Identification of a human orthologue of Sec34p as a component of the cis-Golgi vesicle tethering machinery.

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Abstract

The roles of the components of the Sec34p protein complex in intracellular membrane trafficking, first identified in the yeast *Saccharomyces cerevisiae*, have yet to be characterized in higher eukaryotes. We cloned a human cDNA whose predicted amino acid sequence showed 41% similarity to yeast Sec34p with homology throughout the entire coding region. Affinity-purified antibodies raised against the human SEC34 protein (hSec34p) recognized a cellular protein of 94 kDa in both soluble and membrane fractions. Like yeast Sec34p, cytosolic hSec34p migrated with an apparent molecular weight of 300 kDa on a glycerol velocity gradient, suggesting that it is part of a protein complex. Immunofluorescence microscopy localized hSec34p to the Golgi compartment in cells of all species examined, where it colocalized well with the cis/medial-Golgi marker membrin and partially co-localized with cis-Golgi network (CGN) marker p115 and trans-Golgi marker TGN38. The colocalization with membrin was maintained at 15°C and after microtubule depolymerization with nocodazole. During transport of ts045-VSVG protein through the Golgi, there was significant overlap with the hSec34p compartment. GFP-hSec34 expressed in HeLa cells was restricted to Golgi cisternae and its membrane association was sensitive to Brefeldin A treatment. Taken together, our findings indicate that hSec34p is part of a peripheral membrane protein complex localized on cis/medial Golgi cisternae where it may participate in tethering intra-Golgi transport vesicles.
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Introduction

Protein transport through the secretory pathway occurs via transport vesicles under the direction of a large set of protein components(1). The process can be divided into three stages: (a) vesicle budding, (b) vesicle docking, and (c) membrane fusion, with distinct sets of proteins mediating each stage. The budding stage involves recruitment of coat proteins to the membrane and culminates with the release of coated vesicles(2). Vesicular transport between membrane compartments requires high specificity and tight regulation to deliver cargo molecules in donor vesicles to the correct acceptor organelle and thereby maintain the integrity of distinct compartments. To ensure the proper directionality of membrane flow, the target organelle must possess appropriate molecular machinery allowing for specific recognition and docking of the incoming vesicle. The docking reaction is likely to require a set of integral membrane proteins on the vesicle and target membranes, termed SNAREs. Initially, the SNARE proteins were thought to confer specificity through their pair-wise interactions(3). However, more recent evidence suggests that SNAREs cannot be the only targeting components: some SNAREs can function at several transport steps in vivo (4), SNAREs that faithfully function at different transport steps in vivo can interact promiscuously in vitro, and some SNAREs have been found in multiple SNARE complexes (5). Likewise, members of the Rab GTPase family were at first thought to be the principle determinants for targeting specificity because distinct family members display unique organellar localizations that correlate with their site of action (6). However, it has been demonstrated that a single chimeric Rab protein can function at two transport steps (7), indicating that the Rabs cannot be the sole determinants for specificity in vesicle docking.

Given that neither Rabs nor SNAREs are the sole determinants of targeting specificity, other components must play important roles. Additional candidate include the so-called tethering factors, which are proteins that link donor and acceptor vesicle membranes prior to SNARE-SNARE interactions (8). Tethering factors are a diverse group of proteins with a tendency to form elongated, coiled-coil structures and/or to assemble into large, multimeric complexes (for reviews see (9)). However, because they are peripheral membrane proteins, and therefore must interact with integral membrane components with distinct localizations, once again, tethering factors cannot be the sole determinants of targeting specificity. Taken
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together, these results indicate that specificity in vesicle docking and fusion might be generated by the interaction of several factors in such a way that no individual component plays the dominant role.

A recent genetic screen identified temperature-sensitive alleles of two yeast genes, SEC34 and SEC35, that, when incubated at the restrictive temperature, are defective in ER to Golgi transport and accumulate large numbers of 50-nm vesicles (10). Mutant alleles of these genes can be efficiently suppressed by YPT1, which encodes the Rab GTPase required early in the secretory pathway, or by SLY1-20, which encodes a dominant form of the ER to Golgi SNARE-associated protein Sly1p. Weaker suppression is evident upon overexpression of genes encoding the v-SNAREs SEC22, BET1, or YKT6, a trait shared with all previously characterized ER to Golgi tethering factors (11) (12,13). Based on these data, it was hypothesized that SEC34 and SEC35 might be involved in tethering. This was demonstrated to be the case for SEC34 and SEC35 by the discovery that these genes displayed a genetic interaction with genes involved in tethering and that both Sec34p and Sec35p are required in an in vitro tethering assay (12,13). Using a cell-free assay that measures distinct steps in vesicle transport from the ER to the Golgi, it was shown that Sec34p and Sec35p are required for a vesicle docking stage catalyzed by the tethering factor Uso1p (12). These genetic and biochemical results suggest Sec34p and Sec35p may act with Uso1p to dock ER-derived vesicles to the Golgi.

Sec34p is a peripheral membrane protein with a calculated molecular weight of 93 kD. Fractionation of yeast cytosol indicates that Sec34p and Sec35p exist in an ~450-kD protein complex (13,14). Recently Sec34p has also been described as Grd20p, a protein involved in the proper localization of yeast enzymes to the trans-Golgi network (TGN) (15). A severe defect in TGN localization of Kex2p and missorting of vacuolar carboxypeptidase Y occurs rapidly after loss of Grd20p function. A complete loss of GRD20 function also results in a severe growth defect although the growth defect appears to be independent of the TGN sorting defect. The Grd20/Sec34 protein is only partially localized to the TGN so it is possible that the observed Golgi localization defects of Kex2p are indirect and a manifestation of defects in Golgi recycling. Alternatively, Sec34p might be a general transport factor that is involved in several membrane trafficking steps. Additional experiments will be required to unambiguously address these possibilities.
In this study, we report the cloning and sequencing of a novel membrane-associated human protein, p94. Based on several independent lines of evidence, we propose that p94 is hSec34, the human orthologue of yeast Sec34p. p94 has sequence and structural homology to the yeast vesicle tethering factor Sec34p, it localizes to the cis-Golgi, and like yeast Sec34p, it exists as a component of high molecular complex. Although yeast Sec34p is predicted to act on the same transport step as Uso1p, the localization of hSec34p and its sensitivity to Brefeldin A differ from that of p115, a human orthologue of Uso1p. Therefore we propose that hSec34 may function on the vesicle transport step that is distinct from the one that requires p115.
**Experimental Procedures**

