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Morphological and Functional Association of Sec22b/ERS-24 with the pre-Golgi Intermediate Compartment

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Yeast Sec22p participates in both anterograde and retrograde vesicular transport between the endoplasmic reticulum (ER) and the Golgi apparatus by functioning as a v-SNARE (soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein receptor) of transport vesicles. Three mammalian proteins homologous to Sec22p have been identified and are referred to as Sec22a, Sec22b/ERS-24, and Sec22c, respectively. The existence of three homologous proteins in mammalian cells calls for detailed cell biological and functional examinations of each individual protein. The epitope-tagged forms of all three proteins have been shown to be primarily associated with the ER, although functional examination has not been carefully performed for any one of them. In this study, using antibodies specific for Sec22b/ERS-24, it is revealed that endogenous Sec22b/ERS-24 is associated with vesicular structures in both the perinuclear Golgi and peripheral regions. Colabeling experiments for Sec22b/ERS-24 with Golgi mannosidase II, the KDEL receptor, and the envelope glycoprotein G (VSVG) of vesicular stomatitis virus (VSV) en route from the ER to the Golgi under normal, brefeldin A, or nocodazole-treated cells suggest that Sec22b/ERS-24 is enriched in the pre-Golgi intermediate compartment (IC). In a well-established semi-intact cell system that reconstitutes transport from the ER to the Golgi, transport of VSVG is inhibited by antibodies against Sec22b/ERS-24. EGTA is known to inhibit ER–Golgi transport at a stage after vesicle/transport intermediate docking but before the actual fusion event. Antibodies against Sec22b/ERS-24 inhibit ER–Golgi transport only when they are added before the EGTA-sensitive stage. Transport of VSVG accumulated in pre-Golgi IC by incubation at 15°C is also inhibited by Sec22b/ERS-24 antibodies. Morphologically, VSVG is transported from the ER to the Golgi apparatus via vesicular intermediates that scatter in the peripheral as well as the Golgi regions. In the presence of antibodies against Sec22b/ERS-24, VSVG is seen to accumulate in these intermediates, suggesting that Sec22b/ERS-24 functions at the level of the IC in ER–Golgi transport.

INTRODUCTION

Protein transport along the exocytotic and endocytotic pathways is primarily mediated by various types of transport vesicles that bud from a donor compartment and then fuse with a target compartment (Palade, 1975; Pryer *et al.*, 1992; Rothman, 1994; Hong, 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996). Soluble *N*-ethylmaleimide-sensitive factor (NSF) (or its yeast counterpart, Sec18p) and soluble NSF attachment proteins (SNAPs) (or the yeast counterpart, Sec17p) have been shown to participate in many different transport events (Clary *et al.*, 1990; Graham and Emr, 1991; Griff *et al.*, 1992; Whiteheart and Kubalek, 1995). The action of NSF and SNAP is mediated through SNAP receptors (SNAREs) that play a major role in vesicle docking and fusion events (Rothman, 1994; Whiteheart and Kubalek, 1995; Sutton *et al.*, 1998; Weber *et al.*, 1998). It is generally believed that the specific docking and fusion of vesicles with the target compartment is primarily mediated by interaction between v-SNAREs on vesicles and

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t-SNAREs on the target membrane (Söllner *et al.*, 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994; Scheller, 1995; Südhof 1995; Pfeffer, 1996).

Yeast Sec22p (also referred to as Sly2p) is an integral membrane protein anchored to the endoplasmic reticulum (ER) membrane by its C-terminal hydrophobic membrane anchor (Newman *et al.*, 1990; Dascher *et al.*, 1991; Ossig *et al.*, 1991). Being incorporated into ER-derived transport vesicles, Sec22p, together with Bet1p and Bos1p, functions as a v-SNARE for docking and fusion of the vesicle with the early Golgi subcompartment through an interaction of these v-SNAREs with Sed5p (a t-SNARE on the early Golgi compartment) (Newman *et al.*, 1990; Dascher *et al.*, 1991; Ossig *et al.*, 1991; Hardwick and Pelham, 1992; Banfield *et al.*, 1994; Rexach *et al.*, 1994; Sögaard *et al.*, 1994). Recent studies have established that Sec22p also functions as a v-SNARE for retrograde transport from the early Golgi back to the ER by its interaction with Sec20p and Ufe1p (Sweet and Pelham, 1992; Lewis and Pelham, 1996; Lewis *et al.*, 1997). A rat protein homologous to Sec22p was recently reported and was referrred to as Sec22a. Epitope-tagged Sec22a was shown to be associated with the ER (Hay *et al.*, 1996). Using the amino acid sequence of Sec22a to search the expressed sequence tag (EST) database, we have identified two other mammalian protein homologues to Sec22a and Sec22p. During the course of our study, one of these two proteins was independently identified by two other laboratories and was variously referred to as Sec22b (Hay *et al.*, 1997) and ERS-24 (Paek *et al.*, 1997). Epitope-tagged Sec22b/ERS-24 was shown to be associated with the ER in these studies (Hay *et al.*, 1997; Paek *et al.*, 1997). The other protein was referred to as Sec22c, and epitope-tagged Sec22c was similarly associated with the ER (Tang *et al.*, 1998). Since our recent studies have shown that epitope-tagged SNAREs of the early secretory pathway may not be properly targeted in transfected cells transiently expressing the proteins (Lowe *et al.*, 1997; Zhang *et al.*, 1997), the exact subcellular localization of these mammalian proteins homologous to Sec22p need to be established by investigating the endogenous proteins. In addition, detailed functional studies have not been carefully performed for any of the three distinct proteins homologous to Sec22p.

In this study, we have investigated the subcellular localization of Sec22b/ERS-24 using antibodies specific for the endogenous protein. Our results suggest that, in contrast to the ER localization of the epitope-tagged versions, endogenous Sec22b/ERS-24 is primarily associated with the vesicular structures in the peripheral as well as the Golgi regions and behaves like a protein of the pre-Golgi IC. Furthermore, antibodies against Sec22b/ERS-24 inhibited in vitro ER–Golgi transport at the level of the IC.

MATERIALS AND METHODS

cDNA Cloning and Sequencing

The EST clone (accession number AA023107) that contains the complete open reading frame encoding mouse Sec22b/ERS-24 was generated by the Washington University-MERCK EST project and was obtained from the IMAGE consortium via Research Genetics (Birmingham, AL). The coding region of the EST clone was confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins

For the production of recombinant glutathione-*S*-transferase (GST) fusion proteins, the cytoplasmic regions of Sec22b (residues 1–195) and sec22a (residues 2–186) were retrieved by PCR with oligonucleotides 1 (5′-GGC GCT CTA GAC ATG GTG CTG CTG ACG ATG ATC-3′) and 2 (5′-GC CGC CTC GAG TCA CTT GGC GTA AGT GGA GCG CAT-3′) (Clone AA023107 as the template), or 3 (5′-CCG GGA TCC TCT ATG ATT TTA TCC GCC-3′) and 4 (5′-CGG GGA TCC TTA TTC TAG TCG CTG GTG GGC-3′) (Clone U42209 as the template) (Hay *et al.*, 1996). The PCR products were digested with *Xba*I and *Xho*I for Sec22b/ERS-24, or *Bam*HI for Sec22a. These gel-purified DNA fragments were subcloned into the compatible sites of the bacterial expression vector pGEX-KG (Guan and Dixon, 1991). The ligated DNA was transformed into DH5α cells and ampicillin-resistant colonies expressing the GST-fusion proteins were screened as described (Sambrook *et al.*, 1989). Purification of GST-sec22a and GST-Sec22b was performed as described previously (Lowe *et al.*, 1996; Zhang *et al.*, 1997).

Antibodies

For the preparation of polyclonal antibodies against Sec22b/ERS-24, GST-Sec22b (400 μg) emulsified in complete Freund's adjuvant was injected subcutaneously into local New Zealand white rabbits. Booster injections containing similar amounts of the antigen emulsified in incomplete Freund's adjuvant were performed after 2, 4, 6, 9, and 12 wk. Rabbits were bled 10 d after the second and subsequent booster injections. Affinity purification of specific antibodies was performed using the GST-Sec22b coupled to cyanogen bromide-activated Sepharose (3 mg/ml Sepharose bead). Affinity purification was carried out as described previously (Lowe *et al.*, 1996; Zhang *et al.*, 1997). Monoclonal antibody HFD9 against GS28 (also referred to as GOS-28) has been described previously (Subramaniam *et al.*, 1995, 1996; Nagahama *et al.*, 1996). Polyclonal antibodies against the IC marker p58 (Saraste *et al.*, 1987; Saraste and Svensson, 1991) were kindly provided by J. Saraste. Monoclonal antibody against ERGIC-53, the human counterpart of p58 (Schweizer *et al.*, 1988, 1990), was kindly provided by H.-P. Hauri. Polyclonal antibodies and monoclonal antibody against mammalian KDEL receptor (KDEL R) have been described previously (Tang *et al.*, 1993, 1995a). Monoclonal antibody against Golgi mannosidase II was purchased from Babco (Berkeley, CA). Rabbit antibodies against ribophorin I (Wiest *et al.*, 1990) were kindly provided by D.I. Meyer (University of California, Los Angeles, CA).

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described previously (Subramaniam *et al.*, 1995; Lowe *et al.*, 1996; Zhang *et al.*, 1997). Briefly, cells grown on coverslips were washed twice with PBSCM (PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and then fixed with 3% paraformaldehyde in PBSCM for 30 min at 4°C. Alternatively, cells were fixed with methanol for 5 min that had been chilled at −20°C. After sequential washing with PBSCM, 50 mM NH_4Cl in PBSCM, and PBSCM, cells were permeabilized with PBSCMS (PBSCM containing 0.1% saponin) for 20 min at room temperature. Incubation with primary antibodies (5–10 μg/ml) in

fluorescence dilution buffer (PBSCM with 5% normal goat serum, 5% FBS, and 2% BSA, pH 7.6) was performed for 1 h at room temperature. After washing three times with PBSCMS, cells were incubated with rhodamine- or FITC-conjugated secondary antibodies for 1 h at room temperature. Cells were then washed five times with PBSCMS, mounted with Vectastain (Vector Laboratories, Burlingame, CA), and then viewed. Confocal microscopy was performed using a Bio-Rad MRC1024 scan head (Bio-Rad, Richmond, CA) connected to an axiophot microscope (Carl Zeiss, Thornwood, NY) equipped with epifluorescence optics.

For temperature treatment of cells, NRK cells were incubated at 15°C for 3 h and then incubated at 37°C for 0, 5, 10, and 30 min before processing for immunofluorescence microscopy. Infection of cells with the ts045 strain of vesicular stomatitis virus (VSV) and the subsequent processing for immunofluorescence microscopy were performed as described previously (Tang *et al.*, 1997; Zhang *et al.*, 1997). For the treatment of cells with brefeldin A or nocodazole, cells grown on coverslips were incubated in the presence of brefeldin A (10 μg/ml) or nocodazole (10 μg/ml) for 1 h at 37°C, washed twice with ice-cold PBSCM, and then fixed in 3% paraformaldehyde. Fixed cells were then permeabilized and incubated with antibodies against Sec22b/ERS-24 and monoclonal antibodies against mannosidase II or the KDEL R for 1 h at room temperature. After washing three times with PBSCMS, cells were incubated with rhodamine-conjugated goat anti-mouse IgG (10 μg/ml) and FITC-conjugated sheep antirabbit (10 μg/ml) for 1 h at room temperature. After washing extensively, coverslips were then mounted as described above.

