Isolation and Characterization of a Novel Low Molecular Weight Protein Involved in Intra-Golgi Traffic*

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Analysis of the cytosolic requirements for in vitro intra-Golgi transport led to the characterization of three proteins: N-ethylmaleimide-sensitive fusion protein (NSF), soluble NSF attachment protein (SNAP), and p115, all involved in the docking and fusion of transport vesicles to their target membranes. In the course of determining the minimal cytosolic requirements for intra-Golgi transport in vitro, we identified three additional factors that are sufficient to replace crude cytosol. We describe here the purification and characterization of one of these factors, a novel 16-kDa protein, p16, an essential factor for intra-Golgi protein transport. Based on transport activity, this purification procedure resulted in ~1,400-fold enrichment of p16 to apparent homogeneity. The activity of p16 could be observed in the absence of vesicle formation, suggesting that it may participate in the docking and fusion processes.

Transport of proteins between membrane-bound organelles in eukaryotic cells is a multistage process utilizing soluble and membrane proteins. The molecular machinery mediating this process has been explored biochemically and genetically, leading to the identification and characterization of numerous transport factors (1–3).

Several cytosolic transport factors were purified by the utilization of an in vitro intra-Golgi transport assay. These include N-ethylmaleimide-sensitive factor (NSF) (4); α-, β-, and γ-soluble NSF attachment proteins (SNAPs) (5); and p115 (6). Genetic studies have identified the yeast homologs of NSF, SNAP, and p115 as Sec18p (7, 8), Sec17p (9), and Uso1p (10–12), respectively. NSF and SNAP are considered part of the general docking/fusion apparatus that functions at several transport stages along the secretory pathway including endosome-endosome fusion (13), vacuolar sorting (14), transcytosis (15), and synaptic vesicle fusion (16).

p115 was isolated as a cytosolic factor required for intra-Golgi transport in vitro (6) and was suggested to act together with NSF and SNAP in direct Golgi-Golgi fusion (17). p115 is a peripheral membrane protein localized predominantly in the Golgi apparatus (6) but has also been identified as a component of transcytotic vesicles (15). Recently, p115 has been implicated, together with NSF and SNAP, in the process of reassembly of post-mitotic Golgi fragments into Golgi cisternae (18, 19). Uso1p, the yeast homolog of p115, is required for assembly of the endoplasmic reticulum-Golgi SNARE complex (20). Other cytosolic factors such as Rab proteins and their effectors were also shown to be involved in this process (21–23). It appears, however, that the amount of Rab proteins present on the membrane is sufficient to promote the transport reaction in vitro.

It was demonstrated originally by Clary and Rothman (24) that in addition to NSF and the SNAPs, several other cytosolic factors were required for reconstituting the SNAP-dependent transport assay. The need for additional cytosolic transport factors was demonstrated further for the p115-dependent assay (6) as well as for direct fusion between Golgi stacks (17). Thus, identification and isolation of these novel factors are essential for understanding the exact molecular machinery of intracellular protein traffic. In the present study we describe the purification of a novel 16-kDa transport factor, p16, from bovine brain on the basis of the in vitro intra-Golgi transport assay. Our data suggest that p16 is a transport factor that participates in the docking/fusion reaction.

MATERIALS AND METHODS

General Procedures

Vesicular stomatitis virus (VSV)-G protein-containing donor Golgi membranes from 15B cells and acceptor membranes from wild-type Chinese hamster ovary cells were prepared as described (25). Protein concentration was determined with the Bio-Rad protein assay. The pH values were determined at room temperature. All fractionations were performed at 4 °C. All cytosolic fractions tested in this assay were dialyzed to 25 mM Tris-HCl, pH 7.4, 50 mM KCl, and 1 mM dithiothreitol (dialysis buffer) before their addition to the transport assay.

Cis- to Medial-Golgi Transport Assay

The standard assay mixture (25 μl) contained 0.4 μCi of UDP-[3H]acetylglucosamine (América Radiolabeled Chemical), 5 μM of a 1:1 mixture of donor and acceptor Chinese hamster ovary cellular Golgi membrane, and crude bovine brain cytosol as described (25).

