1500 μM—94%) the reaction in a dose-dependent manner, while liposomes of Gb_3 (not bound by Hsc70—Figure 2A, part ii) were inhibited by <12% (Figure 3A). Quantitative analysis of inhibition was achieved using adaSGC, which caused a dose- and time-dependent reduction of ATPase activity (Figure 3B). Little inhibition was observed within the first 5 min, but the ATPase activity was substantially suppressed within the subsequent 20 min of the assay. A similar inhibitory activity was seen for both adamantyl and norbornaneSGC (72 and 56%, respectively, at 300 μM), whereas neither norbornaneGC nor adamantylGb_3 had an inhibitory effect (Figure 3C). The K_m and V_{max} for Hsc70 ± adaSGC were estimated by fitting a curve of the Michaelis−Menten equation, $Y = V_{max} \times X/[K_m + X]$, to initial linear velocities measured as a function of ATP concentration (Figure 3D). When adaSGC was incubated with Hsc70 and ATP, the K_m was not statistically different (P value = 0.8927), but the V_{max} decreased (P value = 0.0032) indicating that adaSGC is a noncompetitive inhibitor of Hsc70 ATPase activity, a special case of mixed inhibition (Table 1).

Potentiation of adaSGC Inhibition of Hsc70 ATPase by (A) Preincubation: Typically, inhibition of Hsc70 ATPase activity by adaSGC was not observed in the initial phase of the reaction, consistent with dependence on ATP hydrolysis. However, when Hsc70 and adaSGC were preincubated together for 60 min at 25 °C prior to ATP addition, hydrolysis was reduced from the reaction onset (Figure 4A). Without preincubation, a 5 min delay before ATPase inhibition was observed (cf. Figure 3B,C). (B) Hdj-1 addition: In the presence of the cochaperone, DnaJ (Hdj-1), the ATPase rate of Hsc70 was increased 5-fold, and adaSGC inhibition was rapidly observed (Figure 4B). When both Hdj-1 and adaSGC were added to the reaction mixture, inhibition of Hsc70 ATPase activity was measured before 5 min. Hdj-1 stimulation of ATPase activity was retained even when preincubated with Hsc70 and adaSGC (Figure 3B).

DISCUSSION

Hsp70 binding to SGL requires both the 3′sulfogalactose and the lipid backbone (*22*, *30*). SGC liposomes were used to specifically inhibit Hsc70 ATPase activity, but this effect is difficult to analyze. First, at the higher concentrations of lipid there was nonspecific inhibition by globotriaosylceramide (Gb_3) liposomes because of the turbidity of liposome preparation. Second, the liposomes were multilamellar, which does not allow for quantitation of the SGC available for Hsc70 binding. Last, the kinetic analysis is confounded by the two-phase system of a soluble enzyme interacting with essentially a solid inhibitor. We therefore synthesized a water-soluble analogue of native SGC that mimics the structural and functional features of the natural glycolipid as we had previously done for Gb_3 (*36*) by exchanging the hydrophobic fatty acid for a hydrophobic adamantane cage SGC (*32*). We expanded the procedure by generating similar water-soluble derivatives of norbornane, a smaller rigid hydrophobic frame. The addition of the adamantane overcame the insolubility of the glycolipid by weakening the lateral hydrophobic interactions while maintaining some of the rigid crystalline order of the membrane and solvation of the oligosaccharide (*45*). AdaSGC reduced the binding of the testes specific Hsc70 to both 3′ and 4′ SGC (*32*) and in the present study, the binding of bovine brain Hsc70 to SGC. Thus, the adamantylsphingosine moiety mimics the role of the lipid of SGL to facilitate Hsc70 recognition of the carbohydrate. These analogues were designed to retain the interface region (*30*) of the GSL between the hydrophobic and the hydrophilic domains.

