High-affinity binding of the yeast *cis*-Golgi t-SNARE, Sed5p, to wild-type and mutant Sly1p, a modulator of transport vesicle docking

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Abstract Docking of ER-derived vesicles to the *cis*-Golgi compartment in yeast requires vesicle and target membrane receptors (v-SNAREs and t-SNAREs) and the GTPase Ypt1p. The t-SNARE Sed5p is complexed with Sly1p in vivo. The mutant form Sly1-20p rescues Ypt1p-lacking cells from lethality, suggesting an inhibitory function of Sly1p in v-SNARE/t-SNARE interaction. Using surface plasmon resonance spectroscopy, we found that Sed5p binds Sly1p and Sly1-20p with equally high affinity ($K_D = 5.13 \times 10^{-9}$ M and 4.74×10^{-9} M, respectively). Deletion studies show that the N-terminal half of Sly1p rather than the C-terminus (harbouring the E532K substitution in Sly1-20p) is most critical for its binding to Sed5p. These data appear to argue for an active rather than an inhibitory role of Sly1p in vesicle docking.

Key words: Golgi; Sed5 protein; Sly1 protein; Surface plasmon resonance; t-SNARE; Vesicle transport; Yeast; Ypt1 GTPase

1. Introduction

In biosynthetic and endocytic protein trafficking, transport vesicles derived from a given donor compartment dock to a specific acceptor compartment to deliver their cargo. Vesicle docking involves a variety of components which are remarkably high conserved from yeast to man [1–3]. Among them are vesicle and target membrane receptors, termed v-SNAREs and t-SNAREs, respectively. Pairing of these receptors appears to be critical for specific membrane interaction and fusion [4–7]. Other proteins, such as Ypt/Rab GTPases and Sec1 family members, are also needed for vesicle docking most likely to allow or to prevent v-SNARE/t-SNARE interactions [8–10].

Munc-18/N-Sec1 protein was identified and purified from mammalian brain thanks to its high-affinity binding to the target membrane receptor syntaxin 1 [11]. It has been suggested that the N-Sec1 protein prevents the v-SNARE, synaptobrevin, from binding to syntaxin 1 [12]. In yeast, docking of ER-derived vesicles to the Golgi compartment involves the t-SNARE Sed5p [13], v-SNAREs Sec22/Sly2p [14] and Bos1p [15] and the Sec1-related Sly1 protein [14,16]. Sed5p and Sly1p can be isolated from detergent-lysed spheroplasts as a 1:1 complex [6]. Sly1-20p is a mutant form of Sly1p with a single amino acid substitution (E532K) and has been isolated as an efficient suppressor of mutant cells lacking the essential GTPase Ypt1p [14]. From this, it can be inferred that Ypt1p has a direct functional relationship to Sly1p and one might speculate that Sly1-20p is altered in its inhibitory potential for SNARE complex formation. However, the Sed5p/Sly1p (Sly1-20p) binding characteristics reported here are not in favour of such an assumption.

2. Materials and methods

2.1. Strains and vectors

Cloning experiments were performed with the *E. coli* strain DH5α on standard media [17]. Yeast culture techniques including genetic manipulations and molecular biology techniques followed standard protocols [18,19]. The *S. cerevisiae* strain BJ5457 (αura3-51 trp1 lys2-801 leu2Δ1 his3Δ200 pep4(HIS3) prb1Δ1.6R can1 GAL) was used throughout and is referred to as yeast in this paper. Transformants were selected on PM agar plates containing 0.67% Difco yeast nitrogen base with $(NH_4)_2SO_4$ supplemented with 0.5% peptone 140 (GIBCO-BRL) for selecting Ura^+ clones. Glucose (2%) was added as carbon source. A derivative of pEG-KT [20] was used as a glutathione-*S*-transferase (GST) fusion vector. The complete open reading frames of *SLY1* and *SLY1-20* [14] were cloned as *Xba*1/*Hin*dIII fragments. Smaller fragments of *SLY1* were cloned in the same vector. *SED5ΔC* was cloned as a *Bam*H1/*Hin*dIII fragment comprising amino acids 1–319 [13]. *PEP12ΔC* was also cloned as a *Bam*1/*Hin*dIII fragment without the C-terminal transmembrane domain-encoding sequences [21]. For production of His_6-tagged proteins in yeast, the *pEG-SLY1* and *pEG-SLY1-20* vectors were modified such that the *GST* gene was substituted by an oligonucleotide encoding six histidine residues which was inserted into the *Sac*I and *Xba*1 sites in frame with the start-ATG.

