A Novel 115-kD Peripheral Membrane Protein Is Required for Intercisternal Transport in the Golgi Stack

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Abstract. We have used an in vitro Golgi protein transport assay dependent on high molecular weight (>100 kD) cytosolic and/or peripheral membrane proteins to study the requirements for transport from the *cis*- to the *medial*-compartment. Fractionation of this system indicates that, besides the NEM-sensitive fusion protein (NSF) and the soluble NSF attachment protein (SNAP), at least three high molecular weight protein fractions from bovine liver cytosol are required. The activity from one of these fractions was purified using an assay that included the second and third fractions in a crude state. The result is a protein of 115-kD subunit molecular mass, which we term p115. Immunodepletion of the 115-kD protein from a purified preparation with mAbs removes activity. Peptide sequence analysis of tryptic peptides indicates that p115 is a "novel" protein that has not been described previously. Gel filtration and sedimentation analysis indicate that, in its native state, p115 is a nonglobular homo-oligomer. p115 is present on purified Golgi membranes and can be extracted with high salt concentration or alkaline pH, indicating that it is peripherally associated with the membrane. Indirect immunofluorescence indicates that p115 is associated with the Golgi apparatus in situ.

ELUCIDATION of the mechanism by which proteins are transported through the Golgi complex is of fundamental importance in cell biology. In recent years, it has been generally agreed upon that movement involves vectorial vesicular traffic (Rothman et al., 1984; Pfeffer and Rothman, 1987) (for a different view see Klausner et al., 1992; Mellman and Simons, 1992). That is, vesicles bud from one Golgi cisternae (for example, the *cis*-compartment), and then target to, and fuse with, the next Golgi compartment (for example, the *medial*-compartment). This process is repeated for transport from the *medial*- to the *trans*-cisternae. Genetic, biochemical, and morphological analysis of these budding, targeting, and fusion reactions has yielded an overall view of this process (Rothman and Orci, 1990). However, the details of what proteins are involved, and when and how they act, are poorly understood.

One approach to studying this transport process is through the use of cell-free systems that reconstitute movement through the Golgi apparatus (Balch et al., 1984; Rothman, 1987). These in vitro transport systems require ATP, cytosolic proteins, Golgi membrane proteins, and palmitoyl-coenzyme A (Fries and Rothman, 1980; Balch et al., 1984; Glick and Rothman, 1987; Rothman, 1987). Several cytosolic factors have been purified to homogeneity by exploiting the functional *cis* to *medial* transport assay. One, *N*-ethylmaleimide sensitive fusion protein (NSF),[1] acts in vesicle-Golgi membrane fusion (Block et al., 1988; Malhotra et al., 1988). Another set, soluble *N*SF attachment proteins (SNAPS), are required to bind NSF to the Golgi membrane (Weidman et al., 1989; Clary et al., 1990). Several other factors have been defined by kinetic experiments or the assay system's susceptibility to inhibitors (Wattenberg et al., 1986; Wattenberg and Rothman, 1986; Melançon et al., 1987).

Recently, putative structural and regulatory components thought to be involved in vesicular traffic through the Golgi apparatus have also been identified. The most well characterized is β-COP, a component of the vesicle coat (Duden et al., 1991; Serafini et al., 1991*b*) and of the "coatomer" complex (Waters et al., 1991). In addition, the low molecular weight GTP binding protein ARF (ADP-ribosylation factor) has been shown to be a Golgi protein (Stearns et al., 1990) associated with transport vesicles (Serafini et al., 1991*a*). Finally, a *ras*-related small GTP-binding protein, rab6p, has been shown to be specifically associated with the Golgi apparatus (Goud et al., 1990) and postulated to be involved in the regulation of vesicular transport (Goud and McCaffrey, 1991).

Information obtained from studying the proteins involved in transport through the Golgi apparatus has been generally applicable to other vesicular traffic pathways as well. For example, the transport factor NSF was identified as the mam-

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1. *Abbreviations used in this paper*: NSF, *N*-ethylmaleimide-sensitive fusion; SNAP, soluble NSF attachment protein.

malian homologue of Sec18p (Eakle et al., 1988; Wilson et al., 1989), a protein required for ER to Golgi transport in yeast (Novick et al., 1980; Kaiser and Schekman, 1990). Furthermore, a monoclonal anti-NSF antibody inhibits ER to Golgi transport in semi-intact cells (Beckers et al., 1989), and the fusion of endocytic (Diaz et al., 1989) and transcytotic vesicles (E. Sztul, personal communication) in vitro. Another Golgi transport protein, α-SNAP (Clary and Rothman, 1990), is related to Sec17p (Clary et al., 1990), defined genetically by its involvement in ER to Golgi transport in yeast (Novick et al., 1980; Kaiser and Schekman, 1990).

In an attempt to further define the protein machinery involved in vesicular transport, we have modified the Golgi intercisternal transport assay to make it dependent on high molecular weight cytosolic and/or peripheral Golgi membrane proteins. Analysis of this system indicates that at least three distinct high molecular weight components are required for transport. One of these factors has been purified and characterized on the basis of this functional assay.

Materials and Methods

General Procedures

Protein concentrations were determined with the Bio-Rad Laboratories (Richmond, CA) protein assay kit. All stated pH values are at room temperature. Conductivity was converted to salt concentration by using a standard curve of either 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol containing from 0 to 1 M KCl or 25 mM TrisCl, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol containing from 0 to 500 mM KP_i (potassium phosphate). All fractionations were performed at 4°C, unless otherwise indicated. Before assaying transport activity all cytosolic fractions were dialyzed into 25 mM TrisCl, pH 7.4, 50 mM KCl, 1 mM DTT, 10% glycerol, unless otherwise indicated (see Figs. 7 *b* and 8 *b*).

Preparation of Salt Extracted Golgi Membranes

Vesicular stomatitis virus (VSV)-G protein bearing donor membranes from CHO 15B cells and acceptor membranes from wild-type CHO cells were prepared as described (Balch et al., 1984). These membranes were subsequently extracted with 1 M KCl as described (Clary and Rothman, 1990) except that the extraction solution contained 10 mM TrisCl, pH 7.4, and the extraction was done at 0°C.

Preparation of a Low Molecular Weight Fraction of Cytosol

Cytosolic transport factors can be separated into high (Fraction 1) and low (Fraction 2) molecular weight pools, both of which are required for activity (Clary and Rothman, 1990). A crude Fraction 2 pool was prepared as follows: bovine brain cytosol, prepared as described (Waters et al., 1991), was precipitated with 60% saturated ammonium sulfate. The pellet was resuspended in 25 mM TrisCl, pH 7.4, 50 mM KCl, 1 mM DTT (at 50 mg/ml), and loaded at 1 ml/min onto a 350-ml Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column (2.6-cm inner diameter) equilibrated in the same buffer. The column was eluted with the same buffer and 6-ml fractions were collected. The fractions were assayed for Fraction 1 and Fraction 2 activity as described (Clary and Rothman, 1990). Fractions that contained Fraction 2 activity but were devoid of Fraction 1 activity were pooled. This preparation contains cytosolic proteins that are smaller than ~100 kD and had a protein concentration of 5 mg/ml.

Cis- to Medial-Golgi Transport Assay Dependent on High Molecular Weight Components

The assay is a modification of that described previously by Clary and Rothman (1990) with the principal changes being omission of yeast cytosol, inclusion of nucleoside monophosphate kinase (Waldman and Rudnick, 1990), and a KCl concentration of 60 instead of 20 mM. The 25-μl assays contained 0.3 μCi UDP-[^{3}H]*N*-acetylglucosamine (New England Nuclear, Boston, MA), 5 μl of a 1:1 mixture of 1 M KCl extracted donor and acceptor membranes (~2–3 μg protein), 4 μl (20 μg) of a pool of low molecular weight proteins (Fraction 2), 0.2 μl (~5 ng) NSF isolated from CHO cytosol, 12 μl of various cytosolic fractions as indicated in the figure legends, an ATP and UTP regenerating system (Waldman and Rudnick, 1990), and palmitoyl-Coenzyme A. The final conditions, unless otherwise stated in the figure legends, were as follows: 25 mM Hepes-KOH, pH 7.0, 16 mM Tris Cl, pH 7.4, 60 mM KCl, 2.5 mM $Mg(OAc)_2$, 100 μM ATP, 250 μM UTP, 5 mM creatine phosphate, 12 IU/ml creatine kinase, 10 μM palmitoyl-Coenzyme A, 40 μg/ml nucleotide monophosphate kinase, 200 mM sucrose, 4.8% (wt/vol) glycerol, and 0.64 mM DTT. The transport reactions were incubated at 30°C for 2 h. [^{3}H]*N*-acetylglucosamine incorporated into VSV-G protein was detected by immunoprecipitation of VSV-G protein and scintillation counting as described (Balch et al., 1984).

Separation of Bovine Brain Cytosol into Three Active Fractions

350 mg of bovine brain cytosol (Waters et al., 1991) at 5 mg protein/ml in 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT was centrifuged at 10,000 *g* for 10 min to remove particulates and the material was loaded at 2 ml/min onto an 8 ml Mono Q column (Pharmacia Fine Chemicals) equilibrated in 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol. The column was washed with 10 ml of the same buffer, and then eluted with a 5 mM/ml KCl gradient in 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol to 500 mM KCl, and then with a 31 mM/ml KCl gradient to 1 M KCl. 4-ml fractions were collected throughout, dialyzed, and assayed as described above.

