Identification and Characterization of a Novel Golgi Protein, Golgin-67*

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In the course of screening a λgt11 human leukemic T-cell cDNA expression library with an antibody to the mitotic target of Src, Sam68, we identified and cloned a cDNA encoding a novel protein with a predicted molecular mass of 51.4 kDa. Polyclonal antibodies raised to a His6-tagged construct of this protein, detected a ~67-kDa protein in immunoprecipitation experiments, and cytological studies showed that this protein localized to the Golgi complex, through colocalization experiments with specific Golgi markers. Therefore, we designated this protein golgin-67. Sequence analysis revealed that golgin-67 is a highly coiled-coil protein, with potential Cdc2 and Src kinase phosphorylation motifs. It has sequence homologies to other Golgi proteins, including the coatamer complex I vesicle docking protein, GM130. Structurally, golgin-67 resembles, golgin-84, an integral membrane Golgi protein with an N-terminal coiled-coil domain and a single C-terminal transmembrane domain. The C-terminal region of golgin-67, which contains a predicted transmembrane domain, was demonstrated to be essential for its Golgi localization.

The Golgi apparatus is a cytoplasmic organelle that is involved in the processing and sorting of lipids and proteins in the eukaryotic cell. It comprises compartmentalized and polarized stacks of membranous cisternae that reside in the perinuclear region of most mammalian cells. The Golgi complex is involved in the directed movement of newly synthesized proteins from the endoplasmic reticulum to the plasma membrane.

At present, all known resident Golgi proteins are either integral membrane proteins or peripheral membrane proteins located on the cytoplasmic face of the Golgi (1). The integral membrane proteins can either face the lumen of the Golgi or the cytoplasm. The modifying enzymes of the Golgi apparatus, such as the glycosylation proteins, are typical examples of integral membrane proteins that face the interior of the Golgi. The proteins responsible for other processes such as docking, tethering, or budding of vesicles or in the maintenance of Golgi structure (e.g. target SNAREs and GM130) are typically either cytoplasmic-facing integral or peripheral membrane proteins (2, 3).

A common structural feature found among the cytoplasmic-facing integral membrane proteins is a cytoplasmic coiled-coil domain (4) combined with a C-terminal anchor. In addition, a transmembrane domain (TMD) located in the C-terminal end is thought to be critical in localizing and anchoring proteins to the Golgi membrane (1). Golgin 84, a recently identified Golgi protein, has such an arrangement (5).

Cellular Src is a member of a family of nonreceptor tyrosine kinases that are thought to be involved in signal transduction events regulating cell growth and differentiation (for review see Ref. 6). It localizes to the plasma membrane and is involved in a multitude of signaling cascades. However, Src also localizes to endosomes, secretory vesicles, and the perinuclear membrane, although little is known about its function at these sites (7, 8). In an attempt to learn more about the role of Src at these intracellular membranes and to identify new proteins that have the potential to interact with Src family tyrosine kinases, a screening of a cDNA expression library was undertaken, using an antibody specific for a nuclear protein and a mitotic target of Src, known as Sam68 (Src-associated in mitosis) (9–12). In the course of this antibody screening, we isolated a cDNA clone that encodes for a novel Golgi protein, which we have named golgin-67. This protein exhibits the characteristics of a cytoplasmic-facing integral membrane protein, and we demonstrate that its C-terminal end, which contains a predicted TMD, is important for its Golgi localization.

EXPERIMENTAL PROCEDURES

Screening Human T-cell cDNA Libraries—1.25 × 10^6 plaques from a λgt11 human leukemic T-lymphocyte (Molt-4) cDNA expression library (kindly provided by S. Orkin and D. Ginsburg) were screened immunologically with a C-terminal specific Sam68 polyclonal antibody (Santa Cruz). Phage (~250,000 plaque-forming units) were plated on Escherichia coli Y1090hsdR on LB agar in NUNC Bioassay plates (530 cm2/plate). The plated library was preincubated at 42 °C for 6 h, after

The abbreviations used are: TMD, transmembrane domain; PBS, phosphate-buffered saline; kbp, kilobase pair(s); kb, kilobase(s), bp, base pair(s); TBS, Tris-buffered saline; PCR, polymerase chain reaction; GST, glutathione S-transferase; BHK, baby hamster kidney; EGFP, enhanced green fluorescent protein.
which, nitrocellulose filters (Amersham Pharmacia Biotech) impregnated with 10 ml isopropl-β-D-thialigalactoside were placed on top of each plate. After a 4-h incubation at 37 °C, the filters were removed, rinsed once in Tris-buffered saline (TBS), and incubated with blocking solution (1% bovine serum albumin, 0.05% Tween-20 in TBS) for 1 h at room temperature. The primary antibody was incubated at a dilution of 1:2000 in 2% bovine serum albumin, 0.5% Tween-20 in TBS at room temperature, and then washed three times (10 min/wash) with 0.1% Tween-20 in TBS (at room temperature), and incubated for 30 min at room temperature with horseradish peroxidase-labeled secondary antibody (1:2000) (Amersham Pharmacia Biotech) in TBS containing 1% bovine serum albumin, 0.1% Tween-20. Positive plaques were detected with ECL reagents (Amersham Pharmacia Biotech). Eleven clones were purified through three rounds of plaque purification. The cDNA inserts were PCR amplified from the plagues using primers (Qiagen) and subcloned into a TA cloning vector, pCR2.1 (Invitrogen), and sequenced. The insert was also subcloned into EcoRI-digested pBluescript KS (Strategene) and EcoRI-digested pFLAG-CMV-2 (Kodak).

