Glycoconjugate Journal (1997) **14:** 715–722

Expression and sulfogalactolipid binding specificity of the recombinant testis-specific cognate heat shock protein 70

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Immunofluorescent studies with anti-2A antisera, raised specifically against a synthetic C-terminal peptide of native murine P70, the testes-specific cognate heat shock protein 70, demonstrated that the rat homologue of P70 is expressed on the surface of testicular cells. The murine *hsp 70.2* gene, encoding P70, was cloned and expressed in *Escherichia coli*. The recombinant P70 (rP70) protein with a 6Xhistidine affinity tag at its amino terminus was purified from *E. coli* via nickel affinity column chromatography. Monoclonal anti-hsp70 antisera and anti-2A antisera cross-reacted with purified rP70. Binding of rP70 was specific for sulfogalactosylceramide (SGC) and sulfogalactosyglycerolipid (SGG). Binding was not inhibited by the sugar, galactose 3′sulfate, nor was binding observed to desulfated derivatives of SGC and SGG, to other negatively charged lipids or other sulfated lipids. Furthermore, rP70 bound to an SGC-column and was eluted only at high salt in combination with high pH. These results show rP70 to possess a specific sulfatide binding site. Since the biochemical properties and immunoreactivity of rP70 are indistinguishable from native P70 and SLIP1 (testicular sulfoglycolipid immobilized protein 1) rP70 can be employed to examine the role of hsp70-mediated sulfatide binding in fertilization.

***Keywords*: germ cell interaction, sperm surface adhesin, glycolipid receptor**

***Abbreviations*: PAGE, polyacrylamide gel electrophoresis; IPTG, isopropylthio-β-D-galactoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; km, kanamycin; amp, ampicillin; kDa, kilodaltons; kb, kilobases; BHI, brain heart infusion; OPD, O-phenylenediamine dichloride peroxidase substrate; RT, room temperature; h, hours**

Introduction

The sulfated glycolipids, sulfogalactosylceramide (SGC) and the testes-specific sulfogalactoglycerolipid (SGG), have been implicated in various aspects of reproduction; the mechanism of sperm/egg binding [1, 2], zygote implantation [3], and as eukaryotic cell receptors for mycoplasmas [4, 5], infections which have been associated with infertility. We have demonstrated that members of the hsp70 family of stress-induced or 'heat shock' proteins can be expressed on the cell surface, specifically bind SGG and SGC in *in vitro* binding assays and that anti-hsp70 antibodies prevent the attachment of mycoplasma to sulfoglycolipids [6]. Similarly, surface hsp's mediate sulfatide binding by the gastric pathogen *Helicobacter pylori*, induced by exposure to low pH stress [7], and the respiratory pathogen, *Hemophilus influenzae* following heat [8].

These studies highlight the possible function of hsp70 family members as adhesins to mediate sulfoglycolipid-dependent cellular adhesion. Affinity chromatography had identified SLIP1 (sulfoglycolipid immobilized protein 1) [9] as a cell surface testicular sulfogalactolipid binding protein. SLIP 1, or anti-SGG, inhibit sperm/egg binding *in vitro* [2] and vaginal anti-SLIP1 inhibits fertilization *in vivo* [1]. We showed [6] that P70, the product of the testis specific heat-shock gene *hsp70.2* [10], was at least a component of SLIP1. To elucidate the molecular basis of the biochemical and biological function(s) of this protein, fully functional recombinant P70 is required.

In this report we describe the preparation and preliminary characterization of the glycolipid binding specificity of recombinant P70.

Materials and methods

The murine *hsp70.2* gene, harboured in the pBluescript plasmid, pMSP2SM was a gift from Dr L. Moran, Department of Biochemistry, University of Toronto. Taq

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Extender™ and Taq polymerase were purchased from Stratagene (Aurora, On.) and Pharmacia Biotech (Baie D'Urfe, Que.), respectively. All restriction enzymes, ligases and their buffers were also purchased from Pharmacia. DNA and protein standards were purchased from GibcoBRL (Burlington, On.) and BioRad Labs (Richmond, Ca.), respectively. TA cloning and pTrc expression plasmids were purchased from Invitrogen Corp. (San Diego, Ca.). The bovine brain 70 kDa hsp was purchased from StressGen Biotechnologies Corp. (Victoria, BC). Mouse monoclonal anti-hsp 70 (BRM-22) was purchased from Sigma (St Louis, MO). Anti-2A polyclonal rabbit antiserum was prepared against synthetic peptides predicted from the highly conserved C-terminus mouse spermatogenic *hsp70.2* gene sequence [10] and was a generous gift from Dr E.M. Eddy, NIH, Research Triangle Park, North Carolina. This antiserum is highly specific for the P70 mouse spermatogenic cell protein, and does not react with other hsps of spermatogenic or somatic cells [10]. The monoclonal anti-^{MRGS}His antibody, reactive with the N terminal extension of rP70, was purchased from Qiagen Inc. (Chatsworth, Ca.). Goat anti-rabbit and goat anti-mouse immunoglobulin-peroxidase (GAR-HRP, GAM-HRP) were purchased from Bio-Rad Labs. All antisera were used at dilutions optimized for each assay.