Immunoblot Analysis

Total membranes or fractionated membranes derived from rat liver (Subramaniam *et al.*, 1995) were extracted, and the extracts were separated by SDS-PAGE and transferred to a Hybond-C extra nitrocellulose filter (Zhang *et al.*, 1997). Immunoblot analysis was performed as described (Zhang *et al.*, 1997) to detect the respective antigens as indicated.

In Vitro ER–Golgi Transport

The ER-to-Golgi transport assay using semi-intact cells was performed as described previously (Beckers *et al.*, 1987; Balch *et al.*, 1994; Aridor *et al.*, 1995; Zhang *et al.*, 1997). Briefly, NRK cells were grown on 10-cm Petri dishes to form a confluent monolayer and infected with a temperature-sensitive strain of the vesicular stomatitis virus, VSVts045 at 32°C for 3–4 h. The cells were pulse-labeled with ^{35}S-methionine (100 μCi/ml) at the restrictive temperature (40°C) for 10 min and perforated on ice by hypotonic swelling and scraping. Alternatively, labeled cells were incubated at 15°C for 2 h to accumulate the labeled glycoprotein G of VSG (VSVG) in the pre-Golgi IC (Tang *et al.*, 1993, Aridor *et al.*, 1995), and the cells were then perforated. These semi-intact cells were then incubated in a complete assay cocktail of 40 μl containing (in final concentrations) 25 mM HEPES-KOH, pH 7.2, 90 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 μg of cytosol (Davidson and Balch, 1993), and 5 μl (25–30 μg of protein; 1–2 $\times$ 10^5) of semi-intact cells. Additional reagents were added as indicated in RESULTS. For a standard assay, samples were incubated for 90 min at 32°C and transport terminated by transferring to ice. The membranes were collected by a brief spin, solubilized in 60 μl of 0.2% SDS, 50 mM Na citrate (pH 5.5). After boiling for 5 min, the samples were digested overnight at 37°C in the presence of 2.5 U of endoglycosidase H (endo H), and the reaction was terminated by adding 5$\times$ concentrated gel sample buffer. The samples were analyzed on 7.5% SDS-polyacrylamide gels. The transport was quantified using PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For antibody inhibition of transport assay, Sec22b/ERS-24 antibodies were added into the complete assay cocktail and incubated on ice for 60 min to allow the antibodies to diffuse into semi-intact cells. In the case of the two- stage assay, after stage I incubation for 60 min at 32°C in a complete assay cocktail supplemented with 12.5 mM EGTA but without Ca^{++}, membranes were spun for 20 s at full speed in an Eppendorf table top centrifuge and subsequently resuspended in fresh assay cocktail with Ca^{++} by pipetting up and down 10 times with a yellow pipette tip. Additional reagents were added as indicated in RESULTS. Samples were incubated for 30 min at 32°C, and transport was terminated by transferring to ice.

Morphological Analysis of VSVG Transport in Digitonin-permeabilized Cells

This was performed essentially as described previously (Plutner *et al.*, 1992). Briefly, Vero cells were grown to confluency on 12-mm round coverslips. Cells were infected with VSV ts045 at 32°C for 1 h followed by an incubation at 40°C for 3 h. Cells were rapidly transferred to ice and washed immediately with ice-cold washing buffer (50 mM HEPES, pH 7.2, 90 mM KOAC, 2 mM MgOAc). Cells were permeabilized with digitonin (20 μg/ml in the washing buffer) on ice for 5 min. Coverslips were then transferred to 16-mm wells in washing buffer and incubated for 20 min on ice to release the cytosolic components. Coverslips were incubated in transport cocktail supplemented with rat liver cytosol in the presence or absence of indicated antibodies on ice for 1 h followed by an incubation at 32°C for 2 h. Transport was terminated by washing with ice-cold washing buffer and cells were fixed immediately by 3% paraformaldehyde at 4°C for 30 min.

RESULTS

Characterization of Sec22b/ERS-24 Antibodies

The predicted cytoplasmic domain (residues 1–195) of Sec22b/ERS-24 was expressed as a fusion protein to GST (GST-Sec22b) and was used to immunize rabbits. Sec22b/ERS-24–specific antibodies were affinity purified. When the postnuclear fraction of NRK cells was analyzed by immunoblot, a single protein of ~24 kDa was specifically detected by the antibodies (Figure 1A, lane 1). Detection of this 24-kDa protein was abolished by preincubation of the antibodies with GST-Sec22b (lane 3) but not by GST (lane 2) or GST-Sec22a (lane 4). These results suggest that antibodies against Sec22b do not cross-react with Sec22a. Since we have not been able to produce recombinant Sec22c, cross-reactivity of our antibodies with Sec22c could not be determined by this approach. As an alternative, we have found that in vitro translated Sec22c was not immunoprecipitated by Sec22b antibodies, while Sec22b was effectively immunoprecipitated under identical conditions (our unpublished results). These results establish that our antibodies are specific for Sec22b/ERS-24.

When total membranes (TM), microsomal membranes (MM), and Golgi/IC-enriched membranes (GM) derived from rat liver (Subramaniam *et al.*, 1995; Zhang *et al.*, 1997) were analyzed by immunoblot, it was found that Sec22b, like Golgi protein GS28 and IC-enriched proteins KDEL R and p58, is enriched in the GM fraction (Saraste and Svensson, 1991; Tang *et al.*, 1993; Subramaniam *et al.*, 1995; Zhang *et al.*, 1997). In marked contrast, ER membrane protein ribophorin

I (Wiest *et al.*, 1990) is enriched in the MM fraction (Figure 1B). These results suggest that endogenous Sec22b/ERS-24 is not enriched in the ER but rather in the Golgi/IC-enriched membrane fraction.

Association of Sec22b/ERS-24 with the pre-Golgi Intermediate Compartment

The subcellular localization of endogenous Sec22b/ERS-24 was further investigated in NRK cells by indirect immunofluorescence microscopy. As shown in Figure 2, Sec22b/ERS-24 is predominantly detected in vesicular structures located throughout the entire cytoplasm with a higher concentration around the perinuclear Golgi region (panel a). This labeling was totally abolished when the antibodies were preincubated with GST-Sec22b but not with GST-Sec22a (our unpublished results). The perinuclear labeling of Sec22b overlapped to a certain extent with that of Golgi mannosidase II (panels b and c) (Moreman and Robbins, 1991). When cells were treated with brefeldin A, which is known to have differential effects on proteins enriched in the IC as compared with resident Golgi proteins (Klausner *et al.*, 1992; Tang *et al.*, 1995a), Sec22b/ERS-24 was redistributed into spotty structures (panel d). Under the same condition, Golgi mannosidase II was redistributed into ER-like structures (panel e). Redistribution by brefeldin A into spotty structures is a characteristic of proteins associated with the IC, including ERGIC53/p58, the KDEL R, and rbet1 (Saraste and Svensson, 1991; Tang *et al.*, 1993, 1995b, Zhang *et al.*, 1997). When cells were treated with nocodazole, which is known to fragment the Golgi apparatus (Rogalski and Singer, 1984; Turner and Tartakoff, 1989), Sec22b/ERS-24 remained predominantly associated with cytoplasmic spotty structures, although the perinuclear labeling became less obvious (panel g). The Golgi apparatus marked by mannosidase II became fragmented into several patches (panel h). Under this condition, the majority of Sec22b/ERS-24–containing structures was devoid of Golgi mannosidase II labeling (panels g–i). Some Sec22b/ERS-24–containing spotty structures were seen in the vicinity of those marked by mannosidase II (panel i), although they are only partially colocalized, suggesting that this fraction of Sec22b/ERS-24 may be associated with a subregion of the fragmented Golgi apparatus that is not enriched in Golgi mannosidase II. These results suggest that while some Sec22b/ERS24 is associated with structures in the vicinity (most likely the *cis*-region) of the Golgi apparatus, a larger fraction of Sec22b/ERS-24 is associated with structures not marked by Golgi mannosidase II.

Since the response of Sec22b/ERS-24 to brefeldin A is characteristic of markers enriched in the IC, Sec22b/ERS-24 could be associated with the IC. To further confirm this point, we have performed double-labeling of Sec22b/ERS-24 with KDEL R, which has been shown by several studies to be enriched in the pre-Golgi IC (Tang *et al.*, 1993, 1995a,b; Griffiths *et al.*, 1994; Scales *et al.*, 1997; Zhang *et al.*, 1997). As shown in Figure 3, Sec22b/ERS24 is generally colocalized with KDEL R in control cells (panels a–c). When cells were treated with brefeldin A (panels d–f) and nocodazole (panels g–i), colocalization of Sec22b/ERS-24 with KDEL R became more prominent. These results suggest that Sec22b/ERS-24 is indeed enriched in the IC.

To provide additional evidence for the association of Sec22b/ERS-24 with the IC, we have tried double-labeling for Sec22b/ERS-24 and ERGIC-53, the latter being normally enriched in the IC and cycling preferentially between the IC and the ER (Schweizer *et al.* 1988, 1990; Kappeler *et al.*, 1997; Klumperman *et al.*, 1998). Since the

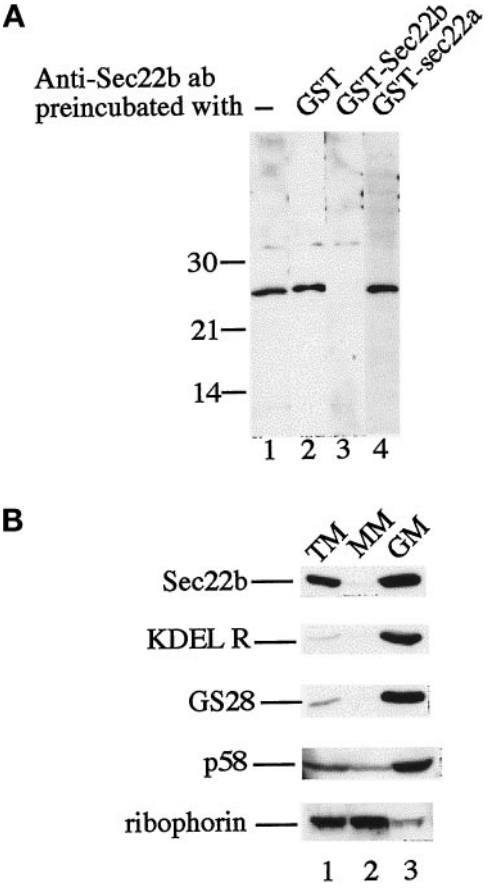

Figure 1. Sec22b/ERS-24 is a 24-kDa protein enriched in the membrane fraction of the Golgi and the IC. (A) The postnuclear total membrane fraction of NRK cells was resolved by SDS-PAGE and transferred to a filter. The filter was incubated with Sec22b/ERS-24 antibodies alone (lane 1), or in the presence of GST (lane 2), GST-Sec22b (lane 3), or GST-Sec22a (lane 4). (B) Total membranes (TM), microsomal membranes (MM), and Golgi/IC-enriched membranes (GM) derived from rat liver were analyzed by immunoblot to detect Sec22b/ERS-24, KDEL receptor (KDEL R), GS28, IC-enriched protein p58, and ER-enriched ribophorin I.

