Article

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Abstract

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Reference

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An adhesion molecule in free-living *Dictyostelium* amoebae with integrin β features

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The study of free-living amoebae has proven valuable to explain the molecular mechanisms controlling phagocytosis, cell adhesion and motility. In this study, we identified a new adhesion molecule in *Dictyostelium* amoebae. The SibA (Similar to Integrin Beta) protein is a type I transmembrane protein, and its cytosolic, transmembrane and extracellular domains contain features also found in integrin β chains. In addition, the conserved cytosolic domain of SibA interacts with talin, a well-characterized partner of mammalian integrins. Finally, genetic inactivation of *SIBA* affects adhesion to phagocytic particles, as well as cell adhesion and spreading on its substrate. It does not visibly alter the organization of the actin cytoskeleton, cellular migration or multicellular development. Our results indicate that the SibA protein is a *Dictyostelium* cell adhesion molecule presenting structural and functional similarities to metazoan integrin β chains. This study sheds light on the molecular mechanisms controlling cell adhesion and their establishment during evolution.

Keywords: adhesion; *Dictyostelium*; integrin; motility; talin

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INTRODUCTION

Cell–substrate adhesion is a crucial step in many biological processes such as development, wound healing, metastasis and phagocytosis. In mammalian cells, several proteins are involved in cellular adhesion, in particular cell-surface receptors, signalling molecules and components of the actin cytoskeleton. Integrin-mediated cell adhesion is one of the most widely studied adhesion mechanisms. Integrins are heterodimeric type I transmembrane proteins composed of one α-subunit and one β-subunit, which bind to the extracellular matrix by their extracellular domain and control cell spreading, migration, proliferation and survival (Schwartz, 2001).

The amoeba *Dictyostelium discoideum* is a widely used model to study cellular adhesion, phagocytosis and the dynamics of the actin cytoskeleton. This unicellular organism is amenable to genetic analysis, mainly owing to its fully sequenced haploid genome (Eichinger *et al*, 2005). Many gene products have been implicated in various aspects of the phagocytic process in *Dictyostelium* on the basis of the analysis of targeted knockout mutants (Bracco *et al*, 2000) or of mutants identified in random genetic screenings (Cornillon *et al*, 2000; Fey *et al*, 2002; Gebbie *et al*, 2004). Nevertheless, the receptors controlling the adhesion of free-living *Dictyostelium* cells to their substrate and to phagocytic particles have not been unambiguously identified.

Here, we report the identification and characterization of a *Dictyostelium* adhesion molecule, named SibA, which presents several features also found in mammalian integrin β chains. These results may shed light on the origin of adhesion molecules in evolution.

RESULTS AND DISCUSSION

SibA, a membrane protein involved in phagocytosis

To identify new genes involved in phagocytosis, we mutagenized *Dictyostelium* cells by random insertion of a plasmid, and selected a mutant defective for phagocytosis of fluorescent latex beads. On the basis of its resemblance to integrins (see below), the corresponding gene was named *SIBA* (Similar to Integrin Beta). In the *sibA* mutant, the mutagenic plasmid is inserted in the DDB0187447 gene (Fig 1A), resulting in the absence of the corresponding transcript (Fig 1B). The SibA protein (1,928 amino-acid residues) has a putative endoplasmic reticulum insertion signal sequence at its amino terminus (position 1–21) and a transmembrane domain towards its carboxyl terminus (position 1859–1881), suggesting that it is a type I transmembrane protein. Indeed, cell-surface biotinylation followed by immunoprecipitation