Plastic backed, silica gel (SIL G) thin layer chromatography (TLC) plates were purchased from Brinkman (Rexdale, Ont.). SGG was isolated from bovine testes [11]. Gangliosides were from bovine brain, Gg_3 and Gg_4 were prepared by acid hydrolysis of GM2 and GM1 respectively [12], neutral glycolipids from human kidney [13], and sulfoglucuronyl paragloboside (SGPG) was kindly provided by Dr R. Yu, Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University. SGC, choloro-1-naphthol, peroxidase, OPD, km, amp, X-Gal and IPTG were purchased from Sigma. Galactose 3′ sulfate [14] was a generous gift from Dr J. Boggs, Department of Biochemistry, Hospital for Sick Children, Toronto. Micro BCA Protein Assay Reagent was purchased from Pierce (Rockford, IL). Protein fractions were concentrated in Centripreps purchased from Amicon Canada Ltd (Oakville, Ont.).

Immunofluorescence

A single cell suspension of testicular germ cells from adult rats was prepared as previously described [15]. For sperm staining, the epididymes were removed, separated into caput and cauda regions and the sperm teased out and washed [15]. Germ cells were fixed in 2% glutaraldehyde, washed 2 × with PBS and incubated for 0.5 h at room temperature in the presence of 1:100 dilution of anti-2A [10] or anti-SLIP 1 [9, 16] antiserum. Cells were washed twice in PBS, incubated in FITC conjugated anti-rabbit IgG, washed again, mounted and cell surface fluorescence observed under incident UV illumination. Non-immune serum and conjugate controls showed background staining as previously reported [15, 17].

Construction of pT70/Babe neo.

An *Xba*I/*Bgl*II fragment, encoding the *hsp70.2* gene, was excised from pMSP2SM and ligated into the *Xba*I/*Bam*HI site of pBluescript. This was possible since *Bam*HI and *Bgl*II have complementary sticky ends. The resulting recombinant vector, pBS/T70, was digested with *Xba*I, blunted with Klenow fragment and linked with *Eco*RI linkers [18]. The *hsp70.2* transcript was excised from pBS/T70 by digesting with *Eco*RI and subcloned into the complementary restriction enzyme site of the retroviral expression vector, pBabe neo [19] generating pT70/Babe neo.

Polymerase chain reaction

Oligonucleotide primers were designed to the 5′(5′-TCAG**GAATTC**ATGTCTGCCCGCGGCCC-3′) and 3′(5′-TGCA**AAGCTT**CTAGTCCACTTCCTCGAT-3′) ends of the *hsp70.2* coding region. Synthetic *Eco*RI and *Hind*III restriction sites (bold nucleotides in primers) were engineered into the upstream and downstream oligonucleotide primers, respectively. PCR was carried out using pT70/Babe neo as template in the Minicycler™ (Fisher) with the following cycling parameters. A 'hot start' at 94 °C for 10 min, followed by an initial cycle of denaturing for 5 min at 94 °C, followed by annealing for 2 min at 58 °C and then extending for 3 min at 72 °C. The 2nd to 29th cycles involved denaturing for 5 min at 94 °C, followed by annealing for 1 min at 58 °C and then increasing the extension time by 5 s for each successive cycle at 72 °C. The 30th and final cycle is similar to the middle cycles except that the final extension time at 72 °C was for 6 min. *Taq* Extender™ was employed as the polymerase for this reaction. *Taq* polymerase was added to the amplified reaction post PCR for 10 min at 72 °C with no cycling.

Cloning and expression of *hsp70.2*

The amplified 1.9 kb open reading frame of *hsp70.2* was ligated into a TA cloning vector (Invitrogen). Competent cells of *E. coli* strain INFα (Invitrogen) were transformed with 50 ng ml^{-1} of DNA from the ligation reaction. Transformed colonies were selected on BHI agar containing 50 μg ml^{-1} of km and 40 μg ml^{-1} of X-Gal. Plasmid DNA was recovered, digested with the restriction enzymes *Eco*RI and *Hind*III [18] and ligated into the complementary restriction enzyme sites of the pTrcHisB expression vector (Invitrogen), yielding the recombinant vector pDMX1.9. The recombinant vector was transformed into competent cells of *E. coli* strain DH5α. Derivatives of this strain were selected on BHI agar containing 50 μg ml^{-1} of amp.