A ZAP II human leukemic T-cell (Jurkat) cDNA library (Strategene) was screened to isolate regions upstream of the first ATG start site found in clone C43. Plaque screening was performed using double lifts onto nitrocellulose filters (Amersham Pharmacia Biotech) from four NUNC Bio assay plates containing ~250,000 plaques on an XLI-Blue E. coli lawn. A pFLAG-C43 DNA insert was digested with EcoRI and XhoI to obtain a 270-bp fragment representing the extreme 5′-end of the C43 clone. This fragment was then [32P]dCTP-labeled DNA labeling kit (Amersham Pharmacia Biotech) and purified with a CENTRI-SEP column (Princeton Separations). Before hybridization the filters were washed for 5 min at room temperature in 2× SSC, washed for 1 h at room temperature, and then washed three times (10 min/wash) with 0.1% Tween-20 in TBS (at room temperature), and incubated for 30 min at room temperature with horseradish peroxidase-labeled secondary antibody (1:2000) (Amersham Pharmacia Biotech). After hybridization, the membrane was exposed for 20 min.

**Fusion Protein Constructs**—The golgin-67 cDNA (clone C43) was used to prepare a GST fusion protein construct that was expressed from the bacterial expression vector pGEX-4TG (Amersham Pharmacia Biotech). The full-length cDNA (clone C43) was inserted in-frame into the EcoRI site of this plasmid. A 400-ml E. coli BL21 culture containing the Golgin-67 expression construct was grown overnight at 30 °C in 2 × TTA medium (16 gl/g litre tryptone, 10 gl/g litre NaCl, 100 μg/ml ampicillin, pH 7.0) and then induced by the addition of 0.2 ml isopropl-β-D-thialigoside for 3 h at 30 °C. Harvesting and purification of the insoluble fusion protein was carried out essentially as described by Frangioni and Neel (13). The fusion protein bound to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) was stored at 4 °C in PBS for no longer than 1 week before use.

A His6 tag protein bearing only the opening frame of golgin-67 was constructed by subcloning a reverse transcription-PCR product of golgin-67 derived from SW480 cells into a pET28 expression vector (Novagen). The reverse transcription-PCR product of golgin-67 was synthesized using a simplified reverse transcription-PCR method as described by Goblet et al. (13). COS cells were transfected with pET28-golgin-67 and proteins were synthesized that corresponded to regions just 5′ and 3′ of the opening frame of golgin-67: forward, 5′-ATAAGAATCTCATGACATCTTCTTCGATATTCT-3′; reverse, 5′-ATAAGAATGGCGGGCTGTATCTCCTTTCTTCTCCGACG-3′. These primers were used for reverse transcription-PCR for 25 cycles at 55 °C using SW480 mRNA (CLONTECH) as the template. After the PCR reaction, the product was digested with EcoRI and NotI and subcloned into corresponding sites in the E. coli expression vector pET28. The construct was transformed into E. coli JM109 for recombinant protein expression. The recombinant proteins were purified with nickel column chromatography and eluted with 8 M urea as described by the manufacturer.

**Rabbit Immunization and Antibody Production**—Antibodies were raised in rabbits against a denatured His6 tag protein containing the full-length golgin-67 protein. Two female New Zealand White rabbits were separately immunized by subcutaneous injections of 0.5 mg of recombinant protein in complete Freund’s adjuvant. The rabbits were given booster injections, and blood samples were collected for analysis. The appearance and titer of Golgi antibodies was monitored by indirect immunofluorescence using serial dilutions on Hep-2 cell substrates (Bio-Enterprises Ltd.).

**35S Labeling of Wil-2 Cells, Immunoprecipitation, and Protein Electrophoresis**—Four 150-mm culture dishes, containing ~4 × 107 Wil-2 cells (American Type Culture Collection), were each labeled with 1 μCi of [35S]methionine (ICN) in methionine-deficient Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum, 4.5 g/ml glucose, and 2 μM glutamine and incubated for 17 h at 37 °C. After harvesting, the cells were washed twice with 0.2 × SSC, 0.1% SDS for 30 min each at 42 °C. Eight clones were purified through four rounds of plaque purification. The insert-containing pBluescript SK(-) phagemids (clones 11, 13, 14, 15, IV2, IV3, III3, and III6) were then excised in *E. coli* XZAP II vectors as described by the manufacturer, purified (Qiagen), and sequenced.

