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# Interaction of 70-kDa heat shock protein with glycosaminoglycans and acidic glycopolymers

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ABSTRACT

Interaction of Hsp70 with natural and artificial acidic glycans is demonstrated based on the native PAGE analysis. Hsp70 interacts with acidic glycopolymers that contain clustered sulfated and di-sialylated glycan moieties on a polyacrylamide backbone, but not with neutral or mono-sialylated glycopolymers. Hsp70 also interacts and forms a large complex with heparin, heparan sulfate, and dermatan sulfate that commonly contain 2-*O*-sulfated iduronic acid residues, but not with other types of glycosaminoglycans (GAGs). Hsp70 consists of the N-terminal ATPase domain and the C-terminal peptide-binding domain. The interaction analyses using the recombinant N- and C-terminal half domains show that the ATPase domain mediates the direct interaction with acidic glycans, while the peptide-binding domain stabilizes the large complexes with particular GAGs. To our knowledge, this is the first demonstration of direct binding of Hsp70 to the particular GAGs. This property may be involved in the physiological functions of Hsp70 at the plasma membrane and extracellular environments.



## 1. Introduction

The 70-kDa heat shock protein (Hsp70) is a family of proteins that are overproduced by cells in response to various stress stimuli, and works as a molecular chaperon in protein folding, assembly, and translocation in the cell [1,2]. Hsp70 binds to unfolded proteins and assists their protein folding [3–5]. On the other hand, Hsp70 is also present in extracellular environment, and appears to function differently than the intracellular Hsp70 [6–9]. The extracellular Hsp70 is known to act as alert signals priming other cells of their immune system [9]. Extracellular Hsp70 has been shown to be in membrane-bound and membrane-free forms, which are either associated with substrate or free from client proteins. However, it has largely remained unclear how Hsp70 is tethered on the membranes or retained in the membrane-free extracellular space. In this regard, it is noted that Hsp70 displays a unique property to bind to sulfatide [10], which is a common glycolipid component in various cellular membranes. In collaboration with the William Lennarz's group, we also demonstrated the sea urchin sperm-binding protein (SBP), a member of Hsp70 superfamily, to be a sialic acid-binding protein [11]. SBP binds in a sialic acid-dependent manner to the major di-sialylated gangliosides that are enriched in the lipid rafts of sea urchin sperm plasma membrane. To characterize the glycolipid binding property of the Hsp70 family in detail, we surveyed various acidic and neutral glycolipids for the interaction with Hsp70, and showed that acidic, but not neutral glycolipids induce the high molecular-weight complex formation with Hsp70 in solution, despite the fact that Hsp70 has an activity to bind both neutral and acidic lipids on the solid surface [12]. Recently the Hsp70-acidic lipid interaction has also been demonstrated by other group using liposome-based binding assay [13]. These results suggest that Hsp70 directly interacts with the acidic glycan moiety of glycolipids. Extracellular acidic glycans

*Abbreviations:* BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; GAG, glycosaminoglycan; Hsp70, 70-kDa heat-shock protein 70; Hsp-C, the peptide-binding domain of Hsp70; Hsp-FL, full-length of Hsp70; Hsp-N, the ATPase domain of Hsp70; Lac, lactose; LacNAc, *N*-acetyllactosamine; PAGE, polyacrylamide gel electrophoresis; PAA, polyacrylamide; *p*Np, para-nitrophenyl; PVDF, polyvinylidene difluoride; SBP, sperm-binding protein.

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are also linked to glycosaminoglycans (GAGs) and sialylated glycoproteins. Therefore, the acidic glycan units of GAG and glycoproteins may also be involved in the interaction with Hsp70, which enables the retention of membrane-free Hsp70 outside cells. In this study, we thus surveyed artificial glycopolymers containing sulfated and sialylated glycans on the polyacrylamide (PAA) backbone and natural GAGs for the interaction with Hsp70.

