

Localization of Sed5, a Putative Vesicle Targeting Molecule, to the *cis*-Golgi Network Involves Both Its Transmembrane and Cytoplasmic Domains

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Abstract. The yeast Sed5 protein, which is required for vesicular transport between ER and Golgi complex, is a membrane protein of the syntaxin family. These proteins are thought to provide the specific targets that are recognized by transport vesicles. We have investigated the mechanism by which Sed5 protein is itself localized. Expression of epitope-tagged versions of the yeast, *Drosophila* and rat Sed5 homologues in COS cells results in a perinuclear distribution; immuno-EM reveals that the majority of the protein is in a tubulo-vesicular compartment on the *cis* side of the Golgi apparatus. A similar distribution was obtained with a chimeric molecule consisting of a

plasma membrane syntaxin with the *Drosophila* Sed5 transmembrane domain. This indicates that the membrane-spanning domain contains targeting information, as is the case with resident Golgi enzymes. However, alterations to the transmembrane domain of *Drosophila* Sed5 itself did not result in its mistargeting, implying that an additional targeting mechanism exists which involves only the cytoplasmic part of the protein. This was confirmed by modifying the transmembrane domain of the yeast Sed5 protein: substitution with the corresponding region from the Ssol protein (a plasma membrane syntaxin homologue) did not affect yeast Sed5 function in vivo.

TRANSPORT of proteins along the secretory pathway of eucaryotic cells involves several steps at which transport vesicles bud from one compartment and fuse specifically with the next. The specificity of these fusion events is thought to be determined by integral membrane proteins on the vesicles and organelles, and candidates for these targeting molecules have recently been identified (Sollner et al., 1993b; Bennett and Scheller, 1993; Südhof et al., 1993; Hardwick and Pelham, 1992; Aalto et al., 1993). On the organellar membranes, several proteins with similar sequences are found: in yeast, these comprise the *SED5* gene product, required for transport between ER and Golgi complex; the *SSO1* and *SSO2* products, required for vesicle fusion with the plasma membrane; and the *PEP12* product, which is involved in the transport of soluble proteases to the vacuole (Hardwick and Pelham, 1992; Aalto et al., 1993; K. Becherer and E. Jones, Carnegie Mellon University, Pittsburgh, PA; GenBank entry YSCPEP12P). The corresponding proteins in animal cells comprise the syntaxin family; syntaxins were originally identified on synaptic plasma membranes, but homologues have since been found in non-

neuronal cells (Bennett et al., 1993). Syntaxin 5 is the homologue of the yeast Sed5 protein and is found in the Golgi complex; all the other syntaxins identified so far are plasma membrane proteins.

The neuronal syntaxins (1A and 1B) have been shown to form complexes that contain SNAP-25 (another plasma membrane-associated protein) and the synaptobrevins (also known as VAMPs), which are found on synaptic vesicles. The soluble fusion-promoting proteins α SNAP and NSF bind to this complex, and in the presence of ATP disrupt it (Sollner et al., 1993a). This data, together with the observation that various neurotoxins that block synaptic vesicle fusion cleave specifically either synaptobrevin, syntaxin, or SNAP-25 (reviewed by Südhof et al., 1993), provides strong evidence that the syntaxin family members are important components of the fusion machinery. Proteins with some similarity to synaptobrevins (the products of the *BET1*, *BOS1*, and *SEC22* genes) are also found on ER-derived transport vesicles in yeast, and are required for these vesicles to fuse with the Golgi complex (Newman et al., 1990; Lian and Ferro-Novick, 1993); they are presumed to interact with the Sed5 protein, although this remains to be proven.

The syntaxin family members are the only integral proteins of the acceptor membranes that are known to be required for vesicle fusion. As such, they are the most plausible candidates for the molecules that define the target for the

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incoming vesicles. It is clearly of great importance that any such proteins are themselves maintained in the correct location, or the organization of the endomembrane system would break down. For the plasma membrane syntaxins, this may not be much of a mechanistic problem, since plasma membrane proteins are thought to require no specific sorting signals to remain in place. However, an efficient mechanism must exist to retain Sed5 (and its homologue syntaxin 5) in the Golgi complex.

The targeting of other Golgi proteins has already been studied. In particular, retention signals for several of the enzymes of the *medial*- and *trans*-Golgi complex, which are all type II membrane proteins, have been mapped to their transmembrane domains (reviewed by Machamer, 1993). In the case of the *trans*-Golgi enzymes, it has been shown that the precise sequence of these domains is not important, but efficient retention requires a relatively short hydrophobic stretch (Munro, 1991; Masibay et al., 1993). How retention of such proteins is achieved is unknown, but the mechanism may depend on the differences in lipid composition and bilayer dimensions that exist between pre- and post-Golgi membranes (Bretscher and Munro, 1993). Since Sed5 proteins are also type II proteins and have short transmembrane (TM) domains, a simple hypothesis is that this domain specifies the location of the protein and hence, by defining the destination of ER-derived transport vesicles, of the Golgi apparatus.

We have tested this idea by expressing derivatives of Sed5 and syntaxin 2 with altered transmembrane domains in COS cells and yeast. In COS cells yeast, *Drosophila* and rat Sed5 homologues appear normally to be restricted to the *cis*-Golgi network (CGN). In chimeric constructs, the *Drosophila* Sed5 TM domain is sufficient to prevent the accumulation of syntaxin 2 on the plasma membrane, although it does not restrict the protein exclusively to the CGN. However, alteration of the Sed5 TM domain does not prevent the correct localization of *Drosophila* Sed5 in COS cells, nor abolish the function of yeast Sed5 *in vivo*. We conclude that the location of Sed5 is only partially determined by its TM domain, and thus that an additional localization mechanism must exist.

Materials and Methods

Plasmids for Mammalian Cell Studies

The mouse epimorphin (syntaxin 2) cDNA clone was obtained from Y. Hirai and M. Takeichi (Kyoto University, Kyoto, Japan) (Hirai et al., 1992) and used as the basis of the plasmids containing syntaxin 2 sequences. A preliminary sequence of *Drosophila* SED5 was provided by I. Dawson (Yale University, New Haven, CT) and S. Roth (Princeton University, Princeton, NJ), and used to design primers for PCR amplification of a full-length clone from *Drosophila* embryonic cDNA (kindly provided by S. Ner, MRC Laboratory for Molecular Biology). Full-length wild type clones were amplified for 25 cycles using "Vent" Polymerase (New England Biolabs, Beverly, MA) in the presence of 2 mM Mg²⁺ and 1 mM dNTPs, and using suitable restriction sites encoded by the primers, cloned into the COS cell expression vector G26 (gift of S. Munro, MRC Laboratory for Molecular Biology). This vector has an SV-40 origin of replication and an expression cassette driven by the adenovirus major late promoter. cDNAs and all fusion constructs were cloned into this vector between the EcoRI and XbaI sites, downstream of sequences encoding the amino acids MEQKLISEEDLNS, which include a 10-amino acid epitope derived from the human *c-myc* pro-

tein and recognized by the monoclonal antibody 9E10 (Munro and Pelham, 1987). All constructs generated by PCR were verified using commercially available "Sequenase" kits (United States Biochemical, Cleveland, OH) and synthetic primers derived from the sequences.

The fusion points for the chimeric COS cell constructs are shown in Fig. 7. Reciprocal fusions of the mouse syntaxin 2 and rat syntaxin 1B transmembrane domains to dSed5, and dSed5 transmembrane domain to syntaxin 2, were made initially by PCR amplification using oligonucleotides encoding the heterologous transmembrane domains (constructs 2, 5, 6, and 7 in Fig. 7). In the case of construct 6, all clones that were amplifiable in *Escherichia coli* contained mutations at the fusion junction and this one was selected as being relatively conservative. Additional fusions near the transmembrane domains were constructed by first introducing KpnI sites into the dSed5 coding region by PCR (changing amino acids 279-281 from KYF to RYL) and into the syntaxin 2 sequence (changing amino acid number 256 from K to R). This site was used as the junction point for constructs 3 and 8; for construct 9 a PCR primer that included a KpnI site was used to make an altered version of dSed5, and the syntaxin 2 terminus added by ligation to the KpnI site.

Fusions at the end of the putative coiled-coil region were made by joining the PCR-generated fragments delineated in constructs 11 and 12 using an EcoRV site encoded by the primers at the junction. The deletions of dSed5 were generated by fusion of the MscI sites at nucleotides 202 and 636 in the coding region (construct 13), or by the fusion of the site at position 636 to the filled in EcoRI site at the junction with the vector (construct 14). The deletion mutant chimera (construct 15) was made by replacing the EcoRI-BglII fragment in construct number 6 with that from number 14.

The CD8 construct used as a cell surface marker consisted of the extracellular domain of CD8 fused at residue 162 to the transmembrane domain of rat dipeptidyl peptidase IV, followed by the sequence KRLK (Chapman and Munro, 1994; a kind gift of S. Munro).

The ySed5 coding region was cloned into the COS cell expression vector G26 following PCR amplification with oligonucleotide primers containing suitable restriction sites. As with dSed5 the ySed5 construct was also tagged at the NH₂ terminus with the *c-myc* epitope (see above). The fusion points for the chimeric ySed5 constructs are shown in Figure 9 (see below for description). Chimeric ySed5 constructs were cloned into G26 by replacing the MscI-BamHI fragment of ySed5 with the corresponding fragment from the chimera.

Plasmids for Yeast Cell Studies

All plasmids tested in yeast were derived from an EcoRI-BamHI PCR-generated fragment corresponding to the yeast *SED5* coding region (Hardwick and Pelham, 1992). This region was cloned into the yeast plasmid vector pRS314 (Sikorski and Hieter, 1989) in front of the TPI promoter. The fusion points for the *SED5* chimeras are shown in Table II. Constructs 17, 18, and 19 were prepared by site-directed mutagenesis (Kunkel et al., 1987) using the *SED5* coding region as template. Reciprocal fusions of the COOH termini were constructed by first introducing either a KpnI or HindIII site into the *SED5* coding region by site-directed mutagenesis. The introduction of the KpnI site changes amino acids 313-315 from DRI to RYQ and the introduction of the HindIII site changes amino acid 325 from V to L. The heterologous hydrophobic COOH termini were made by the PCR using oligonucleotide primers and yeast genomic DNA and joined to the *SED5* sequences via the HindIII or KpnI sites (KpnI constructs: 23, 25; HindIII constructs: 20, 21, 22, and 24). Construct 26 was generated by site-directed mutagenesis using construct 25 as the template for mutagenesis. Construct 27 was generated by site-directed mutagenesis using the *SED5* derivative containing the KpnI site as the template for mutagenesis. All constructs generated by the PCR or site-directed mutagenesis were verified by sequencing.

