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13. ABSTRACT (Maximum 200 Words)

tGolgin-1 is a large, predominantly coiled coil peripheral membrane protein that associates with the *trans* Golgi network (TGN) by virtue of a C-terminal GRIP domain. In the first two funding periods, we (1) used a dominant negative expression approach to show that GRIP domain proteins function in endosome to TGN recycling, and (2) used expression ablation with inhibitory RNA to show that tGolgin-1 is specifically required for linking the Golgi complex to microtubule motors. In the last funding period, we focused more specifically on the mechanism by which tGolgin-1 impacts Golgi positioning. We find that tGolgin-1 is required for recycling of a subset of molecules from endosomes to the TGN, in particular glycosphingolipids. Glycosphingolipids are critically important signalling molecules as well as regulators of cholesterol distribution in the cell. In the absence of tGolgin-1, a subset of glycosphingolipids are mistargeted to and accumulated within late endosomes and lysosomes. These data suggest that the primary function of tGolgin-1 is in regulating lipid distribution within cells, and that Golgi positioning defects are a consequence of continued defects in glycosphingolipid/ cholesterol distribution. These results imply that altered lipid distribution may impact tumorigenesis in cancer cells that lack tGolgin-1 expression.

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INTRODUCTION

This report summarizes the training and research accomplishments over the third year of funding from award # DAMB17-01-1-0365. The award supports research directed toward understanding the function of tGolgin-1 (also known as golgin-245 and trans golgi p230), a peripheral membrane protein associated with the *trans* Golgi network (TGN) of mammalian cells (Erlich et al., 1996; Fritzler et al., 1995; Gleeson et al., 1996). tGolgin-1 is a member of a family of large peripheral membrane proteins that are predicted to consist largely of coiled-coil structure with a C-terminal GRIP domain. The GRIP domain was shown by several groups to mediate localization of these proteins to the cytosolic face of the Golgi complex and/or TGN (Barr, 1999; Brown et al., 2001; Kjer-Nielsen et al., 1999; Luke et al., 2003; Munro and Nichols, 1999). Two groups, including Dr. Chris Burd's group (Univ. of Pennsylvania) in collaboration with us, showed that GRIP domains mediate this function by binding to small Arf-like GTPase called Arl1 (Panic et al., 2003b; Setty et al., 2003). A function for tGolgin-1 or any of the other GRIP domain proteins has not yet been described. In addition to the GRIP domain and a large central coiled-coil region, tGolgin-1 is predicted to contain a small globular domain at the N-terminus.

BODY

We had shown, using a dominant-negative overexpression approach, that GRIP domain proteins contribute to the integrity of the TGN and to protein sorting and processing events that occur within the TGN, likely through regulating recycling of TGN resident proteins from endosomal compartments. Competitive displacement of endogenous GRIP domain proteins from the Golgi resulted in defects in resident integral membrane protein localization to the TGN and in TGN structure and function. Among those proteins that require GRIP-dependent TGN modifications and sorting functions are growth factor receptors associated with breast cancer induction, such as epidermal growth factor receptor and Her2, matrix metalloproteinases required for metastasis of breast tumors, and tumor antigens analogous to the melanoma-associated antigen Pmel17/gp100. Thus, GRIP domain overexpression may influence the growth and metastasis of tumors; moreover, dissecting how GRIP domain protein structure and function are related may reveal novel approaches for treatment of breast tumors. The goals of the experiments as proposed in the *Statement of Work* were to elucidate such structure/function relationships by dissecting functional domains of tGolgin-1 and by characterizing their interacting partners.
Since the inception of the funding from this grant, we have extended and published our preliminary data, accomplished several of the originally proposed aims in the *Statement of Work*, and made some new and very exciting discoveries regarding the function of tGolgin-1 specifically. As discussed in the last two progress reports, we modified the aims in the *Statement of Work* in accordance with these new discoveries. Below we detail our progress in the last funding period.

1. Role of GRIP domain proteins in maintenance of the TGN. A manuscript describing this work was in press at the time of the last funding period, and has now been published with Dr. Yoshino as the first author (Yoshino et al, 2003. J. Cell Sci. **116**: 4441-4454; **see appendix for reprint**). The main findings of the study are that overexpression of GRIP domains results in (1) displacement of endogenous TGN resident proteins, but not residents of the Golgi stack or other subcellular organelles, to peripheral membranes, including multivesicular late endosomes; (2) vacuolization of the Golgi and the TGN; (3) disruption of protein transport of the vesicular stomatitis virus G protein from the Golgi to the plasma membrane; and (4) disruption of furin-dependent cleavage of a substrate protein. We speculated that this implicates a role for GRIP domain proteins or GRIP domain ligands in regulating the recycling of TGN resident proteins from endosomal compartments back to the TGN. As discussed below, this speculation is now supported and extended in the case of tGolgin-1.

2. Functional analysis of tGolgin-1. These studies were initiated during the first funding period (2001-2002) as the result of emerging technology using inhibitory RNAs (RNAi; (Elbashir et al., 2001; Elbashir et al., 2002)), and were the focus of the project in the last funding period. Dr. Yoshino used transfection of cultured cells with small inhibitory RNA duplexes (siRNA) to deplete endogenous tGolgin-1, and then assessed the effects on cell morphology, Golgi/ TGN function, and membrane recycling. Using immunofluorescence and immunoelectron microscopy, Dr. Yoshino showed that in the absence of tGolgin-1 expression, the entire Golgi complex, including the TGN, becomes fragmented and reforms as "mini Golgi" elements. These elements were functional based on the efficient transport of

secretory cargo from the endoplasmic reticulum (ER) to the plasma membrane. Dr. Yoshino found that the mini Golgi stacks accumulated at ER exit sites marked by the accumulation of GFP-tagged Sec13 (a component of the COPII coat involved in ferrying cargo molecules from the ER to pre-Golgi intermediates). This phenotype is similar to that observed in cells in which minus-end-directed transport of pre-Golgi elements along microtubules is disrupted (Burkhardt et al., 1997; Cole et al., 1996), suggesting that tGolgin-1 function is required for efficient transport of pre-Golgi elements from ER exit sites to the microtubule organizing centre (MTOC). Dr. Yoshino used quantitative analysis of the motility of these Golgi elements (using cells expressing a GFP-tagged Golgi marker, GRASP65) to show that the elements were relatively immobile, as in cells with disrupted microtubules. These data indicated that tGolgin-1 is required for activity or recruitment of minus-end-directed microtubule motors rather than for "trapping" of Golgi elements at the MTOC. The data constitute the first evidence that the TGN – via its peripheral membrane protein, tGolgin-1 - is required to maintain the positional integrity of the Golgi complex.

As discussed in the last progress report, Dr. Yoshino showed that the requirement for tGolgin-1 in Golgi motility is likely due to an indirect mechanism rather than through direct binding of dynein/dynactin subunits to tGolgin-1. First, no interactions between dynein/ dynactin and tGolgin-1 were observed using immunoprecipitation or chemical cross-linking from cell lysates or in vitro pull-down assays with recombinant proteins. Second, using immunofluorescence microscopy, pre-Golgi intermediates and TGN elements were found to move independently toward the MTOC in cells recovering from brefeldin A; tGolgin-1 was associated only with the TGN elements, and not the Golgi elements, indicating that the Golgi elements could effectively recruit and activate dynein/ dynactin motors with no associated tGolgin-1. We therefore hypothesized that the disruption of Golgi-associated dynein/dynactin activity in tGolgin-1-depleted cells is the result of a recycling defect, such that a molecule critical for dynein recruitment requires tGolgin-1 for retrieval from post-Golgi membranes to the Golgi complex.

In this last funding period, Dr. Yoshino tested and confirmed this hypothesis. Dr. Yoshino used a fluorescent-tagged form of the Shiga toxin B fragment (STxB) as a probe for endosome to TGN recycling. STxB lacks the toxin activity of the Shiga holotoxin, but is required for transporting the toxic A subunit from the plasma membrane to the ER (from where the A subunit translocates to the cytosol to exert its toxic effect). STxB binds to the glycosphingolipid, Gb3, at the plasma membrane, and is then efficiently inernalized. Normally, plasma membrane bound STxB is efficiently targeted to and accumulated within the Golgi complex within 45 minutes. By contrast, in cells depleted of tGolgin-1, internalized STxB is mistargeted to lysososomes, and accumulation in the Golgi is dramatically inhibited. This mistargeting is specific to the endosome-to-TGN recycling pathway, because tGolgin-1-depletioin has no effect on transferrin recycling or targeting of proteins from the TGN to endosomes. Dr. Yoshino has recenlty obtained evidence that STxB mistargeting may reflect a general defect in glycosphingolipid sorting in tGolgin-1-depleted cells, since a similar mis-accumulation in lysosomes is observed for a fluorescent analogue of glycosphingolipids, lactosyl ceramide. These data indicate that tGolgin-1 functions in glycolipid sorting from the plasma membrane to the Golgi, and thus may indirectly affect the recruitment and/or activation of dynein/dynactin motors to the Golgi.

A manuscript describing these results, on which Dr. Yoshino is first author, is currently under revision after an initial submission to *Molecular Biology of the Cell.* We predict that the paper will be at least in press by the end of the year. This work is important in showing a requirement for the TGN, and perhaps of glycosphingolipids and/or associated cholesterol, in positioning the Golgi complex. Its role in Golgi positioning and glycolipid distribution may affect a number of biologically important functions relevant to breast cancer formation, metastasis and treatment such as maintenance of cell polarity, cell motility, cytotoxic T cell target susceptibility, and cell signalling.

3. Molecular determinants of tGolgin-1 function. As discussed in the last progress report, this was the main thrust of the work proposed in the original *Statement of Work* and significant progress was made over the first two periods. Specifically:

- We found in collaboration with Dr. Chris Burd (Univ. of Pennsylvania) that GRIP domains mediate Golgi localization by virtue of direct binding to the small Arf-like GTPase, Arl1, which itself is recruited to the Golgi upon activation of another Arf-like GTPase, Arl3 (Setty et al., 2003. Curr. Biol. **13**: 401-404). This work was supported by data from two other laboratories

(Lu and Hong, 2003; Panic et al., 2003a; Panic et al., 2003b). Dr. Burd and one of the other laboratories have independently gone on to show that Arl3 is recruited to the Golgi in an unusual manner involving N-terminal acetylation and consequent binding to an integral membrane protein, Sys1 (Behnia et al., 2004; Setty et al., 2004).

- Dr, Yoshino found that the N-terminal domain of tGolgin-1 is capable of localizing to the Golgi complex by itself, and thus must be capable of binding to other macromolecules at the Golgi.

Due to the primary focus on Specific Aim 2 above, little progress was made in the last funding period toward this aim. Dr. Yoshino is trying to identify binding partners for the N-terminal domain at the Riken Institute in Japan, and a new post-doctoral researcher in my laboratory is probing which tGolgin-1 domains are required for tGolgin-1 function in mammalian cells. We anticipate that Dr. Yoshino will be a co-author on at least one more manuscript describing these results within another year.

Additional Training

Dr. Yoshino developed wonderfully as an independent researcher, extending a trend she began in the previous two funding periods. She developed Aims 2 and 3 above nearly completely independently of Drs. Marks and Lemmon, and as a result has made a very significant contribution to the field. Dr. Yoshino gained experience supervising technical staff and a graduate student, and became much more interactive with other lab members, faculty, and other scientists over the last year, networking and initiating new collaborations. Dr. Yoshino has matured phenomentally well in her abilities to develop new techniques on her own, to become enveloped in the literature, and to devise new experimental approaches based on her reading of the literature. Dr. Yoshino continued to improve to top form her presentation skills in group meetings and in personal interactions with members of the Marks, Lemmon, and Burd laboratories. In addition to in-house presentations, Dr. Yoshino presented her work at meetings of the Japanese Biochemical Society every year from 2001-2003, at the Molecular Membrane Biology Gordon Conference in 2001, and at a Keystone Conference on the Golgi in 2004. Her abstract to the Japanese Biochemical Society in 2003 was chosen for an oral presentation, which she delivered superbly according to my Japanese colleagues. Most importantly, Dr. Yoshino developed from a rather inexperienced, robotic student to a full-fledged colleague over the past three years. I am more proud of her accomplishments than of any other student or post-doctoral researcher under my tutelage in 8 years.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of analysis of GRIP domain overexpression
  - mislocalization of TGN resident integral membrane proteins
  - vacuolization of the TGN
  - disruption of TGN function (sorting to plasma membrane and furin-dependent proprotein processing)
  - disruption of endosome to TGN recycling
- Functional analysis of tGolgin-1 using siRNA and live cell imaging
  - dissociation of Golgi complex from microtubule organizing center
  - reformation of functional "mini-Golgis" at endoplasmic reticulum exit sites
  - lack of Golgi motility
  - indirect requirement of tGolgin-1 for minus-end-directed movement of Golgi fragments on microtubules
  - failure to detect interactions between tGolgin-1 and dynein/ dynactin components
  - defect in endosome-to-TGN cycling of glycosphingolipids
- Identification of Arl-1 as ligand for GRIP domains in yeast
- Initial characterization of tGolgin-1 N-terminal domain interactions with cellular factors

REPORTABLE OUTCOMES

1. Published manuscript: D. Cowan, D. Gay, B.M. Bieler, H. Zhao, **A. Yoshino**, J.G. Davis, M.M. Tomayko, R. Murali, M.I. Greene and M.S. Marks (2002). Characterization of mouse tGolgin-1 (golgin-245/ trans golgi p230/ 256kD golgin) and its upregulation during oligodendrocyte development. DNA and Cell Biol. **21**: 505-517.

2. Published manuscript: S.R.G. Setty, M.E. Shin, **A. Yoshino**, M.S. Marks and C.G. Burd (2003). Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 (Arl1p) is regulated by Arf-like GTPase 3 (Arl3p). Curr. Biol. **13**: 401-404.
3. Published manuscripts: **A. Yoshino**, B.M. Bieler, D.C. Harper, D.A. Cowan, S. Sutterwala, D.M. Gay, N.B. Cole, J.M. McCaffery and M.S. Marks (2003). A role for GRIP domain proteins and/or their ligands in structure and function of the *trans* Golgi network. J. Cell Sci. **116**: 4441-4454.
4. Manuscript under revision: **A. Yoshino**, C. Poynton, E.L. Whiteman, A. Saint-Pol, C. G. Burd, L. Johannes, E.L. Holzbaur, J.M. McCaffery and M.S. Marks. A requirement for the TGN and associated tGolgin-1 (p230, golgin-245) in Golgi accumulation at the microtubule organizing center. Under revision.
5. Abstract for Meeting of the Japanese Biochemical Society, 2002: **A. Yoshino**, B.M. Bieler, D.C. Harper, D. Cowan, D. Gay, J.M. McCaffery and M.S. Marks. Regulation of TGN protein localization by GRIP domain proteins.
6. Abstract for Meeting of the Japanese Biochemical Society, 2003: **A. Yoshino**, J.M. McCaffery and M.S. Marks. Characterization of the function of TGN-localized peripheral membrane protein, tGolgin-1.
7. Employment: Dr. Yoshino is now employed as a post-doctoral researcher at RIKEN Research Center for Allergy and Immunology, Yokohama City, Kanagawa, Japan.

CONCLUSIONS

Our research has assigned a function to tGolgin-1 and potentially other GRIP domain proteins, a group of peripheral Golgi-associated proteins for which no function had been previously known. These proteins, particularly tGolgin-1, appear to be involved in a critical step of macromolecule transport from endosomes to the TGN, a subcellular organelle required for protein sorting and modification within both the secretory and endocytic pathways. Disruption of this recycling pathway affects TGN function – such as delivery of critical growth factor receptors and proprotein cleavage of matrix metalloproteinases and other critical metastatic factors. Part of this disruption of function may be a consequence of misrouting of glycosphingolipids, as observed in the absence of tGolgin-1 expression. This latter requirement may explain an indirect requirement for tGolgin-1 in positioning the Golgi complex at the microtubule organizing center. Disruption of its function is thus anticipated to affect cell polarity, migration, and polarized secretion, as well as other cell functions dependent on cholesterol and glycosphingolipid balance. The gene encoding tGolgin-1 has been shown to be deleted in at least one lung carcinoma (Ishikawa et al., 1997), suggesting that manipulation of its function may be important for tumorigenesis. Continued work on the function and physical interactions of tGolgin-1 and other GRIP domain proteins may therefore provide us with insights into manipulation of tumors. More directly, elucidating the function of these mysterious peripheral membrane proteins will help us to understand the biology of intracellular protein transport.

Future work will focus on elucidating the cellular function of tGolgin-1 in glycosphingolipid metabolism, and on identifying interacting factors with the N-terminal domain of tGolgin-1 as a means of elucidating its mechanism of action.

REFERENCES

Barr, F.A. 1999. A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. *Curr. Biol.* 9:381-384.

Behnia, R., B. Panic, J.R.C. Whyte, and S. Munro. 2004. Targeting of the Arf-like GTPase Arl3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p. *Nature Cell Biol.* 6:405-413.

Brown, D.L., K. Heimann, J. Lock, L. Kjer-Nielsen, C. van Vliet, J.L. Stow, and P.A. Gleeson. 2001. The GRIP domain is a specific targeting sequence for a population of trans-Golgi network derived tubulo-vesicular carriers. *Traffic.* 2:336-344.

Burkhardt, J.K., C.J. Echeverri, T. Nilsson, and R.B. Vallee. 1997. Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* 139:469-484.

Cole, N.B., N. Sciaky, A. Marotta, J. Song, and J. Lippincott-Schwartz. 1996. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol. Biol. Cell.* 7:631-650.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494-498.

Elbashir, S.M., J. Harborth, K. Weber, and T. Tuschl. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods Cell Biol.* 26:199-213.

Erlich, R., P.A. Gleeson, P. Campbell, E. Dietzsch, and B.-H. Toh. 1996. Molecular characterization of *trans*-Golgi p230. A human peripheral membrane protein encoded by a gene on chromosome 6p12-22 contains extensive coiled-coil α-helical domains and a granin motif. *J. Biol. Chem.* 271:8328-8337.

Fritzler, M.J., C.-C. Lung, J.C. Hamel, K.J. Griffith, and E.K.L. Chan. 1995. Molecular characterization of golgin-245, a novel Golgi complex protein containing a granin signature. *J. Biol. Chem.* 270:31262-31268.

Gleeson, P.A., T.J. Anderson, J.L. Stow, G. Griffiths, B.H. Toh, and F. Matheson. 1996. p230 is associated with vesicles budding from the *trans*-Golgi network. *J. Cell Sci.* 109:2811-2821.

Ishikawa, S., M. Kai, M. Tamari, Y. Takei, K. Takeuchi, H. Bandou, Y. Yamane, M. Ogawa, and Y. Nakamura. 1997. Sequence analysis of a 685-kb genomic region on chromosome 3p22-p21.3 that is homozygously deleted in a lung carcinoma cell line. *DNA Res.* 4:35-43.

Kjer-Nielsen, L., R.D. Teasdale, C. van Vliet, and P.A. Gleeson. 1999. A novel Golgi-localisation domain shared by a class of coiled-coil peripheral membrane proteins. *Curr. Biol.* 9:385-388.

Lu, L., and W. Hong. 2003. Interaction of Arl1-GTP with GRIP domains recruits autoantigens Golgin-97 and Golgin-245/p230 onto the Golgi. *Mol. Biol. Cell.* 14:3767-3781.

Luke, M.R., L. Kjer-Nielsen, D.L. Brown, J.L. Stow, and P.A. Gleeson. 2003. GRIP domain-mediated targeting of two new coiled coil proteins, GCC88 and GCC185, to subcompartments of the trans-Golgi network. *J. Biol. Chem.* 278:4216-4226.

Munro, S., and B.J. Nichols. 1999. The GRIP domain - a novel Golgi-targeting domain found in several coiled-coil proteins. *Curr. Biol.* 9:377-380.

Panic, B., O. Perisic, D.B. Veprintsev, R.L. Williams, and S. Munro. 2003a. Structural basis for Arl1-dependent targeting of homodimeric GRIP domains to the Golgi apparatus. *Mol. Cell.* 12:863-874.

Panic, B., J.R.C. Whyte, and S. Munro. 2003b. The Arf-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Curr. Biol.* 13:405-410.

Setty, S.R.G., M.E. Shin, A. Yoshino, M.S. Marks, and C.G. Burd. 2003. Golgi recruitment of GRIP domain proteins by ARF-like GTPase 1 (Arl1p) is regulated by Arf-like GTPase 3 (Arl3p). *Curr. Biol.* 13:401-404.

Setty, S.R.G., T.I. Strochlic, A.H.Y. Tong, C. Boone, and C.G. Burd. 2004. Golgi targeting of ARF-like GTPase Arl3p requires its N-acetylation and the integral membrane protein Sys1p. *Nature Cell Biol.* 6:414-419.

APPENDICES

1. Manuscript reprint: Cowan , et al., 2002 (13 pages: pages 10-22).
2. Manuscript reprint: Setty, et al., 2003 (4 pages: pages 23-26).
3. Manuscript reprint: Yoshino, et al., 2003 (14 pages: pages 27-40).
4. Abstract from Meeting of the Japanese Biochemical Society, 2003 (1 page: page 41)

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Characterization of Mouse tGolgin-1 (Golgin-245/*trans*-Golgi p230/256 kD Golgin) and Its Upregulation during Oligodendrocyte Development

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ABSTRACT

As part of an effort to identify gene products that are differentially regulated during oligodendrocyte development, we isolated a mouse cDNA that encodes tGolgin-1, a homolog of the human protein known as golgin-245, *trans*-golgi p230, or 256 kD golgin. Human tGolgin-1 is the target of autoantibodies in patients with Sjögren's syndrome, and is thought to be involved in vesicular transport processes at the *trans*-Golgi network. Sequencing of cDNAs and EST clones comprising the full-length tGolgin-1 transcript predict marked homology with the amino- and carboxy-terminal regions of the human protein, but more limited homology within the central predicted coiled-coil region. Epitope tagged, truncated forms of mouse tGolgin-1, like those of its human homolog, were localized at steady state to the Golgi/*trans*-Golgi network in transfected cells. The tGolgin-1 message was expressed in all tissues examined, but was highly upregulated in oligodendrocyte precursors at a stage just prior to myelination. This expression pattern suggests that tGolgin-1 may play a role in specialized transport processes associated with maturation and/or differentiation of oligodendrocyte precursors.

INTRODUCTION

OLIGODENDROCYTES ARE RESPONSIBLE for the highly complex process of myelination within the central nervous system. Oligodendrocytes arise from undifferentiated neuroepithelial precursors within the ventricular zone of the spinal cord (Orentas and Miller, 1998). These precursors undergo a complex developmental program during which they differentiate first into highly proliferative, pluripotent migratory cells, and then into nonmotile, immature oligodendrocytes that make direct physical contacts with target neurons (Pfeiffer *et al.*, 1993; McMorris and McKinnon, 1996). Mature oligodendrocytes synthesize myelin components, such as myelin basic protein, myelin-associated glycoprotein, myelin/oligodendrocyte protein, and proteolipid protein, which are incorporated into myelin-rich regions of the plasma membrane that enwrap axons of adjacent neurons (Campagnoni, 1988). These developmental changes are marked by the expression of distinct cell surface markers, particularly glycolipids or glycoproteins, and are accompanied by dramatic changes in cellular morphology and physiology. These changes include the extension of cell processes, changes in expression of adhesion molecules and growth factor receptors, and ultimately, the generation and compaction of myelin sheets at great distances from the cell body (Pfeiffer *et al.*, 1993).

The developmental changes in the physiologic and morphologic characteristics of oligodendrocyte precursors likely reflect alterations in the cytoskeleton and in the biosynthetic machinery of the cell. Indeed, unusual microtubule arrays (Lunn *et al.*, 1997) and peripheral Golgi-like structures (de Vries *et al.*, 1993) have been found in the extended processes of oligodendrocyte precursors. Furthermore, oligodendrocytes at later stages of development display a number of features characteristic of polarized cells (Pfeiffer *et al.*, 1993), such as differential sorting of viral glycoproteins (de Vries *et al.*, 1998). The generation of these unusual morphologic features and protein sorting pathways must be orchestrated within the cytoplasm and the central vacuolar system by structural and regulatory proteins, the expression of which is also likely to be developmentally regulated. For example, Rab3a and Rab3c, proteins respectively as-

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sociated with synaptic and endocrine secretory vesicles, are upregulated during later oligodendrocyte development (Madison *et al.*, 1996), and a specialized Rab protein, Rab22b, is expressed selectively in oligodendrocyte lineage cells (Rodriguez-Gabin *et al.*, 2001). It would be expected that Rab effector proteins and other components of the trafficking machinery would also be differentially regulated in these cells.

