Sec34 is implicated in traffic from the endoplasmic reticulum to the Golgi and exists in a complex with GTC-90 and IdlBp

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Running Title: Sec34 forms complex with GTC-90 and IdlBp

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Sec34p/Grd20p has been implicated in endoplasmic reticulum (ER)-to-Golgi transport and/or post-Golgi trafficking events and exists in a protein complex consisting of at least 8 subunits in yeast. Although the mammalian counterpart (Sec34) of Sec34p has been molecularly identified, its role and interacting partners remain undefined. In this study, we have prepared antibodies specifically against the recombinant N-terminal fragment of Sec34 that recognize a polypeptide of about 93 kDa and label the Golgi apparatus. In a well-characterized semi-intact cell assay that reconstitutes transport of the envelope glycoprotein (VSVG) of vesicular stomatitis virus (VSV) from the ER to the Golgi, anti-Sec34 antibodies inhibited the transport in a dose-dependent manner. The inhibition by anti-Sec34 antibodies could be neutralized by a non-inhibitory amount of the antigen. Large-scale immunoprecipitation of rat liver cytosol with immobilized anti-Sec34 antibodies has co-immunoprecipitated GTC-90 and ldIBp, two peripheral Golgi proteins previously shown to exist in separate protein complexes. Two mammalian homologues (Dor1 and Cod1) of the yeast Sec34 complex were similarly recovered in the Sec34 immunoprecipitates. When expressed in transfected cells, epitope-tagged ldICp, and Cod2 were co-immunoprecipitated with anti-Sec34 antibodies with efficiencies comparable to that observed for tagged ldIBp, Dor1, and Cod1. Direct interactions of Sec34 with ldIBp and ldICp were further demonstrated in vitro. These results suggest that Sec34, GTC-90, and ldIBp/ldICp are part of the same protein complex(s) that regulates diverse aspects of Golgi function, including transport from the ER to the Golgi apparatus.
Introduction

Protein transport from the ER to the Golgi in mammalian cells involves several key steps (1-2). Cargo proteins and components necessary for downstream steps are first exported from the ER exit sites (ERES) via the action of COPII components (3-4). COPII vesicles and/or clusters of COPII vesicles are believed to undergo homotypic fusion to form pleiotropic transport intermediates (1, 5). The ERES and transport intermediates have been collectively referred to as the intermediate compartment (IC) or ER-Golgi intermediate compartment (ERGIC) (2, 6). The heterotypic fusion of the transport intermediates with the cis-Golgi results in delivery of cargo proteins to the Golgi apparatus. Many proteins originally identified in yeast have now been shown to participate in ER-to-Golgi transport in mammalian cells (2, 7) and more proteins regulating ER-Golgi transport in mammalian cells are expected to exist.

SEC34 and SEC35 were identified as genes whose products are necessary for protein transport from the ER to the Golgi in yeast *Sc. cerevisiae* (8). More detailed studies of Sec34p and Sec35p have suggested that they function as components of a protein complex that act as a tethering factor to ensure the proper docking and fusion of transport intermediates with the Golgi apparatus (9-12). In addition, the TRAPP protein complex (13) and Uso1p (14-16) have similarly been shown to function in tethering for the same transport event. Although the spatial, temporal and mechanistic relationships among the Sec34/Sec35p complex, the TRAPP complex, and Uso1p are yet to be fully characterized, the importance of the tethering process in ensuring faithful docking and fusion of transport intermediates with the target compartment is becoming more recognized in several trafficking events (17-19). Although the mammalian homologue (Sec34) of Sec34p has been molecularly identified (20), its role in membrane traffic in mammalian cells remains to be established and its interacting partners remain to be explored.
GTC-90 was purified as a component of a novel protein complex in mammalian cells that is required for intra-Golgi transport \textit{in vitro} (21). Although the GTC-90 protein complex is known to include several other different subunits, their identities are unknown and the functional aspects of the GTC-90 complex in other transport events remain to be examined.

Low density lipoprotein (LDL) receptor is responsible for the clearance of serum LDL particles and diverse mutations in its gene are associated with familial hypercholesterolemia (22). Due to its importance, understanding the pathway and mechanism underlying LDL receptor trafficking has been an active area of cell biology. Genetic approaches have been used to create and identify several mutant lines of Chinese hamster ovary (CHO) cells, including ldlA-ldlII (23-25). The genes mutated in some of these mutants (such as ldlA, ldlB, ldlC, ldlD, and ldlF) have been identified (26-30). Biochemical and cell biological characterizations of ldlBp and ldlCp have revealed that they are components of the same protein complex (28, 30). Both ldlBp and ldlCp are necessary for maintaining novel structure and function of the Golgi apparatus, although the precise function remains to be investigated.

