The Capacity to Retrieve Escaped ER Proteins Extends to the *trans*-most Cisterna of the Golgi Stack

Gero Miesenböck and James E. Rothman

Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York 10021

Abstract. To explore how far into the Golgi stack the capacity to retrieve KDEL proteins extends, we have introduced an exogenous probe (the peptide YHPNSTCSEKDEL) into the TGN of living cells. For this purpose, a CHO cell line expressing a c-myc-tagged version of the transmembrane protein TGN38—which cycles between the TGN and the cell surface—was generated. The cells internalized peptides that were disulfide bonded to anti-myc antibodies and accumulated the peptide-antibody complexes in the TGN. Peptides released from these complexes underwent retrograde transport to the ER, as evidenced by the transfer of N-linked carbohydrate to their acceptor site. The KDEL-tagged glycopeptides (~10% of the endocytosed load) behaved like endogenous ER residents: they stayed intracellular, and their oligosaccharide side chains remained sensitive to endoglycosidase H. An option thus exists to extract ER residents even at the most distant pole of the Golgi stack, suggesting that sorting of resident from exported ER proteins may occur in a multistage process akin to fractional distillation.

NEWLY synthesized proteins translocated into the ER represent a complex mixture that is fractionated according to destination as the proteins move farther along the secretory pathway. The prevalent view of this process places the two major sorting steps at the entry and exit faces of the Golgi stack. At the stack's receiving end, the *cis*-Golgi network (CGN)[1] or "salvage compartment," escaped ER residents are thought to be removed from the downstream moving protein mixture and returned to the ER (Warren, 1987; Pelham, 1988, 1989). And at the stack's opposite end, the TGN, lysosomal proteins and proteins of secretory storage granules are similarly diverted from bulk secretory flow (Griffiths and Simons, 1986), leaving behind only secreted and plasma membrane proteins that reach the cell surface by default.

Implicit in this view, however, is a mystery of substantial proportions: if sorting decisions are made mainly at the levels of the CGN and the TGN, why do secretory proteins have to transit, with considerable energy expenditure, the handful of interposed Golgi cisternae? A common answer is that the Golgi stack forms an assembly line for carbohydrate biosynthesis, with each cisterna containing the enzyme equipment for only a few reactions (Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985), and that the vectorial movement of glycoproteins through the stack is used to control the carbohydrate structures assembled on them. However, there is no a priori reason why this biosynthetic pathway should be compartmentalized (substrate specificity of the processing enzymes alone should be sufficient to ensure synthesis of defined oligosaccharides), and the actual distribution of the enzymes among different Golgi cisternae is one of relative dominance rather than of strict mutual exclusion; there is overlap (Nilsson et al., 1993).

Passing the secretory flow through a series of distinct compartments may, however, be necessary for a different reason. Proteins destined for export constitute only a trace fraction of total ER protein (an estimated 1 in 10,000 under physiologic conditions for plasma membrane precursors [Rothman, 1981]; even viral spike proteins do not exceed an ER membrane density of 1 in 1,000 during infection [Quinn et al., 1984]). Transporting these proteins must therefore involve purifying them away from the vast excess of ER residents and appears to be accomplished by a combination of strategies. Cargo proteins are concentrated 5–10-fold in transport vesicles budding from the ER (Quinn et al., 1984; Copeland et al., 1988; Balch et al., 1994), attesting to some degree of selectivity at the port of entry into the secretory pathway. This selectivity is due, at least in part, to retention signals that seem to work by preventing access to vesicles (Rose and Doms, 1988; Hurtley and Helenius, 1989). In addition, those ER proteins that slip through this first selectivity filter are retrieved selectively from one or more downstream compartments. Many lumenal ER residents (and

Address correspondence to James E. Rothman, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Tel.: (212) 639-8598. Fax: (212) 717-3604.

1. *Abbreviations used in this paper*: αMMP, α-methylmannopyranoside; CGN, *cis*-Golgi network; Endo H, endoglycosidase H; MesNa, 2-mercaptoethane sulfonic acid; NHS, *N*-hydroxysuccinimide; OST, oligosaccharyltransferase; SPDP, *N*-succinimidyl 3(2-pyridyldithio)propionate.

also some integral membrane proteins) possess the tetrapeptide sequence KDEL at their COOH termini (Munro and Pelham, 1987) and are thereby recognized and bound by receptors in the Golgi (Lewis and Pelham, 1990, 1992*a*; Hsu et al., 1992). Upon ligand binding, the KDEL receptors move to the ER (Lewis and Pelham, 1992*b*), where the retrieved proteins dissociate. A similar mechanism seems to operate for integral proteins of the ER membrane. These proteins contain, within their cytoplasmic domains, dibasic signals (COOH-terminal double-lysine or NH_2-terminal double-arginine motifs [Nilsson et al., 1989; Jackson et al., 1990; Schutze et al., 1994]) that are recognized by an as yet unidentified sorting machinery that also mediates retrieval from downstream (Jackson et al., 1993; Gaynor et al., 1994).

If a single step of sorting during vesicle budding from the ER is insufficient for removing resident proteins from the export, then multiple budding and retrieval opportunities in combination would be needed. Accordingly, the spillover from the first round of sorting would have to be subjected to a second round, the spillover thereof to a third, and so forth until the necessary level of separation is achieved. In other words, the Golgi stack would operate as a biological distillation tower (Rothman, 1981) in which each seemingly wasteful transport step between successive cisternae in reality provides an essential sorting opportunity.

A central prediction of this hypothesis is therefore that the option to retrieve escaped ER proteins exists all the way through the Golgi stack. An experimental test of this prediction has, however, proven difficult. Analyses of natural or engineered glycoproteins with KDEL or double-lysine termini, for example (Brands et al., 1985; Lewis et al., 1985; Strous et al., 1987; Pelham, 1988, 1989; Dean and Pelham, 1990), have with a few notable exceptions (the endogenous KDEL protein calreticulin [Peter et al., 1992] and KKXX- or KDEL-tagged CD8 chimerae [Jackson et al., 1993]) demonstrated that ER residents lack carbohydrate structures indicative of transit through the medial or the *trans*-Golgi, let alone the TGN. This, however, does not cogently argue that the capacity for retrieval is missing from compartments downstream of the CGN; it may simply be that the first rounds of retrieval are efficient enough to make retrograde transport from later cisternae difficult to detect.

The present work therefore uses a novel strategy: Instead of following cycles of escape and retrieval originating and terminating in the ER (where endogenous marker proteins are placed by way of biosynthesis), we have introduced an exogenous KDEL-tagged probe into the TGN and monitored its retrieval from this compartment to the ER. In this way the anterograde leg of the escape-retrieval cycle has been eliminated, allowing a retrograde leg to be unveiled that indeed spans the secretory pathway in its entirety.

Materials and Methods

mycTGN38: Plasmids and Cell Lines

The following changes were introduced into the cDNA of rat TGN38 (Luzio et al., 1990) by PCR: A nucleotide sequence coding for the c-myc-derived epitope recognized by mAb 9E10 (EQKLISEEDL; Evan et al., 1985) was inserted four codons downstream of the predicted (von Heijne, 1986) signal peptide cleavage site, the initiator codon was placed into an optimal context for initiation of translation by eukaryotic ribosomes (Kozak, 1991), and sites for SalI and XbaI were added as flanking sequences. The PCR product was ligated to a derivative (see the following discussion) of the vector pSV·SPORT1 (Bethesda Research Laboratories, Gaithersburg, MD), which carries the SV-40 early promoter, t-intron region, and polyadenylation signals. Its sequence was verified using Sequenase 2.0 (United States Biochemicals Corp., Cleveland, OH).

For stable expression of mycTGN38, a derivative of pSV·SPORT1 was constructed by inserting, into the vector's unique ClaI site, an expression cassette that contained the coding region of *neo* and regulatory sequences of the murine *Pgk*-1 gene, i.e., an EcoRI–TaqI promoter region and a PvuII–BglII fragment carrying mRNA-processing signals (Boer et al., 1990). The orientation of this expression cassette was chosen such that mycTGN38 and aminoglycoside phosphotransferase were transcribed in opposite directions. The plasmid pSVmycTGN38 was linearized at its unique ScaI site (within the β-lactamase gene) and transfected into CHO-K1 cells (ATCC CCL 61) by electroporation. After 72 h of recovery, neomycin-resistant cells were selected and maintained in complete medium containing G418 (GIBCO BRL, Gaithersburg, MD; 1 mg/ml active compound for a 7-d selection period and 0.5 mg/ml for maintenance thereafter), cloned by serial dilution, and screened for expression of mycTGN38 by immunofluorescence microscopy.