**Sequence Analysis**—A library of nested deletions (12 subclones extending unidirectionally inward at intervals of 150–200 bp) was generated from clone C43 using a double-stranded nested deletion kit (Amersham Pharmacia Biotech). The plasmid, pCR2.1-C43, was digested with *Bam*HI to create a nucleosce-susceptible end and with *Kpn*I to generate the nucleasce-resistant end. Sequencing of these nested deletions was carried out manually with the aid of a T3 sequencing kit (Amersham Pharmacia Biotech). The hot mixed reaction was incubated for 15 min at 95 °C with 100 μl of 10× CGC, 10 ml Tris-HCl (pH 7.5), 1.5 ml MgCl2, 0.5% Nonidet P-40, 0.5% Nonden P-40) was added to each plate for 5 min at room temperature. The lysates were collected and centrifuged at 10,000 × g for 15 min, and the supernatants were stored at −70 °C, until required.

**Immunoprecipitation**—[35S]-labeled Wil-2 cell extracts was performed using protein A-Sepharose beads. The extracts were initially preclared by mixing 1% volume of 10% protein A-Sepharose beads in NET2 + F buffer (0.5% Nonidet P-40, 150 mM NaCl, 5 ml EDTA, 50 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.02% sodium azide, 0.5% deoxycholic acid) containing 0.1% bovine serum albumin (10% protein A-Sepharose stock) for 1 min and then centrifuging for 3 min to collect the supernatant. 10 ml of rabbit serum, 50 μl of preclared, [35S]-labeled cell extract, and 500 μl of NET2 + F buffer were then incubated with 100 μl of 10% protein A-Sepharose stock for 1 h at 4 °C on a tube rotator. The beads were washed five times with NET2 + F buffer, after which 20 ml of Laemmli’s sample buffer was added. Each sample was then boiled for 2 min, resolved by 12.5% SDS-polyacrylamide gel electrophoresis, and analyzed by autoradiography.

**Affinity Purification of Antibody**—Antibodies were purified on a golgin-67 affinity column prepared with glutarddehyde (15). 2 ml of a 50% suspension of GST-golgin-67 immobilized (noncovalently) on glutathione-Sepharose 4B beads was spun down at 214 × g for 5 min. After discarding the supernatant, 4 volumes of 0.25 M sodium bicarbonate buffer (pH 8.8) was added. The beads were incubated for 2.5 h at 4 °C on a tube rotator and spun down, and the supernatant was discarded. Four volumes of 0.25 M sodium bicarbonate buffer containing 0.03% glutar-
aldehyde was then added, after which the beads were incubated for 1 h at room temperature on a tube rotator and centrifuged, and the supernatant discarded. Four volumes of 1 M Tris-HCl (pH 7.8) was then added to the beads and incubated for 1 h at room temperature. The beads were collected by centrifugation, washed with 4 volumes of 1 M NaCl, 0.1% Nonidet P-40, centrifuged, resuspended with Buffer A (10 mM potassium phosphate, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40), and incubated overnight at room temperature. The following day, the beads were collected and resuspended in fresh Buffer A and then stored at 4 °C. Once the antigen was covalently coupled to the bead support, the rabbit serum was affinity purified as described by Harlow and Lane (10). After the purified antibody fractions were pooled, 0.02% sodium azide was added, and the pH was adjusted to ~7.1. The pooled eluate was then buffer exchanged and concentrated in 1× PBS with an Amicon stirred cell using a YM10 membrane (Amicon). The purified antibodies were then used for indirect immunofluorescence analyses.

Indirect Immunofluorescence—Baby hamster kidney (BHK) cells were seeded onto glass covergrips 24–36 h before analysis. After removal of growth medium, the cells were washed twice with PBS, fixed with 3.7% paraformaldehyde/PBS for 20 min, washed three more times with PBS, and then permeabilized with 0.1% Nonidet P-40 in PBS for 20 min. The cell substrates were incubated with primary antibodies for ~1 h at 37 °C in a humidified chamber at the following dilutions: affinity-purified polyclonal golgin-67 antibody (1:50); prototype human anti-giantin serum De (1:50); and monoclonal antibody mannose II (clone 53FC3, kindly provided by B. Burke) (undiluted cell culture supernatant). The covergrips were washed twice with PBS and then incubated with fluorescein isothiocyanate-labeled secondary antibodies in PBS. Rhodamine Red-X (Jackson Laboratories), Cy3- (Jackson Laboratories), and Alanya 488- (Molecular Probes) labeled secondary antibodies were used at the manufacturer’s recommended dilutions. After immunostaining, the coversgrips were washed twice with PBS, counter-stained with 4′,6-diamidino-2-phenylindole (Sigma) in PBS (1 µg/ml) for 10 min, washed two more times, and mounted with Mowiol (Calbiochem) mounting medium. Immunostaining was also performed on commercially prepared monkey testis tissue slides (Medica). The protocol for immunostaining was the same as above, except that no fixation and permeabilization were necessary. Sections were observed and photographed under appropriate illumination with a Leica DMRB Microscope equipped with a 63× Planap objective lens and a MicroMax cooled CCD camera (Prinston Instruments) linked to an Apple Macintosh 8100 PPC IPLab Spectrum (17). Images were combined and aligned using Adobe Photoshop version 4.0.