COS Cell Transfections and Immunofluorescence

These methods have been described previously (Munro and Pelham, 1987; Lewis and Pelham, 1992). Antibody staining was conducted with the mouse monoclonal antibody 9E10 (Evan et al., 1985), the rat monoclonal antibody 23C that recognizes the β subunit of the coatamer complex (a kind gift of K. Willison, Institute of Cancer Research, London; Harrison-Lavoie et al., 1993) and the rat monoclonal antibody Campath 8C (kind gift of G. Hale, University of Cambridge, Cambridge, UK; Bindon et al., 1989), using FITC-conjugated anti-rat Ig (Southern Biotechnologies Associates, Birmingham, AL) and Texas red-conjugated anti-mouse Ig (Amersham Corp., Arlington Heights, IL) as secondary reagents.

1. *Abbreviations used in this paper:* CGN, *cis*-Golgi network; PDI, protein disulphide isomerase; TM, transmembrane.

Electron Microscopy

Transfected COS cells were fixed after 48 h in 0.5% glutaraldehyde in 0.1 M Hepes, pH 7.4, and 0.24 M sorbitol by adding an equal volume of 2× fixative and leaving for 15 min before replacing this with 1× fixative and leaving for 1 h at room temperature. The cells were then rinsed in phosphate-buffered saline and, after scraping with a rubber policeman, were pelleted, and processed for cryoimmunology-EM as described (Rabouille et al., 1993). Small blocks of gelatine-embedded cells were infused overnight in 2.3 M sucrose and frozen in liquid nitrogen.

Ultrathin cryosections were cut and incubated for 30 min with 9E10, followed by a goat anti-mouse antibody conjugated to 10 nm gold particles as described by Nilsson et al. (1993). For double labeling, the sections were first incubated with mAb 1D3 which recognizes protein disulphide isomerase (Vaux et al., 1990; a gift from S. Fuller, EMBL, Heidelberg, Germany), followed by goat anti-mouse antibody coupled to 10 nm gold particles. Sections were then fixed in 3% paraformaldehyde for 15 min to stabilize the antibody complexes. After thorough rinsing, they were incubated with mAb 9E10, followed by goat anti-mouse conjugated to 15 nm gold particles. Single immunolabeling was also performed with 1D3 to check that the labeling was identical. Since each antibody gave the same unique pattern in both double and single label experiments, the possibility of cross-labeling due to incomplete blocking of the first antibody could be ruled out.

NRK cells were fixed and processed in a similar fashion except that the cells were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde for 3 h at room temperature. Cryosections were incubated with mAb 1D3 and anti-peptide antibodies to the syn5 sequence -DSYIQSRADTMQNI- for 30 min followed first by goat anti-rabbit antibody coupled to 10 nm gold particles and, second, goat anti-mouse antibody coupled to 5 nm gold particles. The signal to noise ratio in these experiments was 3:1.

After immunolabeling, ultrathin cryosections were stained with 2% neutral uranyl acetate and embedded in 2% methyl cellulose containing 0.4% uranyl acetate. Sections were examined under a CM10 Philips electron microscope.

Cross-linking of Transfected Cell Membranes

Transfected COS cells were trypsinized 48 h after transfection, and after washing in phosphate-buffered saline, were homogenized in 250 mM sucrose, 20 mM Hepes, pH 7.4, and 1 mM PMSF in a Wheaton dounce homogenizer and after a low-speed spin for 15 min at 7,000 rpm in a Beckmann TL100 centrifuge the supernatant was spun at 55,000 rpm for 20 min.

The resulting pellet was taken up in 50 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM PMSF, and dithiothreitol (succinimidylpropionate) (Pierce Chemical Co., Rockford, IL) added to the concentrations indicated from a 2.5 mg/ml stock in dimethylformamide. The incubation was continued on ice for 20 min and 1/10th volume of 10 mM glycine, pH 7.4, added. After an additional 30 min on ice, an equal volume of 2× SDS sample buffer without reducing agent was added and the sample heated in a boiling waterbath prior to PAGE separation of the proteins.

Proteins were blotted onto nitrocellulose on a semi-dry blotting apparatus and blots probed in 2% nonfat milk/0.5% Tween 20 with mAb 9E10 and horseradish peroxidase-conjugated anti-mouse Ig (Sigma Chemical Co., St. Louis, MO), before detection with an ECL kit (Amersham Corp.).

Construction of Yeast Strains

The viability of yeast strains expressing SED5 mutants and chimera (see Table II) were determined in a yeast strain in which the only Sed5 protein was provided by a galactose inducible version of the yeast gene (Hardwick and Pelham, 1992).

A derivative of the yeast strain SEY6210 (*MAT α ura3-52 his3- Δ 200 leu2-3, -112 trp1- Δ 109 lys2-801 suc2- Δ 9*) expressing a *SED5/SSO1* chimera (construct 26) as its sole *SED5* gene product was prepared via a one step integration. The plasmid used for integration was constructed by subcloning the *MscI*-*Bam*HI fragment of construct 24 (see Table I) into a version of the integration vector pRS306 (Sikorski and Hieter, 1989) from which the *Sac*II site had been removed. To facilitate integration of the *SED5-SSO1* gene fragment at the *SED5* locus the plasmid was linearized using the unique *Sac*II site within the *SED5* coding region. Integration of the plasmid results in a chromosomal structure consisting of the *SED5-SSO1* chimera, followed by the plasmid sequences (including the marker *URA3*), and then by a truncated 3' terminal portion of the original *SED5* gene. After transformation, yeast cells were plated on selective media and incubated at 25°C. After 2–3 d individual colonies were picked and correct integration confirmed by PCR and Southern blot analysis.

Results

Conserved Features of the Sed5 Protein

The *SED5* gene was originally identified in yeast, but detailed investigation of protein localization is difficult in this organism, due to its small size and poorly defined Golgi morphology. We therefore sought homologues of the gene in higher eucaryotes. By chance, a *Drosophila* homologue was isolated by I. Dawson and S. Roth, who provided us with a preliminary sequence. Using appropriate primers for PCR, we generated a cDNA clone and completed the sequence. Subsequently, a rat homologue was isolated by Bennett et al. (1993), using the *Drosophila* clone for cross-hybridization; database searches revealed a further partial sequence of an intron-containing *SED5*-like gene from *Pneumocystis carinii*, adjacent to the folic acid synthase gene (Volpe et al., 1992).

The aligned amino acid sequences of the various Sed5 homologues are shown in Fig. 1. The *Drosophila* and rat proteins are quite similar (56% identity), both being more distantly related to the yeast sequence (31 and 30% identity for the rat and *Drosophila* proteins, respectively). There are four conserved regions: the long coiled-coil motif close to the COOH terminus, two other stretches containing heptad repeats, and a short sequence close to the NH₂ terminus.

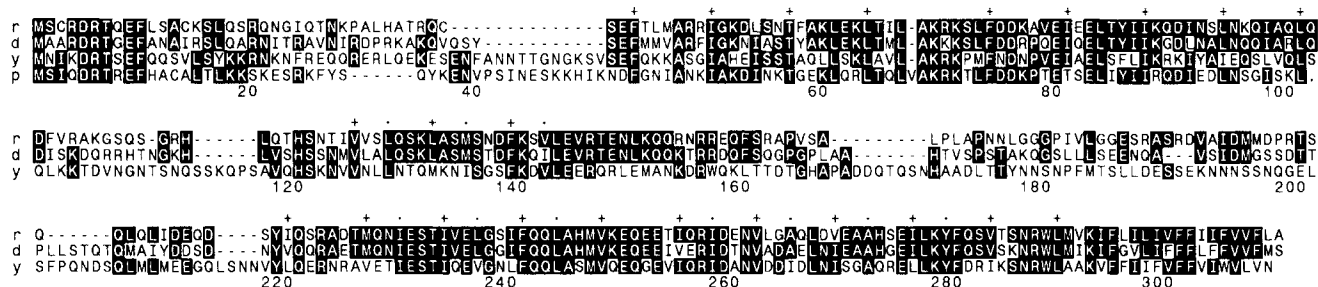


Figure 1. Alignment of the *S. cerevisiae* Sed5 sequence with that of its homologues from *Drosophila*, rat and *Pneumocystis*. Identical residues are highlighted, while plus signs and dots indicate the positions of hydrophobic residues in the coiled-coil motifs. Numbers refer to the *Drosophila* sequence. The cDNA sequence of *Drosophila* Sed5 has been submitted to the EMBL/Genbank databases under accession number X78219.

Protein	C-terminal sequence	TM domain	
		Length	%Phe
syn1A	KAVKYQSKARRKKIMIIICCVILGIIIASTIGGIFG	23	4
syn1B	KAVKYQSKARRKKIMIIICCVLGVVLASSIGGTLGL	24	0
syn2	KAIKYQSKARRKKWIAAVVVAVIAVLALIIIGLTVGK	23	0
syn2'	KAIKYQSKARRKVMFLICVVTLVILGIIILATLS	23	4
syn3	RAMKYQGQARKKLIIVIVVVLLGILALIIIGLSVGLK	25	0
syn4	IALENQKARKKKVMTAICVSVTVLILAVIIGITITVG	25	0
Sso1	KAVKSARKARRNKIRCLIVFAIIVVVVVVVPAVVKTR	22	5
Sso2	KAVKSARKARRNKIRCLIVFAIIVVVVVVVPSVETRK	22	9
Pep12	KAMRYQKRTSRWRVYLLIVLLVMLLFIFLIMKL	18	11
Sed5	KYFDRIKSNRWLAAKVFFIIFVFFVIVVLVN	15	33
dSed5	KYFQSVKSNRWLMIKIFGVLIFFFLFFVVFMS	17	41
syn5	KYFQSVTSNRWLMVKIFLILIVFFIIFVVFPLA	17	29

Figure 2. Comparison of the COOH-terminal sequences of syntaxin family members. The syntaxins (*syn*) are of rat origin, Sso1, Sso2, Pep12, and Sed5 are from yeast, and dSed5 is the *Drosophila* homologue. Syn1-4 and the Sso proteins are implicated in fusion to the plasma membrane, Pep12 is required for transport to the vacuole and Sed5 for transport from ER to Golgi complex.

Similar sequence features are found in the plasma membrane syntaxins, and they probably represent the structural core of this protein family. Of particular relevance to the present study are the COOH-terminal hydrophobic membrane anchors. These diverge in sequence, but their overall characteristics are preserved; compared to the plasma membrane-associated syntaxins, they are consistently shorter and much richer in phenylalanine (Fig. 2). In these respects they are similar to the TM domains of Golgi enzymes (Bretscher and Munro, 1993), although their Phe content is unusually high.

Formation of Sed5 Dimers in COS Cells

We wished to test the possible role of the TM domain in protein targeting. As a first step, we expressed in COS cells a version of the *Drosophila* Sed5 protein (dSed5) that was tagged at the NH₂ terminus with a *c-myc* epitope, and characterized the product. Immunoblotting of transfected cell membranes with an anti-*myc* antibody revealed a single protein with the expected size of about 40 kD (Fig. 3 A), together with trace amounts of a species that was about twice as large. Chemical cross-linking resulted in a greatly increased yield of this apparent dimer, together with traces of larger complexes (Fig. 3 A). Similar results were obtained when cross-linking was performed with intact membranes or after detergent solubilization, implying that the complexes are stable in solution. Confirmation that homodimer formation was occurring was provided by cross-linking of a deletion mutant of dSed5 that lacked most of the NH₂-terminal half of the protein (residues 69-212 deleted) but retained the COOH-terminal heptad repeats. This mutant produced a protein of about 20 kD, which could be cross-linked to form a 40-kD species (Fig. 3 B). We conclude that dSed5 forms membrane-associated dimers (and possibly larger multimers), and that dimer formation is likely to be mediated by the COOH-terminal coiled-coil region.

