

CASP, the Alternatively Spliced Product of the Gene Encoding the CCAAT-Displacement Protein Transcription Factor, Is a Golgi Membrane Protein Related to Giantin

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Large coiled-coil proteins are being found in increasing numbers on the membranes of the Golgi apparatus and have been proposed to function in tethering of transport vesicles and in the organization of the Golgi stack. Members of one class of Golgi coiled-coil protein, comprising giantin and golgin-84, are anchored to the bilayer by a single C-terminal transmembrane domain (TMD). In this article, we report the characterization of another mammalian coiled-coil protein, CASP, that was originally identified as an alternatively spliced product of the CUTL1 gene that encodes CCAAT-displacement protein (CDP), the human homologue of the *Drosophila* homeodomain protein Cut. We find that the *Caenorhabditis elegans* homologues of CDP and CASP are also generated from a single gene. CASP lacks the DNA binding motifs of CDP and was previously reported to be a nuclear protein. Herein, we show that it is in fact a Golgi protein with a C-terminal TMD and shares with giantin and golgin-84 a conserved histidine in its TMD. However, unlike these proteins, CASP has a homologue in *Saccharomyces cerevisiae*, which we call COY1. Deletion of COY1 does not affect viability, but strikingly restores normal growth to cells lacking the Golgi soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor Gos1p. The conserved histidine is necessary for Coy1p's activity in cells lacking Gos1p, suggesting that the TMD of these transmembrane Golgi coiled-coil proteins is directly involved in their function.

INTRODUCTION

The Golgi apparatus occupies a central position in the eukaryotic secretory pathway and provides a sorting point for proteins delivered to, and from, a variety of organelles. The Golgi is itself comprised of several compartments or cisternae typically arranged in a stack (Warren and Malhotra, 1998; Glick, 2000). Although many components of the Golgi have been identified, it remains unclear how the stacked structure is maintained and how the specificity of vesicular traffic between individual cisternae is conferred. Vesicles are targeted to both the *cis*- and *trans*-cisternae from other organelles, and vesicles must also move between cisternae. Newly synthesized proteins arriving from the endoplasmic reticulum (ER) traverse the stack to the *trans*-side for sorting to the plasma membrane, endocytic compartments, and other post-Golgi structures. It is now widely believed that

such proteins move through the stack by maturation of the cisternae, with this perhaps supplemented by some anterograde vesicular transport (Pelham and Rothman, 2000; Barr, 2002). In addition, retrograde transport between cisternae must exist to recycle resident modification enzymes and other membrane proteins to maintain their distribution within the Golgi stack. Because many of these residents are restricted to particular subsets of cisternae, there are likely to be mechanisms to allow vesicles to recycle specifically to many, if not all, of the cisternae within the stack.

The "golgins" are a class of molecules that have emerged as candidates to organize the structure and trafficking pathways of the Golgi (Fritzler *et al.*, 1993, 1995; Seelig *et al.*, 1994; Nakamura *et al.*, 1995; Bascom *et al.*, 1999). These large proteins are predicted to form coiled-coils over most of their length, and as such have a structure suitable either to tether vesicles before fusion, or to hold adjacent cisternae together in what has been termed a Golgi "matrix" or scaffold (Slusarewicz *et al.*, 1994; Orci *et al.*, 1998; Seemann *et al.*, 2000a). Because there is a large number of such proteins, at least 15 in mammalian cells, of which 10 have clear homologues in *Drosophila*, it is possible that they perform more than one general function in the Golgi. Indeed, in vitro

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systems have provided evidence in support of both tethering and organizational functions for a number of these proteins. For instance, Uso1p, and its mammalian homologue p115, are required for ER-to-Golgi transport *in vivo*, and reconstitution of this process *in vitro* has shown that the requirement is at the stage of vesicle tethering to the *cis*-Golgi (Cao *et al.*, 1998; Seemann *et al.*, 2000b; Alvarez *et al.*, 2001). Two other coiled-coil proteins, GM130 (or golgin-95) and giantin (or macrogolgin), have been shown to recruit p115 to Golgi membranes (Sonnichsen *et al.*, 1998; Lesa *et al.*, 2000; Puthenveedu and Linstedt, 2001). These proteins also seem to contribute to cisternal structure *in vivo* and are required in an *in vitro* assay system that reconstitutes the stacking of Golgi cisternae (Shorter and Warren, 1999; Puthenveedu and Linstedt, 2001).

As might be expected for proteins that confer specificity to vesicular transport, or Golgi organization, many golgins are restricted to a subset of cisternae, perhaps performing similar roles in different parts of the Golgi stack (Nakamura *et al.*, 1995; Erlich *et al.*, 1996). In some cases, amino acid sequences or domains have been identified that target these proteins to distinct loci. Most golgins are peripheral membrane proteins and often have short noncoiled-coil regions at either end of the protein that mediate targeting and other interactions. Thus, the C terminus of GM130 binds to GRASP65, a lipid-anchored protein on the *cis*-Golgi (Barr *et al.*, 1998). Similarly, four of the golgins share a C-terminal GRIP domain that is sufficient to target them to the *trans*-Golgi (Barr, 1999; Kjer-Nielsen *et al.*, 1999; Munro and Nichols, 1999). In contrast, two golgins, giantin and golgin-84, are integral membrane proteins anchored to the bilayer via a C-terminal transmembrane domain (TMD), although the functional significance of this is not yet understood (Linstedt *et al.*, 1995; Bascom *et al.*, 1999; Misumi *et al.*, 2001).

Analysis of the targeting and function of Golgi coiled-coil proteins has mainly been performed in mammalian cells, where many were first identified. However, yeast has proven valuable in identifying and analyzing other proteins involved in membrane traffic. *Saccharomyces cerevisiae* has homologues of p115 and GRASP65 and also has a single GRIP-domain-containing protein, Imh1p (Sapperstein *et al.*, 1995; Tsukada *et al.*, 1999). However, neither of the two members of the transmembrane-anchored class of coiled-coil proteins, golgin-84 and giantin, has a clear homologue in the yeast genome.

In this article, we describe an investigation of a human coiled-coil protein, CDP/cut alternatively spliced product (CASP), that was originally identified as an alternatively spliced transcript from the CUTL1 gene that encodes CCAAT-displacement protein (CDP, also termed Cux in mice) (Lievens *et al.*, 1997; Nepveu, 2001). CDP is a transcriptional repressor that was originally identified as a factor that bound to CCAAT elements in the promoter of a sea urchin histone gene, and thereby displaced CCAAT binding protein (Barberis *et al.*, 1987). Cloning of human CDP revealed it to be the homologue of the *Drosophila* homeodomain protein Cut, and both proteins contain three "cut repeat" DNA-binding domains in addition to a homeobox (Neufeld *et al.*, 1992; Ludlow *et al.*, 1996). CDP has been found to repress expression of a wide range of genes during both the cell cycle and development, and analysis of *Drosophila* mutants and mouse knockouts shows that CDP/cut is involved in a

diverse range of cell fate decisions (Blochlinger *et al.*, 1991; Tufarelli *et al.*, 1998; Ellis *et al.*, 2001; Nepveu, 2001; Sinclair *et al.*, 2001). In addition, loss of heterozygosity of the human CUTL1 locus has been observed in a subset of breast and uterine cancers, leading to the suggestion that it acts as a tumor suppressor (Zeng *et al.*, 1999; Neville *et al.*, 2001). During the characterization of human CDP, an alternatively spliced transcript was found that encodes a protein that lacks all of the DNA binding domains of CDP, but instead has a unique C-terminal region (Figure 1A). Called CASP, the function of this protein is unknown, but it was reported to be nuclear (Lievens *et al.*, 1997; confusingly, the name CASP has also been applied to a recently identified scaffolding protein that binds to cytohesin; Mansour *et al.*, 2002; however, this scaffolding protein is unrelated to the CASP described herein). In this article, we show that CASP is in fact a Golgi membrane protein with an overall structure similar to giantin and golgin-84. Moreover, it shares with these proteins hydrophilic residues in its TMD that are conserved between homologues in different species. However, unlike these proteins CASP has a homologue in *S. cerevisiae*. This has allowed us to examine the importance of the conserved TMD residues shared with giantin and golgin-84.