Using antibodies against the recombinant Sec34 N-terminal fragment, evidence is presented to support a role for Sec34 in ER-to-Golgi transport in mammalian cells. Significantly, the observation that antibodies against Sec34 could co-immunoprecipitate GTC-90, ldlBp and others suggests that Sec34, GTC-90, ldlBp and ldlCp are part of the same protein complex(s) that may regulate diverse aspects of the Golgi functions, including ER-Golgi transport.
Materials and Methods

Materials

All cell lines were obtained from the American Type Culture Collection (Rockville, MD). Glutathione Sepharose 4B beads were purchased from Pharmacia (Uppsala, Sweden). Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (IgG) and Texas Red–conjugated goat anti-mouse IgG were from Jackson ImmunoResearch. Restriction enzymes were all purchased from Boehringer Mannheim. Local New Zealand White rabbits were purchased from the Sembawang Laboratory Animals Centre (Singapore). Freund’s adjuvants (complete and incomplete) were from Life Technologies-BRL (Bethesda, MD). The human cDNA clones KIAA1134 and KIAA1381 were kindly provided by Kazusa DNA Research Institute. Human EST clone accession number AA439818 was generated by Washington University-Merck expressed sequence tag (EST) project and made available by IMAGE consortium via Research Genetics Inc. (Huntsville, Alabama). cDNA clone AI492237 was purchased from Life Technologies (Pacific) Ltd. AK026305 cDNA clone was provided by NEDO human cDNA sequencing project. Synthetic oligonucleotides were ordered from Research Genetics (Singapore).

cDNA cloning of human Sec34

Full-length human Sec34 cDNA was assembled firstly by obtaining a 1.8 kb fragment from cDNA clone (AK026305) by digestion with XhoI and NsiI. This fragment which contains the 5’ coding region of Sec34 was ligated to an approximately 3.6 kb fragment obtained from cDNA clone (AA439818) by digestion with XhoI and NsiI, which includes also the vector pT7T3D-Pac. An additional 1.8 kb fragment was obtained from cDNA clone (AA439818) by digestion with NsiI alone. These fragments were ligated to construct an overall 4.3 kb Sec34 cDNA in pT7T3D-Pac vector.
Expression constructs for myc epitope-tagged Sec34, Dor1, Cod1, Cod2, ldlBp, and ldlCp

Myc-Sec34: Sec34 cDNA in pT7T3D-Pac vector was digested with XhoI and NotI and the resulting fragments was digested with BglII to obtain an approximately 2.5 kb fragment which contains the entire coding region of Sec34. This fragment was blunt-ended and ligated to pDMyc-neo vector (31) pre-cut with XbaI and blunt-ended. Myc-Cod2: Primer 1 (5' - GAG CTC GAG CGG GCA GAG GGC AGC GGG GAA GTG) and primer 2 (5' – GGC ATC TGC AAC TTG AGC TCT TAT CTC TAA TTT) were used to amplify a 450 bp fragment from KIAA1134 clone and the resulting PCR product was digested with XhoI and SacI. This fragment was ligated to another fragment retrieved by digesting KIAA1134 with SacI and NotI, together with the pDMyc-neo vector pre-cut with XhoI and NotI. Myc-ldlBp: Primer 3 (5' - GAG CTC GAG CGG GTG GGC GAA CGG TAC) and primer 4 (5’ – TAC ACA TGT GGA TCC ATT TCT GCA GC) were used to amplify an approximately 1 kb fragment from KIAA1381 and the resulting PCR fragment was digested with XhoI and BamHI. This was ligated to a fragment retrieved from KIAA1381 by digestion with BamHI and NotI, together with pDMyc-neo vector pre-cut with XhoI and NotI. Myc-ldlCp: Primer 5 (5’ – GGC CTC GAG CGG GAG AAA AGT AGG) and primer 6 (5’ – GGT CTA GAC GAG AGG CTG CTC TGC TGT TGC) were used to amplify the entire coding sequence of ldlCp from IMAGE clone AI492237 by PCR and the resulting product was digested with XhoI and Xbal and ligated into the corresponding sites of pDMyc-neo vector.

Myc-Dor1 and Myc-Cod1: Plasmids SDOR1M1 and SCOD1M4 which express Dor1 and Cod1, respectively, with triple myc-tags at the C-terminus were kindly provided by Dr. Sean Munro (32).

Expression and purification of recombinant GST fusion proteins For production of recombinant glutathione-S-transferase (GST) fusion proteins, primer 7 (5'- ATT GGA TCC ATT ATG GCG GAG GCG GCG) and primer 8 (5' - CGG CTC GAG CGG ACT TGT GAG GGT CTG TAG TGT GTT) were used to amplify the coding sequence for residues 1-276 of Sec34.
This PCR product was digested with *BamHI* and *XhoI*. Primer 9 (5’ – CTC CCG GGT CAT CAG TTA CTG AAA AGG GAT CCT) and primer 10 (5’ – CGG CTC GAG CGG TCC ATG AAG ATC TGC) were used to retrieve the region coding for residues 277-552 of Sec34. The PCR product was digested with *SmaI* and *XhoI*. Primer 11 (5’ – CCC GGA TCC CCC ATG TGG TAT CCT ACG GTT CGA AGA) and primer 12 (5’ – CGG CTC GAG CGG TTT AGA AAC TGA CAG CAG AAG) were used to amplify the sequence coding for residues 553-828 of Sec34. This PCR product was digested with *BamHI* and *XhoI*. The bacterial expression vector pGEX-4T-1 (Pharmacia Biotech) was digested accordingly and ligated with these PCR fragments. The ligated plasmids were transformed into DH5α cells and ampicillin resistant colonies expressing the GST fusion proteins were screened as described (33). Purification of GST fusion proteins was performed as described (34).