*Cloning of hSec34p* – The sequence encoding the amino-terminal part of the hSec34 was amplified from a human colon cDNA library (Marathon – Ready™ cDNA, Clontech) using the Marathon adaptor primer (5'primer: ccatcctaatacgactcactatagggcc) and an internal hSEC34 specific primer (3' primer: gtaactgacttgtgagggtctgtagtgtg). The polymerase chain reaction was carried out using high fidelity Pfu Turbo™ polymerase from Stratagene (La Jolla, CA). The resulting 850bp DNA fragment was cloned into pBluscript vector digested with NotI and SmaI.

The SEC34 ORF was cloned from a human fetal brain cDNA library using a 2-stage Rapid-Screen™ system (OriGene Technologies Inc., Rockville, MD). The cDNA library was screened by PCR according to the manufacturer's recommendations using HSEC34 gene specific primers (5' primer: gcttccaattgaagacttgtgc and 3' primer: gcaaagcttttagaagacactg). The resulting positive clone in the vector pCMV6-XL4 contained a 4.5kb hSec34 cDNA (pCMV-hSec34).

*Plasmid construction* – To construct a His<sub>6</sub> fusion to the amino-terminus of hSec34p (pHis<sub>6</sub> ΔhSec34), a 843 bp fragment of hSec34 was amplified by PCR creating a BamHI site adjacent to the codon for the first amino acid of hSec34 (5' primer: caggatccatggcggaggcggcgctgttg) and an internal hSEC34 specific primer (gtaactgacttgtgagggtctgtagtgtg). The PCR-product was cloned into the pTOPO vector using a Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen, Carlsbad, CA). Next, an 870bp BamHI – PstI fragment from pTOPO-NhSec34 containing the 5' region of hSec34 was ligated into BamHI and PstI digested pQE30 (Qiagen, Valencia, CA). To generate a green fluorescent protein hSec34 fusion (pEGFP-hSec34), the plasmids pQE30-NhSec34 and pCMV-hSec34 were digested with BamHI/KasI and KasI/PstI, respectively. The resulting 101bp and 2462bp fragments were ligated into pEGFP-C1 from CLONTECH Laboratories (Palo Alto, CA) that has been digested with BglIII/PstI. To generate cyan and yellow color variants, the BspEI – Sall fragment of pEGFP-hSec34 was ligated into BspEI/Sall digested pCFP-C1 and pYFP-C1 vectors to generate pCFP-hSec34 and pYFP-hSec34.
Plasmids encoding CFP-membrin and CFP-Syntaxin5 were from R.H. Scheller (Stanford University School of Medicine, Stanford, CA), the plasmid encoding GFP-Sec13 was from F. Gorelick (Yale University School of Medicine, New Haven, CT), and the plasmid encoding VSVG-ts045-GFP was from M.A. McNiven (Mayo Clinic, Rochester, MN).

Generation of anti-hSec34p antibody – A 247 amino acid protein that corresponding to hSec34p amino acid sequences from 35 to 281 was expressed in bacteria and purified by nickel chelate column chromatography. The purified protein was used to immunize rabbits and specific antibodies were affinity purified from the serum on nitrocellulose strips containing electroblotted His₆ΔhSec34 protein.

Northern analysis – Northern blot analyses were performed using human polyA⁺ RNA blots (MTN) purchased from Clontech (Palo Alto, CA). A 1255 bp EcoRI fragment of hSec34 cDNA corresponding to amino acids 210-423 was labeled with α³²P-dCTP in a random priming reaction and used as the probe. Prehybridization and hybridization were performed in standard hybridization buffer(16) containing 50% formamide at 42°C.

Cell culture and transfection – HeLa cells used in this study were cultured at 37°C in DMEM supplemented with 15mM HEPES, 2.5mM L-glutamine, 10% fetal bovine serum and 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B in a 5% CO₂ incubator. Transfections were performed using the Gene Jammer Transfection Reagent according to the manufacturers recommendations (Stratagene, La Jolla, CA). Cells were split in to 4-chamber coverglass dishes (Nalge Nunc International) at a density of 7 x 10⁴ cells per well 24 hours before transfection.

Cell fractionation – HeLa cells were cultured to confluence in 100mm dishes. Cells were washed 3 times with phosphate buffer saline (PBS) and were scraped in 1 ml of 20mM Heps KOH buffer, pH 7.4 supplemented with a proteinase inhibitor cocktail (Roche, Indianapolis, IN). The cells were disrupted using a Potter homogenizer and cell lysis was verified using phase contrast microscopy. Cytosol and membranes were separated by centrifuging the lysate in a Beckman TLA-55 rotor at 150,000xg (30 min, 4°C). The pellet was resuspended...
in 1 ml of 20mM Hepes KOH buffer, pH 7.4, 0.1 M NaCl and 1% 3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) and incubated on ice for 30 minutes. Insoluble material was removed by centrifugation at 21,000 x g for 5 min, 4°C to yield a solubilized membrane fraction.

**SDS-PAGE and Western blotting** – Protein samples were solubilized in SDS-PAGE sample buffer, heated at 95°C for 5 min and analyzed on 12% gels. For Western blotting, separated proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with 10% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 30 min and were incubated with affinity-purified rabbit anti-hSec34p antibodies (1:500) in 5% BSA TBST for one hour at room temperature. Primary antibody binding was detected using goat anti-rabbit horseradish peroxidase conjugated secondary antibodies diluted in 5% milk TBST for 40 min at room temperature. Secondary antibodies were detected using a chemiluminescence reagent kit (NEN™ Life Science Products, Boston, MA).