We previously described a novel cell surface determinant, termed OIP-1 (Oligodendrocyte Precursor protein-1), that is specifically expressed on developing oligodendrocyte precursors at a stage just prior to the onset of synthesis of myelin components (Gay *et al.*, 1997). In an effort to characterize the OIP-1 antigen, we used an anti-OIP-1 monoclonal antibody (mab) to screen a cDNA expression library from a postnatal day 8 mouse brain. Using this approach, we identified a set of cDNA clones that encode partial, alternatively spliced forms of a single gene product. Sequence analysis of cDNAs spanning the entire coding region identified the gene product as the murine homolog of a human protein previously identified as golgin-245 (Fritzler *et al.*, 1995), *trans*-golgi p230 (Kooy *et al.*, 1992), or 256 kD golgin (H. P. Seelig, Genbank accession #X82834) and referred to here as tGolgin-1. tGolgin-1 is a peripheral membrane protein of unknown function found on the cytosolic face of the *trans*-Golgi network (TGN) and associated vesicles (Gleeson *et al.*, 1996). It is part of a family of peripheral membrane proteins containing a large, central coiled-coil region and a C-terminal "GRIP" domain that is responsible for localization to the Golgi and/or TGN (Barr, 1999; Kjer-Nielsen *et al.*, 1999a, 1999b; Munro and Nichols, 1999; Brown *et al.*, 2001). tGolgin-1 mRNA was ubiquitously expressed, but was dramatically upregulated in oligodendrocyte precursors at the stage of development marked by expression of the OIP-1 determinant. We postulate that upregulation of tGolgin-1 during this stage of development facilitates either the establishment of peripheral Golgi-like structures in oligodendrocyte processes or the synthesis, targeting, or recycling of myelin constituents in preparation for myelin formation.

MATERIALS AND METHODS

Animals and tissue culture conditions

Virus-free BALB/c postnatal day 1 mice (Harlan, Indianapolis, IN) provided cerebral tissue for all primary oligodendrocyte and astrocyte cultures. Oligodendrocyte precursor culture conditions were as described in Gay *et al.* (1997). Cerebral cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 2–4 weeks to generate enriched astrocyte populations. The BALB/C7 fibroblast and HeLa cell lines (ATCC) were maintained in DMEM/10% FBS.

cDNA library production, screening, and sequencing

mRNA was extracted from brain tissue of postnatal day (P)8 mice using the Fastrack system (Invitrogen, Carlsbad, CA), converted into cDNA and directionally cloned using the ZAP Express cDNA II cloning kit (Stratagene, La Jolla, CA). Recombinant fusion proteins were screened for binding to anti-OIP-1 mab with the Picoblue Immunoscreening Kit (Stratagene). These original cDNAs were sequenced using automated sequencing facilities and T3, T7, and internal designed primers. Sequences were analyzed using Geneworks and BLAST software. Additional regions were characterized following identification of mouse EST clones with homology to regions of the human p230 cDNA further 5′ than the original mouse cDNA clones. EST clones with accession numbers (Acc) AA144704, AA561361, and AA389589 were obtained from Genome Systems Inc. (St. Louis, MO). The region between EST clone AA144704 and cDNA clone C91 was amplified by reverse transcriptase (RT) coupled polymerase chain reaction (PCR) from neonatal mouse brain RNA as described (Marks *et al.*, 1995) and cloned using unique NheI and PshAI restriction sites. The region encompassing EST clone AA098411 up to the NdeI site of EST clone AA144704 was also generated by RT-PCR from neonatal mouse brain RNA. The region corresponding to the coding region for the N-terminus of human p230 was generated by RT-PCR from adult mouse brain and liver mRNA using a degenerate oligonucleotide encoding the N-terminal human p230 residues and a reverse primer downstream of the NdeI site. The sequence of the 5′ UTR and the translation start site were inferred from overlapping genbank database entries (Acc BC003268, BF162586, BI151932, and BE304004). See Figure 1 for schematic diagram. Oligonucleotide sequences are available upon request. Sequences of amplified regions were confirmed from at least three cloned products of at least two separate RT-PCR reactions, and only sequences confirmed by two or more database entries are listed for the 5′ UTR. Coiled-coil predictions were done using the MTIDK matrix within the COILS v2.2 program from the Swiss Institute for Experimental Cancer Research (http://www.isrec.isb-sib.ch/software/COILS_form.html)

Northern analysis

RNA was isolated from the indicated tissues of female Balb/c mice using the FastTrack 2.0 kit (InVitroGen) for poly(A)+ RNA or the RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX) for total RNA. RNA samples (25 μg total or 2.5 μg polyA+) were fractionated on 0.8–0.9% agarose gels in formaldehyde/MOPS essentially as described (Sambrook *et al.*, 1989). Gels were soaked in 0.05 M sodium hydroxide for 20 min, then 20× SSC for 1 h prior to transfer onto Immobilon Ny+ filters (Millipore, Bedford, MA) overnight in 20× SSC. Blots were washed in DEPC-treated H_2O, and then baked at 80°C for 2 h. Digoxigenin-labeled probes were generated by PCR using the PCR DIG Probe Synthesis Kit from Boehringer Mannheim-Roche (Palo Alto, CA), and hybridized to filters and developed using the DIG Luminescent Detection Kit according to the manufacturer's instructions. Bands were visualized using a Molecular Dynamics (Sunnyvale, CA) Storm 760 Phosphor Imager and ImageQuant v. 1.1 software. The probe for tGolgin-1 encompassed nt. 110–1172, and the probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) encompassed the entire open reading frame (nt. 47 to 1045).

HA-tagged constructs

HA11 epitope tag sequences were constructed by PCR for ligation onto the N- or C-terminal coding regions of the clone C91 cDNA sequence. For an N-terminal tag, a PCR product including a 5′ Kozak consensus sequence, the coding region for an HA11 tag, and a region contiguous with the 5′ sequence for

C91 was subcloned into the SalI and Sph1 sites of C91 to generate HA-tGolgin-C1247. For a C-terminal tag, a PCR product contiguous with the coding region for the C-terminus of C91 and containing a 3′ HA tag followed by a stop codon was cloned into the Nru1 and Xba1 sites of C91 to generate tGolgin-C1247-HA. Resultant constructs were subcloned into the pCDM8.1 expression vector (Bonifacino *et al.*, 1990). Additional truncation products were generated by PCR from the original clones using similar designed primers, and all amplified products were verified by automated DNA sequencing.

Immunofluorescence microscopy

HeLa cells plated on glass coverslips in six-well dishes were transiently transfected using the calcium phosphate method (Sambrook *et al.*, 1989), fixed with 2% formaldehyde 40 h post-transfection, and processed for indirect intracellular immunofluorescence microscopy as previously described (Marks *et al.*, 1995). Antibodies included a rabbit antiserum to TGN46 (Prescott *et al.*, 1997) (a gift of V. Ponnambalam, Univ. Dundee), a rabbit antiserum to the β-COP subunit of COPI (a gift of Dr. J. Lippincott-Schwartz, National Inst. of Health, Bethesda, MD), anti-HA11 monoclonal antibody (BabCo, Richmond, CA) and secondary fluorochrome-coupled antibodies (Jackson Immunoresearch, West Grove, PA). Slides were analyzed on a Zeiss Axioplan fluorescence microscope (Zeiss, Thornwood, NY), and photographic images were digitized using a Nikon LS-1500 film scanner. In one experiment, images were captured with a Leica TCS laser confocal scanning microscope equipped with a 100×, 1.4 N.A. oil immersion lens and the Leica TCS software package.

Flow cytometry and cell sorting

Precursors from day 4 (populations 1 and 2) or day 9 (population 3) mixed mouse oligodendrocyte cultures were incubated with combinations of anti-OlP-1 (Gay *et al.*, 1997), anti-O4, R-Mab (Ranscht *et al.*, 1982) and O1 (anti-GalC) mabs and prepared for cytofluorography as described (Gay *et al.*, 1997). Cell sorting was accomplished using a FACStar Plus (Becton Dickenson, Franklin Lakes, NJ) and CellQuest software.

Semiquantitative RT-PCR

mRNA was extracted from 10^5 cells using Micro-FastTrack (Invitrogen), converted to cDNA with Superscript II reverse transcriptase (Life Technologies, Rockville, MD) and PCR-amplified using the High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). Semiquantitative PCR was carried out on 5×10^3 cell equivalents with an α^{32}P dCTP tracer using previously specified conditions (Tomayko and Cancro, 1998). Samples (2 μl) were removed at designated cycles, electrophoresed onto a 0.8% agarose gel, vacuum dried and subjected to autoradiography. Nonquantitative PCR was carried out for 40 cycles using mRNA from approximately 10^5 cells. The cDNA 1 PCR product was TA cloned and sequenced to show 100% identity to cDNA clone C91 (unpublished data). The actin primers were as specified (Tomayko and Cancro, 1998). The PLP primers were 5′gactacaagaccaccatctg3′ (nt. 309 to 328 and 5′gaagagggccaatcagt ggca3′ (nt. 923 to 943). The cDNA 1 primers for tGolgin-1 were 5′ggctgggagagctgaggcaga3′ (nt. 3260 to 3280) and 5′gttcaagctcttgtcctccag3′ (nt. 3640 to 3660). The cDNA 2 primers were 5′tggttcagagacttcagcact3′ (nt. 4730 to 4750) and 5′tgtgcagtcctgggaatctg3′ (nt. 5216 to 5235).

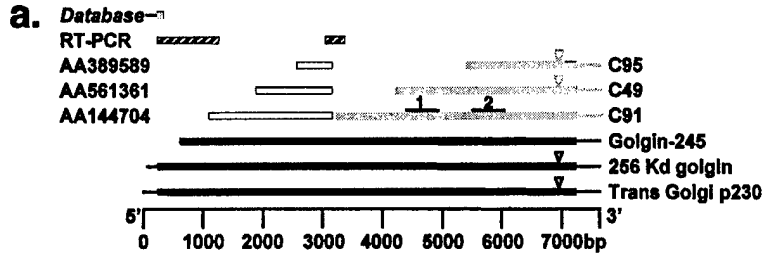
RESULTS

Isolation of a tGolgin-1 cDNA by screening a postnatal mouse brain cDNA library with anti-OlP-1

OlP-1 was identified as a developmentally regulated cell surface determinant specific to premyelinating oligodendrocyte precursors (Gay *et al.*, 1997). Because these cells are most highly represented in the mouse brain during early postnatal development, a postnatal day 8 mouse brain cDNA expression library was screened with anti-OlP-1 mab. Three independent cDNAs (clones C95, C49, and C91) were retrieved ranging in size from 2.2 kb to over 4 kb. Sequencing showed them to be identical except for a 63 bp deletion at the 3′ end of the largest cDNA clone (C91). Figure 1a shows the relative sizes and sequence overlap of these cDNAs. The sequence of C91 contained a single open reading frame of 3741 nucleotides extending to the extreme 5′ end. The 3′ terminus was verified by the presence of multiple stop codons and a poly A tail.

Comparison of the predicted translation product of the C91 open reading frame to available databases identified three entries with considerable homology: golgin 245 (accession number U31906; Fritzler *et al.*, 1995), 256 kD golgin (acc. no. X82834), and *trans*-golgi p230 (acc. no. U41740) (Erlich *et al.*, 1996), corresponding to products of the *GOLGA4* gene. These three molecules bear identical nucleotide sequences except for deletions within the 3′ and/or 5′ regions, and we, therefore, believe that they represent the product of a single gene with a single alternative splice site near the 3′ end (see Discussion). Because of their immunolocalization to the TGN (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. The region of homology extended through the entire predicted translation product of C91, but corresponded to only the C-terminal 1247 amino acids of the 2228–2230 residue full-length tGolgin-1 protein. This suggested that C91 was not a full-length cDNA for mouse tGolgin-1.

A BLAST search of the mouse EST database revealed numerous additional clones with predicted translated amino acid sequence similarity to regions within the N-terminal 1000 residues of human tGolgin-1. Two of these clones, Acc AA098411 and AA144704, were retrieved, and together spanned sequences encoding 878 additional contiguous residues N-terminal to those encoded by C91 (Fig. 1a). cDNA sequences between EST clone AA144704 and C91 were amplified by RT-PCR from postnatal mouse brain RNA, and EST clone AA098411 was reconstructed using RT-PCR from the same source using the available sequence deposited in Genbank. The coding region for the predicted N-terminal region was amplified by RT-PCR of prenatal mouse brain RNA using a degenerate primer corresponding to the coding region for the first six amino acids of human tGolgin-1 and a second reverse primer from within the coding region of EST clone AA144704. The remaining 5′ UTR and coding region for the first six amino acids are inferred from overlapping entries in the Genbank database (see Materials and Methods for details). RT-PCR of mRNA from several sources indicated that all separately identified regions were colinear in a single RNA species (see below). We refer to this composite clone as mouse tGolgin-1.


a.
Database
RT-PCR
AA389589
AA561361
AA144704
C95
C49
C91
1
2
Golgin-245
256 Kd golgin
Trans Golgi p230
5'
3'
0 1000 2000 3000 4000 5000 6000 7000bp

b.

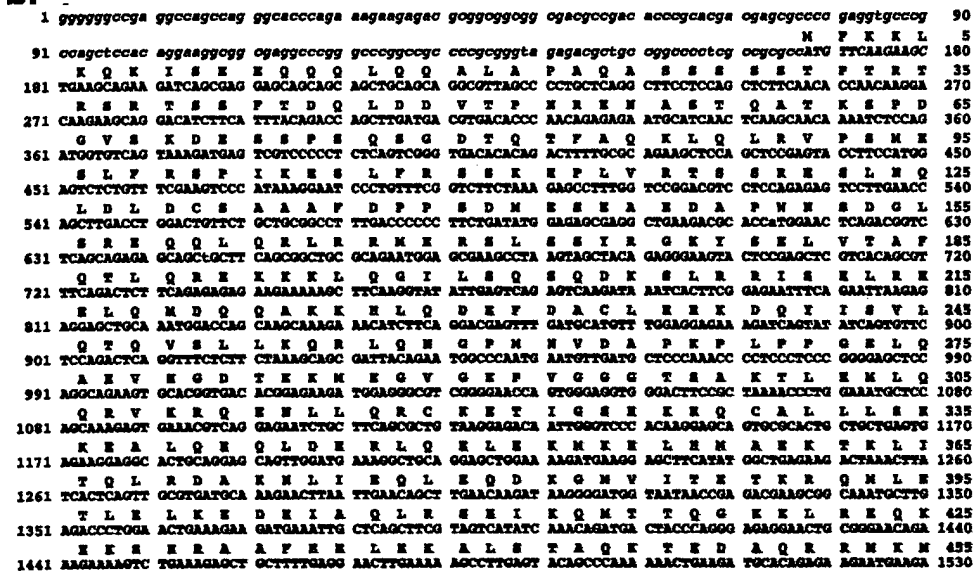


FIG. 1.

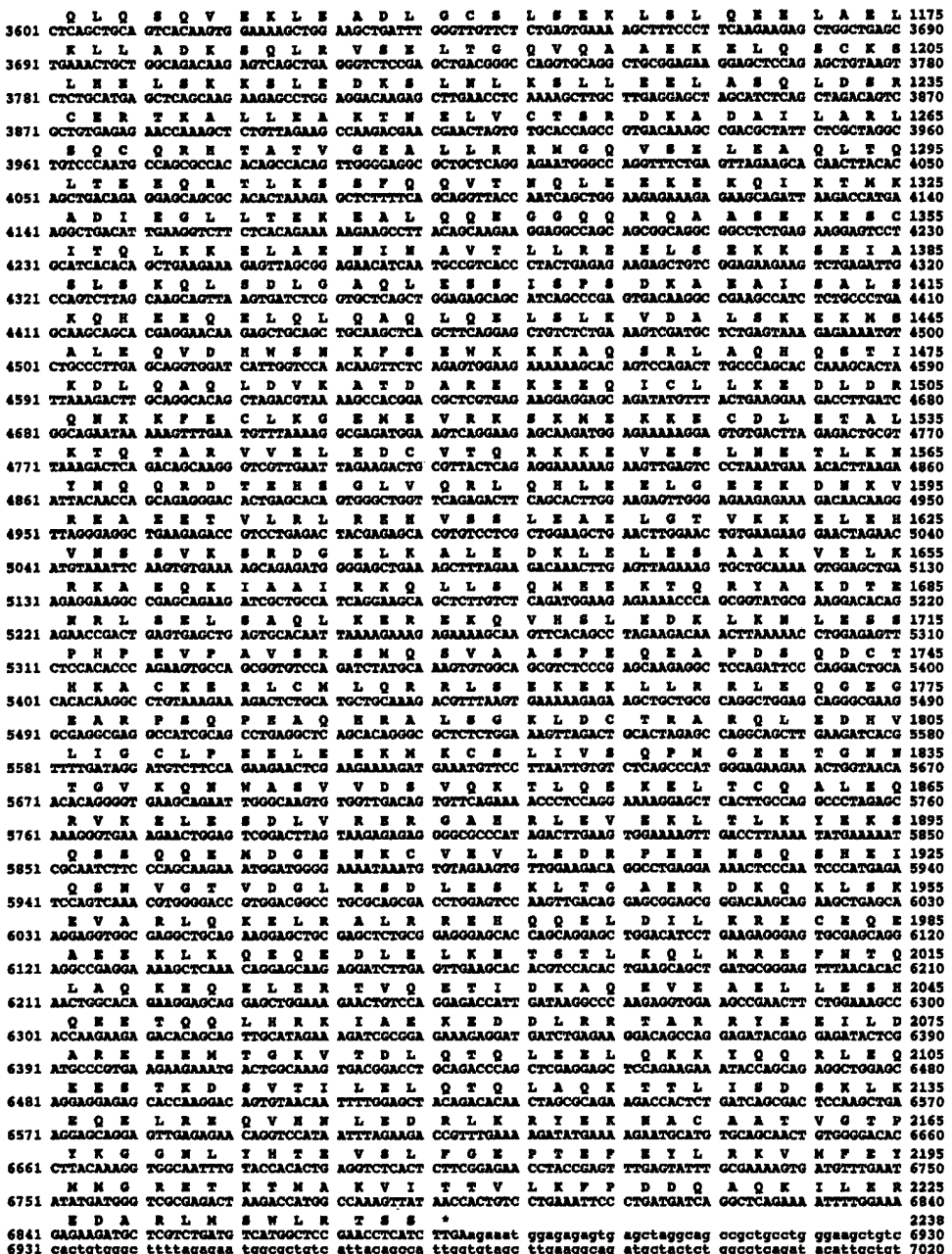


FIG. 1. (*Continued*) Isolation and sequence of mouse tGolgin-1 cDNA. (**a**) Schematic representation of regions of sequence overlap between *trans*-golgi p230, golgin-245, 256 kD golgin, isolated OlP-1 cDNA clones (C91, C49, and C95), EST clones (AA144704, AA561361, and AA389589), and additional regions identified in this work. The triangle represents the 63 bp insertion in p230 and clones C95 and C49. 1 and 2 represent the locations of PCR products used in Figure 7. Regions identified by RT-PCR only are indicated. *Database* represents the 5′ UTR and translation start sequences that were deduced from the deposited sequences of Genbank entries BC003268, BF162586, BI151932, and BE304004; these sequences were not confirmed by our direct sequencing, but represent overlap of the corresponding database entries. (**b**) Nucleotide sequence of mouse tGolgin-1 cDNA and deduced amino acid sequence. Italics represent the inferred sequences from the database entries as indicated above.

The complete cDNA sequence and deduced amino acid sequence of mouse tGolgin-1 is listed in Figure 1b, and the deduced amino acid sequence is compared with that of human tGolgin-1 in Figure 2A. The mouse tGolgin-1 open reading frame encodes 2238 amino acids with a predicted molecular mass of 258 kDa, compared to 2230 amino acids and 261 kDa for the human homolog. The predicted amino acid sequences of the two homologs show 70% identity and 85% similarity. Interestingly, *trans*-golgi p230 and 256 kD golgin, like mouse tGolgin-1 cDNA cones C49 and C95, maintained the 65 bp 3′ nucleotide stretch encoding "FTSPRSGIF" at the C-terminus, whereas golgin-245 and clone C91 had this region deleted, generating an alternative "SWLRTSS" C-terminal end. Both human and mouse proteins are predicted to consist primarily of coiled-coil domains, with short regions of potentially globular structure at the N- and C-termini (Fig. 2B).

To determine whether the sequences we identified corresponded to the only mouse homolog of human tGolgin-1, we searched the dbest database for homologous murine EST sequences using BLAST. We retrieved 145 nucleotide sequences that were greater than 93% identical to mouse tGolgin-1, most of which were mismatched at only one to three residues; given the distribution of the nucleotide differences, we suspect that these clones contain identical sequences to those in mouse tGolgin-1 and that differences reflect sequencing errors and/or potential haplotype variations. Included among the retrieved sequences were clones homologous to regions spanning the entire mouse tGolgin-1 cDNA, with the exception of the short adenosine-rich stretch between C91 and AA144704. Searches of the dbest database for translated sequences homologous to either the human tGolgin-1 proteins or the translated mouse tGolgin-1 sequence resulted in no additional entries other than those identified on the basis of nucleotide similarity. This suggests that no other tGolgin-1 homologues have yet been entered in the EST database. The fact that they were obtained from highly diverse cDNA library sources suggests that they represent an ubiquitous mRNA, as would be expected for a tGolgin-1 homolog (see below).

Significant but low homology was also observed between the mouse tGolgin-1 deduced amino aid sequence and the C-terminal half of myosin II (unpublished data). This homology, which ranged from 23–45% over four distinct regions, correlated specifically with predicted coiled-coil regions as assessed using the MTIDK matrix (Lupas *et al.*, 1991) (Fig. 2B). The GCG computer program "Moment" used to calculate helical dipole moments to predict α-helical regions failed to find long helices within the tGolgin-1 sequence (unpublished data). These data cannot distinguish between the possibilities that tGolgin-1 is supercoiled like the myosin heavy chain C-terminal domain or globular with many small coiled-coil–like domains. However, because myosin II and several other coiled-coil containing Golgi- and endosome-associated peripheral membrane proteins are believed to form dimeric supercoiled structures, we anticipate that tGolgin-1 has a similar structure.

tGolgin-1 localization to the TGN through the C-terminal half of the molecule

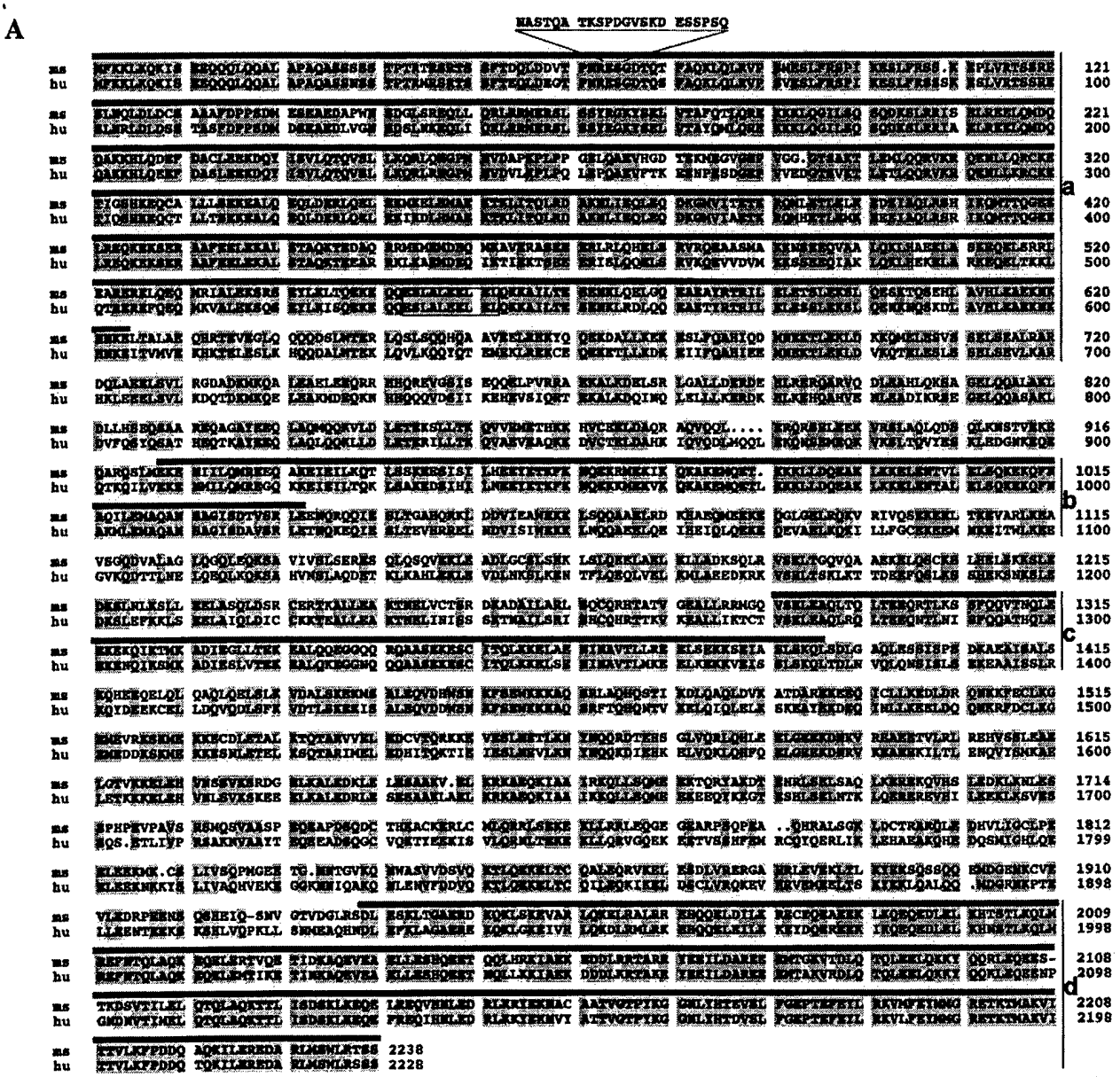
Because human tGolgin-1 has been shown to be peripherally associated with the TGN (Gleeson *et al.*, 1996), we assayed for the intracellular localization of the mouse tGolgin-1 C-terminal fragment encoded by C91. Expression vectors encoding HA11 epitope-tagged forms of the translation product of C91 (HA-C1247, representing the C-terminal 1247 aa of tGolgin-1) were transiently transfected into HeLa cells, and localization was determined by indirect intracellular immunofluorescence microscopy (IFM) using anti-HA11 antibodies. As shown in Figure 3, HA-C1247 localized to a paranuclear, reticular structure reminiscent of the Golgi complex. Parallel staining with an antiserum to the endogenous TGN resident protein, TGN46 (Fig. 3A and B), or to the Golgi stack-associated β-COP subunit of coatomer (Duden *et al.*, 1991) (Fig. 3C and D) showed extensive overlap, suggesting that the C-terminal tGolgin-1 fragment primarily localizes to the TGN and/or the Golgi stacks. Similar results were obtained using C1247 tagged at the C-terminus with the HA11 epitope (unpublished data). Like human tGolgin-1 (Gleeson *et al.*, 1996), the TGN-associated HA-C1247 was partially dispersed upon treatment with brefeldin A (unpublished data). These data further support the notion that we identified the mouse homolog of human tGolgin-1, and that tGolgin-1 localizes to the TGN.