## 2. Materials and methods

### 2.1. Materials

Colominic acid was purchased from Wako Pure Chemicals (Osaka, Japan). ATP-Agarose and Q-Sepharose were from Sigma (St. Louis, MO). Ni–NTA-Agarose was from QIAGEN (Valencia, CA). Peroxidase-conjugated goat antibody against rat IgG was from American Qualex (San Clemente, CA). Acid mucopolysaccharide kit was from Seikagaku Co. (Tokyo, Japan). Sialoglycopolymers were from Glycotech Co. (Rockville, MD, USA). The molecular weight and the degree of glycan substitution of sialoglycopolymers were approximately 30 kDa and 20%mol., respectively. In addition, these polymers were conjugated with biotin at the ratio of 5%mol. *p*-Nitrophenyl 3-*O*-sulfated β-D-galactoside (*p*NP β-3-S-Gal) was prepared as described [14]. A glycopolymer carrying PAA backbone conjugated with 3-*O*-β-D-sulfated Gal (3-S-Gal) was prepared as described [15]. The synthesis of *p*-nitrophenyl sulfated *N*-acetylglucosamine was described [16]. Preparation of glycopolymers carrying PAA conjugated with lactose (Lac), *N*-acetyllactosamine (LacNAc), 6-*O*-sulfated GalNAc (6-S-GalNAc), 3-*O*-sulfated GlcNAc (3-S-GlcNAc), 4-*O*-sulfated GlcNAc (4-S-GlcNAc) and GlcNAc were carried out as previously described [17,18]. PAA(LacNAc) and PAA(Lac) were homopolymers with a molecular weight of approximately 300 kDa. Other glycopolymers are co-polymerized with acrylamide and monomeric glycan and have the glycan substitution of 13–20%mol. Recombinant full-length of Hsp70 (Hsp-FL), the ATPase domain (Hsp-N) and the peptide-binding domain of Hsp70 (Hsp-C) were bacterially expressed and purified as described [12]. Anti-sera against the ATPase domain of Hsp70 (anti-Hsp-N) and the peptide-binding domain (anti-Hsp-C) were prepared as described [12].

### 2.2. Plasmid constructions and protein purifications

The pET32a(+) plasmid encoding a full length of mouse Hsp70.1 (Hsp-FL) and the pQE-30 Xa plasmids encoding the fragments of mouse Hsp70.1 (Hsp-N and Hsp-C) were prepared and used for their expression in *Escherichia coli* BL21 (DE3) pLysS and in SG13009, respectively [12]. Protein purifications were carried out as described [12].

### 2.3. Analysis of the interaction of Hsp-FL, Hsp-N, and Hsp-C with various glycoconjugates by Native PAGE

Interaction of Hsp-FL, Hsp-N, and Hsp-C with various glycoconjugates was assessed as follows. The mixture of the Hsp-FL, Hsp-N, or Hsp-C at 0.1 μM with glycopolymers at 0.45 mg/ml, colominic acid at 0.9 mg/ml or glycosaminoglycans at 0.9 mg/ml was prepared and incubated at 4 °C for 1.5 h followed by incubating at 30 °C for 10 min. After incubation, the mixture was subjected to native PAGE [12,19] or SDS–PAGE, followed by electroblotting onto a PVDF membrane. After blocking with 5% skim milk/PBS-0.05% Tween 20 (PBST) at room temperature for 1 h, the membrane was incubated with anti-Hsp-C antibody for Hsp-FL and Hsp-C (1:5000 dilution) or anti-Hsp-N antibody for Hsp-N (1:2000 dilution) at 37 °C for 1–2 h. After washing three times with PBST, the membrane was incubated with peroxidase conjugated goat anti-rat IgG (1:5000 dilution) at 37 °C for 45 min. After washing three times with PBST, color development was carried out using enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Biosciences).