Preparation of a Fraction 1αβ

1,100 mg of bovine brain cytosol protein, prepared as described (Waters et al., 1991), was adjusted by addition of buffer to 5 mg protein/ml in 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT. After centrifugation at 7,500 *g* for 10 min to remove particulates, 160 mg of the material (32 ml) was loaded at 3 ml/min onto an 8 ml Mono Q column equilibrated in 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol. The column was washed with 8 ml of the same buffer, and then eluted with a 40 mM/ml KCl gradient in 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol (i.e., from 100–320 mM KCl in 5.5 ml), followed by a 10 mM/ml KCl gradient in 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol (i.e., from 320–500 mM KCl in 18 ml). 1 ml fractions were collected throughout. This protocol was repeated 6 more times, collecting the fractions in the same tubes for each run. The fractions were dialyzed and 6 μl were assayed (see Fig. 4) in the presence of 6 μl of Fraction 1γ (see Fig. 3, Fraction 37). The peak fractions were pooled (see Fractions 9–15, Fig. 4) and concentrated by precipitation with 80% saturated ammonium sulfate in the presence of 1 mM EDTA. The precipitate was collected by centrifugation at 25,000 *g* for 20 min, resuspended in 10 ml of 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol, and dialyzed against 25 mM TrisCl, pH 7.4, 50 mM KCl, 1 mM DTT, 10% glycerol. This material is referred to as Fraction 1αβ and had a protein concentration of 33 mg/ml.

Cis- to Medial-Golgi Transport Assay Dependent on Fraction 1γ

This assay was identical to the assay dependent on high molecular weight components except that the 4 μl (20 μg) of Fraction 2 pool was replaced with 4 μl (130 μg) of Fraction 1αβ. This change raised the final glycerol concentration from 4.8 to 6.4% (wt/vol).

Purification of Fraction 1γ

Preparation of Bovine Liver Cytosol. Bovine liver was obtained immediately after slaughtering, placed in ice cold 25 mM TrisCl, pH 7.4, 320 mM sucrose, and kept on ice till use. 450 g of tissue were placed in a glass Waring blender which was then filled to the top (usually 800–810 ml) with 25 mM TrisCl, pH 8.0, 500 mM KCl, 250 mM sucrose, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.5 mM 1,10 phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin (DTT and protease inhibitors were added just before use). The tissue was homogenized two times for 30 s separated by a 2-min cooling period.

The homogenate was centrifuged at 8,500 rpm (7,500 *g*, *k* factor 3,000) in a Sorvall GS3 (Sorvall Instruments, Newton, CT) rotor for 1 h at 4°C. The supernatants were decanted, pooled, and centrifuged at 39,000 rpm in

a Beckman 45Ti rotor (120,000 *g*, *k* factor 180) (Beckman Instruments, Inc., Palo Alto, CA) for 2 h at 4°C. The supernatants were decanted, pooled, and dialyzed in Spectra/por 2 dialysis bags (MW cutoff 12,000–14,000, 6.4 ml/cm) against 30 liters of 25 mM TrisCl, pH 7.4, 50 mM KCl, 1 mM DTT at 4°C. After at least 2 h, dialysis was continued against fresh buffer for 4–12 h. The dialyzed material was collected and clarified by centrifugation in a Sorvall GS3 rotor at 8,500 rpm (Sorvall Instruments) for 1 h at 4°C. The supernatants were pooled. This material is termed bovine liver cytosol and had a protein concentration of 57 mg/ml.

Ammonium Sulfate Precipitation. The protein concentration of the bovine liver cytosol was adjusted to 10 mg/ml by dilution with fresh dialysis buffer, and EDTA was added to 1 mM. The solution was transferred to a 2 liter beaker packed in ice and solid ammonium sulfate was added slowly with stirring to a final concentration of 40% saturation at 0°C (added 0.229 g/ml). After dissolution of the salt, the solution was stirred for 30 min at 0°C and then centrifuged in a Sorvall GS3 rotor at 8,500 rpm for 1 h at 4°C. The supernatants were discarded and the pellets were resuspended in 40 ml of 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol with 10 strokes in a 40-ml Dounce homogenizer with the "B" pestle and then diluted to 300 ml in the same buffer. The conductivity of the solution was measured and then enough resuspension buffer was added so as to bring the final conductivity to the equivalent of 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol. Insoluble material was removed by centrifugation in a Sorvall GS3 rotor at 8,500 rpm for 10 min at 4°C. The resuspended, clarified material had a protein concentration of 6.0 mg/ml and was termed ammonium sulfate precipitate.

DEAE-Cellulose Chromatography. The clarified solution was adjusted to 5 mg/ml with 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol and was loaded at 8 ml/min onto an ~700-ml DEAE-cellulose (Sigma Chemical Co., St. Louis, MO) column (5-cm i.d.) equilibrated in the same buffer. The column was washed with 300 ml of equilibration buffer and then eluted with a 0.3 mM/ml KCl gradient (that is, a 1,300 ml gradient from 25 mM TrisCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10% glycerol to 25 mM TrisCl, pH 7.4, 500 mM KCl, 1 mM DTT, 10% glycerol).

The active fractions, usually from ~190–250 mM KCl, were pooled and termed DEAE pool. This material had a protein concentration of 1.4 mg/ml.

Hydroxylapatite Chromatography. The KCl concentration of the DEAE pool was determined by measuring the conductivity, and then adjusted to 25 mM TrisCl, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol by addition of 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol. Phosphate was added to 10 mM by addition of 1/50 vol of 25 mM TrisCl, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol, 500 mM KP_i.

The solution was loaded at 1.25 ml/min onto an ~140 ml hydroxylapatite (HA Ultrogel, IBF Biotechnics, Savage, MD) column (2.6-cm i.d.) equilibrated in 25 mM TrisCl, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol, 10 mM KP_i. The column was washed with 70 ml of the same buffer and then eluted with a 0.5 mM/ml phosphate gradient in 25 mM TrisCl, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol.

The active fractions, usually from ~65–110 mM KP_i, were pooled and termed hydroxylapatite pool. This material had a protein concentration of 0.6 mg/ml.

Mono Q Chromatography. The hydroxylapatite pool was diluted with 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol to a conductivity equivalent to that of 25 mM TrisCl, pH 7.4, 150 mM KCl, 1 mM DTT, 10% glycerol and then centrifuged in a Sorvall GS3 rotor at 8,500 rpm for 10 min at 4°C. The supernatant was loaded at 3 ml/min onto an 8 ml Mono Q (Pharmacia Fine Chemicals) column equilibrated in 25 mM TrisCl, pH 7.4, 150 mM KCl, 1 mM DTT, 10% glycerol. The protein was eluted with a 2.2 mM/ml KCl gradient in 25 mM TrisCl, pH 7.4, 1 mM DTT, 10% glycerol.

The active fractions, usually from ~350–380 mM KCl, were pooled and termed Mono Q pool. This material had a protein concentration of 1.0 mg/ml.

Isoelectric Precipitation. The Mono Q pool was transferred to Spectra/por 2 dialysis bags and dialyzed extensively against 25 mM NaMES, pH 5.8, 1 mM DTT, 10% glycerol at 4°C. The solution was centrifuged in a Sorvall SS34 rotor at 10,000 rpm (7800 *g*, *k* factor 3,000) for 15 min at 4°C. The supernatant, which had a protein concentration of 0.47 mg/ml, was aspirated. The pellets were saved for purification of coatomer (Waters et al., 1991, 1992).

S-Sepharose Cation Exchange Chromatography. The isoelectric supernatant was adjusted to 25 mM NaMES, pH 5.8, 25 mM NaCl, 1 mM DTT, 10% glycerol (load buffer) by addition of 1/20th vol of 25 mM NaMES, pH 5.8, 500 mM NaCl, 1 mM DTT, 10% glycerol. This solution was loaded at 0.5 ml/min onto a 6-ml S-Sepharose Fast Flow (Pharmacia Fine Chemicals) column (1-cm i.d.) equilibrated in load buffer. The column was washed with the same buffer and the flow-through fractions were collected and pooled. This material is referred to as S-Sepharose flow through and had a protein concentration of 0.08 mg/ml.

Concentration on Hydroxylapatite. The S-Sepharose flow through was brought to 1 mM KP_i by addition of 1/1,000 vol of 1 M KP_i, pH 7.4. The solution was loaded onto a 1-ml hydroxylapatite (Bio-Rad HTP) column (0.5-cm i.d.) at 0.2 ml/min and immediately eluted (i.e., no wash step) with 25 mM Hepes/KOH, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol, 200 mM KP_i. 1/2-ml fractions of the eluate were collected and a protein assay performed. The peak fractions (usually 3–4 fractions) were pooled.

Superose 6 Chromatography. The concentrated S-Sepharose flow-through was centrifuged for 10 min in a microfuge to remove any insoluble material and the supernatant was sieved through onto a 100-ml preparative grade Superose 6 (Pharmacia Fine Chemicals) column (1.6-cm i.d.) equilibrated in 25 mM Hepes/KOH, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol at 0.3 ml/min. 2-ml fractions were collected and aliquots analyzed by electrophoresis and Coomassie staining. In addition, 6.5 μl of each fraction was assayed, without prior dialysis, for Fraction 1γ activity. The activity copurified with a homo-oligomeric complex of 115-kD subunits that gel filters with a Stokes radius of ~83 Å.