**Preparation and affinity-purification of Sec34 antibodies** 400 µg of GST-Sec34/F1 was emulsified with Freund’s complete adjuvant and injected subcutaneously into two local New Zealand White rabbits. Booster injections with the same amount of antigen in Freund’s incomplete adjuvant were administered every two weeks. The rabbits were bled ten days after the second and subsequent boosters. For affinity purification, the antiserum was first diluted with an equal volume of PBS and then incubated for 2 hrs at 4°C with GST-coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech) to remove antibodies against GST. The flow-through was then incubated overnight at 4°C with GST-Sec34/F1-coupled beads. The beads were washed extensively and Sec34 specific antibodies were eluted with low pH elution buffer as described (34).

**Immunoblot analysis** Proteins were separated by SDS-PAGE and electro-transferred onto Hybond C+ nitrocellulose. The blots were then incubated for one hour at 37°C in blocking buffer
(5% skim milk, 5% fetal bovine serum in PBS containing 0.05% Tween-20). The blots were incubated in blocking buffer containing primary antibodies for one hour at room temperature followed by three washes with PBS containing 0.05% Tween-20 for five minutes each. The blots were then incubated with either goat anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase (HRP)(Jackson ImmunoResearch). After three washes in PBS containing 0.05% Tween-20, SuperSignal West Pico Chemiluminescence Substrate (Pierce) was added and the blots were processed as according to the manufacturer’s protocol. For blocking experiment shown in Fig 2A, 50 µg of proteins extracted from Golgi-enriched membranes or cytosol were electrophoresed and transferred to a filter. After blocking, the filter was immunoblotted with 2 µg of affinity-purified Sec34 antibodies; 2 µg of Sec34 antibodies preincubated with 20 µg of recombinant GST-Sec34/F1, GST-Sec34/F2 or GST-Sec34/F3 separately.

**Immunofluorescence microscopy**  Cells were grown on coverslips overnight to a confluency of 50-80% and rinsed twice with PBSCM and processed as described (34-35). 5-10 µg/ml of Sec34 antibodies in fluorescence dilution buffer (PBSCM with 5% normal goat serum, 5% fetal bovine serum and 2% bovine serum albumin, pH 7.6) were used. 0.5 µg of GST-Sec34/F1 was used to neutralize the antibodies.

**In vitro ER-Golgi transport using semi-intact cells**

The ER-Golgi transport assay using semi-intact cells was a modified assay according to previously reported procedure (7, 36) and performed as follows: Briefly, NRK cells grown on 10-cm Petri dishes as a confluent monolayer were infected with a temperature-sensitive strain of the vesicular stomatitis virus, VSVts045, at 32°C for an hour and then at the restrictive temperature of 40°C for another 2 hours. The cells were then subjected to perforation on ice by hypotonic swelling and scraping. These semi-intact cells were then incubated in a complete assay
mixture of 40 µl, containing 25 mM Hepes-KOH, pH 7.2, 90 mM KOAc, 2.5mM MgOAc, 5 mM EGTA, 1.8mM CaCl₂, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 µg of cytosol, and 5 µl (25-30 µg of protein; 1-2 × 10⁵) of semi intact cells. Additional reagents were added as indicated. For a standard assay, samples were incubated for 90 minutes at 32°C and transport terminated by transferring to ice. The membranes were collected by a brief spin, solubilized in 20 µl of 0.2% SDS, 50 mM sodium citrate (pH5.5). After boiling for 5 min, the samples were digested overnight at 37°C in the presence of 2.5 units of endoglycosidase H (endo H) and the reaction was terminated by adding 6× concentrated gel sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels and transferred to a nitrocellulose filter. Immunoblot analysis was performed with anti-VSV antibodies (Boehringer Mannheim). For antibody inhibition of transport assay, Sec34 antibodies were added into the complete assay mixture and incubated on ice for 60 min to allow diffusion of antibodies into the semi-intact cells.