E. coli strain Y100 (derivative of *E. coli* DH5α harbouring pDMX1.9) and *E. coli* strain Top10F′ (Invitrogen) were

grown separately overnight in 2 ml of BHI medium supplemented with 100 μg ml^{-1} of amp. An aliquot of the overnight culture was diluted 1:1000 into 50 ml of LB supplemented with 100 μg ml^{-1} of amp in a 250 ml flask and shaken at 37 °C. When growth of the cultures reached an O.D.$_{600}$ of 0.5, expression of rP70 was induced by adding IPTG to a final concentration of 2 mM.

Electrophoresis and Western blotting

Protein samples were separated on 12% SDS polyacrylamide gels [20]. Separated proteins were detected by staining with Coomassie blue. For Western blotting, separated proteins were transferred to nitrocellulose and blocked with 0.25% (w/v) gelatin in 10 mM Tris, pH 7.4, for 2 h at room temperature. Western blots were probed with the primary antibody, anti-hsp70 (1:5000) (Sigma) or anti-2A (1:2000) or anti-^{MRGS}His (1:2000) overnight at 4 °C. The secondary antibody, peroxidase-conjugated goat anti-mouse (GAM) or goat anti-rabbit (GAR) was incubated with the plate for 2 h at room temperature. Bound antibody was visualized with chloro-1-naphthol [9].

Production of glycolipid affinity matrices

Recombinant P70-glycolipid binding was initially examined using sulfogalactosyl ceramide (SGC)-celite affinity matrices [12]. Briefly, 10 mg of pure SGC in 5 ml of chloroform:methanol (2:1 v/v) was added dropwise to 20 g of dried celite in several volumes of chloroform. The solvent from the SGC-celite slurry was removed using a rotary evaporator. The SGC-celite was resuspended in several volumes of 100 mM TBS, pH 7.4, degassed and poured into a column. The column was washed with 1 l of 100 mM TBS, pH 7.4, at 4 °C. *E. coli* Y100 induced with 2 mM IPTG was denatured with 6 M guanidine hydrochloride. Guanidine hydrochloride was subsequently removed by dialysing against 10 mM TBS, pH 7.4, and the renatured lysate (20 ml) was added to the column. The column was washed with 1 l of 100 mM TBS, pH 7.4, at 4 °C. To elute the recombinant protein, the columns were washed sequentially with 2 M NaCl, Tris (1 M), pH 9.5, and Tris (1 M), pH 11.2. Protein containing fractions were then pooled, concentrated and examined by SDS-PAGE and Western blotting.

TLC overlay

All lipids (1 μg) were separated by TLC using chloroform:methanol:0.88%KCl (60:40:9 v/v). Plates were blocked by incubation with 3% gelatin in water (w/v) for 2 h at 37 °C and washed. Recombinant P70 (5 μg ml^{-1}) in 50 mM Tris-saline, pH 7.6 (TBS), was incubated with the plate for 2 h at 37 °C. The plates were washed and incubated for 2.5 h at RT with rabbit polyclonal anti-2A antisera diluted 1:2000 in 50 mM TBS. The plates were washed and incubated with GAR-HRP at RT for 2 h. Following washing, bound antibody was visualized with chloro-1-naphthol.

TLC plates spotted with 1 μg of purified SGG and SGC were blocked with 3% gelatin in water (w/v) at 37 °C for 2 h then washed with 50 mM TBS, pH 7.4. Recombinant P70 (68 nM) was coincubated with increasing concentrations of galactose 3′ sulfate or galactose for 2 h at 37 °C in 50 mM TBS, pH 7.6. Mixtures (1 ml) of rP70 and the sugars were added to separate plates and incubated overnight at 4 °C. The plates were washed with 50 mM TBS then incubated with anti-2A antisera (1:2000) overnight at 4 °C. After washing, GAR-HRP was added to each well and incubated at RT for 2 h. Following washing, bound antibody was visualized with chloro-1-naphthol. Control wells were incubated with rP70 (68 μM), galactose 3′ sulfate (1 mM), galactose (1 mM) or 50 mM TBS alone and similarly treated to determine background binding.

Results

Cell surface localization of hsp70.2

We have previously shown that hsp 70 proteins are expressed on the surface of spermatozoa and that the sulfogalactolipid binding protein, SLIP1 [9], is cross reactive both with anti-hsp 70 antibodies and with antiserum specific for the testes specific hsp70.2 [6]. In order to confirm that the *hsp70.2* gene product, P70, was expressed on the surface of male germ cells and could thus account for the adhesin functions we have ascribed to SLIP1 [1, 2, 6], staining of testicular germ cells and spermatozoa was performed using anti-2A antiserum. This antiserum has been previously used to localize P70 within the synaptenemal complex within the nucleus of primary spermatocytes [21], but a germ cell surface location for P70 has not been reported.

Our staining results clearly show that the testis specific hsp P70 is expressed on the surface of developing germ cell and spermatozoa (Figure 1). Expression of P70 on spermatozoa was the same as we have reported for SLIP 1 in that in caput cells P70 is found on the concave surface of the sperm head (colocalized with SGG [15]) (Figure 1A), while in cauda sperm, P70 is distributed on both the convex and concave surfaces (Figure 1B, C). On testicular cells P70 was expressed in complex asymmetric surface domains (Figure 1D), identical to those previously reported for SLIP1 [15] which colocalize with cell surface SGG expression.