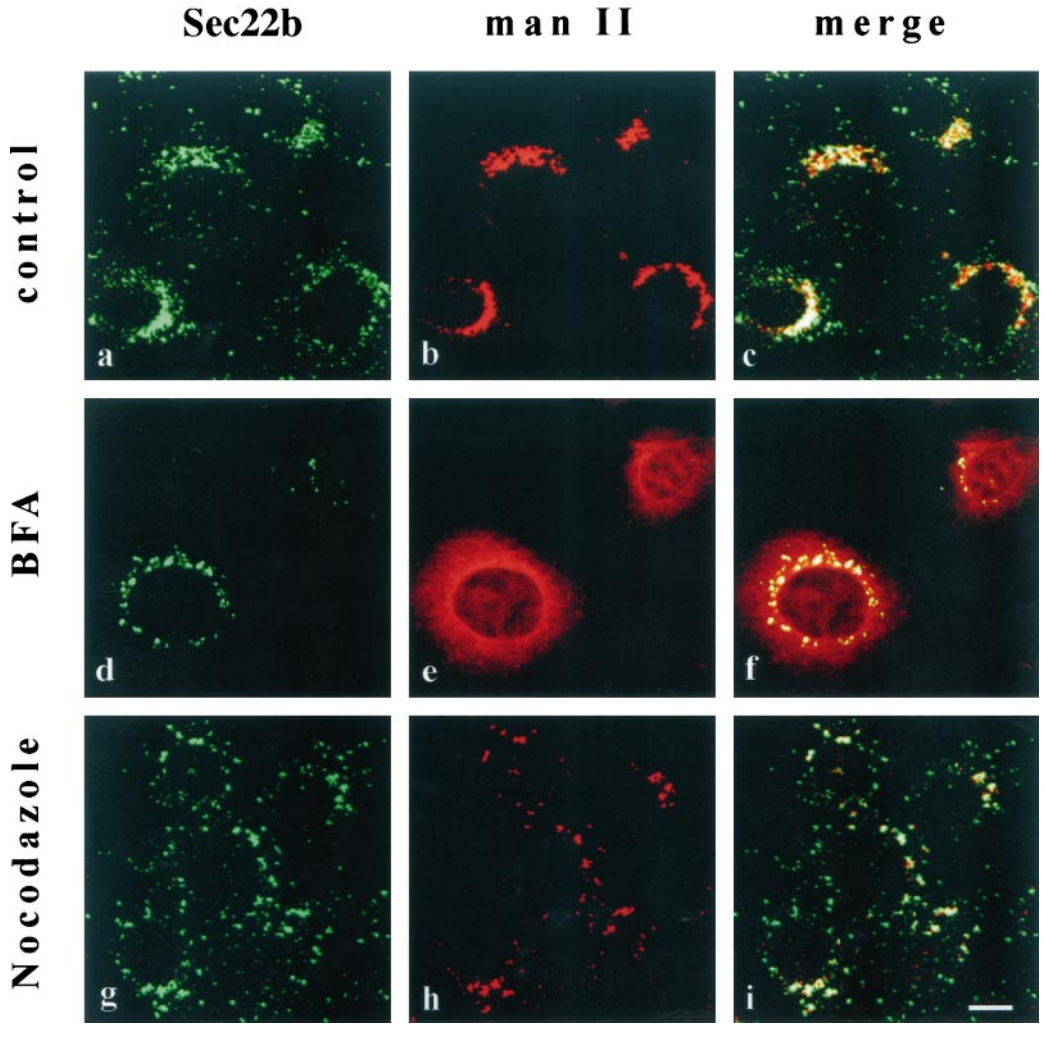

Figure 2. NRK cells under normal conditions (a and b), or treated with 10 μg/ml brefeldin A for 1 h (d and e), or treated with 10 μg/ml nocodazole for 1 h (g and h) were fixed and double-labeled for Sec22b/ERS-24 (a, d, and g) and Golgi mannosidase II (man II) (b, e, and h). The merged images are also shown (c, f, and i). Bar, 10 μm.

monoclonal antibody against ERGIC-53 works only in human or primate cells, we tried double-labeling experiments in Vero cells. However, we found that the monoclonal antibody detects ERGIC-53 only in methanol-fixed cells, while Sec22b/ERS-24 antibodies work only in paraformaldehyde-fixed cells, rendering it impossible to perform successful double-labeling of Sec22b/ERS-24 with ERGIC-53. Since we have both polyclonal as well as monoclonal antibodies against the KDEL R (Tang *et al.*, 1993, 1995a,b), we have provided indirect evidence for the colocalization of Sec22b/ERS-24 with ERGIC-53 (Figure 4). Using monoclonal antibody against the KDEL R (panel a) and polyclonal antibodies against Sec22b/ERS-24 (panel b) in paraformaldehyde-fixed Vero cells, Sec22b/ERS-24 and the KDEL R were perfectly colocalized (panel c). In Vero cells fixed with methanol, the monoclonal antibody against ERGIC-53 revealed labeling of vesicular structures that colocalized well with those marked by polyclonal antibodies against the KDEL R (panels d–f). In addition, some ER labeling was seen for ERGIC-53, consistent with previous observations (Schweizer *et al.* 1988, 1990; Tang *et al.*, 1995a,b). These results, taken together, suggest that the majority of Sec22b/ERS-24 is associated with the IC and behaves like other established proteins enriched in the IC, while some Sec22b/ERS-24 may be associated with the Golgi apparatus.

Dynamic Distribution of Sec22b/ERS-24 between the Peripheral and the peri-Golgi IC

Proteins enriched in the IC are dynamically distributed between the peripheral structures and structures around the Golgi apparatus (peri-Golgi), and cargo

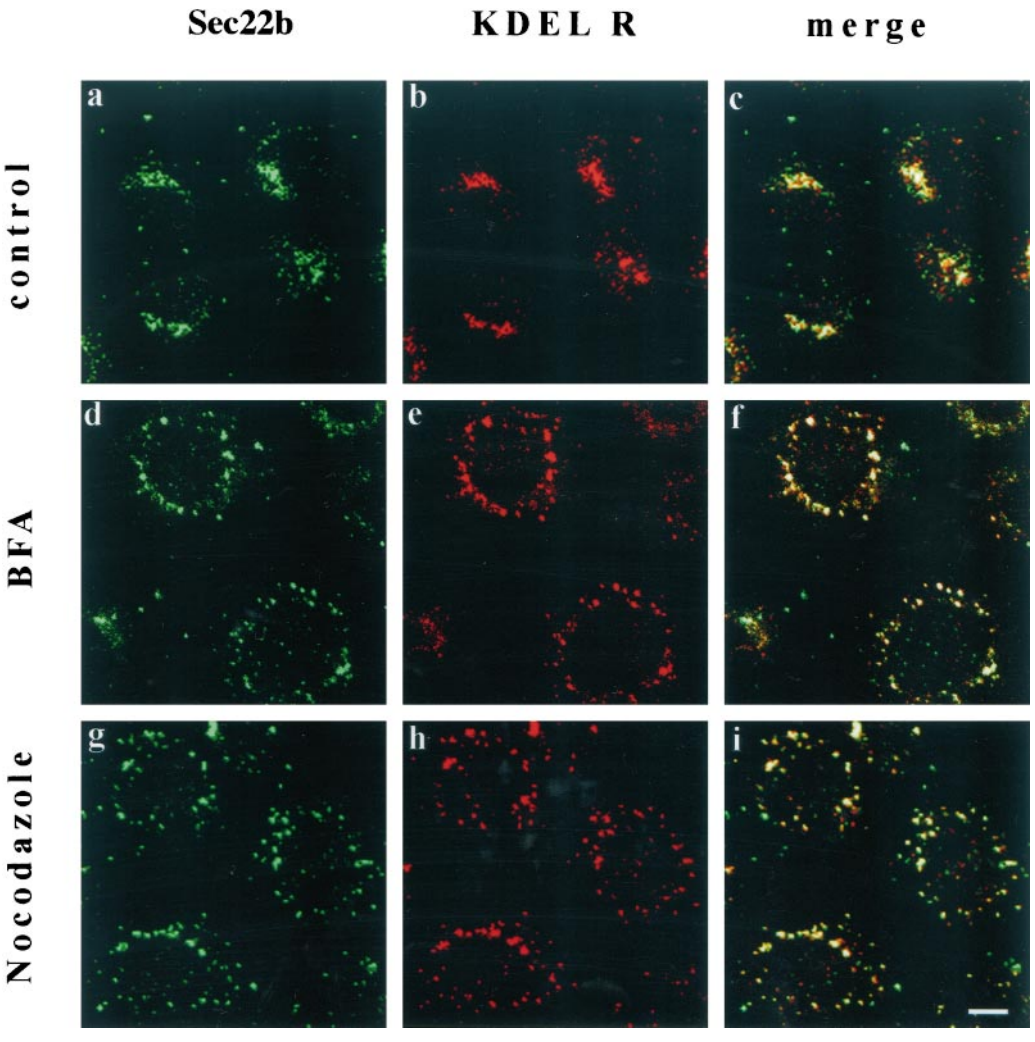

Figure 3. NRK cells under normal conditions (a and b), or treated with 10 μg/ml brefeldin A for 1 h (d and e), or treated with 10 μg/ml nocodazole for 1 h (g and h) were fixed and double-labeled for Sec22b/ERS-24 (a, d, and g) and KDEL R (b, e, and h). The merged images are also shown (c, f, and i). Bar, 10 μm.

proteins in the ER are transported to the Golgi apparatus via migration/maturation of peripheral IC to the peri-Golgi region or directly from the peri-Golgi IC (Saraste and Svensson, 1991; Lotti *et al.*, 1992; Plutner *et al.*, 1992; Lippincott-Schwartz, 1993; Tang *et al.*, 1993, 1995b; Balch *et al.*, 1994; Presley *et al.*, 1997; Scales *et al.*, 1997; Tisdale *et al.*, 1997). Incubation of cells at 15°C is known to block transport from the ER to the Golgi at the level of the IC and also causes an increase in the concentration of IC proteins in the peripheral structures (Saraste and Svensson, 1991; Lotti *et al.*, 1992; Plutner *et al.*, 1992; Lippincott-Schwartz, 1993; Oprins *et al.*, 1993; Tang *et al.*, 1993, 1995a; Balch *et al.*, 1994; Aridor *et al.*, 1995). When cells were preincubated at 15°C, Sec22b/ERS-24 and KDEL R were colocalized in the same vesicular structures (Figure 5, a–c). When 15°C-arrested cells were warmed up to 37°C for 5–30 min, Sec22b/ERS-24 and the KDEL R were seen to shift to the peri-Golgi region (d–l). These results suggest that, like other IC proteins, Sec22b/ERS-24 is dynamically distributed between the peripheral and peri-Golgi IC.