The C-terminal 80 amino acids of human tGolgin-1 encompass a GRIP domain, a modestly conserved region that is necessary and sufficient to mediate localization to the TGN (Barr, 1999; Kjer-Nielsen *et al.*, 1999a, 1999b; Munro and Nichols, 1999). To determine whether the comparable region of mouse tGolgin-1 was sufficient to mediate Golgi localization, we generated N-terminally HA-tagged truncation mutants containing the C-terminal 312, 186, or 81 amino acids of mouse tGolgin-1, and expressed them by transient transfection in HeLa cells. As shown in Figure 4a–f, immunofluorescence microscopy analyses indicated that all of these constructs colocalized well with TGN46. Mutagenesis of a critical conserved tyrosine residue at position 2187 completely disrupted localization, resulting in a diffuse cytoplasmic staining pattern (Figure 4g and h). These results indicate that mouse tGolgin-1 retains a functional GRIP domain, and that the C-terminal 81 amino acids are sufficient to specify localization to the Golgi/TGN.

Expression of mouse tGolgin-1 mRNA: abundant expression in oligodendrocyte precursors

The distribution of human tGolgin-1 mRNA in different tissues has not been evaluated. To assess the tissue distribution of mouse tGolgin-1, RNA was prepared from a panel of mouse tissues and hybridized with a probe derived from the coding region of the mouse tGolgin-1 cDNA (Fig. 5). tGolgin-1 mRNA migrates as a single major band of approximately 7.5 kb in length, as expected from the length of the predicted full-length cDNA and by analogy with the human tGolgin-1 mRNA. Overexposure of the blot reveals additional bands from brain tissue at approximately 9 and 12.5 kb, which may represent incompletely spliced heteronuclear RNAs or alternative splice products; the 9-kb band was only observed in brain tissue (unpublished data). The 7.5-kb mRNA is expressed in all tissues analyzed, as expected for a gene product involved in constitutive transport processes. Relative to the GAPDH controls, no clear enrichment was observed in any tissue analyzed. Although these data do not rule out cell type variation in tGolgin-1 mRNA expression, they indicate that no tissue types are enriched in cells that express particularly high or low levels of tGolgin-1 mRNA.

Because tGolgin-1 was identified using an antibody to OlP-1 expressed on oligodendrocyte precursors, we next sought to



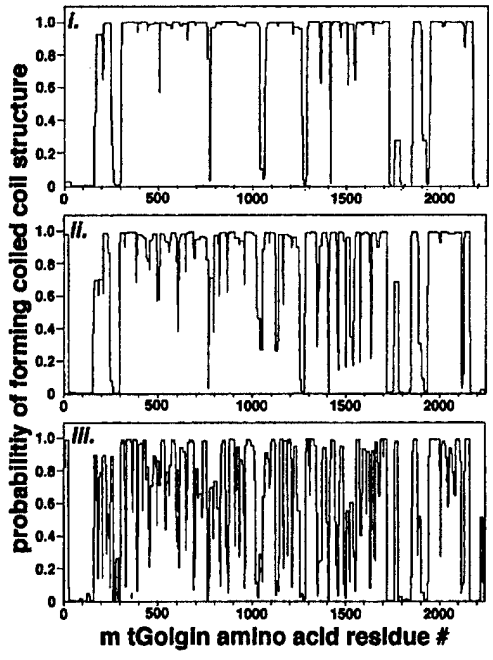


FIG. 2. Comparison of mouse and human tGolgin-1 amino acid sequences and coiled-coil predictions. (**A**) Deduced amino acid sequence of mouse (ms) tGolgin-1 and alignment with that of human (hu) tGolgin-1. The human tGolgin-1 sequence listed includes the N-terminal regions present in *trans*-golgi p230 and 256 kD golgin, but the C-terminal region of golgin-245 containing the region encoded by the 63bp alternatively spliced exon. Amino acid identities are darkly shaded and similarities are lightly shaded. Regions of marked homology between mouse and human sequences are indicated by thick lines above the sequence and by the letters a, b, c, and d to the right of the sequence. Potential granin motifs are boxed. (**B**) Coiled-coil predictions using the MTIDK matrix and windows of 14 (i), 21 (ii), and 28 (iii) residues without weighting of positions 1 and 4. Similar patterns were obtained with weighting of these positions and using the MTK matrix.

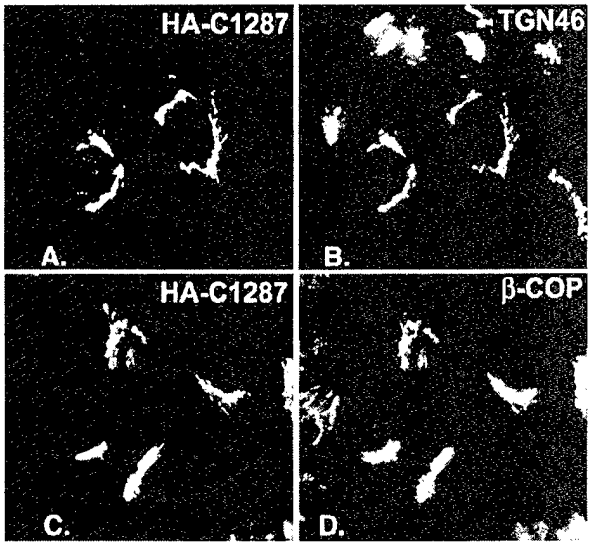


FIG. 3. The HA-tagged gene product of partial tGolgin-1 clone C91 localizes to the TGN region. (**A–D**) HA-C1247, representing the C-terminal 1247 residues of tGolgin-1 tagged at the N-terminus with the HA11 epitope, was expressed in HeLa cells by transient transfection. Cells were fixed and stained with antibodies to HA11 (**A, C**) and to either endogenous TGN46 (**B**) or endogenous β-COP (**D**) and FITC- and Rhodamine Red X-conjugated secondary antibodies. **A, B** and **C, D** show representative immunofluorescence microscopy staining patterns from identical fields of cells. Note the presence of both transfected and untransfected cells in the same fields.

determine whether endogenous tGolgin-1 mRNA was more highly expressed specifically by this cell type. Sorted populations of neonatal mouse brain cells were analyzed by semiquantitative RT-PCR. This assay utilized limited numbers of PCR amplification cycles to assess the relative frequency of different mRNAs. First, oligodendrocyte precursors were sorted into three populations as defined by their expression of well-characterized developmental markers (see Fig. 6A and Gay *et al.*, 1997, for developmental analyses). Population 1 ($O4^+$ R-Mab^-) contains the least mature cells, which express the O4 epitope characteristic of committed oligodendrocyte precursors but not the Ranscht epitope (R-Mab) characteristic of more differentiated, less proliferative cells. Population 2 ($O4^+$R-Mab^+) contains cells at an intermediate stage of development in which R-Mab expression has been initiated but lack expression of myelin components. Population 3 (Gal-C+) contains the most mature cells that express myelin components; the first two populations contained no Gal C^+ cells, as determined by flow cytometry (unpublished data). mRNA from equal cell numbers of each population was subjected to semiquantitative PCR using various primer sets. Using primers corresponding to actin cDNA, a linear increase in accumulation of actin product was seen with increasing rounds of amplification, although both astrocytes and fibroblasts contained much higher amounts of product, most likely reflecting their larger cell size and increased mRNA levels per cell (Fig. 6A).

RT-PCR products corresponding to DM20 and PLP, two temporally regulated proteins encoded by the same gene (Macklin *et al.*, 1987), were assessed to verify the precursor stages under study. During oligodendrocyte development, DM20 transcription precedes that of PLP (Ikenaka *et al.*, 1992; Timsit *et al.*, 1992). As predicted, the smaller DM20 product was found in population 1, whereas both the DM20 and PLP products were found in the more mature populations 2 and 3 (Fig. 6A). Astrocytes also expressed some DM20/PLP products, most likely reflecting a few mature oligodendrocytes in this unsorted cell culture. These data validate that our sorted populations were relatively pure and that the RT-PCR assay faithfully reproduced previous characterizations.

To analyze for the presence of tGolgin-1 transcripts, PCR was performed using primers corresponding to a region of the cDNA sequence present in C91 (see Fig. 1a for PCR product locations along the cDNA sequence). As can be seen in Figure 6A, a PCR product of the appropriate size was obtained from populations 2 and 3. Population 1, astrocytes, and fibroblasts contained very weak bands, which may correspond to low levels of tGolgin-1 transcripts (see below). These data indicate that tGolgin-1 mRNA is expressed most extensively by stage II precursors. This corresponds to the appropriate developmental

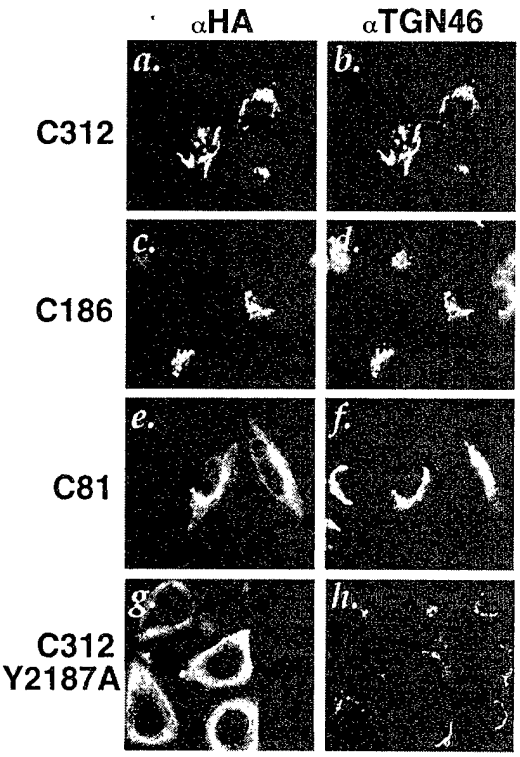


FIG. 4. GRIP domain-dependent localization of C-terminal tGolgin-1 fragments to the TGN. N-terminal HA11-epitope tagged truncations of tGolgin-1 were expressed in HeLa cells by transient transfection, and fixed cells were stained with antibodies to HA11 (**a, c, e, g**) and to endogenous TGN46 (**b, d, f, h**) and fluorophore-tagged secondary antibodies. Cells were analyzed by immunofluorescence microscopy using a conventional (**a–f**) or confocal scanning (**g, h**) microscope. The truncations comprised the C-terminal 312 (C312; **a, b**), 187 (C187; **c, d**), or 81 (C81; **e, f**) residues. In **g, h,** the cells expressed C312 with a mutation changing the conserved tyrosine at position 2187 to an alanine (C312.Y2187A).

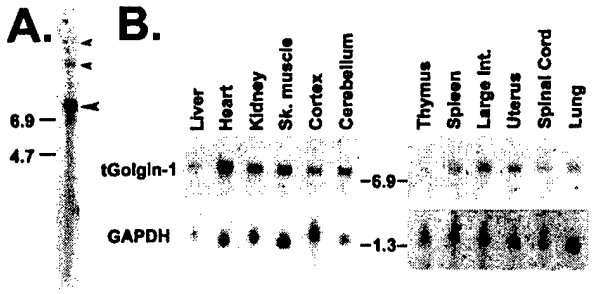


FIG. 5. Northern blot analysis of the tissue distribution of tGolgin-1 mRNA. Five micrograms of polyA+ RNA prepared from neonatal mouse brain (**A**) or 20 μg of total RNA from the indicated tissues (**B**) was fractionated on agarose gels and transferred to nitrocellulose. Blots were developed with digoxigenin-conjugated probes for tGolgin-1 or GAPDH, as indicated, followed by a luminescence detection system and phosphorimaging analysis. The position of RNA markers is indicated. In **A**, the large arrowhead points to the main band estimated at 7.5 kb; this band is also highlighted in the blots in **B**. Small arrowheads in **A** point to minor RNA species only detected in longer exposures, as discussed in the text.

window for OlP-1 expression, and is the first demonstration that transcription of tGolgin-1 may be differentially regulated.

The RT-PCR analyses were extended using oligodendrocyte precursor populations sorted for expression of OlP-1 and R-Mab (Fig. 6B). OlP-1$^-$R-Mab$^-$ (least mature population 1), OlP-1$^+$R-MAb$^+$ (intermediate stage II population 2), and OlP-1$^-$R-Mab$^+$ (late stage II population 3) cells were subjected to identical conditions as in Figure 6A except that a second set of tGolgin-1 primers was also included for analyses. Again, only intermediate and late stage II cells generated PCR products corresponding to the tGolgin-1 sequence, and products correlated precisely with surface OlP-1 expression.

The Northern analyses described above suggested that tGolgin-1 is expressed in all tissues, and hence, in many cell types. To confirm that the RT-PCR assay could detect tGolgin-1 transcripts expressed at lower levels in cells other than oligodendrocyte precursors, two populations found negative by semi-quantitative PCR, astrocytes, and fibroblasts, were subjected to high cycle RT-PCR. At 40 cycles, weak PCR products were observed (Fig. 6C). These data support the view that the tGolgin-1 mRNA is expressed in many cells but is particularly abundant in oligodendrocyte precursors.

DISCUSSION

Identification of the murine tGolgin-1 homolog

Several groups identified and characterized a large, Golgi-localized protein as the target of autoantibodies in patients with Sjögren's Syndrome (Kooy *et al.*, 1992; Fritzler *et al.*, 1995). These groups cloned three homologous cDNAs that putatively encode proteins termed golgin-245 (Fritzler *et al.*, 1995), *trans*-golgi p230 (Erlich *et al.*, 1996), and 256 kD Golgin (submitted to Genbank by H. P. Seelig), all of which reacted with patient antisera, but which differed in sequence at the 5′ and 3′ end. The sequences of these three proteins are nearly identical throughout their coding region; careful analysis of the sequence data indicates that they represent partial or complete clones of the same cDNA, except for the inclusion or exclusion of a 63bp exon near the coding region for the extreme C-terminus. The extreme 5′ terminus of the golgin-245 sequence begins at the coding region for amino acid #130 of the *trans*-golgi p230 sequence, and we therefore believe that this is a 5′-truncated partial clone. The sequence of 256 kD golgin differs from that of *trans*-golgi p230 by the exclusion of a single G nucleotide at position 6666 within a poly-G tract; this shifts the reading frame for the predicted amino acid sequence to differ from those of *trans*-golgi p230 and golgin-245 near the C-terminus, and we therefore believe that this is a sequencing error. Because these proteins were localized to the *trans*-Golgi network and associated vesicles (Gleeson *et al.*, 1996), we refer to them collectively as tGolgin-1. Here, we identify the cDNA for the mouse homolog of tGolgin-1, and show that it is expressed in particularly great abundance in oligodendrocyte precursors.

We previously described a unique cell surface marker on stage II oligodendrocyte precursors that was referred to as OlP-1 (Gay *et al.*, 1997). Using the anti-OlP-1 mab to screen a postnatal mouse brain cDNA expression library, we isolated three overlapping cDNAs that encoded a protein with homology to the C-terminal half of human tGolgin-1. Using the mouse EST database and RT-PCR, we extended these cDNAs to span a 7530 bp region with homology to the entire human tGolgin-1 cDNA. Several data suggest that this clone represents the major or only mouse homolog of human tGolgin-1. First, the mouse tGolgin-1 clones exhibited strong homology to human tGolgin-1, and a similar splice variation of a 63 bp exon occurred at the extreme 3′ end of the coding region. Second, all of the available murine EST sequences with homology to human tGolgin-1 exhibited complete or nearly complete identity with the mouse sequence. These ESTs were derived from diverse embryonic and adult cDNA libraries. Third, like its human counterpart, HA-tagged truncated forms of the mouse protein localized to the TGN in transfected cells and could be partially displaced following treatment with brefeldin A. Finally, comparable to the analyses made of human tGolgin-1 thus far, the mouse mRNA was ubiquitously expressed, albeit at much higher levels in oligodendrocyte precursors. Collectively, this evidence suggests that we have identified the only mouse homolog of human tGolgin-1.

Interestingly, the degree of similarity between the human and mouse proteins varied throughout the coding region. Regions of highest homology (>90% identical) were present at the extreme C-terminus, spanning the GRIP domain and adjacent predicted coiled-coil regions, and within the 600 amino-terminal residues. These were interspersed with regions of modest homology (60–70% identity) throughout the bulk of the protein, within regions predicted to have a coiled-coil structure. We speculate that the less conserved regions retain general structural features that are required for tGolgin-1 function, such as coiled-coil structure, whereas the more highly conserved regions likely mediate specific interactions within a tGolgin-1 oligomer or with other conserved proteins or nonprotein effectors.

It is curious that three independent cDNA clones with overlapping sequences for tGolgin-1 were identified using an anti-

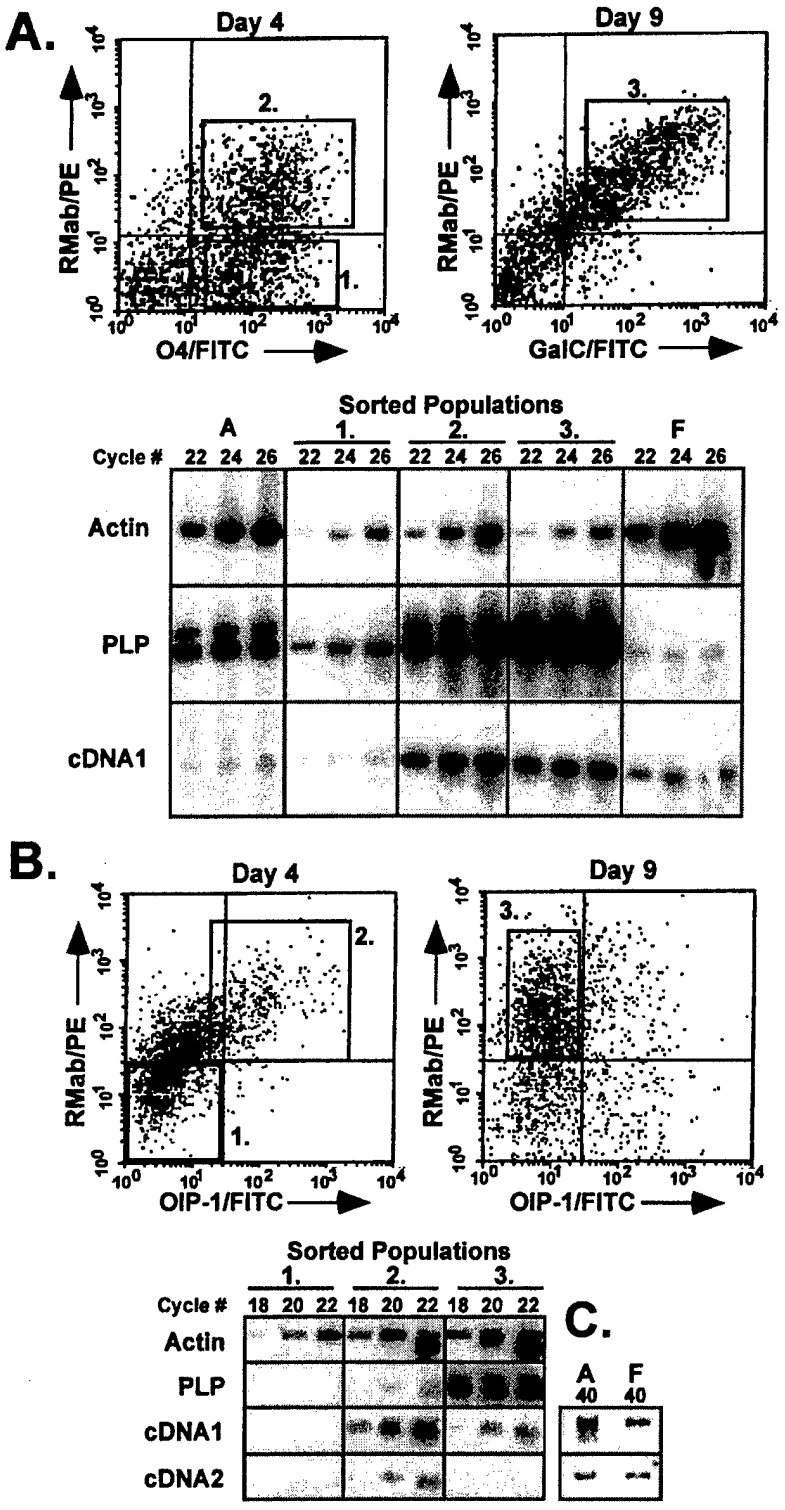


FIG. 6. tGolgin-1 mRNA is highly expressed in oligodendrocyte precursors. Semiquantitative RT-PCR (**A, B**) of sorted oligodendrocyte precursor populations, astrocytes, and fibroblasts and 40-cycle RT-PCR (**C**) of astrocytes and fibroblasts. Top panels represent flow cytometric profiles of cultured day 4 (left) or day 9 (right) mouse oligodendrocyte precursors colabeled with either anti-O4 or anti-GalC antibodies conjugated to FITC and PE-conjugated R-Mab as indicated. Sorted cells are boxed and numbered to indicate populations analyzed by RT-PCR (lower panels). Lower panels show PCR products from sorted populations, astrocytes, and fibroblasts using specific primers against designated proteins. Note that in **A**, PLP and cDNA1 PCR products were close to saturating levels even at the lowest cycle numbers (22, 24, 26 cycles). More representative samples, collected at lower cycle numbers (18, 20, 22 cycles), can be seen in **B**.

body to a cell surface marker on oligodendrocyte precursors. Although we have as yet been unable to express the full-length mouse tGolgin-1 protein in transfected cells, no cell surface expression of the clone C91 gene product could be detected with either anti-HA or anti-OlP-1 antibodies, and antibodies to human tGolgin-1 are nonreactive with the cell surface of human cell lines (unpublished data). Furthermore, except for the initial isolation from bacteriophage plaques of bacterial cultures, we were unable to demonstrate direct reactivity of the tGolgin-1 gene product with the anti-OlP-1 antibody using several assays (unpublished data). Finally, the deduced amino acid sequence contains no apparent signal sequence, consistent with the structure of a peripheral membrane protein. Nevertheless, endogenous tGolgin-1 mRNA levels were greatly upregulated in stage II oligodendrocyte precursors, compared with precursors at other developmental stages or unrelated cells such as astrocytes and fibroblasts; this is the same developmental stage at which OlP-1 surface expression is observed. We speculate that either the OlP-1 determinant represents a small amount of surface expression of tGolgin-1 in cells in which it is highly expressed, or that high levels of tGolgin-1 protein are required to generate a determinant on a distinct molecule that can be recognized by the anti-OlP-1 mab (see below). Surface expression of a normally cytoplasmic protein would be unusual, but not unprecedented (Cleves and Kelly, 1996). Indeed, cellular myosin II, a protein with some homology to tGolgin-1 that also participates in vesicular transport from the TGN (Musch *et al.*, 1997; Stow *et al.*, 1998), has been reported to associate tightly with the neuronal plasma membrane (Li *et al.*, 1994) and to localize to the cell surface (Michelis *et al.*, 1994; Yanase *et al.*, 1997).

A possible function for tGolgin-1 in the vesicular trafficking of glycolipids or proteolipids?