**Glycerol velocity centrifugation** – HeLa cell cytosol or CHAPS solubilized membranes (2 mg total protein) were layered onto a 12 ml linear 10 - 30% glycerol (w/v) gradient in 20mM Hepes KOH, pH 7.4. Molecular weight markers purchased from Sigma (St. Louis, MO) were loaded onto a second gradient (thyroglobulin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; BSA, 66 kDa). The gradients were centrifuged in a Beckman SW40 rotor at 120,000xg for 15 h with slow acceleration and deceleration. Fractions (1ml) were collected through a hole punched in the bottom of the tube and concentrated by TCA precipitation (17).

**Tissue distribution** – Freshly harvested rat tissues (100mg) were homogenized in 200µl 2% SDS, 10mM Tris HCl, pH 7.4, 1mM EDTA using a Potter-Elvehjem homogenizer. The lysates were heated 5 min at 95°C and 20 min at 60°C, then were cleared by centrifugation at 21,000 x g, 10 min, at room temperature. Protein content was measured using the BCA reagent (Pierce, Rockford, IL) to normalize sample loading for western blot analysis.
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Immunofluorescence microscopy - Cells grown on coverslips or in 4-chamber coverglass dishes were fixed with 3% paraformaldehyde in PBS for 10 min. After quenching aldehyde groups with 0.1% of sodium borohydride in PBS, the cells were permeabilized with 0.1% saponin in PBS. Double immunofluorescence labeling was performed with rabbit and mouse antibodies diluted 1:500 in PBS containing 0.2% fish skin gelatin, 1% bovine serum albumin and 0.1% saponin. Reactions contained affinity-purified rabbit anti-hSec34p antibody and either mouse monoclonal anti-membrin antibody (Stressgene, Victoria, BC) or mouse monoclonal anti-p115 (M.G. Waters, Princeton University, Princeton, NJ). Polyclonal affinity-purified anti-TGN38 was a generous gift from M.A. McNiven (Mayo Clinic, Rochester, Minnesota). Secondary goat anti-rabbit-Alexa488, goat anti-rabbit-Texas Red and goat anti-mouse-Alexa594 (Molecular Probes, Eugene, OR) antibodies were used at 1:500 dilution. No cross-reaction was observed in control experiments. Coverslips were mounted in Prolong (Molecular Probes, Eugene, OR) and viewed using a x63 1.4na PlanApochromatic objective fitted to a Zeiss Axiovert S microscope (Thornwood, NY) equipped with filters specific for green and red fluorophores and a Hamamatsu C5985 monochrome chilled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Digitized images were cropped, colored, assembled and labeled in Adobe Photoshop.

The imaging of living cells expressing different proteins fused with green (GFP), cyan (CFP) and yellow (YFP) fluorescent proteins was performed in 4-chamber coverglass dishes (Nalge Nunc International) using the same equipment and the appropriate filter sets. For experiments with Brefeldin A (BfA) and nocodazole, HeLa cells were transfected with pGFP-hSec34. After 24-36 hours, the cells were treated with 1-5 µg/ml BfA at 37°C for 15-30 min or with 10µM nocodazole for 2 hours. For double nocodazole/BfA experiments, the cells were first incubated with 10 µM nocodazole for 2 hours at 37°C, BfA (5 µg/ml final concentration) was then added, and the living cells were imaged at 5 min intervals. VSVG-ts045-GFP - transfected cells were either kept at the non-permissive temperature (39.5°C) or shifted to 15°C, 25°C, or 32°C for 1 hour. After paraformaldehyde fixation, cells were labeled with primary antibodies followed by Alexa 594 –labeled anti-mouse and Texas Red – labeled anti-rabbit secondary antibodies as described above.
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Results

Because many secretory factors are evolutionarily conserved, we determined if the components of the yeast Sec34p/Sec35p complex were also conserved in higher eukaryotes. Searches of GenBank did not yield any Sec35p homologues, but several human expressed sequence tags (ESTs) were discovered with a high degree of similarity to Sec34p.

We used the primary sequence of EST z21242 to design an EST-specific oligonucleotide and used the RACE procedure to amplify a 5' 850 bp long fragment from human colon cDNA. The resulting DNA fragment encoded a 269 amino acid polypeptide highly homologous to Sec34p. A complete 4.5 kb human cDNA was obtained by PCR screening of an arrayed human fetal brain cDNA library with hSec34 specific primers. The complete cDNA for hSec34 was sequenced (GenBank accession number AF349676) and found to contain a 2484 bp open reading frame encoding a 828 amino acid polypeptide homologous to Sec34p with a calculated molecular weight of 94kDa. The hSec34p polypeptide has no predicted transmembrane or other membrane anchoring sequences and contains a predicted coiled-coil domain (amino acid residues 126-205) and a putative ‘leucine-zipper” domain (amino acid residues 399-420). The predicted amino terminal coil-coil domain is evolutionary conserved among all Sec34-like proteins (Figure 1). Several large fragments of the HSEC34 cDNA sequence are identical to to GeneBank AL139326 HTGS draft sequence from chromosome XIII clone RP11-351K3, indicating that HSEC34 gene is on human chromosome XIII.

The strongest homology between the yeast and human proteins is found in a region containing amino acid residues 174 to 280 (32% identity and 54% similarity) and in a region containing amino acid residues 554 to 626 (34% identity and 52% similarity), indicating that the functional elements of the protein could reside in these regions. These regions are also highly conserved in potential homologues of Sec34 found in the Drosophila melanogaster genome data base (AAF51107) and the genomes of Schisosaccharomyces pombe (CAB51337), Candida albicans (unfinished fragment of complete genome Contig6-2488 obtained from the Stanford DNA Sequencing and Technology Center), Arabidopsis thaliana (AAG30981), and Caenorhabditis elegans (computed from the genomic Y71F9AM DNA
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sequence available at the Sanger center (www.sanger.ac.uk). The aligned amino acid sequences of the putative Sec34p-related proteins are shown in Figure 1, and their pairwise sequence identities are shown in Table 1. We also have found that the carboxyl terminal half (amino acid residues 301-807) of hSec34p is similar (21% identical and 40% similar) to the central domain (amino acid residues 610-1106) of the endosomal tethering factor EEA1 (data not shown).