Velocity Sedimentation of p115

p115, after Superose 6 chromatography, was concentrated fourfold in a Centricon 10 microconcentrator (Amicon, Beverly, MA) and then diluted twofold in the same buffer without glycerol so that the final glycerol concentration was 5% (wt/vol). This solution was layered onto a 10–25% linear glycerol gradient in the same buffer. S value standards (bovine plasma α_2-macroglobulin, 20 S; bovine liver catalase, 11.4 S; BSA, 4.6 S) were loaded onto a second gradient. The gradients were centrifuged for 4.5 h in a Beckman SW 55 rotor (Beckman Instruments, Inc.) at 4°C and then fractionated in into ~0.43-ml fractions.

Preparation of mAbs

Mouse mAbs were generated according to standard techniques. Briefly, two 8–12-wk-old BALB/C mice were immunized with 175 μg each of purified p115 (through the concentration step after S-Sepharose) according to the following regimen: day one, 50 μg; day 15, 50 μg; day 35, 50 μg; day 53, 25 μg. On day 56 spleen cells were fused to the myeloma SP2/0 cells and selected in hypoxanthine aminopterin thymidine (HAT) media. Primary tissue culture supernatants were screened by ELISA with 50 ng of pure p115 per well, and an anti-mouse Ig HRP-conjugated secondary antibody for development with 2,2'-azinodi(3-ethyl benzthiazoline sulphonic acid). Hybridomas that were secreting anti-p115 antibodies were expanded. The resulting tissue culture supernatants were screened for the ability to immunoblot pure p115, using an anti-mouse IgG (γ chain specific) HRP-conjugated secondary antibody for development with diaminobenzidine, to identify IgG secreting cell lines. Five cell lines producing antibodies that blotted well and recognized different minor components of the p115 preparation when used at high concentration (presumably proteolytic products of p115) were chosen for subcloning. After subcloning, tissue culture supernatants were again tested by ELISA and immunoblotting and five positive clones (one from each line in the previous screen) were expanded and stored. The result is five hybridomas secreting different IgGs termed MAb-115-1 (an IgG_1) MAb115-2 (an IgG_3), MAb115-3 (an IgG_1), MAb115-4 (an IgG_{2a}), and MAb115-5 (an IgG_1). All mAbs recognize one 115-kD protein in bovine liver cytosol.

Immunoblotting

Proteins were separated by SDS-PAGE and electroblotted onto 0.45 μm nitrocellulose, which was then incubated in 5% nonfat dry milk, PBS, 0.1 Tween 20 (M/PBS/T). The nitrocellulose was incubated for 1 h in various dilutions (in M/PBS/T) of tissue culture supernatants from hybridomas secreting anti-p115 mAbs, washed 4 times for 5 min each in M/PBS/T, incubated in a 1:3,000 dilution in M/PBS/T of goat anti-mouse HRP-conjugated antibody (Bio-Rad Laboratories), for 1 h, and washed again. After washing briefly with PBS, 0.1% Tween 20, the blot was developed with the Amersham Corp. (Arlington Heights, IL) ECL detection kit and exposed to X-AR5 film (Eastman Kodak Co., Rochester, NY) in M/PBS/T.

Immunoprecipitation of Reductively Methylated p115

Formaldehyde (1.8 μl) was added to 200 μl of hydroxylapatite pool (0.76 mg/ml previously dialyzed into 200 mM $NaBO_3$, pH 8.9). Five μl of 120 mM $NaB[^3H]_4$ (5 Ci/mmole) in 0.01 N NaOH was added and incubated at

0°C for 10 min. 10 μl of 500 mM $(NH_4)_2SO_4$ was added and the reaction gel filtered through a 9-ml Sephadex G-25 (Pharmacia Fine Chemicals) column equilibrated in 25 mM Hepes/KOH, pH 7.4, 200 mM KCl, 10% glycerol to remove unincorporated sodium borohydride. The flow-through protein, indicated by a peak of ^3H, was pooled (~1 ml). 75 μl of this material was brought to 0.5% Triton X-100 and incubated for 1 h at 0°C with 75 μl of tissue culture supernatant from hybridomas secreting the indicated antibodies. 20 μl of a 50% suspension of Protein A–Sepharose (Pharmacia Fine Chemicals) in 25 mM Hepes/KOH, pH 7.4, 200 mM KCl was added and mixed for 1 h at 4°C. The Sepharose was pelleted in a microfuge, washed 4 times with 25 mM Hepes/KOH, pH 7.4, 200 mM KCl, 0.5% Triton X-100, and bound protein eluted by incubation in SDS-PAGE sample buffer. The proteins were separated by SDS-8% PAGE, the gel was treated with Enlightning (New England Nuclear), and the proteins visualized by exposure to X-AR5 (Eastman Kodak and Co.).

Preparation of a p115-Affinity Column and Immunodepletion of p115

Cell lines MAb115-2 and MAb115-4 were grown in protein-free hybridoma media (Gibco/BRL, Gaithersburg, MD). The tissue culture supernatants (275 ml for MAb115-2, 100 ml for MAb115-4) were passed via gravity flow over a 2-ml Protein G–Sepharose Fast Flow (Pharmacia Fine Chemicals) column equilibrated in 25 mM TrisCl, pH 7.4, 200 mM KCl. The column was washed with 12 ml of equilibration buffer, and then eluted with 100 mM glycine, pH 3.0. 1/2-ml fractions were collected on top of 10 μl of 2 M Tris base (to immediately neutralize the solution) and aliquots were analyzed by SDS-12% PAGE and Coomassie staining. The antibodies eluted in two or three fractions, which were pooled. We obtained 3.4 mg of MAb115-2 and 1.5 mg of MAb115-4.

Affinity columns containing a covalently coupled mixture of the two antibodies were prepared with the Pierce Immunopure Ag/Ab Immobilization Kit #3 as described in the manufacturers instructions (Pierce Chemical Co., Rockford, IL). The starting material was 3 mg of a 1:1 mixture of MAb115-2 and MAb115-4 which yielded 2 ml of resin containing antibody covalently coupled via their carbohydrate moieties at an approximate concentration of 0.5 mg of antibody/ml of resin. Two 1-ml columns were prepared with this resin.

250 μl of Superose 6 purified p115 at 60 μg/ml in 25 mM Hepes/KOH, pH 7.4, 200 mM KCl, 1 mM DTT, 10% glycerol were supplemented with a final concentration of 125 μg/ml soybean trypsin inhibitor (STI) (a 20-kD protein) so that the flow-through could be located after passage over the anti-p115 column. 200 μl were loaded onto a 1-ml p115 affinity column (above) equilibrated in the same buffer without DTT. 100 μl of equilibration buffer was added to the column and the antigen allowed to interact with the resin for 15 min, after which another 100 μl of buffer were added followed by another 15-min incubation. The column was then eluted with equilibration buffer, 200-μl fractions were collected, and aliquots analyzed by SDS-12.5%PAGE and Coomassie staining. STI eluted predominantly in two fractions, which were pooled. Based on STI staining we estimate the material was diluted threefold by passage over the column and therefore 1 μl of load and 3 μl of flow-through are considered equivalent. There was no p115 detected in the flow-through as determined by immunoblotting with MAb115-2 (the detection limit was <4% of the concentration in the load; therefore, >96% of p115 was removed).

Membrane Extraction of p115

10-μl aliquots of CHO Golgi (see Load in Fig. 9 *c*) in 10 mM TrisCl, pH 7.4, ~30% sucrose were extracted for 30 min at 0°C with 20 μl of 10 mM TrisCl, pH 7.4, buffer containing 0 mM KCl, 90 mM KCl, or 1.5 M KCl or with 20 μl of 150 mM sodium carbonate, pH 11, giving final concentrations of 0 mM, 60 mM, and 1 M KCl, or 100 mM sodium carbonate. The extractions were overlaid onto 170-μl cushions of 20% sucrose containing 0 mM, 60 mM, or 1 M KCl, or 100 mM sodium carbonate, pH 11, and centrifuged in Beckman TLA100 rotor at 50,000 rpm (100,000 *g*, *k* factor 26) for 12 min at 4°C (with slow acceleration and deceleration). The supernatants were aspirated and protein was TCA precipitated in the presence of deoxycholate as described (Waters et al., 1991). The pellets and precipitated supernatants were subjected to SDS-8% PAGE, and immunoblotted with MAb115-5.

Immunofluorescence

Bovine tracheal fibroblast (EBTr cells) were grown in MEM with Earle's balanced salt solution and 10% FCS on 12-mm glass coverslips to ~50% confluency. All subsequent manipulations were performed at room temperature and washes were done for 5 min. The cells were washed briefly in PBS/0.1% BSA (PBS/B) and then fixed in 3% paraformaldehyde in PBS for 30 min. After three PBS/B washes, the cells were incubated in 50 μg/ml wheat germ agglutinin in PBS for 30 min, washed in PBS three times, and then permeabilized for 3 min in −20°C methanol. The coverslips were washed two times with PBS, three times with PBS/2% BSA, and incubated with a 1:50 dilution of MAb115-2 in PBS/B for 1 h. After three PBS/B washes FITC-wheat germ agglutinin (Molecular Probes Inc., Eugene, OR) and Texas red–conjugated goat anti-mouse-IgG (Cappel Laboratories, Malvern, PA) in PBS/B was applied for 1 h. The coverslips were washed three times in PBS/B, twice in PBS, mounted, and viewed on a Nikon microscope (Nikon Inc., Melville, NY) equipped with a Bio-Rad confocal attachment.