Distribution of Sed5 Proteins

The distribution of various syntaxin family members was examined by immunofluorescent staining of transfected COS cells. *Drosophila* Sed5 had a perinuclear distribution, and double labeling with a monoclonal antibody that recognizes

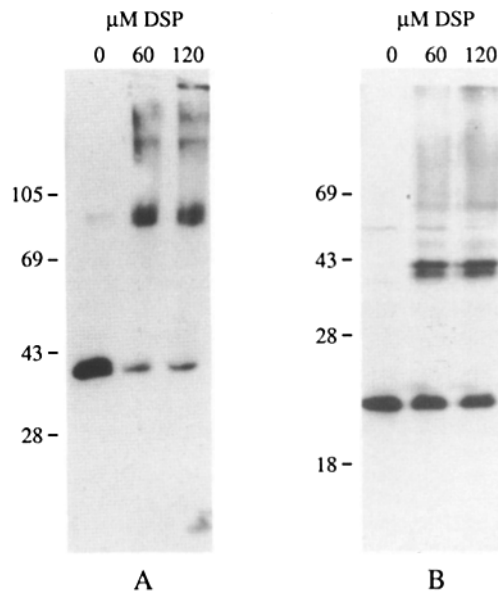


Figure 3. Formation of Sed5 multimers. Membranes from COS cells expressing NH₂-terminally tagged dSed5 (A), or an internal deletion mutant lacking residues 69-212 (B), were treated with the cross-linker DSP or not as indicated, and then analyzed by immunoblotting under nonreducing conditions. The positions and sizes (in kD) of molecular weight markers are indicated.

the β' COP showed that this pattern corresponded to the Golgi complex (Fig. 4, A and B). Similar results were obtained with the rat Sed5 homologue (syntaxin 5; data not shown), as has previously been reported by Bennett et al. (1993). For comparison, we also expressed one of the plasma membrane syntaxins. The gene we used was originally termed epimorphin, and was of mouse origin (Hirai et al., 1992). However, the rat homologue of this has been named syntaxin 2 (Bennett et al., 1993), and for the sake of consistency we shall use the syntaxin nomenclature in this paper. As expected, NH₂-terminal tagged syntaxin 2 was found on the plasma membrane (Fig. 4 C; see also Bennett et al., 1993).

To examine the location of dSed5p more closely, we used immunogold labeling of frozen ultrathin sections. This showed that the protein was predominantly located in a disordered tubular-vesicular compartment adjacent to the nucleus and the Golgi stack. In cells expressing low levels of dSed5 (~10% of the transfected cells), Golgi stacks were visible but essentially unlabeled, the bulk of the labeling being in the reticular structures; an example is shown in Fig. 5 C. In cells with higher levels of expression Golgi stacks were absent, and the protein was restricted to the tubular-vesicular structures, which were particularly prominent (Fig. 5 A; Table I). Double labeling indicated that the structures containing dSed5p co-labeled with mAb 1D3 (see Fig. 5 B for an example); this antibody recognizes the ER enzyme protein disulphide isomerase (PDI; Vaux et al., 1990), which bears a KDEL signal and is likely to cycle through the intermediate compartment or CGN. In single label experiments 1D3 stained both the tubulo-vesicular structures and the cisternal ER. We conclude that dSed5 is located primarily in the CGN, at least under the conditions used in these experiments. Moreover, overexpression of dSed5 seems to cause proliferation of this compartment. NH₂-terminally

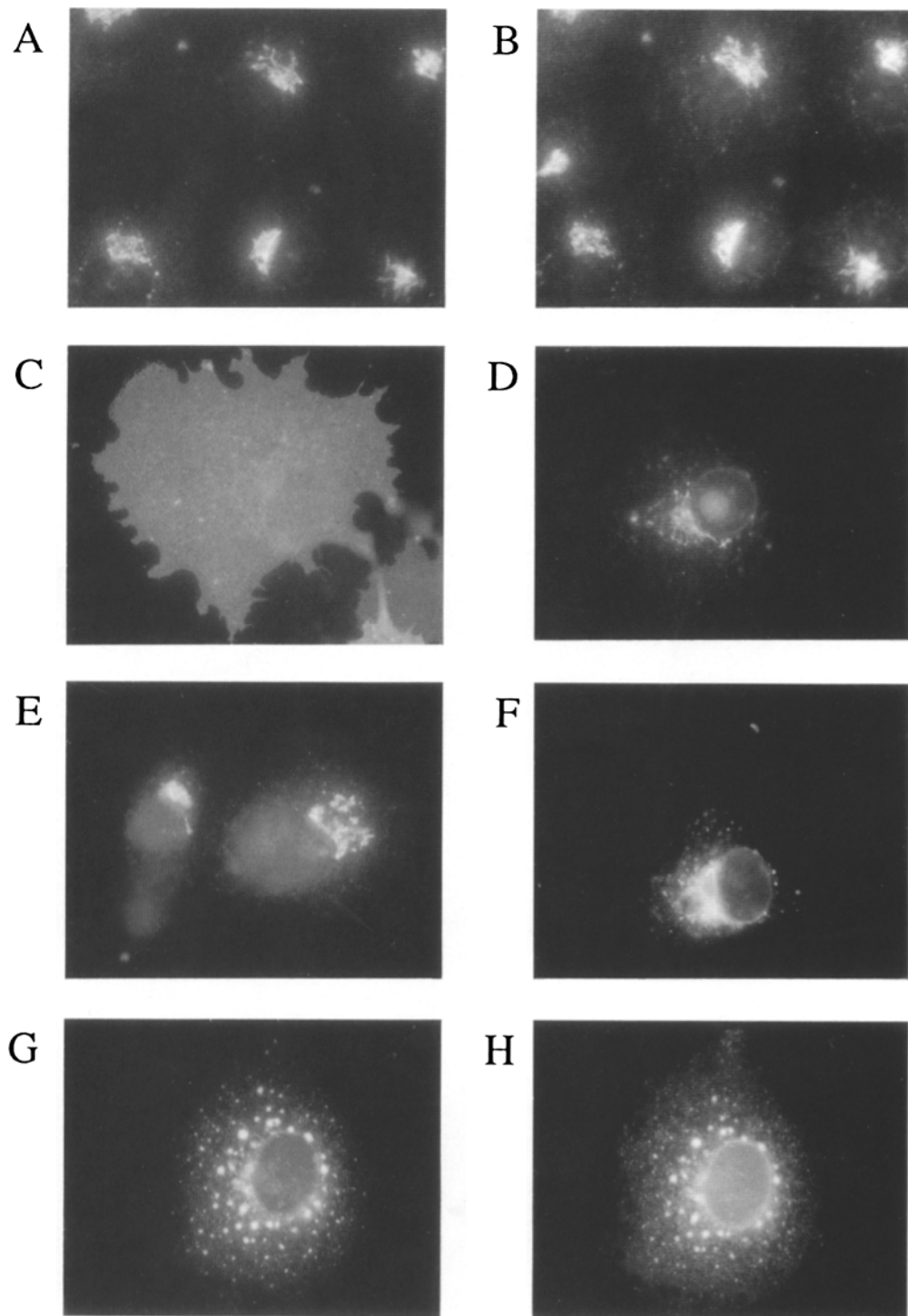


Figure 4. Intracellular distribution of chimeras in COS cells by immunofluorescence. Expressed proteins were detected with mAb 9E10, which recognizes the *myc* tag. (A) dSed5; (B) same field as A, stained for β' COP; (C) syntaxin 2; (D) syntaxin 2 with the dSed5 TM domain (construct 2 of Fig. 7); (E) dSed5 with the syntaxin 2 TM domain (construct 6); (F) dSed5 with syntaxin 1B TM domain (construct 5). G and H show the effects seen in cells expressing high levels of construct 6. This cell was co-transfected with a plasmid expressing a plasma membrane marker (a CD8 derivative); both construct 6 (G) and this marker (H) are present in punctate structures.