Northern blot analysis for HSEC34 revealed a transcript of about 4.5 kilobases in length that is abundantly and ubiquitously expressed in all tissues examined with the highest RNA level observed in pancreas and testis and the lowest level found in lung (Fig. 2a). By contrast the level of a control transcript (β-actin) was more abundant in lung than in pancreas and testis. Ubiquitous expression is indeed a feature expected of an essential molecule like Sec34. Therefore we conclude that the cDNA isolated from the fetal brain library represents a full-length hSec34 RNA molecule.

The sequence encoding amino-terminal part of hSec34p was used to develop protein-specific rabbit polyclonal antibodies. Affinity-purified anti-hSec34p antibodies recognize a 94kDa protein in HeLa cell extracts (Figure 3a, lane 1), numerous rat tissues (Figure 2b) and in extracts from CV-1, HEK 293, and all other mammalian cell lines tested (Figure 3b and data not shown). Interestingly, two different high molecular weight species in addition to the 94kD polypeptide are detected in brain and heart (Figure 2b). This may indicate the presence of a family of hSec34-immunologically-related proteins with different tissue distributions. These proteins may be involved in a specific membrane trafficking pathways in specialized tissues. Additional experiments are required to determine if these immunologically related proteins are functionally and structurally related to the hSec34p.

To determine if the major 94kDa protein was hSec34p, HeLa cells were transfected with hSec34p under control of CMV promoter and analyzed by western blotting. Overexpressed hSec34p comigrated with the endogenous 94kDa immunoreactive protein (Figure 3a, lane 2) indicating that hSec34p is encoded by the HSEC34 cDNA that we cloned. Further, a new immunoreactive 120kDa protein appeared in cells engineered to overexpress a GFP-hSec34p hybrid protein (Figure 3a, lane 3).

We next determined if hSec34p, like its yeast orthologue Sec34, was a peripheral membrane protein. In proliferating HeLa cell cultures (30-50% confluency), 60-90% of the
hSec34p was associated with the membrane pellet (Figure 3b, HeLa, lane M). A similar distribution was observed in CV-1 cells (Figure 3b, CV-1), and several different mammalian tissue extracts (data not shown). A membrane-bound pool of hSec34p was also concentrated on Golgi membranes purified from rat liver (Figure 3c).

To determine if soluble hSec34p exists in a high molecular weight complex, membranes and cytosols were fractionated on glycerol velocity gradients (Figure 3d, C-cytosol, M-membrane). In HeLa cytosols, hSec34p migrated as a 300kDa species, indicated that it is part of a protein complex (Figure 3d). In HeLa membranes, hSec34p was found in even larger protein complex(es), that may indicate associations with cognate t-SNARE membrane receptors. These observations for hSec34p are consistent with those for yeast Sec34p which is also associated with a high molecular weight protein complex (13,14).

hSec34p co-localizes with the v-SNARE membrin in living HeLa cells at 37°C, 15°C and in the presence of nocodazole.

We determined the intracellular localization of hSec34p using affinity-purified anti-hSec34p antibodies (Figure 4a). Indirect immunofluorescence microscopy revealed that hSec34p is primary localized in the juxtanuclear region of HeLa cells along with the v-SNARE membrin, a cis/medial-Golgi localized protein (18) (Figure 4a). Similar co-localization was observed in living HeLa (Figure 4a, middle row) and CV-1 (Figure 4b, upper row) cells co-transfected with plasmids encoding CFP-membrin (19) and YFP-hSec34. To more rigorously assess the colocalization of hSec34p and membrin, a temperature shift experiment was performed.

Incubation of cells at 15°C inhibits anterograde ER-to-Golgi transport (20,21), whereas retrograde transport appears less affected such that the net effect on the localization of a cis-Golgi protein is redistribution from the juxtanuclear region to enlarged peripheral vesicular-tubular compartment (VTC) (22). To determine if hSec34p exhibits such a redistribution in concert with membrin, HeLa cells were incubated at 15°C for 3 h. YFP-hSec34p and CFP-membrin were present on membrane elements scattered throughout the cell and colocalized in all structures (Figure 4a, lower row). We also used a pharmacological approach to explore the colocalization of hSec34p and membrin. Nocodazole treatment induces microtubule depolymerization that leads to fragmentation and dispersal of the Golgi (23). Figure 4b shows that after nocodazole treatment YFP-hSec34 was localized on the
same Golgi fragments as was CFP-membrin (lower row). Thus, in three different situations, hSec34p is localized to the same cis/medial-Golgi compartment as is membrin.

We used CFP-hSec34 to confirm that our CFP, GFP and YFP fusion proteins had biochemical properties consistent with those we have defined for endogenous hSec34p. CFP-hSec34 expressed in CV-1 cells (Figure 3b) and in HeLa cells (data not shown) was found in both cytoplasmic and membrane fractions similar to endogenous hSec34p. CFP-hSec34 was also efficiently incorporated into high molecular weight (~300kDa) complexes as defined by glycerol velocity gradients in both soluble (Figure 3d, CV-1p, C) and membrane (Figure 3d, CV-1p, M) fractions. We suggest that the functional pool of hSec34p contains both CFP-hSec34 and hSec34p (fraction marked *). In addition to this functional pool, there is also a large pool of monomeric CFP-hSec34p in cytosols. This is most likely due to overexpression because monomers of endogenous hSec34p are not detectable in either the HeLa or the CV-1 cytosols.