These results supported our contention that P70 is, at least, a component of SLIP1 and warrant a molecular analysis of the glycolipid binding specificity of P70.

Cloning, expression and purification of *hsp70.2* (P70)

The polymerase chain reaction was employed to amplify the 1.9 kilobase pair (kb) coding region of *hsp70.2*, harboured within pT70/Babe neo. The amplified 1.9 kb product was ligated into the TA cloning vector, pCR^{TM}II. The resulting

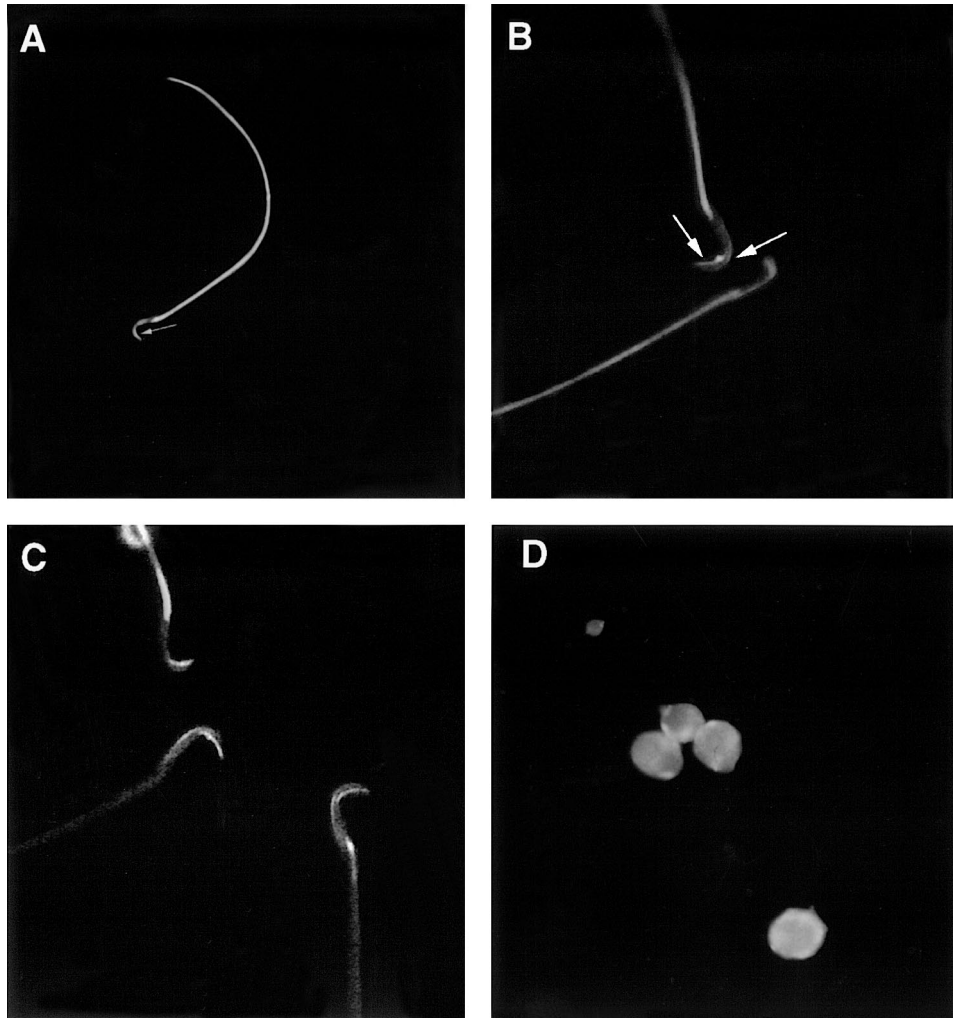

Figure 1. Germ cell surface localization of P70. Anti SLIP1 (not shown) and anti-2A staining of rat male germ cells were compared. Anti-2A stained caput (A), cauda (B, C) spermatozoa and the testicular germ cell (D) surface, in a manner essentially identical to anti-SLIP 1 [15]. Caput sperm were stained within the concave surface of the head (arrowed) while cauda sperm were reactive on both the convex and concave surfaces (arrows in B), consistent with the relocalization of sperm SLIP1 previously reported during epididymal sperm maturation [15]. Primary spermatocytes were stained in complex surface domains. (Magnification: A = 2000, B = 3000, C = 3000, D = 2000x.)

plasmid, denoted pDM1.9, was digested with the restriction enzymes *Eco*RI and *Hind*III, whose recognition sequences were synthetically engineered into the upstream and downstream oligonucleotide primers. The resulting *Eco*RI-*Hind*III 1.9 kb insert was subcloned into the complementary restriction sites of the pTrc-HisB expression vector generating, pDMX1.9. *E. coli* strain DH5α was transformed with pDMX1.9 generating the derivative *E. coli* strain Y100.