The water-soluble SGC analogues we have made inhibited Hsc70 ATPase activity, while similar analogues of GalC and Gb_3, not bound by Hsc70, had no effect. AdaSGC inhibited Hsc70/SGL binding and Hsc70 ATPase activity and is therefore a useful tool to study Hsc70 function. The

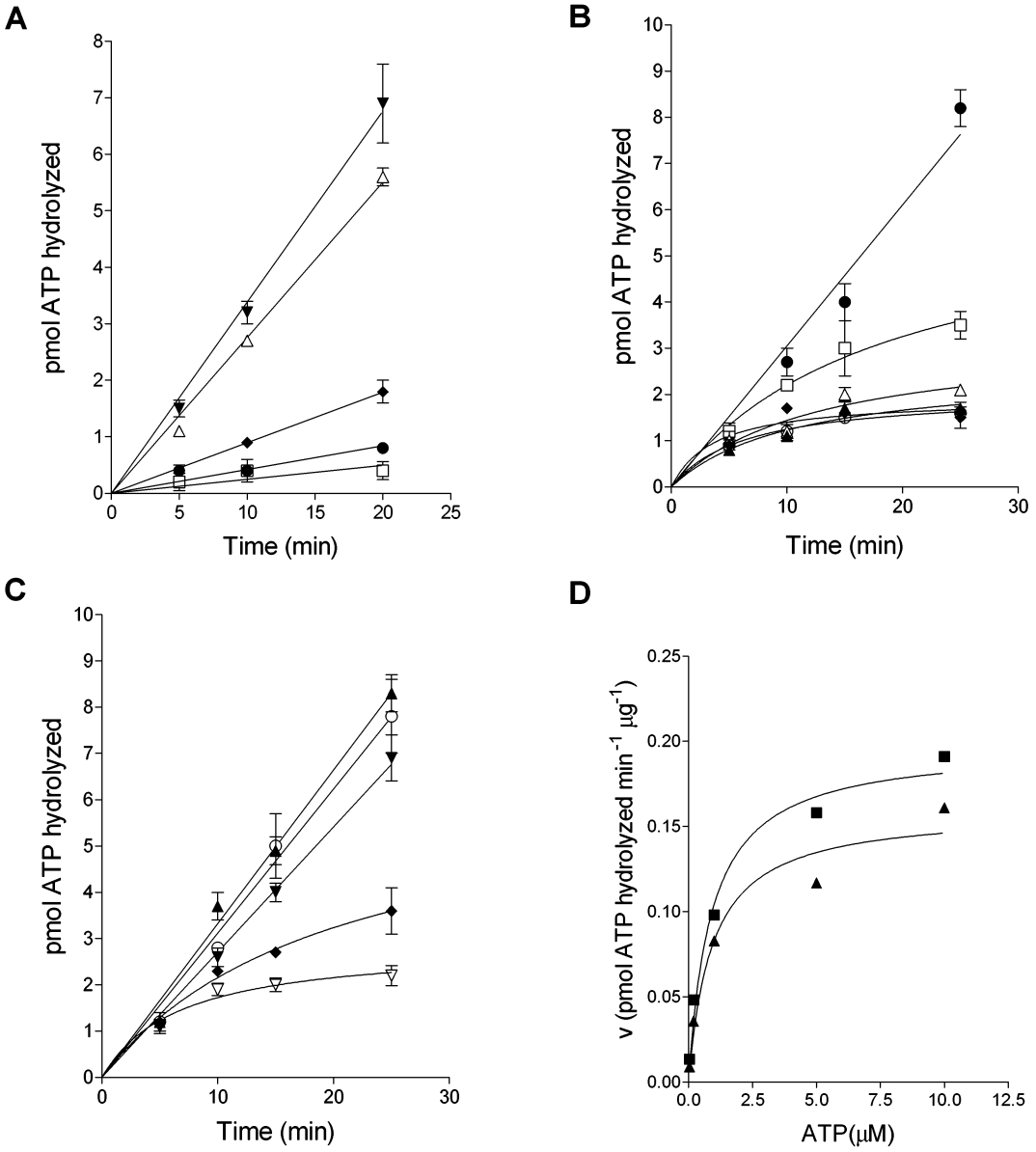
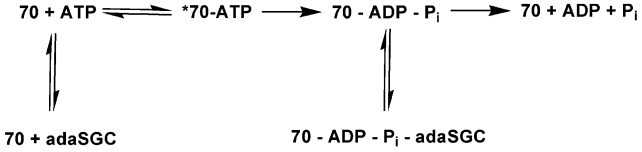