MATERIALS AND METHODS

Plasmids

Full-length and C-terminal regions of human CASP were polymerase chain reaction (PCR) amplified from cDNA, and cloned into COS cell vectors containing the cytomegalovirus promoter, and enhanced green fluorescent protein (CLONTECH, Palo Alto, CA) attached via a myc tag. A full-length cDNA for human CDP was assembled from IMAGE clone 4903642 (residues 749-1446; Human Genome Mapping Project Resource Center, Hinxton, United Kingdom), and PCR products were amplified from cDNA. All PCR products were checked by sequencing. CASP and CDP have two alternative first exons, resulting in the alternative N termini MAAN-VGSMFQYWKRFDLQQLQ or MLCVRGARKL (Rong Zeng *et al.*, 2000). When CDP and CASP constructs described in this article have an intact N terminus, it is always the former version. Full-length CDP was cloned into a COS cell vector containing the cytomegalovirus promoter, with enhanced green fluorescent protein attached to the C terminus via a GAGAGA linker.

Antibody Production

A plasmid expressing a chimera of glutathione S-transferase (GST) and residues 472-618 of human CASP was constructed using an appropriate PCR product and pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ). Fusion proteins were expressed in DH5 α , and the resulting inclusion bodies were washed twice in 0.5% (vol/vol) Triton X-100, 1 mM EDTA in phosphate-buffered saline, resuspended in sample buffer, and separated by SDS-PAGE. The gel was stained briefly in Coomassie Blue, the protein band excised, eluted from the gel by electrophoresis, and recovered by ethanol precipitation. Sera from immunized rabbits were absorbed with GST-Sepharose and then antibodies affinity purified on the GST-CASP antigen coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences).

Immunofluorescence of Mammalian Cells

COS cells were transfected using FuGene (Roche Applied Science, Indianapolis, IN), split onto glass slides, and fixed 30-48 h posttransfection with 4% (wt/vol) paraformaldehyde. Cells were

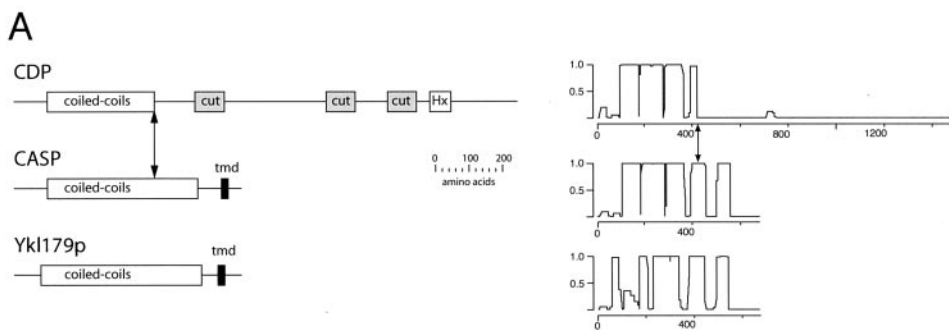
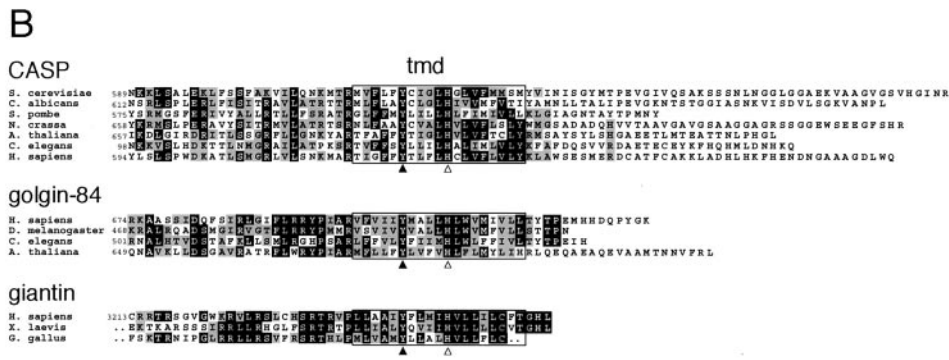
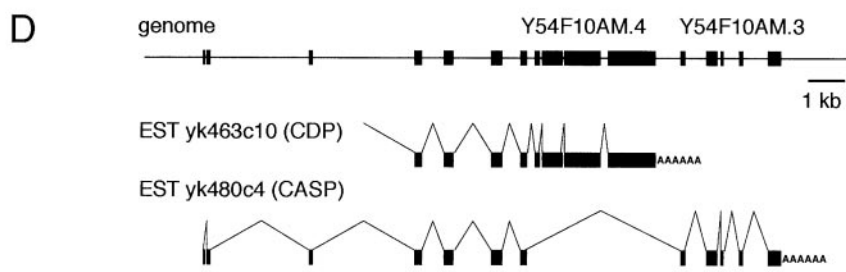


Figure 1. A family of proteins related to CASP, the alternatively spliced product of the CUTL1 gene. (A) Diagrammatic representation of human CDP, CASP, and the product of the yeast ORF Ykl179p. CDP and CASP share an N-terminal region, with the point of divergence indicated by an arrow. The cut repeat and homeobox (Hx) domains in CDP are shown, along with coiled-coil predictions for the proteins (Lupas *et al.*, 1991). (B) Alignment of the C termini of homologues of CASP, golgin-84, and giantin from the indicated species. The putative TMD is boxed and the conserved histidine and tyrosine residues indicated by triangles. The CASP homologues are as in C, plus *Candida albicans* orf6.3753; *S. pombe* SPCC364.04c; *Neurospora crassa* 9G6.340. The giantin homologues are from ESTs, GenBank accession numbers BM486947 and BJ037357. (C) Species distribution of homologues of the indicated proteins, with the gene names given, and the number of residues in brackets. Cux-2 is a neuronal-specific isoform of CDP found in humans and mice that does not seem to have an alternative product analogous to CASP. (D) Structure of two *C. elegans* cDNAs from the region of the predicted genes Y54F10AM.4 and Y54F10AM.3 (cDNAs kindly provided by Yuji Kohara, National Institute of Genetics, Mishima, Japan). The positions in the genome of the exons present in the cDNAs are indicated. The cDNAs share exons but then diverge to encode the *C. elegans* homologues of CDP and CASP.



C

Protein family	Human	Drosophila	C. elegans	Arabidopsis	S.cerevisiae
CDP/cut	CDP (1505) Cux-2 (1486)	cut (2175)	Y54F10AM.4a (1273)	-	-
CASP	CASP (678)	-	Y54F10AM.4b (452) Y54F10AM.3 (169)	At3g18480 (689)	YKL179c (679)
golgin-84	golgin-84 (731)	CG17785 (516)	T24B1.1 (551)	At2g19950 (713) At1g18190 (654)	-
giantin	giantin (3259)	-	-	-	-



permeabilized with 0.5% (vol/vol) Triton X-100 in PBS, blocked with 20% (vol/vol) fetal calf serum/0.25% (vol/vol) Tween 20 in PBS, and probed with antibodies in the same solution. Polyclonal antibodies against CASP, giantin (Seelig *et al.*, 1994), and TGN46 (Prescott *et al.*, 1997), and monoclonals against TGN38 (2F7.1;

Affinity Bioreagents, Golden, CO) and β -COP [23C (TCP-1); Stressgen, Victoria, British Columbia, Canada] were detected with species-specific Alexa-labeled secondary antibodies (Molecular Probes, Eugene, OR), the cells mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and im-