**GFP-VSVG moves though hSec34p-positive compartment.** To provide more direct evidence that hSec34p localized to the cis-face of Golgi apparatus, we used the temperature-sensitive vesicular stomatitis virus G protein, tsO45 VSVG that transits the secretory pathway (24). HeLa cells were co-transfected with pCFP-hSec34 and a plasmid encoding tsO45 VSVG fused with GFP (GFP-VSVG) (25). In cells expressing GFP- VSVG at 39.5°C, newly synthesized protein is trapped in the ER, while CFP-hSec34 is localized in the juxtanuclear region (Figure 5, upper row). When new protein synthesis was inhibited with cycloheximide and cells were incubated at 15°C, the GFP-VSVG preferentially accumulated in pre-Golgi intermediates (26) where its distribution partially overlapped with that of CFP-hSec34p (Figure 5, 15°C row). Some VSVG was observed at ER exit sites at the cell periphery (marked by arrowheads) that lacked CFP-hSec34. Incubation at 25°C led to a redistribution of GFP-VSVG to the Golgi apparatus where it is completely co-localized with CFP-hSec34. Finally, prolonged incubation at 32°C led to redistribution of GFP-VSVG from the CFP-hSec34-positive compartment to the plasma membrane (figure 5, lower row). Thus, a model secretory protein transits the hSec34 compartment.
**GFP-hSec34p localization is distinct from the localization of p115 and TGN38.** The vesicle tethering factor p115 is predominantly associated with vesicular-tubular clusters (VTCs) adjacent to the Golgi stack (27), and cycles extensively between the Golgi and the earlier compartments of the secretory pathway (28). A yeast homologue of p115, Uso1p has been characterized and shown to function in the same step of the secretory pathway as does Sec34p in tethering ER-derived vesicles to cis-Golgi cisternae (11,13,29). Secretory membrane trafficking in mammalian cells has more morphologically distinct steps compared to the yeast secretory pathway (for instance, yeast do not have morphologically identifiable VTCs) and it was therefore important to compare localization of hSec34 and p115. Yeast Sec34p has been partially co-localized with trans-Golgi marker proteins (15). We have tested if hSec34p is co-localized with either human p115 (30,31) or the trans-Golgi marker TGN38 (32,33). In HeLa and CV-1 cells, GFP-hSec34 was generally localized in what appear to be the same juxtanuclear membranes as p115 and TGN38 (Figure 6), but the GFP-hSec34 signal only partially overlapped with p115 and TGN38 in both untreated and nocodazole-treated cells. Interestingly, after a short treatment with nocodazole (30-60 min), GFP-hSec34 bearing Golgi fragments were completely separated from TGN38-positive membranes (data not shown). The distinctive localization of GFP-Sec34 on the p115 and TGN38-negative membranes was even more evident when the Golgi complex was analyzed by confocal microscopy (Figure 6b). Thus, GFP-Sec34 is either localized to distinct Golgi membrane domains devoid of p115 and TGN38 or there is intermingling of distinct hSec34p positive and p115 and TGN38 positive Golgi vesicles in the juxtanuclear compartment.

**hSec34p association to Golgi membranes is sensitive to Brefeldin A.** Treatment of cultured cells with fungal metabolite brefeldin A (BfA) induces the formation of extensive membrane tubules from the Golgi apparatus, trans-Golgi network, and early endosomes in a microtubule-dependent manner (34). This is thought to be due to BfA’s inhibition of nucleotide exchange onto ADP-ribosylation factor (ARF), a low-molecular weight GTP binding protein (35) that prevents assembly of cytosolic coat proteins (including COP I components) onto target membranes. At the same time, extensive retrograde transport of Golgi components to the ER mediated by growth of Golgi tubules occurs in the presence of BfA, leading to a complete loss of Golgi structure (22). After a 30 min BfA treatment,
normally Golgi resident proteins are detected in a characteristic ER pattern, whereas COPII components and tethering factor p115 are found in punctate structures scattered throughout the cell (28,36). In cells treated with BfA, GFP-hSec34 (Figure 7a,c), as well as the endogenous hSec34p (Figure 7b), were displaced from the Golgi cisternae to the cytosol. After incubation with BfA for 30 min a ~7 -fold increase in soluble hSec34 was observed (Figure 7b). The diffuse distribution of hSec34 after BfA treatment was distinct from that of p115 that was found in punctate structures (Figure 7c). In cells first pretreated with Nocodazole and then incubated with BfA (Figure 7a, lower row), GFP-hSec34 was displaced from the Golgi fragments to the cytosol with kinetics similar to that of control cells.

**hSec34 associated with static Golgi cisternae and excluded from transport vesicles.**

Having established that hSec34p is primarily associated with the cis/medial Golgi apparatus, we sought to determine if hSec34 was also present on ER-derived or intra-Golgi vesicles. In yeast cells, Sec34p acts in the tethering of ER-derived vesicles to cis-Golgi. In an in vitro trafficking assay, acceptor (Golgi) membranes prepared from sec34-2 mutant cells were found to be defective (13). The t-SNARE protein Sed5p has also been shown to operate in the same compartment (37). Therefore, we compared the localization of YFP-hSec34 and the t-SNARE CFP-Syntaxin5 in time lapse studies of HeLa cells (Figure 8a). Both molecules were largely localized in the juxtanuclear region, but whereas CFP-Syntaxin5 was also found on rapidly moving vesicular structures (Figure 8a, left column) probably representing anterograde and intra-Golgi vesicles (18), YFP-hSec34 remained localized to relatively static Golgi cisternae (Figure 8a, left column). The GFP-hSec34 was not associated with the β-COP-positive vesicular structures (data not shown). A similar situation was observed when HeLa cells were co-transfected with plasmids encoding YFP-hSec34 and GFP-hSec13. As reported previously(38), GFP-hSec13 was localized to small cytoplasmic vesicles distributed throughout the cytoplasm but most concentrated in a juxtanuclear region (Figure 8b, right panel). By contrast, YFP-hSec34 was exclusively found on the juxtanuclear Golgi stacks (Figure 8b, left panel).
Discussion

In this study, we report the cloning and characterization of a novel membrane-associated human protein, p94. Based on three independent lines of evidence, we propose that the ubiquitously expressed p94 is the orthologue of the yeast Golgi tethering factor Sec34p. p94 has sequence and structural homology to Sec34p; it partially localizes to membraness, it is found in high molecular weight complexes; and it localizes morphologically to the cis/medial-Golgi cisternae. We have therefore named this new protein hSec34p.