Expression of the *hsp70.2* gene product from pDMX1.9 was induced by the addition of 2 mM IPTG to a culture of *E. coli* Y100 in the mid-log phase of growth. Western blotting with monoclonal anti-hsp70 antisera detected the *hsp70.2* gene product, rP70, in SDS solubilized extracts of *E. coli* Y100 (Figure 2). Neither anti-hsp70 nor anti-2A antisera detected rP70 in extracts of *E. coli* Top10F′, which harbours the native pTrc-HisB expression vector without the *hsp70.2* insert (Figure 2). This observation confirmed that anti-hsp70 antisera does not cross react with the native bacterial hsp70, DnaK. The faint 73 kDa band seen in the uninduced sample (Figure 2, lane 2) is likely a result of 'leaky' translation from the Trc promoter.

The His tagged rP70 was purified from the *E. coli* Y100 lysate by nickel affinity chromatography (Figure 2). The reaction of rP70 with the highly specific C-terminal anti-P70 antibody, anti-2A, confirmed that there were no deleterious mutations in the nucleotide sequence of the amplified product and that it was ligated in frame into the pTrc-HisB expression vector. The 6XHis tag is approximately 3000 Da and accounts for the increase in size of rP70 ($M_r = 73$) over native P70 ($M_r = 70$). From a 50 ml bacterial culture, 1.5 mg of rP70 was purified.

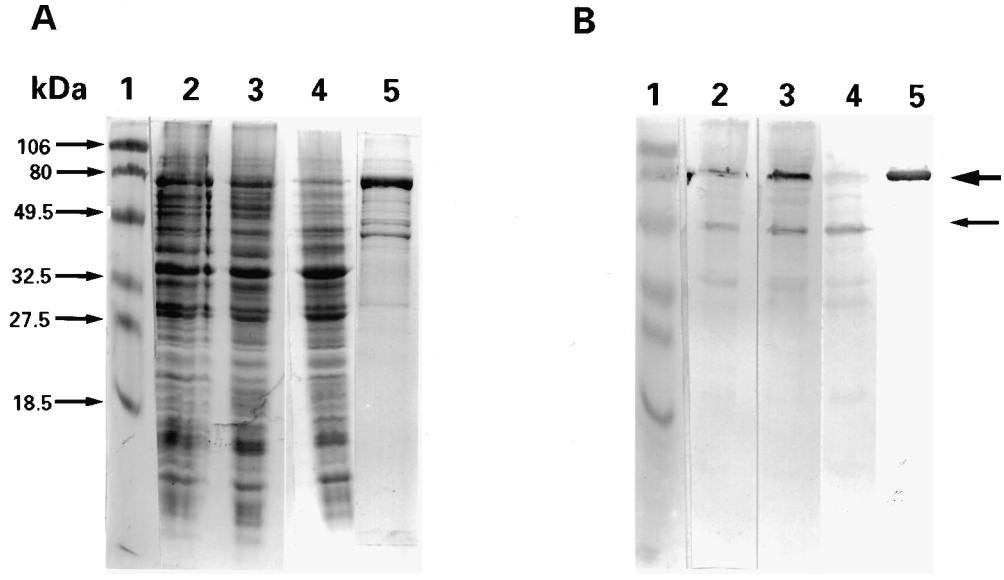

Figure 2. Expression and purification of the recombinant *hsp70.2* gene product, rP70. Expression of rP70 was induced by the addition of 2 mM Isopropylthio-β-D-galactoside (IPTG) to cultures of *E. coli* Y100. Panel A, stained with Coomassie blue. Panel B, lanes (1–4) probed with MAb anti-hsp70, lane (5) probed with anti-2A antisera. Lanes (1) molecular weight markers, (2) uninduced *E. coli* Y100, (3) *E. coli* Y100 induced with 2 mM IPTG for 1 h, (4) *E. coli* Top10F′ (plasmid control, no insert) induced with 2 mM IPTG for 2 h, (5) rP70 purified from *E. coli* Y100 via nickel affinity column chromatography. Large arrow indicates rP70, $M_r = 73$ kDa (the 6XHis tag fused to the amino terminus of rP70 accounts for the increase in molecular weight). Smaller arrow indicates the 44 kDa proteolytic fragment of *E. coli* host protein DnaK (Hsp70).