Table 1. Yeast strains used in this study

BY4741	MAT α <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>
BY4742	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>
BY4743	MAT α / α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0</i>
SEY6210	MAT α <i>ura3-52 his3-Δ200 leu2-3,112 trp1-Δ901 lys2-801 suc2-Δ9</i>
AGY01	BY4742 <i>ykl179cΔ::HIS5Sp</i>
AGY04	BY4743 <i>ykl006c-aΔ::kanMX4/YKL006c-a ykl179cΔ::HIS5Sp/YKL179c</i>
AGY05	BY4743 <i>ymr197cΔ::kanMX4/YMR197c ykl179cΔ::HIS5Sp/YKL179c</i>
AGY06	BY4743 <i>ykl196cΔ::kanMX4/YKL196c ykl179cΔ::HIS5Sp/YKL179c</i>
APY02	SEY6210 <i>ykl179cΔ::YKL179c-HAα3-HIS5Sp</i>
RDY198	MAT α <i>ura3 leu2 sec35-1</i> (R. Duden, Univ. Cambridge, United Kingdom)
RSY279	MAT α <i>ura3-52 his4-619 sec22-3</i> (R. Duden, Univ. Cambridge, United Kingdom)
RSY961	MAT α <i>ura3 leu2 sec34-2</i> (R. Duden, Univ. Cambridge, United Kingdom)
<i>arl1Δ</i>	BY4741 <i>ybr164cΔ::kanMX4</i>
<i>cod3Δ</i>	BY4741 <i>ygl223cΔ::kanMX4</i>
<i>dor1Δ</i>	BY4741 <i>yml071cΔ::kanMX4</i>
<i>gos1-Δ1</i>	BY4741 <i>yhl031cΔ::kanMX4</i>
<i>gos1-Δ2</i>	SEY6210 <i>gos1Δ::HIS3</i> (Lewis <i>et al.</i> , 2000)
<i>ric1Δ</i>	BY4741 <i>yrl039cΔ::kanMX4</i>
<i>rud3Δ</i>	BY4741 <i>yor216cΔ::kanMX4</i>
<i>sec22Δ</i>	BY4741 <i>yrl268wΔ::kanMX4</i>
<i>sft1-11</i>	SEY6210 <i>sft1Δ::LEU2 (CEN HIS3 sft1-11)</i> (M. Lewis, MRC Laboratory of Molecular Biology, United Kingdom)
<i>uso1-ΔC</i>	SEY6210 <i>ydl058wΔ::YDL058w(1-950-GFP)-kanMX4</i>
<i>ypt6Δ</i>	SEY6210 <i>ypt6Δ::HIS3</i> (M. Lewis, MRC Laboratory of Molecular Biology, United Kingdom)

ages obtained on a Radiance confocal microscope (Bio-Rad, Hercules, CA).

Preparation of Golgi Membranes and Analysis of CASP

Golgi membranes were prepared essentially as described previously (Slusarewicz *et al.*, 1994; Hui *et al.*, 1998). Briefly, rat livers were homogenized, equilibrated in phosphate-buffered 0.5 M sucrose, and layered on a phosphate-buffered 0.86 M sucrose cushion, overlaid with phosphate-buffered 0.25 M sucrose and centrifuged at 105,000 \times *g* for 60 min at 4°C. The Golgi stacks were collected from the 0.5 M/0.86 M sucrose interface, diluted to 0.25 M-buffered sucrose, and pelleted by centrifugation at 6,000 \times *g* for 20 min at 4°C. The pellet was washed once and aliquoted for storage at -20°C.

For large-scale immunoprecipitation of CASP, Golgi membranes (1 mg) were solubilized by 2-h rotation in lysis buffer (1% [wt/vol] digitonin, 20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 16,000 \times *g* for 5 min, the supernatant was incubated overnight with either anti-CASP, or rabbit IgG covalently coupled to protein A beads, washed three times with 1 ml of lysis buffer, and once with 1 ml of 5 mM NH₄Ac, pH 6.5. All steps were at 4°C. Bound proteins were eluted with 100 μ l of 0.5 M acetic acid, pH 3.4, lyophilized, and resuspended in SDS sample buffer. After gel electrophoresis and brief staining with Coomassie Blue, the protein bands were excised, digested with trypsin, and analyzed by matrix-assisted laser desorption/ionization mass spectrometry (Shevchenko *et al.*, 1996).

Yeast Strains and Plasmids

The genotypes of yeast strains used are listed in Table 1. YKL179c was tagged at the C terminus in strain SEY6210 by using the PCR method and template plasmid p3xHA-His5 that contains three copies of the hemagglutinin (HA) epitope tag and *Schizosaccharomyces pombe* HIS5 gene (Baudin *et al.*, 1993; Jungmann *et al.*, 1999). YKL179c was deleted in the EUROSCARF parental haploid strain BY4742, and in the diploid strains *vti1 Δ* and *ykt6 Δ* by

transformation with PCR products from p3xHA-His5, to give strains AGY01, AGY05, AGY06, and AGY07, respectively (Table 1). For other genes, AGY01 was mated with haploid EUROSCARF strains *arl1 Δ* , *cod3 Δ* , *dor1 Δ* , *gos1 Δ* , *ric1 Δ* , *rud3 Δ* , and *sec22 Δ* (Brachmann *et al.*, 1998). The resulting diploids were sporulated, tetrads dissected (Singer Instruments, Wachuset, United Kingdom), and spores were incubated on nonselective plates at 30°C for 2 d before testing of marker segregation.

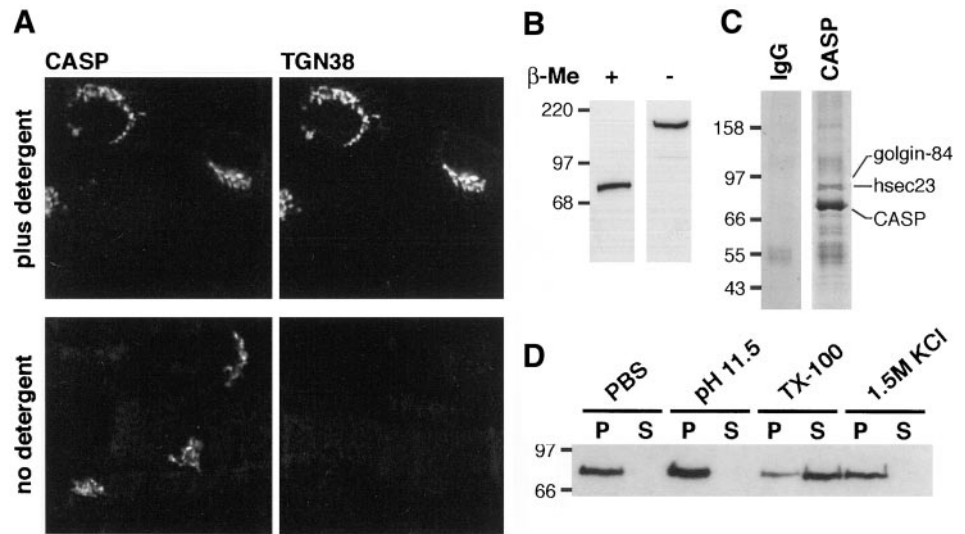
Full-length YKL179c was cloned into the galactose-inducible plasmid pAK, which is pRS416 (CENURA3; Sikorski and Hieter, 1989) containing a *GAL1* promoter and an *ADHI* terminator (gift of Robert Arkowitz, Université de Nice, France), to create pAK-YKL179c. The C terminus of YKL179c was modified by PCR to insert a GAGA linker and a 3xHA tag. For mutation of Y619 to L and H624 to L, PCR products generated using appropriate primers were cloned into the pAK-YKL179c plasmid and the amplified region checked by sequencing. The YKL179c ORF was also cloned into pRS426 (2 μ URA3) with a constitutive *PHO5* promoter to create plasmid pRS426-YKL179c.

Yeast Immunoblotting and Immunofluorescence

Strains transformed with galactose-inducible plasmids were induced in log phase, and total protein samples were prepared by resuspending 1 A₆₀₀ unit/20 μ l of SDS buffer, bead beating for 1 min at 4°C (425–600- μ m glass beads; Sigma-Aldrich, St. Louis, MO), and denaturing at 80°C for 5 min. After gel electrophoresis, proteins transferred onto nitrocellulose were probed with mouse monoclonal 12CA5 to the HA epitope and horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence of formaldehyde fixed cells was carried out as described previously, except for the omission of extraction in methanol/acetone (Holthuis *et al.*, 1998). Affinity-purified rabbit antisera against Anp1p (Jungmann and Munro, 1998) and Tlg1p (Lewis *et al.*, 2000), and 12CA5 were detected with appropriate Alexa⁴⁸⁸ secondary antibodies (Molecular Probes) and images obtained with confocal microscopy.