Results

Development of an Assay for High Molecular Weight Transport Factors

In vitro transport of VSV-G protein from the *cis*- to the *medial*-compartment requires multiple cytosolic factors (Wattenberg and Rothman, 1986; Block et al., 1988; Clary and Rothman, 1990). Recently an assay was developed that uses Golgi membranes stripped of peripheral membrane proteins by extraction with high salt concentration (Clary and Rothman, 1990). Transport in this system can be reconstituted by inclusion of whole cytosol. In addition, fractionation of proteins into low and high molecular weight pools by gel filtration chromatography, indicated that both small (<100 kD) and large (>100 kD) components were required. By assaying fractions of cytosol in the presence of the high molecular weight protein pool, three related proteins of 35, 36, and 39 kD termed α-, β-, and γ-SNAP were purified (Clary and Rothman, 1990). These proteins act to bind another transport factor, NSF, to the Golgi membrane before membrane fusion (Clary et al., 1990).

We have now used the converse of this assay to search for more transport factors. That is, we have included a low molecular weight pool of cytosolic proteins (Fraction 2) (Clary and Rothman, 1990) in our assays, which allowed us to fractionate the high molecular weight components. This assay also differs from the one described previously (Clary and Rothman, 1990) in that yeast cytosol was omitted to potentially reveal requirements for a greater array of high molecular weight components. We found that bovine liver cytosol that had been precipitated with 60% saturated ammonium sulfate stimulated this assay ~ a 13-fold over background (Fig. 1 *a*). Cytosol without ammonium sulfate precipitation stimulates the assay about 6-fold (Fig. 1 *b*), which is likely due to inhibitors in crude cytosol. Despite the lower signal obtained, we have used unfractionated cytosol as a starting material for purification of transport factors so as not to overlook any high molecular weight transport components that may not precipitate with 60% ammonium sulfate.

Cis to Medial Transport Requires At Least Three High Molecular Weight Factors

To determine whether we might be able to purify the high molecular weight activity in cytosol, crude cytosol was loaded on an FPLC Mono Q anion exchanger, protein was eluted with a gradient of KCl (Fig. 2 *a*) and fractions were tested for transport-stimulating activity (Fig. 2, *b–d*). No significant activity was detected in any fraction when assayed alone (Fig. 2 *b*), indicating that either a component was inactivated or multiple components had been separated. If yeast

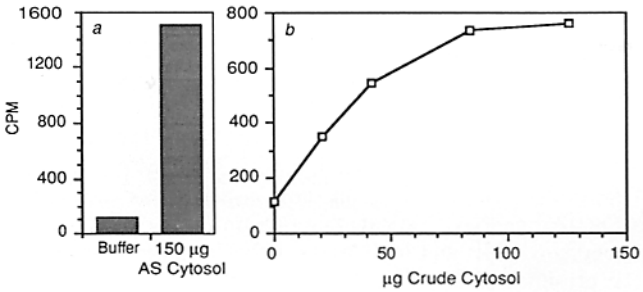

Figure 1. In vitro *cis*- to *medial*-Golgi transport requires high molecular weight cytosolic factors. Transport from VSV-G protein containing CHO mutant 15B Golgi ("donor," lacking *N*-acetylglucosamine transferase 1 activity) was monitored by incorporation of [^{3}H]*N*-acetylglucosamine into VSV-G oligosaccharide upon arrival of this protein in the wild-type CHO Golgi stack ("acceptor"), indicated by *CPM* in this and the following figures. The high molecular weight protein-dependent assay (see Materials and Methods) contained salt-extracted Golgi, purified CHO NSF, Fraction 2 (i.e., low molecular weight protein pool), an ATP and UTP regenerating system, palmitoyl coenzyme A and (*a*) either buffer or 150 µg of a 60% ammonium sulfate fraction of bovine brain cytosol (see Materials and Methods) or (*b*) buffer or the indicated amount of bovine brain cytosol that had not been ammonium sulfate precipitated.

cytosol was included in the assay mix, small peaks of activity (~1.5 times background) were detected, eluting at ~270 and ~380 mM KCl (data not shown). Consequently, we examined these fractions further. Inclusion of the Mono Q fraction eluting at ~270 mM KCl in our assays (Figs. 2, fraction 31) gave a sharp peak of activity at ~380 mM KCl (Fig. 2 *c*, fraction 37). Conversely, inclusion of the ~380 mM KCl fraction (Fig. 2, fraction 37), yielded a peak at ~270 mM KCl (Fig. 2 *d*, fraction 31). In addition, a small peak at ~210 mM KCl (Fig. 2 *d*, fraction 27) was consistently evident in multiple preparations. Since the high molecular weight cytosolic pool required for transport was previously named Fraction 1, we have termed the ~210, ~270, and ~380 mM KCl peaks Fraction 1α, 1β, and 1γ, respectively.

Mixing experiments with the 1α, 1β, and 1γ fractions indicated that all three fractions were required for optimal activity (Fig. 3). Since the activity can be completely reconstituted by addition of fractions 1α, 1β, and 1γ (Fig. 3, compare *Cytosol* and $\alpha+\beta+\gamma$), these fractions most likely contain all the high molecular weight components required for transport in this system. This does not mean that there are only three high molecular weight proteins required however, because some of the fractions may contain more than one activity. The fact that mixtures of two of the fractions (particularly, 1β and 1γ) show some transport activity may be due to contamination by the missing fraction. In fact, the magnitude of the signals with any two factors is consistent with the fact that peaks on Mono Q are often sharp on the low salt side and trail on the high salt side. Finally, transport in the presence of Fraction 1α, 1β, and 1γ was dependent on the presence of Fraction 2 and NSF (data not shown).

Development of an Assay for Fraction 1γ

Since our assay system was most sensitive to the presence of Fraction 1γ (Fig. 3, compare $\alpha\beta$ and $\alpha\beta\gamma$) we sought to develop a convenient assay for purification of this component. This was done by preparation of a fraction that contains both Fraction 1α and 1β, but is devoid of Fraction 1γ. Cytosol was chromatographed on Mono Q and assayed in the presence of Fraction 1γ (Fig. 4 *a*). Two peaks were detected

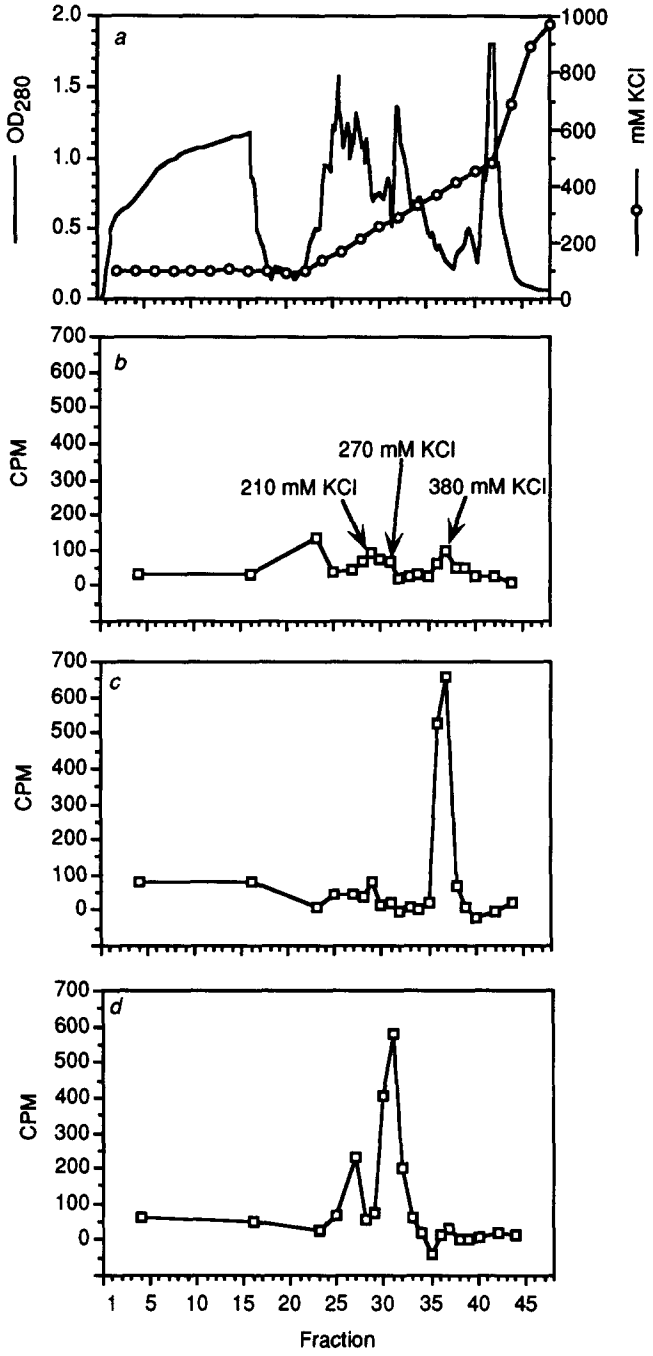

Figure 2. Transport requires multiple high molecular weight factors. Bovine brain cytosol was fractionated (*a*) on FPLC Mono Q anion exchanger by elution with a KCl gradient (see Materials and Methods) and the fractions were assayed in the high molecular weight protein-dependent assay (see Materials and Methods) either with (*b*) 6 µl of each fraction alone, (*c*) 6 µl of the 270 mM eluate (fraction 31) and 6 µl of the indicated column fraction, or (*d*) 6 µl of the 380 mM eluate (fraction 37) and 6 µl of the indicated column fraction. Relevant KCl concentrations are indicated in *b*. Background cpm were subtracted: 116 cpm for buffer alone in *b*, 183 cpm for Fraction 31 alone in *c*, and 215 cpm for Fraction 37 alone in *d*.