**Large scale immunoprecipitation** 300 µl of Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) were washed in PBS and used to bind 500 µg of rabbit anti-goat IgG (Pierce) or 500 µg of anti-Sec34 antibodies separately for 2 hrs at 4°C. The antibodies were cross-linked to the beads with 50 mM dimethyl pimelidate (DMP) in 0.2M Na Borate pH9 overnight at 4°C, and then washed in 0.2 M Na Borate pH 9 and incubated with 0.2 M ethanolamine pH 8 for 2 hr at RT. The beads were washed with PBS and finally in 0.5% TX-100 in gradient buffer (20 mM Hepes, pH7.3, 100 mM KCl, 2 mM EDTA). 50 mg of rat liver cytosol was first incubated with the beads bound with control rabbit IgG in gradient buffer with 0.5% TX-100 for 2 hrs at 4°C. The supernatant was then incubated with the beads bound to Sec34 antibodies overnight at 4°C.
The beads were then washed three times with gradient buffer containing 0.5% TX-100 and then finally with gradient buffer without TX-100.

**Transfection and analytic immunoprecipitation** 293T cells were grown on 60 mm dishes to a confluency of 40-60%. 1 µg of myc-tagged ldlBp, ldlCp, Dor1, Cod1, and Cod2 DNA was used for transfection with Effectene Transfection Reagent, according to manufacturer’s instructions (Qiagen). The next day, the cells were washed twice with PBSCM and 150 µl of PBS, 1% TX-100 and complete EDTA-free protease inhibitor mixture (Roche Diagnostics GmbH) was added, and then scraped with a cell scraper. The cells were rotated at 4°C for 15 min and then spun down at 4 K rpm for 10 minutes. To the supernatant, 5 µg of Sec34 antibodies were added and incubated at 4°C for 2 hours. Next, Protein A-Sepharose CL-4B beads were added and left to bind overnight at 4°C. The beads were then washed thrice with PBS, 1% TX-100 and then with PBS 0.2%TX-100 and finally with PBS and analysed by SDS-PAGE. Immunoblot analysis was performed with anti-myc monoclonal antibodies.

**In Vitro translation and binding experiment** TNT T7 Quick Master Mix (Promega) was used for in vitro translation according to manufacturer’s protocol. In 100 µl reaction, myc-tagged Sec34 and each of myc-tagged ldlBp, ldlCp, and Cod2 were co-translated for 2 hrs at 30°C. 20 µl of each reaction was used for co-immunoprecipitation with 10 µg of anti-myc antibodies (rabbit polyclonal IgG) (Upstate biotechnology) or 10 µg of anti-sec34 antibodies bound to Protein A-Sepharose beads in gradient buffer with 0.1% TX-100. After 1 hr at RT, the beads were washed 5 times with gradient buffer with 0.1% TX-100 and analysed by SDS-PAGE and autoradiography together with 2 µg of in vitro translated product (10% starting material).
Results

**Characterization of antibodies against Sec34**  Due to the established role of Sec34p in ER-Golgi transport in yeast and our general interest in ER-Golgi transport in mammalian cells, we used the amino acid sequence of Sec34p to search for its putative mammalian counterpart by BLAST searches (37). Several cDNA sequences encoding polypeptides homologous to various regions of Sec34p were uncovered. The complete coding region of human Sec34 was assembled from various cDNAs and confirmed by DNA sequencing (GenBank accession number AF332595). During the course of our work, the molecular identification of human Sec34 was independently reported (20). Examination of the deduced 828 amino acid sequences suggests that Sec34 could be roughly divided into two regions (Fig 1A). The N-terminal one third is highly homologous to counterparts from other species such as yeast, fly and worm and has the potential (particularly residues 126-211) to form coiled-coil structures. The C-terminal two thirds is homologous to EEA1 (Fig 1B), a well-established tethering factor that regulates endosome fusion (38). The structural relatedness of Sec34 to a well-defined tethering protein provides some structural evidence for Sec34 to function as a tethering factor. In order to define the functional and biochemical aspects of Sec34, we expressed recombinant Sec34 in three separate fragments (residues 1- 276 as Sec34/F1, residues 277-552 as Sec34/F2, and residues 553-828 as Sec34/F3) fused to GST (Fig 1A). GST-Sec34/F1 was used to raise antibodies against Sec34. As shown in Fig 1C, affinity-purified Sec34 antibodies recognized a polypeptide of about 93 kDa in both membrane (lane 1) and cytosol (lane 5) fractions derived from rat liver. Detection of this polypeptide by the antibodies was abolished by pre-incubation of the antibodies with GST-Sec34/F1 (lanes 2 and 6) but not with GST-Sec34/F2 (lanes 3 and 7) or GST-Sec34/F3 (lanes 4 and 8), suggesting that the antibodies are specific for Sec34 and that Sec34 is present in both cytosolic and membrane fractions. Using these antibodies in immunofluorescence microscopy, Sec34 was seen to be enriched in the Golgi apparatus (Fig 1D, panel a) marked by the Golgi
SNARE GS28 (panel b) (39-40) in HeLa cells. Furthermore, the Golgi labeling of Sec34 (panel d) but not GS28 (panel e) was abolished by pre-incubation of the antibodies with GST- Sec34/F1, further confirming the specificity of the antibodies. These results are similar to those reported previously (20) and suggest that our antibodies are specific for Sec34.