The association of Sec22b/ERS-24 with the peripheral and peri-Golgi IC was further established by colocalization of Sec22b/ERS-24 with VSVG en route from the ER to the Golgi apparatus via the peripheral and peri-Golgi IC (Figure 6). NRK cells were infected with the ts045 strain of VSV, the VSVG of which is mutated in such a way that it cannot exit from the ER at the restricted temperature (40°C). The infected cells were maintained at 40°C to restrict VSVG to the ER. Cells were then processed for double labeling for Sec22b/ERS-24 and VSVG. As shown in Figure 6a, VSVG (b) is mainly distributed in the ER with a sig-

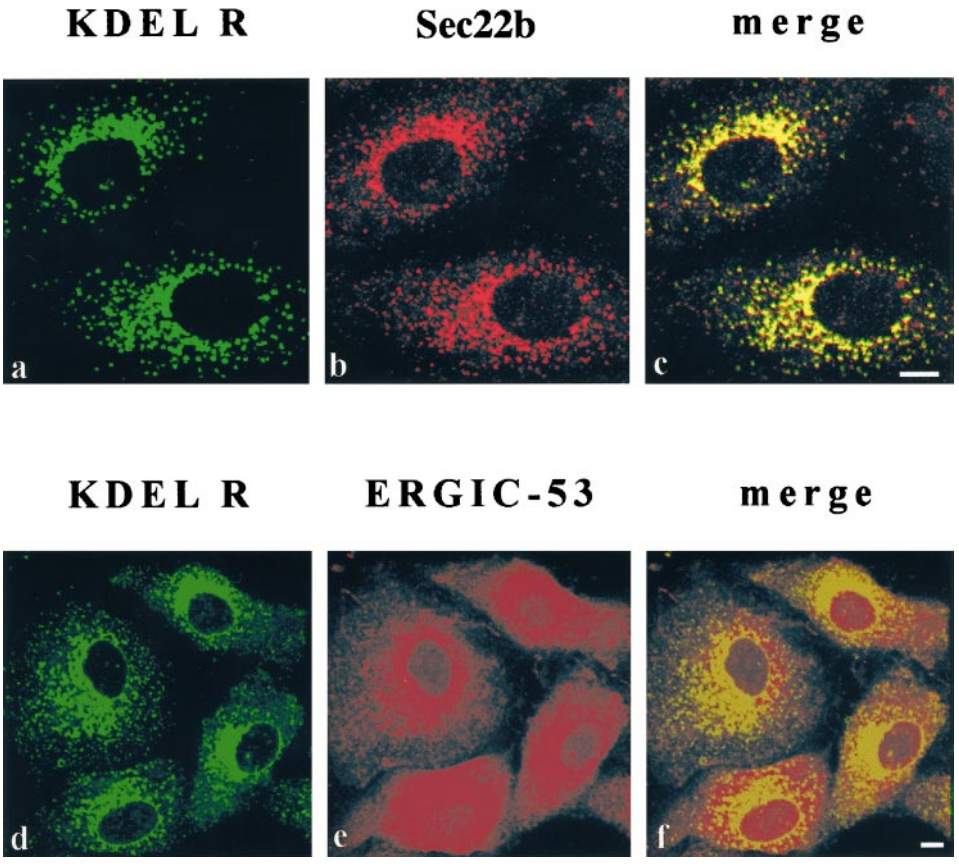

Figure 4. Double labeling with rabbit antibodies against Sec22b/ERS-24 with monoclonal antibodies against KDEL R in paraformaldyhyde-fixed Vero cells (a and b), or rabbit antibodies against KDEL R and monoclonal antibodies against ERGIC-53 in methanol-fixed Vero cells (d and e). The merged images are also shown (c and f). Bar, 10 μm.

nificant amount concentrated in the perinuclear Golgi region, and this may be due to the possibility that VSVG is concentrated in the ER budding sites in the Golgi region. Sec22b/ERS-24 is seen to be associated in the same region (a) with significant colocalization (c). When cells were incubated at 32°C for 5 min to allow export of VSVG from the ER, the majority of VSVG (e) and Sec22b/ERS-24 (d) were colocalized in vesicular tubular structures scattered throughout the entire cytoplasm (f). After incubation at 32°C for 15 min, Sec22b/ERS-24 (g) and VSVG (h) were colocalized in more defined vesicular structures that are enriched in the peri-Golgi region (i). After 30 min of incubation at 32°C, the majority of VSVG had been transported to the Golgi (k), and a significant amount of Sec22b/ERS-24 (i) was colocalized with VSVG in the same region (l). These results suggest that Sec22b/ERS-24 is colocalized with VSVG in the ER-budding sites, peripheral IC, peri-Golgi IC, and the Golgi during VSVG transport from the ER to the Golgi and that Sec22b/ERS-24 is thus a constituent of transport intermediates involved in ER–Golgi transport.

Antibodies against Sec22b/ERS-24 Inhibit ER–Golgi Transport

The enrichment of Sec22b/ERS-24 in the IC and the observation that Sec22b/ERS-24 colocalizes with a cargo protein en route from the ER to the Golgi suggest that Sec22b/ERS-24 may be involved in protein transport from the ER to the Golgi apparatus in mammalian cells. To establish this point, we have examined whether protein transport from the ER to the Golgi can be inhibited by antibodies against Sec22b/ERS-24. The well-established in vitro ER–Golgi transport system using VSV ts045-infected NRK cells (Beckers *et al.*, 1987; Balch *et al.*, 1994) was used. Infected NRK cells were pulse labeled with ^{35}S-Met at 40°C so that the labeled VSVG was restricted to the ER. The plasma membrane was then selectively perforated and the cells depleted of endogenous cytosol. Transport of VSVG from the ER to the Golgi can be reconstituted when such semi-intact cells are incubated at a permissive temperature (32°C) and supplemented with exogenous cytosol (such as rat liver cytosol, rlc) and an ATP-regenerating system. ER–Golgi transport was

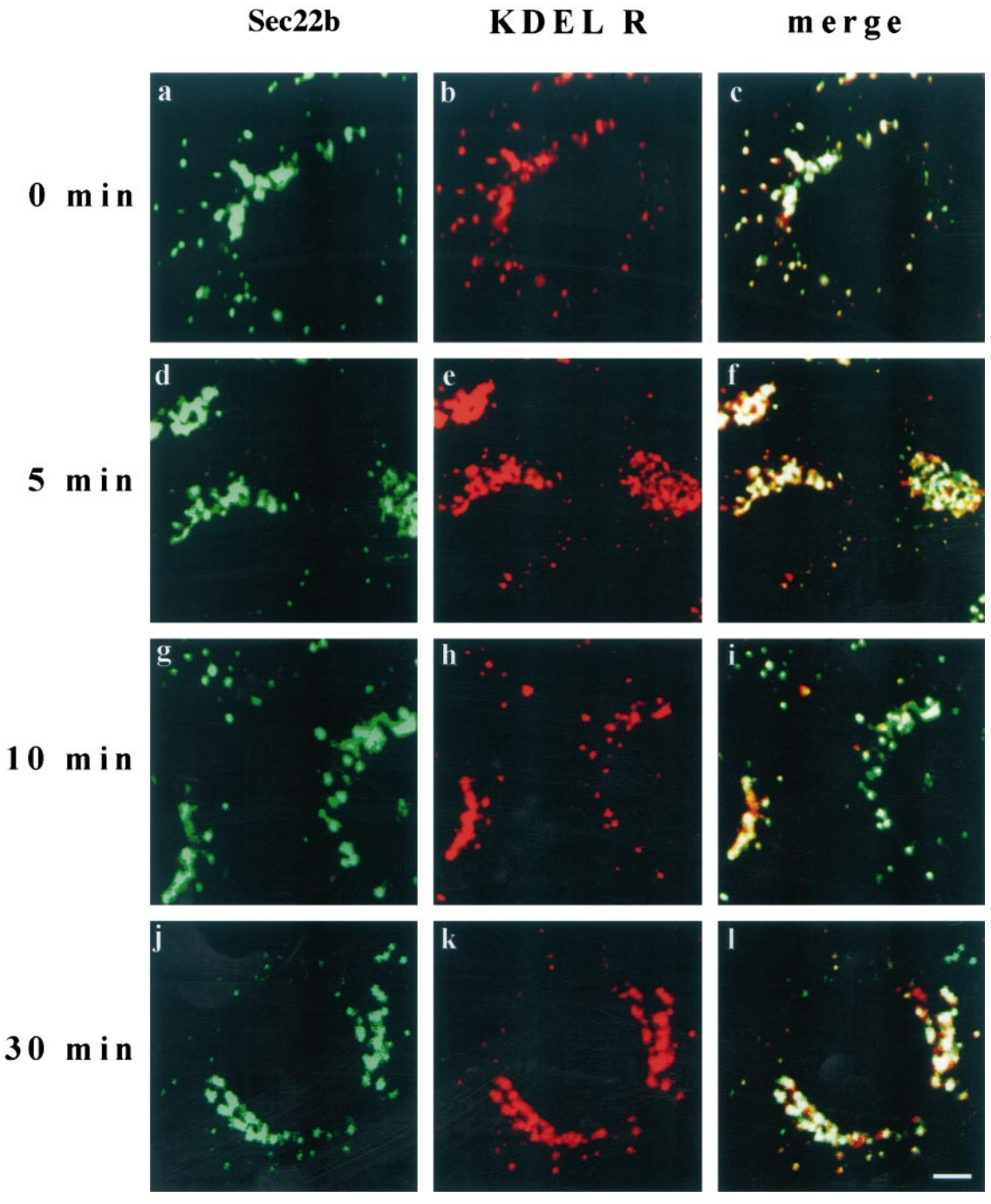

Figure 5. NRK cells were incubated at 15°C for 3 h and then incubated at 37°C for the indicated time. The cells were fixed and double labeled for Sec22b/ERS-24 (a, d, g, and j) and KDEL R (b, e, h, and k). The merged images are also shown (c, f, i, and l). Bar, 10 μm.

measured by following the extent of conversion of ER-restricted endo H-sensitive VSVG into the endo H-resistant Golgi form. As shown in Figure 7A, no transport was detected when semi-intact cells were incubated on ice (lane 1). The majority of VSVG was converted into endo H-resistant Golgi form when incubated at 32°C (lane 2). Transport of VSVG from the ER to the Golgi was however inhibited by antibodies against Sec22b/ERS-24 in a dose-dependent manner (lanes 3–8). Inhibition was not so obvious when only 0.3–0.5 μg of antibodies were added (lanes 3–4). However, about 60% of the VSVG remained in the ER form

