***Staphylococcus aureus* expresses a cell surface protein that binds both IgG and β_2 -glycoprotein I**

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The existence of a second IgG-binding protein, protein Sbi, in *Staphylococcus aureus* has been reported previously. Later data indicated that protein Sbi also bound another serum component. This component has now been affinity-purified on immobilized protein Sbi and identified as β_2 -glycoprotein I (β_2 -GPI), also known as apolipoprotein H. The minimal β_2 -GPI-binding domain was identified by shotgun phage display and the binding was shown to be mediated by a region of 57 amino acids, clearly separated from the IgG-binding domain. It is also shown that protein Sbi, and thus the β_2 -GPI-binding activity, is expressed on the staphylococcal cell surface at levels varying between strains.

Keywords: Sbi, β_2 -glycoprotein I (apolipoprotein H), *Staphylococcus aureus*, IgG, phage display

INTRODUCTION

Staphylococcus aureus is a member of the human commensal flora. However, if mucosal barriers are breached or host immune defences are impaired, *S. aureus* can cause a variety of diseases in humans and animals, including wound sepsis, osteomyelitis, endocarditis and mastitis. With the widespread use of antimicrobials, the incidence and spread of highly antibiotic-resistant, especially methicillin-resistant *S. aureus* strains (MRSA), have increased rapidly in recent years, and constitute a clinical and epidemiological problem in hospitals all over the world (Swartz, 1994). To enable the development of preventive and therapeutic measures, investigations of interactions between the bacterium and the host are required.

S. aureus strains export a number of potential virulence factors into the surroundings, e.g. alpha-, beta-, delta- and gamma-toxins, toxic shock syndrome toxin, enterotoxins, lipase, coagulase and proteases (Cunningham *et al.*, 1996). The bacterium is also known to express adhesins which bind to host matrix and plasma proteins, e.g. fibronectin, fibrinogen, collagen, bone sialoprotein,

vitronectin and immunoglobulins (Cunningham *et al.*, 1996). The binding of IgG is a common property among pathogenic Gram-positive cocci and the IgG-binding protein protein A (SpA; Uhlén *et al.*, 1984) is one of the most studied surface proteins of *S. aureus*. However, it has not been proven that protein A is of major importance for virulence since *spa*-negative mutants are only slightly less virulent than the parent bacteria (Jonsson *et al.*, 1985; Patel *et al.*, 1987). Recently, we reported the existence of a second IgG-binding protein (Sbi) in *S. aureus* (Zhang *et al.*, 1998).

We have shown that shotgun phage display can be used for cloning of genes encoding bacterial adhesins by panning against host proteins (Jacobsson & Frykberg, 1995, 1996, 1998). Recently, panning a phage display library made from *S. aureus* chromosomal DNA against newborn calf serum (NCS) resulted in detection of not only known interactions, but also a novel interaction (Jacobsson & Frykberg, 1998). The majority of the clones isolated after panning against NCS encoded IgG-binding or fibronectin-binding polypeptides. However, most of the other clones contained different overlapping DNA fragments derived from the *sbi* gene. Interestingly, the inserts did not encode the part of protein Sbi that mediates the IgG-binding activity, which strongly suggested that protein Sbi bound a second serum component in addition to IgG. This protein has now been isolated from serum by affinity chromatography

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Abbreviations: β_2 -GPI, β_2 -glycoprotein I; HRP, horseradish peroxidase; NCS, newborn calf serum.

and identified as β_2 -glycoprotein I (β_2 -GPI, also called apolipoprotein H).

METHODS

Bacterial strains, vector and helper phage. Restriction and modifying enzymes were from Amersham or MBI Fermentas.

Escherichia coli strain MC1061 (Wertman *et al.*, 1986) was used for production of fusion protein and *E. coli* strain TG1 (Sambrook *et al.*, 1989) for production of phage stocks. Both strains were cultivated in Luria-Bertani (LB) broth or on LA plates (LB broth with 1.5% w/v, agar) and when required supplied with 50 μ g ampicillin ml⁻¹. Phage R408 (Promega) was used as helper phage. Staphylococcal strains 8325-4, Wood 46, Newman 4 and Cowan I (NCTC 8350) were grown in TSB (Tryptic Soya Broth; Oxoid). *Staphylococcus epidermidis* 247 (Rosendorf & Kayser, 1974) containing the shuttle plasmid pShX1 (Zhang *et al.*, 1998) was grown in TSB supplied with 20 μ g chloramphenicol ml⁻¹. *S. aureus* strain 8325-4 is NCTC 8325 cured of prophages (Novick, 1967). Wood 46 is a protein-A-negative reference strain (Kronvall *et al.*, 1970) and Newman 4 is a spontaneous mutant of strain Newman with enhanced production of fibronectin-binding protein (Jönsson *et al.*, 1991).