2.2. Protein purification from yeast extracts

Strain BJ5457 containing His-tag or GST fusion vectors was grown over night in 5 l of PM medium at 30°C. Subsequently, the cells were centrifuged and resuspended in 5 l of PMGal medium (0.67% yeast nitrogen base with $(NH_4)_2SO_4$, 0.5% peptone 140, 2% galactose) to induce the cloned genes and grown for 14–16 h. For protein purification, 10–15 g wet cells were suspended in 50 mM Tris-HCl (pH 7.5), 100 mM KCl and protease inhibitors. If GST proteins were purified, 5 mM EDTA was added. The cells were disrupted by high-pressure homogenization and proteins were isolated from cytosolic extracts obtained after high-speed centrifugation ($100\,000 \times g$). GST fusion proteins were purified on 1 ml of glutathione–Sepharose (Pharmacia) and His-tag proteins on 1 ml of Talon agarose (Clontech) at 4°C. The affinity purification materials were washed with 30 ml of 50 mM Tris-HCl (pH 7.5) and either 1 M KCl, 5 mM EDTA (for GST proteins) or 0.5 M KCl, 1 mM imidazol (for His-tag proteins). The proteins were eluted with 50 mM Tris-HCl (pH 7.5), 100 mM KCl and 20 mM glutathione (pH 7.5), or with 100 M EDTA (pH 7.5), respectively. We obtained about 200 μg of pure GST fusion protein (whole length) by this single-step purification but significantly less of the truncated versions (10–20 μg). The purification of His-tag proteins included a gel filtration step on a Superdex 200/60 column (Pharmacia) in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM EDTA with a flow rate of 0.4 ml/min. We observed that His_6–Sly1-20p eluted in two major peaks corresponding to monomeric and oligomeric proteins, the latter yielding about 200 μg completely pure protein (Fig. 2).

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2.3. GST–Sly1p/Sed5p co-purification

GST–Sly1p, GST–Sly1-20p and GST fusions of Sly1 protein fragments were isolated from 0.5 l cultures each of galactose-induced cells (density ≈6 at OD_{600}) as described above, except that the cell disruption buffer contained 2% Triton X-100. Glutathione–Sepharose (200 μl of a 50% suspension) was incubated with the 100 000×*g* supernatant for 2 h at 4°C. The suspensions were packed into plastic columns, washed successively with 10 ml of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM EDTA, 2% Triton X-100 and 10 ml of the same buffer containing 0.5 M KCl and finally with 10 ml of 0.5 mM Tris-HCl (pH 7.5). The affinity matrix was suspended in the same volume of SDS-containing electrophoresis buffer and boiled for 5 min. After centrifugation, 0.5 μg of the affinity-purified proteins were separated by SDS-PAGE and either stained with Coomassie brilliant blue or transferred to nitrocellulose and probed for co-purified Sed5p using affinity-purified anti-Sed5p antibodies. Western blot signals were visualized by chemiluminescence (Amersham).

Anti-Sed5p antibodies were raised in rabbits (Eurogentec, Belgium) against gel-purified His_6–Sed5p lacking the C-terminal membrane anchor. The polyclonal antibody was affinity-purified with the antigen immobilized on an AminoLinkflorin column (Pierce).

2.4. Purification of His-tag proteins from E. coli extracts

Genes were expressed in the *E. coli* strain BL21. Three litres of LB medium with 100 μg/ml ampicillin were inoculated with 300 ml of an overnight culture and grown at 37°C for 1 h. After addition of 1 mM IPTG, the cultures continued to grow at 25°C for 3 h. The cells were harvested by centrifugation at 10 000×*g* for 15 min and frozen at −80°C for later use.