Preparation of Cytosolic Factors

Bovine brain cytosol was prepared by the method of Malhotra et al. (26). Recombinant His6-NSF and His6-aSNAP were prepared as described (27). Fraction Iδ was obtained by chromatography of 500 ml of 40% ammonium sulfate precipitate on a 400-ml Fast Flow Q column (Pharmacia Biotech Inc.) equilibrated with 25 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM β-mercaptoethanol, and 10% glycerol. The column was washed with 800 ml of the same buffer and then eluted with a 0.1–0.5 M KCl gradient in 1,200 ml. Nine-m1 fractions were collected, dialyzed to reduce the KCl concentration to 50 mM, and assayed for transport activity in the presence of 0.5 μg of p115, 5 μg of His6-NSF, 60 μg of His6-aSNAP, and 5 μl of Golgi membranes. Typically, a peak of transport activity was eluted at 0.22–0.30 M KCl, and the peak fractions were pooled and concentrated by ultrafiltration using Amicon PM-10 filter. The concentrated pool, designated β, was dialyzed against dialysis buffer and had a protein concentration of about 30 mg/ml. p115 was purified from bovine liver cytosol as described previously (6).

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The assay is a modification of the one described by Waters et al. (6). The 25-μl assay contained 0.4 μCi of UDP-[3H]acetate, 5 μl of a 1:1 mixture of donor and acceptor Chinese hamster ovary cellular Golgi membrane, 100 μg of Ib, 0.5 μg of p115, 5 ng of recombinant NSF, 60 ng of recombinant SNAP, 10 μM palmitoyl-coenzyme A, ATP and UTP regeneration systems, and 10 μl of the various cytosolic fractions as indicated in the figure legends. The transport reactions were incubated at 30 °C for 2 h. N-[3H]Acetylgalactosamine incorporated into VSV-G protein was determined as described previously (25).

**Purification of p16**

**Preparation of Bovine Brain Cytosol**—Bovine brains were obtained immediately after slaughtering and placed on ice-cold 25 mM Tris-HCl, pH 7.4, 340 mM sucrose. The tissue (600 g) was placed on a glass Waring blender, which was then filled with 800 ml of homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 500 mM KCl, 250 mM sucrose, 2 mM EGTA, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μM 1,10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin. The homogenate was centrifuged at 7,500 × g in a Sorvall GS3 rotor for 1 h at 4 °C. The supernatants were pooled and concentrated at 120,000 × g in a Beckman Ti-45 rotor for 1 h at 4 °C. The supernatants were pooled and dialyzed against 25 mM Tris-HCl, pH 7.4, 100 mM KCl, and 10 mM β-mercaptoethanol (buffer A). The dialyzed material was collected and centrifuged by centrifugation at 7,500 × g in a Sorvall GS3 rotor at 1 h at 4 °C. This material was designated bovine brain cytosol and had a protein concentration of about 8 mg/ml.

**Polyethylene Glycol Precipitation**—The protein concentration of bovine brain cytosol was adjusted to 7.5 mg/ml by dilution with fresh dialysis buffer, and KCl was added to the final concentration of 0.5 mM. Polyethylene Glycol (PEG) 4000 was added slowly to a final concentration of 20% PEG. The solution was stirred for 30 min at 4 °C and then centrifuged in a Sorvall SS34 rotor at 8,500 rpm for 30 min at 4 °C. The supernatant was discarded, and the pellets were resuspended in a 100-ml Dounce blender, which was then filled with 800 ml of homogenization buffer and centrifuged at 8,500 × g for 30 min at 4 °C. The supernatant was discarded, and the pellets were resuspended in a 100-ml Dounce homogenizer. The insoluble material was removed by centrifugation at 120,000 × g for 6 min at 4 °C, the protein concentration was adjusted to 7 mg/ml and the salt concentration to 0.1 M KCl. This material was termed polyethylene glycol precipitate.

**Fast Flow Q Chromatography**—The polyethylene glycol precipitate was loaded on a 400-ml Fast Flow Q column equilibrated with buffer A containing 10% glycerol at 3 ml/min. The column was washed with 400 ml of the equilibration buffer and then eluted with a 1,200-ml gradient of KCl at a range of 0.1–0.5 M. Two active fractions were detected, one in the unbound material and the other in fractions eluted from 0.2 to 0.5 M KCl. All unbound protein was pooled and termed flow-through Q; it had a protein concentration of 0.4 mg/ml. The active fractions eluted from the column were termed β (see above).