Plasmid Construction and Expression of EGFP-tagged Golgin-67 Detection Constructs—Golgin-67 cDNA fragment encoding the C-terminal 113 amino acids was isolated from pFLAG-C43 by restriction enzyme digestion. The fragment was digested with PstI and BamHI and inserted in-frame into PstI/BamHI-digested pEGFP-C1 DNA vector (Clontech). This construct (pEGFP-c67[C113]) was then transformed into E. coli DH5α cells and purified (Qiagen) for transfection purposes. Expression of the fusion protein construct in cultured cells was regulated by the cytomegalovirus promoter.

BHK cells were grown on coversgrips in 35-mm culture dishes. At 40–60% confluency the cells were transfected with 1 µg of purified plasmid DNA/11 µl of Lipofectin Reagent (1 mg/ml) (Life Technologies, Inc.) in 1 ml of serum-free Dulbecco’s modified Eagle’s medium. The cells were incubated for 6 h at 37 °C, after which the DNA-containing medium was replaced with 2 ml of medium containing 10% fetal bovine serum. The cells were cultured for another 18 h, fixed for 20 min with 3.7% paraformaldehyde (room temperature), and permeabilized with 0.1% Nonidet P-40. Immunostaining of the cells was performed as described under the indirect immunofluorescence section. As a control, cells were transfected with pEGFP-C1 vector containing no insert.

RESULTS

Cloning of Golgin-67—In an attempt to identify potential new substrates for the Src family tyrosine kinases, as well as learn more about their functions, a human leukemic T-cell (Molt-4) cDNA expression library was screened with a poly-clonal antibody specific for a mitotic target of Src called Sam68 (10, 11), a protein also independently characterized as p70 that interacted with GST fusion proteins containing SH2 and/or SH3 domains of the Src family member Lck (18). Eleven positive clones were isolated from a screening of ~1.3 × 10⁶ plaques. One clone, called C43, represented a novel ~1.9-kbp cDNA. Sequence analysis predicted an open reading frame of 1380 bp (460 amino acids giving a calculated molecular mass of 51.4 kDa). Upon closer examination of the nucleotide sequence, no in-frame stop codon was detected upstream of the first ATG codon. Thus, to verify that the complete coding region for C43 had been identified, an additional screen was performed in a human leukemic T-cell (Jurkat) cDNA library using a probe made from a 270-bp fragment representing the extreme 5′-end of clone C43. (A new T-cell cDNA library was used because the initial Molt-4 cDNA library had a low phage titer, as a result of prolonged storage at 4 °C.) Of ~1 million clones screened, eight positives were identified, two of which contained the upstream sequences necessary to confirm the completeness of the coding region. Clones 11 and 13 both contained an in-frame stop codon 468 nucleotides upstream of the first ATG start site, thus confirming the entirety of the coding region. The combined sequences of clones 11, 13, and C43 represented a 2.73-kbp region of cDNA. The sequence of the cDNA and predicted amino acid sequence are shown in Fig. 1. It is important to note that the coding region for golgin-67 was verified by independent sequences derived from two expressed sequence tag clones and a 5′-rapid amplification of cDNA ends cDNA clone. The two expressed sequence tag clones, clone HFBC48, derived from a human brain cDNA library (accession number M85951), and clone 124493, derived from a human fetal liver/spleen cDNA library (accession number R01949), were obtained from American Type Culture Collection, and their complete cDNA inserts were sequenced and determined to be 2.829 and 0.904 kbp in length, respectively (data not shown). Clone HFBC48, corresponded to the golgin-67 sequence from nucleotide position 1179 to the 3′-end, whereas the cDNA of 124493 matched the 5′-untranslated region of mRNA of golgin-67 up to nucleotide position 857. The sequence in between nucleotides 857 and 1179 was additionally verified by sequencing a 5′-rapid amplification of cDNA ends cloned cDNA (data not shown). Together, these three sequences confirmed the position of the upstream stop codon and confirmed the golgin-67 translational start site. Based on the size of the protein and subsequent cellular localization (see below), we termed this protein golgin-67.

Two Golgin-67 mRNA Transcripts of Differing Molecular Masses Are Expressed in Human Tissues, One of Which Is Abundant in Brain—Golgin-67 mRNA expression was analyzed in 12 human tissues, by probing a human multiple tissue blot with golgin-67 cDNA (Fig. 2). Two prominent transcripts were detected. One was a ~7.5-kb mRNA present in all tissues except skeletal muscle, and the other a ~6.3-kb mRNA observed only in heart and skeletal muscle (lanes 2 and 3, respectively). The presence of two different sized transcripts, suggests that golgin-67 mRNA may be alternatively spliced. The ~7.5-kb transcript was highly abundant in brain tissue (lane 1). Upon shorter exposure times there were no detectable levels of the ~6.3-kb transcript in brain. Longer exposures also revealed no detectable traces of the ~7.5-kb transcript in skeletal muscle; however, they did reveal a weak ~7.5-kb transcript in colon, small intestine, and placenta (lanes 4, 9, and 10, respectively).