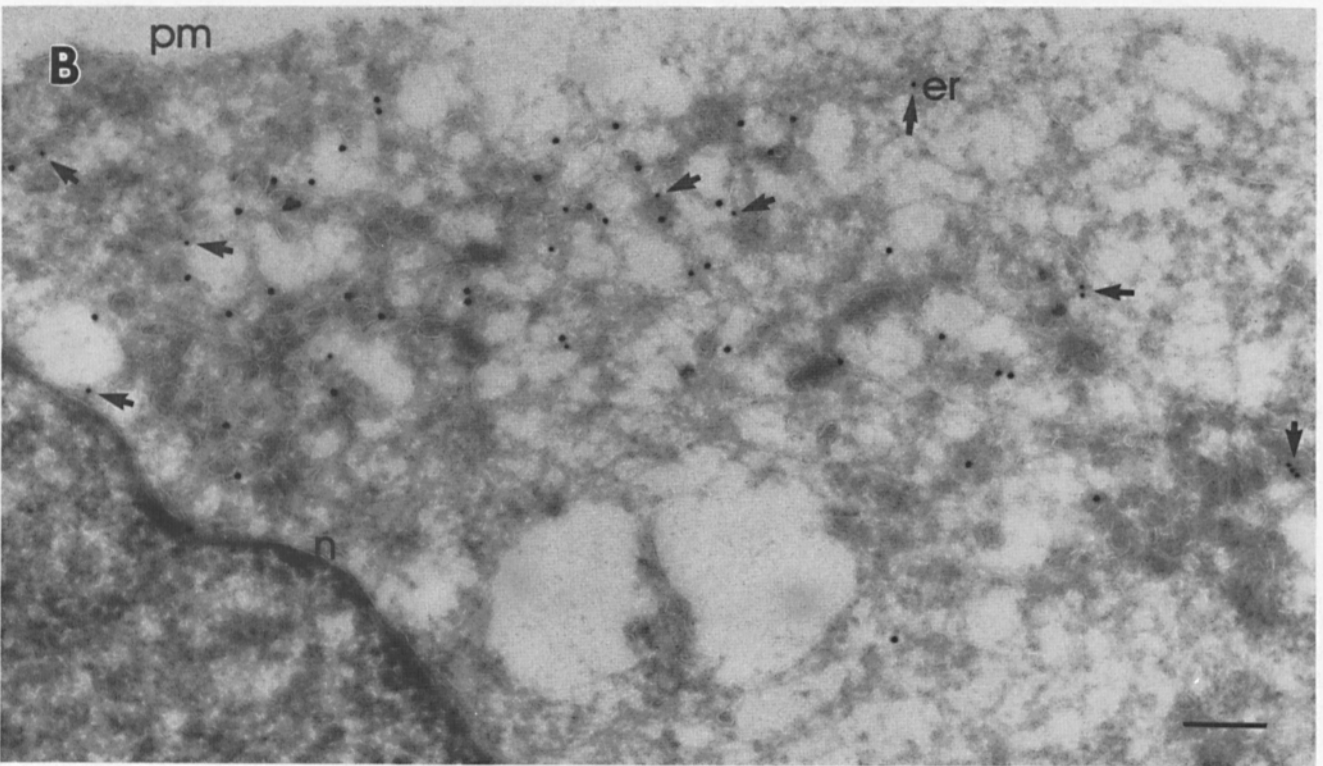
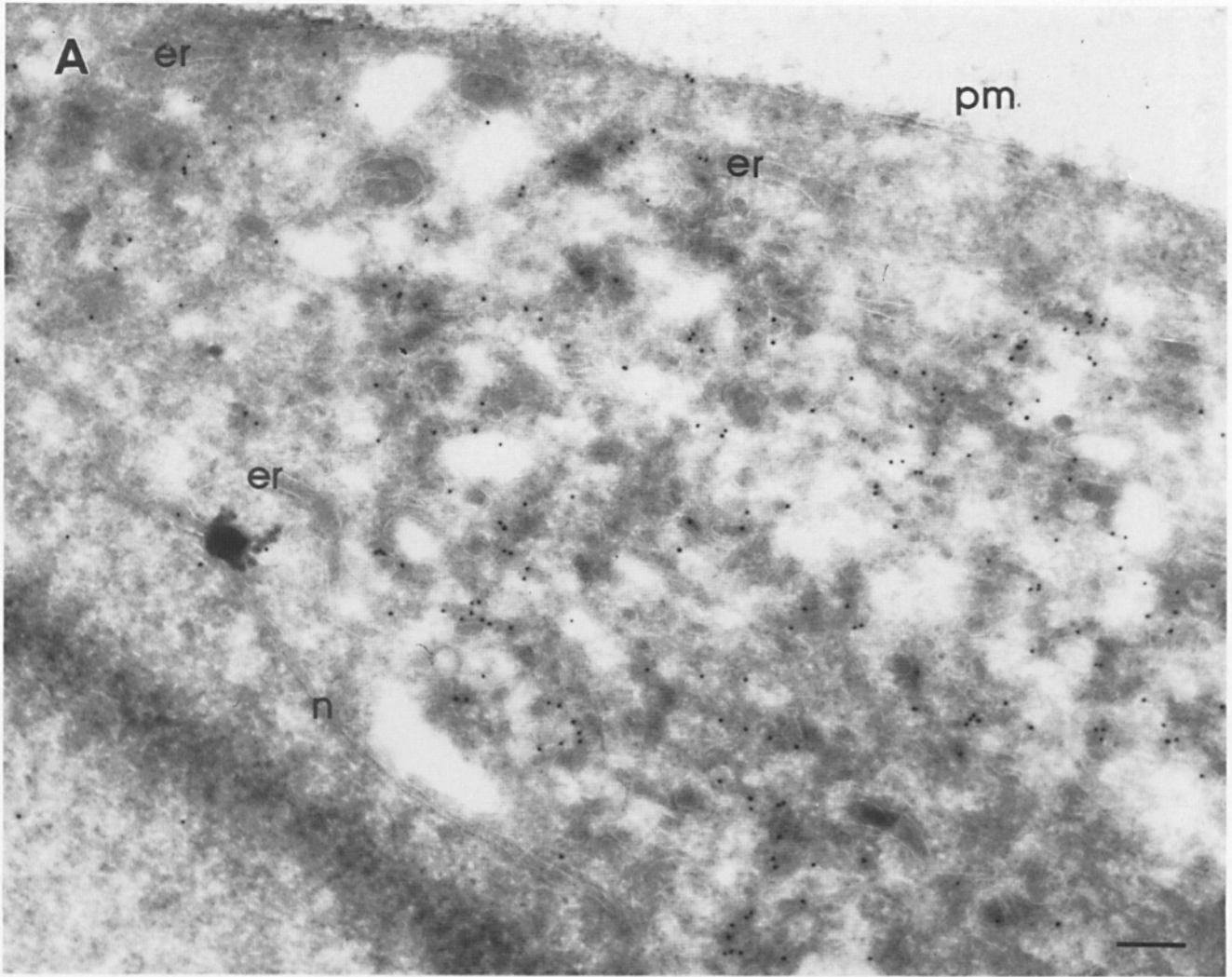
tagged rat syntaxin 5 showed a pattern indistinguishable from *Drosophila* Sed5 (not shown).

To verify that the observed distribution was not restricted to transiently expressed proteins in COS cells, we also labeled rat NRK cells with an affinity-purified antibody raised to a syntaxin 5 peptide. Staining was found in the Golgi region; most of the label was not associated with Golgi stacks, but with CGN-like structures that could also be labeled with mAb 1D3 (Fig. 6). This result confirms the conclusions from the COS cell experiments, and strongly suggests that syn-

taxin 5 is predominantly located in the CGN under normal conditions.

Distribution of dSed5 Chimeras Expressed in COS Cells

We next analyzed chimeric proteins in which the TM domains of dSed5 and a plasma membrane syntaxin were exchanged (see Fig. 7 for details of the sequence changes). When the dSed5 TM domain was attached to syntaxin 2, the protein was no longer found at the cell surface; immunofluorescent staining showed a perinuclear distribution together



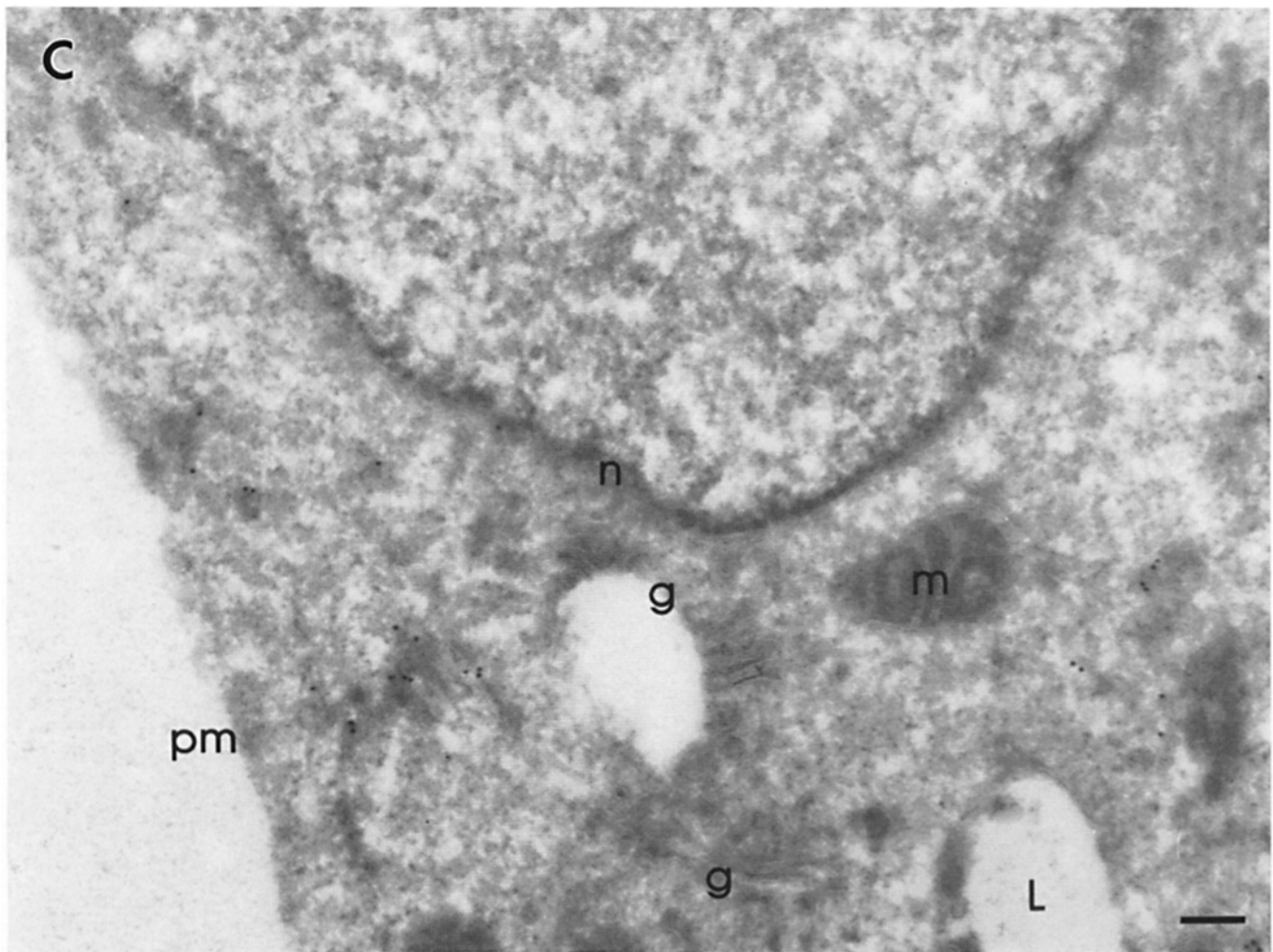


Figure 5.

with some more disperse punctate structures (Fig. 4 *D*). Immuno-EM revealed that the chimeric protein was distributed between the Golgi stacks and the adjacent tubulo-vesicular network, with a small portion being in endosomes (Fig. 8 *C* and *D*; Table I). In contrast to cells expressing dSed5 itself, the syn2/dSed5 chimera did not induce proliferation of the network nor disrupt the Golgi stacks. None of the protein was found on the plasma membrane. Inhibition of protein synthesis with cycloheximide for up to four hours did not noticeably affect this distribution, indicating that the Golgi-associated material was not merely in transit to a later compartment. Under similar conditions NH₂-terminally tagged syntaxin 2 was found almost entirely on the plasma membrane and in endosomes, with only about 1% of the protein being in Golgi stacks (Fig. 8 *A*; Table I).

We conclude from these experiments that the dSed5 TM domain can act as a Golgi-targeting signal when transferred to another protein, a property that is consistent with the short, phenylalanine-rich nature of this domain. On the other hand, this sequence does not appear to be sufficient to generate the tight CGN localization characteristic of dSed5 itself; however, it is difficult to make a precise comparison between the localization of the syn2/dSed5 chimera and that of dSed5, because expression of the latter causes a loss of Golgi stacks, whereas the chimera does not have this property.