It has been postulated that components of the vesicular transport machinery are upregulated to accommodate myelination. Indeed, Rab3a and Rab3c, GTP-binding proteins associated with regulated secretion in neurons and other specialized cell types, have been shown to be upregulated during the late development and maturation of oligodendrocytes (Madison *et al.*, 1996). Oligodendrocytes have been shown to possess features of polarized sorting (de Vries *et al.*, 1998), and express a unique Rab protein, Rab22b, which is thought to be involved in traffic between the TGN and endosomes (Rodriguez-Gabin *et al.*, 2001). We found elevated tGolgin-1 mRNA levels at a point in development slightly earlier than that in which myelin deposits accumulate, that immediately preceding and coincident with high surface expression of R-Mab antigen. These results suggest that expression of tGolgin-1 can be upregulated to accommodate specific vesicular transport functions in oligodendrocyte precursors, such as an increased cargo load or specialized cargo. A role in the transport of specialized cargo is supported by the finding that human tGolgin-1 and another TGN-associated peripheral membrane protein, myosin II, are present on separate vesicles associated with the TGN (Gleeson *et al.*, 1996; Brown *et al.*, 2001).

What might this specialized cargo be? Given the unique role of glycosphingolipids in oligodendrocyte biology, we speculate that tGolgin-1 may be involved in transport or synthesis of glycolipids. Glycolipids, including sulfatides such as the R-Mab antigen, are highly enriched in stage II oligodendrocyte precursors (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992) at the same stage in which we find high levels of tGolgin-1 mRNA. Glycolipids are manufactured and assembled into myelin at the TGN (Simons *et al.*, 2000) and progress to the cell surface through vesicular transport (Burkart *et al.*, 1982; Gow *et al.*, 1994). Glycosphingolipids play a general role in regulating Golgi export (Sprong *et al.*, 2001b), protein sorting processes at the TGN (van Meer, 1998; Sprong *et al.*, 2001a), and retrograde transport from the plasma membrane to the TGN (Falguières *et al.*, 2001; Johannes and Goud, 1998). Moreover, the differentiation of oligodendrocytes has been shown to be associated with distinct glycolipid sorting processes (Watanabe *et al.*, 1999), and maintenance of oligodendrocyte processes requires glycolipid cycling between the plasma membrane and the TGN (Benjamins and Nedelkoska, 1994). We speculate that tGolgin-1 may be involved in either the transport of glycolipids or sulfatides such as the R-Mab antigen (Bansal *et al.*, 1989; Bansal and Pfeiffer, 1992), or in the localization of resident TGN enzymes that facilitate glycolipid or sulfatide synthesis. Perhaps the anti-OlP-1 antibody recognizes a glycolipid that binds to tGolgin-1 and for which cell surface expression or recycling is facilitated by tGolgin-1.

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REFERENCES

BANSAL, R., and PFEIFFER, S.E. (1992). Novel stage in the oligodendrocyte lineage defined by reactivity of progenitors with R-mAb prior to O1 anti-galactocerebroside. J. Neurosci. Res. **32,** 309–316.

BANSAL, R., WARRINGTON, A.E., GARD, A.L., RANSCHT, B., and PFEIFFER, S.E. (1989). Multiple and novel specificities of monoclonal antibodies O1, O4, and R-mAb used in the analysis of oligodendrocyte development. J. Neurosci. Res. **24,** 547–557.

BARR, F.A. (1999). A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. Curr. Biol. **9,** 381–384.

BENJAMINS, J.A., and NEDELKOSKA, L. (1994). Maintenance of membrane sheets by cultured oligodendrocytes requires continuous microtubule turnover and Golgi transport. Neurochem. Res. **19,** 631–639.

BONIFACINO, J.S., SUZUKI, C.K., and KLAUSNER, R.D. (1990). A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. Science **247,** 79–82.

BROWN, D.L., HEIMANN, K., LOCK, J., KJER-NIELSEN, L., VAN

VLIET, C., STOW, J.L., and GLEESON, P.A. (2001). The GRIP domain is a specific targeting sequence for a population of trans-Golgi network derived tubulo-vesicular carriers. Traffic **2,** 336–344.

BURKART, T., CAIMI, L., SIEGRIST, H.P., HERSCHKOWITZ, N.N., and WIESMANN, U.N. (1982). Vesicular transport of sulfatide in the myelinating mouse brain. Functional association with lysosomes. J. Biol. Chem. **257,** 3151–3156.

CAMPAGNONI, A.T. (1988). Molecular biology of myelin proteins from the central nervous system. J. Neurochem. **51,** 1–14.

CLEVES, A.E., and KELLY, R.B. (1996). Rehearsing the ABCs. Protein translocation. Curr. Biol. **6,** 276–278.

DE VRIES, H., SCHRAGE, C., and HOEKSTRA, D. (1998). An apical-type trafficking pathway is present in cultured oligodendrocytes but the sphingolipid-enriched myelin membrane is the target of a basolateral-type pathway. Mol. Biol. Cell. **9,** 599–609.

DE VRIES, H., SCHRAGE, C., HOEKSTRA, K., KOK, J.W., VAN DER HAAR, M.E., KALICHARAN, D., LIEM, R.S., COPRAY, J.C., and HOEKSTRA, D.J. (1993). Outstations of the Golgi complex are present in the processes of cultured rat oligodendrocytes. Neurosci. Res. **36,** 336–343.

DUDEN, R., GRIFFITHS, G., FRANK, R., ARGOS, P., and KREIS, T.E. (1991). β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. Cell **64,** 649–665.

ERLICH, R., GLEESON, P.A., CAMPBELL, P., DIETZSCH, E., and TOH, B.-H. (1996). Molecular characterization of *trans*-Golgi p230. A human peripheral membrane protein encoded by a gene on chromosome 6p12-22 contains extensive coiled-coil α-helical domains and a granin motif. J. Biol. Chem. **271,** 8328–8337.

FALGUIÈRES, T., MALLARD, F., BARON, C., HANAU, D., LINGWOOD, C., GOUD, B., SALAMERO, J., and JOHANNES, L. (2001). Targeting of shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. Mol. Biol. Cell. **12,** 2453–2468.

FRITZLER, M.J., LUNG, C.-C., HAMEL, J.C., GRIFFITH, K.J., and CHAN, E.K.L. (1995). Molecular characterization of golgin-245, a novel Golgi complex protein containing a granin signature. J. Biol. Chem. **270,** 31262–31268.

GAY, D., LAVI, E., ZHAO, H., MUMIN, A., and BHANDOOLA, A. (1997). OIP-1, a novel protein that distinguishes early oligodendrocyte precursors. J. Neurosci. Res. **50,** 591–604.

GLEESON, P.A., ANDERSON, T.J., STOW, J.L., GRIFFITHS, G., TOH, B.H., and MATHESON, F. (1996). p230 is associated with vesicles budding from the *trans*-Golgi network. J. Cell Sci. **109,** 2811–2821.

GOW, A., FRIEDRICH, V.L.J., and LAZZARINI, R.A. (1994). Intracellular transport and sorting of the oligodendrocyte transmembrane proteolipid protein. J. Neurosci. Res. **37,** 563–573.

IKENAKA, K., KAGAWA, T., and MIKOSHIBA, K. (1992). Selective expression of DM-20, an alternatively spliced myelin proteolipid protein gene product, in developing nervous system and in nonglial cells. J. Neurochem. **58,** 2248–2253.

JOHANNES, L., and GOUD, B. (1998). Surfing on a retrograde wave: How does Shiga toxin reach the endoplasmic reticulum? Trends Cell Biol. **8,** 158–162.

KJER-NIELEN, L., TEASDALE, R.D., VAN VLIET, C., and GLEESON, P.A. (1999a). A novel Golgi-localisation domain shared by a class of coiled-coil peripheral membrane proteins. Curr. Biol. **9,** 385–388.

KJER-NIELSEN, L., VAN VLIET, C., ERLICH, R., TOH, B.-H., and GLEESON, P.A. (1999b). The Golgi targeting sequence of the peripheral membrane protein p230. J. Cell Sci. **112,** 1645–1654.

KOOY, J., TOH, B.H., PETTITT, J.M., ERLICH, R., and GLEESON, P.A. (1992). Human autoantibodies as reagents to conserved Golgi components. Characterization of a peripheral, 230-kDa compartment-specific Golgi protein. J. Biol. Chem. **267,** 20255–20263.

LI, D., MILLER, M., and CHANTLER, P.D. (1994). Association of a cellular myosin II with anionic phospholipids and the neuronal plasma membrane. Proc. Natl. Acad. Sci. USA **91,** 853–857.

LUNN, K.F., BAAS, P.W., and DUNCAN, I.D. (1997). Microtubule organization and stability in the oligodendrocyte. J. Neurosci. **17,** 4921–4932.

LUPAS, A., VAN DYKE, M., and STOCK, J. (1991). Predicting coiled coils from protein sequences. Science **252,** 1162–1164.

MACKLIN, W.B., CAMPAGNONI, C.W., DEININGER, P.L., and GARDINIER, M.V. (1987). Structure and expression of the mouse myelin proteolipid protein gene. J. Neurosci. Res. **18,** 383–394.

MADISON, D.L., KRUGER, W.H., KIM, T., and PFEIFFER, S.E. (1996). Differential expression of rab3 isoforms in oligodendrocytes and astrocytes. J. Neurosci. Res. **45,** 258–268.

MARKS, M.S., ROCHE, P.A., VAN DONSELAAR, E., WOODRUFF, L., PETERS, P.J., and BONIFACINO, J.S. (1995). A lysosomal targeting signal in the cytoplasmic tail of the β chain directs HLA-DM to the MHC class II compartments. J. Cell Biol. **131,** 351–369.

MCMORRIS, F.A., and MCKINNON, R.D. (1996). Regulation of oligodendrocyte development and CNS myelination by growth factors: Prospects for therapy of demyelinating disease. Brain Pathol. **6,** 313–329.

MICHELIS, D., KOUNNAS, M.Z., ARGRAVES, W.S., SANFORD, E.D., BORCHELT, J.D., and WRIGHT, J.R. (1994). Interaction of surfactant protein A with cellular myosin. Am. J. Respir. Cell Mol. Biol. **11,** 692–700.

MUNRO, S., and NICHOLS, B.J. (1999). The GRIP domain—A novel Golgi-targeting domain found in several coiled-coil proteins. Curr. Biol. **9,** 377–380.

MUSCH, A., COHEN, D., and RODRIGUEZ-BOULAN, E. (1997). Myosin II is involved in the production of constitutive transport vesicles from the TGN. J. Cell Biol. **138,** 291–306.

ORENTAS, D.M., and MILLER, R.H. (1998). Regulation of oligodendrocyte development. Mol. Neurobiol. **18,** 247–259.

PFEIFFER, S.E., WARRINGTON, A.E., and BANSAL, R. (1993). The oligodendrocyte and its many cellular processes. Trends Cell Biol. **3,** 191–197.

PRESCOTT, A.R., LUCOCQ, J.M., JAMES, J., LISTER, J.M., and PONNAMBALAM, S. (1997). Distinct localisation of TGN46 and β1,4-galactosyltransferase in HeLa cells. Eur. J. Cell Biol. **72,** 238–246.

RANSCHT, B., CLAPSHAW, P.A., PRICE, J., NOBLE, M., and SEIFERT, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc. Natl. Acad. Sci. USA. **79,** 2709–2713.

RODRIGUEZ-GABIN, A.G., CAMMER, M., ALMAZAN, G., CHARRON, M., and LAROCCA, J.N. (2001). Role of rRAB22b, an oligodendrocyte protein, in regulation of transport of vesicles from trans Golgi to endocytic compartments. J. Neurosci. Res. **66,** 1149–1160.

SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

SIMONS, M., KRAMER, E.M., THIELE, C., STOFFEL, W., and TROTTER, J. (2000). Assembly of myelin by association of proteolipid protein with cholesterol- and galactosylceramide-rich membrane domains. J. Cell Biol. **151,** 143–154.

SPRONG, H., DEGROOTE, S., CLAESSENS, T., VAN DRUNEN, J., OORSCHOT, V., WESTERINK, B.H.C., HIRABAYASHI, Y., KLUMPERMAN, J., VAN DER SLUIJS, P., and VAN MEER, G. (2001a). Glycosphingolipids are required for sorting melanosomal proteins in the Golgi complex. J. Cell Biol. **155,** 369–380.

SPRONG, H., VAN DER SLUIJS, P., and VAN MEER, G. (2001b). How proteins move lipids and lipids move proteins. Nat. Rev. Mol. Cell Biol. **2,** 504–513.

STOW, J.L., FATH, K.R., and BURGESS, D.R. (1998). Budding roles for myosin II on the Golgi. Trends Cell Biol. **8,** 138–141.

TIMSIT, S.G., BALLY-CUIF, L., COLMAN, D.R., and ZALC, B. (1992). DM-20 mRNA is expressed during the embryonic development of the nervous system of the mouse. J. Neurochem. **58,** 1172–1175.

TOMAYKO, M.M., and CANCRO, M.P. (1998). Long-lived B cells are distinguished by elevated expression of A1. J. Immunol. **160,** 107–111.

VAN MEER, G. (1998). Lipids of the Golgi membrane. Trends Cell Biol. **8,** 29–33.

WATANABE, R., ASAKURA, K., RODRIGUEZ, M., and PAGANO, R.E. (1999). Internalization and sorting of plasma membrane sphingolipid analogues in differentiating oligodendrocytes. J. Neurochem. **73,** 1375–1383.

YANASE, K., SMITH, R.M., PUCCETTI, A., JARETT, L., and MADAIO, M.P. (1997). Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1. J. Clin. Invest. **100,** 25–31.

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Golgi Recruitment of GRIP Domain Proteins by Arf-like GTPase 1 Is Regulated by Arf-like GTPase 3

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Summary

Golgins are Golgi-localized proteins present in all molecularly characterized eukaryotes that function in Golgi transport and maintenance of Golgi structure. Some peripheral membrane Golgins, including the yeast Imh1 protein, contain the recently described GRIP domain that can independently mediate Golgi localization by an unknown mechanism [1–3]. To identify candidate Golgi receptors for GRIP domain proteins, a collection of *Saccharomyces cerevisiae* deletion mutants was visually screened by using yeast, mouse, and human GFP-GRIP domain fusion proteins for defects in Golgi localization. GFP-GRIP reporters were localized to the cytosol in cells lacking either of two ARF-like (ARL) GTPases, Arl1p and Arl3p. In vitro binding experiments demonstrated that activated Arl1p-GTP binds specifically and directly to the Imh1p GRIP domain. Arl1p colocalized with Imh1p-GRIP at the Golgi, and Golgi localization of Arl1p was regulated by the GTPase cycle of Arl3p. These results suggest a cascade in which the GTPase cycle of Arl3p regulates Golgi localization of Arl1p, which in turn binds to the GRIP domain of Imh1p and recruits it to the Golgi. The similar requirements for localization of GRIP domains from yeast, mouse, and human when expressed in yeast, and the presence of Arl1p and Arl3p homologs in these species, suggest that this is an evolutionarily conserved mechanism.

Results and Discussion

When expressed in mammalian tissue culture cells, isolated GRIP domains from yeast and mammalian proteins localize to the Golgi, and high-level expression of isolated GRIP domains displaces endogenous GRIP domain-containing proteins from the Golgi [1, 3]. These data suggest that a limiting, evolutionarily conserved receptor at the Golgi is responsible for recruitment of GRIP domain proteins. The yeast genome encodes a single protein, Imh1p, with a GRIP domain, and a GFP-Imh1p GRIP domain fusion protein localizes to punctate organelles that likely correspond to the Golgi [1]. We expressed the Imh1p GRIP domain as a fusion to a variant red fluorescent protein (RFP), DsRedT.4 [4], in cells coexpressing Sec7p-GFP, a pan Golgi marker protein [5], and Chs3p-GFP, which is localized predominantly to the late Golgi/post-Golgi endosome [6]. Nearly perfect colocalization was observed with Chs3p-GFP, and substantial colocalization was observed with Sec7p-GFP (Figure 1). Identical results were obtained with full-length Imh1p, and deletion of the GRIP domain from Imh1p resulted in cytosolic localization (data not shown), indicating that its GRIP domain mediates localization of the full-length protein, as is the case for human p230/tGolgin-1 [7]. In control experiments, no colocalization was observed in yeast expressing a GFP-FYVE domain protein (derived from human EEA1) that labels prevacuolar endosomes (data not shown) [8]. These results definitively demonstrate that the Imh1p GRIP domain localizes to the yeast Golgi and preferentially to late Golgi compartments.

To determine if the yeast Golgi contains a receptor that can be recognized by mammalian GRIP domains, we expressed GFP fusions to the mouse tGolgin-1 [9] and human Golgin-97 [10] GRIP domains in wild-type yeast cells. Both GRIP domains localized to punctate organelles (Figure 2), indicating that whatever element of the Golgi serves to recruit GRIP domain proteins is conserved between yeast and mammals. We hypothesized that since *IMH1* is not an essential gene, the Golgi receptor that recruits Imh1p might also be encoded by a nonessential gene(s) or activity. Thus, to identify a candidate Golgi-localized receptor for GRIP domain proteins, we expressed the GFP-GRIP domain of yeast Imh1p in approximately 250 yeast strains with deletions of genes encoding nonessential, known, or predicted Golgi-localized proteins or proteins known to regulate traffic to, through, or out of the Golgi, and we screened these strains by fluorescence microscopy for loss of Golgi localization. In two strains, *arl1*Δ and *arl3*Δ, the GFP reporter was no longer localized to the Golgi and appeared cytosolic (Figure 2). Localization of mouse tGolgin-1 and human Golgin-97 GFP-GRIP domains in these mutant strains was similarly affected (Figure 2). Arl1p and Arl3p are divergent members of the ARF family of GTPases, referred to as ARF-like or ARL GTPases, and they are highly conserved with the human ARL1 and ARF-related protein (ARP) GTPases, respectively [11].

Yeast *arl1*Δ, *arl3*Δ, and *imh1*Δ mutants have minor defects in sorting proteins to the lysosome-like vacuole; these defects suggest a role for these proteins in biosynthetic protein sorting in the late Golgi or in endosomes [12–16]. Moreover, human ARL1 has been localized to the Golgi of transfected cells and has been implicated in regulating Golgi structure and Golgi protein sorting [17, 18]. These findings, and the general observation that localization of many peripheral membrane proteins is often regulated by a Ras-related GTPase [19], led us to explore the possibility that Arl1p or Arl3p might serve as a receptor for GRIP domains on the Golgi. To test this, we purified recombinant Arl1p and Arl3p and assayed for binding to a GST fusion protein containing the yeast Imh1p GRIP domain. As shown in Figure 3, the constitu-

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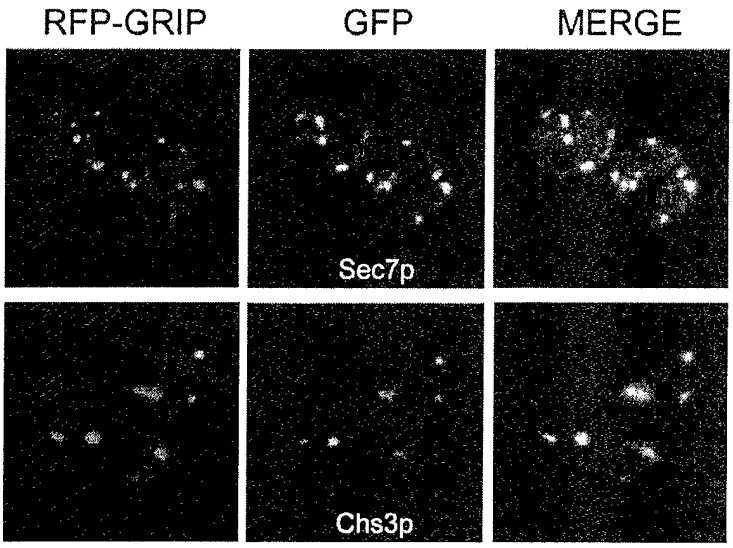

Figure 1. Imh1p GRIP Domain Localizes to Golgi Compartments

A red fluorescent protein (RFP)-Imh1p-GRIP fusion (encoding amino acids 735–912) was expressed in wild-type strains (BY4741 or BY4742) expressing Sec7-GFP or Chs3p-GFP, which both localize to Golgi compartments. Colocalization can be observed as yellow in the panels on the right.

tively active (GTPase-deficient, Q72L) form, but not the GTP binding-defective (GDP-locked, T32N) form, of Arl1p bound to GST-GRIP. The binding was specific, as no binding to Arl3p, either in the GTP- or GDP-locked forms (Q78L, T31N), or by recombinant yeast Arf3/Arl2p or the Rab GTPases, Ypt31p, Ypt7p, or Vps21p, was observed (data not shown). Furthermore, coincubation of Arl3p had no effect on Arl1p binding. These results indicate that the Imh1p GRIP domain binds directly and specifically to Arl1p-GTP. Because only a small proportion of Arl1p (approximately 5% of input) bound to the Imh1p GST-GRIP domain fusion protein, it is possible that myristoylation of Arl1p influences this interaction, or that within the cell, additional factors, such as other Golgi-localized proteins and/or lipids, contribute to Imh1p Golgi localization.

If Arl1p is a Golgi receptor for GRIP domain proteins, then the Imh1p GRIP domain and Arl1p should colocalize at the Golgi. To test this, we tagged the chromosomal *ARL1* locus with GFP so that it served as the only source of Arl1p and transformed this strain with the RFP-Imh1p-GRIP plasmid. Arl1p-GFP localized to punctate organelles and colocalized perfectly with RFP-Imh1p-GRIP, indicating that Arl1p is indeed localized to the Golgi (Figure 4). In contrast to the results with Arl1p, in cells expressing Arl3p-GFP, very few cells exhibited a punctate signal and very weak cytoplasmic fluorescence was observed (see Figure S1 in the Supplementary Material available with this article online). Because membrane targeting of most characterized ARF GTPases is regulated by their GTPase cycle, we examined localization of GFP-tagged, constitutively active Arl3p(Q78L) and

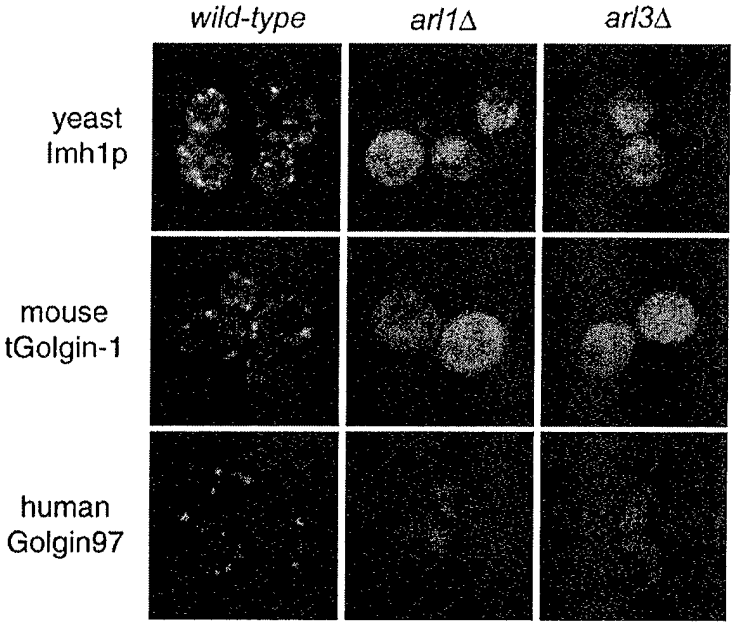

Figure 2. Golgi Localization of Yeast Imh1p, Mouse tGolgin-1, and Human Golgin-97 GRIP Domains Is Disrupted in *arl1*Δ and *arl3*Δ Mutants

GFP fusions to the Imh1p GRIP domain, the mouse tGolgin-1 GRIP domain (amino acids 2053–2238), or the human Golgin-97 GRIP domain (amino acids 588–767) were expressed in wild-type, *arl1*Δ, and *arl3*Δ strains and were photographed.

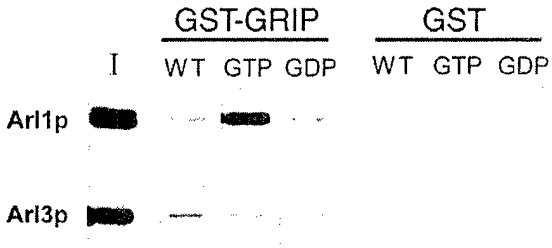

Figure 3. Activated Arl1p Binds the GST-Imh1p GRIP Domain

A fusion protein consisting of GST coupled to the Imh1p GRIP domain, or GST as a control, was captured on GSH Sepharose and mixed with purified, recombinant wild-type ("WT"), GTPase-deficient ("GTP"), or GTP binding-defective ("GDP") Arl1p or Arl3p. After incubation, the beads were washed twice, and bound material was visualized by immunoblotting with antibodies to epitope-tagged Arl GTPases. Approximately 10% of the added input of each Arl GTPase is shown in the "I" lane (only the activated ["GTP"] Arl input lanes are shown).

inactive Arl3p(T31N) mutants expressed in *arl3*Δ cells from a single copy vector. A faint punctate signal was observed for constitutively active Arl3p(Q78L)-GFP (Figure 4), but Arl3p(T31N)-GFP was apparently localized to the cytosol (Figure S1). These observations suggest that Arl3p is localized to the Golgi in a nucleotide-dependent manner, although the low Arl3p-GFP signal precluded definitive colocalization with Golgi markers. Interestingly, Arl3p and its human ortholog ARP lack the consensus Glycine that is myristoylated in all characterized ARF GTPases, and this may explain in part why Arl3p is poorly localized to the Golgi at steady state.