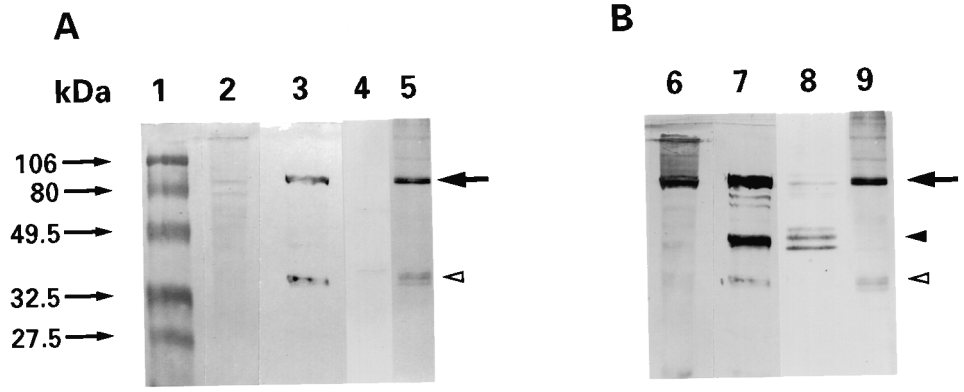

Figure 3. SGC column eluted proteins probed with anti-^{MRGS}His and anti-2A antisera. Lysate containing soluble, denatured proteins of *E. coli* strain Y100 was added to an SGC-celite affinity column. Panel A, Western Blot with anti-^{MRGS}His antisera. Panel B, re-blotted panel A with anti-2A antisera. Lanes: (1) molecular weight markers; (2, 6) *E. coli* Y100 whole cell extract; (3, 7) *E. coli* Y100 whole cell extract treated with guanidine hydrochloride; (4, 8) SGC column wash with 100 mM TBS, pH 7.4; (5, 9) SGC column elute with Tris (1 M), pH 11.2. Large arrow indicates rP70; black arrowhead indicates C-terminal 44 kDa fragment; open arrowhead indicates N-terminal 33 kDa fragment.

We found rP70 to localize in both the soluble and insoluble cellular fractions of *E. coli* Y100 (data not shown). To increase the yield of soluble rP70, an IPTG induced culture of *E. coli* Y100 was denatured with 6 M guanidine hydrochloride. Denatured rP70 should not bind any other proteins, as might be expected from the chaperone function of native hsps. The denaturation of *E. coli* Y100, however, resulted in the partial cleavage of rP70, yielding an additional C-terminal 44 kDa and an N terminal 33 kDa fragment (Figure 3 lanes 3, 7).

Glycolipid binding specificity of rP70

a) Affinity chromatography

The guanidine HCl treated lysate containing soluble proteins of *E. coli* strain Y100 was renatured by dialysis against TBS and added to an SGC-celite affinity column. The column fractions were analysed by western blot with anti-^{MRGS}His and anti-2A antisera (Figure 3). The C-terminal 44 kDa fragment of rP70, as detected by anti-2A antisera (resolved as a doublet), was found only in the unbound

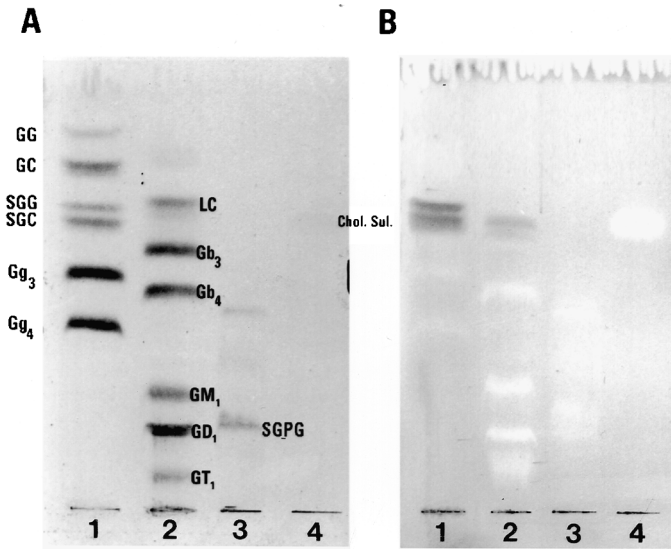

Figure 4. Tlc overlay assay of binding specificity of rP70. (A) Orcinol treatment of tlc plates for chemical visualization of glycolipids. Approximately 1 μg of the glycolipids indicated were run. Position of cholesterol sulfate is masked in lane 4. (B) rP70 overlay demonstrating binding specificity to SGG and SGC in lane 1 (trace SGC binding is also detected in lane 2).