The pG8SAET phagemid vector is a derivative of the pG8SPA1 vector (Jacobsson & Frykberg, 1998), in which the serum-albumin-binding domain has been exchanged for the E-tag (Pharmacia Biotech). The oligonucleotides E-tagS 5' GGT GCG CCG GTG CCG TAT CCG GAC CCA CTG GAA CCG CGT GCC TAG G 3' and E-tagR 5' GAT CCC TAG GCA CGC GGT TCC AGT GGG TCC GGA TAC GGC ACC GGC GCA CC 3' were synthesized by Pharmacia Biotech. The pG8SPA1 vector was digested with *Bam*HI and *Sma*I to remove the region encoding the albumin-binding domain and ligated with the oligonucleotides using the Ready-To-Go ligation kit from Pharmacia Biotech.

Antibodies. Antibodies against MAL-Sbi (aa 33–436 in protein Sbi; Zhang *et al.*, 1998) developed in chicken were obtained through Immunsystem (four repeated immunizations with 50 μ g MAL-Sbi). The antibodies were affinity-purified on MAL-Sbi, immobilized on CNBr-activated Sepharose 4B (Pharmacia Biotech) and labelled with horseradish peroxidase (HRP) (Boehringer Mannheim). Rabbit polyclonal antibodies against human β_2 -GPI were from Chemicon International and HRP-labelled goat anti-rabbit antibodies were from Santa Cruz Biotechnology. The mAb against the E-tag was from Pharmacia Biotech and the HRP-labelled horse anti-mouse antibodies were from Vector Laboratories.

Serum protein purification and N-terminal amino acid sequencing. MAL-Sbi Δ fusion protein, lacking the IgG-binding region (aa 1–143), was purified on an amylose resin as described by Zhang *et al.* (1998). MAL-Sbi Δ (10 mg) was coupled to 0.5 g dry CNBr-activated Sepharose 4B according to the manufacturer's instructions. The 4.5 ml column was equilibrated with 20 mM Tris/HCl, pH 7.4, with 0.2 M NaCl, whereafter 10 ml NCS (Life Technologies) was passed over the column. After washing with the same Tris/HCl buffer until a steady baseline was obtained, the bound protein was eluted in 6 ml 0.25 M acetic acid, pH 2.2. After extensive dialysis against distilled H₂O the eluted proteins were lyophilized and dissolved in 100 μ l PBS-D (140 mM NaCl, 8 mM Na₂HPO₄, 1.7 mM KH₂PO₄, 2.7 mM KCl, pH 7.4). The N-terminal amino acid sequence was determined using the Applied Biosystems 476A Protein Sequencer according to the

manufacturer's instructions. The SWISS-PROT and PIR databases were searched for sequence homologies.

The same protocol was also used for purification of the serum component from human and rat serum.

Electrophoresis and Western blotting. Proteins purified from bovine, human and rat serum were analysed by non-reducing SDS-PAGE using the Phast system (Pharmacia Biotech) with PhastGel Gradient 8–25% (w/v) gels and PhastGel SDS Buffer Strips. Purified protein (2 μ l) was mixed with 2 μ l 2 $\times$ sample buffer (1 $\times$ buffer = 62.5 mM Tris/HCl, pH 6.8; 10%, v/v, glycerol; 2%, w/v, SDS; and 0.01%, w/v, bromophenol blue), boiled for 2 min, after which 1 μ l was loaded on the gel. As a positive control, 0.2 μ g commercially available human apolipoprotein H (ICN Pharmaceuticals) was used. The gels were run in duplicate and the proteins were blotted onto nitrocellulose filters (Schleicher & Schuell). Protein was detected using either rabbit polyclonal antibodies against human β_2 -GPI and HRP-labelled goat anti-rabbit antibodies, or MAL-Sbi [15 μ g ml⁻¹ in PBS-T (PBS-D containing 0.05% v/v, Tween 20)] and HRP-labelled antibodies against MAL-Sbi. Bound antibodies were detected with 4-chloro-1-naphthol (Serva).