FIGURE 3: Sulfogalactolipid inhibition of Hsc70 ATPase activity. (A) Inhibition by native SGC. ▼ Hsc70; △ Hsc70 + 300 μM Gb_3; ◆ Hsc70 + 300 μM SGC; ● Hsc70 + 600 μM SGC; □ Hsc70 + 1500 μM SGC. Preincubation of Hsc70 with SGC liposomes significantly inhibited Hsc70 ATPase activity, but Gb_3 liposomes do not. Error bars represent the standard deviation of the mean of three independent experiments in triplicate. (B) Inhibition by adamantylSGC. ● Hsc70; □ Hsc70 + 100 μM adaSGC; △ Hsc70 + 200 μM adaSGC; ◆ Hsc70 + 300 μM adaSGC; ▲ Hsc70 + 400 μM adaSGC; and ○ Hsc70 + 500 μM adaSGC. The ATPase activity of Hsc70 is inhibited 57 and 74% by the addition of 100 and 200 μM adaSGC, while 300−500 μM adaSGC inhibited the ATPase activity by an average of 80%. Error bars represent the standard deviation of the mean of three independent experiments in triplicate. (C) Specificity of inhibition of Hsc70 ATPase Activity by GSL derivatives. ○ Hsc70; ▼ Hsc70 + 300 μM $adaGb_3$; ▽ Hsc70 + 300 μM adaSGC; ◆ Hsc70 + 300 μM norbornaneSGC; and ▲ Hsc70 + 300 μM norbornaneGC. Only the SGC derivatives are inhibitory, with adaSGC being somewhat more effective at 72% as compared to the 56% inhibition by norbSGC. The nonsulfated form of norbSGC, norbornaneGC showed 0% inhibition, and $adaGb_3$ only inhibited by 12%. Error bars represent the standard deviation of the mean of three independent experiments in triplicate. (D) AdaSGC inhibition as a function of ATP concentration. ■ Hsc70 and ▲ Hsc70 + 300 μM adaSGC. The curve fit with the Michaelis−Menten equation, $Y = V_{max} \times X/[K_m + X]$ was used to estimate the ATPase K_m and V_{max} ± adaSGC. The data are representative of four independent experiments. The data presented in panels A−D were generated using recombinant bovine brain Hsc70 from one batch.

preferential partitioning of adaSGC into water clearly shows a remarkable increase in water solubility of the adamantylSGC as opposed to the native SGC species.

AdaSGC behaved as a noncompetitive inhibitor of Hsc70 ATPase activity, a special case of mixed inhibition, since the K_m was not significantly different, and the V_{max} decreased (*46*). According to this model, adaSGC does not change the affinity of the enzyme for substrate but affects the catalytic conversion of substrate to product. This special example of mixed inhibition requires that adaSGC can bind equally to the enzyme (Hsc70) and the enzyme/substrate complex (Hsc70−ADP−P_i) according to the following scheme:



Thus, adaSGC can interact at two stages within the ATPase cycle, but it is intuitively unlikely that adaSGC would bind