**Isoelectric Precipitation**—The flow-through Q pool was transferred to Spectra/por 3 dialysis bags and dialyzed against 10 mM potassium phosphate, pH 6.6, and 10 mM β-mercaptoethanol. The insoluble material was removed by centrifugation in a Sorvall SS334 rotor at 1,800 × g for 20 min at 4 °C. The supernatant, which had a protein concentration of about 0.21 mg/ml, was concentrated about 10-fold by ultrafiltration on an Amicon PM3 filter.

**Superdex 75 Chromatography**—The concentrated isoelectric supernatant was centrifuged for 10 min in a microcentrifuge to remove insoluble material, and the supernatant was chromatographed on a 24-ml Superdex 75 HR 10/30 column (Pharmacia) equilibrated in 25 mM Tris-HCl, pH 7.4, 150 mM KCl, 10 mM β-mercaptoethanol, and 10% glycerol at 0.3 ml/min. Fractions of 0.7 ml were collected and tested for transport activity. A single peak of activity was detected at an elution volume corresponding to a molecular mass of 14–16 kDa as determined by low molecular weight calibration kit (Pharmacia). This material had a protein concentration of about 0.08 mg/ml.

**Mono S Chromatography**—The Superdex 75 pool was adjusted by dialysis to 10 mM phosphate buffer, pH 6.5, 10 mM β-mercaptoethanol, and 10% glycerol and loaded onto a 1-ml Mono S HR 5/5 (Pharmacia) column equilibrated by the same buffer at 0.5 ml/min. The column was washed with the equilibration buffer and eluted with 25 ml of a 0–400 mM KCl gradient. Fractions (1 ml) were collected, and aliquots were analyzed for transport activity and by electrophoresis followed by Coomassie Blue staining. The fraction purified in this way was referred to as p16.

**FIG. 1. Chromatography of 12.5% polyethylene glycol precipitate of bovine brain cytosol on Fast Flow Q column.** Panel A, cytosolic proteins (850 mg) were pelleted with 12.5% polyethylene glycol, loaded on a 400-ml Fast Flow Q column, and eluted by an increasing KCl gradient. Panel B, fractions were analyzed for transport activity in the fractionated cytosol-dependent assay (see “Materials and Methods”). Panel C, transport activity of 200 μg of crude cytosol or pooled fractions eluted from the Fast Flow Q column; samples of 150 μg of α (fractions 7–12) and 175 μg of β (fractions 26–29) were tested as indicated. Assays were done in duplicate, and the mean is plotted with the error bar representing the higher value.

**RESULTS**

**Identification of Novel Cytosolic Factors Involved in Intra-Golgi Transport**—The cell-free system that reconstitutes intra-Golgi transport has been used in recent years for the characterization and isolation of several factors involved in intracellular trafficking (2). Proteins such as NSF, SNAPs, and p115 were isolated by this transport assay in which the activity of each factor could be assessed specifically. One such an experimental design is a complementation assay where saturating levels of different crude cytosolic fractions are added to the transport assay, and a signal is observed only upon the addition of the protein of interest. To identify novel soluble factors required to reconstitute intra-Golgi transport in vitro, we analyzed the transport activity of different cytosolic fractions in the presence of saturating levels of NSF, SNAP, and p115. Fig. 1A describes the fractionation of 12.5% polyethylene glycol precipitate of bovine brain cytosol protein on a Q-Sepharose anion exchange column. This chromatography step separated two soluble factors with considerable transport activity (Fig. 1B). A peak of transport activity could be detected in the unbound material and was tentatively termed α; another factor that showed transport activity was eluted as a single peak between 0.22 and 0.30 M KCl and was tentatively termed β (Fig. 1B). Each of these cytosolic factors reconstituted only part of the transport activity observed in the presence of crude cytosol, whereas both factors together recovered the full transport activity (Fig. 1C).