Figure 2. CASP behaves as a type II integral membrane protein of the Golgi apparatus. (A) Immunofluorescence localization of CASP in normal rat kidney fibroblasts. Cells fixed in 4% paraformaldehyde were permeabilized by freeze-thaw and either treated with 0.5% Triton X-100 in PBS (top) or left untreated (bottom). Cells were probed with antibodies against CASP and the Golgi protein TGN38 as described in MATERIALS AND METHODS, with the omission of Tween 20. (B) Anti-CASP protein blot of rat liver Golgi membranes (5 μ g/lane) solubilized in SDS sample buffer with or without 5% β -mercaptoethanol (β -ME). (C) Coomassie Blue-stained protein gel of immunoprecipitates from rat liver Golgi membranes solubilized in digitonin, by using anti-CASP or a control IgG. Proteins identified by mass spectrometry are indicated. (D) Anti-CASP protein blot of rat liver Golgi membranes extracted with PBS containing either nothing (PBS), 1.5 M KCl, 100 mM sodium carbonate, pH 11.5 or 1% Triton X-100. Membranes (1 mg/ml) were extracted for 30 min at 4°C, centrifuged (105,000 \times g for 40 min), and equivalent amounts of pellet (P) and supernatant (S) were analyzed by SDS-PAGE and protein blotting.



RESULTS

CASP Defines a Conserved Family of Coiled-Coil Proteins, Related to Giantin and Golgin-84

The mammalian Golgi membrane proteins giantin and golgin-84 do not have clear homologues in yeast. However, when the C-terminal region of an Arabidopsis homologue of golgin-84 was used to search the GenBank database with the iterative program PSI-BLAST, homologues of the uncharacterized *S. cerevisiae* gene YKL179c were found to have a similarity only just below the default cut-off for significance ($p = 0.005$). This yeast gene encodes a protein that is predicted to contain a C-terminal TMD and extensive regions of coiled-coil, the same overall structure as giantin and golgin-84 (Figure 1A). However, the product of the YKL179c gene cannot be a distant yeast homologue of either of these proteins because it is clearly related to another family of proteins represented by a single gene in the genomes of higher eukaryotes (Figure 1, B and C). The human member of this family is the protein CASP that is produced from an alternatively spliced transcript from the CUTL1 gene that also encodes the transcriptional repressor CDP (Lievens *et al.*, 1997) (Figure 1, A and C). CASP was reported to have homologues in mouse, chicken, and yeast, but the presence of a putative TMD near the C terminus was not noted (Lievens *et al.*, 1997). In *C. elegans*, a predicted gene Y54F10AM.3 encodes a small protein related to the C terminus of CASP. However, the adjacent gene Y54F10AM.4 encodes a homologue of CDP. We thus sequenced two *C. elegans* expressed sequence tags containing the 3' end of these two genes, and found that they share 5' exons, indicating that the structure of the human CDP/CASP gene is conserved in *C. elegans* (Figure 1D).

Although most of the sequence of CASP, and of its relatives, is predicted to form regions of coiled-coil, this is not so

for the N- and C-terminal regions. Database searches with either of these regions shows significant similarity to all known homologues in other species, but to no other proteins. Figure 1B shows an alignment of the C-terminal region of these homologues, and the program TMHMM predicts a TMD in this region for all of these proteins (Krogh *et al.*, 2001). In addition, some of the TMD residues are conserved, and a tyrosine and histidine in the middle of the TMD are invariant across species. Histidine is of course charged and is, not surprisingly, very rare in the TMDs of single-span proteins (Landolt-Marticorena *et al.*, 1993). Strikingly, comparing this putative TMD region to those of golgin-84 and giantin reveals that these two residues are also well conserved in both of these proteins (Figure 1B). These similarities suggest that CASP could be a Golgi membrane protein that has properties in common with golgin-84 and giantin but, unlike these proteins, is conserved in yeast. However, the original characterization of CASP reported that the protein was localized in the nucleus, although the data were not shown (Lievens *et al.*, 1997). In the light of the above-mentioned homologues, and conservation of a putative TMD, we decided to reinvestigate the localization of CASP.

CASP Is a Golgi-localized Membrane Protein

To examine the subcellular distribution of CASP, polyclonal antibodies were raised against residues 472–618 of the human protein, a section not present in CDP. The affinity-purified antiserum recognized a single prominent band on blots of total cellular proteins, and of purified Golgi membranes, with an apparent size of 80 kDa (Figure 2B; our unpublished data). When this antiserum was used for immunofluorescence of various tissue culture cells, including COS, normal rat kidney, human embryonic kidney 293, and HeLa, in every case a juxtannuclear ribbon-like staining was

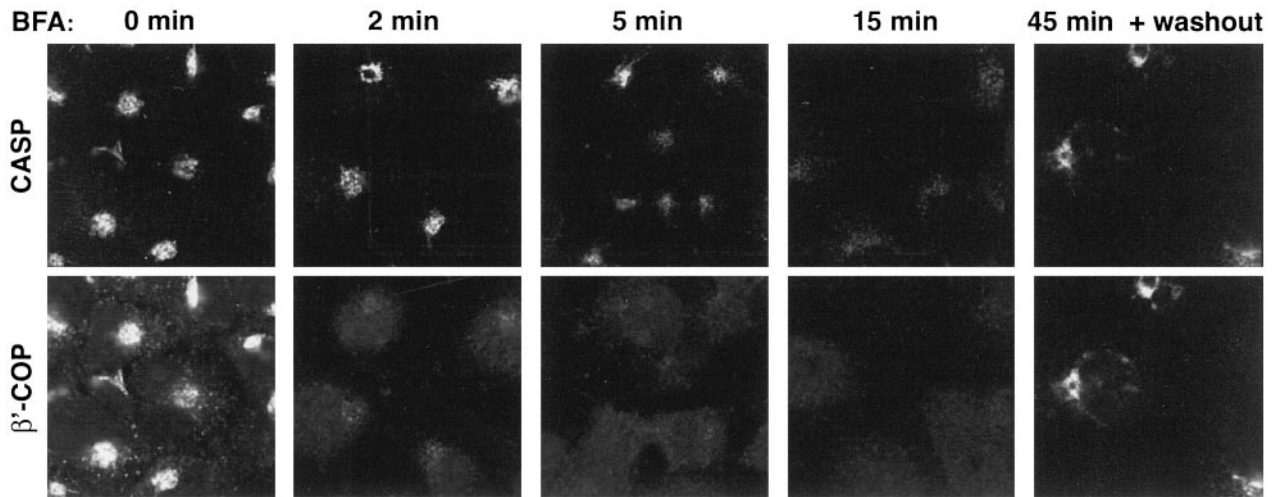


Figure 3. Redistribution of CASP after treatment with brefeldin A (BFA). Immunofluorescence micrographs of COS cells treated with 10 $\mu\text{g}/\text{ml}$ BFA at 37°C for the times indicated, and then fixed, permeabilized, and stained with antibodies against CASP and β -COP. In the right-hand panels cells were treated for 45 min with BFA, washed thoroughly, and allowed to recover for 60 min.

observed that colocalized with Golgi markers (Figures 2A and 3; our unpublished data).

To examine the possibility that CASP is an integral membrane protein, Golgi membranes were extracted with various disruptive agents. Figure 2D shows that CASP remained Golgi-associated after treatment with high salt (1.5 M KCl), or high pH (carbonate pH 11.5), but upon addition of detergent (1% Triton X-100) much of the protein was solubilized. Thus, CASP behaves as an integral membrane protein. Moreover, when CASP was analyzed by protein blotting in nonreducing conditions, the gel mobility of the protein was reduced to an apparent molecular mass of 160–180 kDa (Figure 2B). This is consistent with a disulfide-linked dimer and implies that at least part of the molecule has been exposed to the oxidizing environment of the ER lumen. To confirm that CASP dimerizes with itself, rather than with another protein of a similar size, it was immunoprecipitated under nonreducing conditions from detergent-solubilized Golgi membranes. As shown in Figure 2C, the precipitate contained a single abundant protein, and mass spectrometric analysis of tryptic peptides confirmed that the band solely comprised CASP. Two other proteins present at substoichiometric levels were identified as golgin-84 and hSec23, a subunit of the COPII coat.

To investigate the orientation of CASP in the bilayer, the anti-CASP antiserum was used to probe cells in which the plasma membrane had been permeabilized by freeze-thaw, a procedure known to leave Golgi membranes intact (Seaman *et al.*, 1993). The antiserum was raised against a region of CASP located on the N-terminal side of the predicted TMD and showed a clear signal both in the absence and presence of detergent (Figure 2A). In contrast, antibodies against a luminal epitope of the transmembrane protein TGN38 only gave a signal in the presence of detergent (Figure 2A). This indicates that CASP has a type II orientation with its C terminus in the Golgi lumen. There are two cysteine residues in the region C terminal to the putative

TMD that could participate in the formation of intermolecular disulfide bridges in a CASP dimer.