Immunofluorescence Microscopy

The localization of mycTGN38, as visualized with mAb 9E10 and Texas red–conjugated anti–mouse IgG antibodies, was compared with that of endogenous TGN38, which was detected with a rabbit polyclonal antiserum (Wilde et al., 1992) and FITC-conjugated secondary antibodies (both fluorochrome-labeled antibodies were from Molecular Probes, Inc., Eugene, OR). Cells grown on coverslips were fixed and permeabilized in methanol at −20°C, blocked in PBS containing 0.2% gelatin, and stained with appropriate dilutions of primary and secondary antibodies in 0.2% gelatin. The coverslips were mounted with 2.5% 1,4-diazabicyclo(2,2,2)-octane (Aldrich Chemical Co., Milwaukee, WI) in Mowiol (Calbiochem Corp., La Jolla, CA) and examined by wide-field or confocal immunofluorescence microscopy.

Peptides and Peptide–9E10: Synthesis and Labeling

Peptides were synthesized using automated 9-fluorenylmethoxycarbonyl (fmoc) chemistry, purified by HPLC, and dissolved in water at concentrations of 1 mM. To prevent their oxidation during peptide iodination, cysteine residues were protected in mixed disulfides with glutathione; the pertinent disulfide exchange reactions involved a 100-fold molar excess of glutathione disulfide over thiol-containing peptide in 100 mM Tris, pH 8.5, for 48 h at 40°C. The glutathionylated peptides were purified by reverse-phase HPLC, lyophilized, and redissolved in water at 1 mM. The purity of all peptide stocks exceeded 97%, as determined by HPLC and mass spectrometry of material from the major chromatographic peak.

For radioiodinations, 20 nmol of peptide (20 μl of a 1-mM aqueous stock solution) were mixed with 50 μl of 0.5 M sodium phosphate buffer, pH 7.5, in an IODOGEN-coated tube (Pierce, Rockford, IL). Labeling was initiated with the addition of 5 mCi of $Na^{125}I$ (New England Nuclear, Boston, MA). The reaction was allowed to proceed for 20 min at room temperature and terminated by transferring the sample to 100 μl of 0.05 M sodium phosphate buffer, pH 7.5, containing 2 mM carrier NaI and 200 mM DTT to remove the protective glutathione groups. The labeled, deprotected peptide was purified on a Sep-Pak C_{18} cartridge (Waters Chromatography, Milford, MA). Specific radioactivities ranged from 40 to 130 Ci/mmol.

Iodinated peptides were coupled to mAb 9E10 with the heterobifunctional cross-linker, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce). The mAb was purified from ascites on protein G–Sepharose Fast Flow (Pharmacia Diagnostics AB, Uppsala, Sweden), dialyzed against PBS, pH 7.6, and reacted with the N-hydroxysuccinimide (NHS) ester function of SPDP under conditions to introduce between 2.2 and 3.7 mol of cross-linker per mol of 9E10. Free NHS esters were inactivated with 0.2 M ethanolamine, pH 8.0, and removed on a desalting column equilibrated with 20 mM MOPS/NaOH, pH 6.5, 150 mM NaCl, 1 mM EDTA. Derivatized 9E10 was combined with labeled peptide such that the number of peptide cysteines in the mixture exceeded twice that of antibody-linked pyridine-2-thiones, and the disulfide exchange process was allowed to reach equilibrium. An average of 1.7–2.9 mol of peptide was cross-linked to each mol of 9E10 under these conditions. Unreacted pyridine-2-thiones were quenched with 0.2 mM cysteine, and the peptide–9E10 conjugate was isolated by gel filtration on a Sephacryl S-200 HR column (Pharmacia Diagnostics AB) equilibrated with 20 mM MOPS/NaOH, pH 7.0, 150 mM NaCl, 1 mM EDTA.

Peptides and Peptide–9E10: Interactions with mycTGN38, KDEL Receptors, and Oligosaccharyltransferase In Vitro

To determine the pH dependence of the mycTGN38–9E10 association, mRNA encoding mycTGN38 was transcribed in vitro from XbaI-linearized pSVmycTGN38 and used to program a rabbit reticulocyte lysate (Promega Corp., Madison, WI). Aliquots of the translation reaction were diluted fivefold with immunoprecipitation buffer (50 mM sodium cacodylate plus 50 mM sodium acetate, adjusted to the pH indicated in Fig. 2; 100 mM KCl, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5% (vol/vol) Triton X-100) and rotated at 4°C for 2 h with 30 μl of a 50% (vol/vol) suspension of protein G–Sepharose Fast Flow beads to which 1 mg/ml 9E10 had been cross-linked with dimethylsuberimidate (Pierce). After five washes with immunoprecipitation buffer, the bound proteins were eluted in SDS sample buffer and analyzed by 12% SDS-PAGE. Immunoprecipitated mycTGN38 was quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

For KDEL receptor binding experiments (Wilson et al., 1993), Golgi membranes from CHO cells (Balch et al., 1984) were extracted on ice with 100 mM Na_2CO_3, pH 11.5, pelleted, and resuspended in 10 mM Hepes/NaOH, pH 7.4. Binding assays contained, in a final volume of 25 μl, Na_2CO_3-washed Golgi membranes (1 μg of protein), 0.1 × 10^6 cpm labeled peptide or peptide–9E10, and the indicated concentrations of unlabeled competitor peptide in 50 mM sodium cacodylate, pH 5.0, 20 mM NaCl, 250 μg/ml BSA, 5 mM DTT, unless stated otherwise. After 20 min on ice, the membranes were pelleted, and bound radioactivity was quantified.

Rough microsomes from chicken liver (Walter and Blobel, 1983) served as the source of both oligosaccharyltransferase (OST) and $Glc_3Man_9GlcNAc_2$-PP-dolichol in experiments to determine the ability of peptides to accept N-linked carbohydrate. The microsomes (80 μg of protein per 100-μl assay) were permeabilized with 0.625% (wt/vol) recrystallized digitonin (Calbiochem Corp.) in 50 mM Tris, pH 7.4, 2 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 3% (wt/vol) glycerol on ice for 20 min. Labeled peptide was added to a final concentration of 3 μM, and the incubation was continued at 25°C for the indicated times. The reactions were stopped with 1 ml of ice-cold Con A buffer (25 mM Tris, pH 7.4, 250 mM NaCl, 1.5 mM $CaCl_2$, 1.5 mM $MnCl_2$, 1 mM DTT, 0.5% [vol/vol] hydrogenated Triton X-100 [Calbiochem Corp.]). After 10 min on ice, the samples were boiled for 5 min in the presence of 50 mM DTT and clarified by centrifugation (15,000 *g*, 15 min). The aqueous and detergent supernatants were combined and rotated at 4°C for 30 min with 50 μl of a 50% (vol/vol) suspension of Con A–Sepharose beads (Pharmacia Diagnostics AB) in Con A buffer. Bound glycopeptide was eluted with 0.5 M α-methylmannopyranoside (αMMP; Sigma Chemical Co., St. Louis, MO) in Con A buffer and counted.

Experiments on CHO Cells Expressing mycTGN38

Transformed CHO cells were grown and all experiments on them were performed in suspension with gentle stirring. To avoid the extracellular cleavage of peptide–9E10, the growth medium (αMEM with 10% FBS and 0.5 mg/ml G418) was removed, and the cells were resuspended at a density of 10 × 10^6 per ml in buffered, cysteine-free medium (DMEM with 10% dialyzed bovine serum and 20 mM Hepes/NaOH, pH 7.4).

Surface binding and internalization data represent duplicate measurements taken in the absence and presence of a 50-fold molar excess of unlabeled antibody. In binding experiments, the cells were maintained at 4°C for 30 min before the addition of peptide–9E10 and for 2 h thereafter; unbound material was removed with wash buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 2 mg/ml BSA). In internalization experiments, uptake of 9E10 was allowed to proceed for 2 h at 37°C, and the intracellular content of labeled peptide was determined after cleavage of surface-bound peptide–9E10 with 2-mercaptoethane sulfonic acid (MesNa; Sigma Chemical Co.) at a concentration of 20 mM for 1 h at 4°C. To probe the stability of peptide–9E10 in the cell interior and to delineate conditions to compromise this stability in a controlled way, 20 × 10^6 transformed CHO cells that had been loaded with peptide–9E10 for 2 h were resuspended in medium containing variable concentrations of DTT or MesNa. After 10 min at 37°C, the reductants were quenched with 50 mM iodoacetamide for 1 h at 4°C, and the cells were lysed on ice in CHAPS buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CHAPS [Boehringer Mannheim, Mannheim, Germany]). The lysates were clarified by centrifugation and chromatographed on a Sephacryl S-100 HR column to separate free and 9E10-bound peptide. Addition of 20 mM DTT was found to be necessary and sufficient for cleavage of >90% of the internalized peptide–9E10.