Cell pellets were resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM KCl and protease inhibitors and disrupted in a high-pressure homogenisator. To obtain a cytosolic extract, the lysate was centrifuged for 15 min at 10 000×*g* and the resulting supernatant for 1 h at 100 000×*g*. The high-speed supernatant was incubated with 2 ml of Ni–NTA–agarose (Quiagen) for 3 h at 4°C. The gel was successively washed with 30 ml of 50 mM Tris-HCl (pH 7.5), 1 M KCl, 2 mM imidazol and 10 ml of 50 mM Tris-HCl (pH 7.5), 100 mM KCl. Finally, the proteins were eluted with 4 ml of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 100 mM EDTA (pH 7.5). They were concentrated to 1 ml in an ultrafiltration chamber (Amicon) and purified in a second step by size exclusion chromatography as described for the His-tag proteins isolated from yeast.

2.5. Surface plasmon resonance spectroscopy (SPR)

The experiments were performed on a BIAcore2000 apparatus (Pharmacia). In the following the immobilized protein is denoted ligand and the soluble protein analyte. His_6–Sed5ΔCp or His_6–Pep12ΔCp expressed in *E. coli* and purified to homogeneity were immobilized on a sensor chip by coupling the amino groups with 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride and *N*-hydroxysuccinimide according to the manufacturers recommendations. Coupling was stopped as soon as 500–600 RU (relative units) were immobilized. The modified sensor chip surface was stable for long series of experiments although ligand analyte complexes were dissociated by a fast (1 min) 100 mM HCl pulse. The analytes were purified to homogeneity as described above and injected while a continuous flow rate of 10 μl/min was applied. The running buffer was 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM EDTA. Three flow cells were connected in series: the first was used as a blank control, the second was occupied by His_6–Sed5ΔCp and the third by the control protein His_6–Pep12ΔCp.

The results were evaluated after the response curves from chamber two and three had been subtracted from the blank run (flow cell 1). Interaction constants were determined by fitting the curves to the following equations: $R_{(t)} = R_{eq}[1-\exp-(k_a C+k_d)(t-t_0)]$ for the association reaction and $R_{(t)} = R_0 \exp-k_d(t-t_0)$ for the dissociation reaction. In these equations the following abbreviations are used: $R_{(t)}$ = relative response at a given time, R_{eq} = response at steady level, R_0 = initial response, t = time (s), t_0 = start time of association or dissociation, C = molar concentration of the analyte, k_a = association rate constant, k_d = dissociation rate constant. The computer fits reflected a simple 1:1 interaction of the analyte and the ligand with only one binding site for the ligand. The equilibrium dissociation constants were calculated according to the relationship $K_D = k_d/k_a$.

3. Results

In a first attempt to study the binding characteristics to the integral membrane Sed5 protein, Sly1p, Sly1-20p and truncated versions of Sly1p were produced as GST fusions in yeast from the GAL10 promoter-controlled, plasmid-borne genes. Although the Sly1 wild-type protein was still synthesized in all transformants and was nearly exclusively associated with membranes, presumably with Sed5p [6], easily detectable amounts of the Sed5 t-SNARE were co-purified with GST–Sly1 wild-type and the GST–Sly1-20 mutant protein under stringent washing conditions (0.5 M KCl) of the glutathione affinity matrix (Fig. 1, lanes 2 and 3). Although to a lesser extent, Sed5p co-purified also with Sly1p fragments comprising the N-terminal half but not with the C-terminal half of the protein (Fig. 1, lanes 4–9). These results showed that GST–Sly1 fusion proteins interacted with Sed5p in vivo and that Sed5p was not fortuitously co-purified with GST or the GST fusion proteins. The findings also indicated that the N-terminal half of Sly1p (amino acids 1–294) is the critical region of the protein for its binding to Sed5p.