The observations that GRIP domains are mislocalized in the *arl3*Δ strain, but that Arl3p does not appear to bind the Imh1p GRIP domain, led us to hypothesize that Arl3p might regulate localization of Arl1p. To test this hypothesis, we examined localization of GFP-tagged Arl1p in *arl3*Δ mutant cells and found that it was localized to the cytosol in *arl3*Δ cells, indicating that Arl3p functions to promote stable association of Arl1p with the Golgi (Figure 4). To determine if the nucleotide cycle of Arl3p regulates Arl1p-dependent Golgi localization of GRIP domains, we next examined localization of GFP-Imh1p-GRIP and Arl1p-GFP in *arl3*Δ mutant cells transformed with single copy plasmids expressing Arl3p(T31N) or Arl3p(Q78L) as the sole sources of Arl3p. As can be seen in Figure 4 and in Figure S1, both GFP-Imh1p-GRIP and Arl1-GFP were localized to the Golgi when activated Arl3p(Q78L) was present, but not when Arl3p(T31N) was present. Thus, Arl3p indirectly affects Arl1p-dependent localization of GFP-GRIPs because, in *arl3*Δ cells, Golgi localization of the GRIP domain receptor, Arl1p-GTP, is abrogated.

These results lead us to propose a model in which the nucleotide cycle of Arl3p regulates Golgi localization of Arl1p, which, when activated by nucleotide exchange, recruits Imh1p to the Golgi via its GRIP domain. Orthologs of yeast Arl3p, Arl1p, and Imh1p are present in other eukaryotes, and because localization of mammalian GRIP domains in yeast requires the same proteins as yeast Imh1p, the *ARL3/ARL1/IMH1* pathway appears to be conserved throughout eukaryotic evolution. Other components of this pathway are likely to include guanine nucleotide exchange factors (GEFs) and GTPase activating factors (GAPs) that regulate the nucleotide cycles of Arl3p and Arl1p. Mon2p (also called Ysl2p) was reported to be related to Sec7 family ARF GEFs, and high-level expression of *ARL1*, but not *ARL3*, was found to suppress the growth defect of a *mon2*Δ deletion mutant [20], raising the possibility that Mon2p may be a GEF for Arl1p. However, localization of Arl1p-GFP and GFP-Imh1p-GRIP is not substantially affected in a *mon2*Δ mutant (data not shown), although genetic evidence does implicate Mon2p as a component of the *ARL3/ARL1/IMH1* pathway. Analysis of Golgi protein sorting

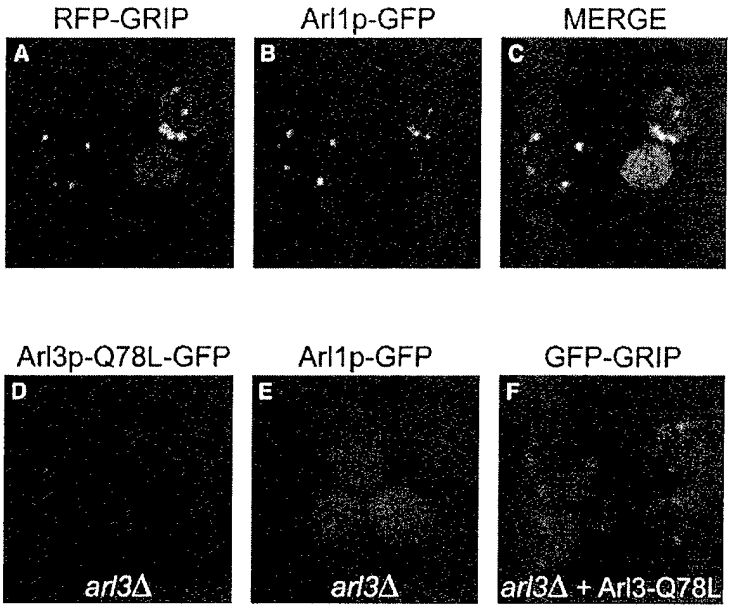

Figure 4. The GTPase Cycle of Arl3p Regulates Golgi Localization of Arl1p and GFP-Imh1p-GRIP

(A–C) RFP-Imh1p-GRIP and Arl1p-GFP were visualized by fluorescence microscopy individually (A and B), and then the two micrographs were overlayed (C).

(D) To visualize Arl3p within the cell, the GFP-tagged wild-type, inactive Arl3p(T31N) or constitutively active Arl3p(Q78L) were expressed from single copy vectors in the *arl3*Δ strain and were photographed (see Figure S1).

(E) Localization of Arl1p in *arl3*Δ mutant cells was determined by expressing GFP-tagged wild-type Arl1p from a single copy vector that had been transformed into the *arl3*Δ strain. To determine if the GTPase cycle of Arl3p regulates Arl1p-dependent Golgi localization of the Imh1p GRIP domain, the *arl3*Δ strain was cotransformed with single copy vectors expressing wild-type or mutant *arl3* genes and the GFP-Imh1p-GRIP plasmid.

(F) Golgi localization of GFP-Imh1p-GRIP was observed in wild-type and Arl3p(Q78L) cells, but not in Arl3p(T31N) cells (Figure S1).

in *imh1*, *arl1*, and *arl3* mutants, and identification of genetic interactions between mutations in *imh1* and mutations affecting the Ypt6 Rab GTPase, suggest that it may serve in the retention and/or retrieval of proteins to the late Golgi [16, 20, 21, 22]. We have found that *arl1*Δ, *arl3*Δ, and *mon2*Δ alleles lead to synthetic lethality when combined with a deletion of *ypt6* (Figure S2), as has been reported for an *imh1*Δ *ypt6*Δ double mutant [23]; this finding is consistent with these genes functioning together in a genetic pathway that is essential in the absence of Ypt6p.

Our findings are consistent with a cascade of ARF-like GTPases that function in a sequential manner to regulate protein localization in the Golgi. A cascade of sequentially acting yeast Rab GTPases that regulates vectoral transport of cargo to and through the Golgi has recently been described [24, 25], and it may be that an Arl GTPase cascade functions in an analogous manner to regulate recycling of proteins to the Golgi from endosomal compartments. This work establishes functions for the two yeast ARF-like GTPases and should open the door to a better understanding of the roles of this family of enzymes in regulating Golgi function.

Supplementary Material

Supplementary Material including a description of the Experimental Procedures used to construct and visualize the GFP- and RFP-tagged proteins and to perform the GRIP domain binding experiments is available at http://images.cellpress.com/supmat/supmatin.htm.

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References

1. Munro, S., and Nichols, B.J. (1999). The GRIP domain—a novel Golgi-targeting domain found in several coiled-coil proteins. Curr. Biol. *9*, 377–380.
2. Barr, F.A. (1999). A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. Curr. Biol. *9*, 381–384.
3. Kjer-Nielsen, L., Teasdale, R.D., van Vliet, C., and Gleeson, P.A. (1999). A novel Golgi-localization domain shared by a class of coiled-coil peripheral membrane proteins. Curr. Biol. *9*, 385–388.
4. Bevis, B.J., and Glick, B.S. (2002). Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). Nat. Biotechnol. *20*, 83–87.
5. Seron, K., Tieaho, V., Prescianotto-Baschong, C., Aust, T., Blondel, M.O., Guillaud, P., Devilliers, G., Rossanese, O.W., Glick, B.S., Riezman, H., et al. (1998). A yeast t-SNARE involved in endocytosis. Mol. Biol. Cell *9*, 2873–2889.
6. Valdivia, R.H., Baggott, D., Chuang, J.S., and Schekman, R.W. (2002). The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins. Dev. Cell *2*, 283–294.
7. Kjer-Nielsen, L., van Vliet, C., Erlich, R., Toh, B.H., and Gleeson, P.A. (1999). The Golgi-targeting sequence of the peripheral membrane protein p230. J. Cell Sci. *112*, 1645–1654.
8. Burd, C.G., and Emr, S.D. (1998). Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol. Cell *2*, 157–162.
9. Cowan, D.A., Gay, D., Bieler, B.M., Zhao, H., Yoshino, A., Davis, J.G., Tomayko, M.M., Murali, R., Greene, M.I., and Marks, M.S. (2002). Characterization of mouse tGolgin-1 (golgin-245/trans-golgi p230/256 kD golgin) and its upregulation during oligodendrocyte development. DNA Cell Biol. *21*, 505–517.
10. Griffith, K.J., Chan, E.K., Lung, C.C., Hamel, J.C., Guo, X., Miyachi, K., and Fritzler, M.J. (1997). Molecular cloning of a novel 97-kd Golgi complex autoantigen associated with Sjogren's syndrome. Arthritis Rheum. *40*, 1693–1702.
11. Pasqualato, S., Renault, L., and Cherfils, J. (2002). Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front-back' communication. EMBO Rep. *3*, 1035–1041.
12. Bonangelino, C.J., Chavez, E.M., and Bonifacino, J.S. (2002). Genomic screen for vacuolar protein sorting genes in *Saccharomyces cerevisiae*. Mol. Biol. Cell *13*, 2486–2501.
13. Rosenwald, A.G., Rhodes, M.A., Van Valkenburgh, H., Palanivel, V., Chapman, G., Boman, A., Zhang, C.J., and Kahn, R.A. (2002). ARL1 and membrane traffic in *Saccharomyces cerevisiae*. Yeast *19*, 1039–1056.
14. Huang, C.F., Buu, L.M., Yu, W.L., and Lee, F.J. (1999). Characterization of a novel ADP-ribosylation factor-like protein (yARL3) in *Saccharomyces cerevisiae*. J. Biol. Chem. *274*, 3819–3827.
15. Lee, F.J., C.F. Huang, Yu, W.L., Buu, L.M., Lin, C.Y., Huang, M.C., Moss, J., and Vaughan, M. (1997). Characterization of an ADP-ribosylation factor-like 1 protein in *Saccharomyces cerevisiae*. J. Biol. Chem. *272*, 30998–31005.
16. Tsukada, M., Will, E., and Gallwitz, D. (1999). Structural and functional analysis of a novel coiled-coil protein involved in Ypt6 GTPase-regulated protein transport in yeast. Mol. Biol. Cell *10*, 63–75.
17. Lu, L., Horstmann, H., Ng, C., and Hong, W. (2001). Regulation of Golgi structure and function by ARF-like protein 1 (Arl1). J. Cell Sci. *114*, 4543–4555.
18. Van Valkenburgh, H., Shern, J.F., Sharer, J.D., Zhu, X., and Kahn, R.A. (2001). ADP-ribosylation factors (ARFs) and ARF-like 1 (ARL1) have both specific and shared effectors: characterizing ARL1-binding proteins. J. Biol. Chem. *276*, 22826–22837.
19. Munro, S. (2002). Organelle identity and the targeting of peripheral membrane proteins. Curr. Opin. Cell Biol. *14*, 506–514.
20. Jochum, A., Jackson, D., Schwarz, H., Pipkorn, R., and Singer-Kruger, B. (2002). Yeast Ysl2p, homologous to Sec7 domain guanine nucleotide exchange factors, functions in endocytosis and maintenance of vacuole integrity and interacts with the Arf-Like small GTPase Arl1p. Mol. Cell. Biol. *22*, 4914–4928.
21. Siniossoglou, S., and Pelham, H.R. (2001). An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. EMBO J. *20*, 5991–5998.
22. Bensen, E.S., Yeung, B.G., and Payne, G.S. (2001). Ric1p and the Ypt6p GTPase function in a common pathway required for localization of trans-Golgi network membrane proteins. Mol. Biol. Cell *12*, 13–26.
23. Siniossoglou, S., Peak-Chew, S.Y., and Pelham, H.R. (2000). Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p. EMBO J. *19*, 4885–4894.
24. Ortiz, D., Medkova, M., Walch-Solimena, C., and Novick, P. (2002). Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast. J. Cell Biol. *157*, 1005–1015.
25. Wang, W., and Ferro-Novick, S. (2002). A ypt32p exchange factor is a putative effector of ypt1p. Mol. Biol. Cell *13*, 3336–3343.

A role for GRIP domain proteins and/or their ligands in structure and function of the trans Golgi network

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Summary

tGolgin-1 (golgin-245, trans golgi p230) and golgin-97 are members of a family of peripheral membrane proteins of unknown function that localize to the trans Golgi network (TGN) through a conserved C-terminal GRIP domain. We have probed for GRIP protein function by assessing the consequences of overexpressing isolated GRIP domains. By semi-quantitative immunofluorescence microscopy we found that high level expression of epitope-tagged, GRIP domain-containing fragments of tGolgin-1 or golgin-97 specifically altered the characteristic pericentriolar distribution of TGN integral membrane and coat components. Concomitantly, vesicular transport from the TGN to the plasma membrane and furin-dependent cleavage of substrate proteins in the TGN were inhibited. Mutagenesis of a conserved tyrosine in the tGolgin-1 GRIP domain abolished these effects. GRIP domain overexpression had little effect on the distribution of most Golgi stack resident proteins and no effect on markers of other organelles. Electron microscopy analyses of GRIP domain-overexpressing cells revealed distended perinuclear vacuoles and a proliferation of multivesicular late endosomes to which the TGN resident protein TGN46 was largely mislocalized. These studies, the first to address the function of GRIP domain-containing proteins in higher eukaryotes, suggest that some or all of these proteins and/or their ligands function in maintaining the integrity of the TGN by regulating resident protein localization.

Key words: Golgin-245/p230, Golgin-97, TGN46, Endosomes, VSV-G

Introduction

The trans Golgi network (TGN) is a series of interconnected tubules and vesicles at the trans face of the Golgi stack that functions in the processing and sorting of glycoproteins and glycolipids at the interface of the biosynthetic and endosomal pathways (Griffiths and Simons, 1986; Traub and Kornfeld, 1997). TGN structure is dynamic, subject to constant influx and efflux of membrane from and to both secretory and endosomal compartments. Such dynamics require efficient membrane recycling to maintain a constant steady state composition of lipids and proteins. Hence, TGN resident integral membrane proteins, including glycosyl modifying enzymes (Geuze and Morre, 1991), proprotein processing enzymes (Seidah and Chretien, 1997; Varlamov and Fricker, 1998), SNARE proteins (Lewis et al., 2000; Mallard et al., 2002; Siniossoglou and Pelham, 2001), and putative cargo binding proteins such as TGN38/TGN46 (Banting and Ponnambalam, 1997), maintain a steady state accumulation within the TGN by active retention and recycling (Bryant and Stevens, 1997; Ghosh et al., 1998; Mallet and Maxfield, 1999; Ponnambalam et al., 1994). The best characterized recycling pathways involve retrieval of resident proteins and glycolipids from endosomes (Molloy et al., 1999; Rohn et al., 2000). The molecular mechanisms that regulate both the efflux of membrane to the cell surface and the retrieval from endosomes are only beginning to be understood, and include sorting signals on cargo proteins and specific cytoplasmic components to effect cargo movement (Mallard et al., 2002; Rohn et al., 2000). Among these components there are likely to exist as yet unidentified tethering factors (Lowe et al., 1998; Pfeffer, 1999).

In an effort to elucidate effectors regulating TGN biogenesis, we have focused on a group of large peripheral membrane proteins of unknown function characterized by an extensive predicted coiled-coil structure and a conserved C-terminal GRIP (Golgin-97, RanBP2α, Imh1p and trans golgi p230) domain (Barr, 1999; Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b; Munro and Nichols, 1999). The GRIP domain confers localization to the TGN and associated vesicles for all four mammalian GRIP domain-containing proteins (GRIP proteins): tGolgin-1 [(Cowan et al., 2002) also known as trans golgi p230 (Erlich et al., 1996), golgin-245 (Fritzler et al., 1995) and 256 kDa golgin (H. P. Seelig, GenBank accession no. X82834)], golgin-97, GCC88 and GCC185 (Brown et al., 2001; Gleeson et al., 1996; Luke et al., 2003). The single yeast GRIP protein, Imh1p, similarly localizes to the late Golgi (Panic et al., 2003; Setty et al., 2003). No function has yet been assigned to any GRIP protein, but indirect evidence supports a role in vesicular traffic at the TGN. Several GRIP domains bind to the small Arf-like GTPase, Arl1 (Lu et al., 2001; Panic et al., 2003; Setty et al., 2003; Van Valkenburgh et al., 2001), which itself may regulate Golgi and/or TGN structure (Lu et al., 2001; Van Valkenburgh et al., 2001; Panic et al., 2003). tGolgin-1 is associated with a class of vesicles released from Golgi membrane fractions in an in vitro budding assay (Brown et al.,

2001; Gleeson et al., 1996). Finally, IMH1 displays both multicopy suppression and synthetic lethality with mutations in genes encoding the yeast Rab6 homologue, YPT6, and its nucleotide exchange factor, RIC1 (Li and Warner, 1996; Siniossoglou et al., 2000; Tsukada and Gallwitz, 1996; Tsukada et al., 1999), which themselves are implicated in endosome to TGN recycling (Bensen et al., 2001; Siniossoglou and Pelham, 2001; Tsukada et al., 1999). Given these data, and their structural similarities to known tethering proteins (Pfeffer, 1999), we hypothesized that mammalian GRIP proteins function in regulating protein recycling from endosomes to the TGN.

Overexpression of isolated GRIP domains by transfection competitively displaces endogenous GRIP proteins from Golgi membranes (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), presumably by competition for a limiting GRIP domain binding site. Such displacement would be expected to interfere with the function of endogenous GRIP proteins. We show here that in cells in which GRIP domain-containing fragments from tGolgin-1 or golgin-97 are overexpressed, the structure, resident protein localization and function of the TGN are largely disrupted. The results suggest that GRIP proteins or their ligands function in the maintenance of TGN integrity, probably through regulating TGN membrane protein localization.

Materials and Methods

Plasmids

cDNA clones encoding mouse tGolgin-1 and plasmids encoding N-terminally HA11 (influenza hemagglutinin 11) epitope-tagged truncated forms of tGolgin-1 encoding the C-terminal 312, 186, 81 or 50 amino acids (C312, C186, C81, C50), and a point mutant of C312, C312(Y2187A) (in which the codon for the critical GRIP domain tyrosine residue was altered to that for alanine) in the mammalian expression vector pCDM8.1 (Bonifacino et al., 1990) are described elsewhere (Cowan et al., 2002). T7 epitope-tagged C312 in pCDM8.1 was prepared by two-step polymerase chain reaction (Higuchi et al., 1988) using primers encoding the epitope tag and a Kozak consensus start site. A fragment encoding the C-terminal 179 amino acids of golgin-97 (G97-C179) was amplified by reverse transcriptase coupled PCR (RT-PCR) from RNA isolated from a human melanoma, MNT-1, and subcloned into pCDM8.1-HA11. Sequences of all PCR-derived inserts and of junctions of subcloned fragments were verified by automated dideoxy sequencing. Details of the sequence of PCR primers used for plasmid construction are available upon request. The following constructs have been described previously as indicated: Tac in pCDM8.1 (Leonard et al., 1984); Tac chimeric proteins with the cytoplasmic domains of TGN38 [TGG (Humphrey et al., 1993)], furin [TTF (Voorhees et al., 1995)] or Lamp1 [TTL1 (Marks et al., 1996)], or the di-leucine-based sorting signal of CD3γ [Tac-DKQTLL (Letourneur and Klausner, 1992; Marks et al., 1996)] in pCDM8.1; Pmel17 in pCI (Berson et al., 2001); furin with a C-terminal HA11 epitope tag in pXS (Bosshart et al., 1994); and the ts045 variant of the vesicular stomatitis virus glycoprotein conjugated to enhanced green fluorescent protein [VSV-G-EGFP (Presley et al., 1997)].

Cell culture and transfections

HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were transfected using calcium phosphate precipitation as described previously (Marks et al., 1996). For most experiments, cells grown on coverslips in six-well dishes were transfected with 7 μg of total DNA; in cases where moderate expression levels were desired, 50-100 ng of the desired expression construct were used. Except where noted, cells were analyzed 2-3 days following transfection. Stable transfectants of the CHO cell variant TRVb-1 expressing Tac chimeric proteins TGG and TTF (Ghosh et al., 1998; Mallet and Maxfield, 1999), provided by Dr F. R. Maxfield (Weil Medical College of Cornell University, New York, NY), were transiently transfected using Superfect (Qiagen, Valencia, CA) or GenePORTER (Gene Therapy Systems, San Diego, CA) according to the manufacturers' instructions.

Antibodies

Antibodies were to the following molecules (sources are given in parentheses). HA11 epitope (mAb 16D12, Covance Research Products, Richmond, CA; mAbs 12CA5 and 3F10, Roche Molecular Biochemicals, Indianapolis, IN); TGN46 [rabbit anti-TGN46 (Prescott et al., 1997); sheep anti-TGN46, Serotec, Oxford, UK)]; cation-independent mannose 6-phosphate receptor (mAb αMPR300, Affinity BioReagents, Golden, CO); Tac (Santini et al., 1998); golgin-97 (rb αgolgin-97 and mAb αgolgin-97, E. K. L. Chan, Scripps Res. Inst., La Jolla, CA); giantin (Linstedt and Hauri, 1993); Rab6 (C-19 rb αRab6, Santa Cruz Biotechnology, Santa Cruz, CA); ERGIC-53 (Schweizer et al., 1988); mannosidase II (Moremen and Touster, 1985); galactosyltransferase (Berger and Hesford, 1985); MG160 (Gonatas et al., 1989); AP-1 (Ahle et al., 1988); Lamp1 (Carlsson et al., 1988; Mane et al., 1989); Lamp2 (Mane et al., 1989); CD63 (mAb αCD63, Beckman Coulter, Fullerton, CA); transferrin receptor (mAb B3/25, Roche Molecular Biochemicals); T7 epitope (mAb from Novagen, Madison, WI). mAbs to EEA1, human tGolgin-1 (p230), GM130, p115 and syntaxin 6 were from Becton Dickenson/Transduction Laboratories (San Diego, CA). Secondary antibodies to mouse, rabbit, sheep or rat IgG conjugated to rhodamine red X (RRX), fluorescein isothiocyanate (FITC), aminomethylcoumarin acetate (AMCA), or gold (6 nm and 12 nm) were from Jackson ImmunoResearch (West Grove, PA). FITC- and Texas Red-conjugated secondary antibodies to mouse IgG isotypes were from Southern Biotechnology (Birmingham, AL).

Immunofluorescence microscopy

Cells were fixed with 2% formaldehyde and stained as described previously (Marks et al., 1995). Where indicated, cells were treated with 1 mg/ml leupeptin or 10-50 μg/ml cycloheximide (CHX) prior to fixation. For transferrin uptake, cells on coverslips were incubated with FITC-transferrin (from Sigma; 20 μg/ml) in serum-free medium containing 0.5% bovine serum albumin for 15-20 minutes, then rinsed in warm PBS prior to fixation. Cells were analyzed on a Leica (Bannockburn, IL) DM IRBE microscope using a Hamamatsu (Hamamatsu, Japan) Orca digital camera and Improvision (Lexington, MA) OpenLab software. Semiquantitative analyses were done with the Measurements module using raw images taken at constant exposure times pre-determined to be sub-saturating for the brightest samples. Outlines were drawn around individual cells, and total fluorescence from each cell was determined by multiplying the total number of pixels within the outline by the average value per pixel (after subtracting a background value per pixel, taken from an area within the photographed field in which there were no cells). Images shown were obtained using the Volume Deconvolution module from a series of raw images in different z-axis planes.

Metabolic pulse/chase and immunoprecipitation

Cells transfected with C312, C312(Y2187A), or control vector together with pCI-Pmel17 were metabolically labeled for 30 minutes with [^{35}S]methionine/cysteine and chased for various periods of time. Cell lysates in Triton X-100 were immunoprecipitated with anti-Pmel17, immunoprecipitates were fractionated by SDS-PAGE and gels were analyzed by phosphorimaging as described previously (Berson et al., 2001).