In addition to hSec34p, Sec34 homologues are encoded in genomes of different eukaryotic species ranging from yeast (Candida and Schizosaccharomyces), worms (Caenorhabditis), plants (Arabidopsis) and flies. Interestingly, all of the Sec34-like proteins are very similar in length with sizes ranging from 735 to 884 amino acids. The strongest homology between the different Sec34-related proteins is found in two regions, one located in the amino-terminal region of the protein (amino acid residues 174 to 280 in hSec34p) and another in the carboxyl-terminal region (amino acid residues 554 to 626 in hSec34p). In addition, in all Sec34-related proteins, the polypeptide sequence immediately adjacent or partially overlapping the amino terminal homology domain (amino acid residues 126 to 206 in hSec34p) is predicted to form a coiled-coil structure. The predicted coiled-coil domain and the amino-terminal homology domain are both present in temperature-sensitive truncated forms of yeast Sec34p (14) that are functional at permissive temperatures. This region may therefore represent a unique feature of Sec34-protein family that is responsible for Golgi binding by the Sec34p-complex in mammalian cells. The carboxyl-terminal part of the protein, which is dispensable in yeast, may play a structural role in stabilizing Sec34p complex (VL and ES, unpublished observations). Interestingly, mammalian database searches using the human Sec34p sequence revealed that the carboxyl-terminal two-thirds of the protein (amino acid residues 301 to 810) is 40% similar to the early endosomal tethering factor EEA1. EEA1 has been shown to physically interact with rab5 and the endosomal t-SNARE syntaxin 6 (39,40). In the yeast cell, Sec34p and the Rab protein Ypt1p play a role in tethering ER-derived vesicles to the cis-Golgi (13). Thus, it is possible that the evolutionary conserved domains in Sec34p may function to connect Rab proteins and SNAREs to facilitate vesicle tethering to the cis-Golgi in all eukaryotic cells.
We have determined that hSec34p is a peripheral membrane protein localized to the cis/medial cisternae of Golgi apparatus. On these membranes it colocalizes with the cis-Golgi operating v-SNARE membrin and its t-SNARE partner syntaxin 5. Unlike the SNAREs, which are present on both the relatively static Golgi cisternae and on rapidly moving transport vesicles (18), hSec34p only localizes to the Golgi cisternae, a feature that is in good agreement with a proposed role for hSec34p as component of vesicle tethering machinery.

Characterization of the soluble and membrane-bound pools of hSec34p on glycerol velocity gradients demonstrate that the protein is a part of a high molecular weight complex(es). Similarly, yeast Sec34p behaves as a component of a high molecular weight complex when soluble proteins are separated on gel filtration columns (13,14) and on glycerol velocity gradients (our unpublished observation). In this complex, yeast Sec34p is stably associated with another tethering protein Sec35p (12-14). However, searches of GenBank did not reveal any human Sec35p homologues. Nearly half of Sec35p is predicted to form a coil-coil structure (12), a feature common for all proteins involved in vesicle tethering. HSec34p may well interact with proteins other than a Sec35p homologue in both soluble and membrane-bound fractions. Indeed, immunoprecipitations from the cytoplasmic and from detergent-solubilized membrane fractions isolated from [35S]methionine-labeled cells reveal several polypeptide bands with molecular sizes distinct from what we would expect for a Sec35p homologue (data not shown).

When the dynamics of hSec34p were analyzed together with anterograde transport of ER-arrested VSVG, the picture was also largely in agreement with the proposed Golgi tethering role for the hSec34 protein complex. Transfected cells incubated for 6 h at 39.5°C showed distinct ER staining of VSVG-GFP. At 15°C a fraction of VSVG clearly moved to ER-exit sites and to scattered Golgi elements where it colocalized with CFP-hSec34p. Colocalization of hSec34p with the anterograde cargo was even more evident after incubation at 25°C, then all VSVG moved to the Golgi apparatus. However, after prolonged incubation at the permissive temperature of 32°C, VSVG moved to plasma membrane where it no longer colocalized with hSec34p.

In *Saccharomyces cerevisiae*, SEC34 genetically interacts with another vesicle tethering factor USO1 (11,13,29). Uso1p in yeast is clearly involved in movement of anterograde ER-
derived vesicles to Golgi apparatus (11,13,29) and full-length Uso1 protein is required for the assembly of functional ER-Golgi SNARE complexes (11,13,29). The phenotype of sec34 mutants is more complex. Besides the ER to Golgi trafficking defects (11,13,29), sec34/grd20 mutants mislocalize several Golgi-resident proteins and underglycosylate the secretory enzyme invertase (11,13,29). This feature was previously observed for mutant vti1, a v-SNARE involved in the retrograde intra-Golgi trafficking (11,13,29). In addition, Sec34p physically interacts with the sub-set of yeast SNARE proteins involved in retrograde trafficking (our unpublished observations).

The mammalian orthologue of Uso1p, p115, participates in the assembly and maintenance of normal Golgi structure and is required for ER to Golgi vesicle trafficking at a pre-Golgi stage (41,42). We find that p115 is only partially co-localized with hSec34p on Golgi cisternae and on scattered Golgi fragments in cells treated with nocodazole. Although both proteins appear to be localized on the same membrane elements, they are apparently preferentially enriched in different membrane sub-domains. As is the case in yeast, it is possible that in mammalian cells both p115 and the hSec34 protein complex are regulating different sides of cis-Golgi-oriented membrane trafficking. In this model, p115 would tether anterograde ER-derived vesicles while the hSec34p complex would regulate proper targeting of retrograde intra-Golgi vesicles. Additional experiments using in vitro transport assays and the cloned hSec34 we have characterized will be required to test this model.
Acknowledgements

We are grateful to Fred Gorelick, Mark McNiven, Richard Scheller and Gerry Waters for their generous gifts of antibodies and GFP-tagged proteins.
References

Human homologue of Sec34p

Human homologue of Sec34p

Figure Legends

Figure 1. Protein sequence analysis of Sec34p homologues.
Shown are the deduced amino acid sequences of S. cerevisiae Sec34 (NP_011084), human hSec34 (AF349676), D. melanogaster AAF51107, S. pombe CAB51337, A. thaliana AAG30981, C. albicans (translation of the unfinished fragment of complete genome Contig6-2488 obtained from the Stanford DNA Sequencing and Technology Center) and C. elegans (translated from the genomic Y71F9AM.2-4 DNA sequence available at the Sanger center [www.sanger.ac.uk]). Blackened boxes denote identical residues, and gray shading denotes conserved residues. The conserved amino-terminal and carboxyl-terminal domains are boxed. Optimal alignment was produced with the ClustalW program.