We next sought to examine the interaction parameters of Sly1p and Sed5p using purified proteins in a biosensor analysis. GST–Sly1 and GST–Sly1-20 fusion proteins were produced in yeast, and by a single purification step on glutathione–Sepharose highly purified proteins were obtained (Fig. 2). As the removal of the GST moiety by thrombin cleavage led to appreciable protein degradation, the complete fusion proteins were analyzed. The ligand, His_6-tagged Sed5p lacking the C-terminal membrane anchor, was purified from *E. coli*. Surface plasmon resonance spectroscopy measurements revealed that the Sly1-fusion protein binds Sed5p with high affinity. Most importantly, neither on nor off rates were significantly different for GST–Sly1 and GST–Sly-1-20 protein binding to immobilized Sed5 receptor (Fig. 3A,B and Table 1).

Different controls verified the specificity of Sed5p/Sly1p interactions. No interactions of GST–Sly1p could be observed

Table 1
Interaction constants of immobilized His_6–Sed5ΔCp and fusion proteins of Sly1p and Sly1-20p

Analyte	Source of analyte	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
GST–Sly1p	yeast	$(1.52 \pm 0.53)\times 10^4$	$(7.80 \pm 1.90)\times 10^{-5}$	5.13×10^{-9}
GST–Sly1-20p	yeast	$(1.96 \pm 0.95)\times 10^4$	$(9.30 \pm 1.75)\times 10^{-5}$	4.74×10^{-9}
His_6–Sly1p	yeast	$(1.12 \pm 0.09)\times 10^5$	$(1.17 \pm 0.02)\times 10^{-3}$	1.04×10^{-8}
His_6–Sly1-20p	yeast	$(1.38 \pm 0.17)\times 10^5$	$(1.23 \pm 0.03)\times 10^{-3}$	8.91×10^{-9}

The constants have been determined from two independent protein preparations each of which was analyzed four times.
Given are mean values and standard errors for constants determined in a reproducible series of $n = 8$–16 measurements.
k_a, association rate constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant.

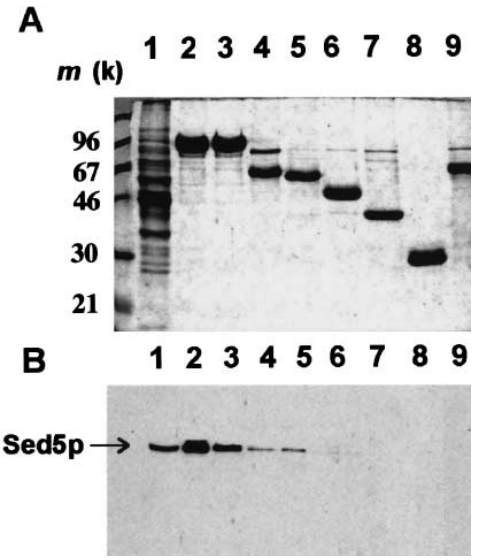

Fig. 1. Sed5p is associated with GST–Sly1 fusion proteins isolated from yeast cell extracts. A: Coomassie brilliant blue-stained SDS gel of Sly1 proteins purified on glutathione Sepharose: 1, yeast crude extract; 2, GST–Sly1p; 3, GST–Sly1-20p; 4, GST–Sly1p$^{1-340}$; 5, GST–Sly1p$^{1-294}$; 6, GST–Sly1p$^{1-197}$; 7, GST–Sly1p$^{1-107}$; 8, GST; 9, GST–Sly1p$^{340-666}$. The superscribed numbers indicate the start and end positions of the amino acids as referred to the wild-type Sly1p which is composed of 666 amino acids. Marker proteins (m) were run in the left lane, their mass is given in kDa (k). B: Proteins shown in (A) were transferred to a nitrocellulose filter and probed with anti-Sed5p antibodies.

with the structural Sed5 homologue Pep12p that was immobilized in one flow chamber of the sensor chips as a His_6-tagged version lacking the C-terminal membrane-spanning domain (Fig. 3C). Furthermore, purified GST protein did not bind to the immobilized Sed5p.