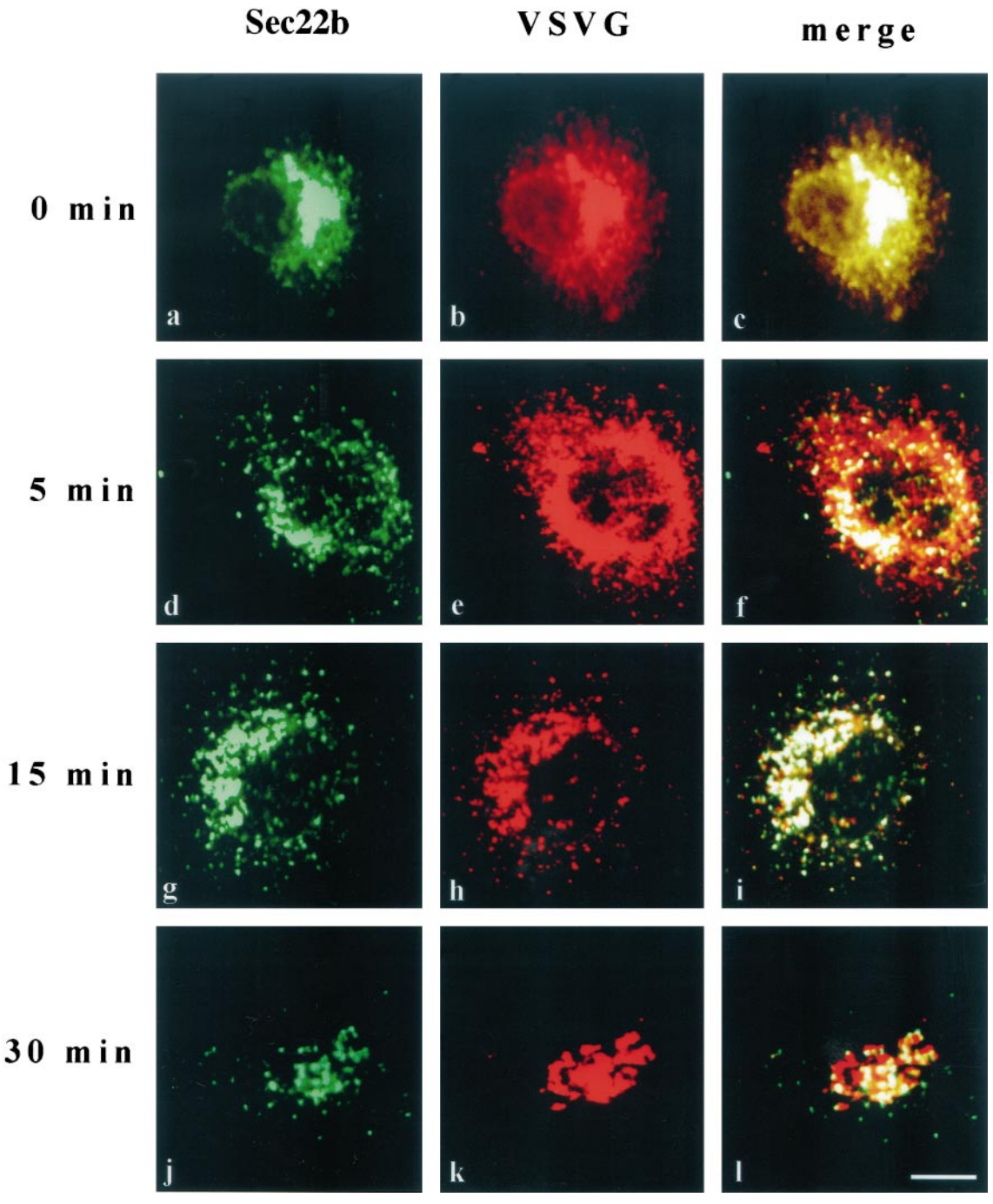

Figure 6. Vero cells were infected with VSV ts045 and incubated at 40°C for 4 h. Cells were then shifted to 32°C for the indicated time, fixed, and double labeled for Sec22b/ERS-24 (a, d, g, and j) and the envelope protein of VSV (VSVG) (b, e, h, and k). The merged images (c, f, i, and l) are also shown. Bar, 10 μm.

when 1 μg of antibodies was added (lane 5). Transport of VSVG was almost completely inhibited when 3 μg (lane 6) or more (lanes 7–8) of antibodies were added. The inhibition was specific because the same amount of heat-denatured antibodies had no effect on transport (compare lane 4 with lane 3 of Figure 7B). Comparable amounts of antibodies against the KDEL R (Tang *et al.*, 1997) and several other control antibodies had no effect on ER–Golgi transport of VSVG (our unpublished results; also see Subramaniam *et al.*,

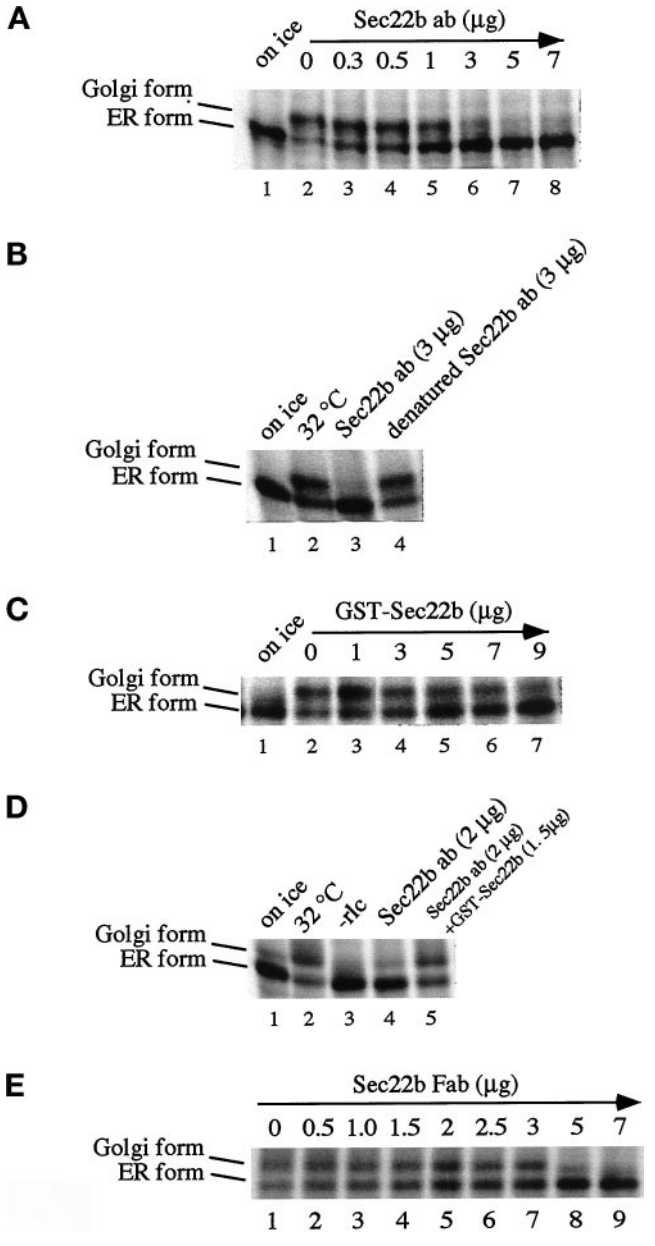

Figure 7. Sec22b/ERS-24 antibodies specifically inhibit in vitro ER–Golgi transport. (A) In vitro ER–Golgi transport was performed either on ice (lane 1) or at 32°C (lanes 2–8) supplemented with the indicated amounts of Sec22b/ERS-24 antibodies. (B) In vitro transport was performed either on ice (lane 1) or at 32°C (lanes 2–4) supplemented with 3 μg of Sec22b/ERS-24 antibodies (lane 3) or heat-inactivated antibodies (lane 4). (C) In vitro transport was performed either on ice (lane 1) or at 32°C (lanes 2–7) supplemented with indicated amounts of GST-Sec22b. (D) In vitro transport was performed either on ice (lane 1) or at 32°C (lanes 2–5) in the absence (lane 3) or the presence of rat liver cytosol (rlc) (lanes 1, 2, 4, and 5) supplemented with 2 μg of Sec22b antibodies (lane 4), or 2 μg of Sec22b antibodies and 1.5 μg of GST-Sec22b (lane 5). (E) In vitro transport was performed at 32°C in the absence (lane 1) or the presence of indicated amounts of the Fab fragment of Sec22b antibodies (lanes 2–9).

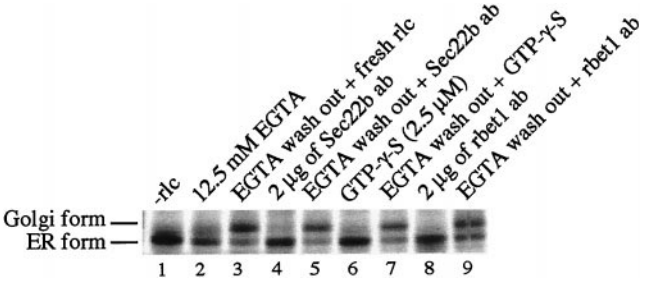

Figure 8. Sec22b/ERS-24 antibodies must be present before the EGTA-sensitive stage to exhibit the inhibitory effect on in vitro ER–Golgi transport. In vitro ER–Golgi transport was performed at 32°C in the absence (lane 1) or presence of rat liver cytosol (rlc) (lanes 2–9). For lanes 3, 5, 7, and 9, transport assay was first performed in the presence of 12.5 mM EGTA to arrest the transport at the EGTA-sensitive stage followed by a washing step and second incubation at 32°C to continue the transport. Reagents were supplemented as indicated.

1996). Importantly, VSVG transport from the ER to the Golgi was also inhibited by recombinant GST-Sec22b in a dose-dependent manner (Figure 7C). A clear inhibition by GST-Sec22b could be seen when 5 μg (lane 5) or more (lanes 6–7) of the recombinant protein were added to the transport assay. Furthermore, inhibition exhibited by antibodies against Sec22b/ERS-24 could be neutralized by noninhibitory amounts of GST-Sec22b (Figure 7D). VSVG transport to the Golgi was almost completely inhibited by 2 μg of Sec22b/ERS-24 antibodies (lane 4). However, preincubation of Sec22b/ERS-24 antibodies with 1.5 μg (lane 5) of GST-Sec22b resulted in ~50% of VSVG being converted into the Golgi form. To rule out the possibility that the observed inhibition of in vitro ER–Golgi transport by Sec22b/ERS-24 antibodies is due to cross-linking of Sec22b/ERS-24 on the membrane, we have also examined the effect of the Fab fragment of Sec22b antibodies on the in vitro ER–Golgi transport (Figure 7E). As shown, significant inhibition of in vitro ER–Golgi transport could be seen when 3 μg of the Fab fragment was included in the transport assay (lane 7), and transport was completely inhibited when 5 μg or more of the Fab fragment was included (lanes 8–9). These results suggest that inhibition of ER–Golgi transport by Sec22b/ERS-24 antibodies occurs by specific association with endogenous Sec22b/ERS-24, and that the inhibition is not due to cross-linking of Sec22b/ERS-24 on the membrane.