The Sequence and Structural Analysis of Golgin-67—A BLAST search of the GenBank™ data base showed that the golgin-67 cDNA sequence had a 98–100% identity (over a span of 2250 nucleotides) with a recently sequenced but uncharacterized human cDNA coding for a protein designated KIAA0855 (accession number AB020662) (19). Although these two sequences were very similar, there was one significant difference found between them. Golgin-67 cDNA was shown to have a stop codon 468 nucleotides upstream of the first ATG codon, whereas the KIAA0855 cDNA lacked a stop codon upstream of this ATG and thus was predicted to code for a much
larger protein. The difference between these two sequences is due to a 46-nucleotide insertion present only in the golgin-67 cDNA (nucleotides 488–533), which contains this in-frame stop codon. The insert also contained an ApaI restriction site (nucleotides 518–523), which upon digestion with the ApaI enzyme resulted in the predicted cleavage of the golgin-67 cDNA (data not shown).

The 5′-most ATG found within the coding region of the golgin-67 cDNA serves as the start site for translational initiation, even though the sequence flanking this ATG (TGTATCACATGAAA) does not conform very well with the Kozak consensus sequence (20, 21). In vitro transcription and translation of the golgin-67 cDNA yielded a 67-kDa protein (data not shown), which matches the authentic protein size seen by the immunoprecipitation experiment using Wil-2 cell lysates (see below). This suggests that the cDNA encodes the full-length golgin-67 protein and that most likely, translational initiation starts from this 5′-most ATG codon. No polyadenylation signal (AATAAA) nor poly(A) tail was detected in the cDNA sequence.

Analysis of the deduced amino acid sequence for golgin-67

Fig. 1. Human golgin-67 cDNA sequence and its deduced amino acid sequence (GenBank™ accession number AF163441). The largest open reading frame encodes a 460-amino acid protein with a predicted molecular mass of ~51 kDa. The underlined nucleotides are the first upstream in-frame stop codon, the sequence flanking the methionine start site, and the stop codon, respectively. The nucleotides in bold type represent the 46 nucleotide insertion found in golgin-67 cDNA but lacking in the KIAA0885 cDNA. The underlined amino acid residues are the proline-rich region, the leucine zipper motif, the putative di-acidic sorting signal, and the predicted TMD, respectively. The amino acid residues in bold type represent potential Src (YFEEE) and Cdc2 (SPGAP) kinase phosphorylation motifs. The first 866 bases of 5′-untranslated region of mRNA sequence was derived from clones I1 and I3 (screen of Jurkat cDNA library); the remaining 1.9 kb, shown after the dashed line, was obtained from clone C43 (screen of Molt-4 cDNA library).
revealed a number of interesting features. First, a proline-rich motif (amino acids 143–149) and a potential C-terminal transmembrane domain (amino acids 437–455) (see below) were detected. Second, a putative di-acidic sorting signal required for efficient endoplasmic reticulum export (22), was identified. The signal consists of amino acids aspartate or glutamate separated by a variable residue ([(D/E)]X[(D/E)]) and is found in the cytoplasmic tail of a number of transmembrane proteins (23).

Lastly, in terms of structure, golgin-67 closely resembles a structural protein of the Golgi matrix, that is most likely involved in vesicle docking and mitotic fragmentation of the Golgi (3, 26, 27). Because of the high sequence homology with GM130 and Golgin-95 (accession number Q08379), GM130 (accession number AAC50434), golgin-97 (accession number NP_002068), and golgin-160 (accession number P55937). Sequence similarities were primarily associated with the ~200-residue coiled-coil domain and a ~90-residue region near the C-terminal end of the protein (amino acids 356–450).

Golgin-95, a human protein of unknown function that was initially identified as an antigen in the autoimmune disease systemic lupus erythematosus (25), exhibited the highest degree of homology to golgin-67 (BLAST p value = 2.3e-70). The other Golgi protein, with which golgin-67 exhibited a high degree of homology, was the cis-Golgi matrix protein, GM130 (BLAST p value = 2.5e-63). GM130 is a tightly associated peripheral membrane protein, originally identified as a structural protein of the Golgi matrix, that is most likely involved in vesicle docking and mitotic fragmentation of the Golgi (3, 26, 27).

Because of the high sequence homology with GM130 and knowing that GM130 can be phosphorylated by Cdc2 kinase (27) a closer re-examination of the golgin-67 amino acid sequence was undertaken. Interestingly, it revealed a potential Cdc2 kinase phosphorylation motif. The golgin-67 sequence, SPQAP (amino acids 377–381), was found to be almost identical to a site within GM130, SPGAP (amino acids 25–29), which becomes phosphorylated on the serine by Cdc2 kinase, an event thought to be critical in mitotic Golgi fragmentation (27).

In addition, golgin-67 also contains a YFEEE sequence found at its N terminus (amino acids 7–11) that closely resembles the consensus Src tyrosine kinase phosphorylation motif (28).

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novel Golgi integral membrane protein thought to be involved in vesicle tethering/docking (5). It comprises a C-terminal transmembrane domain and a cytoplasmic coiled-coil region, which is 43% similar (21% identical) to the coiled-coil sequence of golgin-67.