Electron microscopy analyses

HeLa cells cotransfected with C312 or C312(Y2187A) (42 μg/10 cm dish) and pCDM8.1-Tac (4.2 μg) (Marks et al., 1995) were harvested in PBS/5mM EDTA, washed and stained with anti-Tac antibodies conjugated to phycoerythrin (Beckman Coulter, Fullerton, CA). Fluorescently labeled cells were harvested using a FACStar Plus cell sorter. Sorted cells were fixed as described previously (McCaffery and Farquhar, 1995) after a 15 or 30 minutes incubation at 37°C with 5 mg/ml horseradish peroxidase (Sigma). For conventional electron microscopy (EM), cells were fixed in 100 mM cacodylate buffer, pH 7.4, containing 3% formaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose for 1 hour, washed, and osmicated at 4°C in Palade's fixative containing 1% OsO_4. Cells were then washed, treated with tannic acid, stained with uranyl acetate, dehydrated through a graded series of ethanol, and embedded in epon. 80 nm sections were cut on a LEICA UCT ultramicrotome and analyzed on a Philips 420 TEM at 80 kV. For immunogold labeling of ultrathin cryosections, cells were fixed in PBS containing 4% paraformaldehyde for 1 hour, washed and harvested. Cell pellets were cryo-protected in 2.3 M sucrose containing 20% polyvinyl pyrollidone, mounted on aluminum cryo-pins, and frozen in liquid nitrogen. Ultrathin cryosections were then cut on a Reichert UCT ultramicrotome equipped with an FCS cryo-stage, and sections were collected onto 300 mesh, formvar/carbon-coated nickel grids. Grids were washed, blocked in 10% FCS, and incubated overnight with primary antibodies (10 μg/ml). After washing, grids were incubated with gold-conjugated secondary antibodies for 2 hours, washed, and embedded in a mixture containing 3.2% polyvinyl alcohol (10×10^3 mol. mass), 0.2% methyl cellulose (400 centiposes), and 0.2% uranyl acetate. Sections were analyzed on a Philips EM 410 transmission electron microscope.

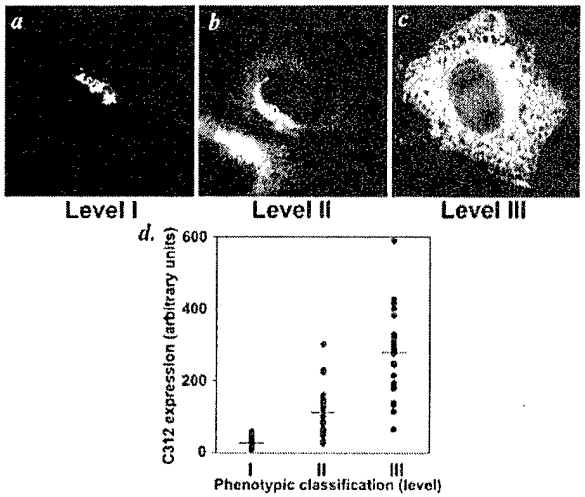

Fig. 1. Saturation of tGolgin-1 C312 localization in HeLa cells. HeLa cells transiently transfected with plasmid expressing HA-tagged C312 were analyzed 2 days later by IFM with anti-HA and RRX-conjugated secondary antibodies. (a-c) Examples of cells with different expression levels of C312 classified as levels I, II and III as indicated. (d) Comparison of semiquantitative total cell fluorescence levels with phenotypic classification. Total fluorescence from individual cells (in arbitrary units) was measured using OpenLab software as indicated in Materials and methods, and plotted on the y axis. Each dot represents the measurement from a single cell; the bar represents the median expression level from all cells.

Results

Disruption of TGN protein localization by overexpression of GRIP domains

In earlier work, HA-epitope-tagged, truncated forms of mouse tGolgin-1 containing the C-terminal 1247, 312, 186 or 81 amino acids, encompassing the GRIP domain, were efficiently localized to the Golgi when expressed at low levels by transfection in HeLa cells or other cell lines (Cowan et al., 2002). To probe GRIP protein function, we used immunofluorescence microscopy (IFM) to analyze whether high level expression of GRIP domain-containing fragments in transiently transfected HeLa cells affected the distribution of TGN resident proteins. HA-tagged C312 (corresponding to the C-terminal 312 amino acids of mouse tGolgin-1) was used in most experiments because of its enhanced expression and stability relative to the other truncated products, but similar results were observed using all GRIP domain-containing tGolgin-1 fragments. As the expression levels of C312 increased, pericentriolar anti-HA staining gave way to a diffuse staining pattern throughout the cytoplasm (Fig. 1b,c). We defined three qualitative fluorescence patterns: level I, a narrow ribbon-like structure consistent with Golgi localization; level II, a more diffuse paranuclear/pericentriolar structure with additional cytoplasmic staining; and level III, an intense cytoplasmic staining that may mask underlying structure. The C312 distribution in these cells was often reticular, perhaps reflecting association with ER membranes or cytoskeletal elements. Semiquantitative analysis of expression levels showed a correlation between total fluorescence intensity and the staining pattern observed (Fig. 1d). Consistent with previous reports (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), cells with level II or III staining patterns showed reduced or eliminated pericentriolar staining for endogenous tGolgin-1 and golgin-97 (see Fig. 3), likely reflecting competition for a limited GRIP domain binding site at the TGN (Barr, 1999; Kjer-Nielsen et al., 1999b).

Surprisingly, the localization of several TGN resident integral membrane proteins was disrupted in HeLa cells with high (level III) C312 expression (Fig. 2). TGN46, which localizes to a tight pericentriolar structure in untransfected cells or cells expressing low levels (level I) of C312 (Fig. 2, stars), was present in diffuse, vacuolated structures in cells with level III C312 expression (Fig. 2a-b′); in some cells expressing extremely high levels, pericentriolar staining was completely absent (not shown). Similarly, HA-tagged furin, which localizes to the TGN in transfected HeLa cells (Bosshart et al., 1994), was mislocalized to a diffuse and 'expanded' pericentriolar structure in cells with level III expression of T7-epitope tagged C312 (Fig. 2e,e′). These effects were independent of cell-type and a function of TGN localization rather than of unrelated functions of the TGN46 and furin lumenal domains. This was evident in transiently transfected CHO cell variants by the C312-induced redistribution of the chimeric proteins TGG and TTF, which bear the cytoplasmic domains of TGN38 or furin, respectively, and the lumenal and transmembrane domains of an irrelevant protein, Tac (Fig. 2g-h′,j-k′). Furthermore, AP-1, which associates with the TGN as a peripheral membrane protein, also failed to accumulate in the pericentriolar region in C312-overexpressing cells (Fig. 2m-n′). Since TGN46/TGG and furin/TTF localize to the TGN via distinct cytoplasmic targeting signals (Humphrey et al., 1993; Voorhees et al., 1995) and recycling pathways (Ghosh et al.,

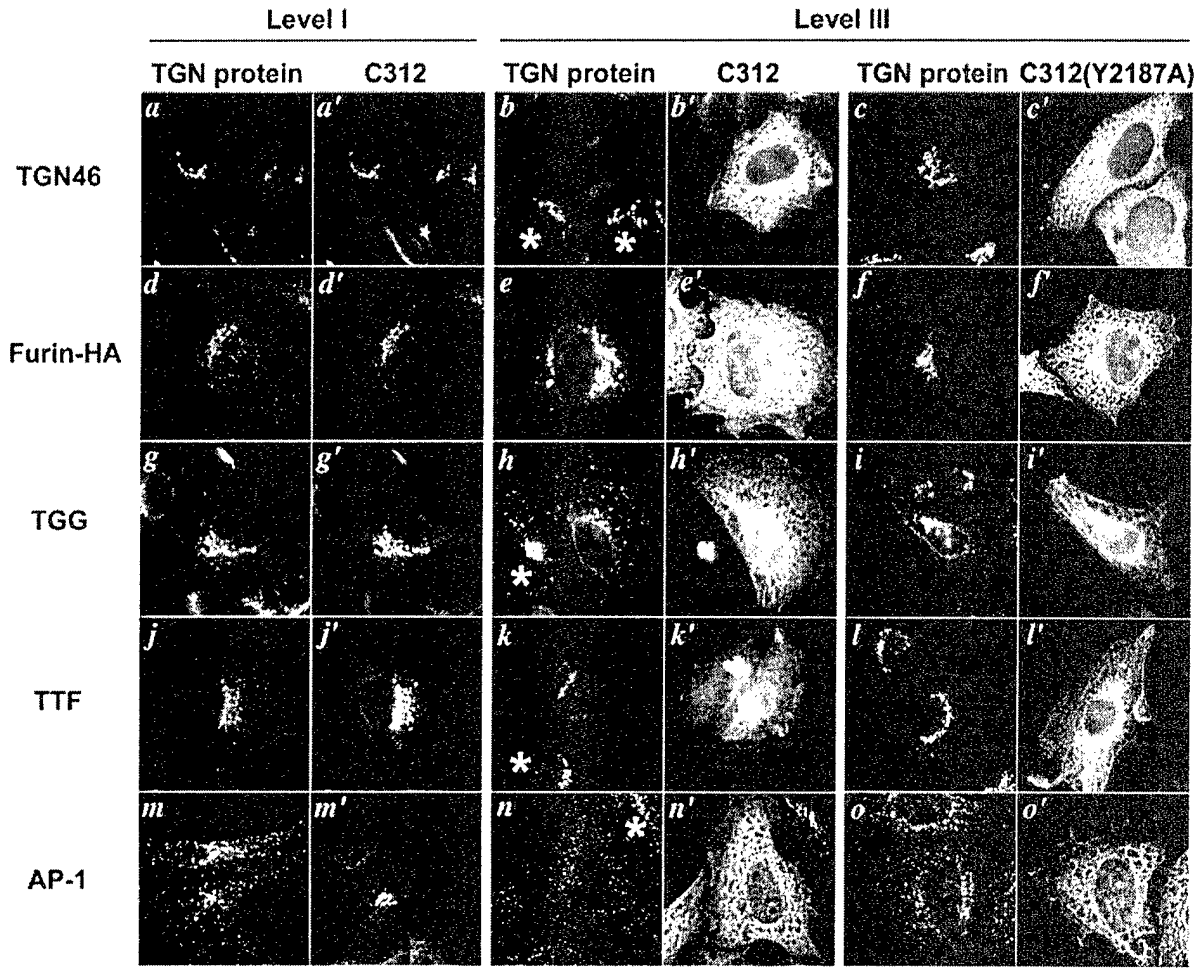

Fig. 2. Overexpression of C312 disrupts the localization of TGN resident proteins. HeLa cells (a-f,m-o) or stable transfectants of TRVb-1 cells expressing TGG (g-i) or TTF (j-l) were transiently transfected with C312 or C312(Y2187A), as indicated, and analyzed by IFM using antibodies to the indicated proteins and to the HA tag (′ columns) and appropriate secondary RRX- and FITC-conjugated secondary antibodies. The cells in d-f were cotransfected with furin-HA (50-100 ng/six-well dish) and T7-epitope-tagged C312 (5-7 μg/six-well dish); anti-HA was used to detect furin and anti-T7 to detect C312. (a,d,g,j,m) Cells with low C312 expression (level I); (b,e,h,k,n) cells with high expression (level III);(c,f,i,l,o) selected cells in which semiquantitative analyses showed levels of HA staining comparable to those in b, e, h, k, and n.

1998; Mallet and Maxfield, 1999), and AP-1 localization is mediated by independent mechanisms (Page and Robinson, 1995; Seaman et al., 1996), these data show that high level expression of C312 disrupted TGN localization mediated by multiple pathways. Similar results were obtained in COS, MOP8, and NRK cells (unpublished data). Note that the distribution of syntaxin 6 and the cation-independent mannose 6-phosphate receptor did not overlap significantly with the TGN in our untransfected HeLa cells, and thus no change in their distribution could be observed in cells overexpressing C312 (unpublished data).

Three approaches were used to show that the displacement of TGN proteins was due to overexpression of an intact GRIP domain. First, cells were transfected with HA-tagged C312 in which a tyrosine conserved in all GRIP domains was replaced by alanine [C312(Y2187A)]; this fragment failed to localize to the Golgi in transfected cells at any expression level (Cowan et al., 2002) (see also Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999). Expression of C312(Y2187A) at levels comparable to those sufficient for the level III phenotype of intact C312 (assessed by semiquantitative analysis of anti-HA fluorescence intensity) failed to disrupt the localization of any analyzed TGN resident protein (Fig. 2c,f,i,l,o), indicating that the GRIP domain must be intact to effect TGN disruption. Second, TGN46 mislocalization was observed upon overexpression of a C-terminal fragment of golgin-97, g97.C179, containing an independent GRIP domain (Fig. 3m,n). Finally, using semiquantitative IFM analyses in triple stained HeLa cells, we found that comparable levels of C312 expression induced both the mislocalization of TGN46 and the displacement of the endogenous GRIP proteins, tGolgin-1 and golgin-97, from the Golgi (Fig. 3a-l). Taken together, these data suggest that saturation of GRIP domain binding sites on TGN membranes or on a GRIP effector molecule results in disruption of the steady state distribution of TGN resident proteins.

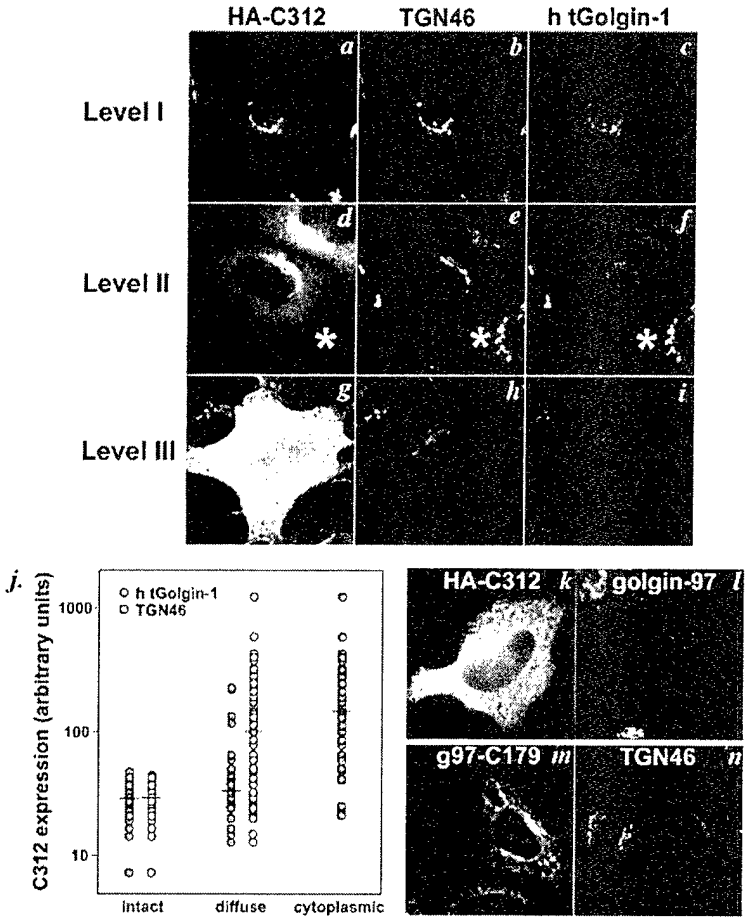

Fig. 3. Parallel displacement of TGN46 and endogenous GRIP domain proteins at similar expression levels of C312. (a-j) HeLa cells that were transiently transfected with C312 were analyzed by three-color IFM using rat anti-HA, mouse anti-tGolgin-1 and sheep anti-TGN46 with AMCA-, RRX- and FITC-conjugated species-specific antibodies, respectively. (a-i) Representative images of staining patterns for HA-C312 (a,d,g), TGN46 (b,e,h) and tGolgin-1 (c,f,i) obtained with C312 expressed at level I (a-c), II (d-f) and III (g-i). (j) Semiquantitative analyses of total cell expression level of AMCA fluorescence (representing C312) in cells characterized as having a tight pericentriolar Golgi staining pattern (intact), diffuse paranuclear staining (diffuse), or diffuse cytoplasmic distribution (cytoplasmic) for TGN46 and endogenous tGolgin-1. C312 expression is plotted in arbitrary units on a log scale on the y axis; circles represent values for individual cells, and bars represent the median of all analyzed cells. (k,l) Cells transiently transfected with C312 were analyzed by IFM using antibodies to HA (k) and golgin-97 (l). (m,n) Cells transiently transfected with g97-C179 were analyzed by IFM using antibodies to HA (m) and to TGN46 (n).

To determine whether GRIP domain overexpression interfered with a dynamic process, we tested whether the change in TGN46 distribution was reversible. Because C312 has a short half-life (Fig. 4a; see also Fig. 7), it could be rapidly depleted by CHX treatment of transfected HeLa cells; treatment for 1-4 hours increased the fraction of transgene-positive cells with level I staining at the expense of cells with level III staining (Fig. 4b), indicating a loss of cells with high level C312 expression. Analysis of this same population of cells for TGN46 localization indicated that the fraction of cells with a wild-type pericentriolar TGN46 staining pattern increased over time of CHX treatment (Fig. 4c). Thus, the effect of GRIP domain overexpression on TGN46 distribution is reversible.

Specificity of protein localization defects in GRIP domain overexpressing cells

To determine whether the effects of GRIP domain overexpression were limited to the TGN, we analyzed the distribution of residents of other organelles in cells expressing high levels of C312. Staining patterns for Golgi stack integral (giantin and mannosidase II; ManII) and peripheral (GM130 and p115) membrane proteins were unaffected by overexpression of C312 (Fig. 5A,a-h) or of other GRIP domain-containing fragments (unpublished data). Furthermore, there were no consistent effects on the distribution of actin filaments, cell surface proteins, or markers of the endoplasmic reticulum (ER), ER/Golgi intermediate compartment, early endosomes, or late endosomes/lysosomes (Table 1). Pericentriolar staining for β1,4 galactosyltransferase (GalT), an enzyme that localizes to both the TGN and the trans Golgi cisternae, was disrupted in about half of the cells that overexpressed C312, but not in cells that overexpressed C312(Y2187A) (Fig. 5Ba-f). This effect was observed less consistently than for TGN46 or other TGN resident proteins (Fig. 2) and did not correlate with C312 expression level (unpublished data), perhaps reflecting the distribution of GalT to both the TGN and Golgi stacks (Nilsson et al., 1993; Rabouille et al., 1995) and cycling between these compartments (Cole et al., 1998; Miesenböck and Rothman, 1995). The results indicate that GRIP domain overexpression affects primarily TGN structure and/or protein localization, with minimal effects on the Golgi stacks and no discernible effects on other membranous organelles.

Rab6 has been implicated as a binding partner for the GRIP domains of tGolgin-1 and golgin-97 (Barr, 1999). Rab6 localization to the Golgi was only marginally affected in C312-overexpressing cells (Fig. 5g-l and Table 1); as for GalT, loss of tight pericentriolar Rab6 staining was observed in only a fraction (25.7%) of C312-overexpressing cells and did not correlate with C312 expression levels. This suggests that Rab6 localization to the Golgi is not dependent on binding of any particular GRIP-domain containing protein, and that excess GRIP domain cannot sequester Rab6 from membranes. Similar results were obtained with a more established GRIP domain binding partner, Arl1 (data not shown), which in yeast is known to regulate – but not to be regulated by – GRIP domain Golgi localization (Panic et al., 2003; Setty et al., 2003).

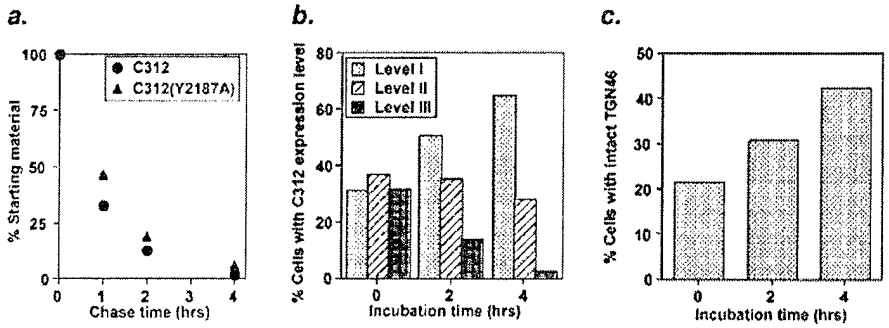

Fig. 4. Reversibility of TGN46 displacement induced by GRIP domain overexpression. (a) HeLa cells transiently transfected with C312 or C312(Y2187A) or control untransfected cells were metabolically labeled with [^{35}S]methionine/cysteine for 30 minutes and then chased for 0, 1, 2, or 4 hours. C312 or C312(Y2187A) was immunoprecipitated from cell lysates at each time point using anti-HA antibodies, fractionated by SDS-PAGE, and total C312 levels were determined by phosphorimaging analysis of the 40 kDa band that was absent in the controls (see Fig. 7). The amount at time 0 was set to 100%, and the percentage remaining at each time point is plotted. A representative of 3 experiments is shown. (b,c) HeLa cells from the same well transiently transfected with C312 were treated with 10 μg/ml CHX for 0, 2 or 4 hours as indicated, fixed, and then analyzed by IFM using antibodies to the HA-epitope (b) and to TGN46 (c). (b) The percentage of cells in a representative experiment that were positively stained with anti-HA (n=238 to 269 per time point in this experiment) were characterized for phenotypic expression level of C312 expression. (c) The percentage of cells in the same experiment that were positively stained with anti-HA were characterized for phenotypic appearance of TGN46 staining; 'intact' TGN46 indicates a tight Golgi ribbon as in Fig. 2a.

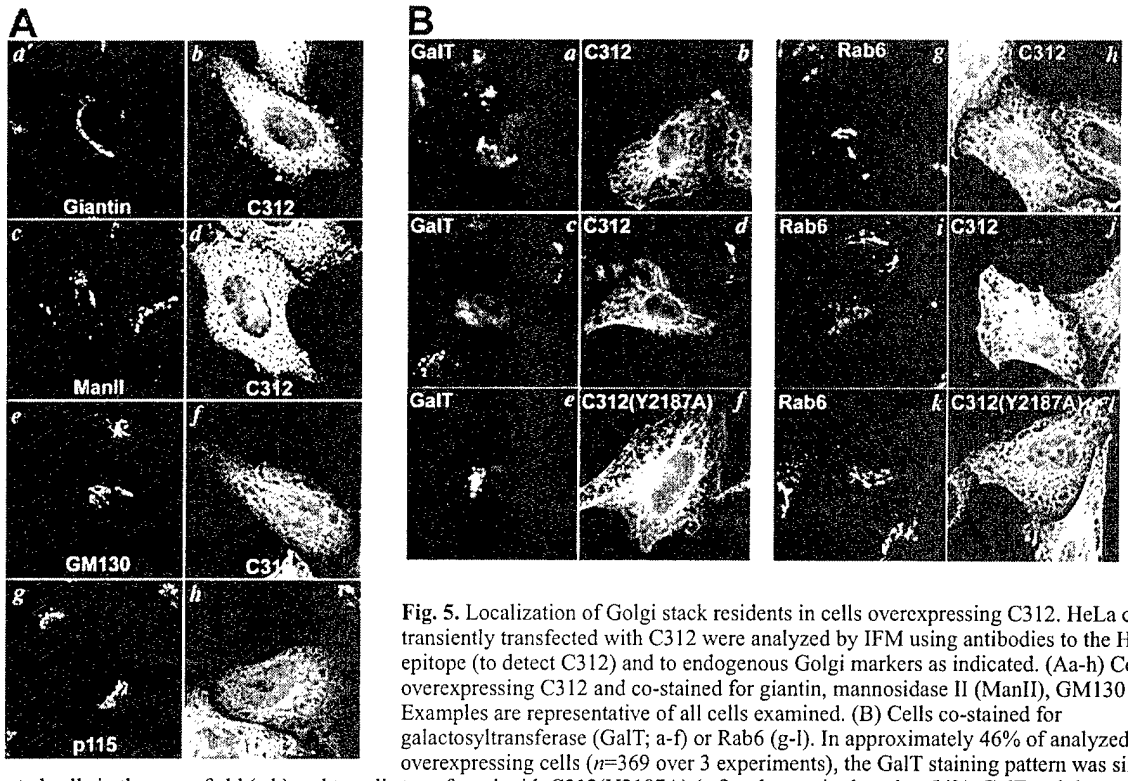

Fig. 5. Localization of Golgi stack residents in cells overexpressing C312. HeLa cells transiently transfected with C312 were analyzed by IFM using antibodies to the HA-epitope (to detect C312) and to endogenous Golgi markers as indicated. (Aa-h) Cells overexpressing C312 and co-stained for giantin, mannosidase II (ManII), GM130 or p115. Examples are representative of all cells examined. (B) Cells co-stained for galactosyltransferase (GalT; a-f) or Rab6 (g-l). In approximately 46% of analyzed C312-overexpressing cells (n=369 over 3 experiments), the GalT staining pattern was similar to untransfected cells in the same field (a,b) and to cells transfected with C312(Y2187A) (e,f), whereas in the other 54%, GalT staining was diffuse (c,d). A similar disparity was seen for Rab6; in approximately 74% of analyzed C312-overexpressing cells (n=175 over 2 representative experiments), Rab6 staining was similar to controls (g,h compared with k,l) and in 26%, Rab6 staining was diffuse (i,j).