In a standard retrieval experiment, peptide–9E10 was offered to 50 × 10^6 transformed CHO cells in 5 ml of cysteine-free medium at a concentration of 150 nM over 2 h. After uptake of peptide–9E10, the cells were resuspended in medium containing 20 mM DTT and kept at 37°C for 10 min to cleave the endocytosed peptide–9E10. The reductant was then removed by washing with four 50-ml portions of medium, and the cells were resuspended in 10 ml of medium for an additional incubation of 2.5 h at 37°C. To terminate the experiment and to quantify glycopeptide formation, the cells were solubilized for 30 min on ice in 2 ml of Con A buffer containing 4.5% (vol/vol) Triton X-100. The lysates were clarified by centrifugation, boiled in 50 mM DTT for 10 min, and again clarified; the media were made 0.5% (vol/vol) in Triton X-100, 1.5 mM in $CaCl_2$ and $MnCl_2$, and 50 mM in DTT and then boiled and clarified. Aqueous and detergent supernatants from clarifying spins were combined and loaded onto 0.4-ml Con A–Sepharose columns. The columns were washed until the radioactivity of the effluent dropped to <15 cpm/ml and then eluted with 0.5 M αMMP and counted.

For endoglycosidase H (Endo H) digestions, glycopeptide was similarly solubilized and bound to Con A–Sepharose. After washing with 30 column volumes of detergent-free Con A buffer, the bound glycopeptide was eluted with 0.5 αMMP in water. The eluates were concentrated and washed repeatedly with 100 mM sodium citrate, pH 5.5, 10 mM DTT, in Centricon-3 microconcentrators (Amicon Corp., Beverly, MA). Glycopeptide samples were incubated with or without 20 mU/ml Endo H (Boehringer Mannheim) for 1 h at 37°C and fractionated on a 16.5%T/6%C polyacrylamide gel in a discontinuous Tricine/Tris buffer system (Schägger and von Jagow, 1987).

Results

Experimental Strategy

The type I transmembrane protein TGN38 (Luzio et al., 1990) contains two targeting signals mapped to its cytoplasmic and transmembrane domains (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994). The cytoplasmic signal slows—probably in concert with the membrane-spanning domain—the protein's exit from the TGN to the cell surface. It also acts as an endocytosis motif, permitting those TGN38 molecules that have escaped TGN retention to be retrieved from the cell surface. The combined effect of the two sorting signals is to confer the eponymous TGN localization on TGN38, with a low but detectable level of recycling via the plasma membrane (Bos et al., 1993; Humphrey et al., 1993, Reaves et al., 1993; Chapman and Munro, 1994).

We attempted to exploit this recycling pathway to introduce, in a process reminiscent of receptor-mediated endocytosis, membrane-impermeable, KDEL-tagged reporter peptides into the TGN of living cells (Fig. 1). To this end, we appended the c-myc–derived epitope that is recognized by mAb 9E10 (Evan et al., 1985) to the ectodomain of mature TGN38 and expressed the recombinant protein in CHO cells. Reporter peptides were disulfide bonded to 9E10 and thus could reversibly associate with mycTGN38. When added to the culture medium of transformed CHO cells, the 9E10 adaptors tethered the peptides to, and allowed their internalization in complex with, mycTGN38 "receptors" appearing at the cell surface (Fig. 1). Since 9E10 binds the myc epitope at moderately acidic pH (Fig. 2), its association with mycTGN38 is expected to remain stable within endosomes, and endocytosed peptide–9E10 conjugates should accumulate within the TGN. Addition of the membrane-permeable reductant DTT, whose effects on the secretory pathway are

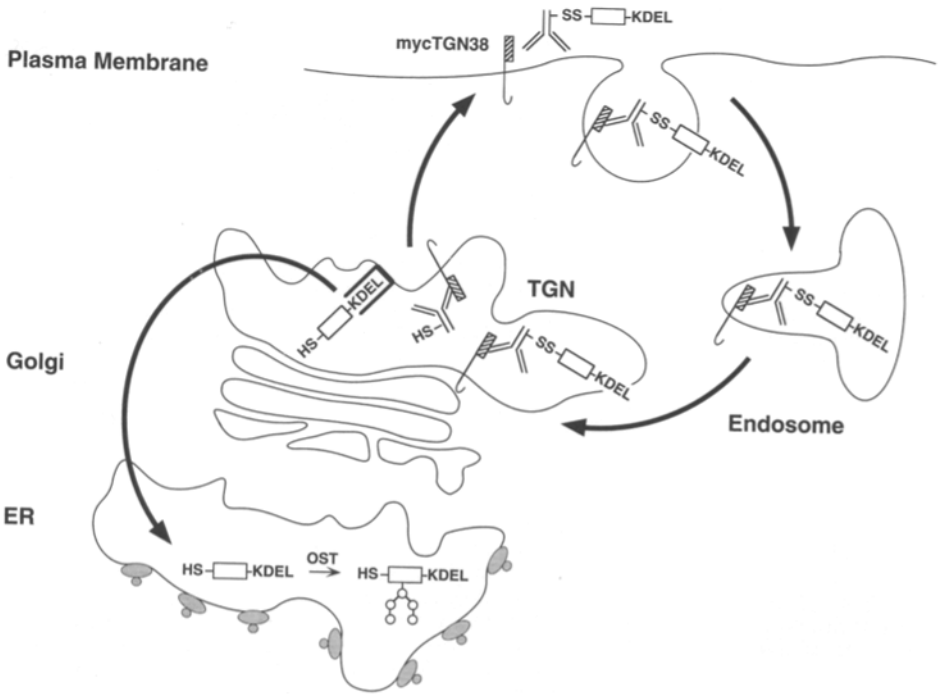

Figure 1. Experimental strategy. Myc-tagged TGN38 that has escaped TGN retention appears at the cell surface and binds anti-myc antibodies added to the cell culture medium. Reversibly coupled to these antibodies (via disulfide bonds) are synthetic peptides with the COOH-terminal sequence KDEL. Endocytosis of mycTGN38 results in its return to the TGN and in the concomitant introduction of antibody–peptide conjugates into the lumen of this compartment. Reduction of the disulfide bonds liberates the KDEL-tagged peptides from their complexes with the anti-myc antibodies. This makes them available for binding to KDEL receptors. Receptor binding in turn triggers retrograde transport to the ER, where OST can add N-linked carbohydrate to the peptides' acceptor sites.

fully reversible within minutes (Braakman et al., 1992; Chanat et al., 1993; Lodish and Kong, 1993), should cleave the disulfide bond of internalized peptide–9E10 and thus release the reporter peptides into the lumen of the TGN. Provided that functional KDEL receptors reach the distal end of the Golgi stack, they should bind the reporter peptides and retrieve them into the ER. Such retrieval events can be conveniently detected if the peptides contain an acceptor sequence for N-linked glycosylation, a process that occurs in the ER (Czichi and Lennarz, 1977; Kornfeld and Kornfeld, 1985).

Peptides as Probes for Retrograde Transport

Four structural characteristics were incorporated into peptides designed to serve as probes for retrograde transport: a retrieval signal comprising the 6 COOH-terminal amino acids of the mammalian ER resident BiP (SEKDEL; this particular sequence had been shown to confer ER retention when transplanted to secreted proteins [Munro and Pelham, 1987]), a consensus site for N-linked glycosylation (NXT or NXS), a tyrosine residue for radioiodination, and a cysteine residue to form the reversible cross-link with 9E10. This cysteine residue was restricted to the position immediately NH_2-terminal of the SEKDEL motif in an attempt to keep the peptides unavailable for binding to KDEL receptors as long as the cross-link with 9E10 remained intact.

The peptide sequence that accommodated these structural features in the most condensed form was YNCSEKDEL. The peptide bound to KDEL receptors exposed on carbonate-extracted Golgi membranes (Wilson et al., 1993) with an apparent dissociation constant of 95 nM (Fig. 3 *A*) but proved to be a very poor substrate for OST in digitonin-permeabilized rough microsomes (Fig. 4 *A*). We therefore attempted to modify the peptide's glycosylation site according to the preferences of OST (for reviews, see Kornfeld and Kornfeld, 1985; Kaplan et al., 1987). The enzyme appears to favor NXT over NXS (Bause and Legler, 1981), a small aliphatic or hydroxyl amino acid in position X (Kaplan et al., 1987), and a β turn conformation of the peptide segment carrying the glycosylation site, with the carbohydrate-accepting asparagine residue in position i+2 (Aubert et al., 1976, 1981; Bause, 1983). To redesign the peptide according to these specifications, we used a proline residue, which dominates the position i+1 of naturally occurring β turns (MacArthur and Thornton, 1991), as the nucleus for turn

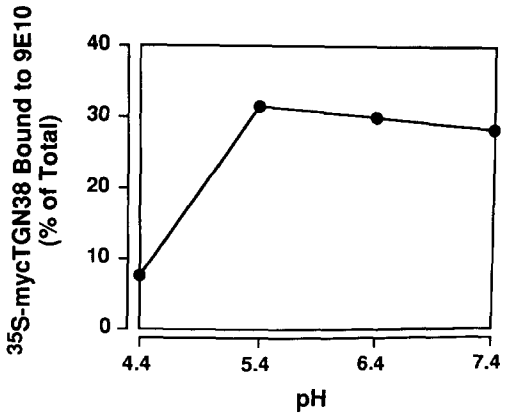

Figure 2. mAb 9E10 binds mycTGN38 at moderately acidic pH. mRNA encoding mycTGN38 was translated in 70% nuclease-treated reticulocyte lysate in the presence of 0.8 mCi/ml [^{35}S]methionine. Aliquots of the translation reaction were diluted fivefold with immunoprecipitation buffer adjusted to the indicated pH and incubated with 9E10 that had been covalently coupled to protein G beads. Immunoprecipitates were analyzed by 12% SDS-PAGE, and mycTGN38 was quantified with a PhosphorImager.