Finally, the localization of CASP was examined after treatment with the Golgi-disrupting drug brefeldin A. Figure 3 shows that CASP began to redistribute to the ER after 2–5 min of drug treatment, kinetics slower than the Golgi peripheral coat protein COPI, but similar to that of known integral membrane proteins giantin and golgin-84 (Seelig *et al.*, 1994; Bascom *et al.*, 1999). After 5 min, intermediate tubules decorated with CASP were clearly visible, and by 15 min CASP was completely dispersed from the Golgi region. The dispersed signal was too weak to confirm that it corresponded to the ER, but it was at least clear that there was no concentration of staining in the vesicular tubular clusters labeled by GM130 and p115 (our unpublished data). On removal of the drug the Golgi reassembled and CASP returned. Taken together, these data indicate that CASP is a membrane protein of the Golgi apparatus with a large N-terminal region protruding into the cytoplasm. This is the same structure as found for both golgin-84 and giantin (Linstedt and Hauri, 1993; Bascom *et al.*, 1999).

Identification of Regions of CASP Involved in Golgi Targeting

Although CASP is predicted to adopt a coiled-coil conformation over the majority of its length, there are regions of ~150 residues at both the N and C termini that seem to be not coiled-coil. To determine whether either of these regions is sufficient to target CASP to the Golgi apparatus, green fluorescent protein (GFP)-tagged chimeras encoding CASP residues 1–137 [CASP(1–137)-GFP] or residues 519–678 [GFP-CASP(519–678)] were expressed in COS cells. The C-terminal region of CASP is sufficient to target GFP to the Golgi apparatus, whereas the N-terminal chimera was found diffusely distributed throughout the cytoplasm (Figure 4A; our unpublished data).

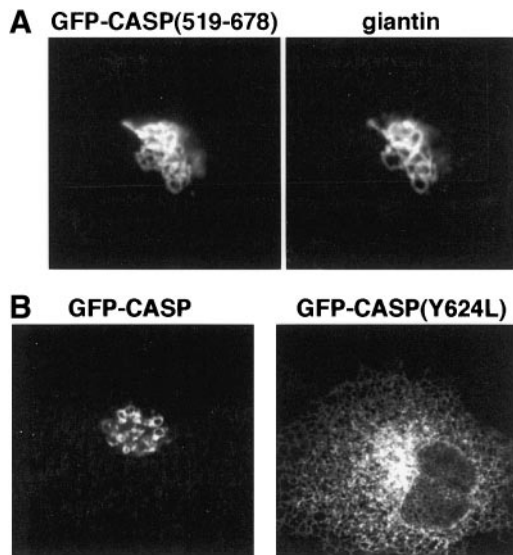


Figure 4. The C terminus of CASP contains a TMD important for Golgi targeting. Confocal micrographs of COS cells expressing GFP fusions to the C-terminal 160 residues of CASP [GFP-CASP(519–678)] (A), or full-length CASP (GFP-CASP), or to CASP with a TMD mutation [GFP-CASP(Y624L)] (B). In A, the cells were fixed, permeabilized, and stained with antibodies against the Golgi marker protein giantin.

The TMD residues of CASP Y624 and H629 (numbering for human sequence) seem to be invariant across species, and to determine whether either is important for the intracellular localization of CASP, both were mutated independently to leucine in the context of the full-length protein. Wild-type GFP-CASP localized to the Golgi apparatus of transfected COS cells, providing further support for the proposed Golgi localization of the endogenous protein (Figure 4B). The mutant GFP-CASP(H629L) also localized to the Golgi apparatus, but in contrast, the Y624L mutant accumulated in the ER (Figure 4B; our unpublished data). Protein blot analysis of extracts prepared in the absence or presence of reducing agent showed that the mutant proteins accumulated to levels comparable with wild type and were still capable of forming disulfide-linked dimers (our unpublished data). This implies that the mutations do not affect protein folding, and indicate that Y624 may contribute to ER exit, but that H629 is not required for exit from the ER or localization to the Golgi.

CASP Does Not Seem to Interact with CDP *In Vivo*

The results mentioned above indicate that CASP is localized to Golgi membranes, raising the question of what function it performs in this organelle. Because CASP shares >300 residues of coiled-coil with CDP, one possibility is that the proteins can heterodimerize, or associate by formation of four-helical bundles or lateral association of coiled-coils as occurs with filamentous myosins (McLachlan and Karn, 1982). CDP has previously been localized to the nucleus, as expected for a transcription factor (Ellis *et al.*, 2001), but interaction with CASP could conceivably serve to sequester

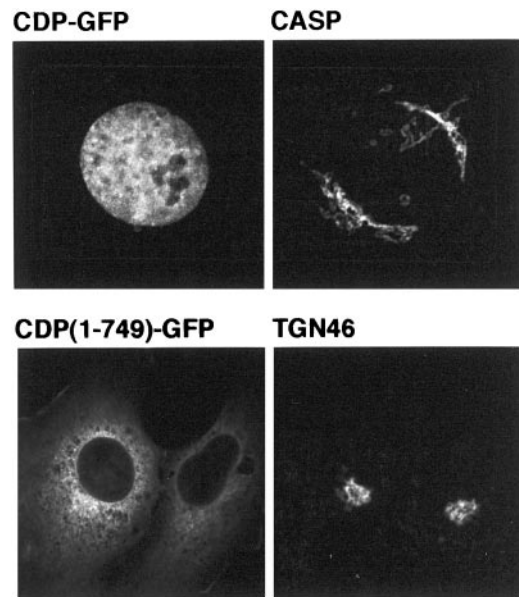


Figure 5. Subcellular localizations of CDP and CASP are distinct. Confocal micrographs of COS cells expressing either full-length CDP fused to GFP (CDP-GFP), or the N-terminal 749 residues fused to GFP [CDP(1–749)-GFP]. Cells were also stained with antibodies to endogenous CASP or TGN46.

a proportion of CDP in the cytoplasm. However, when full-length CDP was expressed with GFP fused to its C terminus, only nuclear staining was observed with no detectable colocalization with endogenous CASP (Figure 5). It has been reported that a truncated version of mouse CDP that only extends to the second of the cut repeats, accumulates in the cytoplasm, presumably because the nuclear targeting signal is located near the C terminus (Ellis *et al.*, 2001). When an N-terminal portion of human CDP comprising the coiled-coil region and the first cut repeat (residues 1–749) was expressed as a GFP fusion it also accumulated in the cytoplasm, but no Golgi localization was observed (Figure 5). These results suggest that CASP does not readily recruit CDP to Golgi membranes. Moreover, the nuclear targeting signal of CDP may be located in the C terminal region to ensure that the cell does not place both proteins in the nucleus.

Characterization of YKL179c, the Yeast Homologue of Mammalian CASP

To investigate the function of CASP, we examined its homologue in the yeast *S. cerevisiae*. The protein is encoded by the open reading frame (ORF) YKL179c, that was shown to be nonessential in a global analysis of the yeast genome, but is otherwise uncharacterized (Winzeler *et al.*, 1999). Initially, three copies of the HA epitope tag were inserted at the C terminus of the YKL179c ORF by homologous recombination. Probing blots of total cellular proteins with anti-HA antibodies revealed a 70-kDa protein, indicating that the gene is expressed under laboratory growth conditions (Figure 6A). Immunofluorescence showed that the tagged

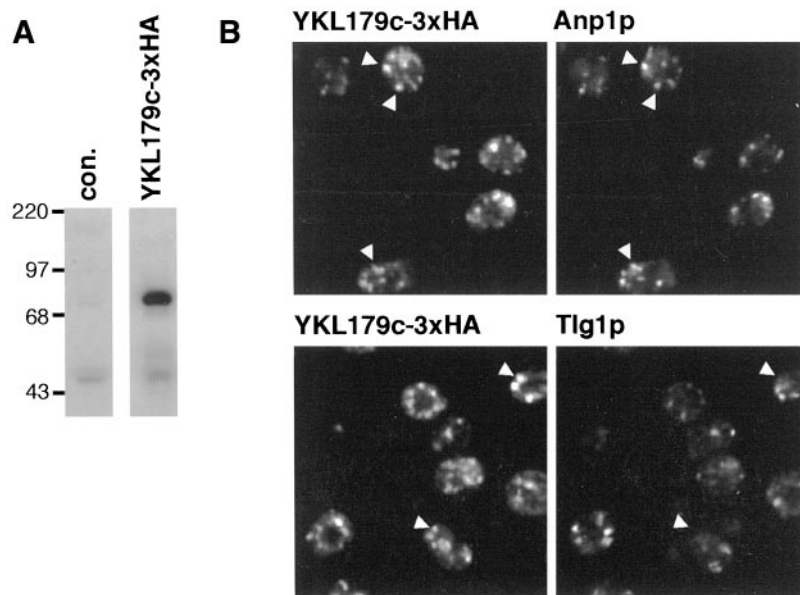


Figure 6. Yeast ORF YKL179c is expressed under normal growth conditions and encodes a protein that localizes to the early Golgi. (A) Anti-HA protein blots of total protein from yeast strain APY02 in which the YKL179c ORF is followed by a triple HA epitope tag (YKL179c-3xHA) or from the parental strain SEY6210 (con.). (B) Confocal micrographs of APY02 double labeled with anti-HA monoclonal 12CA5 and antibodies against either the early Golgi protein Anp1p or the late Golgi protein Tlg1p.