Sec22b/ERS-24 Antibodies Must Be Present before the EGTA-sensitive Stage to Achieve Inhibition

In vitro ER–Golgi transport can be inhibited by EGTA at a stage after docking of transport intermediates but before the actual fusion event (Rexach and Schekman, 1991; Balch *et al.*, 1994; Pind *et al.*, 1994; Aridor *et al.*,

1995; Lupashin *et al.*, 1996; Subramaniam *et al.*, 1996). We have found that Sec22b/ERS-24 antibodies must be added before the EGTA-sensitive stage to exhibit an inhibitory effect (Figure 8). In this experiment, in vitro ER–Golgi transport was first performed in the presence of EGTA to arrest transport at the EGTA-sensitive stage. Semi-intact cells were then washed, resuspended in complete transport cocktail, and reincubated to resume the second stage of transport involving events between the EGTA-sensitive stage to the actual membrane fusion. As shown, VSVG remained in the endo H-sensitive ER form after the first stage of incubation (lane 2). A second incubation in fresh cytosol and complete transport cocktail allowed almost complete conversion of the EGTA-arrested ER form into the endo H-resistant Golgi form (lane 3). Inclusion of 2 μg of Sec22b/ERS-24 antibodies in standard transport assay inhibited the transport to the background level (lane 4). However, when Sec22b/ERS-24 antibodies were included only during the second stage of incubation, almost complete transport was achieved (lane 5), suggesting that Sec22b/ERS-24 antibodies could no longer inhibit the transport when included only in the second stage of the transport assay. As shown previously (Aridor *et al.*, 1995), GTP-γ-S (lanes 6–7) and rbet1 antibodies (Zhang *et al.*, 1997) (lanes 8–9) were also no longer inhibitory to ER–Golgi transport when included only at the second stage of incubation. These results suggest that either Sec22b/ERS-24 antibodies cannot gain access to Sec22b/ERS-24 or that Sec22b/ERS-24 is no longer required after the EGTA-sensitive stage.

Inhibition of ER–Golgi Transport at the Intermediate Compartment Stage by Sec22b/ERS-24 Antibodies

VSVG transport from the ER to the Golgi can also be followed morphologically in digitonin-permeabilized Vero cells (Plutner *et al.*, 1992; Balch *et al.*, 1994; Aridor *et al.*, 1995). Previous studies have shown that VSVG is transported from the ER to the Golgi via small vesicular structures distributed throughout the cytoplasm. These structures correspond to the peripheral IC because they are enriched in IC markers such as ERGIC-53/p58 (Plutner *et al.*, 1992; Balch *et al.*, 1994; Aridor *et al.*, 1995). VSVG was restricted to the ER in the digitonin-permeabilized cells (Figure 9A, panel a). When incubated in the absence of cytosol (w/o rlc), VSVG was similarly distributed in the ER (panel b). VSVG was transported to the Golgi apparatus when cells were incubated in complete transport cocktail (panel c). Transport of VSVG to the Golgi was inhibited by GTPγS (panels d and e) and EGTA (panel f). VSVG was arrested by GTPγS in numerous fine dotted structures that could correspond to budding vesicles. In the presence of EGTA, the majority of VSVG was seen in larger vesicular structures in the Golgi region with some in smaller vesicular structures at the periphery, consistent with the known effect of EGTA on in vitro ER–Golgi transport (Rexach and Schekman, 1991; Balch *et al.*, 1994; Pind *et al.*, 1994; Aridor *et al.*, 1995; Lupashin *et al.*, 1996; Subramaniam *et al.*, 1996). rbet1 has been shown to be enriched in the IC and involved in ER–Golgi transport (Zhang *et al.* 1997). When the transport assay was performed in the presence of antibodies against rbet1, VSVG was accumulated in both peri-Golgi large vesicular as well as small peripheral vesicular structures (Figure 9B, panels a–c), which are also labeled with antibodies against rbet1. The effect of rbet1 antibodies is comparable to that of EGTA. Interestingly, when cells were incubated in the presence of Sec22b/ERS-24 antibodies, VSVG was primarily located in small vesicular structures that were scattered evenly in the entire cytoplasm, and these structures also contained Sec22b/ERS-24. The larger vesicular structures in the peri-Golgi region that accumulated in cells incubated with EGTA or rbet1 antibodies were not obvious in the presence of Sec22b/ERS-24 antibodies. Since peripheral vesicular structures are known to mature and migrate into the larger peri-Golgi structures, these results indicate that Sec22b/ERS-24 may play a role in maturation/migration of peripheral IC into the peri-Golgi IC.

To gain additional insight of the structures in which VSVG was accumulated in the presence of rbet1 or Sec22b antibodies, we have performed double labeling of rbet1 and Sec22b/ERS-24 marked by the respective inhibitory antibodies with GS28 (Subramaniam *et al.*, 1995, 1996) or ERGIC-53 (Schweizer *et al.*, 1988, 1990) (Figure 10). As shown in Figure 10A, panel a, the majority of rbet1 is associated with perinuclear vesicular structures that colocalized well with GS28 (Figure 10A, panels b and c), suggesting that rbet1 and therefore VSVG have been transported to the Golgi region in the presence of rbet1 antibodies. In marked contrast, the majority of Sec22b is still associated with peripheral vesicular structures (Figure 10A, panel d). These structures have not been transported to the Golgi region as they are not colocalized with GS28 (Figure 10A panels e and f) in the presence of Sec22b/ERS-24 antibodies, suggesting that Sec22b/ERS-24 and therefore VSVG are associated with peripheral vesicular structures, and these structures fail to be transported to the Golgi region. ERGIC-53 is known to cycle primarily between the ER and the intermediate compartment (Schweizer *et al.*, 1988, 1990; Tang *et al.*, 1995b; Kappeler *et al.*, 1997; Klumperman *et al.*, 1998). We have also performed double labeling of rbet1 and Sec22b/ERS-24 marked by the respective inhibitory antibodies with ERGIC-53 in the morphological transport assay. As shown in Figure 10B, panel b, ERGIC-53 was distributed both in the ER and some perinuclear spotty structures, and the perinuclear structures colo-

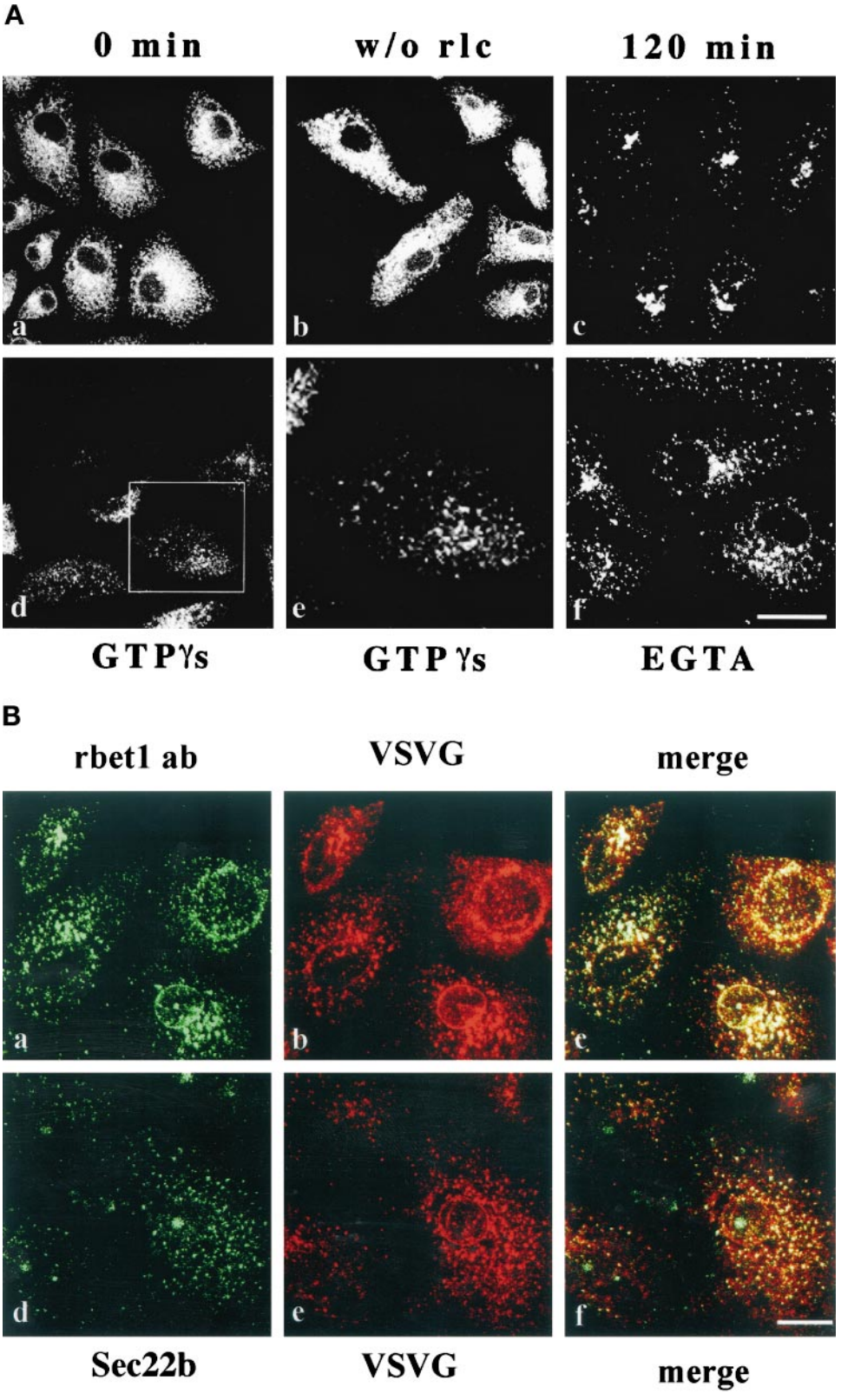
A
0 min
w/o rlc
120 min
a
b
c
d
e
f
GTPγs
GTPγs
EGTA
B
rbet1 ab
VSVG
merge
a
b
c
d
e
f
Sec22b
VSVG
merge

Figure 9.

calized only partially with that of rbet1 (Figure 10B, panels a and c). This suggests that segregation of ERGIC-53 from anterograde cargo in the IC (Aridor *et al.*, 1995; Tang *et al.*, 1995b) and recycling of ERGIC-53 back to the ER are not affected by the presence of rbet1 antibodies. However, in the presence of Sec22b antibodies, ERGIC-53 (Figure 10B, panel e) is primarily associated vesicular structures that colocalized well with Sec22b (Figure 10B, panels d and f), suggesting that segregation of ERGIC-53 from anterograde cargo (such as VSVG) in the IC, recycling of ERGIC-53 from the IC back to the ER, and/or mobilization of peripheral transport intermediates to the Golgi region are inhibited by Sec22b antibodies. These results thus strengthen the possibility that Sec22b/ERS-24 but not rbet1 may participate in the maturation/migration of peripheral IC into the peri-Golgi IC.