100 mM TBS, pH 7.4 wash fraction (Figure 3 lane 8). No protein was eluted with 1 M Tris, pH 9.5 (not shown). rP70 and the N-terminal 33 kDa fragment were bound and eluted from the SGC column by 1 M Tris, pH 11.2 (Figure 3, lanes 5, 9).

b) Thin layer chromatogram overlay

Tlc overlay assays demonstrated that rP70 bound specifically to the sulfated glycolipids, SGC and SGG, and not to the desulfated derivatives, GC and GG (Figure 4, lane 1). In addition, no binding was observed to other negatively charged glycolipids (GM_1) or to their asialo derivatives (Gg_3, Gg_4) (Figure 4, lanes 1 and 2, respectively). rP70 did not bind to glycolipids of the globo series (Gb_3 and Gb_4) (Figure 4, lane 2), nor to cholesterol sulfate (Figure 4, lane 4). Binding of rP70 to traces of SGC contaminating the ganglioside sample were observed (Figure 4, lane 2). Interestingly, the sulfoglycolipid SGPG (sulfoglycuronyl paragloboside) [22] was not recognized (Figure 4, lane 3) suggesting that sulfated galactose is necessary for binding. However, galactose 3′sulfate itself (or galactose) was not an effective inhibitor of rP70 sulfoglycolipid binding as monitored by dot tlc overlay (Figure 5) even at concentrations representing a 30 000-fold molar excess over protein.

Discussion

In this report we describe the cloning and expression of the murine testis specific cognate *hsp70.2* transcript in *E. coli* and demonstrate that the recombinant gene product, P70 (rP70), maintained the glycolipid binding specificity (for SGC and SGG) of native P70 and the testicular sulfolipid immobilized protein 1 (SLIP1) [6].

Previous immunofluorescence studies have shown that intracellularly, P70 is associated with the nuclear synaptonemal complex of primary spermatocytes [21]. Although we [6] and others [23] have previously shown that hsp70s are expressed on the surface of male germ cells, in order to fully support our inference that P70 and SLIP 1 are the same, it was necessary to show that the testis specific hsp 70, P70, was also germ cell surface located. This is of additional interest since the P70-specific antiserum is directed against the C-terminal 17 amino acid sequence. The essentially identical asymmetric germ cell surface staining pattern of anti-2A (Figure 1) and anti-SLIP1 [15] supports our assignment of P70 as SLIP1 and indicates that the C-terminus is available at the cell surface.

PCR amplified the 1899 base pair (bp) coding region of *hsp70.2* [24] from the full length 2.3 kb transcript on pT70/Babe neo. The synthetic restriction enzyme sites engineered into the 5′ and 3′ ends of the oligonucleotide primers facilitated the subcloning of the amplified product from pDM1.9 into complementary restriction sites of a pTrc-HisB expression vector, pDMX1.9.

SGG column affinity chromatography was first used to identify SLIP1 from rat testicular cells [15]. Since we have

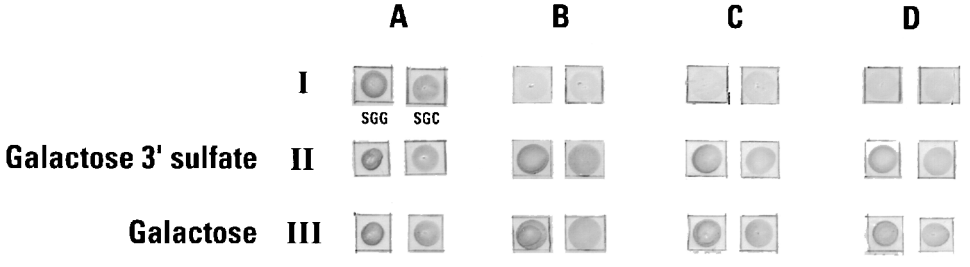

Figure 5. Inhibition of rP70 sulfogalactolipid binding. rP70 was demonstrated to bind SGG and SGC in the absence (I.A) or presence of increasing concentrations of galactose 3′sulfate (II.A-D). Increasing concentrations of galactose (III.A-D) had no effect on the binding specificity of rP70 to SGG and SGC. Wells in rows II.A-D and III.A-D had increasing concentrations of 50 μM, 100 μM, 1 mM and 2 mM of galactose 3′sulfate and galactose, respectively. Wells I B-D represent controls incubated with 1 mM galactose 3′sulfate, 1 mM galactose or TBS respectively, in the absence of rP70.

demonstrated that hsp70s from a variety of sources specifically bind sulfatides [6], SGC column affinity chromatography might be useful in the purification of other members of the hsp70 family. We found that rP70 bound sulfatide and could be readily purified from *E. coli* lysate by SGC column affinity chromatography. High salt (1 M Tris) in combination with high pH was required to elute rP70 from the SGC column which suggests that rP70 contains a high affinity SGC binding site.

The SGC binding demonstrated by affinity chromatography was confirmed by tlc overlay. Nickel affinity purified recombinant P70 (Figure 2) bound specifically to sulfated glycolipids (SGC and SGG) and not their desulfated derivatives, nor to other negatively charged glycolipids/lipids (Figure 4). Interestingly, rP70 did not bind to the sulfoglycolipid, SGPG, indicating the importance of sulfated galactose in binding. The fact that cholesterol sulfate is not bound, confirms the importance of the galactose but the finding that binding is not inhibited by free galactose 3′sulfate (Figure 5) suggests that the lipid component plays a significant role in hsp-sulfogalactolipid binding. This may be a similar effect as we have reported for verotoxin binding to its glycolipid receptor globotriaosyl ceramide, in which the free oligosaccharide is also an ineffective inhibitor [25] and alterations in the lipid moiety of the glycolipid can have significant effects on the ability of the toxin to bind the carbohydrate moiety of this glycolipid [26, 27].