More surprisingly, we found that the localization of dSed5p itself was not affected by changes in its membrane anchor. Substitution of the TM domain with that of syntaxin 1B or syntaxin 2 did not abolish the perinuclear immunofluorescence pattern (Fig. 4, *E* and *F*) or the CGN localiza-

Figure 5. Immunolocalization of dSed5 in transiently transfected COS cells. Ultrathin cryosections of COS cells expressing *myc*-tagged dSed5 were analyzed. (*A*) Single immunolabeling with 9E10 anti-*myc* antibody followed by goat anti-mouse Ig coupled to 10 nm gold particles. A large reticulo-vesicular network close to the nucleus showed some apparent continuity with the endoplasmic reticulum (*er*). The plasma membrane (*pm*) was not labeled. (*B*) Double immunolabeling with anti-*myc* antibody followed by goat anti-mouse Ig conjugated to 15 nm gold and with monoclonal antibody 1D3 (anti-protein disulphide isomerase) followed by goat anti-mouse Ig conjugated to 10 nm gold (*arrows*). The two labels colocalize in the network region. (*C*) An example of a cell expressing low levels of dSed5, labeled as in *A*. Golgi stacks are visible, but are unlabeled. The white region that covers part of one Golgi stack is a sectioning artefact. *n*, nuclear envelope; *m*, mitochondrion; *l*, lysosome. Bar, 0.2 μ m.

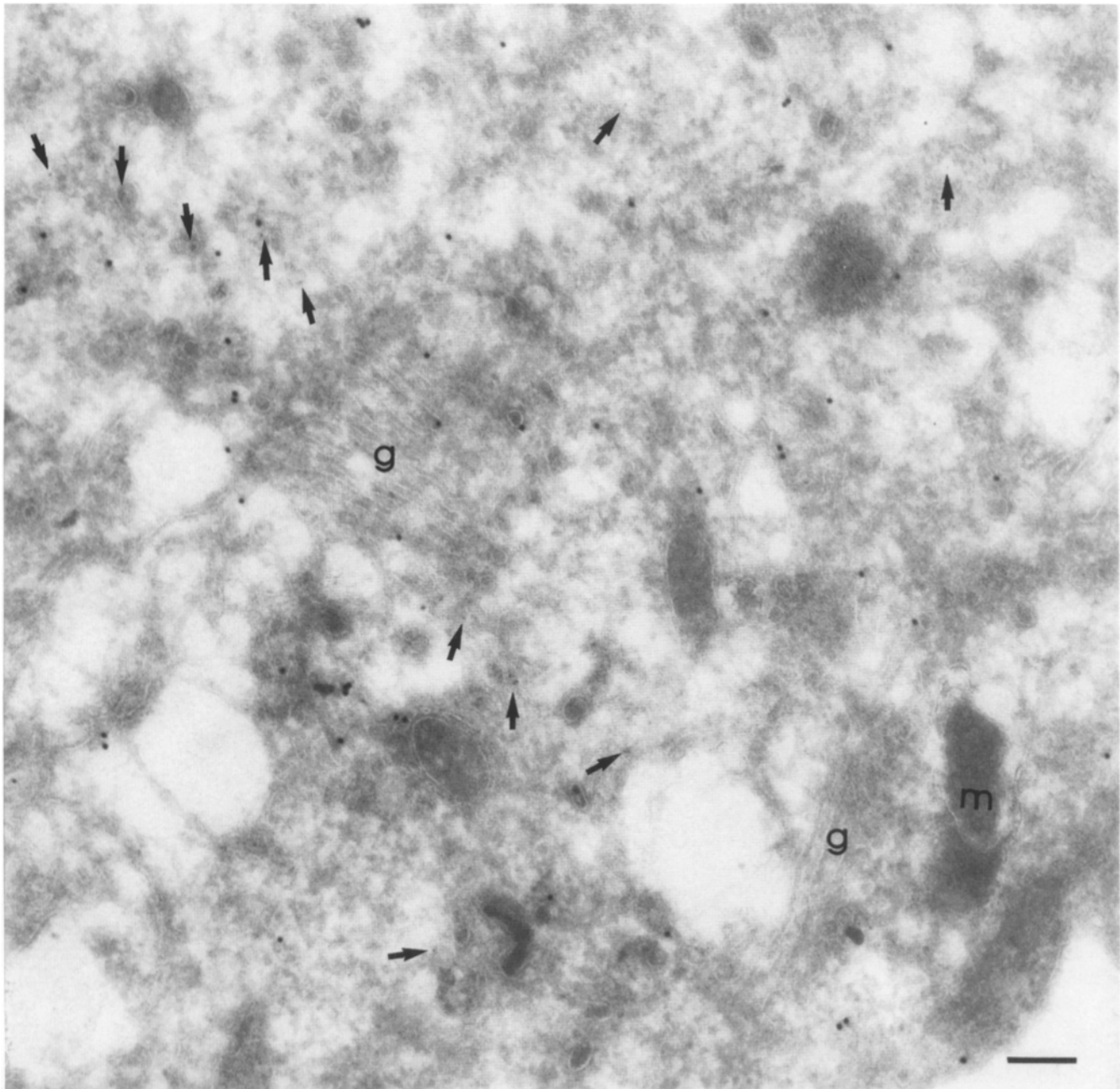


Figure 6. Localization of syntaxin 5 in NRK cells. Cells were double labeled with anti-peptide antibodies against syntaxin 5 (10 nm gold) and with mAb 1D3 (5 nm gold, *arrowed*). Golgi stacks are visible, but are only weakly labeled for syntaxin 5. Most of the label is seen on nearby tubular-vesicular structures which fill much of the area of the micrograph. 1D3 labeling is found in the same region, suggesting that these structures correspond to the CGN. *g*, Golgi stack; *m*, mitochondria. Bar, 0.2 μ m.

tion as judged by immuno-EM (Fig. 8 *B*; Table I). In a few cells, however, a different pattern was observed: the altered dSed5 protein was in discrete spots throughout the cytoplasm. When such cells were co-stained with anti- β' COP antibody, no discrete Golgi complex could be detected (not shown); moreover, if the cells were co-transfected with a plasmid expressing an unrelated plasma membrane protein, this marker was also found in the dSed5-containing structures (Fig. 4, *G* and *H*). This unusual phenotype correlated with a high level of expression of the dSed5/syntaxin chi-

mera, which apparently leads to a block in the secretory pathway at or shortly after exit from the ER. Although it was more commonly observed with the chimeric protein, this same phenotype could also be generated when *myc*-tagged dSed5 was over-expressed, and it is thus unlikely to be a specific consequence of the alteration to the TM domain. We conclude that the properties of the chimera differ slightly from those of dSed5 itself, but that it is not significantly mis-localized.

A variety of other dSed5 derivatives were tested (see Fig.

Table 1. Relative Distribution of Proteins Expressed in COS Cells

Construct	Percent of gold particles over				
	Golgi stack	Network	Vacuolar endosome	Plasma membrane	Other
1 (syn2)	1 ± 1	0	10 ± 5	85 ± 7	4 ± 2
2 and 3					
(syn2/sed5)*	31 ± 7	60 ± 12	8 ± 5	0	1 ± 1
4 (dSed5)	— [‡]	97 ± 5 [§]	1.5 ± 1	0	1.5 ± 1
6 (sed5/syn2)	— [‡]	96 ± 4 [§]	2 ± 1	0	1 ± 1

Cells transiently expressing the indicated constructs for 48 h were either fixed immediately (constructs 2, 4, and 6) or incubated for 2 h with 10 µg/ml cycloheximide before fixation (constructs 1 and 3). Results are expressed as the mean ± SEM and the number of gold particles counted was 200, 660, 220, 2,040, and 1,756 for constructs 1, 2, 3, 4, and 6, respectively.

* Constructs 2 and 3 gave very similar results, and the data were pooled for statistical purposes.

[‡] Golgi stacks were only visible at low levels of expression (10–15 gold particles per Golgi region), and were not labeled.

[§] Double labeling showed that this network contained protein disulphide isomerase, and thus corresponds to the ER-Golgi intermediate compartment.

^{||} The linear density of gold particles was 1.37 ± 0.3 per µm.

7). These included chimeras containing both TM and flanking sequences from syntaxin 2 (constructs 8 and 9), and a mutant in which the lysine residue on the cytoplasmic side of the TM was changed to a leucine, thus increasing the length of the hydrophobic region from 17 to 22 residues (construct 10). All of these proteins were associated with membranes and could be cross-linked into dimers, but none of them could be detected in post-Golgi compartments by immunofluorescence. This suggests that an efficient retention mechanism exists that is independent of the TM domain and the immediately adjacent sequences.

Further attempts to map a localization signal were unsuccessful. Chimeras in which the NH₂-terminal half of dSed5 were joined to the COOH-terminal heptad repeat region and TM of syntaxin 2 (Fig. 7, constructs 11 and 12) remained Golgi complex associated. Moreover, an NH₂-terminal truncation of dSed5 that left only the COOH-terminal heptad repeats also failed to pass beyond the Golgi complex, even when its TM domain was changed to that of syntaxin 2 (constructs 14 and 15). Thus either there are multiple targeting signals within the dSed5 molecule, or these hybrid proteins have global properties (such as a misfolded structure) that prevent their efficient transport through the Golgi complex.

All these experiments were performed with tagged proteins, and we were concerned that the presence of the tag might interfere with the targeting of dSed5. To address this, we expressed intact untagged versions of dSed5 and a derivative with the syntaxin 2 TM domain (construct 6). These proteins could be detected, albeit inefficiently, with antibodies raised against yeast Sed5; their distribution was indistinguishable from that of the corresponding tagged proteins. Thus the failure of the dSed5-syntaxin 2 chimera to reach the plasma membrane is not due to tag-induced misfolding.

Functional Analysis of SED5 Mutants in Yeast

Although the COS cell experiments allowed a rapid analysis of protein localization, they suffered from a number of limitations. In particular, it is not clear whether any of the expressed proteins are functional in these cells. To overcome

Construct	Structure	Sequence
1	syn2	..akeetkkaikyqskarrkkwiiiaavavavlavlaliiglsvqk
2	syn2/dSed5	..akeetkkaikyqskarrkkwLMIKIFGVLIFFFLFFVFM
3	syn2/dSed5	..akeetkkaikyqskarrkkwLMIKIFGVLIFFFLFFVFM
4	dSed5	..ieeahgeilkfyqsvsknrwlmikifgvlifffiffvfvfms
5	dSed5/syn1B	..ieeahgeilkfyqsvsknrTMIITCCVVLGVVLISSIGGTLGL
6	dSed5/syn2	..ieeahgeilkfyqsvsknrwTAAVAVAVIAVLALIIGLSVQK
7	dSed5/syn2	..ieeahgeilkfyqsvsknrwTAAVAVAVIAVLALIIGLSVQK
8	dSed5/syn2	..ieeahgeilRYQSKARRKKWIAAVAVAVIAVLALIIGLSVQK
9	dSed5/syn2	..ieHAKSETKKAIRYQSKARRKKWIAAVAVAVIAVLALIIGLSVQK
10	dSed5 K→L	..ieeahgeilkfyqsvsknrwlmikifgvlifffiffvfvfms
11	dSed5/syn2	(dSed5 1-211)-GY-(syn2 186-289)
12	dSed5/syn2	(dSed5 1-208)-YLGNSI-(syn2 178-289)
13	dSed5 del	(dSed5 1-68)-(dSed5 213-311)
14	dSed5 del	(dSed5 213-311)
15	dSed5 del/syn2	(dSed5 213-281)-RLQNR-(syn2 266-289)

Figure 7. Structures of dSed5 chimeras tested in COS cells. The sequences of the COOH-terminal regions of the various constructs are shown. Small letters correspond to the normal sequence of syntaxin 2 (constructs 1-3) or dSed5 (constructs 4-10); capital letters indicate the altered sequences, and the TM domains are underlined. For constructs 11-15, the residues of the parental proteins that are contained in the constructs are indicated, with junction sequences in capital letters. All proteins were tagged with the myc epitope at the NH₂ terminus, except where noted in the text.

this limitation, we investigated the importance of the TM domain for Sed5 function in yeast. Plasmids expressing various mutants were introduced into a strain in which the only Sed5 protein is provided by a galactose-inducible version of the yeast gene, and the cells spread on glucose-containing plates to inhibit expression of the wild-type protein. Only plasmids expressing functional versions of yeast Sed5 protein (ySed5) could give rise to colonies in this assay.