Golgin-67 Is a 67-kDa Protein That Localizes to the Golgi—To study the cellular characteristics of the golgin-67 protein, antibodies were raised in rabbit against a His$_6$-tagged construct containing the full-length golgin-67 protein, made in E. coli. The immune serum immunoprecipitated a ~67-kDa protein from $^{35}$S-labeled Wil-2 cell lysates (Fig. 4, lane 2), whereas the corresponding preimmune serum did not (Fig. 4, lane 1). The immune serum also immunoprecipitated in vitro translated $^{35}$S-labeled golgin-67 protein, whereas the preimmune serum had no effect (data not shown).

Affinity-purified golgin-67 antibodies were used for indirect immunofluorescence studies on BHK substrates. Each interphase cell showed a single, large perinuclear patch of staining characteristic of the Golgi (Fig. 5A). To confirm that this perinuclear fluorescence corresponded to the Golgi apparatus a double label experiment was performed using the affinity-purified golgin-67 antibody and an autoimmune serum shown to react with the 400-kDa Golgi protein, giantin (29). Fig. 5B, illustrates that these two proteins, golgin-67 (green) and giantin (red), colocalize, although they do not occupy identical regions throughout the Golgi complex.

To determine whether golgin-67 was a constitutive component of the Golgi apparatus, we examined sections of monkey testis tissue by indirect immunofluorescence. Fig. 5C illustrates that golgin-67 (green) could be detected in seminiferous tubule sections and that it was particularly abundant in the Golgi of cells undergoing spermiogenesis. In contrast, giantin (red) was found in the Golgi of all the cells of the testis, including those staining for golgin-67. Together, these data suggest that unlike giantin, golgin-67 is restricted to specific types of Golgi complexes.

The C-terminal 113 Residues of Golgin-67 Contain a Golgi Targeting Signal and a Putative Membrane Insertion Sequence—Using the program TMpred (30), golgin-67 was predicted to have a single TMD very close to its C terminus at amino acid residues 437–455 (Fig. 6A). To confirm that the C-terminal end of golgin-67 was responsible for its Golgi localization, a chimera was constructed between the C-terminal 113-amino acid region of golgin-67 and enhanced green fluorescent protein (EGFP-g67(C113)) (Fig. 6B) and expressed in cultured BHK cells.

EGFP expression alone resulted in a signal throughout the cell (Fig. 6C). In contrast, the EGFP-g67(C113) construct localized to the Golgi. Golgi localization was confirmed by immunostaining with antibodies specific for the Golgi enzyme mannosidase II. No fluorescent signal was detected at the plasma membrane, thus confirming the specificity of Golgi membrane targeting by the C-terminal 113 residues, which contains the putative TMD.

DISCUSSION

Golgin-67 is a novel Golgi protein with a C-terminal TMD and an extensive coiled-coil domain at its N terminus. Structurally, golgin-67 resembles a recently identified novel Golgi protein, golgin-84, a C-terminally anchored type II integral membrane protein with an extensive cytoplasmic coiled-coil domain (5). EGFP fusion experiments with the golgin-67 protein confirmed that a Golgi targeting signal was present in the last 113 residues of the protein. Based on this evidence, as well as the structural similarity between golgin-67 and golgin-84, it is likely that golgin-67 exhibits a similar type II topology, with the C terminus anchored at the Golgi membrane and the N-terminal coiled-coil region exposed to the cytoplasm.

TMDs of mammalian Golgi proteins are on average five residues shorter than those of plasma membrane proteins (31). One of the models for retention of proteins at the Golgi is the lipid sorting or “bilayer thickness” model (1). Because of the
mannosidase II.

Golgi. Golgi targeting of EGFP-g67(C113) was confirmed by immuno- and cytoplasmic signal. However, EGFP-g67(C113) was targeted to the membrane domain.

cresidues of golgin-67. The g67(C113) is a fusion protein construct between EGFP and the last 113 wildtype and mutant EGFP-tagged golgin-67 proteins. EGFP-helices between 17 and 35 residues in length.

(30). The prediction parameter was set to search for transmembrane transmembrane score against the amino acid number, using TMpred to its C terminus (amino acids 437–455). A profile was plotted as the main.

Golgi targeting signal and a putative membrane spanning do-
gion for golgin-67 is too short for plasma membrane insertion TMD of golgin-84 (5), suggesting that the transmembrane region of the C terminus and apparently involves two critical aromatic residues (35). Upon visual inspection of the C-terminal 113 amino acid residues of golgin-67, no evidence of the GRIP domain was found, further suggesting that golgin-67 is an integral membrane protein and that the predicted C-terminal TMD is responsible for its Golgi targeting.

Structural analysis of golgin-67 predicted a coiled-coil domain spanning almost the entire N-terminal half of the molecule. The presence of discontinuities within the domain suggests that the protein assumes a rod-like structure with several joints or fixed bends (37). The coiled-coil region and more specifically the leucine zipper domain may allow golgin-67 to form dimers with itself or other proteins. The oligomerization of Golgi proteins or “kin recognition” is theorized to be a second mechanism for Golgi protein retention (1). Coiled-coils are also a characteristic of many structural proteins of the Golgi, such as GM130, which is a component of the Golgi matrix (3). The high degree of structural and sequence homologies shared between GM130 and golgin-67 suggests that golgin-67 may too play an organizational role in the Golgi apparatus.