A role for Sec34 in ER-Golgi transport  Since evidence for a functional role for Sec34 in ER-Golgi transport in mammalian cells is lacking, we investigated the potential transport function of Sec34 by using a modified semi-intact cell assay that reconstitutes protein transport from the ER to the Golgi (see Materials and Methods for details). NRK cells were infected with a temperature sensitive mutant vesicular stomatitis virus (VSV ts045) at 32°C for 1hr followed by incubation at 40°C for 2 hr to accumulate its envelope protein (VSVG) in the ER. Cells were then permeabilized by scraping in hypotonic buffer and used to reconstitute transport of VSVG by supplementing with exogenous rat liver cytosol and an ATP-regenerating system at 32°C. Transport of VSVG to the Golgi was measured by monitoring the conversion of its endo-H sensitive glycans to endo-H resistant forms of the entire population of ER-arrested VSVG molecules as revealed by immunoblot analysis. There are two advantages in this modified assay as compared to the original protocol (7). The first is that no radioactive materials were used. The other is that, instead of measuring a small fraction (the radiolabeled pool) of total VSVG, this assay measures the synchronized transport to the Golgi of almost all VSVG molecules accumulated in the ER. As shown in Fig 2, VSVG remained as the endo-H sensitive ER form when the transport reaction was performed on ice (Fig 2A, lane 1; Fig 2B, lane 1) or in the absence of cytosol (Fig 2A, lane 2; Fig 2B, lane 2). Between 60-90% of total VSVG was converted into the endo-H resistant form when the transport assay was conducted in the presence of complete cocktail at 32°C (Fig 2A, lane 3; Fig 2B, lane 3). Addition of antibodies against GST (Fig 2A, lanes 4-6) did not inhibit this transport, while addition of antibodies against Sec34 (Fig
in the presence of 3 µg or more of anti-Sec34 antibodies and the transport was almost completely inhibited by 7 µg of antibodies (Fig 2A, lane 10). The inhibition by Sec34 antibodies is specific because it could be neutralized by a non-inhibitory amount of GST-Sec34/F1 (Fig 2B, lanes 5 and 7) but not GST (Fig 2B, lanes 4 and 6). These results suggest that Sec34 is important for ER-Golgi transport in mammalian cells.

Co-immunoprecipitation of GTC-90, ldlBp, Dor1 and Cod1 from total cytosol by anti-Sec34 antibodies In order to define the molecular mechanism underlying the action of Sec34, we have performed large-scale immunoprecipitations using rat liver cytosol (Fig 3). As compared to rabbit anti-goat IgG (lane 2), antibodies against Sec34 immunoprecipitated several distinct polypeptides (lane 3) as resolved by SDS-PAGE. We focused our mass spectrometric analyses on polypeptides that are of sizes above that of the IgG heavy chain, as polypeptides with smaller sizes are commonly detected by large-scale immunoprecipitations due to non-specific interactions of abundant cytosolic proteins. As shown in Fig 3, GTC-90 (21) and ldlBp (28), in addition to Sec34, were identified in the Sec34 immunoprecipitates. The intensities of ldlBp and GTC-90, as revealed by Coommasie blue staining, were stronger than that of Sec34, suggesting that the Sec34 polypeptide might be poorly stained or that other subunits are present at higher abundance in the complex. The quantitative and specific recoveries of ldlBp and GTC-90 by anti-Sec34 antibodies from total cytosol suggest that Sec34, GTC-90 and ldlBp are components of the same protein complex(s). Furthermore, two mammalian homologues (Dor1 and Cod1) of the yeast Sec34p/Sec35p complex (32) were also detected in the Sec34 immunoprecipitates (Fig3), supporting the hypothesis that they are part of the mammalian complex. In order to rule out the possibility that some interesting components may be overlooked, we have also analyzed other polypeptides migrating faster than that of IgG heavy chain. These mainly represented abundant
cytosolic proteins such as actin, three ribosomal subunits, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and glutathione peroxidase. In addition, an uncharacterized protein with Genbank accession number XP_34431 was identified. While we cannot exclude the possibility that some of these proteins may be involved in Sec34 function, the presence of abundant cytosolic proteins such as actin, GAPDH, and ribosomal proteins in this low molecular weight region indicates that their presence is likely due to non-specific interactions.