Fig. 9 summarizes the various mutant forms of ySed5 that were tested. Three constructs that did not abolish function are particularly striking. In the first, analogous to one of the mutants of dSed5 tested in animal cells, a Lys to Leu change effectively extended the hydrophobic portion of the transmembrane domain by five residues (construct 19). In the second, the TM and flanking sequence of ySed5 was replaced with that of Pep12, the yeast syntaxin-like protein involved in transport to the vacuole (construct 23). Finally, replacement of the TM domain with that from Ssol, a yeast plasma membrane syntaxin, could also be tolerated (construct 24). These results establish that the short, Phe-rich nature of the Sed5 TM domain is not essential for function in vivo.

Not all changes to the TM domain were tolerated; for example, it could be replaced with the Bet1 TM domain but not with those from Bos1 or Sec22 (Fig. 9, constructs 20-22). Furthermore, only one of three different fusions to Ssol was functional (constructs 24-26), even though they differed only in the sequences adjacent to the membrane, which did not themselves appear to be critical for ySed5 function (construct 27). These negative results are difficult to interpret, because there are many possible reasons for the inactivity of the altered proteins. Unfortunately, further studies of the nonfunctional proteins was precluded by the observation that they had strong dominant lethal effects on wild-type yeast strains, possibly due to interaction with the endogenous ySed5 protein.

In these experiments, the ySed5 derivatives were expressed from a strong promoter. As a more stringent test, the

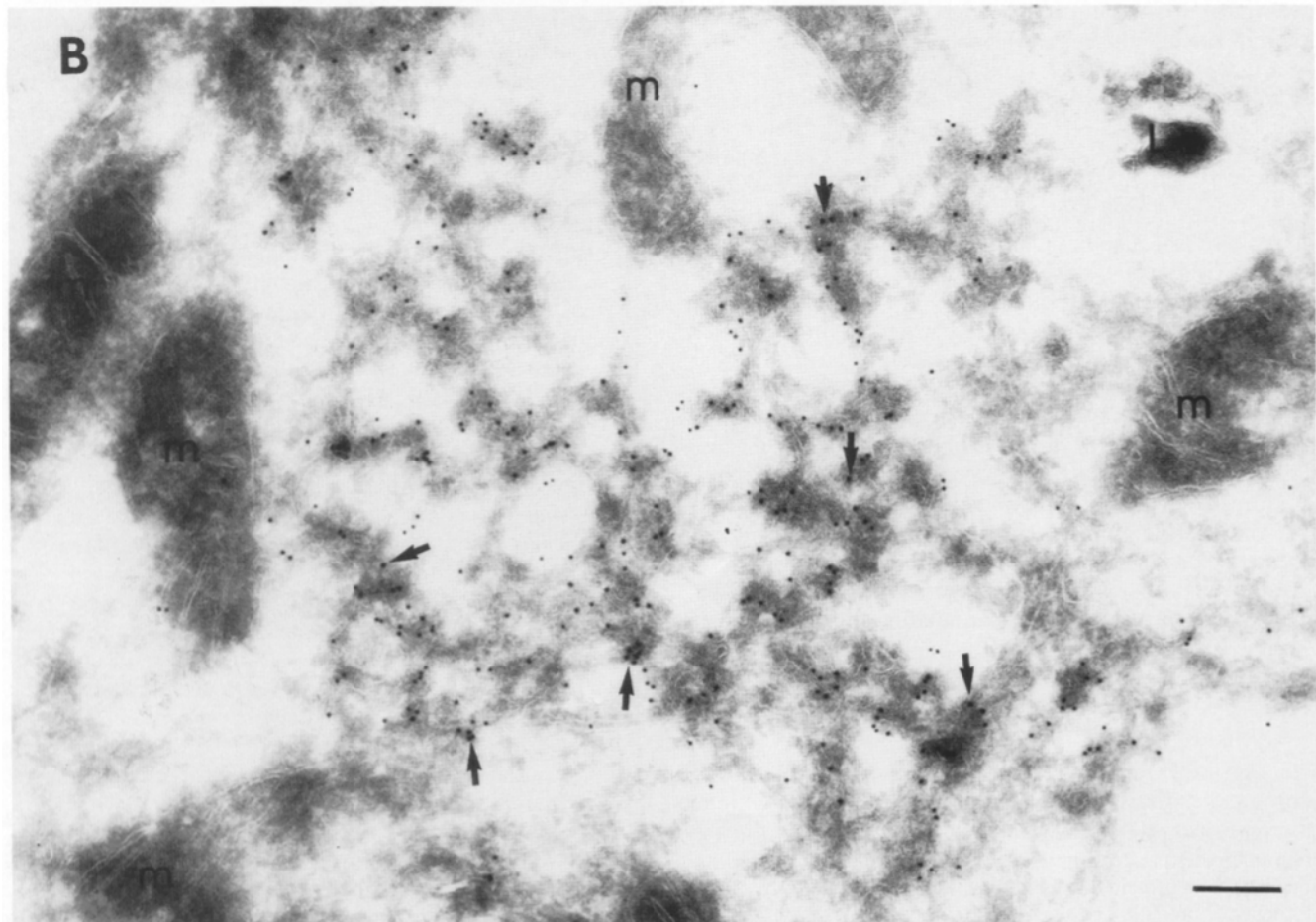
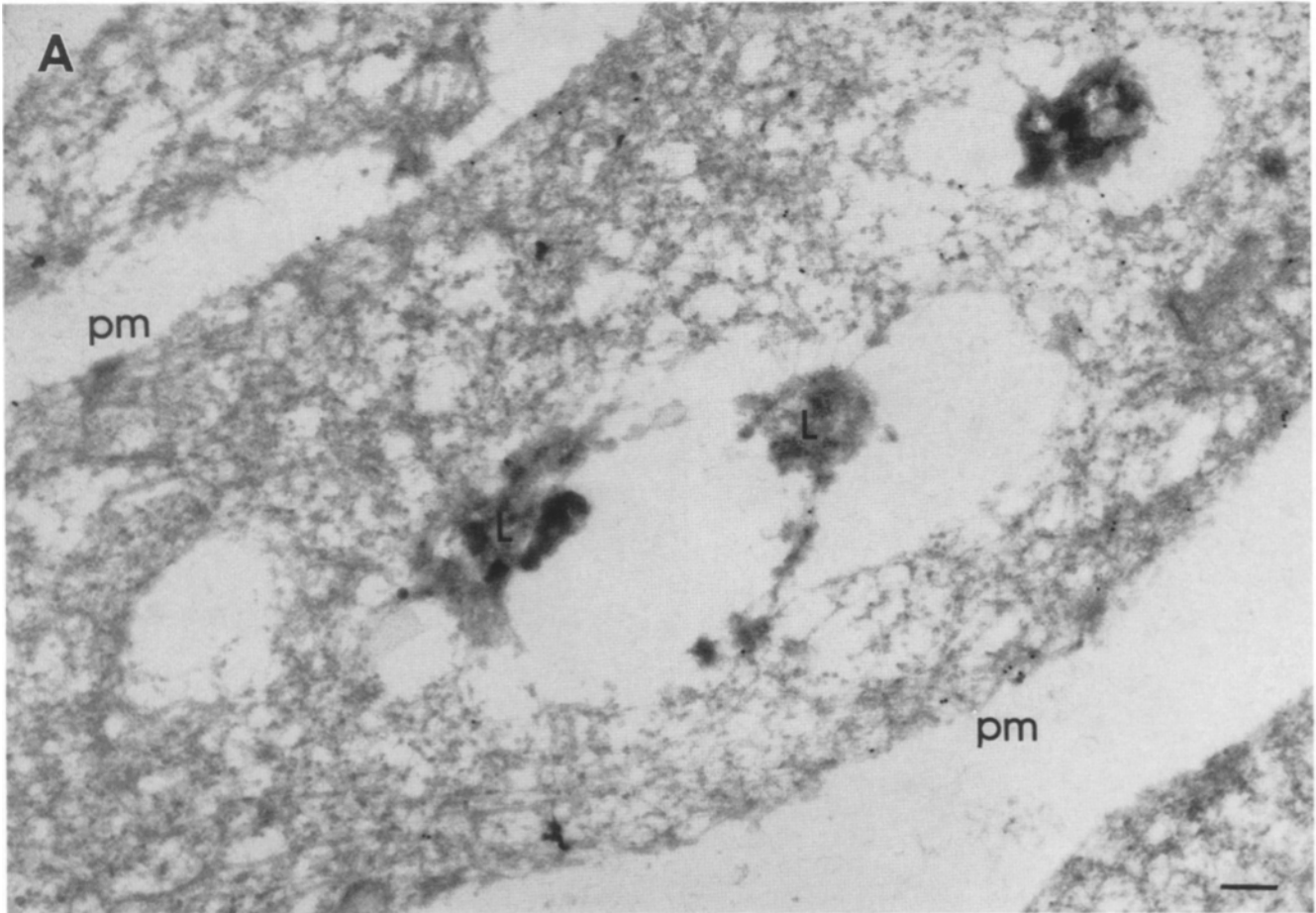
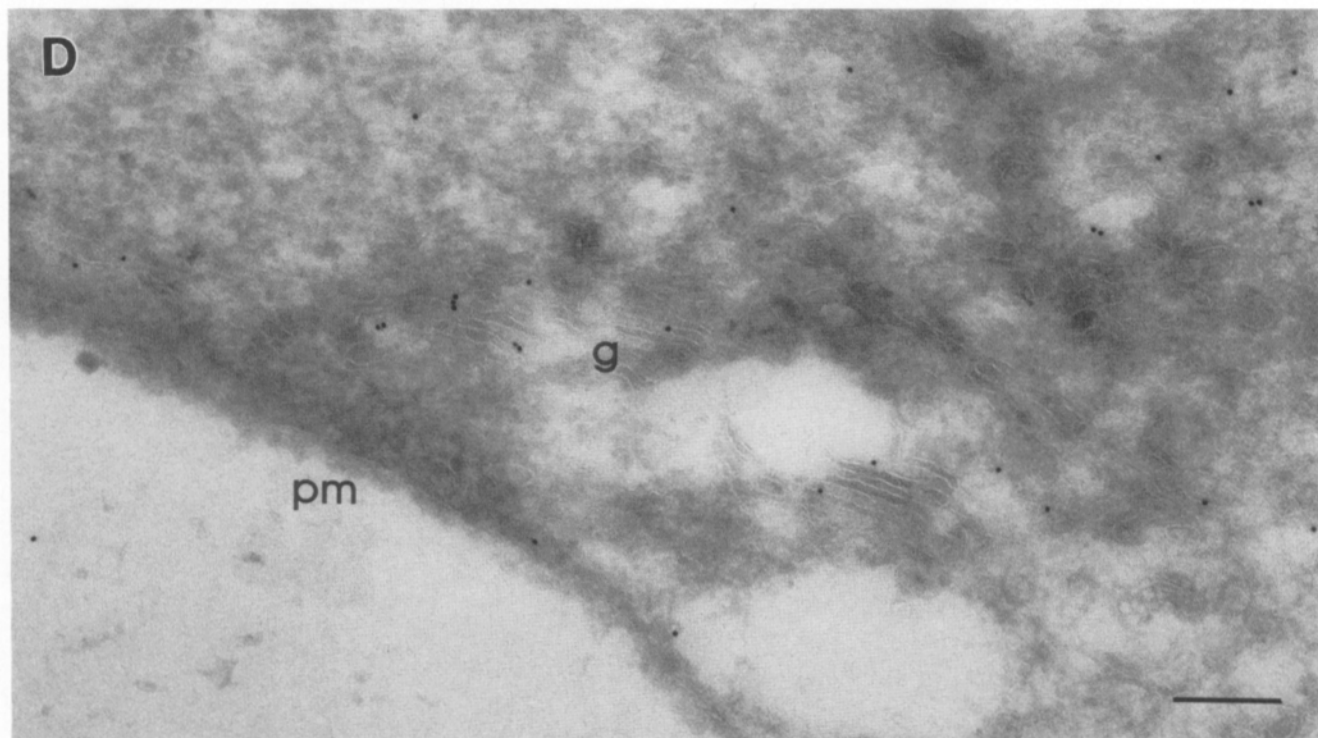
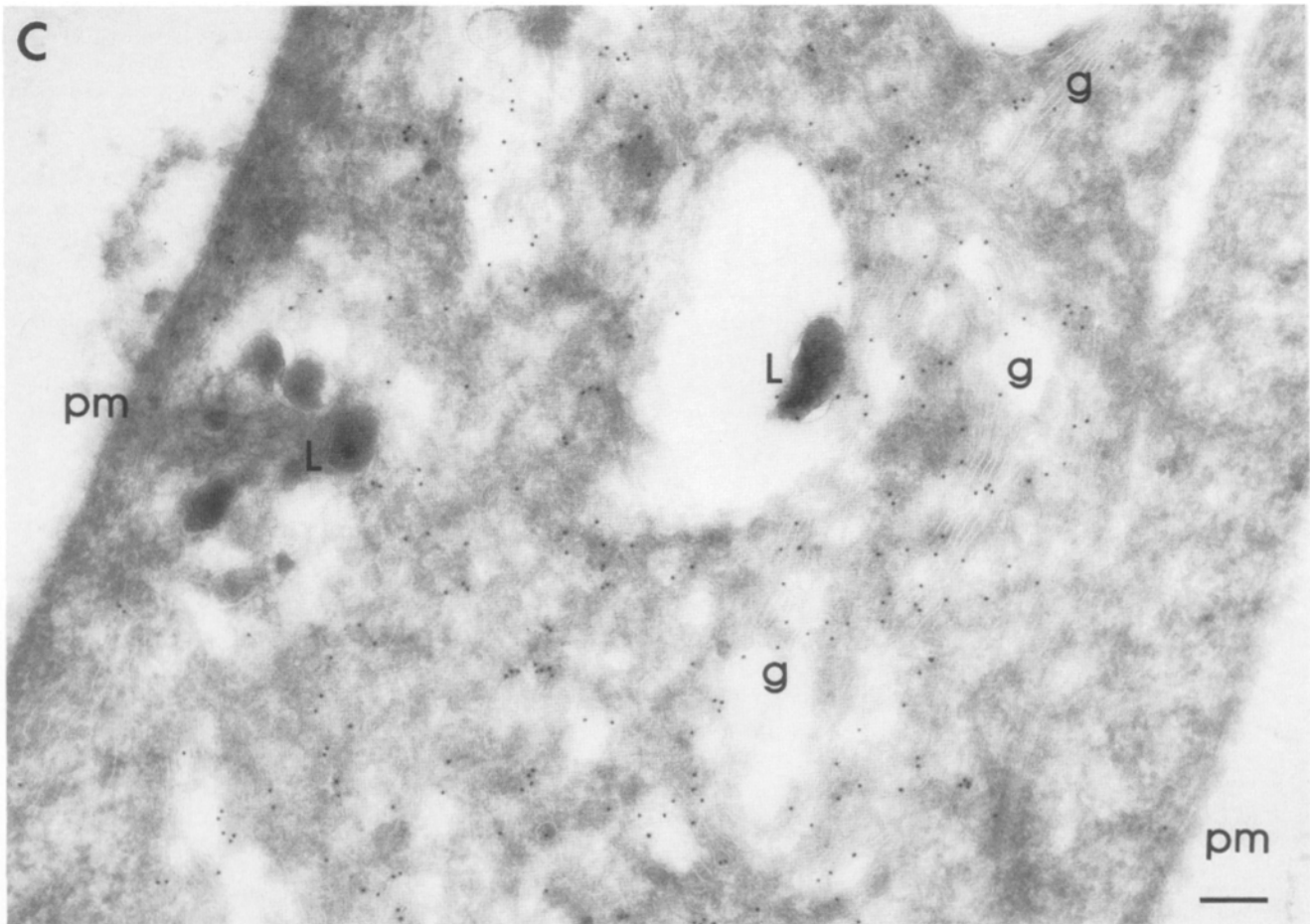