## 3. Results

### 3.1. Hsp-FL interacts with sulfated glycopolymers in solution

We previously showed that Hsp70 forms the large-size complexes with sulfatide, but not galactosylceramide, glucosylceramide and lactosylceramide [12]. To examine whether the glycan part in sulfatide is directly involved in the complex formation with Hsp70, the interaction of Hsp-FL with PAA(3-*S*-Galβ), an artificial glycopolymer that contains clustered 3-*S*-Galβ structures along PAA backbone, was analyzed by native PAGE (Fig. 1A). In this experiment, the large-size complex that stays on the upper gel and gel top is designated as Type I, and the medium-size complex that migrates at upper region of the lower gel as Type II. Consistent with previous results [12], Hsp-FL gave smear bands at the Type I and II regions in the presence of sulfatide (3-*S*-Galβ-ceramide) (Fig. 1B), while only mono- and dimer of Hsp-FL were observed in the absence of sulfatide or glycopolymers (Fig. 1B, none). When Hsp-FL was incubated with PAA(3-*S*-Galβ), the Type I complex and broadly diffused bands migrating around and faster than mono- and dimer of Hsp-FL were observed. These results indicate that Hsp-FL does interact with PAA(3-*S*-Galβ) and forms the large-size complexes. In contrast, no interaction was observed with glycopolymers containing no sulfate groups, such as PAA(Lacα) and PAA(GlcNAcβ). For PAA(LacNAcβ), although there observed weak, broad smears at the Type I and II regions, main staining pattern corresponds to mono- and dimer bands of Hsp-FL, matching negative control results (Fig. 1A, none). Thus, it is indicated that the sulfate group is important in the interaction. Neither was *p*Np β-3-*S*-Gal, corresponding to a monomeric unit of PAA(3-*S*-Galβ), showing that clustered 3-*S*-Gal structure is required for the interaction with Hsp-FL. These results indicate that Hsp-FL directly interacts with the clustered 3-*S*-Gal moiety of PAA(3-*S*-Galβ) and forms the large-size complex with the glycopolymer. Broadly diffused bands displayed by Hsp-FL in the presence of PAA(3-*S*-Galβ) were also obtained in the presence of other sulfated glycopolymers, PAA(3-*S*-GlcNAcβ) and PAA(4-*S*-GlcNAcβ) (Fig. 1A). PAA(6-*S*-GalNAcβ) showed a little different, but basically the same profile to PAA(3-*S*-Galβ). Taken all together, it is concluded that in solution, Hsp-FL can bind to sulfated glycopolymers, but not to neutral ones, It should be noted that the migration profile of the Hsp70-sulfated glycopolymer complexes slightly differed depending on the structure of the sulfated glycan moiety. Differences in the binding affinity of Hsp70 to these sulfated glycopolymers may reflect the size of the complex.

It has been reported that stress-inducible Hsp70 from HEK293T cells binds to GlcNAc-conjugated beads and *O*-GlcNAc modified proteins [20]. We tested if Hsp-FL interacted with the glycopolymer PAA(GlcNAcβ); however, no broadly diffused bands or large- and medium-size complexes was observed in our assay method (Fig. 1A). Hsp-FL may be interacting with GlcNAc-conjugated polymers, but the interaction is not so strong as the one obtained with sulfated glycan-conjugated polymers.

### 3.2. Hsp-FL interacts with di-sialylated glycopolymer in solution

We then examined the interaction of Hsp-FL with sialoglycopolymers to ask if sialic acid residues are involved in the interaction like sulfate groups (Fig. 2A). In the presence of the