TGN functional defects induced by GRIP domain overexpression

We next tested whether the alterations in TGN protein distribution observed upon overexpression of GRIP domains underlie TGN functional defects. First, we assayed protein transport from the ER to the plasma membrane using an EGFP-tagged form of the temperature-sensitive ts045 variant of VSV-G (Bergmann et al., 1981; Presley et al., 1997). VSV-G-EGFP misfolds at the restrictive temperature of 39°C and is thus retained within the ER of transfected HeLa cells, producing a reticular pattern throughout the cytoplasm visible by fluorescence microscopy (Fig. 6a) (Presley et al., 1997). When shifted to 32°C, accumulated VSV-G-EGFP synchronously exits the ER and traverses the Golgi complex en route to the plasma membrane. VSV-G-EGFP largely accumulates at the Golgi by 30 minutes and at the plasma membrane by 60

Table 1. Extent of protein mislocalization caused by the overexpression of the mouse tGolgin-1 C312 fragment

	C312*	C312(Y2187A)*
ER		
Calnexin	No	No
Intermediate compartment		
ERGIC-53	No	No
Golgi stack		
Giantin	No	No
GM130	No	No
p115	No	No
Mannosidase II	No	No
Galactosyl transferase	46% affected	No
Rab6	26% affected	No
Trans-Golgi network		
TGN46	Yes	No
Furin-HA (transgene)	Yes	No
TGG (transgene)	Yes	No
TTF (transgene)	Yes	No
AP-1	Yes	No
tGolgin-1	Yes	No
Golgin-97	Yes	No
Early endosome		
EEA1	No	No
Transferrin receptor	No	No
Internalized transferrin	No	No
Late endosome/lysosome		
Lamp1	No	No
Lamp2	No	No
CD63	No	No
TTL1 (transgene)	No	No
Tac-DKQTLL (transgene)	No	No
Cell surface		
MHC class I	No	No
Tac (transgene)	No	No

*Table tabulates whether changes were observed in the IFM pattern for the indicated marker proteins upon level III expression of either C312 or C312(Y2187A), as indicated. 'No' indicates no alteration in >95% of examined cells with level III expression pattern in at least three separate experiments; 'Yes' indicates alteration in >95% of examined cells with level III expression pattern. For Rab6 and GalT, patterns were changed in a fraction of cells (alterations did not correlate with expression level of C312). TGG, TTF and TTL1 are chimeric proteins containing the lumenal domain of Tac and the cytoplasmic domains of TGN38, furin, and lamp1 respectively; TGG contains the TGN38 transmembrane domain, whereas TTF and TTL1 contain the Tac transmembrane domain. Tac-DKQTLL contains the entire Tac coding sequence appended with the di-leucine-based sorting signal of CD3γ.

minutes (Fig. 6b-c). Coexpression of VSV-G-EGFP with very high levels of C312(Y2187A), which lacks a functional GRIP domain, had no effect on the kinetics of VSV-G-EGFP transport (compare Fig. 6a-c to d-f). In cells expressing comparable levels of C312, VSV-G-EGFP staining was also similar to the controls at 39°C and after 30 minutes at 32°C (Fig. 6g,h), indicating normal folding kinetics and ER to Golgi transport. However, after 60-120 min, most C312-expressing cells retained VSV-G-EGFP within the Golgi region (Fig. 6i,j), indicating a block in Golgi to plasma membrane transport. By 3 hours, some VSV-G-EGFP reached the plasma membrane in most cells (Fig. 6k), indicating that the block was not absolute, but still a substantial proportion of cells displayed prominent pericentriolar fluorescence not observed in the controls. The Golgi to plasma membrane block was dependent on C312 expression level; in cells expressing lower levels of the C312, transport proceeded normally such that plasma membrane staining was apparent by 60 minutes (Fig. 6l), whereas in cells expressing extremely high levels of C312, no plasma membrane fluorescence was observed even after 3 hours (Fig. 6m).

As a second test of TGN function, we assessed the ability of proprotein convertases of the furin family to cleave a substrate protein. Furin localizes predominantly to the TGN (Shapiro et al., 1997) where it cleaves target proproteins at dibasic recognition sites (Nakayama, 1997). Pmel17, a glycoprotein found in melanosome precursors in pigment cells, is cleaved by furin or a related proprotein convertase en route to late endosomes in transfected HeLa cells (Berson et al., 2003). To determine whether furin cleavage is compromised in cells overexpressing GRIP domains, Pmel17 processing was assessed by metabolic pulse/chase and immunoprecipitation in transfected HeLa cells expressing Pmel17 without or with excess C312 or C312(Y2187A). In cells expressing Pmel17 alone, a single ~100 kDa band (P1) present in cell lysates from pulse-labeled cells matured first to a slower migrating species (P2) by oligosaccharide processing in the Golgi, and then to two faster migrating species (Mα and Mβ) by proprotein convertase cleavage (Fig. 7b, lanes 1-4) as observed previously (Berson et al., 2001; Berson et al., 2003) and outlined schematically in Fig. 7a. By 4 hours, most of the P1 and P2 was converted to Mα and Mβ (lane 4). The kinetics of processing was virtually unchanged in cells co-expressing C312(Y2187A) (lanes 9-12). However, in cells co-expressing C312 at comparable levels (Fig. 7c), P2 accumulated and Mα and Mβ were not substantially generated (lanes 5-8). The formation of P2, which is resistant to digestion by endoglycosidase H (Berson et al., 2001; Berson et al., 2003), indicates that these cells had no, or minimal, defects in oligosaccharide processing within the Golgi stack. However, P2 accumulation coupled with Mα and Mβ depletion indicate that proprotein convertase cleavage of Pmel17 was blocked. These data indicate that overexpression of GRIP domains compromises either enzymatic functions or the interactions between enzyme and substrate within the TGN.

Despite these defects, some sorting functions normally ascribed to the TGN appeared to remain intact in C312-overexpressing cells. Transport to late endosomes was unaltered relative to controls as assessed by the steady state localization of several endogenous late endosomal and lysosomal proteins and the transport kinetics of cotransfected chimeric proteins (Table 1). These results suggest that C312 overexpression affects only certain TGN subdomains or the cycling of only certain cargo.

Ultrastructural defects and mislocalization of TGN46 in cells overexpressing GRIP domains

To determine the effects of C312 overexpression on TGN morphology in more detail, cells were analyzed by EM. Cells expressing C312 or C312(Y2187A) were enriched by cell sorting following cotransfection with a plasma membrane marker, Tac; by IFM, >90% of sorted cells expressed C312 or C312(Y2187A), the majority at high levels. Sorted cells were incubated with horseradish peroxidase (HRP) for 15-30 minutes at 37°C as a marker of fluid phase endocytosis prior to fixation, and then analyzed by EM either for morphology

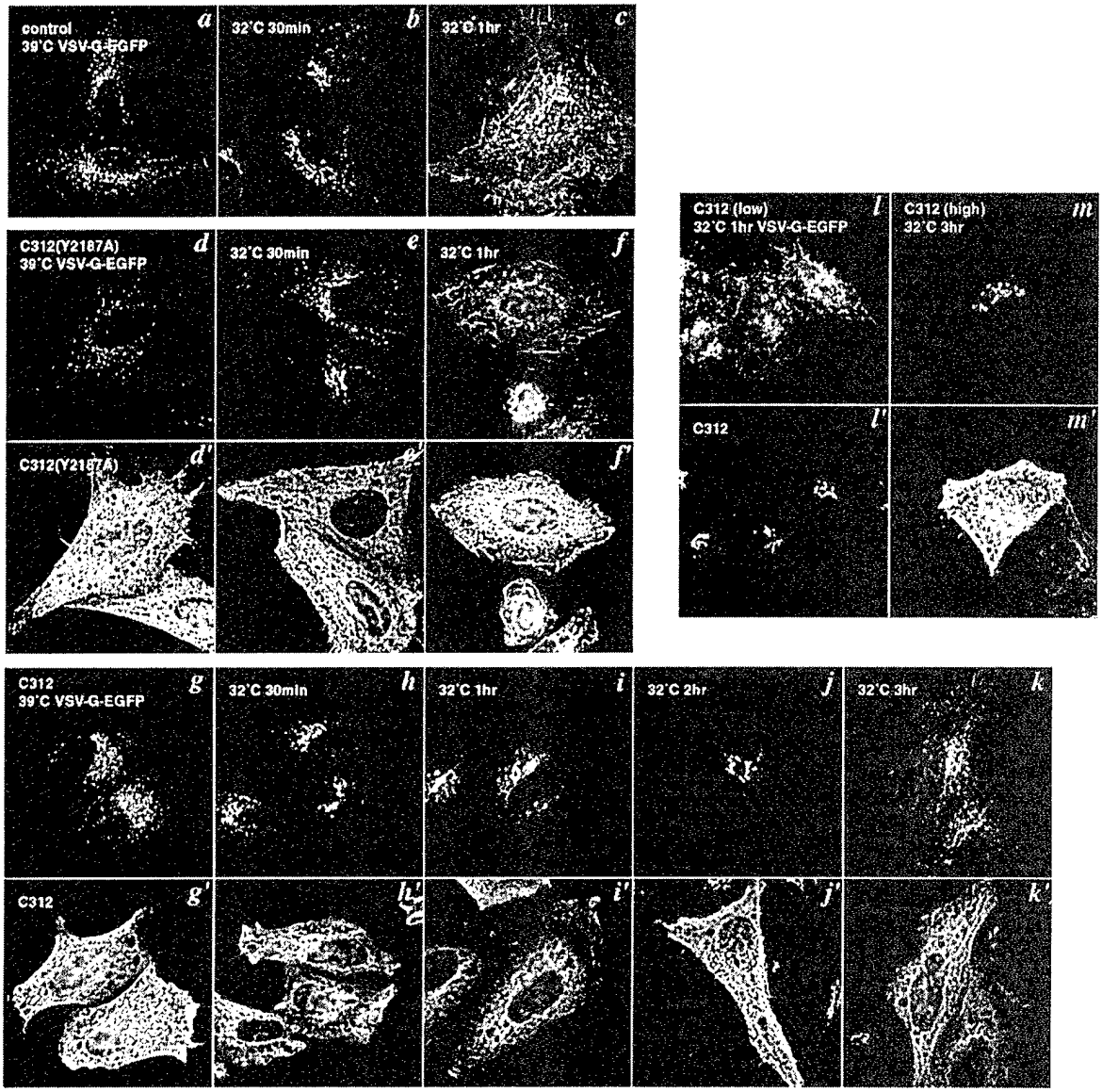

Fig. 6. GRIP domain overexpression affects Golgi to plasma membrane transport. HeLa cells were transiently transfected with expression vectors for VSV-G-EGFP (2.5 μg/six-well dish) and either vector alone (a-c′), C312(Y2187A) (d-f′) or C312 (g-m′) at 5μg/six-well dish. Cells were grown at 39°C for 1 day and then fixed either before (a,d,g) or after shifting to 32°C for the indicated times. Cells were analyzed by fluorescence microscopy after immunostaining with anti-HA and RRX-conjugated secondary antibodies (′ panels); EGFP was visualized directly (non-′ panels). (g-k′) Examples of cells with similar anti-HA staining intensities to those shown in d-f′; (l,l′) an example of a cell with low C312 expression, and (m,m′) an example of a cell with very high C312 expression. All ′ panels were taken at the same exposure time on samples prepared and analyzed on the same day. The 'spotty' appearance of VSV-G-EGFP localization in a, d and g may result from a fixation artifact.

after embedding in epon or for immuno-EM after indirect immunogold labeling of ultrathin cryosections with antibodies to HA, HRP, and/or TGN46.

HeLa cells transfected with C312(Y2187A) displayed a relatively normal morphology, similar to untransfected cells, with intact, flattened pericentriolar Golgi cisternae (Fig. 8a). Cells transfected with C312 were more heterogeneous, but displayed general defects in Golgi morphology. Intact Golgi cisternae were difficult to find in most C312-expressing cells. They were replaced by large, pericentriolar vacuolated structures (Fig. 8b,c). The vacuoles varied in number, reflecting an apparent proliferation of Golgi/TGN rather than simply engorged cisternae. The structures often displaced a large fraction of the cytoplasm, and some of the vacuoles contained internal membrane sheets. Multivesicular endosomes (mve) were also more abundant in cells expressing C312 than those expressing C312(Y2187A) (e.g., see Fig. 8c). These data indicate that GRIP domain overexpression results in a large scale disruption of Golgi/TGN architecture and alterations in the abundance of late endosomes.

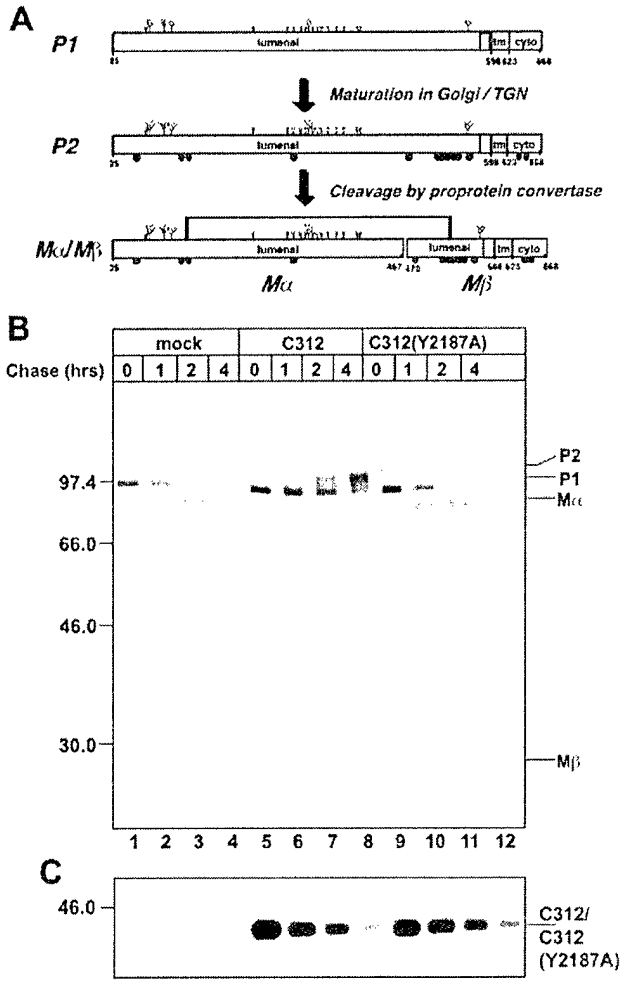

Fig. 7. GRIP domain overexpression affects proprotein convertase activity on a substrate protein, Pmel17. (a) Schematic diagram of Pmel17 primary structure and processed forms, as shown by Berson et al. (Berson et al., 2001). (b) HeLa cells were transiently transfected with expression vectors for Pmel17 (10 μg/10 cm dish) and either vector alone (lanes 1-4), C312 (lanes 5-8) or C312(Y2187A) (lanes *9-12*) at 39 μg/10 cm dish. Two days post-transfection cells were harvested and pulse labeled with [^{35}S]methionine/cysteine for 30 minutes, and then chased for the indicated times. Pmel17 was immunoprecipitated from cell lysates at each time point, fractionated by SDS-PAGE, and visualized by phosphorimaging analysis. (c) Anti-HA immunoprecipitates from the same samples analyzed in the same way. Only the relevant portion of the gel encompassing C312 or C312(Y2187A) transgene products is shown.

Immuno-EM analyses (Fig. 9) revealed the basis for the phenotypes observed by IFM. In cells expressing high levels of C312(Y2187A), TGN46 was localized as expected to cisternae and tubulovesicular structures at the trans side of the Golgi stack (Fig. 9A). A similar pattern was observed in cells expressing low levels of C312, in which TGN46 and C312 were colocalized in these structures (unpublished data). In cells expressing high levels of C312, however, TGN46 was instead detected primarily in two types of membrane compartments.

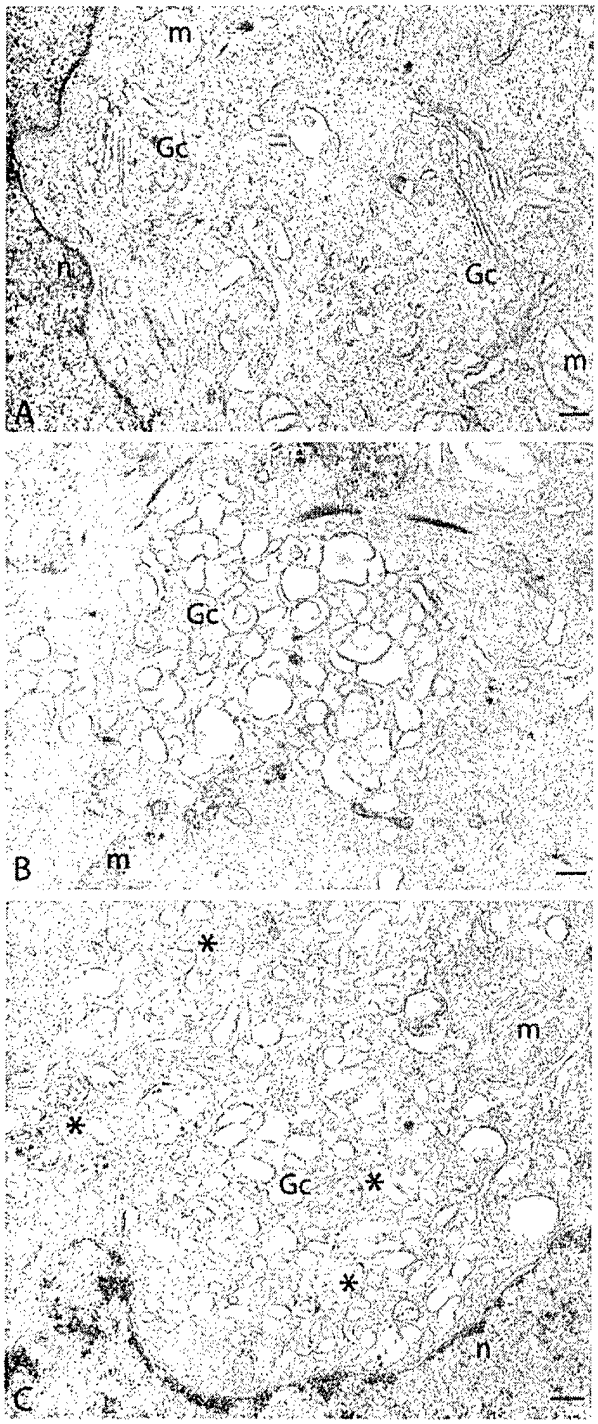

Fig. 8. GRIP domain overexpression disrupts Golgi morphology. HeLa cells transfected with expression vectors for Tac and for (A) C312(Y2187A) or (B,C) C312 were sorted for Tac cell surface staining, exposed to HRP for 15-30 minutes at 37°C, and then fixed and embedded in epon for conventional EM analyses. Note the flattened stacks of Golgi complex (Gc) cisternae in A and their absence in B and C. m, mitochondrion; n, nucleus. In C, stars are placed next to multivesicular bodies, which were abnormally abundant in most profiles from C312-transfected cells. Bars, 0.1 μm.

One type comprised large vacuolated structures with few internal membranes (Fig. 9B,C), probably corresponding to those observed by conventional EM (Fig. 8). These structures were labeled abundantly on the cytoplasmic face by anti-HA antibodies detecting the C312 transgene (anti-HA labeling was also observed throughout the cytoplasm and in large, electron dense inclusions; unpublished data). The second class of compartments comprised multivesicular structures with numerous intralumenal vesicles (Fig. 9D-F). These structures were mve, since they were also labeled by HRP following internalization for 15 or 30 min (Fig. 9E,F). TGN46 labeling in mve was found both on the limiting and intralumenal membranes, suggesting targeting for degradation in lysosomes. Since TGN46 normally bypasses the mve and trafficks through recycling endosomes en route to the TGN (Ghosh et al., 1998), these data suggest that GRIP domain overexpression results in TGN46 mistargeting to both late endocytic organelles and enlarged, vacuolated Golgi-derived structures, thus interfering with the normal delivery of TGN46 from the endocytic pathway to the TGN.

Discussion

Mammalian GRIP protein function has remained undefined, and a role for the yeast GRIP protein, Imh1p, in Golgi maintenance is inferred only from indirect genetic interactions. We have shown that overexpression of isolated GRIP domains in cultured mammalian cells results in specific disruption of TGN morphology, protein localization and function. The data provide evidence that at least one GRIP protein and/or GRIP ligand functions in TGN maintenance, probably by regulating recycling from endosomes.

Specificity of GRIP-domain-dependent disruption of vesicular transport

The displacement of endogenous GRIP proteins by expression of increasing doses of exogenous GRIP domains had been shown previously (Kjer-Nielsen et al., 1999a; Kjer-Nielsen et al., 1999b), but this is the first report to describe a concomitant

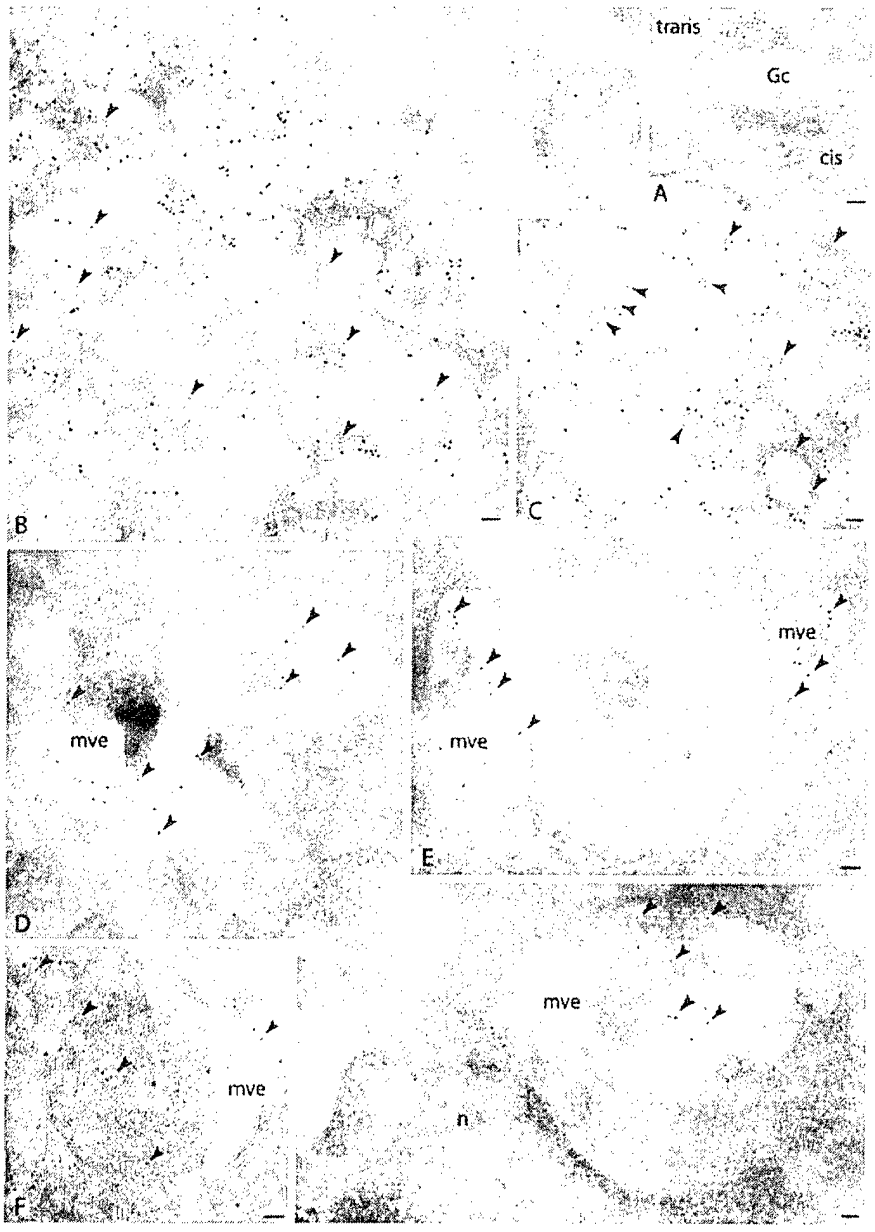

Fig. 9. TGN46 localizes to vacuoles and to multivesicular bodies in C312-overexpressing cells. HeLa cells transfected with expression vectors for Tac and for (A) C312(Y2187A) or (B-F) C312 were sorted for Tac cell surface staining. Positive cells were exposed to HRP for 15-30 minutes at 37°C, then fixed, and ultrathin cryosections were labeled with antibodies to HA, TGN46 and or HRP and gold-conjugated secondary antibodies for immuno-EM analyses. (A-C) Sections were labeled with anti-HA and 10 nm gold-conjugated anti-mouse immunoglobulin, and anti-TGN46 and 5 nm gold-conjugated anti-sheep immunoglobulin. Arrowheads in B and C point to 5 nm gold particles. Note the labeling of the trans face of the Golgi complex by both anti-HA and anti-TGN46 in A, and the dense labeling of vacuoles with anti-HA in B and C. These examples show TGN46 in the vacuolated structures. (D-F) Sections were labeled with anti-HRP and 10 nm gold-conjugated anti-rabbit immunoglobulin, and anti-TGN46 and 5 nm gold-conjugated anti-sheep immunoglobulin. Arrowheads point to 5 nm gold particles (TGN46) in multivesicular endosomes (mve) that are also labeled by anti-HRP (10 nm gold). Bars, 0.1 μm.

redistribution of all TGN resident proteins. That TGN disruption required a functional GRIP domain was confirmed by (1) induction of a similar phenotype by hyper-expression of all GRIP-domain containing fragments of tGolgin-1 and golgin-97, (2) the inactivity of N-terminal tGolgin-1 fragments that lack the GRIP domain, and (3) the inactivity of a tGolgin-1 GRIP domain fragment with a mutation in the critical tyrosine residue. The innate instability of C-terminal fragments of tGolgin-1 (see Figs 4, 7) and the requirement for high level expression of GRIP domain-containing fragments to disrupt TGN localization may explain why this phenotype was not previously noted. Although TGN homeostasis was affected by overexpression of both tGolgin-1 and golgin-97 GRIP domains, subtle differences in the IFM staining pattern of the residual TGN and in the effects on some Golgi stack residents suggest some specificity in the function of individual GRIP domains.