Figure 2. mRNA and protein expression levels of hSec34p in different mammalian tissues. A. Northern blot analysis of hSec34p and β-actin was performed with human mRNA samples (MTN blot, Clontech). B. To determine the expression pattern of Sec34p, homogenates were prepared from different rat organs. Each sample (50 µg of protein) was separated on 12% gels and the rat Sec34p was visualized by immunoblotting using affinity purified anti-hSec34p antibodies.

Figure 3. hSec34p is a peripheral Golgi protein existing in a large complexes. A. Affinity purified rabbit antibodies raised against recombinant hSec34p recognized a protein of ~94 kDa in whole cell lysates of HeLa cells (lane 1) and in lysates from cells transfected with pCMV-hSec34 (lane 2) and pGFP-hSec34 (lane 3). The GFP-hSec34 fusion protein migrated as a 120 kDa protein. For this assay, cells from one 100mm dish were lysed in 200 µl of preheated (95°C) SDS sample buffer. The lysates were clarified and 5 µl analyzed by immunoblotting after separation on 12% gels. B. To estimate the distribution of hSec34p between cytosolic and membrane fractions, HeLa and CV-1 (wild type and transfected with pGFP-hSec34) cells were grown to 50% confluency collected in PBS, and lysed by vortexing with glass beads. Cytosol and membrane fractions were separated centrifugation at 150,000 xg using a Beckman TLA55 rotor. The volumes for both fractions were adjusted.
Human homologue of Sec34p

and equivalent proportions of each fraction analyzed by SDS-PAGE and immunoblotting using anti-hSec34p. C. To determine is hSec34p is enriched in Golgi fractions, rat liver Golgi membranes were isolated from a light mitochondrial fraction on a discontinuous sucrose gradient (43). The total liver lysate (total) and Golgi membranes (Golgi) (20µg of protein each) were separated on 12% gels, and immunoblotted using affinity purified anti-hSec34p antibodies. D. Cytosol (C) and CHAPS-solubilized membranes (M) from wild type HeLa cells (HeLa) and from pCFP-hSec34 transfected CV-1 cells (CV-1p) were fractionated on 10-30% glycerol (w/v) gradients. The fractions were precipitated with trichloroacetic acid and equivalent amounts of each fraction were analyzed by SDS-PAGE and immunoblotting using anti-hSec34 antibodies. Fractions at which the molecular weight markers peaked are indicated. The asterisk indicates the deduced position of the functional hSec34p complex.

Figure 4. hSec34p is co-localized with the v-SNARE membrin on Golgi membranes. A., Upper row. HeLa cells were fixed, permeabilized and incubated with affinity-purified rabbit anti-hSec34p (1:100) and mouse anti-membrin (1:500) antibodies. Secondary goat anti-rabbit-Alexa488 and goat anti-mouse-Alexa594 (Molecular Probes) were used at the 1:500 dilution. Cytoplasmic “spotty” staining visible in cells labeled with anti-hSec34p was nonspecific since it was observed in cells incubated without primary antibodies (not shown). Nuclear DNA was stained blue with DAPI. Middle row. Cells were co-transfected with pYFP-hSec34 and pCFP-membrin, incubated for 24 h at 37°C and observed with the yellow (left panel) or cyan (middle panel) filter set. Lower row. Cells were transfected as before, grown for 24 h at 37°C and then incubated for 3h at 15°C. B. CV-1 cells were co-transfected with pYFP-hSec34 and pCFP-membrin, incubated for 24 h at 37°C and observed with the yellow (left panel) or cyan (middle panel) filter set. Upper row. The specific fluorescence for both molecules was observed in the untreated living cells on the same elaborated Golgi membranes. Lower row. Cells were treated with nocodazole for 120 min. The third picture of each row was generated by merging of the images in red and green (A, upper row), or yellow and cyan (other rows) channels. The right insert in B (upper row) shows a twofold magnified view of Golgi region. Bar, 10µm.
Figure 5. hSec34p colocalizes with a secretory cargo that moves through the Golgi. HeLa cells were co-transfected at the restrictive temperature (39.5°C) with plasmids encoding tsO45 VSVG-GFP and CFP-hSec34p. The cells were kept at 39.5°C (top panel) or shifted to either 15°C for 1 hour, (15°C panel), 25°C for 1 hour, (25°C panel) or 32°C for 2 hours( bottom panel). Living cells were viewed using yellow (for GFP) or cyan (for CFP) filter sets. As shown on the merged images at 25°C, but not at 39.5°C or 32°C, hSec34p overlapped with VSVG in the Golgi structures. At 15°C hSec34p and VSVG showed partial overlap with noticeable absence of CFP-hSec34p on peripheral ER exit sites (marked by arrowheads). Bar, 10µm.

Figure 6. GFP-hSec34p localization is distinct from p115 and TGN38. HeLa cells transfected with GFP-hSec34p plasmid were treated with 10µM nocodazole for 2 hours, then fixed and labeled with mouse monoclonal anti-p115 (upper panel) or with affinity-purified rabbit anti-TGN38 antibodies. Secondary goat anti-rabbit-Texas Red and goat anti-mouse-Alexa594 were used at a 1:500 dilution. A. Epifluorescent images of untreated and nocodazole-treated cells. The third picture of each row was generated by pseudocoloring and merging of the images in red and green channels. The right insert in shows a threefold-magnified view of the Golgi region. Bar, 10µm. B. Confocal images of Golgi apparatus in fixed untreated HeLa cells. Bar, 5µm.