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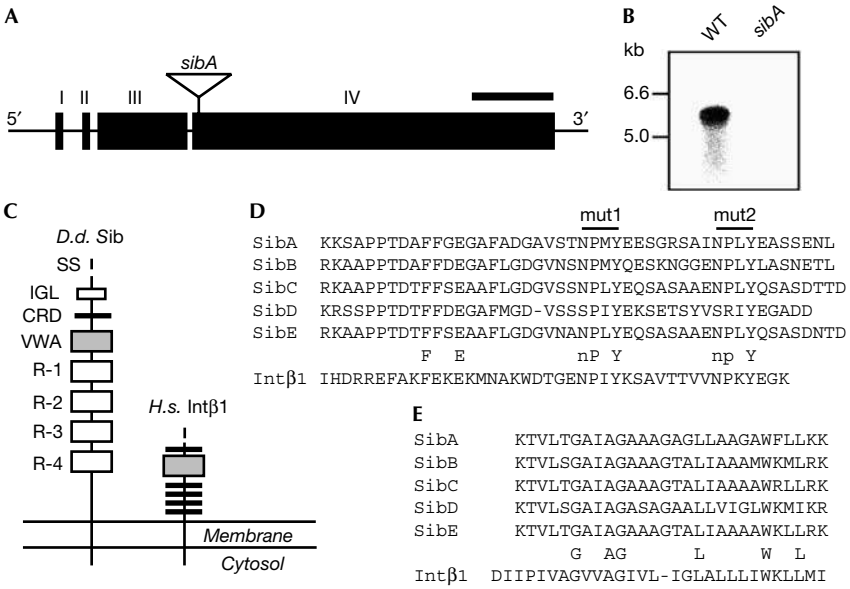

Fig 1 | Structure of Sib proteins. (**A**) Organization of the *SIBA* gene. Exons are shown as numbered black boxes (I–IV). The position of the vector insertion site in *sibA* mutant cells is indicated (nucleotide 1235 of the coding sequence). Scale bar, 1 kb. (**B**) A 6-kb *SIBA* transcript was detected by northern blot in wild-type (WT) but not in *sibA* mutant cells. (**C**) Structure of the SibA protein, and of human integrin β. CRD, cystein-rich domain; *D.d.*, *Dictyostelium discoideum; H.s.*, *Homo sapiens*; IGL, immunoglobulin-like fold; R, repeated motif; SS, cleavable signal sequence; VWA, von Willebrand factor type A domain. (**D**) The cytoplasmic domain of Sib proteins contains a conserved motif also found in integrin β chains. (**E**) The transmembrane domain of Sib proteins contains a GxxxG motif also present in integrin β chains.

confirmed that SibA is present at the cell surface (Fig 2A). Four genes encoding homologues of SibA are present in the *Dictyostelium* genome (DDB0187821, DDB0187822, DDB0219318, DDB0187788), and positioned, like *SIBA*, on chromosome V. The first three are adjacent and arranged in tandem, and the fourth is in close proximity, suggesting a recent expansion of this gene family. Consistent with this hypothesis, the overall structure of Sib proteins is almost identical.

The extracellular domains of Sib proteins contain an immunoglobulin-like fold (E-set, CL-0159, positions 174–235), a cysteine-rich domain (positions 394–461) and a von Willebrand factor type A domain (VWA, positions 520–691; Fig 1C). These three domains are commonly found in the extracellular domains of secreted or membrane-bound proteins and in many instances have been implicated in cell–substrate adhesion. VWA domains are ubiquitous in cytoplasmic proteins, but are also specifically deployed in several metazoan extracellular proteins, with integrin β subunits as very early evolutionary representatives (Whittaker & Hynes, 2002). Although lacking several features characteristic of metazoan integrin β chains, the Sib VWA domains show particularly high homology to integrin β VWA domains (supplementary information 1 online).

In addition, Sib extracellular domains contain four repeats of about 200 amino-acid residues (R1–R4; Fig 1C; supplementary Fig S2 online) also found in several surface or secreted bacterial proteins, in particular VCBS (IPR010221) and RTX (Lally *et al*, 1999) proteins. These bacterial proteins are involved in the interaction of bacteria with their substrate or with host cells, suggesting that these repeats could have a role in binding to certain substrates. We could not detect similar repeats in any eukaryotic protein other than *Dictyostelium* Sib proteins. Sib proteins thus represent hybrid structures associating bacterial-like repeats and metazoan-like features (an extracellular VWA domain), suggesting that these bacterial repeats might have been acquired from bacterial genomes by horizontal transfer.

The Sib transmembrane and cytosolic domains are particularly conserved among Sib proteins. The transmembrane domain is unusually rich in glycine residues, forming notably a GxxxG motif (Fig 1E). A GxxxG membrane motif is important for the oligomerization of several homo- and hetero-oligomeric membrane complexes (Cosson & Bonifacino, 1992; Russ & Engelman, 2000) and is a key feature of integrins' structure (Schneider & Engelman, 2004). The cytosolic domain contains two highly conserved NPxY motifs separated by eight amino-acid residues (Fig 1D), a motif also found in integrin β chains.