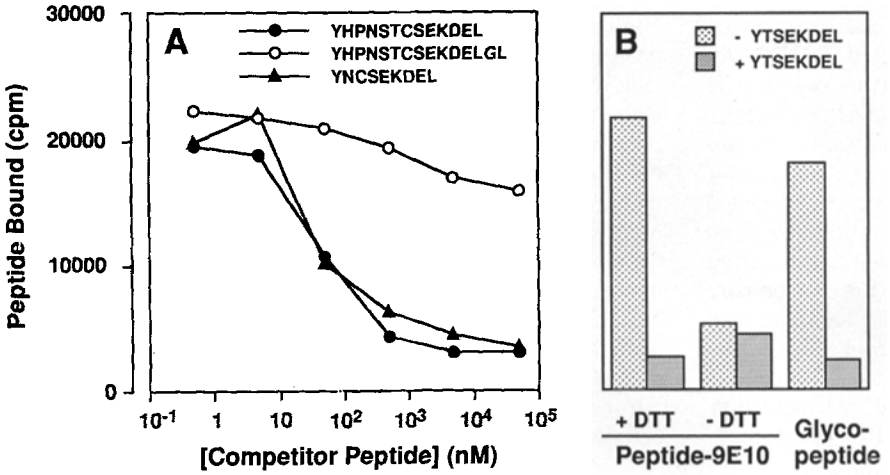

Figure 3. Reporter peptides bind to KDEL receptors in vitro. (*A*) KDEL receptor binding of the test peptide ^{125}I-YTSEKDEL (Wilson et al., 1993) in the presence of the indicated competitor peptides. Na_2CO_3-extracted CHO Golgi membranes (1 μg of protein) were incubated on ice, at pH 5.0, with 0.1 × 10^6 cpm radiolabeled test peptide and the indicated concentrations of unlabeled competitor. The membranes were pelleted, and bound radioactivity was quantified. (*B*) KDEL receptor binding of the test peptide ^{125}I-YHPNSTCSEKDEL after cross-linking to 9E10 or N-linked glycosylation. Assays were performed as in *A*, in the absence or presence of 5 × 10^4 nM unlabeled YTSEKDEL, to ascertain specific binding. Peptide–9E10 was preincubated with or without 20 mM DTT in 20 mM Tris, pH 8.0, at 37°C for 30 min before assembly of the binding assays on ice; additional DTT was omitted from the buffer in these assays. N-glycosylated ^{125}I-YHPNSTCSEKDEL was synthesized by digitonin-permeabilized rough microsomes in vitro. The glycopeptide was solubilized with Triton X-100, bound to Con A–Sepharose, eluted with αMMP in detergent-free Con A buffer, and concentrated and desalted in a Centricon-3 microconcentrator.

formation. Drawing on statistical and spectroscopic data (Aubert et al., 1976; Chou and Fasman, 1978; Gratwohl and Wüthrich, 1981; Kaplan et al., 1987; Dyson et al., 1988) as the arbiter for amino acid selection at all positions where the sequence was not dictated by the glycosylation consensus, YHPNSTCSEKDEL emerged as the optimized peptide. When compared with the original peptide, YHPNSTCSEKDEL fully retained the affinity for KDEL receptors (Fig. 3 *A*) but was indeed a superior substrate for OST (Fig. 4 *A*). A control peptide, YHPNSTCSEKDELGL, in which the KDEL signal was masked by an additional two amino acids at the COOH terminus, was glycosylated at the same rate (Fig. 4 *A*) but failed to bind to KDEL receptors (Fig. 3 *A*). The peptides were unable to cross membranes by simple diffusion, as permeabilization of the microsomal membrane was absolutely required for glycosylation to occur (Fig. 4 *B*).

The peptides were labeled with ^{125}I and coupled to 9E10. With its cysteine cross-linked to the antibody, YHPNSTCSEKDEL was unable to bind to KDEL receptors; binding, however, was promptly restored after reductive cleavage of the cross-linker (Fig. 3 *B*). A core oligosaccharide attached to the peptide's asparagine residue, in contrast, did not interfere with receptor binding (Fig. 3 *B*). This somewhat surprising finding permitted the strong prediction that if YHPNSTCSEKDEL is bound by KDEL receptors in the TGN, transported into the ER, and glycosylated, the glycopeptide should be retained by the cells (owing to its functional KDEL signal) rather than resecreted like a bulk flow marker devoid of sorting signals (Wieland et al., 1987).

Endocytosis of Peptide–9E10 Conjugates

In stably transformed CHO cells, mycTGN38 was found, by confocal scanning microscopy, to colocalize with endogenous TGN38 in the presumptive TGN (Fig. 5, compare *A* and

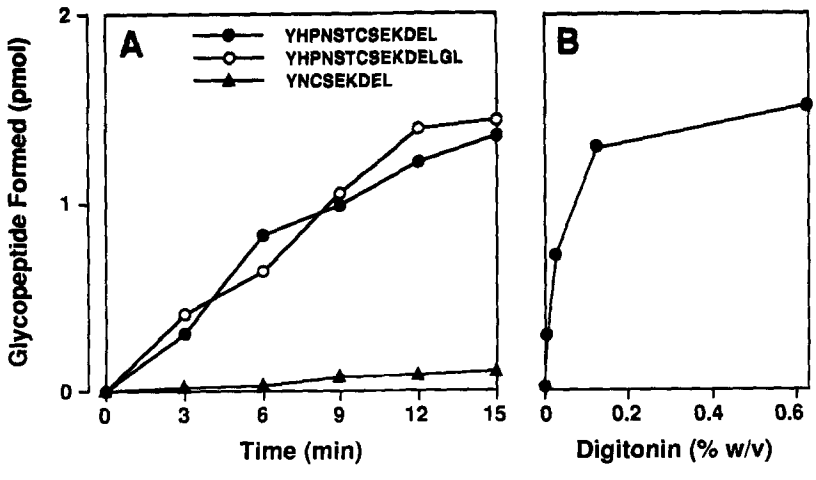

Figure 4. Reporter peptides are glycosylated in vitro. (*A*) Time course of glycosylation. Rough microsomes from chicken liver were permeabilized with 0.625% digitonin and incubated with the indicated ^{125}I-labeled peptides at 25°C, in a final volume of 100 μl; peptides were present at 3 μM. The glycosylation reactions were stopped on ice with 0.5% (vol/vol) Triton X-100; glycopeptides were isolated by batch adsorption to Con A–Sepharose, eluted with αMMP, and counted. The background signals observed after 15-min incubations on ice corresponded to ~10% of the maximum assay signals obtained with proline-containing peptides and to between 60 and 70% of the signal obtained with YNCSEKDEL. Data are plotted after subtraction of these background values. (*B*) Permeabilization of rough microsomes is required for peptide glycosylation. Rough microsomes in complete assay buffer were incubated on ice with the indicated concentrations of digitonin for 20 min. ^{125}I-YHPNSTCSEKDEL was added to 3 μM thereafter. The reactions were then shifted to 25°C for 15 min, and glycopeptide formation was quantified as in *A*. A membrane-independent signal of 2,815 cpm, corresponding to 11% of the maximal signal, was subtracted from the data. The signal obtained in the presence of microsomes, but in the absence of digitonin, was 2,843 cpm.

B). (The polyclonal antiserum used in these experiments recognizes both endogenous and myc-tagged TGN38. Attempts to stain endogenous protein exclusively by using an antiserum against an epitope unique to TGN41, an isoform of TGN38 [Wilde et al., 1992], were unsuccessful. The antiserum failed to recognize TGN41 in CHO cells.) Similar staining of the TGN was observed when antibodies directed against the myc epitope were added to the culture medium of intact cells, and material internalized over a 2-h period was decorated with fluorescent antibodies (Fig. 5, compare *C* and *D*): its round trip itinerary to the plasma membrane indeed enabled mycTGN38 to ferry 9E10 from the cell surface to the TGN.