YKL179c was present in punctate structures characteristic of the yeast Golgi (Figure 6B). These showed substantial overlap with the early Golgi marker protein Anp1p, and little with the late Golgi t-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Tlg1p (Holthuis *et al.*, 1998; Jungmann and Munro, 1998). Because the protein encoded by YKL179c is related to CASP, and seems also to be in the Golgi apparatus, we suggest that the gene be named *COY1*, for CASP of yeast.

Genetic Interactions of COY1/YKL179c and Genes Whose Products Are Involved in the Secretory Pathway

It has been reported that *COY1/YKL179c* is not required for viability, and we also deleted the gene in both the SEY6210 and BY4742 strain backgrounds and found no effect on growth, or temperature sensitivity (Winzeler *et al.*, 1999; our unpublished data). However, a number of genes encoding well conserved Golgi proteins only show a growth phenotype when deleted in combination with mutations in other genes involved in membrane traffic (Tsukada *et al.*, 1999; Bensen *et al.*, 2000; Siniosoglou *et al.*, 2000). This synthetic lethality may reflect redundancy of components acting in a given transport step, or the loss of a particular transport step being compensated for by the increased use of other steps. We therefore deleted *COY1* from diploid strains lacking a number of genes involved in ER-Golgi and intra-Golgi traffic, and examined the viability of double mutants by sporulation and tetrad analysis. Loss of *COY1* had no effect on the growth rate of spores that also lacked the nonessential genes *RUD3* (a Golgi coiled-coil protein), *COD3* and *DOR1* (subunits of the COG [or Sec34/35] complex), *ARL1* (an ARF-like GTPase), or *RIC1* (the GTP exchange factor for Ypt6p) (VanRheenen *et al.*, 1999; Siniosoglou *et al.*, 2000; Whyte and Munro, 2001). In contrast, analysis of spores from diploids lacking a single copy of *COY1* in combination with loss of

either of the Golgi SNAREs *Gos1p* or *Sec22p* revealed a striking genetic interaction. As previously reported, spores that lack *GOS1* alone are viable, but grow very slowly after germination (Figure 7A, e.g., A4; McNew *et al.*, 1998). However, when the spores lacked *COY1* in addition to *GOS1*, growth was restored to wild-type levels (Figure 7A, e.g., A2). This surprising result was reproduced with an independent Δ *gos1* allele, and the absence of *Gos1p* was confirmed by protein blotting (our unpublished data). A similar effect was seen with spores lacking *SEC22*, but the effect was more subtle because loss of *Sec22p* has a less severe effect on growth (Figure 7B). Deletion of *COY1* did not restore growth to spores lacking the essential SNAREs *Vti1p* or *Ykt6p*. *Sec22p* and *Gos1p* are both implicated in ER-Golgi transport, and *Gos1p* is additionally involved in intra-Golgi traffic.

Overexpression of Coy1p Has Distinct Effects on Loss of ER-Golgi SNAREs Gos1p and Sec22p

To obtain further evidence for a role of *Coy1p* in Golgi function we examined the effect of overexpressing *Coy1p* in strains carrying temperature-sensitive or deletion mutations of various Golgi proteins involved in membrane traffic. Overexpression of *Coy1p* from a multicopy 2 μ plasmid had no effect on the temperature sensitivity or growth of *ypt6* Δ , *sec35-1*, or *sec34-2*, partially suppressed the temperature sensitivity of *uso1* Δ *Csft1-11* and *sec22-3* (Figure 7C; our unpublished data). In contrast, *Coy1p* overexpression severely compromised the viability of the *gos1* Δ -2 strain at all temperatures tested. Although strains lacking *GOS1* grow slowly after sporulation, they eventually acquire near normal growth rates, suggesting some process of adaptation (McNew *et al.*, 1998; Niedenthal *et al.*, 1999). Nonetheless, overexpression of *Coy1p* slowed growth of cells lacking *Gos1p* but had no effect on the wild type, consistent with the removal of *Coy1p* improving growth in the absence of the SNARE (Figure 7D).