Figure 7. Brefeldin A treatment displaces hSec34 from Golgi membranes. A. CV-1 cells transiently transfected with pGFP-hSec34, grown for 24 h at 37°C and then incubated for time indicated with Brefeldin A (5µg/ml)(upper row), or incubated first with nocodazole (10µM for 120 min) and then with BfA (lower row). Bar, 10µm. B. HeLa cells were grown to 50% confluency were treated with BfA for 30 min at 37°C. After incubation cells were immediately washed with ice-cold PBS, collected and lysed with glass beads. Cytosol (C) and membranes (M) were separated by centrifugation (150,000xg, TLA 55) and an equivalent volume of each fraction was loaded on 12% gels and analyzed by immunoblotting using anti-hSec34 antibodies. C. HeLa cells transfected with pGFP-hSec34, grown for 24 h at 37°C and then incubated for 30 min with BfA (5µg/ml). Cells were fixed with 2% paraformaldehyde and labeled with monoclonal anti-p115. The third picture was
Human homologue of Sec34p

generated by merging of the images in red and green channels. The right insert in shows a threefold-magnified view of juxtanuclear region. Bar, 10µm.

Figure 8. hSec34 is localized on static Golgi membranes and not on transport vesicles like Syntaxin 5 and hSec13.

A. HeLa cells were transiently co-transfected with pYFP-hSec34 and pCFP-Syntaxin5, incubated for 24 h at 37°C and observed with a cyan (left panel) or yellow (middle panel) filter set. Fluorescent images were taken every 10 sec. Syntaxin5 positive vesicles are marked by arrowheads.

B. HeLa cells were transiently co-transfected with pYFP-hSec34 and pGFP-hSec13, incubated for 24 h at 37°C and observed with the yellow (left panel) or green (middle panel) filter sets. Sec13 positive ER-derived COPII vesicles are marked by arrowheads. Bar, 10µm.
### Table 1. Relative sequence identity/similarity (%) of Sec34p-related proteins

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<th>S. pombe</th>
<th>C. albicans</th>
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<th>C. elegans</th>
<th>D. melanogaster</th>
<th>H. sapiens&lt;sup&gt;d&lt;/sup&gt;</th>
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*The percentage of amino acid identity/similarity of putative Sec34p-related proteins is displayed.

Optimal alignments were produced by the BLASTP program (BLOSUM62 matrix) available on NCBI Web page ([http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi](http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi)).

<sup>b</sup> translated from unfinished fragment of complete genome Contig6-2488 obtained from the Stanford DNA Sequencing and Technology Center

<sup>c</sup> computed from the genomic Y71F9AM DNA sequence available at the Sanger center ([www.sanger.ac.uk](http://www.sanger.ac.uk))

<sup>d</sup> GenBank accession number AF349676
**Human homologue of Sec34p**

**Fig. 1**

| H. sapiens | 1 |
| D. melanogaster | 77 |
| A. thaliana | 1 |
| S. cerevisiae | 1 |
| C. albicans | 1 |
| S. pombe | 1 |

| H. sapiens | 11 |
| D. melanogaster | 89 |
| A. thaliana | 206 |
| S. cerevisiae | 65 |
| C. albicans | 37 |
| S. pombe | 80 |

| H. sapiens | 212 |
| D. melanogaster | 133 |
| A. thaliana | 307 |
| S. cerevisiae | 171 |
| C. albicans | 142 |
| S. pombe | 189 |

| H. sapiens | 313 |
| D. melanogaster | 297 |
| A. thaliana | 383 |
| S. cerevisiae | 275 |
| C. albicans | 250 |
| S. pombe | 248 |

| H. sapiens | 409 |
| D. melanogaster | 393 |
| A. thaliana | 381 |
| S. cerevisiae | 356 |
| C. albicans | 379 |
| S. pombe | 381 |

| H. sapiens | 505 |
| D. melanogaster | 489 |
| A. thaliana | 468 |
| S. cerevisiae | 479 |
| C. albicans | 459 |
| S. pombe | 476 |

| H. sapiens | 605 |
| D. melanogaster | 571 |
| A. thaliana | 538 |
| S. cerevisiae | 585 |
| C. albicans | 530 |
| S. pombe | 540 |

| H. sapiens | 712 |
| D. melanogaster | 675 |
| A. thaliana | 640 |
| S. cerevisiae | 691 |
| C. albicans | 632 |
| S. pombe | 644 |

| H. sapiens | 813 |
| D. melanogaster | 786 |
| A. thaliana | 750 |
| S. cerevisiae | 788 |
| C. albicans | 721 |
| S. pombe | 724 |
Human homologue of Sec34p

Fig 2.

A. [Diagram showing various tissues and their corresponding protein expressions, with arrows indicating Sec34 and β-actin bands.]

B. [Diagram showing samples labeled 1 to 9 with corresponding Sec34p bands.]
Fig 3.

A. α-hSec34p

B. HeLa and CV-1p

C. Total and Golgi

D. HeLa and CV-1p

MW (kD): 660kD and 200kD

Markers: CFP-hSec34p and hSec34p

Glycerol content: 30% and 10%

* indicates a specific marker or condition.
Fig. 4
A.

B.
Human homologue of Sec34p

Figure 5.

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<tr>
<td>25°C</td>
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<tr>
<td>32°C</td>
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</table>
Fig. 6
A.

B.
Human homologue of Sec34p

Fig. 7
A.

B. [BfA] 0 1µg/ml 5µg/ml

C.
Fig. 8
A.

B.
Identification of a human orthologue of Sec34p as a component of the cis-Golgi vesicle tethering machinery
Elena S. Suvorova, Richard C. Kurten and Vladimir V. Lupashin

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