**Co-immunoprecipitation of ldlCp and Cod2** While our study was in progress, the yeast Sec34p/Sec35p complex was characterized in detail (32). It contains 8 subunits: Sec34p, Sec35p, Dor1p, Cod1p, Cod2p, Cod3p, Cod4p and Cod5p. Cod4p corresponds to GTC-90 in mammalian cells. Some structural relatedness between Sec35p and ldlCp was noticed, indicating that Sec35p could be a candidate for the functional counterpart of mammalian ldlCp (32). Mammalian homologues of Dor1p, Cod1p and Cod2p but not Cod3p or Cod5p were also identified and the epitope-tagged mammalian Dor1 and Cod1 were detected in the Golgi apparatus upon transient expression by transfection (32). No apparent structural or functional yeast counterpart of ldlBp was found in the yeast Sec34p/Sec35p complex. Since GTC-90, ldlBp, Dor1 and Cod1 were recovered in the anti-Sec34 immunoprecipitates, we examined whether other potential subunits (ldlCp, and Cod2) of the mammalian complex(s) could be immunoprecipitated by anti-Sec34 antibodies (Fig 4). 293T cells were transiently transfected with constructs expressing Myc epitope-tagged ldlBp, ldlCp, Dor1, Cod1, or Cod2. Cell lysates were immunoprecipitated with anti-Sec34 antibodies and analyzed by immunoblot to detect the tagged proteins. As positive controls, cells transiently expressing myc-ldlBp, myc-Dor1 and myc-Cod1 were immunoprecipitated with anti-Sec34. About 5% of myc-ldlBp (lanes 1 and 2), myc-Dor1 (lanes 5-6), or myc-Cod1 (lanes 7-8) could be recovered by anti-Sec34 antibodies. Under identical conditions, they were not co-immunoprecipitated by other control antibodies (data not shown). These results further support our conclusion that they are specifically co-immunoprecipitated by
Sec34 antibodies. Importantly, myc-ldlCp (lanes 3 and 4), and myc-Cod2 (lanes 9 and 10) were co-immunoprecipitated by anti-Sec34 antibodies with efficiencies comparable to that observed for myc-ldlBp, myc-Dor1 and myc-Cod1, supporting the notion that these other proteins are indeed subunits of the same mammalian complex(s). Consistent with our interpretation that polypeptides with sizes smaller than that of IgG heavy chain were present in the Sec34 immunoprecipitates due to their high abundances and non-specific interactions, myc-tagged XP_034431 was not co-immunoprecipitated by anti-Sec34 from lysates prepared from transfected cells (data not shown).

Direct interaction of Sec34 with ldlBp or ldlCp  We next investigated whether Sec34 could interact directly with any of the other subunits. Myc-Sec34 was co-translated with myc-ldlBp, myc-ldlCp, or myc-Cod2 using the in vitro translation system. The translation reactions were then subjected to immunoprecipitation using Sec34 antibodies or anti-myc antibodies. As shown in Fig 5, myc-Sec34 and myc-Cod2 were both effectively immunoprecipitated with anti-myc antibodies (lane 3). However, myc-Cod2 was not co-immunoprecipitated by anti-Sec34 under the conditions that myc-Sec34 was efficiently immunoprecipitated (lane 2), suggesting that myc-Cod2 and myc-Sec34 do not interact directly. Interestingly, both myc-ldlBp and myc-ldlCp, upon co-translation with myc-Sec34, could be efficiently (30-50% of co-expressed protein) co-immunoprecipitated by anti-Sec34 antibodies (lanes 5 and 8 respectively), suggesting that both ldlBp and ldlCp could interact directly with Sec34.
Discussion

Sec34, GTC-90, and ldlBp/ldlCp have been independently identified by different approaches. Sec34 was identified based on a genomic approach to search for the mammalian homologue of Sec34p (20 and this study), which has a well-defined role in ER-Golgi transport in yeast (9-12). GTC-90 was previously identified as a component of a novel protein complex that participates in intra-Golgi transport using an
in vitro
biochemical assay (21). ldlBp and ldlCp were originally identified by a genetic approach (22-25) and subsequently shown to exist in the same protein complex that regulates Golgi structure and function (28, 30). One of the most important discoveries of the present study is that Sec34, GTC-90 and ldlBp/ldlCp are components of the same protein complex (s) (we tentatively refer to this as Sec34/GTC-90/ldlBp complex). This is based on several lines of observations. Firstly, GTC-90 and ldlBp (as well as Dor1 and Cod1) could be efficiently and specifically co-immunoprecipitated from rat liver cytosol by antibodies against Sec34. Secondly, upon expression by transient transfection, myc-tagged ldlBp, ldlCp, Dor1, Cod1 and Cod2 could similarly be co-immunoprecipitated by anti-Sec34 antibodies. Finally, direct interaction of Sec34 with ldlBp and ldlCp could be demonstrated in the absence of other subunits using in vitro translated proteins. While this work was in progress, the yeast Sec34p/Sec35p complex was shown to consist of eight subunits: Sec34p, Sec35p, Dor1p, and Cod1p, Cod2p, Cod3p, Cod4p, and Cod5p. Mammalian homologues for Dor1p, Cod1p, Cod2p and Cod4p but not Cod3p or Cod5p have been also identified (32), and GTC-90 appears to be the counterpart of Cod4p. An approximately 110 amino acid region of ldlCp was found to be homologous to Sec35p, indicating some relatedness between ldlCp and Sec35p (32).