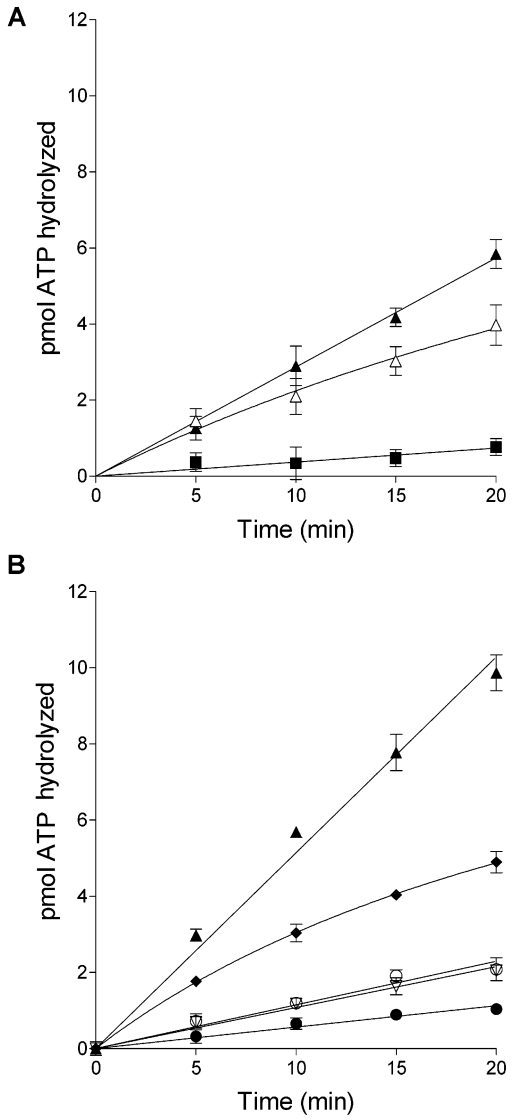


FIGURE 4: Effect of preincubation and the cochaperone Hdj-1 on adaSGC inhibition of Hsc70 ATPase. (A) adaSGC preincubation: ▲ Hsc70; △ Hsc70 + 100 μM adaSGC; and ■ Hsc70 preincubated with 100 μM adaSGC for 1 h at 25 °C prior to assay. Preincubation of Hsc70 and adaSGC removed the initial lag for inhibition and increased the inhibition of Hsc70 ATPase activity by adaSGC from 26 to 87%. Error bars represent the standard deviation of the mean of three independent experiments in triplicate. (B) Hdj-1 and Hsc70 were mixed with ATP and adaSGC as in Experimental Procedures: ▽ Hsc70; ▲ Hsc70 + 0.8 μM Hdj-1; ◆ Hsc70 + 0.8 μM Hdj-1 + 300 μM adaSGC; ○ Hsc70 + 0.8 μM Hdj-1 preincubated with 300 μM adaSGC for 1 h at 25 °C; and ● Hsc70 preincubated with 300 μM adaSGC for 1 h at 25 °C. Hdj-1 stimulated Hsc70 ATPase activity by approximately 5-fold but only by 2-fold in the presence of adaSGC. Error bars represent the standard deviation of the mean of three independent experiments in triplicate.

equally to both the enzyme and the enzyme/substrate complex as required for noncompetitive inhibition. To clearly distinguish these possibilities, direct binding experiments are required to obtain the K_d of adaSGC and are required to measure the affinity of adaSGC for Hsc70 as compared to Hsc70−ADP−P_i.

Mutation of Arg^{342} in rP70 showed significantly reduced binding to SGL in in vitro binding assays (*31*). The π-electrons of the guanidinium groups of Arg^{272} and Arg^{342} of Hsc70 form hydrophobic interactions with the adenosine ring of ATP to stabilize binding (*47*). If the binding site for adaSGC centers around Arg^{342}, adaSGC could compete with ATP for Hsc70 binding, and the observed decrease in V_{max} indicates that adaSGC binding to Hsc70 slows the conversion of substrate to product and the release of ADP. It would be of interest to study the effect a nucleotide exchange factor, such as Bag-1, would have on adaSGC inhibition of Hsc70 ATPase activity. The Hsc70/Bag-1 cocrystal (*47*) shows that Bag-1 forms electrostatic interactions with subdomains IB and IIB of the N-terminal ATPase domain of Hsc70 inducing a rotation of subdomain IIB and opening of the binding pocket allowing the exchange of ADP for ATP. Thus, the activity of Bag-1 could increase the dissociation of adaSGC from Hsc70 resulting in decreased inhibition.