The cytosolic domain of Sib proteins interacts with talin

The conserved cytoplasmic domain of integrin β chains mediates interactions with several cytosolic proteins. One of these, talin, has a crucial role in integrin-mediated adhesion (Calderwood, 2004). However, integrin β cytosolic domains contain a tryptophan residue crucial for this interaction, which is not conserved in Sib proteins. To test the ability of the SibA cytosolic domain to interact with talin, we expressed a fusion protein of glutathione *S*-transferase (GST) with the cytosolic domain of SibA in bacteria, purified it and immobilized it on Sepharose beads and then incubated the beads with a *Dictyostelium* cellular lysate. Talin bound to GST–SibA, but not to GST alone (Fig 2B). As expected, no signal was detected when a lysate from *talin* knockout cells was used. In addition, talin also bound to GST–SibB, GST–SibC,

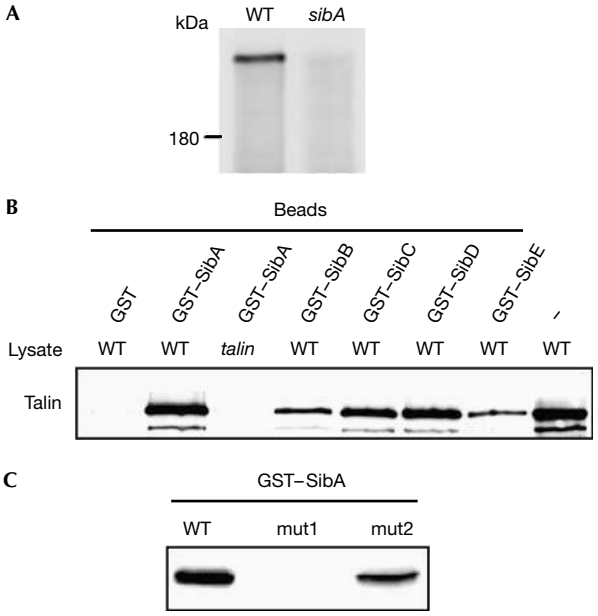

Fig 2 | Interaction of SibA with talin. (**A**) SibA is localized at the cell surface. The cell surface of wild-type (WT) and *sibA* cells was biotinylated. Biotinylated SibA was immunoprecipitated and then observed with avidin–horseradish peroxidase. (**B**) Fusion proteins of glutathione *S*-transferase (GST) with the cytosolic domain of each Sib protein were produced in bacteria, purified and immobilized on beads. The beads were incubated with *Dictyostelium* cell lysates, washed and the bound talin was shown by immunoblotting. A lysate from *talin* mutant cells was used as a control (third lane). The *Dictyostelium* lysate loaded on the gel (right lane) corresponds to 10% of the amount of lysate incubated with each GST fusion protein. (**C**) Mutating the membrane-proximal NPxY SibA motif (mut1; see Fig 1D) abolished the interaction with talin.