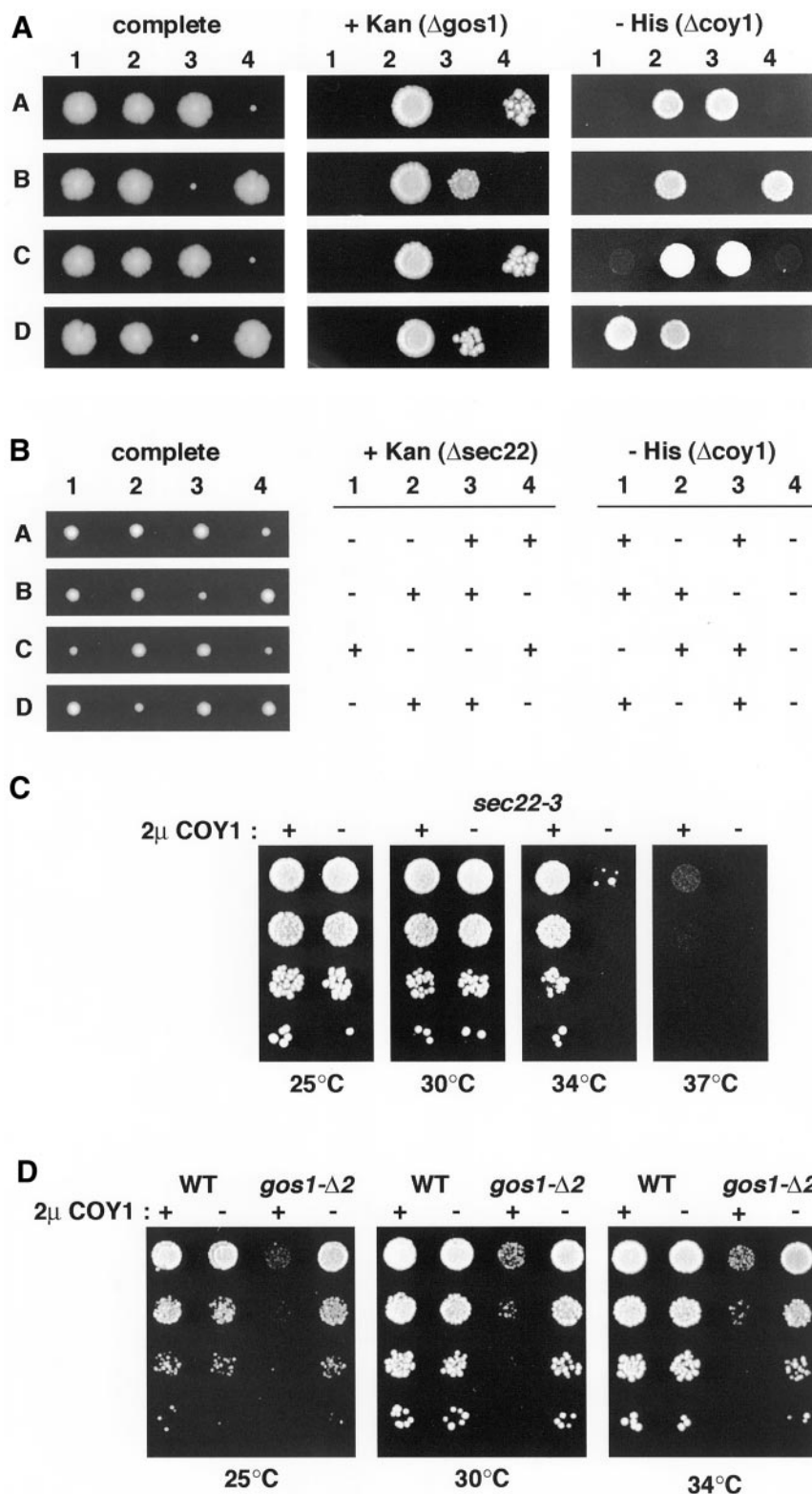


Figure 7. Genetic interactions between COY1/YKL179c and the SNARE-encoding genes GOS1 and SEC22. (A) Tetrad analysis of spores from a *gos1 Δ ::kanMx/GOS1*, *ykl179c Δ ::HIS5/YKL179c* diploid strain. Tetrads were dissected and incubated on YEPD plates at 30°C for 3 d (complete), and markers were then tested by replica plating on kanamycin (+ Kan) and minus-histidine plates (- His). Four representative tetrads are shown. The small segregants in each tetrad are Kan^{res}, but segregants that are both Kan^{res} and HIS⁺ do not display any growth or germination defect. (B) Tetrad analysis as in A of spores from a *sec22 Δ ::kanMx/SEC22*, *ykl179c Δ ::HIS5/YKL179c* diploid strain (AGY03). Segregants that contained *sec22 Δ ::kanMx* alone showed a small but reproducible growth defect, which was not seen in those that also contained *ykl179c Δ ::HIS5*. (C) Growth at the indicated temperatures of the temperature-sensitive *sec22-3* strain containing the 2μ plasmid pRS426 with (+) or without (-) full-length Coy1p expressed from a constitutive *PHO5* promoter. Ten-fold serial dilutions of cells were spotted onto selective plates and incubated at the indicated temperatures for 3 d. (D) Growth of parental strain SEY6210 (WT) or *gos1- Δ 2* containing 2μ plasmids as in C.

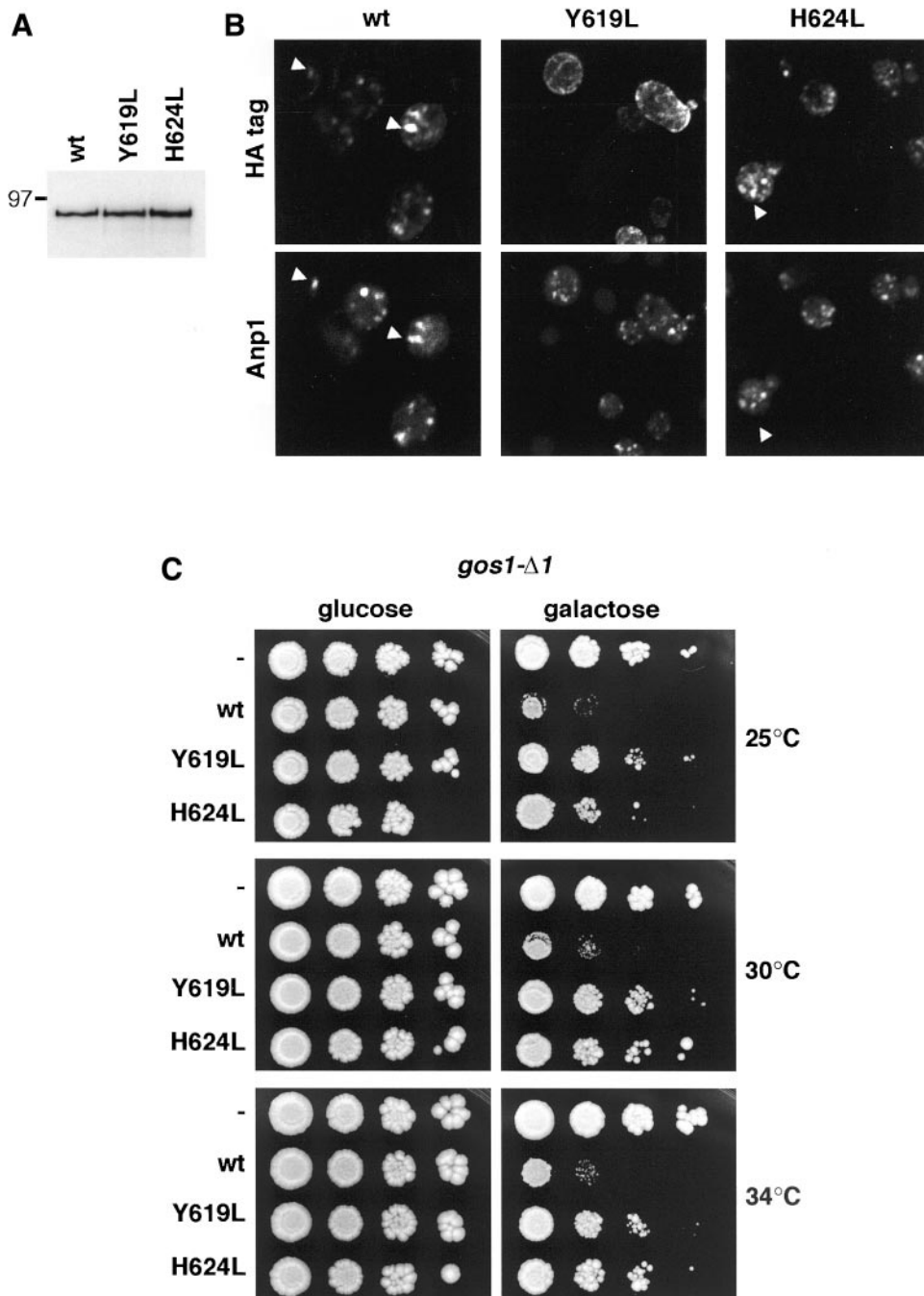


Figure 8. Role of the conserved TMD residues in the Coy1p function. (A) Anti-HA protein blots of total protein from yeast expressing HA-tagged Coy1p (wt) or the same protein with the indicated TMD mutations. Proteins were expressed in strain *gos1-Δ1* from CEN plasmids under the control of the galactose-regulated *GAL1* promoter and induced with 2% galactose for 3 h at 30°C. (B) Confocal micrographs of yeast as in A. After induction as in B, cells were fixed, permeabilized, and stained with antibodies against the HA epitope and the Golgi marker Anp1p. (C) Effect of TMD mutants on the ability of Coy1p to perturb growth of the *gos1-Δ1* strain. Cells were transformed with plasmids as in A but expressing Coy1p (wt) without an epitope tag, or the same protein with the indicated TMD mutants, or no gene (-). Yeast were grown overnight in media containing 2% raffinose, spotted onto selective plates containing either 2% glucose or 2% galactose, and incubated at the indicated temperatures for 2–3 d.

Conserved TMD Residues in Coy1p Are Important for Function

The slow growth phenotype induced by Coy1p when over-expressed in cells lacking the Golgi SNARE Gos1p provides an indirect assay for at least some aspect of Coy1p function. This assay was used to investigate the importance of the tyrosine and histidine residues conserved in the TMD. Versions of full-length Coy1p with either Y619 or H624 mutated to leucine were expressed from a low copy centromeric

plasmid under the control of the galactose-inducible *GAL1* promoter. We initially compared the levels and localization of wild-type and mutant proteins that were tagged at the C terminus with three copies of the HA epitope. Figure 8A shows that the mutations had no effect on the level of Coy1p that accumulated after 2 h of galactose induction. Immunofluorescence localization of the induced proteins showed that both wild type and Coy1p(H624L) were in punctate structures that colocalized with the Golgi marker Anp1p

(Figure 8B). In contrast, Coy1p(Y619L) showed nuclear envelope and peripheral staining characteristic of the ER, consistent with the localization of human CASP carrying the equivalent mutation. The activity of the mutant proteins was then assayed in a *gos1* Δ strain, by comparing growth on glucose and galactose plates. When Coy1p expression was suppressed by glucose, the *gos1* Δ strain grew well at all temperatures tested, whereas induction of wild-type Coy1p on galactose decreased viability as expected (Figure 8C). In contrast, induction of either Coy1p(Y619L) or Coy1p(H624L) only slightly inhibited growth (Figure 8C). These results indicate that the TMD of Coy1p is important for both efficient exit of the protein from the ER, and for the function of the protein because mutation of the conserved histidine affects activity without any apparent effect on localization. This suggests that the TMD of Coy1p, and hence its human homologue, serves a role in the function of the protein beyond that of providing an attachment to the lipid bilayer.