Inhibition of VSVG Transport from the 15°C-arrested Structures to the Golgi Requires More Sec22b/ERS-24 Antibodies

VSVG is accumulated in the IC when cells are incubated at 15°C (Saraste and Kuismanen, 1984; Bonatti *et al.*, 1989; Saraste and Svensson, 1991; Lotti *et al.*, 1992; Plutner *et al.*, 1992; Lippincott-Schwartz, 1993; Oprins *et al.*, 1993; Tang *et al.*, 1993, 1995b; Balch *et al.*, 1994; Aridor *et al.*, 1995). Transport of the 15°C-arrested VSVG from the IC to the Golgi in semi-intact cells occurs rapidly (Aridor *et al.*, 1995). As shown in Figure 11A, VSVG transport in the standard assay from the ER to the Golgi occurred only after a lag period of about 20–25 min. However, transport of 15°C-arrested VSVG from the IC to the Golgi occurred essentially without a lag period, and similar levels of transport were generally achieved about 20–25 min earlier than the standard assay. As shown in Figure 11B, transport of 15°C-arrested VSVG from the IC to the Golgi was dependent on cytosol (lane 3) and energy (lane 4). This transport could be inhibited by GTPγS (lane 5) and EGTA (lane 6). The majority of VSVG was arrested in the ER form by about 2 μg of Sec22b/ERS-24 antibodies in the standard assay (Figure 7D, lane 4 and Figure 11D, lane 3). In contrast, transport of the 15°C-arrested VSVG from the IC to the Golgi was essentially unaffected by the same amount of Sec22b/ERS-24 antibodies (Figure 11C, lane 3). However, transport of the 15°C arrested VSVG from the IC to the Golgi could be completely inhibited by higher doses of Sec22b/ERS-24 antibodies (Figure 11C, lanes 4 and 5). These results suggest that transport of 15°C-arrested VSVG from the IC to the Golgi still requires Sec22b/ERS-24 and imply that Sec22b/ERS-24 at this stage may be in a different conformation or in a protein complex so that it is less accessible to its antibodies. Although transport of the 15°C-arrested VSVG from the IC to the Golgi was also inhibited by antibodies against rbet1 or syntaxin 5, the inhibitory amounts of these antibodies were similar to that which inhibited VSVG transport from the ER to the Golgi in the standard assay (our unpublished results). Among the three SNAREs (rbet1, syntaxin 5, and Sec22b/ERS-24) known to participate in ER–Golgi transport, Sec22b/ERS-24 is unique in that it becomes less accessible to its antibodies in transport of the 15°C-arrested VSVG from the IC to the Golgi.

DISCUSSION

Association of Sec22b/ERS-24 with the IC

It was previously concluded that Sec22b/ERS-24 is primarily associated with the ER based on localization studies of the epitope-tagged forms of this protein (Hay *et al.*, 1997; Paek *et al.*, 1997). Using highly specific antibodies against Sec22b/ERS-24, we have investigated in detail the subcellular localization of endogenous Sec22b/ERS-24 in NRK and Vero cells. Our results suggest that Sec22b/ERS-24 is enriched in the IC and behaves like other proteins known to be enriched in the IC, such as ERGIC-53/p58 (Schweizer *et al.* 1988, 1990; Kappeler *et al.*, 1997), KDEL R (Tang *et al.*, 1993, 1995a,b; Griffiths *et al.*, 1994; Scales *et al.*, 1997), and rbet1 (Zhang *et al.*, 1997). This conclusion is based on several lines of evidence. First, unlike ribophorin I, which is enriched in the ER-enriched microsomal fraction as assessed by immunoblot analysis, Sec22b/ERS-24 is preferentially present at high levels in a membrane fraction enriched for membranes derived from the Golgi apparatus and the IC. Second, indirect immunofluorescence labeling with antibodies against Sec22b/ERS-24 revealed that Sec22b/ERS-24 is distributed in vesicular structures scattering throughout the entire cell but more enriched in the Golgi region. Labeling of the ER was not detected. When cells are treated with nocodazole to fragment the Golgi apparatus, the majority of Sec22b/ERS-24 is present in vesicular structures devoid of Golgi mannosidase II. These Sec22b/ERS-24-containing structures are marked by KDEL R, which has been shown

Figure 9 (facing page). (A) VSV ts045-infected Vero cells grown on coverslips were digitonin permeabilized. Cells were either kept on ice (a) or at 32°C (b–f) for 120 min in transport buffer without (b) or with (a, c–f) rlc. Other reagents were added as indicated. Cells were fixed and labeled with antibodies against VSVG. The square box in panel d is enlarged and shown as panel e. (B) VSV ts045-infected Vero cells grown on coverslips were digitonin permeabilized and incubated for 120 min at 32°C in complete transport cocktail buffer supplemented with either rbet1 (a–c) or Sec22b/ERS-24 (d–f) antibodies. The inhibitory antibodies (panels a and d) and VSVG (panels b and e) were visualized by indirect immunofluorescence microscopy. The merged images are also shown (c and f). Bars, 10 μm.

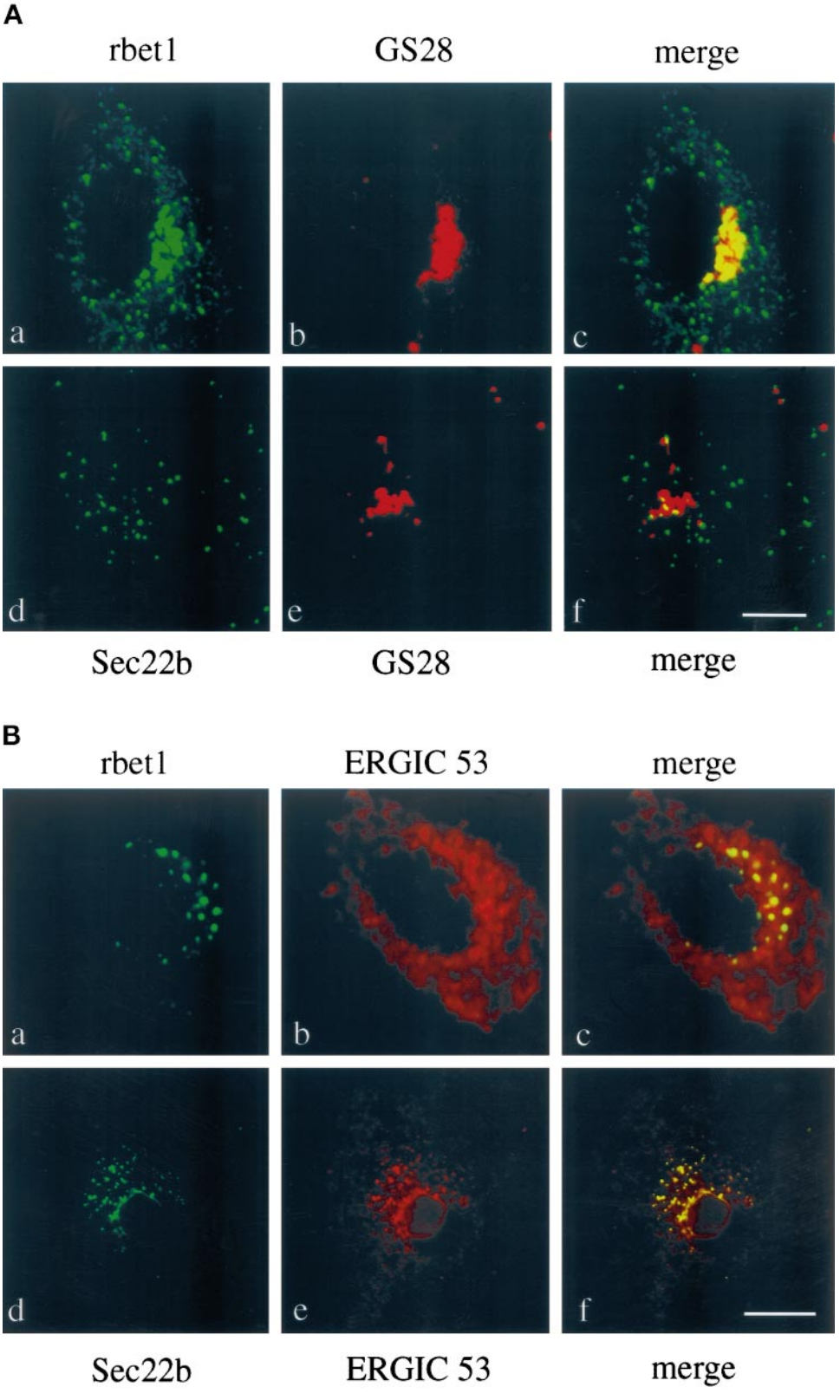
A
rbet1
GS28
merge
a
b
c
d
e
f
Sec22b
GS28
merge
B
rbet1
ERGIC 53
merge
a
b
c
d
e
f
Sec22b
ERGIC 53
merge

Figure 10.

to be enriched in the IC by several studies, suggesting that the majority of Sec22b/ERS-24 segregates together with the IC rather than the Golgi when cells are treated with nocodazole. Brefeldin A is known to have differential effects on markers of the Golgi apparatus and the IC (Klausner *et al.*, 1992; Tang *et al.*, 1995a). When cells are treated with brefeldin A, markers of the Golgi apparatus are redistributed into the ER, while markers such as ERGIC-53/p58, KDEL R, and rbet1 are redistributed into distinct vesicular structures. Unlike Golgi mannosidase II, which is redistributed into the ER by brefeldin A, Sec22b/ERS-24 is redistributed into distinct vesicular structures that colocalize well with the KDEL R, suggesting that Sec22b/ERS-24 behaves like a marker of the IC when cells were treated with brefeldin A. Third, Sec22b/ERS-24 colocalizes well with VSVG en route from the ER to the Golgi apparatus, suggesting that Sec22b/ERS-24 is a constituent of ER–Golgi transport intermediates. When ER–Golgi transport of VSVG is inhibited by antibodies against Sec22b/ERS-24, VSVG is accumulated in distinct pre-Golgi vesicular structures, and the majority of Sec22b/ERS-24 is similarly associated with these structures, further supporting this notion. These results, taken together, suggest that the majority of Sec22b/ERS-24 is associated with the pre-Golgi IC, which functions as an intermediate in ER–Golgi transport. A recent study has shown that Sec22b/ERS-24 is associated with ER-derived COPII-coated transport vesicles (Rowe *et al.*, 1998). Although this study did not address the subcellular localization of the majority of Sec22b/ERS-24 in the cell, the demonstration of its association with transport vesicles involved in ER–Golgi transport complements our study.

A Role for Sec22b/ERS-24 in ER–Golgi Transport

The amino acid sequence homology of Sec22a, Sec22b/ERS-24, and Sec22c with yeast Sec22p suggests that one or more of these mammalian proteins may function as the mammalian equivalent of Sec22p in ER–Golgi transport. The enrichment of Sec22b/ERS-24 in the IC suggests that it may play a role in ER–Golgi transport. Several other lines of evidence suggest that Sec22b/ERS-24 is indeed important for ER–Golgi transport. First, VSVG transport in semi-intact cells is inhibited by antibodies against Sec22b/ERS-24 in a dose-dependent manner. This inhibition is specific because the same amount of antibodies denatured by boiling does not inhibit ER–Golgi transport of VSVG. Furthermore, the inhibitory effect can be neutralized by preincubation of antibodies with non-inhibitory amounts of recombinant GST-Sec22b. Second, recombinant GST-Sec22b also exhibites dose-dependent inhibition of ER–Golgi transport of VSVG, although GST-Sec22b is not as potent as its antibodies. The lower potency of GST-Sec22b is a possibility due to only a fraction of the recombinant protein being in a properly folded state. Furthermore, in vitro ER–Golgi transport was inhibited by the Fab fragment of Sec22b antibodies in a dose-dependent manner. Sec22b/ERS-24 has been recently shown to exist in a SNARE complex that also contains rbet1, syntaxin 5, and GS28/GOS-28 (Hay *et al.*, 1997; Paek *et al.*, 1997). Since rbet1, syntaxin 5, and GS28/GOS-28 have been shown to play a role in ER–Golgi transport (Dascher *et al.*, 1994; Subramaniam *et al.*, 1996; Zhang *et al.*, 1997; Rowe *et al.*, 1998), the association of Sec22b/ERS-24 with these SNAREs supports a role for Sec22b/ERS-24 in ER–Golgi transport.