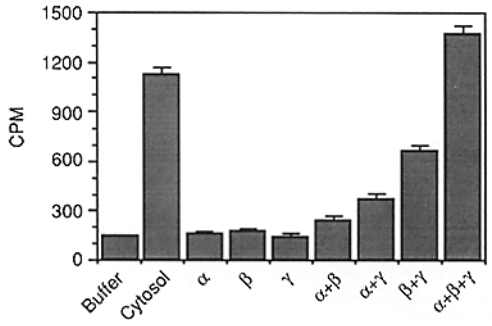

Figure 3. Transport requires at least three high molecular weight factors. High molecular weight protein-dependent transport assays (see Materials and Methods) were performed that contained either buffer, 12 μl (126 μg) of crude bovine brain cytosol (i.e., the column load in Fig. 2), or the indicated combinations of fractions from the Mono Q column in Fig. 2 eluting at 210 mM KCl (α, fraction 27), 270 mM KCl (β, fraction 31), or 380 mM KCl (γ, fraction 37), 4 μl each. Assays were done in duplicate. The mean is plotted, with the error bar representing the higher value.

at salt concentration corresponding to the elution positions of Fraction 1α and 1β. The region of the column encompassing the Fraction 1α and 1β peaks was pooled and termed Fraction 1αβ. Transport was dependent on the amount of Fraction 1αβ added (Fig. 4 *b*). In the subsequent purification of Fraction 1γ we added 4 μl of Fraction 1αβ per assay.

Unlike the case when separate 1α, 1β, and 1γ fractions were used (Fig. 3), transport in the presence of crude Fraction 1αβ and Fraction 1γ was only partially dependent on NSF and independent of Fraction 2 (the low molecular weight pool, data not shown). Immunoblots of Fraction 1αβ with an anti-NSF mAb, an anti-peptide antibody that recognizes both α- and β-SNAP, and an anti-peptide antibody that recognizes γ-SNAP indicate that Fraction 1αβ contains both α- and γ-SNAPs and high levels of NSF (data not shown). The presence of SNAPs is consistent with the absence of a requirement for Fraction 2, while the abundance of NSF (which was probably partially inactivated by the absence of ATP) (Block et al., 1988) explains the partial NSF dependence. Since Fraction 2 (the low molecular weight pool) was no longer required in our new assay we did not include it during purification. Consequently our assay system consisted of salt-extracted Golgi membranes, NSF, Fraction 1αβ (which contains α- and γ-SNAPs), and the fraction to be tested for Fraction 1γ activity.

Purification of Fraction 1γ

The activity in Fraction 1γ was purified from bovine liver cytosol by ammonium sulfate precipitation, DEAE-cellulose anion exchange chromatography, hydroxylapatite chromatography, FPLC Mono Q anion exchange chromatography, isoelectric precipitation, S-Sepharose cation exchange

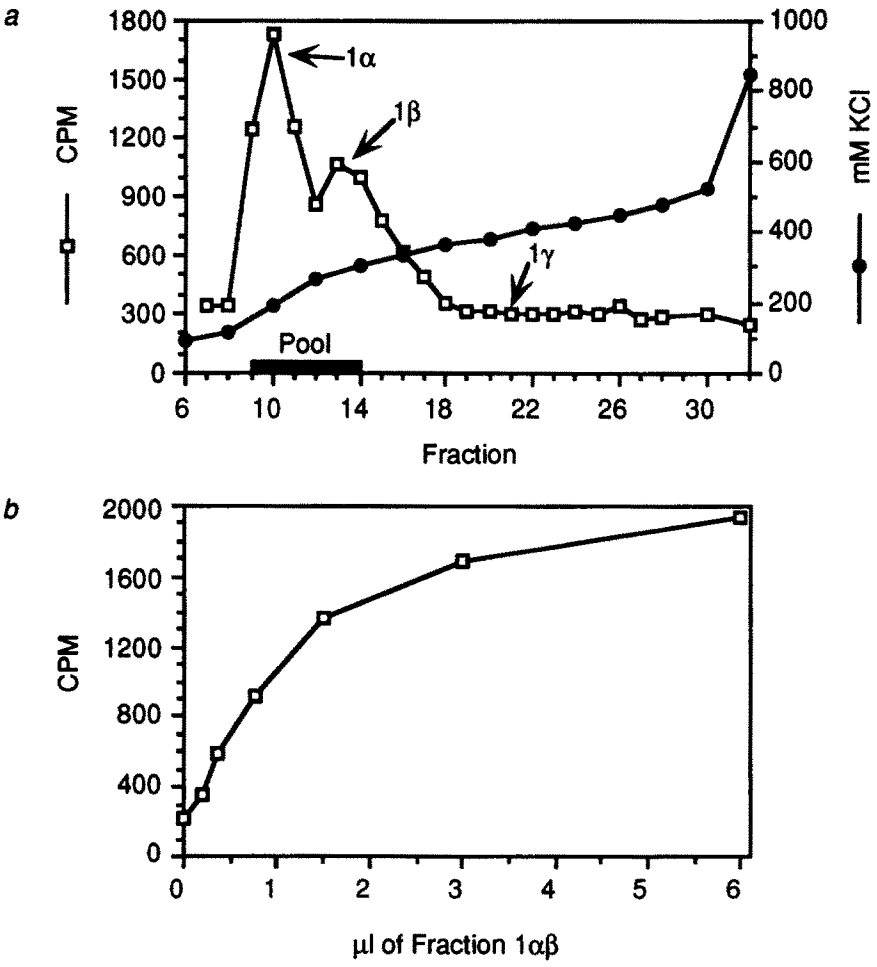

Figure 4. Preparation of a Fraction 1αβ. (*a*) Bovine brain cytosol was fractionated on FPLC Mono Q anion exchanger with a steep KCl gradient through the Fraction 1α and 1β region followed by a shallow gradient through the Fraction 1γ region. 6-μl aliquots were assayed in the high molecular weight protein-dependent assay (see Materials and Methods) in the presence of 6 μl of Fraction 1γ from the previous column (Fig. 2, 380 mM KCl eluate, fraction 37). Fractions 9 through 15 were pooled, termed Fraction 1αβ, and used as a complementing fraction for purification of Fraction 1γ. (*b*) Varying amounts of Fraction 1αβ were assayed in the presence of 6 μl of Fraction 1γ from the previous column. We included 4 μl of Fraction 1αβ in assays for the purification of the Fraction 1γ activity.

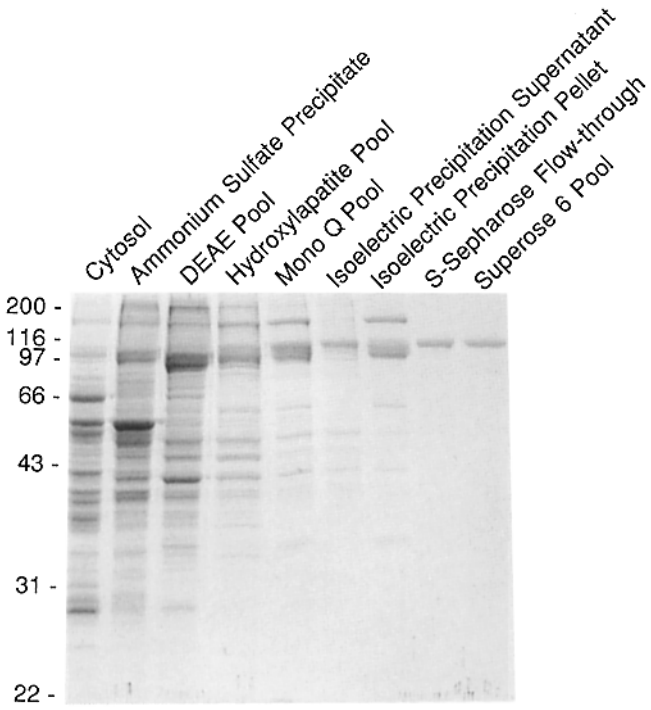

Figure 5. Protein profiles of fractions through the purification of p115 from bovine liver. Proteins were separated by SDS-10% PAGE, and visualized with Coomassie blue. Lanes contain 10 μg of cytosol, ammonium sulfate precipitate, or DEAE pool, 5 μg of hydroxylapatite pool, 3 μg of Mono Q pool, 2 μg of isoelectric precipitation supernatant or pellet, 0.8 μg of S-Sepharose flow through, and 0.4 μg of Superose 6 pool. Molecular weight markers (Bio-Rad) are myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b 97 kD; BSA, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor 22 kD.

chromatography, and gel filtration on Superose 6. We have described the purification from bovine liver cytosol, but the procedure works equally well for bovine brain cytosol. Protein profiles of the pools from each purification step are shown in Fig. 5 and quantitation of the purification is shown in Table I. The increase in total activity in the early purification steps is presumably due to removal of components that interfere with the assay. A typical purification results in about 0.4 mg of a 115-kD protein (on SDS-PAGE) with an ~1,400-fold increase in specific activity and a 4% activity yield. We have termed this protein p115.