Upon analysis of the golgin-67 cDNA sequence, the translational start site for golgin-67 was found to lie in poor context with respect to the Kozak consensus sequence (38). However, three lines of evidence suggest that the entire coding region for golgin-67 had been obtained and that translation does initiate from this particular start site. First, the upstream stop codon and translational start site were verified and confirmed independently from two expressed sequence tag sequences and a 5′-rapid amplification of cDNA ends of a region internal to them. Second, there were no significant differences found with respect to the sequence flanking the ATG start site nor the 5′-untranslated region of mRNA. Third, in vitro translated product (data not shown) matched the size (67 kDa) of the in vivo protein immunoprecipitated by rabbit golgin-67 serum from 35S-labeled Wil-2 cell lysates.

Coiled-coil integral membrane proteins, similar in structure to golgin-67 and termed SNAREs, have been implicated in the docking of transport vesicles to target membranes (2, 39). Although, golgin-67 is not a SNARE (it lacks the target SNARE homology domain (40)), it is structurally related to two type II C-terminally anchored membrane proteins with an extensive cytoplasmic coiled-coil domain (that also lack target SNARE homology), golgin-84 and giantin (5, 29). Both of these proteins have been implicated in vesicle tethering/docking. In particular, giantin, a large 400-kDa protein has been observed to tether coatamer complex I vesicles to Golgi membranes (29, 41). Golgin-67 also resembles golgin-95 and GM130. GM130, is a tightly associated peripheral membrane protein (mentioned earlier in the discussion) that is involved in the mitotic fragmentation of the Golgi (3, 27). During mitosis GM130 is phosphorylated by Cdc2 kinase, which prevents a vesicle docking protein, p115, from binding its N terminus (27). This is believed to lead to vesicle transport cessation and ultimately to coatamer complex I-mediated vesiculation of the Golgi cister-
nae (42). GM130 was shown to be serine phosphorylated by Cdc2 kinase at its N terminus (27). Golgin-67 was observed to have a very similar phosphorylation site, suggesting that it too might be phosphorylated by Cdc2 kinase during mitosis. Together, the structural and sequence similarities of golgin-67, with these varied Golgi proteins, suggest that in addition to being possibly involved in Golgi structure maintenance, this protein may also play a role in docking/tethering of vesicles.

Lastly, golgin-67 was identified during a search for potential differences in thickness among the various membranes found within the cell, the lipid sorting model theorizes that specific membrane targeting is dependent on the length of the TMD of the protein. The plasma membrane, which is rich in sphingo-
lipids and sterols, forms a thicker bilayer (32) than Golgi membranes and as a result, the Golgi proteins containing the shorter TMDs are prevented from trafficking forward and instead are retained at the Golgi. Consistent with this theory is the observation that a synthetic TMD of 17 leucines gives Golgi retention, whereas one of 23 leucines does not and instead results in transport to the plasma membrane (33). Interestingly, the predicted transmembrane spanning region for golgin-67 was 19 residues long, identical in length to the predicted TMD of golgin-84 (5), suggesting that the transmembrane region for golgin-67 is too short for plasma membrane insertion but optimal for Golgi retention.

Recently, a novel Golgi localization domain, known as the “GRIP” domain, has been identified in several peripherally associated coiled-coil proteins (34–36). This conserved domain (about 50 amino acids in length) is found in the noncoiled-coil region of the C terminus and apparently involves two critical aromatic residues (35). Upon visual inspection of the C-terminal 113 amino acid residues of golgin-67, no evidence of the GRIP domain was found, further suggesting that golgin-67 is an integral membrane protein and that the predicted C-terminal TMD is responsible for its Golgi targeting.

FIG. 6. The C-terminal 113 residues of golgin-67 contain a Golgi targeting signal and a putative membrane spanning do-

A, golgin-67 (g67), was predicted to have a single TMD very close to its C terminus (amino acids 437–455). A profile was plotted as the transmembrane score against the amino acid number, using TMpred (30). The prediction parameter was set to search for transmembrane helices between 17 and 35 residues in length. B, schematic diagram of wildtype and mutant EGFP-tagged golgin-67 proteins. EGFP, g67(C113) is a fusion protein construct between EGFP and the last 113 residues of golgin-67. The shaded box represents the C-terminal transmembrane domain. C, BHK cells expressing EGFP alone had a nuclear and cytoplasmic signal. However, EGFP-g67(C113) was targeted to the Golgi. Golgi targeting of EGFP-g67(C113) was confirmed by immuno-

staining the cells with antibodies against the Golgi marker protein mannosidase II. Bar, 5 μm.
new targets of Src using an antibody specific to a mitotic substrate of Src, Sam68, which contains a proline-rich motif that closely resembles a similar motif identified in golgin-67 (amino acids 143–149). Notably, this proline-rich motif is thought to mediate Src SH3 domain interactions. In addition, golgin-67 also contains a putative Src tyrosine phosphorylation motif. Together, these observations suggest that golgin-67 is a potential target of Src. Preliminary experiments indicate that Src associates with and tyrosine phosphorylates golgin-67, in vitro. These relationships are currently under further investigation.