We then followed the transport activity present in the flow-through material of the Fast Flow Q column (α, see Figs. 1 and 2D). Fig. 2 describes the chromatography of factor α on a CM-Sepharose cation exchange column. The transport activity of the different fractions eluted from this column was deter-
mined in the presence or absence of β. Transport activity was clearly detected in the unbound material and was termed Iα (Fig. 2B). Significant transport activity was also observed in fractions eluted at 0.15–0.25 M KCl, tentatively termed IIα (Fig. 2B). When the different fractions obtained from the CM-Sepharose column were tested in the presence of β, the transport activity of both bound and unbound materials was increased significantly (Fig. 2B).

We next tested whether all three factors, Iα, IIα, and β, are required to reconstitute the transport assay to a level comparable to that observed with crude cytosol. As shown in Fig. 2C, factors Iα, IIα, and β all showed low transport activity when each was added separately to the transport assay containing NSF, αSNAP, and p115. When either Iα or IIα was added together with β, a synergistic effect was obtained leading to about 80% of the signal observed with the cytosol. When both Iα and IIα were added in the absence of β only a small increase in transport activity was observed. Together, Iα, IIα, and β reconstituted the full transport activity. These experiments demonstrate the involvement of at least three different cytosolic factors (as illustrated in Fig. 2D) in intra-Golgi transport in addition to NSF, SNAP, and p115.

**Characterization of a IIα-dependent Assay**—In the present study we focused on the purification of the transport factor present in IIα. To assure that IIα acts as part of the known transport machinery, we tested whether the signal obtained with the different factors was NSF-, SNAP- and p115-dependent. Golgi membranes treated with N-ethylmaleimide were tested for transport activity in the presence of the indicated fractions. Panel C, increasing concentrations of IIα were added into a transport reaction containing NSF, αSNAP, p115, and β, in the absence (open circles) or the presence of 150 μM brefeldin A (open circles). The ion exchange steps used in this process are framed.

![Fig. 2. Chromatography of pool Iα on a Fast Flow CM column. Panel A, fractions 7–12 eluted from the Fast Flow Q column were loaded onto a 150-ml Fast Flow CM column and eluted by a KCl gradient. Panel B, fractions were analyzed for transport activity in the absence (open circles) or presence (filled circles) of pool Iβ. Panel C, the different cytosolic pools were tested for intra-Golgi transport as indicated in the presence of NSF, αSNAP, and p115. Panel D, schematic presentation of the procedure that led to the identification and separation of Iα, IIα, and β, three novel cytosolic factors involved in intra Golgi transport. The ion exchange steps used in this process are framed.

![Fig. 3. Characterization of IIα-dependent assay. Panel A, Golgi membranes were treated with 1 mM N-ethylmaleimide as described (34) and then tested for transport activity in the presence of the indicated fractions. Panel B, increasing concentrations of IIα were added into a transport reaction containing NSF, αSNAP, p115, and β, in the absence (open circles) or the presence of 150 μM brefeldin A (open circles).]
observed, indicating that IIα is acting in conjunction with the known transport factors.

Increasing concentrations of IIα in a transport reaction containing NSF, αSNAP, p115, and β resulted in a saturable signal (Fig. 3B). This signal could be observed in the presence of 150 μM brefeldin A, a drug that prevents budding of transport vesicles and promotes uncoupled fusion (17, 28) (Fig. 3B). Apparently, IIα is involved in docking and fusion and not in vesicle budding. The IIα-dependent assay was used further for the purification of the protein responsible for this transport activity.

**Purification of a Novel 16-kDa Protein from Pool IIα**—Using the IIα-dependent assay a 16-kDa protein, p16, was purified from bovine brain cytosol by the following steps: (i) ammonium sulfate precipitation; (ii) Q-Sepharose anion exchange chromatography; (iii) CM-Sepharose cation exchange chromatography; (iv) gel filtration on a Superdex 75; and (v) Mono S cation exchange chromatography. Fractions obtained from each chromatography step were tested in the IIα-dependent assay. The purification of the protein responsible for this transport activity is summarized in Table I. This signal could be observed in the presence of 150 μM brefeldin A, a drug that prevents budding of transport vesicles and promotes uncoupled fusion (17, 28) (Fig. 3B). Apparently, IIα is involved in docking and fusion and not in vesicle budding. The IIα-dependent assay was used further for the purification of the protein responsible for this transport activity.