Additional understanding of the involvement of Sec22b/ERS-24 in ER–Golgi transport was revealed by the demonstration that Sec22b/ERS-24 antibodies must be present before the EGTA-sensitive stage to achieve an inhibition. Since EGTA inhibits ER–Golgi transport after docking of transport intermediates but before the actual membrane fusion event (Rexach and Schekman, 1991; Balch *et al.*, 1994; Pind *et al.*, 1994; Aridor *et al.*, 1995; Lupashin *et al.*, 1996; Subramaniam *et al.*, 1996), our results indicate that once transport intermediates have docked onto the *cis*-Golgi membrane, Sec22b/ERS-24 antibodies are no longer inhibitory in the transport assay. This observation can be explained in two alternative ways. The first is that Sec22b/ERS-24 is only important for the docking process but not for the fusion event. Alternatively, once transport intermediates have docked onto the *cis*-Golgi membrane, Sec22b/ERS-24 becomes incorporated into a large SNARE complex in such a way that Sec22b/ERS-24 is no longer accessible to the antibodies. In view of the recent demonstration that SNAREs are not only involved in the docking but also participate in the fusion event by forming a SNAREpin structure (Sutton *et al.*, 1998, Weber *et al.*, 1998), we favor the later possibility. Similarly, antibodies against rbet1 (Zhang *et al.*, 1997) or syntaxin 5 (our unpublished observations) must be added before the EGTA-sensitive stage to exhibit an inhibition on ER–Golgi transport. Since Sec22b/ERS-24, rbet1, and syntaxin 5 have

Figure 10 (facing page). (A) VSV ts045-infected Vero cells grown on coverslips were digitonin permeabilized and incubated at 32°C in complete transport cocktail buffer supplemented with either rbet1 or sec22b/ERS-24 antibodies for 120 min. Cells were fixed in paraformaldyhyde. Panels of inhibitory antibodies are shown in green. Panels of GS28 are shown in red. Also shown are the merged images. Bar, 5 μm. (B) VSV ts045-infected Vero cells grown on coverslips were digitonin permeabilized and incubated at 32°C in complete transport cocktail buffer supplemented with either rbet1 or sec22b/ERS-24 antibodies for 120 min. Cells were fixed in methanol. Panels of inhibitory antibodies are shown in green. Panels of ERGIC53 are shown in red. Also shown are the merged images. Bar, 5 μm.

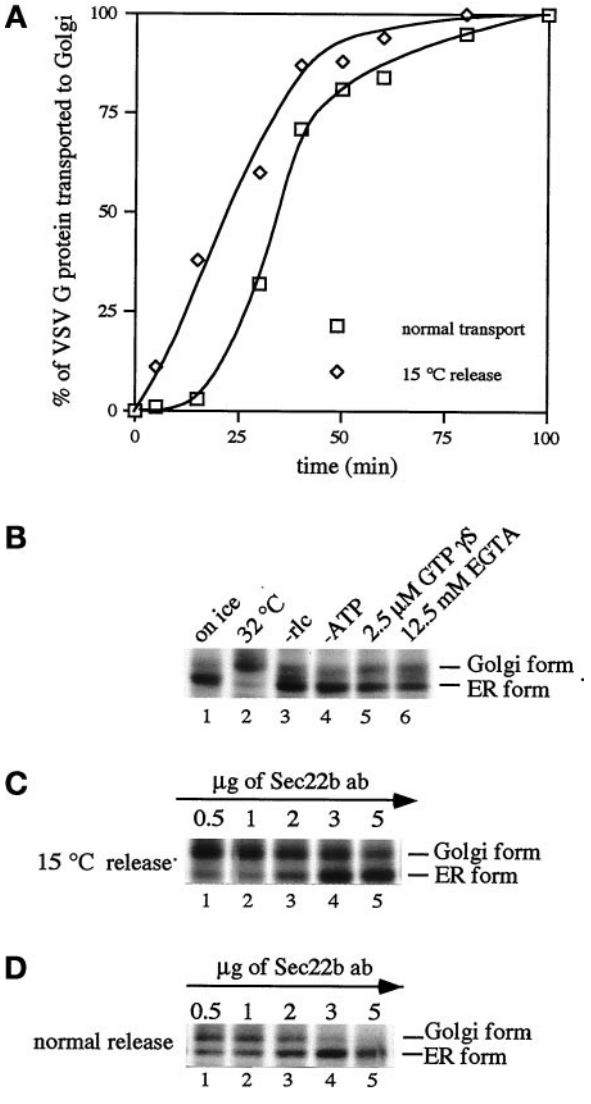

Figure 11. More Sec22b/ERS-24 antibodies are required for inhibition of transport of 15°C-arrested G protein from the IC to the Golgi. (A) Transport reactions using semi-intact cells in which G protein has been arrested in the ER (square) or the IC (diamond) (by preincubating cells in vivo for 90 min at 15°C before the preparation of semi-intact cells) were incubated for the indicated time at 32°C. In each transport, the final rate of G protein transported to Golgi was set as 100%. (B) In vitro transport from the IC to the Golgi was performed either on ice (lane 1) or at 32°C (2–6) in the absence (lane 3) or presence (lane 1, 2, 4–6) of rlc. The reagents were omitted or added as indicated. (C) In vitro IC–Golgi transport, supplemented with the indicated amounts of Sec22b/ERS-24 antibodies, was performed at 32°C. (D) In vitro ER–Golgi transport was performed at 32°C supplemented with the indicated amounts of Sec22b/ERS-24 antibodies.

been shown to exist in a protein complex in Golgi detergent extracts (Hay *et al.*, 1997; Paek *et al.*, 1997), it seems that these three proteins may be incoporated into a SNAREpin structure once transport intermediates have docked onto the cis-Golgi membrane while subsequent fusion is prevented by EGTA. Because of the compact structure of SNAREpin and the fact that the SNAREpin is sandwiched in between the two membranes undergoing fusion (Sutton *et al.*, 1998, Weber *et al.*, 1998), it is conceivable that components (Sec22b/ERS-24, rbet1, and syntaxin 5) of the SNAREpin are no longer accessible to their antibodies. Alternatively, the requirement of Sec22b/ERS-24 before the EGTA-sensitive stage could be explained by proposing that Sec22b/ERS-24 is only required for ER-IC transport and that events downstream of the EGTA-sensitive stage could be mediated by other homologues (such as Sec22a and/or Sec22c) of Sec22p. Further experiments are needed to explore these possibilities.

Cargo molecules such as VSVG are accumulated in the pre-Golgi IC when cells are incubated at 15°C (Saraste and Svensson, 1991; Lotti *et al.*, 1992; Plutner *et al.*, 1992; Lippincott-Schwartz, 1993; Oprins *et al.*, 1993; Tang *et al.*, 1993, 1995b; Balch *et al.*, 1994; Aridor *et al.*, 1995). Under this condition, VSVG has already been exported from the ER and exists in numerous COPII-coated vesicles in the form of vesicular-tubular clusters (VTC), which are adjacent to the budding sites of the ER, scattered throughout the peripheral as well as peri-Golgi regions (Lotti *et al.*, 1992; Balch *et al.*, 1994; Aridor *et al.*, 1995). The 15°C-accumulated VTCs (inmature VTCs) are incompetent for fusion with the Golgi apparatus and must undergo a maturation step. Although the ER export of VSVG and other cargo molecules requires only COPII coat proteins, the maturation event requires both COPII and COPI coat proteins (Aridor *et al.*, 1995; Scales *et al.* 1997; Aridor *et al.*, 1998). The IC marker ERGIC53/p58 has recently been shown to be a major component of ER-derived COPII vesicles/immature VTCs (Rowe *et al.*, 1996; Tisdale *et al.*, 1997). The maturation of immature VTCs involves exchange of COPII for COPI coat proteins (Aridor *et al.*, 1995; Scales *et al.*, 1997; Aridor *et al.*, 1998). Association of the COPI coat with the immature VTCs is coupled to the dissociation of the COPII coat. Once recruited, the COPI coat then mediates segregation of proteins destined for anterograde transport (such as VSVG) from those for retrograde transport back to the ER (such as ERGIC53/p58) (Aridor *et al.*, 1995; Tang *et al.*, 1995b). During this maturation process, VTCs may undergo homotypic fusion to form larger structures (Bannykh *et al.*, 1996; Bannykh and Balch, 1997). Furthermore, VTCs in the peripheral region must migrate toward the Golgi region along microtubules by using the microtubule minus-end–directed motor complex of dynein/dynactin (Presley *et al.*, 1997; Scales *et al.*, 1997). Transport of VSVG from 15°C-arrested immature VTCs to the Golgi can be inhibited by antibodies against Sec22b/ERS-24, rbet1 (Zhang *et al.*, 1997), or syntaxin 5 (Rowe *et al.*, 1998, and our unpublished observations), suggesting that these SNAREs partici-

pate in events downstream of the formation of the immature VTCs. Consistent with this interpretation, VSVG export is not affected because it is accumulated in small vesicular structures characteristic of immature VTCs distributed throughout the entire cytoplasm in the presence of antibodies against Sec22b/ERS-24. Interestingly, more Sec22b/ERS-24 antibodies are required to inhibit VSVG transport from the 15°C-accumulated VTCs to the Golgi as compared with VSVG transport from the ER to the Golgi. Under identical conditions, VSVG transport from either the 15°C-accumulated VTC or the ER to the Golgi can be inhibited by the same amounts of antibodies against rbet1 or syntaxin 5 (our unpublished results). These observations suggest that Sec22b/ERS-24, but not rbet1 and syntaxin 5, becomes less accessible to its antibodies during the 15°C incubation. This could be explained by proposing that Sec22b/ERS-24 adopts a different conformation or is incorporated into a protein complex during the 15°C incubation in such a way that it is less accessible to its antibodies. This observation implies that Sec22b/ERS-24 may play a role in the early maturation events (such as homotypic fusion, segregation of retrograde and anterograde cargoes, or migration from the peripheral to the Golgi region) of the immature VTCs. Consistent with this interpretation, VSVG is seen to be accumulated in vesicular structures that are distributed evenly throughout the cytoplasm in the presence of antibodies against Sec22b/ERS-24, while significant amounts of VSVG are accumulated in larger vesicular structures in the Golgi region when VSVG transport is inhibited by rbet1 antibody or EGTA. Furthermore, the structures containing Sec22b/ERS-24 and therefore VSVG do not colocalize with the Golgi appparatus marked by GS28. These results suggest that antibodies against Sec22b/ERS-24, but not rbet1, have an inhibitory effect on the migration/maturation of peripheral VTCs to the Golgi region. These results, taken together, indicate that Sec22b/ERS-24 may participate in the maturation/migration of pre-Golgi VTCs. Furthermore, Sec22b/ERS-24, together with rbet1 (Zhang *et al.*, 1997), syntaxin 5, GS28/GOS-28, and Ykt6 (Sögaard *et al.*, 1994; Nagahama *et al.*, 1996; Subramaniam *et al.*, 1996; McNew *et al.*, 1997; Rowe *et al.*, 1998), may be involved in the docking and fusion of mature VTCs with the cis-Golgi membrane, although the possibility does exist that SNAREs may be involved only in either the stable attachment of membrane (docking), initiation of lipid bilayer mixing (fusion), or even just signaling events that regulate vesicular transport.

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