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Note Added in Proof—Recently, additional clones of golgin-67 isolated from other cDNA libraries have been characterized independently at the Scripps Research Institute (AF164622), and autoantibodies to golgin-67 have been identified in a subset of patients with anti-Golgi reactivity (Eystathioy, T., Jakymiw, A., Fujita, D. J., Fritzler, M. J., and Chan, E. R. L. (2000) J. Autoimmun., in press).

REFERENCES

1. Munro, S. (1998) Trends. Cell Biol. 8, 11–15
2. Pfeiffer, S. R. (1996) Annu. Rev. Cell Dev. Biol. 12, 441–461
3. Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995) J. Cell Biol. 131, 1715–1726
4. Lupas, A. (1996) Trends. Biochem. Sci. 21, 375–382
5. Bascom, R. A., Srinivasan, S., and Nussbaum, R. L. (1999) J. Biol. Chem. 274, 2953–2962
6. Biscardi, J. S., Tice, D. A., and Parsons, S. J. (1999) Adv. Cancer Res. 76, 61–119
7. David-Pfeuty, T., and Nouvian-Douguè, Y. (1990) J. Cell Biol. 111, 3097–3116
8. Kaplan, K. B., Swedlow, J. R., Varmus, H. E., and Morgan, D. O. (1992) J. Cell Biol. 118, 321–333
9. Weng, G., Muller, O., Clark, R., Conroy, L., Moran, M. F., Polakis, P., and McCormick, F. (1992) Cell 69, 551–558
10. Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtneidge, S. A. (1994) Nature 368, 871–874
11. Taylor, S. J., and Shalloway, D. (1994) Nature 368, 867–871
12. McBride, A. E., Schlegel, A., and Kirkegaard, K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2296–2303
13. Frangioni, J. V., and Neel, B. G. (1993) Anal. Biochem. 210, 179–187
14. Golbet, C., Prost, E., and Whalen, R. G. (1989) Nucleic Acids Res. 17, 2144
15. Kowal, R., and Parsons, R. G. (1980) Anal. Biochem. 102, 72–76
16. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 313–315, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Bastos, R., Ribas, d. P., Enarson, M., Bodoor, K., and Burke, B. (1997) J. Cell Biol. 137, 989–1000
18. Vogel, L. B., and Fujita, D. J. (1995) J. Biol. Chem. 270, 2506–2511
19. Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Hirozawa, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998) DNA Res. 5, 355–364
20. Kozak, M. (1984) Nucleic Acids Res. 12, 857–872
21. Kozak, M. (1986) Cell 44, 283–292
22. Nishimura, N., and Balch, W. E. (1997) Science 277, 556–558
23. Bannykh, S. I., Nishimura, N., and Balch, W. E. (1998) Trends Cell Biol. 8, 21–25
24. Lupas, A., Van, D. M., and Stock, J. (1991) Science 252, 1162–1164
25. Fritzler, M. J., Hamel, J. C., Ocha, R. L., and Chan, E. K. (1993) J. Exp. Med. 178, 49–62
26. Nakamura, N., Lowe, M., Levine, T. P., Rabouille, C., and Warren, G. (1997) Cell 89, 445–455
27. Lowe, M., Rabouille, C., Nakamura, N., Watson, R., Jackman, M., Jansa, E., Rahman, D., Pappin, D. J., and Warren, G. (1998) Cell 94, 783–793
28. Zhou, S., Carraway, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., and Eng, C. (1995) Nature 373, 536–539
29. Linstedt, A. D., and Hauri, H. P. (1993) Mol. Biol. Cell 4, 679–693
30. Hoffman, K., and Stoffel, W. (1993) Biol. Chem. Hoppe-Seyler 374, 166
31. Munro, S. (1995) Biochem. Soc. Trans. 23, 527–530
32. Ren, J., Lew, S., Wang, Z., and London, E. (1997) Biochemistry 36, 10213–10220
33. Munro, S. (1995) EMBO J. 14, 4695–4704
34. Munro, S., and Nichols, B. J. (1999) Curr. Biol. 9, 377–380
35. Kjer-Nielsen, L., Teasdale, R. D., van Vliet, C., and Gleeson, P. A. (1999) Curr. Biol. 9, 385–388
36. Barr, F. A. (1999) Curr. Biol. 9, 381–384
37. Oas, T. G., and Endow, S. A. (1994) Trends. Biochem. Sci. 19, 51–54
38. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
39. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318–324
40. Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3046–3051
41. Sonnichsen, B., Lowe, M., Levine, T., Jansa, E., Dirac-Svejstrup, B., and Warren, G. (1998) J. Cell Biol. 140, 1013–1021
42. Lowe, M., Nakamura, N., and Warren, G. (1998) Trends. Cell Biol. 8, 40–44
43. Henikoff, S., and Henikoff, J. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10915–10919
44. Appel, R. D., Bairouch, A., and Hochstrasser, D. F. (1994) Trends Biochem. Sci. 19, 258–260

2 E. Raharjo, A. Jakymiw, and D. J. Fujita, unpublished results.