![Figure 4](http://www.jbc.org/)

**TABLE I**

**Quantitation of p16 purification**

| Stage                  | Protein | Specific transport activity | Purification Yield |
|-----------------------|---------|----------------------------|--------------------|
| Differential centrifugation | 8,500   | 14                         | 1                  |
| PEG precipitation      | 1,200   | 88                         | 6                  |
| Flow-through Q         | 216     | 424                        | 29                 |
| Isoelectric precipitation | 150    | 525                        | 36                 |
| CM-Sepharose           | 20      | 2,100                      | 144                |
| Superdex 75            | 2       | 8,400                      | 576                |
| Mono S FPLC           | 0.3     | 18,000                     | 1440               |

**Discussion**

We have utilized the well-characterized intra-Golgi cell-free transport assay to detect yet unidentified cytosolic transport factors. This work describes the identification of three cytosolic factors and the purification of a novel 16-kDa protein required for intra-Golgi transport.

In the process of identifying novel cytosolic factors required for intra-Golgi protein transport in vitro, we characterized three different protein pools each exhibiting low transport activity in the presence of NSF, SNAP, and p115, yet together they constituted the full transport activity observed with crude cytosol. Previously, Waters et al. (6) identified two crude cytosolic factors required to reconstitute intra-Golgi transport in vitro in addition to NSF, SNAP, and p115. Factors α and β described in the present study could be related to these factors. In this study we were able to separate further the α factor into two distinct activities. Pool β significantly stimulated the signal of either Iα or IIα, whereas combining Iα and IIα in the absence of β resulted in only a slight increase in the assay signal (Fig. 2C). Conceivably, both α factors could share the active protein component. However, further purification of Iα based on its transport activity revealed that the active component in this pool was a 56-kDa polypeptide, whereas in the present study we show that the transport activity of Iα was attributed to p16. It could still be possible that both crude fractions in the early stages of the purification might include small amounts of reciprocal activity.

We described here a purification procedure based on a functional intra-Golgi transport assay, which led to the isolation of a novel low molecular weight protein, p16. Using this purification procedure, p16 was enriched by about 1,400-fold to apparent homogeneity. Similar enrichment of transport activity was required for the other soluble transport factors, such as αSNAP.
and p115, indicating that the activity of p16 in the cytosol is comparable to these other transport factors. Amino acid sequence analysis of five different tryptic peptides derived from the pure 16-kDa protein indicates that it is a novel protein (data not shown). We have recently cloned the p16 encoding cDNA from bovine brain, and all sequences of the peptides obtained from the endogenous protein are represented in the putative cDNA p16 amino acid sequence. This clearly demonstrates that the 16-kDa protein in the pure fraction is a single polypeptide. We also found that a recombinant p16 was active in the intra-Golgi transport cell-free assay, strongly supporting the notion that the 16-kDa polypeptide described here is the active component of the pure fraction.

Several well defined steps are required for intracellular vesicular protein traffic, including budding, targeting, docking, and fusion of vesicles with their target membranes, each requiring a different set of cytosolic factors. It has been demonstrated previously that ARF-1 and the coat proteins are the only cytosolic factors required for the production of Golgi-derived COPI (31). Vesicle targeting involves interaction between integral membrane proteins, the v-SNAREs located on vesicles and the t-SNAREs present on the target membrane (32, 33). The v-SNARE-t-SNARE complex then binds SNAP and NSF which, in turn, catalyze the disassembly of the SNARE complex, thus initiating fusion (32). This core machinery of protein transport probably requires the participation of additional accessory factors. Our data suggest that all three factors described in this study, Iα, β, and p16, are part of the targeting and fusion machinery. Analysis of the intra-Golgi transport assay revealed that in the absence of the coat proteins the assay measures mainly fusion between the Golgi cisternae (17). This uncoupled fusion reaction requires known components such as NSF, SNAP, and p115 and is resistant to GTPγS. We demonstrated here that the p16-dependent assay was not inhibited by GTPγS or brefeldin A, indicating that it represents an uncoupled fusion. Based on these results we suggest that p16 plays a role in docking or fusion of vesicles rather than being part of the budding apparatus.

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4 It should be noted that affinity-purified anti-p16 antibodies directed against the recombinant protein specifically inhibited the cell-free intra-Golgi transport assay in the presence of crude cytosol, hence demonstrating that p16 is required for intra-Golgi transport under conditions in which the cytosolic factors coatamer and ARF are present.
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