Identification of the β_2 -GPI-binding domain. A gene fragment phage display library was constructed essentially as described by Jacobsson & Frykberg (1995). In short, DNA from plasmid pPX1 (pUC18 containing the cloned *sbi* gene; Zhang *et al.*, 1998) was sonicated until fragments from 0.1 to 1 kb in size were obtained. The fragments were made blunt-ended and, using the Ready-To-Go ligation kit (Pharmacia Biotech), were ligated into the gene-VIII-based phagemid pG8SAET, previously digested with *Sna*BI and dephosphorylated with calf intestine alkaline phosphatase. Electrotransformation into *E. coli* strain TG1 yielded 8 $\times$ 10⁵ individual transformants, and after infection with helper phage, the final library had a titre of 1 $\times$ 10¹¹ c.f.u. ml⁻¹.

Two hundred microlitre portions of the library were panned against NCS (diluted 1/20 in 50 mM NaHCO₃, pH 9.7) or human β_2 -GPI (10 μ g ml⁻¹) immobilized in microwells as described earlier (Jacobsson & Frykberg, 1995). Pannings against NCS were carried out in the presence of human IgG (100 μ g ml⁻¹; Kabivitrum) to inhibit the enrichment for IgG-binding phage. Colonies obtained after infection of *E. coli* TG1 with the phage particles eluted at pH 2.0 were screened for expression of the E-tag using an mAb against the E-tag and a HRP-labelled horse anti-mouse antibody. Plasmid DNA was isolated from clones expressing the E-tag using the Qiaprep Miniprep kit (Qiagen). The nucleotide sequence of the inserted DNA was determined with the Thermo Sequenase dye terminator cycle sequencing kit from Amersham and the samples were analysed using the ABI 377 DNA Sequencer (Perkin Elmer).

Separation of the IgG- and β_2 -GPI-binding domains. Phage stocks were made from clones SbiS12 and SbiS13 (Fig. 1) as described previously (Zhang *et al.*, 1998) and the titres were adjusted to 5 $\times$ 10⁸ c.f.u. ml⁻¹. The stocks were panned against human IgG, β_2 -GPI and fibronectin (Sigma), coated at concentrations of 10 μ g ml⁻¹, as described by Jacobsson & Frykberg (1995). The number of bound phagemid particles was determined as c.f.u. after infection of *E. coli* TG1 cells with the phage eluted at pH 2.0.

Binding of ¹²⁵I-labelled β_2 -GPI and anti-Sbi antibodies to different *S. aureus* strains. Human β_2 -GPI was ¹²⁵I-labelled according to Bolton & Hunter (1973) using the Bolton + Hunter reagent from Amersham. Anti-Sbi antibodies

Fig. 1. Schematic drawing of protein Sbi aligned with the polypeptides isolated by panning. (a) Clones isolated after panning a library made from staphylococcal chromosomal DNA against NCS (Jacobsson & Frykberg, 1998). (b, c) Panning of an *sbi* gene fragment library against serum and β_2 -GPI, respectively. Amino acids (aa) show the exact position of the peptides in protein Sbi (Zhang *et al.*, 1998). Black box, signal peptide; first hatched box, IgG-binding domain; second hatched box, putative IgG-binding domain; dotted box, β_2 -GPI-binding domain; striped box, proline repeat region. (d) Amino acid sequence of the minimal β_2 -GPI-binding domain deduced by phage display.

were ^{125}I -labelled using the IODO-BEADS Iodination Reagent kit (Pierce) according to the manufacturer's instructions.

Cells from overnight cultures were collected by centrifugation and washed in PBS-D. One hundred microlitres of ^{125}I -labelled β_2 -GPI (approx. 65 000 c.p.m., specific activity 1.1×10^6 c.p.m. μg^{-1}) or 100 μl labelled anti-Sbi antibodies (approx. 125 000 c.p.m., specific activity 1.2×10^8 c.p.m. μg^{-1}) in PBS-T with 0.1% (w/v) BSA was mixed with 5×10^8 cells and incubated at room temperature for 1 h. The cells were pelleted and washed three times in PBS-T with 0.1% (w/v) BSA. Finally, the radioactivity of the pellets was measured in an LKB gamma counter.