It should be noted that dialysis of the Mono Q pool into pH 5.8 buffer results in precipitation of a complex of proteins related to the coat of Golgi transport vesicles (Waters et al., 1991) which we have termed coatomer (Fig. 5, *Isoelectric Precipitation Pellet*). This material can be further purified as described (Waters et al., 1991, 1992).

Amino acid sequence analysis of six tryptic peptides of p115 yielded sequences of 15–25 amino acids that are unique, that is, they do not exhibit significant homology to known proteins or putative gene products.

Preparation of mAb to p115

Purified p115 (after the S-Sepharose step) was used as an immunogen in mice for production of mAbs. Five different monoclonal IgGs were obtained which we term MAb115-1, MAb115-2, MAb115-3, MAb115-4, and MAb115-5. All recognize a 115-kD protein on immunoblots of bovine liver cytosol (Fig. 6 *a*). MAb115-2, MAb115-3, and MAb115-4 recognize native bovine protein as evidenced by their ability to immunoprecipitate p115 radiolabeled by reductive methylation (Fig. 6 *b*). On immunoblots of purified CHO Golgi, only MAb115-5 recognizes a 115-kD protein (see Fig. 9 *c*) and therefore may be the most broadly reactive mAb.

Table I. Quantitation of p115 Purification

	Protein	Specific activity	Purification	Percent activity yield
	mg	*CPM/μg*	*fold*	
Differential centrifugation	13,000	14	[1]	[100]
Ammonium sulfate precipitation	2,400	98	7	130
DEAE-cellulose	290	850	61	140
Hydroxylapatite	71.0	1,300	93	51
Mono Q FPLC	14.0	3,200	230	25
Isoelectric precipitation	6.7	6,200	440	23
S-Sepharose	1.2	15,000	1,100	10
Superose 6 gel filtration	0.38	20,000	1,400	4

Aliquots of the pools through the p115 purification were dialyzed, a twofold dilution series was made (down to 64-fold diluted), and 6 μl were assayed in the Fraction 1γ dependent transport assay. Specific activities were estimated from the slope (cpm/mg protein) in the linear region of the curves. Yields were corrected for sampling during the purification.

Immunodepletion of p115 Removes Activity

To demonstrate conclusively that the 115-kD protein in our purified preparation was the active component, we depleted our pure preparation of p115 by passage over an affinity column that contained a covalently coupled mixture of MAb115-2 and MAb115-4. Soybean trypsin inhibitor was added to the pure p115 as a marker of the loaded material. The flow-through was pooled and equivalent amounts of the load and flow-through (based on Coomassie staining of trypsin inhibitor) were examined. Immunoblots indicate that the column removed >96% of the p115 in the load (data not shown). Equivalent amounts of p115, p115 supplemented with trypsin inhibitor (i.e., the column load), and the column flow-through were assayed for transport activity in the Fraction 1γ dependent assay (Fig. 6 *c*). The purified p115 was highly active and addition of soybean trypsin inhibitor had no effect. In contrast, the flow through had only background activity indicating that the 115-kD band recognized by the mAbs, not some minor contaminant, is responsible for the Fraction 1γ activity.

p115 is a Homo-oligomer

The final purification step of p115 uses gel filtration where the protein (Fig. 7 *a*) and corresponding activity (Fig. 7 *b*) elute with a Stokes radius of about 83 Å. For a globular protein, an 83 Å Stokes radius corresponds to ~700 kD. To assess whether p115 is indeed globular we performed velocity sedimentation of purified p115. We found that p115, as measured by reactivity with an anti-p115 mAb (Fig. 8 *a*), and by activity (Fig. 8 *b*), has a sedimentation coefficient to about 6.8 S, which is much lower than the expected value for a 700-kD globular protein. Knowing both the Stokes radius and the S value allowed us to estimate (Siegel and Monty, 1966) that

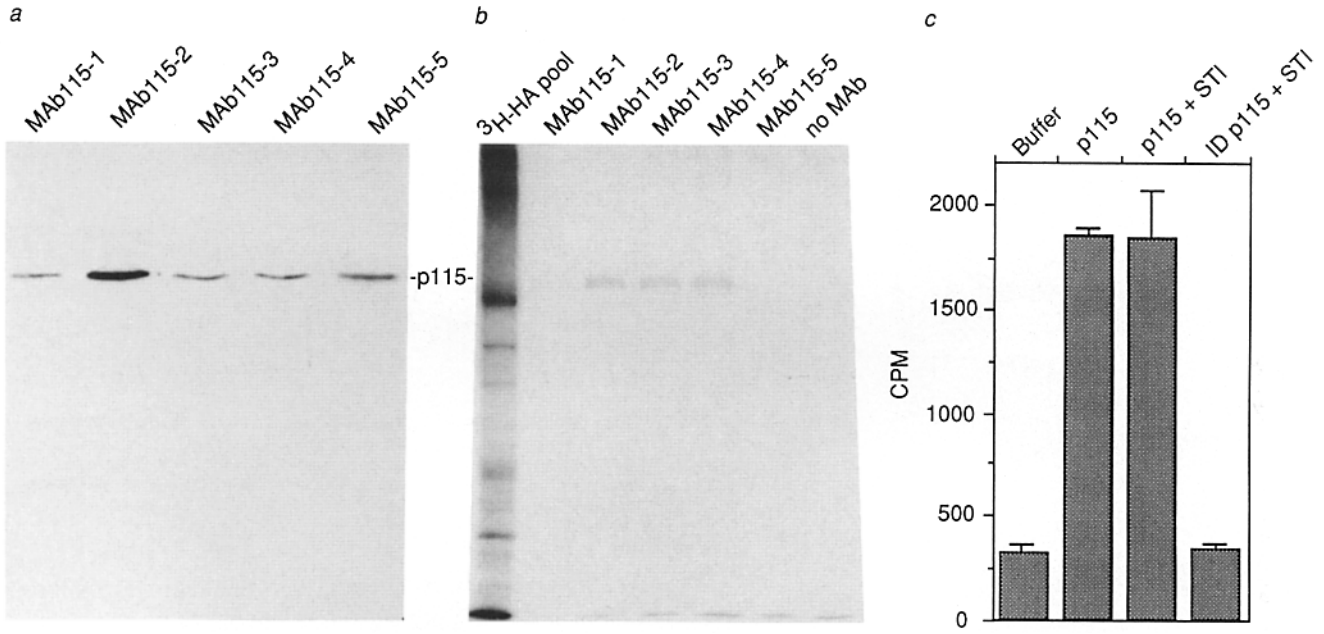

Figure 6. Anti-p115 mAbs remove Fraction 1γ activity. (*a*) Immunoblot of 20 μg of bovine liver cytosol protein with different anti-p115 mAbs. (*b*) Immunoprecipitation of p115 from 75 μl of radiolabeled hydroxylapatite pool (^{3}H-HA pool, 7.5 μl loaded) with the same antibodies or with buffer (no mAb). For *a* and *b* proteins were separated on an SDS-8% PAGE. (*c*) Removal of p115 activity by immunoaffinity chromatography with anti-p115 mAbs (MAb115-2, MAb115-4). Buffer, 120 ng (2 μl) of Superose 6 pure p115 (p115), 120 ng (2 μl) of Superose 6 pure p115 supplemented with soybean trypsin inhibitor (p115 + STI), or an equivalent amount of material (6 μl, based on soybean trypsin inhibitor content) after immunodepletion of p115 (ID p115 + STI) were assayed in duplicate in the Fraction 1γ-dependent Golgi transport assay. The mean is plotted, with the error bar representing the higher value.