Figure 8. Immunolocalization of chimeric proteins in COS cells. Ultrathin cryosections of transiently transfected cells are shown, fixed either before (*B* and *C*) or after (*A* and *D*) a 2-h incubation with 10 $\mu\text{g/ml}$ cycloheximide. (*A*) Tagged syntaxin 2 detected with mAb 9E10



followed by goat anti-mouse Ig coupled to 10 nm gold. The plasma membrane was labeled as well as endosomal/lysosomal structures (L). Some gold particles were not associated with any particular defined organelle. (B) The dSed5/syn2 chimera (construct 6) was detected with mAb 9E10 and 10 nm gold particles. Protein disulphide isomerase was detected with mAb 1D3 and 15 nm gold. This chimera colocalized with protein disulphide isomerase in a reticulo-vesicular network (arrows) and had virtually the same distribution as dSed5. m, mitochondrion. (C) The syn2/dSed5 chimera (construct 2) detected as above. The Golgi stacks (G) were heavily labeled in addition to the same reticulo-vesicular network. Some labeling of endosomal/lysosomal structures (L) was also observed. The plasma membrane (PM) was devoid of labeling. (D) A second syn2/dSed5 chimera (construct 3) was also localized and gave the same distribution. Bars, 0.2 μ m.

Construct	Structure	Sequence	Function
16	ySed5	..yfdriksnrwlaakvffiiifvffv1wv1vn	+
17	ySed5 (Fs→Vs)	..yfdriksnrwlaakvVVi1Vvffv1wv1vn	+
18	ySed5 (Fs→Vs)	..yfdriksnrwlaakvffiiifvVv1Vv1vn	+
19	ySed5 (K→L)	..yfdriksnrwlaaLvffiiifvffv1wv1vn	+
20	ySed5/Bet1	..yfdriksnrwlaakLWLIIFFMVGVLFVWVLT	+
21	ySed5/Bos1	..yfdriksnrwlaakLVFWALILLIIGIYVLLKWLK	-
22	ySed5/Sec22	..yfdriksnrwlaakLLISQYAPIVVAVFFVLEFWIFLK	-
23	ySed5/Pep12	..yFRYQKRSRWRVLLVLLVLMLEFIFLIMKL	+
24	ySed5/Sso1	..yfdriksnrwlaakLWLVFAIIVVVVVVVVPAVVKTR	+
25	ySed5/Sso1	..yFRYQKARKNKIRWLVFAIIVVVVVVVVPAVVKTR	-
26	ySed5/Sso1	..yFRYQSRNRWLAQKLVFAIIVVVVVVVVPAVVKTR	-
27	ySed5/Sso1/ySed5	..yFRYQKARKNKakvffiiifvffv1wv1vn	+

Figure 9. Sed5 mutants tested in yeast. The conventions are the same as in Fig. 6. Also indicated are the results of functional assays for Sed5 function. Those constructs that could support growth of a yeast strain in the absence of any other Sed5 protein are indicated by a +.

chromosomal copy of *SED5* was modified so as to replace the sequences encoding the TM domain with the equivalent region of *SSO1* (see Materials and Methods for details). Cells carrying only this modified chromosomal gene grew at the same rate as wild-type cells. Moreover, we could not detect any substantial mislocalization of the ySed5 protein: immunofluorescent staining revealed a faint punctate pattern that was indistinguishable from the normal ySed5 distribution described previously (data not shown).

Expression of Yeast Sed5 Derivatives in COS Cells

As a final test, we expressed ySed5 derivatives in COS cells. NH₂-terminally tagged yeast Sed5 was found in the CGN by immuno-EM (not shown). Immunofluorescence showed a typical Golgi-like pattern (Fig. 10, *A* and *B*). The functional chimeras containing the Pep12 or Sso1 TM domains gave a similar pattern (Fig. 10 *C–F*); thus, as with *Drosophila* Sed5, there was no indication that altering the TM domain of yeast Sed5 caused mislocalization. Chimeras that did not function in vivo, such as the Bos1 and Sec22 fusions, also were localized to the Golgi region but gave much fainter staining, suggesting that they were inefficiently incorporated into membranes, or were unstable for some other reason. An exception was construct 25, which was often found in a punctate pattern centered on the Golgi region (Fig. 10, *G* and *H*). Even in this case, no sign of plasma membrane staining was apparent, despite the presence of the TM and flanking region from Sso1.

In these COS cell experiments we used NH₂-terminally myc-tagged derivatives of ySed5. To ensure that the tag did not induce grossly abnormal behavior of the protein, we also tested the ability of tagged ySed5 to support yeast growth. Strains expressing only the tagged protein grew well. Thus, tagging does not abolish the function of ySed5.