Quantitative data for surface binding and internalization were obtained with ^{125}I-labeled peptide–9E10 conjugates. High affinity binding to transformed CHO cells at 4°C approached saturation at an IgG concentration of ~250 nM (Fig. 6 *A*). A Scatchard analysis (Fig. 6 *A*, *inset*) revealed a binding constant of 49 nM and a single type of binding site present at 5,800 copies per cell surface. When cells to which peptide–9E10 had bound were maintained at 4°C for 2 h and then treated with the membrane-impermeant reducing agent MesNa (Schmid and Smythe, 1991), >90% of the initially cell-associated radioactivity was removed (data not shown), indicating that the conjugates had remained at the cell surface during extended incubation at 4°C (the radiolabel resided in the conjugate's peptide moiety and thus was liberated after cleavage of the cross-linker). In contrast, when a 2-h incubation at 37°C preceded the exposure to MesNa, a certain fraction of peptide–9E10 became resistant to the

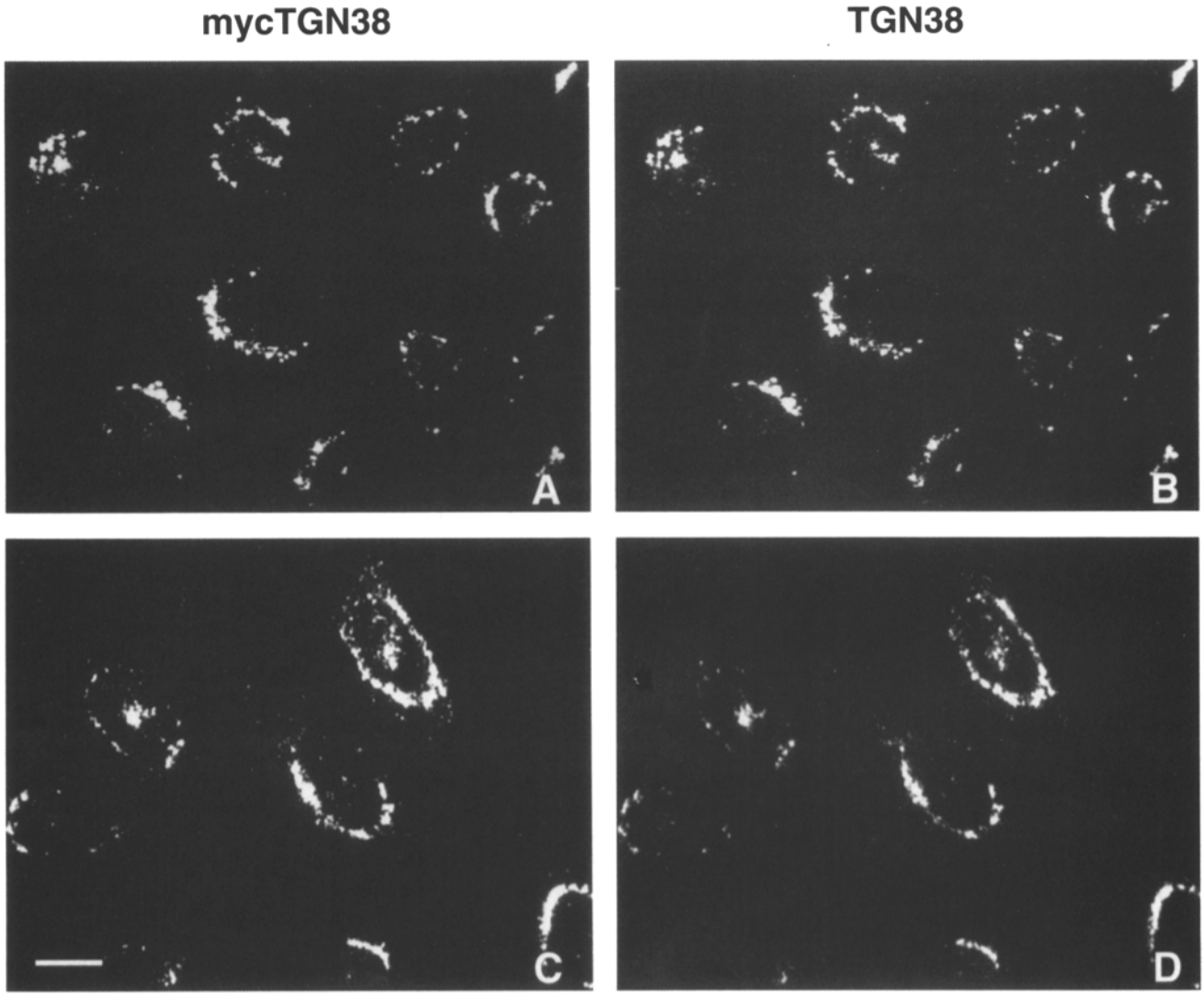

Figure 5. mycTGN38 cycles between the TGN and the cell surface of transformed CHO cells. (*A* and *B*) Stably transformed CHO cells expressing mycTGN38 were double-labeled with a polyclonal antiserum against TGN38 and the anti-c-myc mAb 9E10. The polyclonal antiserum gives rise to comparably faint and intense signals from TGN38 and mycTGN38, respectively. Attempts to stain exclusively the endogenous protein, using an antiserum against an epitope unique to the TGN38 isoform TGN41 and not present in mycTGN38, were unsuccessful; the antiserum failed to recognize TGN41 in CHO cells. (*C* and *D*) Transformed CHO cells grown on coverslips were incubated with 150 nM 9E10 in complete medium for 2 h at 37°C, followed by a 15-min chase in medium without 9E10. Compare the distribution of internalized 9E10, which was visualized with Texas red–conjugated secondary antibodies (*C*), with the steady-state localization of endogenous TGN38 and mycTGN38, as revealed by indirect immunostaining with the polyclonal antiserum (*D*). All images were acquired by laser scanning confocal microscopy. Bar, 10 μm.

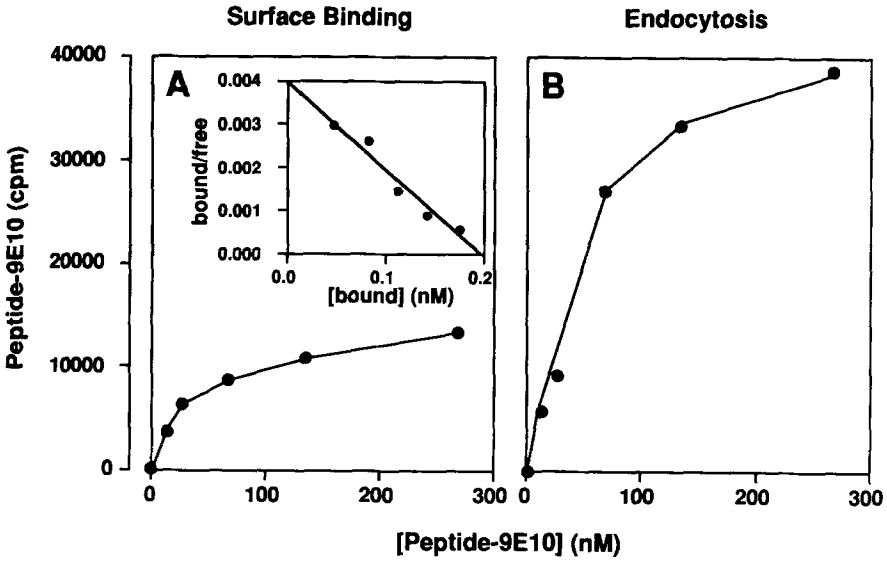

Figure 6. Transformed CHO cells bind and internalize peptide-9E10. Transformed CHO cells (5×10^6 cells per data point) were incubated in 0.5 ml of cysteine-free medium with the indicated concentrations of 9E10 to which ^{125}I-YHPNSTCSEKDEL had been coupled with SPDP. (*A*) Surface binding of peptide-9E10. For each peptide-9E10 concentration, measurements of cell-bound radioactivity were taken after a 2-h incubation at 4°C in the absence or presence of a 50-fold molar excess of unlabeled 9E10. The differences between these measurements are displayed. The inset shows a Scatchard analysis of the same data. (*B*) Endocytosis of peptide-9E10. For each peptide-9E10 concentration, measurements of MesNa-resistant radioactivity were taken after a 2-h incubation at 37°C in the absence or presence of a 50-fold molar excess of unlabeled 9E10. The differences between these measurements are displayed.

reductant (Fig. 6 *B*). MesNa resistance can be observed only after sealing of the susceptible disulfide in a membrane-bound compartment (Bretscher and Lutter, 1988; Schmid and Smythe, 1991) and thus provides an index for endocytic uptake.