The inhibition of Hsc70 ATPase activity by adaSGC was time-dependent but observed only after 5 min. Kinetic analysis of the Hsc70 ATPase cycle reported the binding and hydrolysis of ATP by Hsc70 to have a mean duration of approximately 5 min (*48*, *49*) and has been associated with a conformational change in the protein. It is possible that adaSGC binds to the ADP—in preference to the ATP—conformer of Hsc70, which would explain why no significant inhibition was detected in the first 5 min of the ATPase assay. As more Hsc70 binds and hydrolyzes ATP to give Hsc70−ADP−P_i, this would increase adaSGC binding and ATPase inhibition. By adding adaSGC before ATP, any competition for adaSGC binding to Hsc70 was removed. When ATP was added, adaSGC would hinder ATP binding to Hsc70 leading to the more rapid reduction of ATP hydrolysis. This is also consistent with the results obtained when Hdj-1 was added to the assay. The cochaperone, Hdj-1, increases the rate of ATP hydrolysis by Hsp70 leading to the increased production of Hsc70−ADP−P (*10*), which would result in more effective binding of adaSGC and a reduction in the lag period before inhibition of ATPase activity. The maintenance of Hdj-1 stimulated Hsp70 ATPase activity in the presence of adaSGC, and the retained sensitivity to inhibition are consistent with the binding of DnaJ to the more distal surface of the nucleotide binding cleft (*41*).

SGL binding is a new function of the N-terminal ATPase domain of Hsp70s (*30*). We have shown that SGL binding decreases ATP hydrolysis and thus may provide a new independent mechanism for the regulation of Hsp70. In addition, SGL binding allows for membrane association of an otherwise soluble protein, a regulation mechanism used effectively by other systems (*50*, *51*). The physiological relevance of this inhibition has yet to be shown. In any event, SGL binding is a new tool that can be used to investigate ATPase regulated Hsp70 chaperone activity in vitro and may provide a new mode for the exogenous regulation of Hsp70. However, cellular glycolipids are membrane components synthesized in the ER/Golgi lumen and expressed in the endomembrane system and at the cell surface. If this potential mechanism of regulation of Hsp70 has any cellular role, and this remains to be shown, it would be expected to operate primarily for chaperones in the secretory endomembrane system. Kar2, yeast BiP, also specifically binds to SGC and SGG (H. Whetstone, unpublished). Lysosomal Hsp70 (*16*, *52*) is also appropriately located to interact with SGC within the lysosomal membrane.

SGLs are expressed at the greatest levels in tissues considered exposed to high levels of stress (e.g., gastrointestinal mucosa, ref *53*). If the mechanism we have demon-

strated has physiological relevance in such tissues, it might be that SGLs serve to selectively inhibit the endomembrane located Hsp70s involved in the degradation of misfolded proteins (*54*). 3′Sulfogalactose is found only in glycolipids. We have, however, shown that 4′sulfogalactosyl ceramide is effectively bound by Hsc70 (*32*). This extent of binding flexibility might be sufficient to allow other sulfoglycoconjugates to interact with Hsc70 by this mechanism. It is relevant to note SGC has been detected immunologically in the cytoplasm of astrocytes (*55*), neurons (*56*), pericytes of the eye (*57*), and secretory granules of pancreatic islet cells (*58*), suggesting a greater potential scope for Hsp70−SGC interaction.