DISCUSSION

The results presented herein show that the two proteins produced by the human CUTL1 locus have strikingly different locations: a nuclear transcription factor CDP and a Golgi-localized membrane protein CASP. The choice between the two alternative transcripts encoding these proteins seems to be controlled by use of a site for transcript termination and poly(A) addition (Lievens *et al.*, 1997; Rong Zeng *et al.*, 2000; Figure 1D). If the termination site is used then the transcript is spliced to produce CDP, but if it is read through then the transcript includes further exons, which results in an alternative splice and the production of CASP. We have confirmed that this unusual gene structure is conserved in the nematode *C. elegans*, suggesting that it has persisted for a long period of evolution and raising the question of whether it has any functional significance. However, we have been unable to find any evidence that the region shared between CDP and CASP results in targeting of the former to the Golgi. When CASP was originally described it was reported to associate with CDP when the two proteins were translated together in vitro (Lievens *et al.*, 1997). However, the authors stated that they did not observe any such association when the proteins were expressed in transfected cells. This would agree with our observations and suggests that mechanisms may exist in vivo to ensure that homodimeric coiled-coil domains only associate with translation products from the same polysome.

An origin for this unusual gene structure is suggested by the fact that CASP seems more evolutionarily ancient than CDP because only the former is present in plants and fungi. Moreover, both the C-terminal TMD and the N-terminal region of the protein are conserved between all CASP homologues. Thus, it is possible that during evolution the CDP DNA-binding domains were rearranged into a CASP intron, allowing the coiled-coil region to be shared by the two proteins. The resulting dimerization of CDP may have proved valuable and difficult to lose during evolution. Interestingly, the golgins GMAP-210 and golgin-84 have both been found to participate in oncogenic rearrangements that attach their N-terminal mostly coiled-coil regions to the tyrosine kinase domains of the platelet-derived growth factor receptor β and the Ret oncogene, respectively (Abe *et al.*, 1997; Klugbauer *et al.*, 1998). This pre-

sumably results in constitutive dimerization and altered activity of the tyrosine kinase domains, although in these cases the imposed dimerization is not of any evolutionary benefit.

Irrespective of the evolutionary history of CDP, it is clear that CASP has a function on the Golgi that is independent of CDP. This function seems likely to be related to that of giantin and golgin-84 because the proteins share an overall structure and conserved TMD residues. Indeed, all the eukaryotes shown in Figure 1C have at least one member of this trio, although none of the three is universal. Thus, in *Drosophila*, the *cut* locus does not seem to produce a homologue of CASP (although the N terminus of the cut protein has short regions predicted to form coiled-coils) but there is a homologue of golgin-84. In contrast, *C. elegans* has CASP, but no obvious golgin-84, whereas plants have both (Figure 1C). This suggests that the proteins may have been able to substitute for one another when lost during evolution. Only CASP shares an N-terminal domain with yeast Coy1p, but even so it cannot be assumed that the two proteins are precise functional homologues. Because mammals also have giantin and golgin-84, it is possible that yeast Coy1p additionally performs functions associated with these other proteins. Nonetheless, the results presented herein suggest that the TMD residues shared by the proteins serve in both efficient ER exit and in Golgi function, and indeed the tyrosine and histidine residues examined are predicted to be on opposite sides of an α -helix. Efficient ER exit may be important to prevent the protein forming interactions with other Golgi components before it is correctly localized.

What then can be said of the function of CASP? The lack of a growth phenotype when *COY1* is deleted from yeast indicates that the protein is not essential for anterograde transport through the Golgi. However, a similar situation is found with the yeast homologue of GRASP65 and with the Golgi coiled-coil proteins Imh1p and Rud3p (Tsukada *et al.*, 1999; VanRheenen *et al.*, 1999; Winzeler *et al.*, 1999). Thus, it may be that under normal growth conditions there is redundancy between vesicle tethers or between transport routes in the Golgi. Indeed, a number of nonessential Golgi proteins show genetic interactions with known trafficking proteins. For example, Imh1p shows synthetic lethality with the yeast rab GTPase Ypt6, or its exchange factors (Tsukada and Gallwitz, 1996; Siniouoglou *et al.*, 2000). In this article, we find that *COY1* shows genetic interactions that suggest a role in membrane traffic. In particular *COY1* shows a striking interaction with the gene encoding the Golgi SNARE Gos1p, in that deletion of *COY1* restores normal growth to *gos1* Δ cells, whereas overexpression has the opposite effect. This SNARE is well conserved in evolution with a homologue in mammals called GOS-28 or GS28 (Nagahama *et al.*, 1996; Subramaniam *et al.*, 1996; McNew *et al.*, 1998). Antibodies to GOS-28 inhibit in vitro assays for both intra-Golgi transport and ER-to-Golgi transport, although the latter effect could be an indirect consequence of perturbed intra-Golgi traffic. In both yeast and mammals the protein has been found to form complexes with the Golgi t-SNARE Sed5p (syntaxin-5) (Hay *et al.*, 1997; McNew *et al.*, 1998). This syntaxin plays an essential role in ER-to-Golgi and intra-Golgi transport, and participates in the formation of at least two SNARE complexes, and possibly several (Banfield *et al.*, 1995; Tsui *et al.*, 2001). The individual components of these complexes do not all have the same distribution. Thus, in mammalian cells GOS-28 is found through-

out the Golgi stack, whereas other syntaxin-5-interacting SNAREs such as rBet1 are concentrated toward the *cis*-side of the Golgi (Hay *et al.*, 1998; Orci *et al.*, 2000).

The results reported herein show that Coy1p has a deleterious effect on cells lacking Gos1p, which might suggest that the protein has a negative rather than positive role in transport. However, this effect does not preclude a positive role for Coy1p such as the tethering of vesicles, as proposed for other coiled-coil proteins. Vesicle tethering is likely to be beneficial only if it is productive. If a component required downstream of tethering is missing then the posttethering steps might be slowed and proteins such as SNAREs become sequestered, with the result that transport elsewhere in the Golgi would be compromised. Thus, if Coy1p and Gos1p act in the same transport step in intra-Golgi traffic then removal of Coy1p in a *gos1Δ* strain could reduce the tethering of vesicles that are dependent on Gos1p, and so release frustrated vesicles or sequestered trafficking components to function elsewhere in the Golgi in steps that are independent of Gos1p. It is at least unlikely that the toxicity of deleting *GOS1* reflects Gos1p normally masking some toxic part of Coy1p, because increasing the levels of Coy1p is not detrimental to wild-type cells.

Our results also suggest that the function of CASP, and by implication those of giantin and golgin-84, involves a specific interaction with residues in the TMD. This is perhaps unexpected because all the functional evidence so far for giantin and other long coiled-coil proteins of the Golgi has shown that they are involved in attaching vesicles or soluble proteins to the Golgi (Nakamura *et al.*, 1997; Cao *et al.*, 1998; Linstedt *et al.*, 2000). Functional residues in the TMD suggest an additional interaction within the lipid bilayer. So far, we have not found any other membrane protein associating stoichiometrically with either rat CASP or yeast Coy1p. A small fraction of the former protein coprecipitated with hSec23, and we have found a similar interaction in yeast (our unpublished data), which may reflect an interaction with the COPII coat required for ER exit, as was recently reported for another Golgi membrane protein Sys1p (Votsmeier and Gallwitz, 2001). Rat CASP also associated with a small amount of golgin-84, but because this protein is not present in yeast this interaction cannot be obligatory, and so its significance is unclear. It may be that a stoichiometric interaction with another membrane protein was destabilized by detergent solubilization, or alternatively any interactions may be transient. Such an interaction could serve to mediate communication between the tethering process and events in the cisternal bilayer. What is clear is that the TMD of CASP, and presumably those of giantin and golgin-84, is doing more than simply providing an anchor to the Golgi membrane. The identification of a member of this family in yeast now provides the opportunity to use genetic approaches to identify the components that interact with this class of proteins.

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