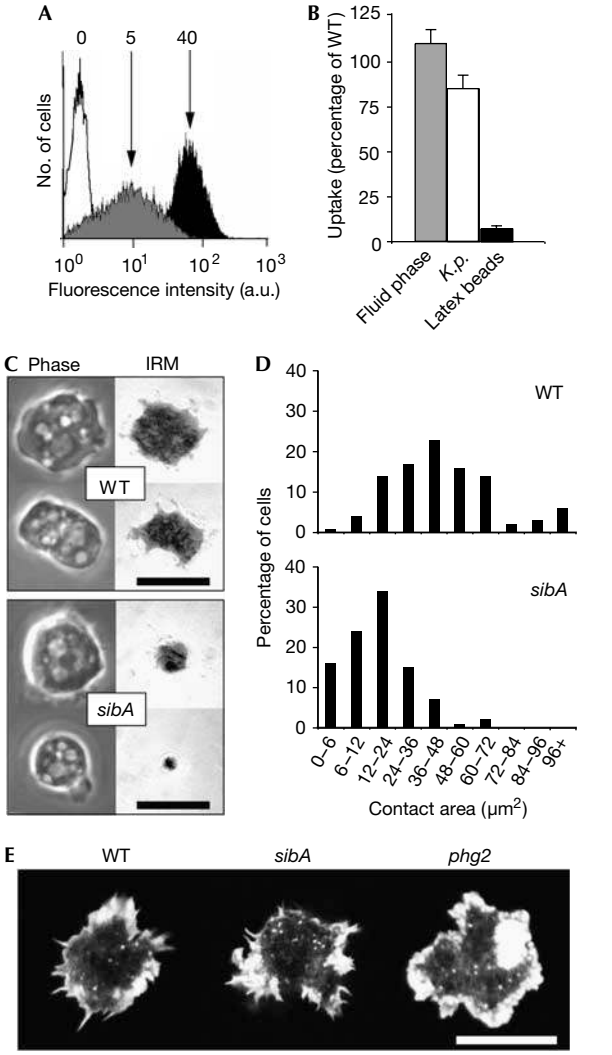

Fig 3 | *sibA* mutant cells show specific adhesion defects. (**A**) *sibA* mutant cells phagocytose latex beads poorly. Wild-type (WT, black curve) or *sibA* cells (grey curve) were incubated for 20 min in the presence (or absence: white curve) of fluorescent latex beads. The amount of internalized fluorescence was determined with a fluorescence-activated cell sorter. The average number of beads incorporated per cell is indicated above each curve. (**B**) Cells were incubated with fluorescent latex beads, *Klebsiella* bacteria (*K.p.*) or dextran for 20 min. The internalization in *sibA* cells is expressed as a percentage of WT internalization. The mean and s.e.m. of at least five experiments are indicated. (**C**) The attachment of *sibA* mutant cells to a glass substrate is defective. WT and *sibA* cells attached to glass coverslips were observed by phase-contrast microscopy (Phase) and by IRM (interference reflection microscopy). (**D**) The areas of 100 individual cells in contact with the coverslip were measured. (**E**) The actin cytoskeleton is unaffected in *sibA* cells. WT, *sibA* or *phg2* cells were fixed and stained with phalloidin. As described (Gebbie *et al*, 2004), abnormal actin aggregates form in *phg2* mutant cells. Scale bar, 10 μm.

GST–SibD and GST–SibE (Fig 2). Mutational analysis showed that the membrane-proximal NPxY motif (mutated in mut1) is essential for binding to talin, whereas the membrane-distal motif (mut2) is dispensable (Fig 2C). A similar finding was previously reported for integrin β chains (Calderwood, 2004).

Cellular adhesion of *sibA* mutant cells is defective

In several phagocytosis mutants characterized so far (Fey *et al*, 2002; Gebbie *et al*, 2004), the phagocytosis defect is caused by a defect in cellular adhesion to particles. For *sibA* mutant cells, phagocytosis of latex beads was altered (Fig 3A), but macropinocytosis, which was determined by measuring the uptake of fluid phase, was unaffected (Fig 3B). In addition, *sibA* mutant cells phagocytosed *Klebsiella* bacteria almost normally (Fig 3B), indicating that the machinery responsible for engulfment of particles was still functional and suggesting that the primary cause of the phagocytosis defect in *sibA* cells might be a specific defect in cell adhesion. To confirm this hypothesis, we measured the ability of *sibA* cells to establish a tight contact with their substrate. Cells were incubated on a glass substrate, and their zone of contact with the glass was visualized and measured by interference reflection microscopy (IRM; Fig 3C). The contact area was markedly smaller for *sibA* cells (average 14 μm^2) than for wild-type cells (41 μm^2; Fig 3D), suggesting that *sibA* mutant cells adhered less readily to their substrate.

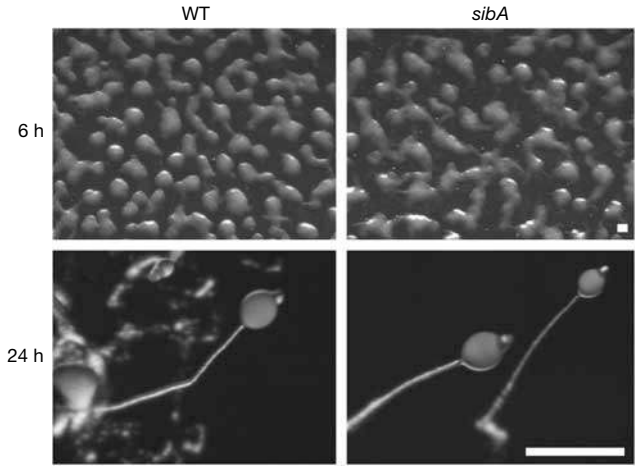

Fig 4 | Development of *sibA* mutant cells is normal. Wild-type (WT) or *sibA* cells were starved to induce development. Cellular aggregates were observed after 6 h and fruiting bodies after 24 h. Scale bar, 0.5 mm.