RESULTS

Isolation of the second serum protein bound by protein Sbi

Earlier results suggested that protein Sbi binds a second serum component in addition to IgG (Jacobsson & Frykberg, 1998). This component has now been isolated from NCS on an affinity matrix containing a truncated protein Sbi lacking the IgG-binding domain (MAL-Sbi Δ ; Zhang *et al.*, 1998). The eluted proteins were concentrated by lyophilization and analysed by SDS-PAGE under non-reducing conditions. Staining with Coomassie blue showed one major protein of about 50 kDa (Fig. 2a, lane 3). N-terminal sequencing of this protein gave the peptide sequence Gly-Arg-Thr-Cys-Pro-Lys-Pro-Asp. Homology searches showed that this sequence was identical to the N-terminus of the mature β_2 -GPI. The molecular mass of bovine β_2 -GPI deduced from the nucleotide sequence (Gao *et al.*, 1993) is 36 kDa, but is reported to migrate at approximately 50 kDa in SDS-PAGE, which agrees well with the size of

the protein we isolated from NCS. A protein of similar size could also be purified from human and rat serum.

The identity of the purified proteins was confirmed using antibodies directed against human β_2 -GPI. Fig. 2(b) shows that the proteins affinity-purified from the three different sera (lanes 2–4) and the commercially available human β_2 -GPI (lane 5) are recognized by a polyclonal antibody against human β_2 -GPI. Fig. 2(c) shows an identical blot incubated with the MAL-Sbi fusion protein, which was detected with HRP-labelled antibodies against protein Sbi.

In both Western blots, a second larger protein is visible in the commercially available human β_2 -GPI (Fig. 2b, c, lane 5) and is believed to be a dimer. A second, significantly larger protein, is also visible in the β_2 -GPI purified from human serum (Fig. 2b, c, lane 4). This protein is stronger in the blot detected with the MAL-Sbi fusion protein (Fig. 2c) than in the one detected with antibodies against human β_2 -GPI (Fig. 2b). This protein is most likely either a complex of β_2 -GPI and an unidentified serum component co-purified with β_2 -GPI, or a multimer of β_2 -GPI. A protein of the same size is also visible on the Coomassie-blue-stained gel after purification from rat serum (Fig. 2a, lane 2), but is not recognized by either the MAL-Sbi fusion protein or the polyclonal antibody in Western blots (Fig. 2b, c). Strangely, the larger component purified from human serum, but not from rat serum, reacts strongly with the MAL-Sbi protein. In contrast, the antibodies against β_2 -GPI react only weakly with the larger protein band. However, from bovine serum only one form of the protein was obtained after affinity purification on protein Sbi.

Fig. 2. Purification of β_2 -GPI from different mammalian sera. (a) SDS-PAGE. Also shown are Western blots detected with (b) polyclonal antibodies against human β_2 -GPI and (c) MAL-Sbi as described in Methods. Lanes: 1, size marker (97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa); 2, β_2 -GPI purified from rat serum; 3, β_2 -GPI purified from bovine serum; 4, β_2 -GPI purified from human serum; 5, commercially available human β_2 -GPI (ICN).

Table 1. Specific binding of phage displaying the β_2 -GPI- or IgG-binding domain of protein Sbi to different ligands

Data represent the means $\pm$ SD of three parallel experiments.

Stock	Phagemid particles eluted after panning against the respective ligand ($10^{-2} \times$ c.f.u.)		
	β_2 -GPI	IgG	Fibronectin
SbiS12	3.0 ± 1.9	15000 ± 2000	8.8 ± 4.0
SbiS13	590 ± 220	2.6 ± 0.2	3.9 ± 0.3

Identification of the β_2 -GPI-binding domain

The β_2 -GPI-binding domain was identified using shotgun phage display. A phage display library was constructed from the cloned *sbi* gene. The resulting library, consisting of phages expressing different parts of the gene, was panned against human β_2 -GPI or bovine serum. By sequencing the inserted DNA in clones obtained after panning and aligning the encoded polypeptides with protein Sbi, the β_2 -GPI-binding domain was identified. As shown in Fig. 1, the domain is positioned downstream of the IgG-binding domain and is completely separated from this. Although the panning against NCS was carried out in the presence of free IgG to inhibit the isolation of IgG-binding phage, one clone containing the IgG-binding region (SbiS12) was recovered.