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REFERENCES

1. Hartl, F. U. (1996) *Nature 381*, 571−580.
2. Bukau, B., and Horwich, A. L. (1998) *Cell 92*, 351−366.
3. Feder, M. E., and Hofmann, G. E. (1999) *Annu. Rev. Physiol. 61*, 243−282.
4. Fink, A. (1999) *Physiol. Rev. 79*, 425−449.
5. Buchberger, A., Theyssen, H., Schroder, H., McCarty, J. S., Virgallita, G., Milkereit, P., Reinstein, J., and Bukau, B. (1995) *J. Biol. Chem. 270*, 16903−16910.
6. Barthel, T., Zhang, J., and Walker, G. (2001) *J. Bacteriol. 183*, 5482−5490.
7. Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 5452−5457.
8. Brehmer, D., Rudiger, S., Gassler, C. S., Klostermeier, D., Packschies, L., Reinstein, J., Mayer, M. P., and Bukau, B. (2001) *Nature Struct. Biol. 8*, 427−432.
9. Hohfeld, J., Minami, Y., and Hartl, F. (1995) *Cell 83*, 589−598.
10. Minami, Y., Hohfeld, J., Ohtsuka, K., and Hartl, F.-U. (1996) *J. Biol. Chem. 271*, 19617−19624.
11. Nollen, E. A., Kabakov, A. E., Brunsting, J. F., Kanon, B., Hohfeld, J., and Kampinga, H. H. (2001) *J. Biol. Chem. 276*, 4677−4682.
12. Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aime-Sempe, C., Xie, Z., Morimoto, R. I., and Reed, J. C. (1997) *Biochem. J. 328*, 807−813.
13. Ballinger, C., Connell, P., Wu, X., Hu, Z., Thompson, L., Yin, L., and Patterson, C. (1999) *Mol. Cell Biol. 19*, 4535−4545.
14. Haas, I. G. (1994) *Experientia 50*, 1012−1020.
15. Munro, S., and Pelham, H. R. (1986) *Cell 46*, 291−300.
16. Agarraberes, F. A., Terlecky, S. R., and Dice, J. F. (1997) *J. Cell Biol. 137*, 825−834.
17. Triantafilou, K., Triantafilou, M., Ladha, S., Mackie, A., Dedrick, R. L., Fernandez, N., and Cherry, R. (2001) *J. Cell Sci. 114*, 2535−2545.
18. Kishi, A., Ichinohe, T., Hirai, I., Kamiguchi, K., Tamura, Y., Kinebuchi, M., Torigoe, T., Ichimiya, S., Kondo, N., Ishitani, K., Yoshikawa, T., Kondo, M., Matsuura, A., and Sato, N. (2001) *Immunogenetics 53*, 401−409.
19. Banumathy, G., Singh, V., and Tatu, U. (2002) *J. Biol. Chem. 277*, 3902−3912.
20. Blachere, N., Li, Z., Chandawarkar, R., Suto, R., Jaikaria, N., Basu, S., Udono, H., and Srivastava, P. (1997) *J. Exp. Med. 186*, 1315−1322.
21. Lingwood, C. A. (1985) *Can. J. Biochem. Cell Biol. 63*, 1077−1085.
22. Mamelak, D., and Lingwood, C. (1997) *Glycoconjugate J. 14*, 715−722.
23. Lingwood, C. A., Schramayr, S., and Quinn, P. (1990) *J. Cell. Physiol. 142*, 170−176.
24. Lingwood, C. A., Quinn, P. A., Wilansky, S., Nutikka, A., Ruhnke, H., and Miller, R. B. (1990) *Biol. Reprod. 43*, 694−697.
25. Boulanger, J., Faulds, D., Eddy, E. M., and Lingwood, C. A. (1995) *J. Cell. Physiol. 165*, 7−17.
26. Huesca, M., Borgia, S., Hoffman, P., and Lingwood, C. A. (1996) *Infect. Immun. 64*, 2643−2648.
27. Huesca, M., Goodwin, A., Bhagwansingh, A., Hoffman, P., and Lingwood, C. A. (1998) *Infect. Immun. 66*, 4061−4067.
28. Hartmann, E., and Lingwood, C. A. (1997) *Infect. Immun. 65*, 1729−1733.
29. Hartmann, E., Lingwood, C., and Reidl, J. (2001) *Infect. Immun. 69*, 3438−3441.
30. Mamelak, D., Mylvaganam, M., Whetstone, H., Hartmann, E., Lennarz, W., Wyrick, P., Raulston, J., Han, H., Hoffman, P., and Lingwood, C. (2001) *Biochemistry 40*, 3572−3582.