We then directly measured the adhesion of mutant cells to their substrate. Cells attached to a glass substrate were subjected to a flow of medium, and the speed of the flow necessary to detach 50% of the cells was determined. The strength necessary to detach the cells can be extrapolated and represents the strength of the cellular adhesion to the substrate (Decave *et al*, 2002). The adhesion of *sibA* mutant cells (0.1 ± 0.05 Pa) was significantly weaker than that of wild-type cells (0.6 ± 0.05 Pa), indicating that SibA is necessary for efficient cell–substrate adhesion.

Defects in cellular adhesion can be associated with alterations of the actin cytoskeleton, resulting in a range of anomalies in cell shape and migration, or in cytokinesis (Gebbie *et al*, 2004). No alteration in the organization of the cytoskeleton was seen in *sibA* cells (Fig 3E). Motility of *sibA* cells (2.7 ± 0.2 μm/min) was also identical to that of wild-type cells (3.1 ± 0.2 μm/min). Finally, only a small proportion of *sibA* cells were multinucleate (5.9 ± 0.4%), indicating that there was no significant defect in cytokinesis. Thus, besides their defect in cellular adhesion, *sibA* mutant cells did not show any important alteration in the organization and function of the actin cytoskeleton.

Social amoebae, such as *Dictyostelium*, undergo multicellular development in nutrient-free medium. Here, we observed that *sibA* mutant cells undergo normal development, with the formation of tight aggregates after 6 h of starvation, and of fruiting bodies after 24 h (Fig 4). This indicates that SibA does not have an essential role in the cell-to-cell interactions that allow multicellular development. Analysis of messenger RNA levels by real-time PCR indicated that SibA, SibB, SibC and SibE are expressed in vegetative as well as in starved cells (S.C., unpublished data), suggesting that for certain cellular processes SibB, SibC and SibE may compensate for the lack of SibA.

Similarity between β integrins and Sib proteins

In summary, several features found in *Dictyostelium* Sib proteins are also present in metazoan integrin β chains. First, like integrin β chains, the extracellular domains of Sib proteins contain a VWA domain. Second, the transmembrane and cytosolic domains of Sib proteins are very similar to those found in integrin β chains. In metazoan proteins, this specific configuration (a transmembrane domain containing a GxxxG motif and two conserved cytosolic NPxY motifs) is present only in integrin β chains. Third, like integrin β chains, the cytosolic domain of Sib proteins binds to talin. Finally, loss of SibA causes a specific alteration in cellular adhesion.

There are, however, clear differences between Sib proteins and metazoan β integrins: several domains typical of metazoan integrins are not found in Sib proteins (Psi domain, repeated EGF-like cystein-rich domains), and we have not identified a Sib-associated subunit that would represent the equivalent of α integrins. Several features found in Sib proteins (for example, Sib bacterial-like repeats) are also absent from β integrins. Thus, a phylogenetic relationship between Sib proteins and β integrins remains highly speculative (see below).

Irrespective of their putative phylogenetic relationship, our observations suggest a certain degree of functional similarity in the way in which integrin β chains and Sib proteins connect the extracellular space with the cytosolic machinery. This reinforces the notion that cellular adhesion mechanisms are similar in *Dictyostelium* and in mammalian cells, and that observations in the *Dictyostelium* model system can be extrapolated meaningfully to mammalian cells. A more detailed analysis of the structure and function of Sib proteins will be necessary to determine further the extent of the similarity between β integrins and Sib proteins.