To confirm this result, phage stocks were prepared from two different clones, clone SbiS13 expressing the β_2 -GPI-binding region (aa 196–293) and clone SbiS12 expressing the IgG-binding region (aa 20–109). The phage stocks were panned in triplicate against human IgG, β_2 -GPI and fibronectin, included as a negative control. The number of eluted phage particles was used as a measure of the ability to bind the respective ligand. As shown in Table 1, phages expressing aa 20–109 only bind IgG, while phages expressing aa 196–293 only bind β_2 -GPI.

Table 2. Binding of 125 I-labelled antibodies against protein Sbi or 125 I-labelled β_2 -GPI to staphylococcal cells

Data represent the means $\pm$ SD of three parallel binding assays.

Strain	125 I- α Sbi	125 I- β_2 -GPI
<i>S. aureus</i>		
8325-4	1846 ± 153	1311 ± 108
Cowan I	2951 ± 164	1459 ± 5
Newman 4	14018 ± 352	6305 ± 75
Wood 46	92 ± 24	580 ± 80
<i>S. epidermidis</i>		
247	336 ± 39	519 ± 38
247 with pShX1	2061 ± 34	1043 ± 12

Expression of protein Sbi and binding of β_2 -GPI to *S. aureus*

125 I-labelled β_2 -GPI and anti-Sbi antibodies were used to show that protein Sbi is expressed on the cell surface in different strains of *S. aureus*. The antibodies were developed in chicken since *S. aureus* does not bind chicken IgG. Binding of labelled β_2 -GPI to staphylococcal cells required labelling with Bolton + Hunter reagent to avoid reduction of the disulfide bridges in β_2 -GPI. In addition, a high concentration of BSA in the binding buffer was required to inhibit non-specific binding, which also has been reported for binding of β_2 -GPI to phospholipids (Wurm, 1984). However, the results obtained for the negative control *S. epidermidis* indicate that the background binding of β_2 -GPI could not be completely eliminated. The results in Table 2 show that high levels of protein Sbi were found in *S. aureus* strain Newman 4 whereas strains 8325-4 and Cowan I produced low amounts. Strain Wood 46 did not bind more antibodies or β_2 -GPI than the negative control, *S. epidermidis* strain 247. However, *S. epidermidis* strain 247 harbouring a shuttle plasmid containing the *sbi* gene (pShX1) expressed similar

amounts of protein Sbi as strains 8325-4 and Cowan I (Table 2).

DISCUSSION

Using immobilized protein Sbi, the second interacting serum component has now been purified from bovine serum and identified as β_2 -GPI by N-terminal amino acid sequencing. The identity has further been confirmed with polyclonal antibodies against β_2 -GPI. As shown in Fig. 2, both commercially available β_2 -GPI and β_2 -GPI purified by affinity chromatography on protein Sbi are recognized by the antibodies against β_2 -GPI and by protein Sbi. All SDS-PAGE was run under non-reducing conditions as preceding experiments showed that, in a Western blot, protein Sbi did not recognize the purified proteins and the polyclonal antibodies only showed a weak binding, after electrophoresis under reducing conditions (data not shown). This was not unexpected since β_2 -GPI contains 11 disulfide bonds (Kato & Enjyji, 1991; Bendixen *et al.*, 1992), which points at the importance of the secondary structure for the recognition by protein Sbi. A similar observation was reported by Mehdi *et al.* (1994), who found that treatment of β_2 -GPI with a reducing agent destroyed the ability to bind to a recombinant hepatitis B surface antigen.