When the GST–Sly1 fusion protein was subjected to size exclusion chromatography it eluted as a sharp peak with an apparent molecular mass of close to 300 kDa (Fig. 4), suggesting a trimer. The Sed5p binding activity perfectly coincided with the apparently multimeric form. To examine the influence of the 26.5 kDa GST portion of the chimeric Sly1 protein on its interaction with Sed5p, we also produced His_6-tagged Sly1 and Sly1-20 proteins in yeast. Monomeric and multimeric forms of the proteins were detected on Superdex 200 chromatography (data not shown), and the purified monomeric forms were analyzed for its binding to Sed5p.

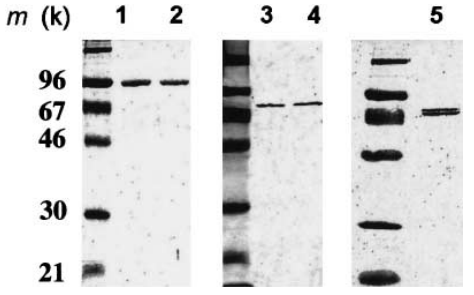

Fig. 2. Purifications of Sly1 proteins by affinity chromatography. 1, GST–Sly1p; 2, GST–Sly1-20p; 3, His_6–Sly1p; 4, His_6–Sly1-20p; 5, His_6–Sly1p. Proteins were produced in yeast (1-4) or in *E. coli* (5). Shown are Coomassie brilliant blue-stained SDS gels. Lanes showing molecular mass markers (see Fig. 1) are not numbered.

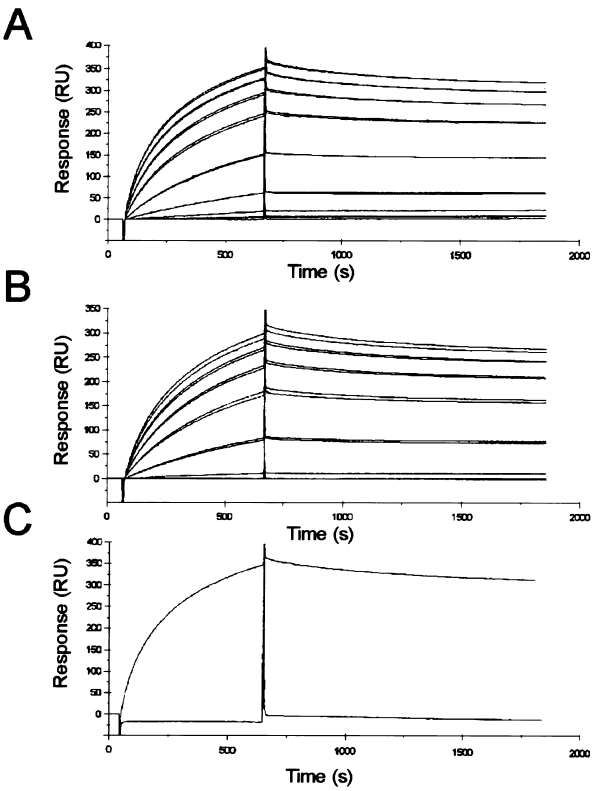

Fig. 3. SPR sensorgram of immobilized His_6–Sed5ΔCp or His_6–Pep12ΔCp and soluble GST–Sly1p or GST–Sly1-20p. A: ligand = His_6–Sed5ΔCp; analyte = GST–Sly1p (duplicate measurements from the top to the bottom, with 500, 400, 300, 200, 100, 50, 25, 12.5 nM). B: Ligand = His_6–Sed5ΔCp; analyte = GST–Sly1-20p (duplicate measurements from the top to the bottom, with 450, 310, 220, 130, 75, 37.5, 18.5 nM). C: ligand = His_6–Sed5ΔCp (upper curve) or His_6–Pep12ΔCp (lower curve); analyte = GST–Sly1p (500 nM). RU, relative units.