With few exceptions, the disrupting effects of GRIP domain overexpression were limited to the TGN, with the strongest effects on TGN46 distribution. The effects did not extend to other organelles, consistent with the localization of GRIP proteins to the TGN (Brown et al., 2001; Gleeson et al., 1996; Luke et al., 2003). Effects on the Golgi stack were mixed. The distribution of most Golgi resident proteins by IFM was unaltered in GRIP overexpressing cells relative to controls, and Golgi function was largely unaffected based on the pericentriolar accumulation of ER-released VSV-G and on the largely unchanged kinetics of acquisition of Golgi modifications to Pmel17. Other effects on the Golgi may have been secondary to TGN disruption, perhaps as a consequence of cycling of stack residents through the TGN (Johnston et al., 1994). Two Golgi stack residents that also localize to the TGN (Martinez et al., 1994; Rabouille et al., 1995), GalT and Rab6, were redistributed in only a fraction of cells that expressed GRIP domain fragments at a threshold level sufficient to disrupt TGN46 distribution and not in a manner that correlated with the expression level of GRIP. The failure to observe characteristic Golgi stacks by EM in cells overexpressing GRIP domains was probably because they were obscured by the bloated TGN membranes and/or because of slight Golgi cisternal dilation proximal to the centriole. Finally, the redistribution of the early Golgi v-SNARE, GS28, in cells overexpressing the tGolgin-1-derived C312 (unpublished data) may, as for Rab6 and GalT, reflect a more general distribution of this vSNARE throughout the Golgi and TGN (Nagahama et al., 1996).

How might TGN dynamics be disrupted by GRIP domain overexpression? The expansion of membrane observed by EM and the failure to sort VSV-G from the Golgi to the plasma membrane suggest a defect in TGN export, consistent with in vitro budding of tGolgin-1-bound membranes from purified Golgi stacks (Gleeson et al., 1996). However, disrupted Golgi export is probably a secondary consequence of a primary failure to properly localize TGN resident proteins for several reasons. First, defective TGN export would not explain the redistribution of TGN resident proteins to peripheral structures. Second, this redistribution occurred at GRIP expression levels similar to those required to displace tGolgin-1 from the Golgi, suggesting that TGN46 mislocalization was a primary effect of competition for GRIP domain binding sites. Third, TGN46 was largely mislocalized to endosomes, from which TGN46 is normally recycled to the TGN (Ghosh et al., 1998; Mallet and Maxfield, 1999). We thus favor the interpretation that competition for GRIP domain binding sites interferes with the recycling of TGN46 from endosomes to the TGN (Fig. 10). The secretory defect could then result secondarily from the depletion of factors from the TGN that follow a similar recycling pathway and that are required for subsequent budding of plasma membrane-bound cargo, such as cargo recruitment proteins (Rojo et al., 1997) or v-SNAREs (Gurunathan et al., 2000; Salem et al., 1998; Springer and Schekman, 1998) (Fig. 10). Displacement of TGN resident proteins that follow a different recycling pathway, such as GalT and furin, might also be secondary to changes in TGN architecture. This model would explain why we did not observe redistribution of endosomal residents (Table 1), which rely on distinct cargo recruitment proteins and SNAREs with distinct recycling pathways. Our model predicts that only the post-endocytic endosome-to-TGN recycling step would be blocked by GRIP domain overexpression, consistent with qualitative assessments that failed to detect defects in internalization, recycling, and late endocytic delivery of several internalized cargo proteins (unpublished data).

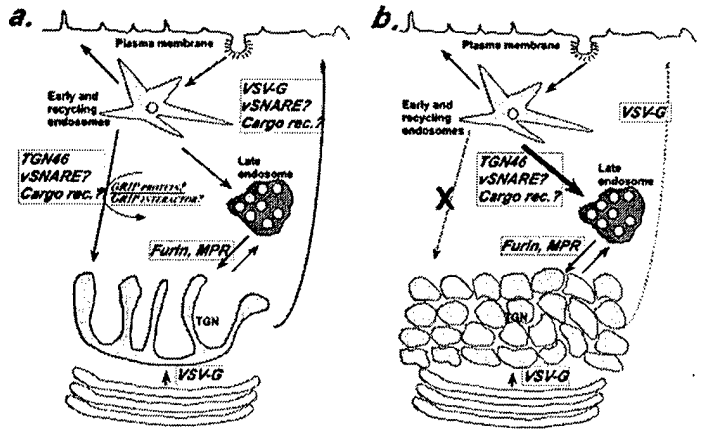

Fig. 10. Model for functional effects of GRIP domain overexpression. (a) Simplified model of vesicular traffic in and out of the TGN in HeLa cells, showing relevant endosomal compartments, the TGN, and the Golgi stack. Early and recycling endosomes are grouped together for simplicity. Cargo following the indicated pathways between organelles are boxed. GRIP proteins or GRIP-interacting molecules are shown to facilitate recycling of proteins from early/recycling endosomes to the TGN. (b) Perturbation of these pathways by overexpression of GRIP domain proteins.

Potential mechanisms of TGN disruption by GRIP domain overexpression

What might be the molecular basis for the effects of GRIP domain overexpression? One potential explanation is competitive displacement from GRIP domain binding sites of endogenous GRIP proteins that are required for TGN maintenance. The GRIP proteins may play a direct role in maintaining TGN homeostasis, or serve as regulators of an effector (or effectors) of TGN maintenance such that overexpression of the GRIP domain would saturate

binding sites and block effector function. The structural similarity of GRIP proteins to tethering factors involved in membrane fusion events at other sites within the secretory and endosomal system (reviewed by Pfeffer, 1996; Pfeffer, 1999) or to components of the 'Golgi matrix' required for stacking Golgi cisternae (Warren and Shorter, 2002) would be consistent with such a function. A second potential explanation for the effects is that overexpressed GRIP domains sequester GRIP domain ligands that function in TGN maintenance. Such ligands may interact with additional effectors, and thus their sequestration by excess GRIP domains could block functions in which GRIP proteins are not directly involved. The best candidate for such a ligand is Arl1. GTP-bound Arl1 associates with GRIP domains and other effectors in vitro (Lu et al., 2001; Panic et al., 2003; Setty et al., 2003; Van Valkenburgh et al., 2001) and in yeast is required for GRIP protein recruitment to the TGN (Panic et al., 2003; Setty et al., 2003). Moreover, the vacuolization of the Golgi and TGN observed here is similar to that observed upon overexpression of a predicted GTP-locked form of Arl1 (Lu et al., 2001; Van Valkenburgh et al., 2001), and another putative Arl1 effector in yeast, the VFT complex (Panic et al., 2003), has been implicated in regulating recycling from endosomes to the TGN (Siniossoglou et al., 2001; Siniossoglou et al., 2002). Another candidate GRIP ligand Rab6 (Barr, 1999), is a known effector of endosome to TGN recycling (Mallard et al., 2002) but less likely to be responsible for the observed phenotype; a GTP-locked form of Rab6 induces TGN46 redistribution (Martinez et al., 1994), but only at extremely high levels of expression and the effect is phenotypically distinct from that induced by GRIP overexpression (unpublished data). GRIP domains might also conceivably sequester lipid ligands, such as phosphatidylinositol phosphates which are known to regulate the morphology and function of the TGN and Golgi (Godi et al., 1999; Munro, 1998). Biochemical analyses of GRIP domain binding to Golgi membranes should help to identify potential GRIP effectors and the mechanism of GRIP overexpression-induced TGN disruption.

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References

Ahle, S., Mann, A., Eichelsbacher, U. and Ungewickell, E. (1988). Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.* **7**, 919-929.

Banting, G. and Ponnambalam, S. (1997). TGN38 and its orthologues: roles in post-TGN vesicle formation and maintenance of TGN morphology. *Biochim. Biophys. Acta* **1355**, 209-217.

Barr, F. A. (1999). A novel Rab6-interacting domain defines a family of Golgi-targeted coiled-coil proteins. *Curr. Biol.* **9**, 381-384.

Bensen, E. S., Yeung, B. G. and Payne, G. S. (2001). Ric1p and the Ypt6p GTPase function in a common pathway required for localization of trans-Golgi network membrane proteins. *Mol. Biol. Cell* **12**, 13-26.

Berger, E. G. and Hesford, F. J. (1985). Localization of galactosyl- and sialyltransferase by immunofluorescence: evidence for different sites. *Proc. Natl. Acad. Sci. USA* **82**, 4736-4739.

Bergmann, J. E., Tokuyasu, K. T. and Singer, S. J. (1981). Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus en route to the plasma membrane. *Proc. Natl. Acad. Sci. USA* **78**, 1746-1750.

Berson, J. F., Harper, D., Tenza, D., Raposo, G. and Marks, M. S. (2001). Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. *Mol. Biol. Cell* **12**, 3451-3464.

Berson, J. F., Theos, A. C., Harper, D. C., Tenza, D., Raposo, G. and Marks, M. S. (2003). Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. *J. Cell Biol.* **161**, 521-533.

Bonifacino, J. S., Suzuki, C. K. and Klausner, R. D. (1990). A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. *Science* **247**, 79-82.

Bosshart, H., Humphrey, J., Deignan, E., Davidson, J., Drazba, J., Yuan, L., Oorschot, V., Peters, P. J. and Bonifacino, J. S. (1994). The cytoplasmic domain mediates localization of furin to the trans-Golgi network en route to the endosomal/lysosomal system. *J. Cell Biol.* **126**, 1157-1172.

Brown, D. L., Heimann, K., Lock, J., Kjer-Nielsen, L., van Vliet, C., Stow, J. L. and Gleeson, P. A. (2001). The GRIP domain is a specific targeting sequence for a population of trans-Golgi network derived tubulo-vesicular carriers. *Traffic* **2**, 336-344.

Bryant, N. J. and Stevens, T. H. (1997). Two separate signals act independently to localize a yeast late Golgi membrane protein through a combination of retrieval and retention. *J. Cell Biol.* **136**, 287-297.

Carlsson, S. R., Roth, J., Piller, F. and Fukuda, M. (1988). Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan. *J. Biol. Chem.* **263**, 18911-18919.

Cole, N. B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. (1998). Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* **140**, 1-15.

Cowan, D. A., Gay, D., Bieler, B. M., Zhao, H., Yoshino, A., Davis, J. G., Tomayko, M. M., Murali, R., Greene, M. I. and Marks, M. S. (2002). Characterization of mouse tGolgin-1 (golgin-245/trans golgi p230/256kD golgin) and its upregulation during oligodendrocyte development. *DNA and Cell Biol.* **21**, 505-517.

Erlich, R., Gleeson, P. A., Campbell, P., Dietzsch, E. and Toh, B.-H. (1996). Molecular characterization of trans-Golgi p230. A human peripheral membrane protein encoded by a gene on chromosome 6p12-22 contains extensive coiled-coil α-helical domains and a granin motif. *J. Biol. Chem.* **271**, 8328-8337.

Fritzler, M. J., Lung, C.-C., Hamel, J. C., Griffith, K. J. and Chan, E. K. L. (1995). Molecular characterization of golgin-245, a novel Golgi complex protein containing a granin signature. *J. Biol. Chem.* **270**, 31262-31268.

Geuze, H. J. and Morre, D. J. (1991). Trans-Golgi reticulum. *J. Electron Microsc. Tech.* **17**, 24-34.

Ghosh, R. N., Mallet, W. G., Soe, T. T., McGraw, T. E. and Maxfield, F. R. (1998). An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. *J. Cell Biol.* **142**, 923-936.

Gleeson, P. A., Anderson, T. J., Stow, J. L., Griffiths, G., Toh, B. H. and Matheson, F. (1996). p230 is associated with vesicles budding from the trans-Golgi network. *J. Cell Sci.* **109**, 2811-2821.

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D. and De Mattels, M. A. (1999). ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P_2 on the Golgi complex. *Nature Cell Biol.* **1**, 280-287.

Gonatas, J. O., Mezitis, S. G., Stieber, A., Fleischer, B. and Gonatas, N. K. (1989). MG-160. A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J. Biol. Chem.* **264**, 646-653.

Griffiths, G. and Simons, K. (1986). The trans Golgi network: sorting at the exit site of the Golgi complex. *Science* **234**, 438-443.

Gurunathan, S., Chapman-Shimshoni, D., Trajkovic, S. and Gerst, J. E.

(2000). Yeast exocytic v-SNAREs confer endocytosis. *Mol. Biol. Cell* **11**, 3629-3643.

Higuchi, R., Krummel, B. and Saiki, R. K. (1988). A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351-7367.

Humphrey, J. S., Peters, P. J., Yuan, L. C. and Bonifacino, J. S. (1993). Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. *J. Cell Biol.* **120**, 1123-1135.

Johnston, P. A., Stieber, A. and Gonatas, N. K. (1994). A hypothesis on the traffic of MG160, a medial Golgi sialoglycoprotein, from the trans-Golgi network to the Golgi cisternae. *J. Cell Sci.* **107**, 529-537.

Kjer-Nielsen, L., Teasdale, R. D., van Vliet, C. and Gleeson, P. A. (1999a). A novel Golgi-localisation domain shared by a class of coiled-coil peripheral membrane proteins. *Curr. Biol.* **9**, 385-388.

Kjer-Nielsen, L., van Vliet, C., Erlich, R., Toh, B.-H. and Gleeson, P. A. (1999b). The Golgi targeting sequence of the peripheral membrane protein p230. *J. Cell Sci.* **112**, 1645-1654.

Leonard, W. J., Depper, J. M., Crabtree, G. R., Rudikoff, S., Pumphrey, J., Robb, R. J., Kronke, M., Svetlik, P. B., Peffer, N. J., Waldmann, T. A. et al. (1984). Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature* **311**, 626-631.

Letourneur, F. and Klausner, R. D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell* **69**, 1143-1157.

Lewis, M. J., Nichols, B. J., Prescianotto-Baschong, C., Riezman, H. and Pelham, H. R. (2000). Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. *Mol. Biol. Cell* **11**, 23-38.

Li, B. and Warner, J. R. (1996). Mutation of the Rab6 homologue of Saccharomyces cerevisiae, YPT6, inhibits both early Golgi function and ribosome biosynthesis. *J. Biol. Chem.* **271**, 16813-16819.

Linstedt, A. D. and Hauri, H. P. (1993). Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679-693.

Lowe, M., Nakamura, N. and Warren, G. (1998). Golgi division and membrane traffic. *Trends Cell Biol.* **8**, 40-44.

Lu, L., Horstmann, H., Ng, C. and Hong, W. (2001). Regulation of Golgi structure and function by ARF-like protein 1 (Arl1). *J. Cell Sci.* **114**, 4543-4555.

Luke, M. R., Kjer-Nielsen, L., Brown, D. L., Stow, J. L. and Gleeson, P. A. (2003). GRIP domain-mediated targeting of two new coiled coil proteins, GCC88 and GCC185, to subcompartments of the trans-Golgi network. *J. Biol. Chem.* **278**, 4216-4226.

Mallard, F., Tang, B. L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B. and Johannes, L. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *J. Cell Biol.* **156**, 653-664.

Mallet, W. G. and Maxfield, F. R. (1999). Chimeric forms of furin and TGN38 are transported with the plasma membrane in the trans-Golgi network via distinct endosomal pathways. *J. Cell Biol.* **146**, 345-359.

Mane, S. M., Marzella, L., Bainton, D. F., Holt, V. K., Cha, Y., Hildreth, J. E. K. and August, J. T. (1989). Purification and characterization of human lysosomal membrane glycoproteins. *Arch. Biochem. Biophys.* **268**, 360-378.

Marks, M. S., Roche, P. A., van Donselaar, E., Woodruff, L., Peters, P. J. and Bonifacino, J. S. (1995). A lysosomal targeting signal in the cytoplasmic tail of the β chain directs HLA-DM to the MHC class II compartments. *J. Cell Biol.* **131**, 351-369.

Marks, M. S., Woodruff, L., Ohno, H. and Bonifacino, J. S. (1996). Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. *J. Cell Biol.* **135**, 341-354.

Martinez, O., Schmidt, A., Salamero, J., Hoflack, B., Roa, M. and Goud, B. (1994). The small GTP-binding protein rab6 functions in intra-Golgi transport. *J. Cell Biol.* **127**, 1575-1588.

McCaffery, J. M. and Farquhar, M. G. (1995). Localization of GTPases (GTP-binding Proteins) by indirect immunofluorescence and immunoelectron microscopy. *Methods Enzymol.* **257**, 259-279.

Miesenböck, G. and Rothman, J. E. (1995). The capacity to retrieve escaped ER proteins extends to the trans-most cisterna of the Golgi stack. *J. Cell Biol.* **129**, 309-319.

Molloy, S. S., Anderson, E. D., Jean, F. and Thomas, G. (1999). Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. *Trends Cell Biol.* **9**, 28-35.

Moremen, K. W. and Touster, O. (1985). Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. *J. Biol. Chem.* **260**, 6654-6662.

Munro, S. (1998). Localization of proteins to the Golgi apparatus. *Trends Cell Biol.* **8**, 11-15.

Munro, S. and Nichols, B. J. (1999). The GRIP domain – a novel Golgi-targeting domain found in several coiled-coil proteins. *Curr. Biol.* **9**, 377-380.

Nagahama, M., Orci, L., Ravazzola, M., Amherdt, M., Lacomis, L., Tempst, P., Rothman, J. E. and Söllner, T. H. (1996). A v-SNARE implicated in intra-Golgi transport. *J. Cell Biol.* **133**, 507-516.

Nakayama, K. (1997). Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.* **327**, 625-635.

Nilsson, T., Pypaert, M., Hoe, M. H., Slusarewicz, P., Berger, E. G. and Warren, G. (1993). Overlapping distribution of two glycosyltransferases in the Golgi apparatus of HeLa cells. *J. Cell Biol.* **120**, 5-13.

Page, L. J. and Robinson, M. S. (1995). Targeting signals and subunit interactions in coated vesicle adaptor complexes. *J. Cell Biol.* **131**, 619-630.

Panic, B., Whyte, J. R. C. and Munro, S. (2003). The Arf-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Curr. Biol.* **13**, 405-410.

Pfeffer, S. R. (1996). Transport vesicle docking: SNAREs and associates. *Ann. Rev. Cell Dev. Biol.* **12**, 441-461.

Pfeffer, S. R. (1999). Transport-vesicle targeting: tethers before SNAREs. *Nature Cell Biol.* **1**, E17-E22.

Ponnambalam, S., Rabouille, C., Luzio, J. P., Nilsson, T. and Warren, G. (1994). The TGN38 glycoprotein contains two non-overlapping signals that mediate localization to the trans-Golgi network. *J. Cell Biol.* **125**, 253-268.

Prescott, A. R., Lucocq, J. M., James, J., Lister, J. M. and Ponnambalam, S. (1997). Distinct localisation of TGN46 and β1,4-galactosyltransferase in HeLa cells. *Eur. J. Cell Biol.* **72**, 238-246.

Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J. M. and Lippincott-Schwartz, J. (1997). ER-to-Golgi transport visualized in living cells. *Nature* **389**, 81-85.

Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E. G., Warren, G. and Nilsson, T. (1995). Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. *J. Cell Sci.* **108**, 1617-1627.

Rohn, W. M., Rouillé, Y., Waguri, S. and Hoflack, B. (2000). Bi-directional trafficking between the trans-Golgi network and the endosomal/lysosomal system. *J. Cell Sci.* **113**, 2093-2101.

Rojo, M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R. G. and Gruenberg, J. (1997). Involvement of the transmembrane protein p23 in biosynthetic protein transport. *J. Cell Biol.* **139**, 1119-1135.

Salem, N., Faundez, V., Horng, J.-T. and Kelly, R. B. (1998). A v-SNARE participates in synaptic vesicle formation mediated by the AP3 adaptor complex. *Nature Neurosci.* **1**, 551-556.

Santini, F., Marks, M. S. and Keen, J. H. (1998). Endocytic clathrin-coated pit formation is independent of receptor internalization signal levels. *Mol. Biol. Cell* **9**, 1177-1194.

Schweizer, A., Fransen, J. A., Bachi, T., Ginsel, L. and Hauri, H. P. (1988). Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J. Cell Biol.* **107**, 1643-1653.

Seaman, M. N. J., Sowerby, P. J. and Robinson, M. S. (1996). Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network. *J. Biol. Chem.* **271**, 25446-25451.

Seidah, N. G. and Chretien, M. (1997). Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr. Opin. Biotechnol.* **8**, 602-607.

Setty, S. R. G., Shin, M. E., Yoshino, A., Marks, M. S. and Burd, C. G. (2003). Golgi recruitment of GRIP domain proteins by ARF-like GTPase 1 (Arl1p) is regulated by Arf-like GTPase 3 (Arl3p). *Curr. Biol.* **13**, 401-404.

Shapiro, J., Sciaky, N., Lee, J., Bosshart, H., Angeletti, R. H. and Bonifacino, J. S. (1997). Localization of endogenous furin in cultured cell lines. *J. Histochem. Cytochem.* **45**, 3-12.

Siniossoglou, S., Peak-Chew, S. Y. and Pelham, H. R. B. (2000). Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p. *EMBO J.* **19**, 4885-4894.

Siniossoglou, S. and Pelham, H. R. B. (2001). An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. *EMBO J.* **20**, 5991-5998.

Siniossoglou, S. and Pelham, H. R. B. (2002). Vps51p links the VFT complex to the SNARE Tlg1p. *J. Biol. Chem.* **277**, 48318-48324.

Springer, S. and Schekman, R. (1998). Nucleation of COPII vesicular coat complex by endoplasmic reticulum to Golgi vesicle SNAREs. *Science* **281**, 698-700.

Traub, L. M. and Kornfeld, S. (1997). The trans-Golgi network: a late secretory sorting station. *Curr. Opin. Cell Biol.* **9**, 527-533.

Tsukada, M. and Gallwitz, D. (1996). Isolation and characterization of SYS genes from yeast, multicopy suppressors of the functional loss of the transport GTPase Ypt6p. *J. Cell Sci.* **109**, 2471-2481.

Tsukada, M., Will, E. and Gallwitz, D. (1999). Structural and functional analysis of a novel coiled-coil protein involved in Ypt6 GTPase-regulated protein transport in yeast. *Mol. Biol. Cell* **10**, 63-75.

Van Valkenburgh, H., Shern, J. F., Sharer, J. D., Zhu, X. and Kahn, R. A. (2001). ADP-ribosylation factors (ARFs) and ARF-like 1 (ARL1) have both specific and shared effectors. Characterizing Arl1-binding proteins. *J. Biol. Chem.* **276**, 22826-22837.

Varlamov, O. and Fricker, L. D. (1998). Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. *J. Cell Sci.* **111**, 877-885.

Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M. S., Peters, P. J. and Bonifacino, J. S. (1995). An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. *EMBO J.* **14**, 4961-4975.

Warren, G. and Shorter, J. (2002). Golgi architecture and inheritance. *Annu. Rev. Cell Dev. Biol.* **18**, 379-420.

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Characterization of the function of TGN-localized peripheral membrane protein, tGolgin-1.

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Abstract:

tGolgin-1 (also known as trans golgi p230) is a large Golgi-associated peripheral membrane protein of unknown function. Here, we report the effect of specific gene silencing of tGolgin-1 using RNA interference (RNAi) in cultured cells. Upon transfection of HeLa cells with siRNA duplexes, the expression of tGolgin-1 was drastically reduced as assessed by immunofluorescence microscopy (IFM) and immunoblotting. Interestingly, the distribution of the Golgi and TGN was disrupted by gene silencing of tGolgin-1, resulting in the loss of a pericentriolar ribbon and the appearance of punctate, dispersed structures, which were localized in close proximity to ER exit sites and retained the characteristic *cis/ trans* polarity of the Golgi complex. This phenotype is similar to that observed in cells either in which microtubules are disrupted by nocodazole-treatment or in which dynein/ dynactin function is disturbed by dynamitin-overexpression. Importantly, microtubules remain intact in RNAi cells, and the peripheral Golgi-like structures align along the microtubules. These data suggest that tGolgin-1 is required for minus-end-directed motility of Golgi elements along microtubules and thus to maintain Golgi/TGN structure in close proximity to the MTOC. Now we further examine the vesicular motility in living cells.

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