A comparison of high affinity binding and endocytosis at saturating peptide-9E10 concentrations (Fig. 6) showed that the mycTGN38 pool at the cell surface was internalized only about three times in 2 h, corresponding to a $t_{1/2}$ for endocytosis of ~20 min. This exceeds that for recycling cell surface receptors by about fivefold (Steinman et al., 1983)—a kinetic limit on endocytic capacity that further aggravated the effect of the low surface density of mycTGN38, as inferred from the binding experiments displayed in Fig. 6 *A*. Consequent to these restrictions on mycTGN38-mediated endocytosis, the nonspecific fluid-phase uptake of peptide-9E10 (measured in the presence of a 50-fold excess of unlabeled 9E10) appeared relatively prominent in our system. At a level of 0.013% of the load administered to 5×10^6 CHO cells over 2 h, it was somewhat lower than previous estimates for fibroblasts (Steinman et al., 1974; 1983) but still exceeded mycTGN38-mediated uptake about threefold at a peptide-9E10 concentration of 150 nM, the standard in our experiments. This uptake of material not bound to mycTGN38 will deserve some consideration in attempts to gauge the efficiency of retrograde transport from the TGN to the ER (see the legend to Fig. 7).

Movement of Peptides Beyond the TGN

YHPNSTCSEKDEL coupled to 9E10 was offered to transformed CHO cells for targeted delivery and release into the lumen of the TGN. Release of the peptide from its complex with 9E10 is the prerequisite for binding to KDEL receptors (Fig. 3 *B*). Binding, in turn, should trigger retrograde transport of receptor and ligand to the ER (Lewis and Pelham, 1992*b*) and thus place the peptide within the reach of OST. This scenario ostensibly hinges upon the presence of KDEL receptors in the TGN and upon the ability of the machinery for retrograde transport to retrieve them from the distal end of the secretory pathway—two premises that KDEL-dependent glycosylation of the reporter peptide would substantiate.

Glycopeptide was formed indeed. After reductive release of YHPNSTCSEKDEL (a 10-min pulse of 20 mM DTT was necessary and sufficient to cleave >90% of the endocytosed peptide-9E10 load [see Materials and Methods] and was required for maximum glycopeptide formation during subsequent incubation at 37°C), the cells accumulated ^{125}I-labeled material that bound to immobilized Con A and could be eluted from the lectin with the hapten sugar αMMP (Fig. 7). Two independent pieces of evidence confirm that the Con A-bound material was indeed peptide that had undergone N-linked glycosylation. First, treatment of the cells with tunicamycin (to inhibit synthesis of the dolichol-linked oligosaccharide that OST uses for transfer to polypeptides) diminished the Con A-bound fraction about fivefold (Fig. 8, compare columns *1* and *5*). Second, SDS-PAGE of the Con A eluate demonstrated a large reduction in peptide mobility—the expected consequence of adding a bulky carbohydrate chain to a small molecule—and its reversal by Endo H (Fig. 7, *inset*).

The glycopeptide's sensitivity to Endo H indicated that the high mannose structure of the core oligosaccharide transferred in the ER had been more or less preserved during the experiment. As the conversion of N-linked carbohydrates into Endo H-resistant structures is thought to be initiated in the medial Golgi (Dunphy et al., 1985; Kornfeld and Kornfeld, 1985), lack of such processing suggested that the major fraction of KDEL-tagged glycopeptide had not reached this compartment. And this is the result predicted from the observation that YHPNSTCSEKDEL retained its affinity for KDEL receptors after glycosylation (Fig. 3 *B*).

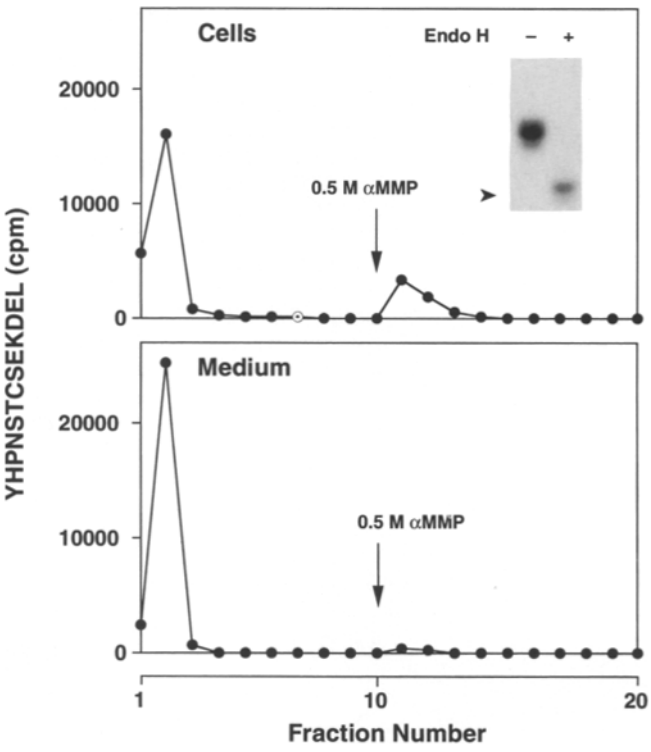

Figure 7. Transformed CHO cells glycosylate a fraction of the internalized peptide load and retain the glycosylated species. To obtain an estimate of retrieval efficiency from the TGN, our standard conditions (see Materials and Methods) were modified in this experiment. The aim was to reduce or eliminate fluid-phase uptake of peptide–9E10 and thus the introduction of radiolabeled peptide into compartments that may be unaccessible to the KDEL system. Transformed CHO cells (100×10^6 cells in 10 ml of cysteine-free medium) were allowed to bind peptide–9E10 on ice for 30 min. The cells were then collected through a cushion of cold medium, resuspended, and shifted to 37°C for 2 h to permit endocytosis of prebound peptide–9E10. After a 10-min pulse with 20 mM DTT and a 2.5-h chase at 37°C, cells and medium were separated and processed for Con A–Sepharose chromatography. The columns were loaded and washed in Con A buffer; the switch to elution buffer containing 0.5 M αMMP is indicated. Fraction size is 1 ml. To facilitate a comparison of the two chromatograms, the noninteracting material in the medium (which, owing to the larger sample volume, eluted in 22 column fractions) has been plotted in a way that its elution volume matches that of the flow-through from the cell sample. The Con A–binding material retained by the cells is characterized in the inset. Aliquots of the αMMP eluate from an incubation with 100×10^6 transformed CHO cells under standard conditions as described in Materials and Methods were either mock digested (–) or digested (+) with Endo H and fractionated by 16.5%T/6%C SDS-PAGE in a discontinuous Tricine/Tris buffer system (Schägger and von Jagow, 1987). ^{125}I-labeled peptides were visualized by autoradiography. The arrowhead indicates the migration position of unmodified YHPNSTCSEKDEL.

A corollary to efficient retention early in the secretory pathway was that virtually no glycopeptide should be secreted into the medium. When passed over Con A–Sepharose, the medium indeed contained almost exclusively noninteracting ^{125}I-labeled material (Fig. 7), comprising between 55 and 70% of the originally internalized YHPNSTCSEKDEL load. Of the 30–45% remaining intracellular, 20–25% received an N-linked carbohydrate chain, the diagnostic of a passage through the ER. Thus, 5–10% of the total endocytosed peptide load typically underwent glycosylation, a figure setting a lower limit to the efficiency of retrograde transport from the TGN to the ER. This figure might, however, be an underestimation of the true efficiency of retrieval: First (and trivially), not all peptide molecules that reached the ER may actually have undergone glycosylation. Second, some mycTGN38 molecules transporting peptide–9E10 conjugates may not have migrated to the TGN when the cross-linker was cleaved. The peptides released from these conjugates scored as internalized despite being unavailable for retrieval (see the following discussion) and thus decreased the apparent efficiency of the process.

Retrieval Occurs from the TGN and Requires a Functional KDEL Signal

Although the aforementioned experiments show that an endocytosed KDEL-tagged peptide can eventually reach the ER, they do not show that the peptide's movement from the cell surface to the ER can in fact be decomposed into the two steps outlined in Fig. 1, i.e., delivery from the cell surface to the TGN (via the interaction of peptide–9E10 with mycTGN38) and retrieval from the TGN to the ER (via the interaction of KDEL with its receptor). To address this point, we performed two sets of control experiments. In the first set, mycTGN38-mediated uptake of YHPNSTCSEKDEL was eliminated, either by blocking mycTGN38 at the cell surface with an excess of 9E10 or by using free peptide instead of peptide coupled to 9E10. Because of the relative prominence of fluid-phase vis a vis receptor-mediated uptake, the total amount of radiolabel internalized by the cells was attenuated only slightly under these conditions (to ~70% of the original value) but glycopeptide formation dropped dramatically (to <20%; Fig. 8, compare columns *2* and *3* with column *1*): placing the peptide into the appropriate cellular compartment, i.e., a compartment served by the KDEL system, was a prerequisite for retrieval. The fact that internalized TGN38 (which is sorted out of the endocytic pathway and returned to the TGN [Chapman and Munro, 1994]) but not bulk-phase endocytosis could mediate access to this compartment strongly suggests that the starting point for KDEL-mediated retrieval was indeed the TGN.