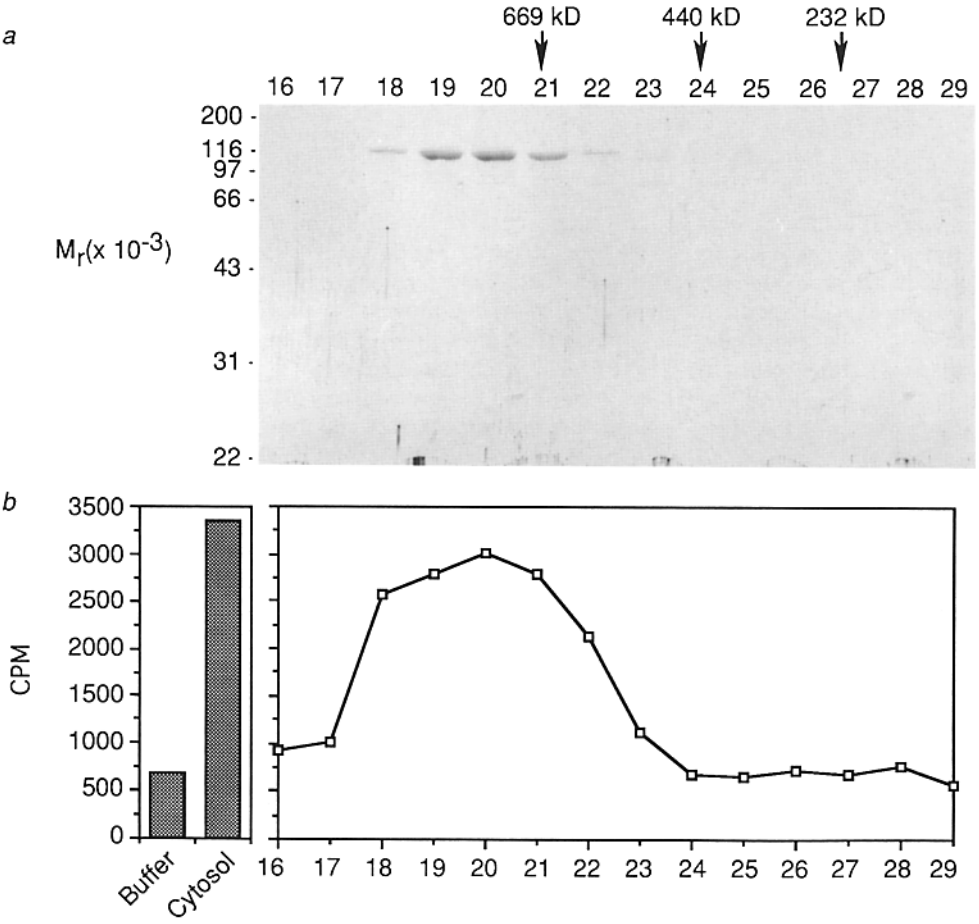

Figure 7. Superose 6 gel filtration of p115. Flow-through from the S-Sepharose column was concentrated and chromatographed on an FPLC Superose 6 column (see Materials and Methods). (*a*) Aliquots (6 μl) of fractions were separated by SDS-10% PAGE and visualized with Coomassie blue. Molecular size standards (Pharmacia Fine Chemicals) for gel filtration were bovine thyroid thyroglobulin (*669 kD*, 85 Å Stokes radius), horse spleen ferritin (*440 kD*, 61 Å), bovine liver catalase (*232 kD*, 52.2 Å). Molecular weight standards for SDS-PAGE were as in Fig. 5. (*b*) Buffer, 150 μg of a 60% ammonium sulfate fraction of bovine brain cytosol or 6.5-μl aliquots of fractions were assayed in the Fraction 1γ-dependent assay. The fractions were not dialyzed before use, therefore the final conditions of the following components were: 31 mM Hepes-KOH, pH 7.0, 4 mM Tris Cl, pH 7.4, 4.2% (wt/vol) glycerol, and 0.42 mM DTT.

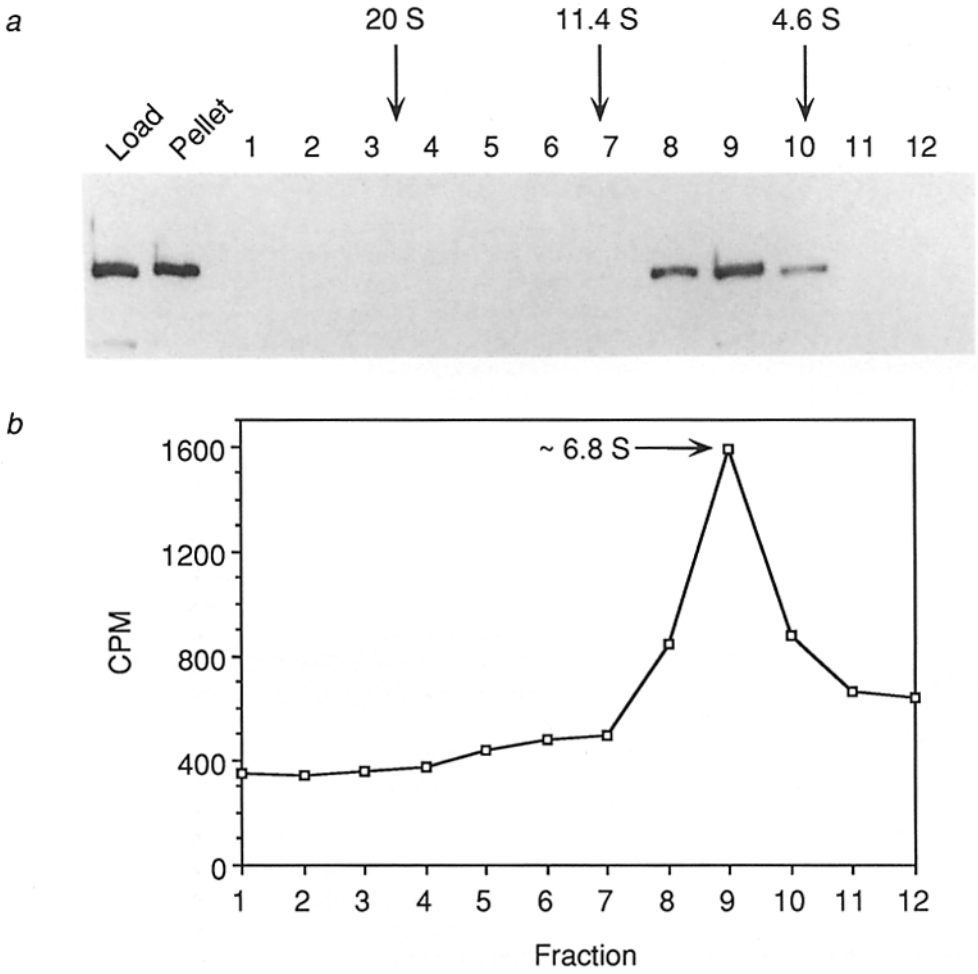

Figure 8. Velocity sedimentation of p115. Purified p115 (i.e., after Superose 6 chromatography, Fig. 7) was sedimented through a glycerol gradient. Fractions were collected and p115 was detected by (*a*) SDS-8% PAGE of 1/40 vol of the load and fractions, or the whole pellet (presumably a small amount of aggregated material), followed by immunoblotting with MAb115-2. S value standards were detected by Coomassie staining. (*b*) 6.5 μl of undialyzed fractions were assayed in the Fraction 1γ-dependent assay. The final conditions were as described in Materials and Methods except that the final glycerol concentration varied from ~4.2–8.1% (wt/vol).

the native molecular mass of p115 is ~360 kD. These data suggest that p115 is a nonglobular homo-oligomer, possibly a trimer.

p115 Is a Golgi Peripheral Membrane Protein

Since the assay used for purification of p115 uses salt-extracted membranes that were depleted of peripheral membrane proteins, it was possible that p115 is either a cytosolic or a peripheral membrane protein. Fig. 9 *a* shows that when salt-extracted membranes are used the assay is dependent on added p115. The assay plateaus at ~400 ng p115 (16 μg/ml, 44 nM using 360 kD as the molecular mass). ~50 ng (2 μg/ml, 6 nM) is sufficient for half-maximal stimulation. On a molar basis the requirement for p115 in our assay is similar to that for α-SNAP (~23 nM to saturate, ~5 nM at half-maximal stimulation) (Clary and Rothman, 1990) in a similar assay system. In contrast to salt-extracted Golgi, when unextracted Golgi membranes are used there is no requirement for additional p115 (Fig. 9 *b*), and therefore it must be present on the membrane. These data indicate that p115 is present on Golgi membranes and is either inactivated, or removed from the membrane by salt extraction.

To test whether p115 is physically extracted from the membrane (as opposed to inactivated) we used a monoclonal anti-bovine p115 antibody (MAb115-5) that cross-reacts with the endogenous p115 on CHO Golgi to probe supernatant and pellet fractions prepared under various conditions (Fig. 9 *c*). Purified CHO Golgi (Load CHO Golgi) were extracted with buffer alone (0 mM KCl), buffer containing 60 mM KCl, buffer containing 1 M KCl, or sodium carbonate, pH 11, a treatment known to remove peripheral membrane proteins (Fujiki et al., 1982). p115 was efficiently extracted by both 1 M KCl and alkali indicating it is a peripheral membrane protein. p115 also dissociates from the Golgi membrane, albeit less efficiently, at low salt concentration. Little p115 was extracted from the Golgi when 60 mM KCl was used. It is interesting to note that KCl optimum of the assay used for purification of p115 is 60 mM (data not shown) suggesting that p115 performs its transport function as a peripheral membrane protein.

To determine where p115 is localized in vivo, we performed double-label indirect immunofluorescence with an anti-p115 mAb (MAb115-2) and wheat germ agglutinin in bovine tissue culture fibroblasts (EBTr). Fluorescent wheat germ agglutinin yields a distinct perinuclear crescent typical of Golgi localization (Fig. 10 *a*) that colocalizes with the p115 staining (Fig. 10 *b*).