Western blot of SGC column eluted proteins with anti-2A and anti-^{MRGS}His antisera suggest that rP70 has a cleavage site distinct from the proteolytic cleavage site of bovine Hsc70 uncoating ATPase [28]. The major tryptic fragment of Hsc70 comprises the conserved 44 kDa N-terminal domain, containing the ATPase activity but devoid of clathrin binding activity [29]. Western blots (Figure 3) indicate that the 44 kDa fragment of rP70 is a C-terminal peptide. Consistent with this deduction, the anti-^{MRGS}His antisera, specific for the first 10 amino acids of the N-terminal fusion protein, detected a fragment of approximately 33 kDa (Figure 3). The fact that the N-terminal r33 kDa rP70 breakdown product (but not the C terminal 44 kDa fragment) is co-eluted with rP70 from the SGC column (Figure 3, lanes 5, 9) indicates that the SGC binding site is contained entirely within this ATPase containing fragment.

Hsp70.2 is first expressed at early meiosis in pachytene spermatocytes and throughout the remainder of spermatogenesis [24, 30]. It has been speculated that the gene products of hsp transcripts are involved in spermatogenesis and germ cell differentiation [31]. Indeed, transgenic mice in which the *hsp70.2* gene has been inactivated are infertile and spermatogenesis is arrested at the primary spermatocyte stage due to extensive apoptosis [32].

SLIP1 [9], murine P70 [10] and rat P70 [33] have all been found to first appear during the early pachytene stage of spermatogenesis. This is also the stage that SGG is synthesized [34], which serves as a marker for germ cell differentiation [35]. It is possible that P70/SLIP1-SGG binding plays a role in intracellular communication between the male germ cells during spermatogenesis. SLIP1 is colocalized with SGG in asymmetric cell surface domains on testicular germ cells and at the ventral surface of the caput sperm head [15]. During epididymal sperm maturation, SLIP1 and SGG separate such that SLIP1 is now found on the dorsal surface of cauda sperm [15], and is thus available at the site on mature sperm which makes contact with the egg zona pellucida. Murine fertilization studies have demonstrated that purified SLIP1 inhibited sperm/egg binding *in vitro* [2] while anti-SLIP1 antibody inhibited fertilization *in vivo* [1]. In addition, mycoplasma species associated with infertility bind and degrade sperm sulfated glycolipids [4, 5]. Such binding is prevented by coincubation with anti-hsp70 antisera [6]. These findings indicate the adhesin function of surface hsps and emphasize the importance of hsp70-sulfoglycolipid binding in fertilization [5].

Heat shock proteins have traditionally been described as intracellular chaperones which facilitate the correct folding of proteins or mediate the degradation of improperly folded ones via ATP dependent binding [36, 37]. We have recently demonstrated that brief heat shock or exposure to low pH of the gastric pathogen *Helicobacter pylori* results in the surface expression of surface heat shock proteins which mediate binding to sulfated glycolipids *in vitro* [7]. Similarly, brief heat shock of *Hemophilus influenzae* results in the induction of cell surface hsp 70-mediated sulfoglycolipid binding [8].

Since hsps lack a leader sequence or putative transmembrane regions, the mechanism by which they transit the cytoplasm and remain membrane bound is speculative. Surface expression of hsps might result from a non-classical type III secretory pathway in bacteria [38], autolysis and adsorption of cytoplasmic proteins on the surface [39], or co-export with other membrane proteins (*eg* proacrosin [33] or urease [40]). Rapid and selective release of hsps from eukaryotic cells without involving the classical secretion pathway has been described [41].

It is conceivable that P70 could be retained on the male germ cell surface via SGG binding. In which case, the peptide binding domain could be available for the binding of a second ligand. Alternatively the peptide binding domain could retain P70 at the cell surface. In this regard it is interesting to note that our original procedure for the release of SLIP1 from the male germ cell surface was by treatment with ATP [9] which should release chaperone-bound peptides.

The availability of this rP70 expression system will allow investigation of the molecular basis of sulfatide binding. Such studies will be relevant, not only to reproduction, but to the adhesion of several bacterial pathogens including mycoplasma [6], *Helicobacter pylori* [7], and *Hemophilus influenzae* [8]. Our studies indicate that cell surface hsp 70

proteins may provide a new family of adhesins which mediate the attachment of both prokaryote and eukaryotes to sulfogalactolipids.

Acknowledgements

This work was supported by MRC grant No MT 12559.

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Received 12 September 1996, revised and accepted 1 November 1996