Interestingly, no homologue for ldlBp was identified in yeast, suggesting that either there is another subunit yet to be uncovered in the yeast Sec34p/Sec35p complex or that ldlBp could be a functional counterpart of either Cod3p or Cod5p. Although we favor the possibility that ldlBp (980 amino acids for mouse protein and 962 amino acids for human protein) is not a functional
counterpart of Cod3p (417 amino acids) or Cod5p (279 amino acids) due to the lack of similarity of their amino acid sequences and the huge differences in sizes, further studies are needed to establish this point. Since myc-tagged ldlCp can be co-immunoprecipitated with Sec34 upon expression by transient transfection, and that a direct interaction between Sec34 and myc-ldlCp could be observed, ldlCp is indeed a component of the Sec34/GTC-90/ldlBp complex. Since ldlCp (731 and 738 amino acids for mouse and human protein, respectively) is much larger than Sec35p (275 amino acids) and the observed sequence homology (24% identity over a region of 110 amino acids) is limited, additional evidence is required to resolve whether ldlCp is the structural and/or functional counterpart of yeast Sec35p. The direct interaction of ldlBp or ldlCp with Sec34 suggests that Sec34 harbors structural information for direct interaction with ldlBp and ldlCp in the absence of other subunits.

The other potential subunits (Dor1, Cod1 and Cod2) of the mammalian Sec34/GTC-90/ldlBp complex were similarly confirmed immunologically and/or biochemically as components of the Sec34-containing protein complex(s). Both Dor1 and Cod1 were recovered in Sec34 immunoprecipitates. Epitope-tagged versions of Dor1, Cod1 and Cod2, upon expression by transient transfection, could be co-immunoprecipitated by anti-Sec34 antibodies at efficiencies comparable to those observed for ldlBp and ldlCp under similar conditions. In summary, six other proteins (ldlBp, ldlCp, Dor1, Cod1, Cod2, and GTC-90/Cod4,) in mammalian cells are shown here to be present in the Sec34-containing protein complex(s) with ldlBp and ldlCp having the ability to interact directly with Sec34. It remains possible that these proteins, together with others that are yet to be discovered, may form distinct complexes or sub-complexes. If we assume that a functional counterpart of ldlBp exist in yeast, then at least two other proteins (Cod3 and Cod5) remain to be discovered for the mammalian complex(s). However, if ldlBp is a functional counterpart of Cod3 or Cod5, then we have uncovered all but one components of the mammalian complex.
The function of the Sec34/GTC-90/ldlBp complex in mammalian cells remains to be explored. In view of all the available results, it could be suggested that the Sec34/GTC-90/ldlBp complex may have a general role in the Golgi apparatus that regulates several trafficking events, including ER-Golgi transport, various intra-Golgi transports, and possibly endosome-to-TGN traffic. The role of Sec34p and Sec35p in ER-to-Golgi transport has been well established in yeast (9-12). Using a modified assay that reconstitutes synchronized transport of almost all ER-arrested VSVG to the Golgi, it was shown that anti-Sec34 antibodies could exhibit dose-dependent inhibition. Furthermore, the inhibition could be neutralized by a non-inhibitory amount of the antigen. We have thus provided evidence that Sec34 plays a similar role in ER-Golgi transport in mammalian cells, although the temporal and other mechanistic aspects of its action remain to be explored. Although a role for Sec34p or other subunits in intra-Golgi transport in yeast has yet to be investigated, the purification of GTC-90 complex using an in vitro intra-Golgi transport assay suggests that this complex could be intimately involved in intra-Golgi transport (21). A role for Sec34p in endosome-to-TGN transport has been reported and this was the basis for which Sec34p was independently identified as Grd20p (41). Although it has been suggested that the role of Sec34p/Grd20p in endosome-to-TGN transport could be due to an indirect effect due to its role in ER-to-Golgi transport (10, 12), the recent identification of the Sec34p complex and the relatedness of this complex with Ypt6p function indicate that Sec34p/Grd20p complex might have a direct role in endosome-to-TGN transport (32). Detailed studies of this complex and its individual subunits using genetic, biochemical and cell biochemical approaches in both yeast and mammalian cells will provide additional understanding on its function and mechanism as well as the general organization and regulation of Golgi structure and function.
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References


Figure legends

**Figure 1.** Characterization of Sec34 antibodies. (A) Domain organization of Sec34. The 828-residue Sec34 can be divided into the N-terminal one third that is conserved among proteins from various species and has the potential to form coiled-coil structures; while the C-terminal two thirds is homologous to EEA1. The regions from which various GST-fusion proteins were derived are indicted below. (B) The C-terminal two thirds of Sec34 is homologous to EEA1. Residues 301-810 of Sec34 were aligned with residues 610-1109 of EEA1. Identical residues are shown in red, while conserved residues are shown in pink. (C) Antibodies raised against GST-Sec34/F1 recognize specifically a 93 kDa protein. The 50 µg of Golgi-enriched membrane fractions (lanes 1-4) and 50 µg of total cytosol (lanes 5-8) derived from rat liver were resolved by SDS-PAGE and transferred to a filter. The filters were incubated with either Sec34 antibodies alone (lanes 1 and 5), or in the presence of GST-Sec34/F1 (lanes 2 and 6), GST-Sec34/F2 (lanes 3 and 7), or GST-Sec34/F3 (lanes 4 and 8). (D) Anti-Sec34 antibodies label the Golgi apparatus. HeLa cells were fixed, permeabilized, and double-labeled with Sec34 antibodies (panels a and d) and monoclonal antibodies against Golgi SNARE GS28 (panels b and e). The Golgi labeling of Sec34 (panel d) but not GS28 (panel e) was abolished by prior incubation of the antibodies with GST-Sec34/F1. The merged images are shown in panels c and f. Bar, 10 µm.