Discussion

We have defined a novel protein that functions in vesicular

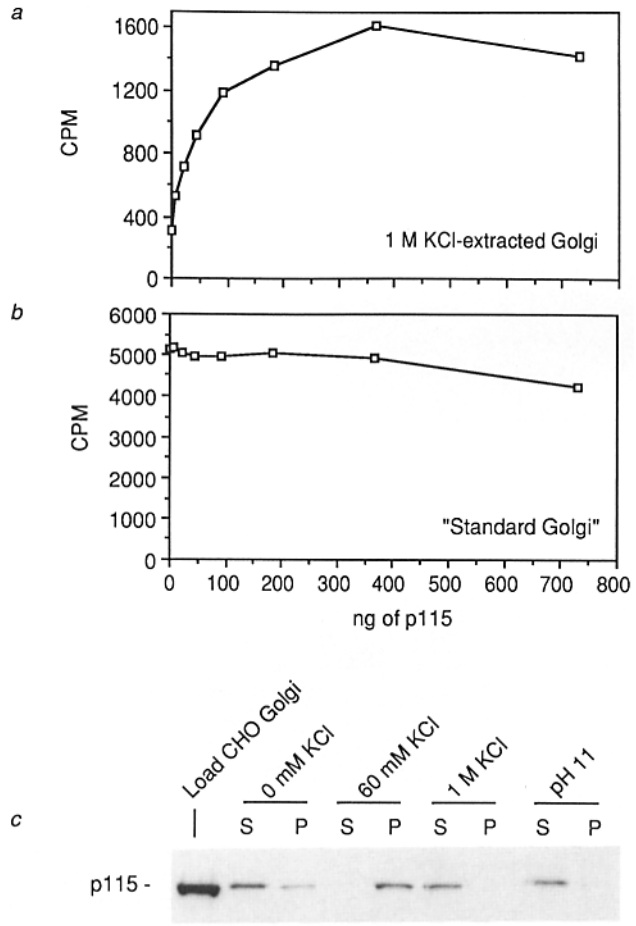

Figure 9. p115 is a Golgi peripheral membrane protein. (*a*) Purified p115 (after Superose 6 chromatography, Fig. 7) was titrated into the Fraction 1γ–dependent assay. (*b*) A 1:1 mixture of donor and acceptor membranes before salt extraction were used instead of the salt-extracted Golgi apparatus used in *a*. (*c*) CHO Golgi membranes (*Load*) were extracted under various conditions, separated into supernatant and pellet fractions by centrifugation, and p115 was detected by immunoblotting with an anti-bovine p115 antibody that recognizes CHO p115 (MAbl15-5).

transport through the Golgi apparatus. The protein is a homo-oligomer of 115-kD subunits and has a nonglobular shape. This transport factor, which we term p115, is a peripheral membrane protein localized to the Golgi apparatus in vivo. In addition to p115, we have found that transport from the *cis*- to *medial*-compartment is dependent on at least two other high molecular mass (>100 kD) factors. These components remain to be purified.

Vesicular transport can be divided into discrete stages involving budding of transport vesicles from the "donor" cisternae, specific targeting of the vesicles to the next compartment, and finally fusion of the vesicle with the "acceptor" cisternae (Rothman and Orci, 1990). The assay system we have used to identify and purify p115 measures the overall transport process, and as such, defines p115 as a functional component. To further refine the role of p115 in transport, it would be useful to develop partial reactions that separately monitor the stages of budding, targeting, and fusion (Groesch et al., 1990; Rexach and Schekman, 1991). Another approach is morphological analysis of transport reactions performed in the presence of function inhibiting anti-p115 antibodies. None of the five monoclonal anti-p115 antibodies presently available exhibit this property.

Vesicular transport through the Golgi apparatus requires components that act catalytically, while other components are thought to play structural roles. Transport factors that act catalytically would be expected to be required at significantly lower concentrations than those that have a structural function. The fusion of transport vesicles with the Golgi is catalyzed by NSF and SNAPs (Malhotra et al., 1988; Clary et al., 1990). Other components, such as coatomer (Duden et al., 1991; Serafini et al., 1991*b*; Waters et al., 1991) and ARF (Stearns et al., 1990; Donaldson et al., 1991; Serafini et al., 1991*a*), are thought to have a structural role in formation of transport vesicles from the Golgi stack. We have found that a low level of p115 (6 nM) is required to obtain half-maximal transport in our assay system. This value is similar to that for α-SNAP (5 nM) in a similar system (Clary and Rothman, 1990), suggesting that p115 plays a catalytic role in the vesicular transport process.

During the development of the p115 purification scheme we discovered a complex of seven polypeptides thought to form the coat of Golgi transport vesicles (Malhotra et al., 1989), which we have termed coatomer (Waters et al., 1991). One of the coatomer polypeptides is β-COP, which exhibits homology to a protein of clathrin-coated vesicles, termed β-adaptin (Duden et al., 1991; Serafini et al., 1991*b*). In addition, β-COP has been shown to rapidly dissociate from the Golgi apparatus upon treatment with the drug brefeldin A (Donaldson et al., 1990). Since p115 and coatomer copurify through four purification steps we were interested in determining whether they physically interact. Immunoblots of column fractions from the p115 purification with anti-coatomer and anti-p115 antibodies (data not shown) indicate that although the chromatographic behavior of p115 and coatomer are similar, they are not identical. That is, although the peaks overlap, they are not exactly coincident, indicating that the copurification of p115 and coatomer is not due to direct physical interaction. This interpretation is supported by the absence of coatomer components in p115 immunoprecipitates (Fig. 6 *b*).

We anticipate that further study of p115 and the two other transport components we have defined will provide insight into the mechanism of vesicular transport through the Golgi apparatus and, by extension, other vesicular transport steps as well.

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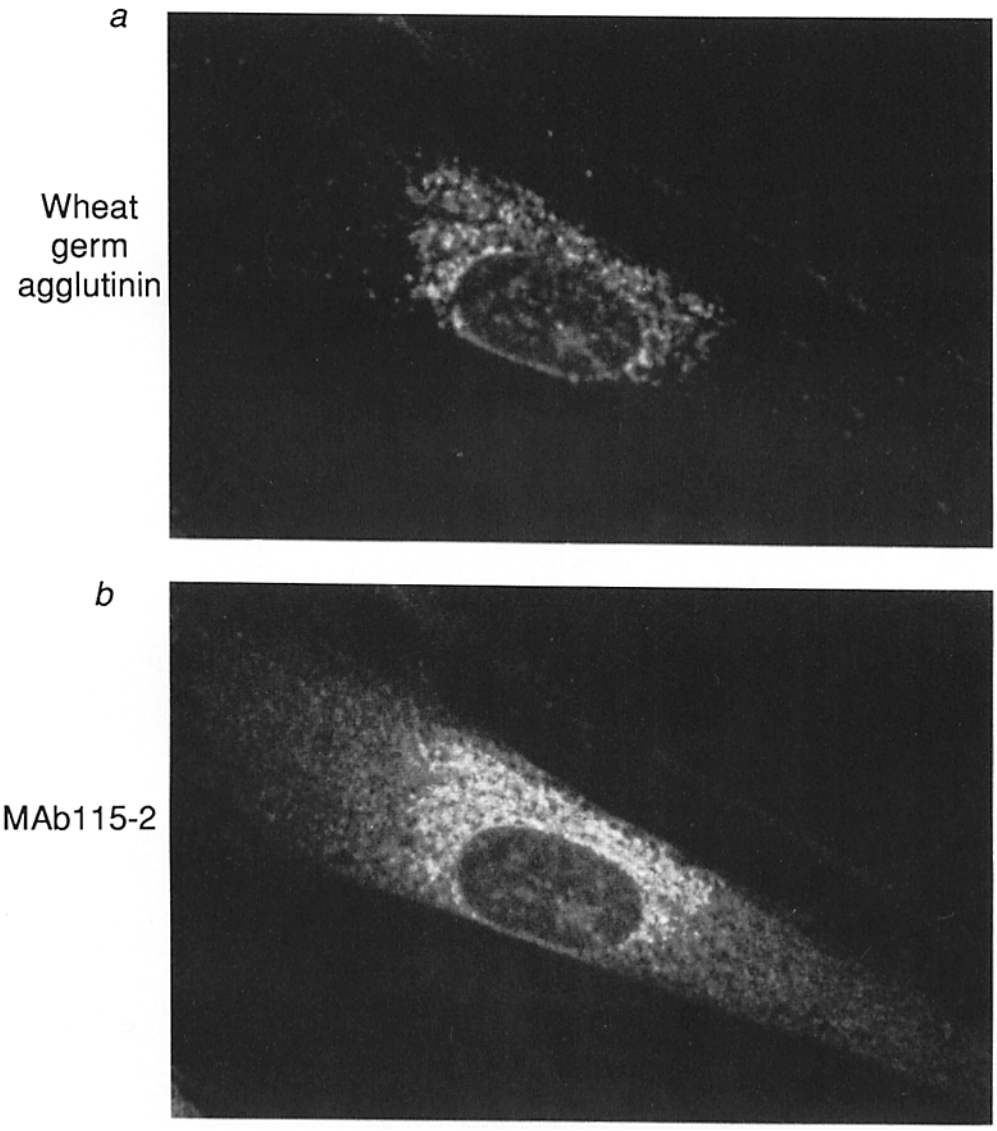

Figure 10. p115 is associated with the Golgi complex in vivo. (*a*) FITC-wheat germ agglutinin and (*b*) MAb115-2 followed by a Texas red–conjugated secondary antibody were used for double-labeling in a bovine fibroblast line (EBTr). The probes colocalize to a perinuclear crescent, characteristic of Golgi complex staining. No staining was evident when the anti-p115 antibody was omitted (not shown). Confocal imaging was done by Joseph Goodhouse.

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