Alignment with protein Sbi of the polypeptides expressed by the phage display clones obtained after panning against NCS or β_2 -GPI shows one β_2 -GPI-binding domain. This domain is located downstream of the IgG-binding domain and is clearly separated from this (Fig. 1). The minimal β_2 -GPI-binding domain deduced from isolated clones consists of 57 aa, similar in size to the minimal IgG-binding domain (52 aa) identified earlier. Immediately downstream of the IgG-binding domain there is a region with homology to the IgG-binding repeats in protein A, but no IgG-binding clones covering only this region were isolated (Zhang *et al.*, 1998). Four clones isolated after panning against serum and β_2 -GPI (NCS17, SbiS8, SbiS27 and SbiA96) extend approximately 70 aa further upstream than the majority of the clones (Fig. 1). This suggests that an extended N-terminus may contribute to a more favourable conformation that increases the affinity for β_2 -GPI, or may even indicate the presence of a second β_2 -GPI-binding domain. Thus, it can not be excluded that protein Sbi contains two IgG- and two β_2 -GPI-binding domains, but with a difference in affinity for the respective ligand, which would explain why only one domain is isolated by shotgun phage cloning.

This is the first report of a bacterial protein that binds the serum glycoprotein β_2 -GPI. Although β_2 -GPI was described in 1961 and despite its rather high concentration in serum (0.15–0.30 mg ml⁻¹) (Cleve & Rittner, 1969), the biological function is not clear. The protein binds to negatively charged substances such as heparin, dextran sulfate and phospholipids. β_2 -GPI is structurally related to regulators of the complement system, and consists of five repetitive domains, four of

which contain a motif called the complement control protein repeat or Sushi-domain (Kato & Enjyji, 1991; Steinkasserer *et al.*, 1991; Bendixen *et al.*, 1992). β_2 -GPI has been reported to have several biological functions when bound to negatively charged lipids, such as promoting clearance of liposomes and maybe foreign particles from the bloodstream (Chonn *et al.*, 1992) and inhibiting blood coagulation by affecting the pro-thrombinase activity of, for example, non-activated or lysed platelets (Nimpf *et al.*, 1986). The relevance of the staphylococcal- β_2 -GPI interaction *in vivo* is not obvious. It is possible that bacteria in wounds can benefit from inhibition of the blood clotting. In addition, the interaction of cell-wall-bound β_2 -GPI with negatively charged lipid membranes may be a means for attachment to host cells or entering cells for intracellular growth of the staphylococci.

β_2 -GPI has attracted a lot of attention during the last decade since it has been implicated to be involved in the development of autoimmune disease. For example, in anti-phospholipid antibody syndrome (APS), the auto-immune antibodies are often not directed against phospholipids alone, but against phospholipids bound to β_2 -GPI or β_2 -GPI alone (Galli *et al.*, 1990; McNeil *et al.*, 1990; Verrier Jones *et al.*, 1992). It is not known what causes the induction of these antibodies but it has been suggested that a change in conformation of β_2 -GPI may be responsible. Interestingly, anti-phospholipid antibodies (aPL) can be induced by immunization with heterologous β_2 -GPI (Pierangeli & Harris, 1989; Gharavi *et al.*, 1992, 1995; Aron *et al.*, 1995; García *et al.*, 1997). Phospholipids as such are poor immunogens, but when coupled to a carrier such as BSA or *S. aureus* cells, they can induce production of aPL (Rauch *et al.*, 1984). Thus, it is tempting to speculate that *S. aureus* infections may be one of the mechanisms for induction of APS. The bacteria may simply act as a carrier of β_2 -GPI and trigger the production of aPLs. Alternatively, the binding of β_2 -GPI to protein Sbi might cause a conformational change in β_2 -GPI which activates the immune response.

The biological importance of protein Sbi in the virulence of *S. aureus* has not yet been determined. IgG binding is found in virtually all *S. aureus* strains and has been considered to be important for virulence. Still, *S. aureus* mutants lacking protein A were only slightly less virulent than the parental strain (Jonsson *et al.*, 1985; Patel *et al.*, 1987). The fact that protein Sbi binds both IgG and β_2 -GPI increases the likely importance of Sbi in virulence.

PCR analysis with a single primer pair has shown that the *sbi* gene was present in 28 of 29 human clinical isolates (data not shown). In addition, using antibodies against protein Sbi expression levels similar to that of strain 8325-4 were detected in 23 of the 24 isolates tested (data not shown). Thus, in general the expression level of protein Sbi is low. Interestingly, strain Newman 4, originally isolated as a spontaneous mutant of strain Newman that expresses high levels of fibronectin-binding proteins, also is high in expression of protein Sbi, indicating a common regulatory mechanism.

Further investigation of the regulation of protein Sbi expression is under way.

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