Discussion

Location of Sed5/Syntaxin 5

Genetic evidence in yeast suggests that Sed5 is required for ER-derived transport vesicles to reach the Golgi apparatus, and one would therefore expect it to be present in the earliest part of the Golgi complex. Previous yeast immunofluores-

cence data is consistent with a location for Sed5 that is distinct from the conventionally defined Golgi compartments: wild-type ySed5 protein is found in structures that are clearly separate from the ER, yet do not contain a marker for the early part of the Golgi complex (the *MNT1*-encoded mannosyl transferase). The immuno-EM data in this paper leads to a similar conclusion: when expressed in COS cells, *Drosophila*, rat, and yeast Sed5 proteins are all found in tubulo-vesicular structures close to the Golgi stack. Double labeling with an anti-PDI antibody strongly suggests that these correspond to the intermediate compartment or CGN. Although these studies were largely performed with epitope-tagged molecules, we consider it unlikely that the presence of the tag significantly alters the location of the proteins. This is because: (a) tagging of the yeast protein does not inactivate its function in vivo; (b) tagged and untagged versions of dSed5 had similar distributions in COS cells as judged by immunofluorescence; and (c) immunolocalization at the EM level of the endogenous rat protein in NRK cells also revealed a CGN-like distribution. Given the presumed role of Sed5 as a vesicle receptor, it follows that this convoluted structure represents the first distinct post-ER compartment.

This conclusion conflicts with previous suggestions that the intermediate compartment is simply an extension of the ER. It does contain PDI, which is normally considered an ER marker, but since this protein (like other luminal ER proteins) can be retrieved from later compartments by the KDEL receptor, its presence does not prove that there is a direct connection to the ER. Such connections have, however, been seen in electron micrographs of virus-infected cells, implying that vesicular transport is not necessary at this step (Krijnse-Locker et al., 1994). It may be that the connections are transient, and/or do not constitute the primary mode of transport out of the ER. Alternatively, it could be that the role of Sed5 in the secretory pathway is more subtle than has been assumed; for example, it could function in retrograde transport instead of, or as well as, in forward transport.

A related issue is whether Sed5 protein is present in the rest of the Golgi complex. Our analysis is complicated by the fact that overexpression of Sed5 perturbs Golgi structure, but it is clear from the weakly staining COS cells and from the NRK cell staining, as well as from yeast immunofluorescence, that the bulk of the Sed5 protein is not found in the main part of the Golgi apparatus. If Sed5 is absent, could there be other syntaxin-like targeting molecules for the medial and *trans*-Golgi cisternae? Several members of the syntaxin family have now been identified, and in at least three cases they were found by accident rather than selection, yet so far no candidate has been found for a molecule involved in transport from one part of the Golgi complex to another. Have these proteins been missed, or could it be that such transport does not involve syntaxin-like molecules? There is evidence in yeast that intra-Golgi transport involves Sec18 (the NSF fusion protein homologue) (Graham and Emr, 1991), and one would therefore expect SNAPs (the NSF attachment proteins) and SNAREs (SNAP receptors such as the syntaxins) also to be involved, but it remains possible that a special targeting and fusion machinery exists for these steps.

We cannot exclude the possibility that Sed5 is present throughout the Golgi apparatus at levels sufficient to allow

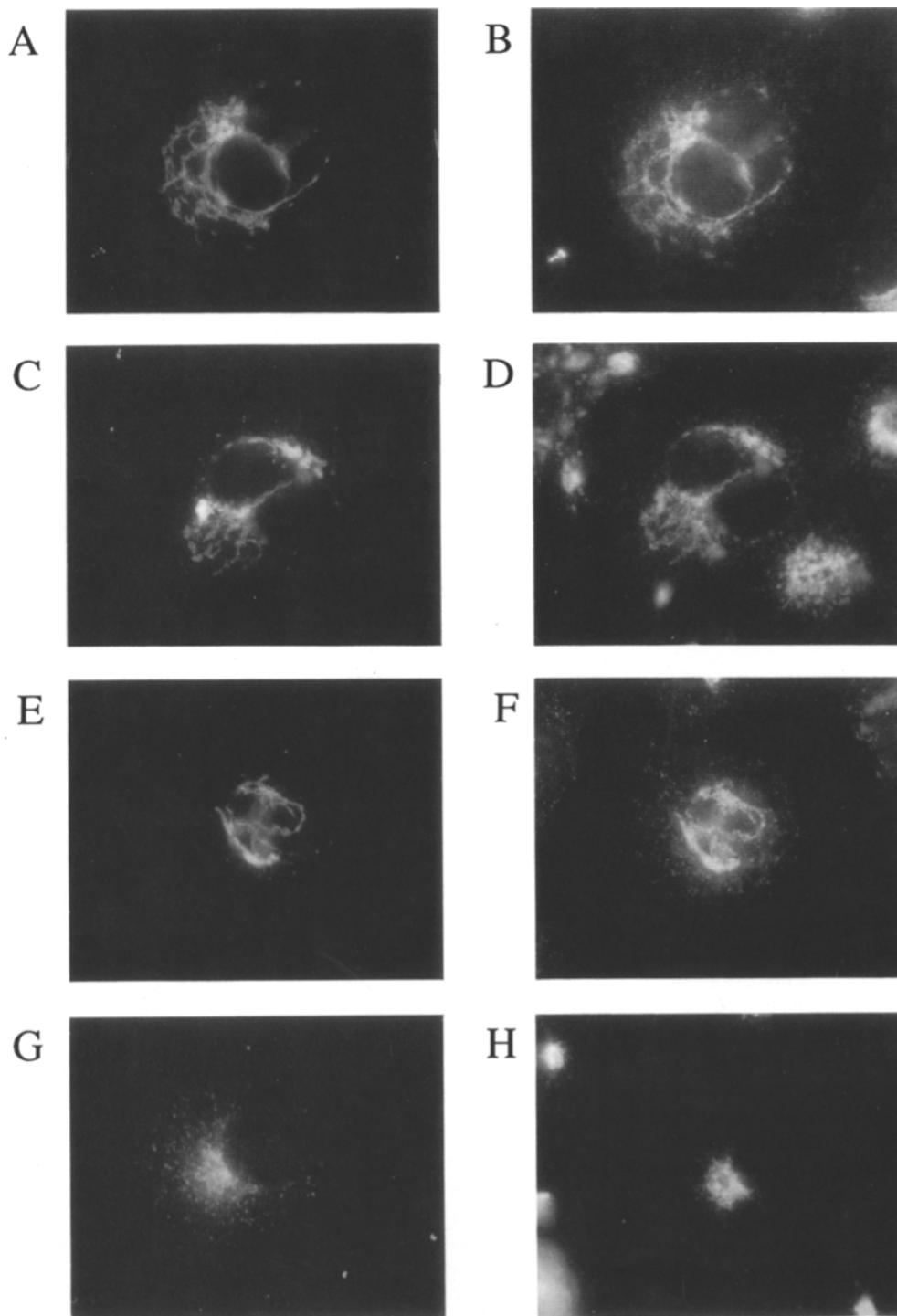


Figure 10. Expression of yeast Sed5 chimeras in COS cells. The proteins were tagged at the NH₂ terminus and detected with mAb 9E10 (A, C, E, and G); the cells were also stained with anti-β' COP as a Golgi marker (B, D, F, and H). The proteins shown are: (A and B) yeast Sed5 (construct 16); (C and D) Sed5/Pep12 (construct 23); (E and F) Sed5/Ssol (construct 24); (G and H) Sed5/Ssol (construct 25).

vesicle fusion. If this were so, however, it would still be necessary to postulate some targeting mechanism other than mere recognition of this protein to account for the polarized nature of traffic through the Golgi stack.

How is Sed5 Localized?

Retention of Golgi enzymes is mediated by their transmembrane domains, and although Sed5 is in a different compartment from the medial and trans enzymes that have been studied so far, it seemed possible that it was localized by a similar

mechanism. Indeed, we found that the dSed5 TM could retain a plasma membrane syntaxin in the Golgi complex. However, our data suggest that the dSed5 TM does not specify a tight CGN localization. Moreover, its short, phenylalanine-rich nature is not required for normal localization of dSed5 in COS cells nor for the function of the yeast homologue. There must therefore be some other mechanism that allows retention of this protein.

The most obvious possibility is that Sed5 binds to another protein that provides a localization signal. The plasma membrane syntaxins are known to bind to at least two other

proteins—SNAP-25 and a Sec1/unc-18 homologue—but neither of these proteins have a transmembrane domain, and thus they do not suggest any obvious retention mechanism (Hata et al., 1993). Possibly, an as yet undiscovered integral membrane partner of Sed5 exists. Our cross-linking studies showed that dSed5 and a truncated form of the protein formed dimers when expressed in COS cells, in contrast to the reported behavior of bacterially expressed members of the syntaxin family (Hata et al., 1993). However, the bulk of the protein appeared not to form hetero-dimers with endogenous Sed5, and there were no abundant cross-linked complexes that might contain another associated protein, although we would not have detected one that was inefficiently cross-linked.

An alternative mechanism might be provided by recycling of Sed5 from later compartments. Interestingly, the best-known constituent of the CGN/intermediate compartment, ERGIC53, is thought to recycle through the ER. This protein carries a KKXX retrieval signal on its cytoplasmic tail, which probably mediates this recycling (Schindler et al., 1993; Jackson et al., 1993). So far, we have not been able to identify any discrete cytoplasmic signal on Sed5, but more detailed studies might reveal some such signal; if one exists, it would appear to be functionally well conserved, since both yeast and *Drosophila* Sed5 had a location in COS cells that was indistinguishable from that of rat syntaxin 5. The presence of two distinct targeting signals in a Golgi protein would not be without precedent: the protein TGN38 seems to be localized by a combination of a retention signal in its transmembrane domain and a recycling signal in the cytoplasmic tail (Ponnambalam et al., 1994).

In this discussion, we have assumed that Sed5 and the other syntaxin family members are inserted into the ER membrane and transported through the secretory pathway in the normal manner. However, the unusual structure of these proteins, with the TM domain at the extreme COOH terminus, means that they cannot be inserted cotranslationally as are other membrane proteins. It is possible that they use a novel insertion mechanism, which need not be restricted to the rough ER membrane. Indeed, we saw very little ER staining, even when dSed5 was expressed at high levels, and thus we cannot rule out the possibility that this protein is inserted directly into the smooth ER or CGN. This could in part explain why so many of the chimeras tested are found in the CGN; they might simply fail to move from their insertion site. It seems less likely that the other syntaxins are inserted directly into the plasma membrane; we observed Golgi staining with these constructs that could be chased away in the presence of cycloheximide, implying passage of the proteins through the Golgi complex. In any case, the TM domains of syntaxin 2 and Ssol are not sufficient to target Sed5 chimeras to the plasma membrane.

In summary, we have shown that Sed5 proteins are tightly confined to the *cis* side of the Golgi complex, as predicted from their function. This localization is achieved by at least two distinct mechanisms. One is mediated by the TM domain, and is presumably analogous to the retention mechanism for Golgi enzymes. The other, which is sufficient for Sed5 function, involves only the cytoplasmic portion of the protein. A full understanding of this second mechanism will require more detailed knowledge of the proteins with which Sed5 interacts *in vivo*.

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