31. Mamelak, D., and Lingwood, C. (2001) *J. Biol. Chem. 276*, 449−456.
32. Mamelak, D., Mylvaganam, M., Tanahashi, E., Ito, H., Ishida, H., Kiso, M., and Lingwood, C. (2001) *Carbohydr. Res. 335*, 91−100.
33. Pellizzari, A., Pang, H., and Lingwood, C. A. (1992) *Biochemistry 31*, 1363−1370.
34. Boyd, B., Zhiuyan, Z., Magnusson, G., and Lingwood, C. A. (1994) *Eur. J. Biochem. 223*, 873−878.
35. Kiarash, A., Boyd, B., and Lingwood, C. A. (1994) *J. Biol. Chem 269*, 11138−11146.
36. Mylvaganam, M., and Lingwood, C. (1999) *Biochem. Biophys. Res. Commun. 257*, 391−394.
37. Lennarz, W., and Hart, G. (1994) *Methods Enzymol. 230*, 348−389.
38. Lingwood, C. A., Murray, R. K., and Schachter, H. (1980) *J. Immunol. 124*, 769−774.
39. Ha, J. H., Hellman, U., Johnson, E. R., Li, L., McKay, D. B., Sousa, M. C., Takeda, S., Wernstedt, C., and Wilbanks, S. M. (1997) *J. Biol. Chem. 272*, 27796−27803.
40. Wilbanks, S. M., Deluca-Flaherty, C., and McKay, D. B. (1994) *J. Biol. Chem. 269*, 12893−12898.
41. Gassler, C. S., Buchberger, A., Laufen, T., Mayer, M. P., Schroder, H., Valencia, A., and Bukau, B. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 15229−15234.
42. Koshy, K., and Boggs, J. (1983) *Chem. Phys. Lipids 34*, 41−53.
43. Fredman, P., Mattsson, L., Andersson, K., Davidson, P., Ishizuka, I., Jeansson, S., Mansson, J., and Svennerholm, L. (1988) *Biochem. J. 251*, 17−22.
44. Sadis, S., and Hightower, L. (1992) *Biochemistry 31*, 9406−9412.
45. Mylvaganam, M., and Lingwood, C. A. (in press) A preamble to aglycone reconstruction for membrane presented glycolipid mimics, in *Carbohydrate-based drug discovery* (Wong, C.-H., Ed.), Wiley Press.
46. Voet, D., Voet, J. G., and Pratt, C. W. (1998) *Fundamentals of Biochemistry*, John Wiley and Sons Inc., New York.
47. Sondermann, H., Scheufler, C., Schneider, C., Höfeld, J., Hartl, F.-U., and Moarefi, I. (2001) *Science 291*, 1553−1557.
48. Ha, J., and McKay, D. (1994) *Biochemistry 33*, 14625−14635.
49. Ha, J.-H., and McKay, D. B. (1995) *Biochemistry 34*, 11635−11644.
50. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J. Biol. Chem. 270*, 503−506.
51. Tamanoi, F. K.-S., J. Jiang, C., Machado, I., Thapar, N. (2001) *J. Cell. Biochem. Suppl. 37*, 64−70.
52. Agarraberes, F. A., and Dice, J. F. (2001) *J. Cell Sci. 114*, 2491−2499.
53. Natomi, H., Sugano, K., Takaku, F., and Iwamori, M. (1990) *J. Clin. Gastroenterol. 12*, S52−57.
54. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) *Nat. Cell Biol. 3*, 100−105.
55. Berntson, Z., Hansson, E., Rönnbäck, L., and Fredman, P. (1998) *J. Neurosci. Res. 52*, 559−568.
56. Pernber, Z., Molander-Melin, M., Berthold, C.H., Hansson, E., Fredman, P. (2002) *J. Neurosci. Res. 69*, 86−93.
57. Buschard, K., Horn, T., Aaen, K., Josefsen, K., Persson, H., and Fredman, P. (1996) *Diabetologia 39*, 658−666.
58. Fredman, P., Månsson, J.-E., Rynmark, B.-M., Josefsen, K., Ekblond, A., Halldner, L., Osterbye, T., Horn, T., and Buschard, K. (2000) *Glycobiology 10*, 39−50.

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