**Figure 2.** Sec34 antibodies specifically inhibit ER-Golgi transport *in vitro*. (A) In vitro ER-Golgi transport assay was performed either on ice (lane 1), or at 32°C (lanes 2-10), in the absence (lane 2) or presence of rat liver cytosol (lanes 1, 3-10), and supplemented with the indicated amounts of GST antibodies (lanes 4-6) or Sec34 antibodies (lanes 7-10). The upper form represents VSVG whose N-lined glycans are resistant to endo-H digestion, while the lower form represents the ER form whose-N-linked glycans have been removed by endo-H. (B) *In vitro* transport was performed either on ice (lane 1), or at 32°C (lanes 2-7) in the absence (lane 2) or presence of rat liver cytosol (lanes 1, 3-7). The inhibition exhibited by anti-Sec34 (7 µg) was neutralized by a noninhibitory amount of GST-Sec34/F1 (3 µg) (lanes, 5 and 7) but not by GST (3 µg) (lanes 4 and 6).

**Figure 3.** Co-immunoprecipitation of GTC-90 and ldlBp (as well as Dor1 and Cod1) by anti-Sec34 antibodies. Rat liver cytosol (50 mg) was immunoprecipitated with 500 µg of Sec34 antibodies (lane 3) or rabbit anti-goat IgG (lane 2) immobilized on Protein A-Sepharose beads. The immune complexes collected on the beads were washed extensively. The
immunoprecipitated proteins were released by boiling in SDS-PAGE sample buffer and then resolved on a 10% SDS-PAGE. Molecular size markers are shown on lane 1. Polypeptides with sizes above the IgG heavy chain were first excised and subjected to mass spectrometric analysis. The amino acid sequences of tryptic peptides of ldlBp, GTC-90, Dor1 and Cod1 are indicated on the right. The identity of the Sec34 band was established by immunoblotting analysis. Mass spectrometric analysis of polypeptides with sizes smaller than that of IgG heavy chain were similarly analysed and revealed the presence of abundant cytosolic proteins that could be due to non-specific interactions.

**Figure 4.** ldlCp and Cod2 are present in Sec34-containing protein complex. 293T cells were transiently transfected with constructs expressing myc epitope-tagged ldlBp (lanes 1-2), ldlCp (lanes 3-4), Dor1 (lanes 5-6), Cod1 (lanes 7-8) or Cod2 (lanes 9-10). Cell lysates were immunoprecipitated with Sec34 antibodies. 5% of each lysate (odd numbered lanes) and the immunoprecipitates (even numbered lanes) were resolved by SDS-Page and analyzed by immunoblot with anti-myc antibodies to detect the co-immunoprecipitated proteins. These myc-tagged proteins were not co-immunoprecipitated by control rabbit IgG or antibodies against Bet3 (not shown).

**Figure 5.** Direct interaction of Sec34 with ldlBp and ldlCp. Myc-Sec34 was co-translated with myc-Cod2 (lanes 1-3), myc-ldlBp (lanes 4-6) or myc-ldlCp (lanes 7-9) by *in vitro* translation reactions in the presence of 35S-Met. The translation products were either immunoprecipitated with Sec34 antibodies (lanes 2, 5 and 8) or anti-myc antibodies (lanes 3, 6 and 9). The immunoprecipitates and 10% the respective translation reactions (lanes 1, 4 and 7) were analysed by SDS-PAGE and fluorography.
**A**

Coiled-coil domain (AA 126-211)

EEA1-homology domain (AA 301-810)

**B**

RAAAPKVRILEQDLRSEKIPVYDDLNDHOCYLDQRELLLGPSCQ1VAELTSQNNRNGHALVRSQCAFVHVCGDOEHLHEYFTKPSKLIQDELTEKL------CVSLYDVFRPL1 Sec34

RAAQOVRVLTSQKELNQLSKEKVSQSLIGIKDKATELLLSSAEEAKTCRGRNQHOHIDAO------NALQDKOHGEL------KTTGQDQYTAKLQDKOECRQDLESSLQEKYEY EEA1

**C**

**D**

Sec34

GS28

merge

Fig1
Fig 4
Sec34 is implicated in traffic from the endoplasmic reticulum to the Golgi and exists in a complex with GTC-90 and ldlBp

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