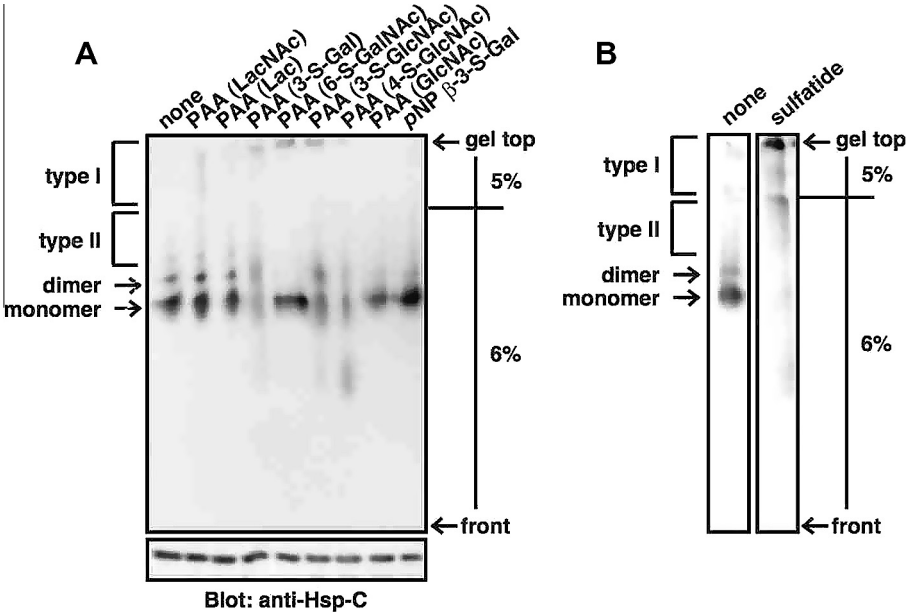


**Fig. 1.** Hsp-FL forms the complex with sulfated glycopolymers. (A) Hsp-FL (0.1 μM) was incubated with 0.45 mg/ml of indicated glycopolymers, followed by native PAGE (stacking gel, 5% and separating gel, 6%; *upper panel*) or SDS–PAGE (*lower panel*) analysis. For native-PAGE, whole gel from the top to front was shown. Hsp-FL was detected by immunostaining with anti-Hsp-C antibody. SDS–PAGE analysis (*lower panel*) indicated that the same amount of Hsp-FL was used in these experiments. Representative results of three independent experiments are shown. (B) Hsp-FL (0.1 μM) was incubated with or without 250 μM of sulfatide at 4 °C for 1.5 h followed by incubating at 30 °C for 10 min. The mixture was subjected to native PAGE (stacking gel, 5% and separating gel, 6%).

mono-sialylated and desialylated glycopolymers tested, Hsp-FL exhibited essentially the same staining pattern as negative control (Fig. 2A, none), suggesting that Hsp-FL does not interact with mono-sialylated or neutral glycopolymers. These results indicate that sialylated glycans are not so effective as sulfated glycans in the interaction with Hsp-FL. In contrast, a di-sialylated glycopolymer, PAA(NeuAcα2,3(NeuAcα2,6)GalNAcα), formed the Type I and II complexes with Hsp-FL, suggesting that the presence of two sialic acid residues on the GalNAc residue in PAA(NeuAcα2,3(NeuAcα2,6)GalNAcα) is important for the formation of the large- and medium-size complexes with Hsp-FL. Thus, the intra-molecular cluster of sialic acid residue appears to be essential for interaction with Hsp-FL. However, colominic acid, a tandem-linked polymer of α2,8-Neu5Ac, did not exhibit any complex with Hsp-FL (Fig. 2B),

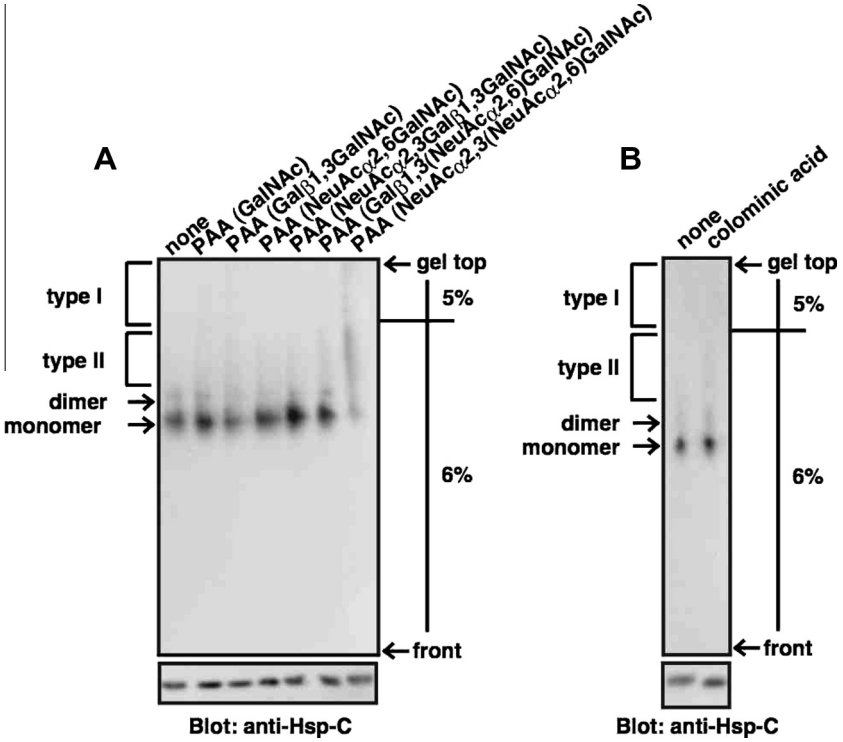


**Fig. 2.** Hsp-FL forms the complex with di-sialoglycopolymer. Hsp-FL (0.1 μM) was incubated with 0.45 mg/ml of indicated glycopolymers (A) or with 0.90 mg/ml of colominic acid (B), followed by native-PAGE (stacking gel, 5% and separating gel, 6%; *upper panel*) or SDS–PAGE (*lower panel*) analysis. See also the Fig. 1 legend.

suggesting that not only the number of sialic acid residue, but also the inner glycan structure or the linkage of the sialic acid residue is important in the complex formation.