The second set of control experiments was aimed at the role of the KDEL system itself. To this end, we introduced YHPNSTCSEKDELGL, a reporter peptide that does not bind to KDEL receptors (Fig. 3 *A*), into the TGN of transformed CHO cells. Although YHPNSTCSEKDELGL and YHPNSTCSEKDEL were internalized to virtually the same extent (cellular uptake of the two peptides differed by <15%), and were equally good substrates for OST in vitro (Fig. 4 *A*), only the peptide equipped with a functional KDEL signal received N-linked carbohydrate in vivo (Fig. 8, compared columns *1* and *4*). The failure of YHPNSTCSEKDELGL to undergo glycosylation in vivo is thus, in all likelihood, due to its failure to reach the ER.

The strict KDEL dependence of peptide glycosylation bears an additional important consequence. Assume for a moment that YHPNSTCSEKDEL became glycosylated only because it bypassed membrane-bound transport intermediates and reached the ER via alternative pathways such as sim-

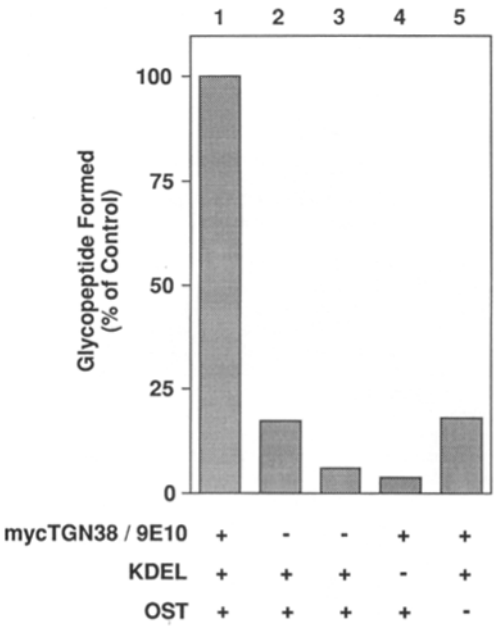

Figure 8. Requirements for glycosylation of reporter peptides in vivo. Glycopeptide formation was quantified by chromatography of cell lysates and media on a Con A–Sepharose, followed by elution of bound radioactivity with αMMP. Since under all experimental conditions the media contained only minute (and invariant) amounts of Con A–binding material, only data from cell lysates are displayed. With the exceptions of the experiments shown in columns *2* and *3*, cellular uptake of peptide–9E10 was unaffected by the different experimental conditions; the cell-associated specific radioactivities measured after reduction of peptide–9E10 were within 15% of that shown in column *1* (standard conditions as described in Materials and Methods). (Columns *2* and *3*) The mycTGN38–9E10 interaction required for targetting YHPNSTCSEKDEL–9E10 to the TGN was eliminated by adding a 50-fold molar excess of unlabeled 9E10 to the cell culture medium during endocytosis (column *2*), or by using free peptide instead of peptide–9E10 (column *3*). (Column *4*) YHPNSTCSEKDELGL, a reporter peptide with a nonfunctional KDEL signal, was used instead of YHPNSTCSEKDEL, under otherwise standard conditions. (Column *5*) Cells were treated with 10 μg/ml tunicamycin throughout the experiment to inhibit N-linked glycosylation by OST.

ple diffusion or leakage into the cytosol, followed by reimport into the ER lumen via a peptide pump, or because it was transported in complex with some (hypothetical) constitutively recycling component of the secretory pathway. If any of these KDEL-independent pathways were open to YHPNSTCSEKDEL, however, the same would be expected to hold true for the structurally very similar peptide YHPNSTCSEKDELGL. The diametrically opposed behavior of the two reporter peptides therefore provides strong evidence that retrieval from the far end of the Golgi stack uses the same mechanism that is understood to operate from the near end: receptor-mediated vesicular transport.

Discussion

Circumstantial support for the proposition, originally made on theoretical grounds (Rothman, 1981), that retrograde transport routes coexist with anterograde ones throughout the Golgi stack has gradually mounted during recent years. The suspicion that proteins marked for ER residence might penetrate the Golgi complex beyond the "salvage compartment" and nevertheless be retrieved became concrete with the case of calreticulin, a KDEL-tagged glycoprotein that carries N-linked oligosaccharides with terminal galactose residues (Peter et al., 1992). Other possible cases are certain bacterial and herbal toxins that are thought to use the protein import and folding machinery of the ER for translocation into the cytosol (Pastan et al., 1992; Pelham et al., 1992). To do so, the toxins would have to reach the ER lumen in the first place and to this end were postulated to exploit sequentially the processes of receptor-mediated endocytosis and KDEL-mediated retrieval, switching between the two in the TGN, where the endocytic and the secretory pathways intersect. In fact, a few of the toxins in question possess the COOH-terminal tetrapeptide KDEL or a related sequence (Chaudhary et al., 1990; Pelham et al., 1992), and addition of KDEL to those that lack the signal tends to increase their toxicity (Wales et al., 1993).

The experiments reported here provide more direct evidence for KDEL receptor–mediated retrograde transport from the TGN to the ER and thus constitute a functional complement to morphologic studies indicating the presence of KDEL receptors throughout the Golgi stack (Griffiths et al., 1994). To define the starting point for retrieval as precisely as possible, KDEL-tagged reporter peptides were targeted to the TGN in complex with a marker protein for this compartment, TGN38 (Luzio et al., 1990). To define the end point, the peptides were equipped with a glycosylation site probing for encounters with an ER marker, OST (Czichi and Lennarz, 1977). KDEL-dependent addition of N-linked carbohydrate to the peptides' acceptor sites, as observed, suggests that retrieval pathways can indeed originate from cisternae throughout the Golgi stack, including those at the stack's *trans*-most pole. The stack would then provide multiple opportunities to extract escaped ER residents, consistent with the idea that high fidelity sorting of secreted from ER proteins is achieved in a multistage process akin to fractional distillation (Rothman, 1981).

If this notion is correct, however, would not an ER resident's repeated exposure to Golgi enzymes (including those concentrated at the distal pole) ultimately result in a detectable imprint on its carbohydrate structure? We believe that, owing to the very nature of iterative sorting, this is true only to a limited degree. Consider a multistage sorting device in which the efficiency of each individual step is constant and on the order of 98% (in the case of the Golgi complex this would imply that 1 of 50 ER residents slips through at each successive retrieval opportunity; the figure is taken from studies on receptor-mediated endocytosis [Anderson et al., 1977], the only receptor-mediated process for which data on sorting efficiency are available). The probability for an escaped ER resident to encounter a certain Golgi enzyme then decreases exponentially with the enzyme's distance from the ER: from 0.02 for one retrieval step interposed between the ER and the enzyme's compartment of residence to $(0.02)^2$ for two interposed retrieval steps to $(0.02)^3$ or 8 in 10^6 for three interposed steps. If the ER resident undergoes about five escape–retrieval cycles per day (a number based on the secretion rate for a truncated, KDEL-less version of BiP [Munro and Pelham, 1987]), the cumulative probability for becoming modified by an enzyme located a single retrieval

step downstream is ~10%, whereas that for becoming modified by an enzyme located two or more steps downstream is <0.2%. Carbohydrate modifications introduced by proximal Golgi enzymes should thus become readily detectable in prolonged pulse-chase experiments, whereas those introduced by distal enzymes should not. And this is exactly what is observed (Jackson et al., 1993).

The lack of discernible traces of a *trans*-Golgi passage in the bulk of ER residents therefore does not necessarily contradict a model of iterative sorting in the Golgi complex. In fact, the elusiveness of distal Golgi modifications could simply reflect the dramatic refinement of protein composition across the stack that such a model predicts. In a strict sense, however, despite strong evidence that resident proteins continually leave the ER and have to be retrieved from downstream (Pelham 1988, 1989; Lewis and Pelham, 1992*b*; Jackson et al., 1993; Gaynor et al., 1994), despite a clear appreciation of the magnitude of the sorting problem at the ER-Golgi interface and the elegance and economy of a multistage solution (Rothman, 1981), and despite our demonstration that the potential for such a solution exists, the question as to the extent to which it is actually realized must remain undecided. Only the notoriously difficult demonstration that ER residents (in addition to calreticulin) use retrieval opportunities from the distal Golgi stack, and that eliminating these opportunities has functional consequences, could provide a definitive answer.

We wish to thank Tobias Hohl for help with the OST assay, Martin Wiedmann for advice on microsome preparation and in vitro translation, J. Paul Luzio for rat TGN38 cDNA, George Banting for antisera against TGN38 and TGN41, and Zvulun Elazar, Morten Søgaard, Thomas Söllner, and Gary Tanigawa for criticism.