Speculation

Metazoan integrin β chains and *Dictyostelium* Sib proteins could be the products of convergent evolution. However, in view of the similarities demonstrated in this study, we speculate that they might stem from the same ancestral integrin β-like protein. This ancestral β integrin would represent one of the first evolutionary attempts to use VWA domains in extracellular domains, as proposed previously (Whittaker & Hynes, 2002). This hypothesis would place the appearance of β integrins long before the appearance of Metazoa, as recent analysis of the *Dictyostelium* genome indicated that *Dictyostelium* diverged from the line leading to animals after the plant–animal split, but before the divergence of fungi (Eichinger *et al*, 2005). The higher rate of evolutionary change along the fungal lineage may explain why, so far, no fungal integrin β-related molecules have been identified.

METHODS

Cell culture and mutagenesis. Cells were cultured at 21 °C in HL5 medium (Cornillon *et al*, 1998). To initiate multicellular development, cells were plated on 1% agarose Petri dishes in nutrient-free medium (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5; Sussman, 1987).

The *sibA* mutant was isolated following a procedure described previously (Cornillon *et al*, 2000). The plasmid rescued from the original mutant was used to inactivate the *SIBA* gene by homologous recombination in a DH1-10 wild-type strain (Caterina *et al*, 1994; Cornillon *et al*, 2000), and these *sibA* knockout cells were used for further characterization. Reverse transcription of RNA from wild-type cells followed by PCR amplification (Access Quick™ RT–PCR system, Promega, Madison, WI, USA) and sequencing showed that the 5′ coding sequence of *SIBA* differed from the coding sequence predicted by

the genome sequencing centre, with two short exons instead of one. For northern blot analysis, a *SIBA* probe was obtained by PCR amplification of the genomic DNA using the primers CCAACTCCAGGTTCATCTGG and GCACCATCAGCAAAAGCTCC. The probe was radiolabelled and northern blot was carried out as described (Cornillon *et al*, 1998). The *SIBA* sequence can be found at www.dictybase.org (DDB0187447). Analysis of protein sequences was carried out with SMART (Schultz *et al*, 1998), Blast (Altschul *et al*, 1990) and MULTALIN (Corpet, 1988) programs.

Cellular assays. Phagocytosis and fluid-phase uptake were measured by incubating cells for 20 min at 21 °C in HL5 containing 0.5-μm-diameter Fluoresbrite YG carboxylate microspheres (Polysciences, Warrington, PA, USA), fluorescently labelled *Klebsiella* (Cornillon *et al*, 2000) or 10 μg/ml Alexa647-labelled dextran (Molecular Probes, Eugene, OR, USA). The internalized fluorescence was measured in a fluorescence-activated cell sorter after two washes with HL5 containing 0.1% sodium azide (Cornillon *et al*, 2000). Velocity measurements and staining of nuclei and of the actin cytoskeleton were also performed as described (Gebbie *et al*, 2004).

To examine cell–substrate adhesion, cells were incubated for 4 h in HL5 on a glass coverslip, then observed by phase-contrast microscopy and IRM (Verschueren, 1985), using an Axiovert 100 M microscope (Carl Zeiss AG) coupled to a Hamamatsu Photonics camera, Openlab 3.0.6 software; contact surfaces were determined using the MetaMorph Offline software.

Biochemical methods. To express GST–Sib fusion proteins, the sequence coding for each Sib cytosolic domain was cloned into the bacterial expression vector pGEX-3X. The expression and purification of GST fusion proteins on glutathione–Sepharose beads was carried out as described (Smith & Johnson, 1988). *D. discoideum* cells (5×10^6) were lysed in 1 ml of PBS containing 1% Triton X-100 and protease inhibitors (Ravanel *et al*, 2001). Lysates were cleared by centrifugation (15 min, 10,000*g*, 4 °C), and supernatants preincubated twice with glutathione–Sepharose beads before incubation for 16 h at 4 °C with GST fusion proteins immobilized on Sepharose beads. Beads were washed five times in lysis buffer and once in PBS, and bound proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Talin was observed as described (Gebbie *et al*, 2004), with monoclonal anti-talin antibodies (169.477.5; Kreitmeier *et al*, 1995).

To immunoprecipitate surface SibA, 6×10^6 cells were biotinylated, lysed and immunoprecipitated with a rabbit anti-SibA polyclonal antibody raised against the cytoplasmic domain of SibA. Biotinylated SibA was shown with horseradish peroxidase-coupled avidin as described (Ravanel *et al*, 2001).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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