We previously showed that a mono-sialylated ganglioside GM3, Neu5Acα2,3Galβ1,4Glcβ-ceramide, and a di-sialylated ganglioside GD3, Neu5Acα2,8Neu5Acα2,3Galβ1,4Glcβ-ceramide, interact with Hsp-FL and that these two glycolipids make the Type II complex [12]. This observation appears to be contrasted with our new findings with regard to the mono- and di-sialylated glycopolymers (Fig. 2A). We do not know why gangliosides and sialoglycopolymers differently interacts and make complexes with Hsp-FL, even though they contain the same number of sialic acid residues. However, it is possible that the inner glycan structure or the aglycone structure may affect the interaction and complex formation with Hsp-FL. In this regard, it should be noted that gangliosides form micelles above its critical micelle concentration. In micelles, clustered sialic acid resides are densely exposed on the micelle surface. PAA(NeuAcα2,3(NeuAcα2,6)GalNAcα) may mimic the ganglioside micelle, because it also shows the clustered sialic acid residues along the polymer backbone.

### 3.3. Hsp-FL interacts with heparin, heparan sulfate and dermatan sulfate in solution

GAGs are natural sulfated glycopolymers that have various sulfation patterns on the co-polymer of particular disaccharide units. We examined if GAGs are also involved in the interaction with Hsp-FL. As shown in Fig. 3, the Type II complex was obviously observed in the presence of heparin, heparan sulfate and dermatan sulfate. However, the complex formation was not detected with chondroitin, chondroitin sulfate A and C, keratan sulfate, keratan polysulfate, and hyaluronic acid. The formation of the Type II complex was the most prominent with heparin. These results indicate that the complex formation of Hsp-FL appears to depend on sulfated glycan structures. Notably, heparin, heparan sulfate, and dermatan sulfate, but not other GAGs tested, commonly contain 2-*O*-sulfated iduronic acid residues. Hsp70 may thus recognize the specific glycan structure including 2-*O*-sulfated iduronic acid

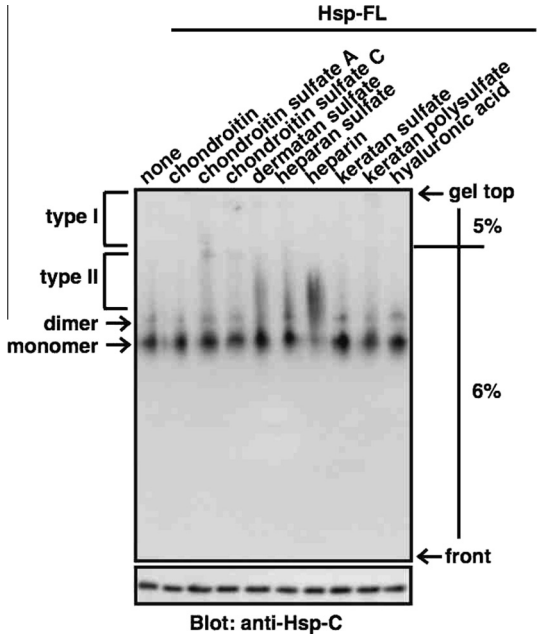


**Fig. 3.** Hsp-FL forms the complex with heparin, heparan sulfate and dermatan sulfate. Hsp-FL (0.1 μM) was incubated with 0.90 mg/ml each of the indicated GAGs, followed by native-PAGE (tacking gel, 5% and separating gel, 6%; *upper panel*) or SDS–PAGE (*lower panel*) analysis. See also the Fig. 1 legend.

in heparin, heparan sulfate and dermatan sulfate. The strong binding of Hsp70-FL with heparin was also confirmed by co-elution of Hsp-FL with heparin or heparin dimer prepared by glutaraldehyde-assisted cross-linking, when the mixtures were applied to Sephacryl S-100 chromatography (data not shown).