This work was supported by grants from the National Institutes of Health and by the Mathers Charitable Foundation. G. Miesenböck is an Erwin Schrödinger Fellow of the Austrian Science Foundation.

Received for publication 12 September 1994 and in revised form 23 January 1995.

References

Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351–364.

Aubert, J. P., G. Biserte, and M. H. Loucheux-Lefebvre. 1976. Carbohydrate-peptide linkage in glycoproteins. *Arch. Biochem. Biophys.* 175:410–418.

Aubert, J. P., N. Helbecque, and M. H. Loucheux-Lefebvre. 1981. Circular dichroism studies of synthetic Asn-X-Ser/Thr-containing peptides: structure-glycosylation relationship. *Arch. Biochem. Biophys.* 208:20–29.

Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell.* 39:405–416.

Balch, W. E., J. M. McCaffery, H. Plutner, and M. G. Farquhar. 1994. Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell.* 76:841–852.

Bause, E. 1983. Structural requirements of N-glycosylation of proteins: studies with proline peptides as conformational probes. *Biochem. J.* 209:331–336.

Bause, E., and G. Legler. 1981. The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the N-glycosylation step during glycoprotein biosynthesis. *Biochem. J.* 195:639–644.

Boer, P. H., H. Potten, C. N. Adra, K. Jardine, G. Mullhofer, and M. W. McBurney. 1990. Polymorphisms in the coding and noncoding regions of murine Pgk-1 alleles. *Biochem. Genet.* 28:299–308.

Bos, K., C. Wraight, and K. K. Stanley. 1993. TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2219–2228.

Braakman, I., J. Helenius, and A. Helenius. 1992. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1717–1722.

Brands, R., M. D. Snider, Y. Hino, S. S. Park, H. V. Gelboin, and J. E. Rothman. 1985. Retention of membrane proteins by the endoplasmic reticulum. *J. Cell Biol.* 101:1724–1732.

Bretscher, M. S., and R. Lutter. 1988. A new method for detecting endocytosed proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4087–4092.

Chanat, E., U. Weiss, W. B. Huttner, and S. A. Tooze. 1993. Reduction of the disulfide bond of chromogranin B (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathway. *EMBO (Eur. Mol. Biol Organ.) J.* 12:2159–2168.

Chapman, R. E., and S. Munro. 1994. Retrieval of TGN proteins from the cell surface requires endosomal acidification. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2305–2312.

Chaudhary, V. K., Y. Jinno, D. FitzGerald, and I. Pastan. 1990. Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 87:308–312.

Chou, P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47:45–148.

Copeland, C. S., K. P. Zimmer, K. R. Wagner, G. A. Healy, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell.* 53:197–209.

Czichi, U., and W. J. Lennarz. 1977. Localization of the enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. *J. Biol. Chem.* 252:7901–7904.

Dean, N., and H. R. B. Pelham. 1990. Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* 111:369–377.

Dunphy, W. G., and J. E. Rothman. 1985. Compartmental organization of the Golgi stack. *Cell.* 42:13–21.

Dunphy, W. G., R. Brands, and J. E. Rothman. 1985. Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell.* 40:463–472.

Dyson, H. J., M. Rance, R. A. Houghten, R. A. Lerner, and P. E. Wright. 1988. Folding of immunogenic peptide fragments of proteins in water solution. I. Sequence requirements for the formation of a reverse turn. *J. Mol. Biol.* 201:161–200.

Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 4:2843–2850.

Gaynor, E. C., S. te Heesen, T. R. Graham, M. Aebi, and S. D. Emr. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653–665.

Grathwohl, C., and K. Wüthrich. 1981. NMR studies of the rates of proline cis-trans isomerization in oligopeptides. *Biopolymers.* 20:2623–2633.

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

Griffiths, G., M. Ericsson, J. Krijnse-Locker, T. Nilsson, B. Goud, H.-D. Söling, B. L. Tang, S. H. Wong, and W. Hong. 1994. Localization of the Lys, Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells. *J. Cell Biol.* 127:1557–1574.

Hsu, V. W., N. Shah, and R. D. Klausner. 1992. A brefeldin A-like phenotype is induced by the overexpression of a human ERD-2-like protein, ELP-1. *Cell.* 69:625–635.

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

Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277–307.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121:317–333.

Kaplan, H. A., J. K. Welply, and W. J. Lennarz. 1987. Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly. *Biochim. Biophys. Acta.* 906:161–173.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.

Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115:887–903.

Lewis, M. J., and H. R. B. Pelham. 1990. A human homologue of the yeast HDEL receptor. *Nature (Lond.).* 348:162–163.

Lewis, M. J., and H. R. B. Pelham. 1992*a*. Sequence of a second human KDEL receptor. *J. Mol. Biol.* 226:913–916.

Lewis, M. J., and H. R. B. Pelham. 1992*b*. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell.* 68:353–364.

Lewis, M. J., S. J. Turco, and M. Green. 1985. Structure and assembly of the endoplasmic reticulum: biosynthetic sorting of endoplasmic reticulum proteins. *J. Biol. Chem.* 260:6926–6931.

Lodish, H. F., and N. Kong. 1993. The secretory pathway is normal in dithiothreitol-treated cells, but disulfide proteins are reduced and reversibly retained in the endoplasmic reticulum. *J. Biol. Chem.* 268:20598–20605.

Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network (TGN38). *Biochem. J.* 270:97–102.

MacArthur, M. W., and J. M. Thornton. 1991. Influence of proline residues on protein conformation. *J. Mol. Biol.* 218:397–412.
Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
Nilsson, T., M. R. Jackson, and P. A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707–718.
Nilsson, T., M. Pypaert, M. H. Hoe, P. Slusarewicz, E. G. Berger, and G. Warren. 1993. Overlapping distribution of two glycosyltranserases in the Golgi apparatus of HeLa cells. *J. Cell Biol.* 120:5–13.
Pastan, I., V. Chaudhary, and D. J. Fitzgerald. 1992. Recombinant toxins as novel therapeutic agents. *Annu. Rev. Biochem.* 61:331–354.
Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.
Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1–23.
Pelham, H. R. B., L. M. Roberts, and J. M. Lord. 1992. Toxin entry: how reversible is the secretory pathway? *Trends Cell Biol.* 2:183–185.
Peter, F., P. N. Van, and H.-D. Söling. 1992. Different sorting of Lys-Asp-Glu-Leu proteins in rat liver. *J. Biol. Chem.* 267:10631–10637.
Ponnambalam, S., C. Rabouille, J. P. Luzio, T. Nilsson, and G. Warren. 1994. The TGN38 glycoprotein contains two non-overlapping signals that mediate localization to the trans-Golgi network. *J. Cell Biol.* 125:253–258.
Quinn, P., G. Griffiths, and G. Warren. 1984. Density of newly synthesized plasma membrane proteins in intracellular membranes. II. Biochemical studies. *J. Cell Biol.* 98:2142–2147.
Reaves, B., M. Horn, and G. Banting. 1993. TGN38/41 recycles between the cell surface and the TGN: brefeldin A affects its rate of return to the TGN. *Mol. Biol. Cell.* 4:93–105.
Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257–288.
Rothman, J. E. 1981. The Golgi apparatus: two organelles in tandem. *Science (Wash. DC).* 213:1212–1219.
Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.
Schmid, S. L., and E. Smythe. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J. Cell Biol.* 114:869–880.
Schutze, M.-P., P. A. Peterson, and M. R. Jackson. 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1696–1705.
Steinman, R. M., J. M. Silver, and Z. A. Cohn. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. *J. Cell Biol.* 63:949–969.
Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1–27.
Strous, G. J., P. van Kerkhof, R. Brok, J. Roth, and D. Brada. 1987. Glucosidase II, a protein of the endoplasmic reticulum with high mannose oligosaccharide chains and a rapid turnover. *J. Biol. Chem.* 262:3620–3625.
von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683–4690.
Wales, R., L. M. Roberts, and J. M. Lord. 1993. Addition of an endoplasmic reticulum retrieval sequence to ricin A chain significantly increases its cytotoxicity to mammalian cells. *J. Biol. Chem.* 268:23968–23990.
Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84–93.
Warren, G. 1987. Signals and salvage sequences. *Nature (Lond.).* 327:17–18.
Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell.* 50:289–300.
Wilde, A., B. Reaves, and G. Banting. 1992. Epitope mapping of two isoforms of a trans Golgi network specific integral membrane protein TGN38/41. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 313:235–238.
Wilson, D. W., M. J. Lewis, and H. R. B. Pelham. 1993. pH-dependent binding of KDEL to its receptor in vitro. *J. Biol. Chem.* 268:7465–7468.
Wong, S. H., and W. Hong. 1993. The SXYQRL sequence in the cytoplasmic domain of TGN38 plays a major role in trans-Golgi network localization. *J. Biol. Chem.* 268:22853–22862.