As shown in Table 1, the on and off rates measured differed by approximately one order of magnitude from those deter-

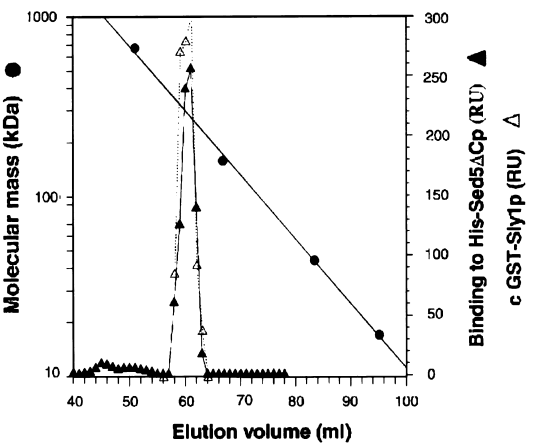

Fig. 4. Size exclusion chromatography of purified GST–Sly1p isolated from yeast. GST–Sly1p was chromatographed on Superdex 200 and individual fractions were subjected to SPR and SDS-PAGE. The Sed5p-binding activity is given as the maximum of the SPR response curve. The relative protein concentration was determined by scanning the density of bands on SDS gels. The molecular mass standards are thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa). RU, relative units.

mined for the GST fusion proteins. However, the affinities differed only by a factor of two, and they were not significantly different for Sly1 wild-type or Sly1-20 mutant protein binding to Sed5p, i.e. 10.4 nM and 8.9 nM respectively.

4. Discussion

In agreement with previous observations showing that the Sly1 protein in yeast can be co-immunoprecipitated with the Golgi-localized transport vesicle receptor Sed5p [6], we have shown in this report that both proteins bind to each other with nanomolar affinity in a bimolecular reaction. It is worth noting here that comparable binding constants have been determined in a biosensor analysis for the interaction of mammalian N-Sec1p and syntaxin 1A ($k_a = 4.4 \times 10^4$ $M^{-1}s^{-1}$; $k_d = 2.4 \times 10^{-4}s^{-1}$; $K_D = 5.7 \times 10^{-9}$ M) [12]. For the interaction studies described here, the analytes (Sly1p and Sly1-20p) were produced in yeast to avoid possible folding problems. To ease their purification, the N-terminus of both proteins was fused to either GST or a stretch of six histidines. Depending on the N-terminal extension, the kinetics but not the affinity of Sly1 protein binding to Sed5p differed markedly. This was most likely due to trimer (or dimer) formation of GST–Sly1 fusion proteins caused by the GST portion which is known to dimerize [22,23]. As the GST fusion proteins might not be globular it appears well possible that they actually were dimers although they behaved like trimers on size exclusion chromatography. Regardless of this, the GST–Sly1 fusion proteins may expose more than one binding site to the ligand Sed5p resulting in decelerated dissociation of such complexes as compared to the dissociation rate of monomer/monomer complexes.

The main purpose of this study was, however, to compare the affinities of wild-type and mutant Sly1p to the t-SNARE Sed5p. A comparison of the binding kinetics and the affinities to Sed5p showed that they were not significantly different for either the monomeric His_6-tagged or the di- or trimeric GST–Sly1 and –Sly1-20 fusion proteins. Obviously, the single amino acid substitution (E532K) in Sly1-20p through which the protein acquires its activity to suppress the loss of Ypt1p function [14] does not influence its high-affinity binding to Sed5p, at least not in the absence of other components. This seems understandable as our studies also show that the N-terminal rather than the C-terminal half of Sly1p interacts with Sed5p in vivo. The C-terminal half of Sly1p may thus contain binding site(s) for proteins other than Sed5p which in concert with the Ypt1 GTPase influence Sly1p's function.

We propose that Sly1p may positively engage in transport vesicle docking to the *cis*-Golgi compartment rather than simply prevent vesicle docking by occupying the target membrane receptor. Such a view seems more appealing to us in the light of the facts that Sly1p has an essential role for yeast cell viability [14,16], that anti-Sly1p antibodies inhibit ER-to-Golgi transport in vitro (our unpublished observations) and that Sly1p and Sed5p can be found in a 1:1 stoichiometry in isolated docking complexes [6].

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