It was reported that Hsp70 contained a heparin-binding motif in the ATPase domain [21]. However, it should be noted that interactions with heparan sulfate and dermatan sulfate have never been reported. Chondroitin sulfate and dermatan sulfate are similar structures to each other, except that glucuronic acid residues in chondroitin sulfate are isomerized at 6-position into iduronic acid residues in dermatan sulfate. It is interesting to note that Hsp-FL interacted with dermatan sulfate, but not with chondroitin sulfate (Fig. 3) The isomerization from glucuronic acid to iduronic acid causes conformational changes of the backbone structures of these GAGs, which may change the orientation of sulfate groups along the polymers. In this context, the orientation of sulfate groups may be implicated in the interaction. The 6-*S*-GalNAcβ structure is the common residue between PAA(6-*S*-GalNAcβ) and chondroitin sulfate C. Although PAA(6-*S*-GalNAcβ) formed the Type I complex with Hsp-FL (Fig. 1A), chondroitin sulfate C showed no effects on the complex formation (Fig. 3). PAA(6-*S*-GalNAcβ) contains multiple external 6-*S*-GalNAcβ residues in the polymer, while chondroitin sulfate C exclusively contains internal 6-*S*-GalNAcβ residues in the repeated disaccharide unit 6-*S*-GalNAcβ1,4GlcAcβ1,3. It is thus suggested that the presence of clusters of non-reducing terminal 6-*S*-GalNAcβ residue is important in the interaction with Hsp-FL. Taken together, the orientation and appropriate clustering of sulfated glycans on the polymer appear to be important for the interaction and complex formation with Hsp70.

### 3.4. Determination of the domain of Hsp70.1 involved in the complex formation with glycopolymers and GAGs

Hsp70 consists of two domains, an N-terminal ATPase domain (Hsp-N) and a C-terminal peptide-binding domain (Hsp-C). It has been shown that Hsp-N has the activity to form the large- and medium-size complexes with acidic lipids [12]. Then, we asked which domain of Hsp70 is responsible for the interaction with glycopolymers and GAGs by native PAGE analysis (Fig. 4A). Broadly diffused bands of Hsp-N were observed around and below the Hsp-N monomer band in the presence of the glycopolymers and GAGs that were shown to interact with Hsp-FL, *i.e.*, PAA(3-*S*-Galβ), PAA(3-*S*-GlcNAcβ), PAA(4-*S*-GlcNAcβ), PAA(NeuAcα2,3(NeuAcα2,6)GalNAcα), heparin, heparan sulfate and dermatan sulfate (Figs. 1A, 2A and 3). The interaction of Hsp-N with heparan sulfate and dermatan sulfate was considerably weak, as compared with heparin, consistent with the results with Hsp-FL. In contrast, Hsp-C did not show the broadly diffused or large- and medium-size bands with any compounds (Fig. 4B). These results indicate that Hsp-N, but not Hsp-C, is responsible for the interaction with these acidic glycopolymers and GAGs. Notably, whereas these acidic glycopolymers and GAGs apparently formed the Type I complexes with Hsp-FL (Figs. 1A, 2A and 3), Hsp-N showed only broadly diffused bands (Fig. 4A). This result suggests that the Hsp-C domain is dispensable for the direct interaction with the acidic moieties, but is required for forming and stabilizing the large- and medium-size complex with the acidic glycopolymers.

## 4. Discussion

In this study, we have examined the interaction and the large- and medium-size complex formation of Hsp70 with sulfated and

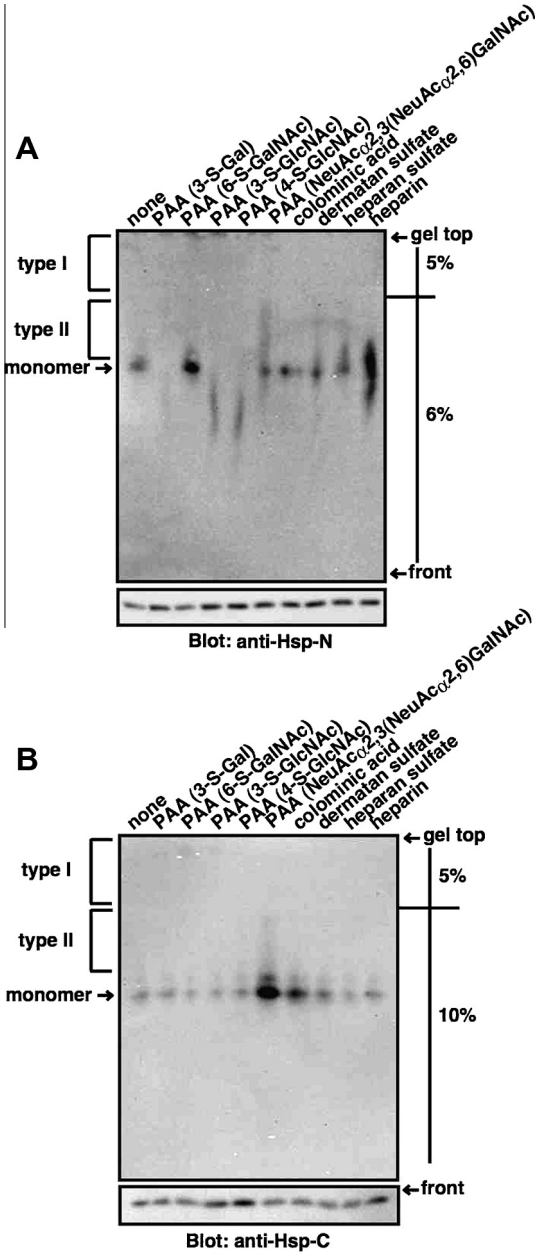


**Fig. 4.** Determination of the domain of Hsp70 involved in the complex formation with glycopolymers and GAGs. Hsp-N (A) or Hsp-C (B) (0.1 μM each) was incubated with indicated glycopolymers (0.45 mg/ml each), GAGs (0.90 mg/ml each) or colominic acid (0.90 mg/ml), and subjected to the native-PAGE (stacking gel, 5% and separating gel, 6% for Hsp-N or 10% for Hsp-C) (*upper panel*) or SDS–PAGE (*lower panel*). Hsp-N and Hsp-C antibodies were detected by immunostaining with anti-Hsp-N and anti-Hsp-C, respectively. SDS–PAGE analysis (*lower panel*) indicates that the same amount of Hsp-N or Hsp-C was used in these experiments.

sialylated glycopolymers and GAGs. We showed that Hsp70 has an activity to directly interact with di-sialylated and sulfated glycans in artificial PAA-glycopolymers and GAGs. The orientation and appropriate clustering of acidic glycans in these polymers appear to be important for the interaction with Hsp70. The ATPase domain is responsible for the binding with di-sialylated and sulfated glycans, but is not enough for forming large- and medium-size complex formation. On the other hand, the C-terminal domain is dispensable for the direct binding with the acidic glycans. Notably, heparin, heparan sulfate, and dermatan sulfate can form the large- and medium-size complexes with Hsp-FL, but not with Hsp-N. The fact that the lack of the C-terminal domain of Hsp70 impaired the large- and medium-sized complex formation with acidic glycans suggests that Hsp-C domain is important for forming and stabilizing the large- and medium-size complexes. In addition, it is particularly interesting to note that Hsp70 can bind to heparan sulfate and dermatan sulfate more strongly than chondroitin sulfate or keratan sulfate, because it seems that the presence of 2-*O*-sulfated iduronic acid residue, but not the acidity or the size of GAG, is important for the interaction. To our knowledge, this is the first indication of the complex formation between Hsp70 and a particular group of glycosaminoglycans (proteoglycans). Heparan sulfate and chondroitin sulfate are found on the cell surface of macrophages [22–24]. It is therefore attractive to speculate that interaction of Hsp70 with the GAGs may be involved in the extracellular functions of Hsp70. Hsp70 binds to macrophages and enhances the macrophage-mediated antigen uptake and the presentation of the antigens by MHC class-II molecule [25,26]. Therefore, it is suggested that Hsp70 may utilize heparan sulfate as a scaffold on the cell surface of macrophages for efficient endocytosis of the antigen into the antigen presentation pathway. Further study will be necessary to demonstrate the involvement of GAGs in the extracellular Hsp70 function.

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