Retrograde Transport of Golgi-localized Proteins to the ER

Nelson B. Cole,* Jan Ellenberg,* Jia Song,* Diane DiEuliis,‡ and Jennifer Lippincott-Schwartz*

*Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development; and ‡National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Abstract. The ER is uniquely enriched in chaperones and folding enzymes that facilitate folding and unfolding reactions and ensure that only correctly folded and assembled proteins leave this compartment. Here we address the extent to which proteins that leave the ER and localize to distal sites in the secretory pathway are able to return to the ER folding environment during their lifetime. Retrieval of proteins back to the ER was studied using an assay based on the capacity of the ER to retain misfolded proteins. The lumenal domain of the temperature-sensitive viral glycoprotein VSVGtsO45 was fused to Golgi or plasma membrane targeting domains. At the nonpermissive temperature, newly synthesized fusion proteins misfolded and were retained in the ER, indicating the VSVGtsO45 ectodomain was sufficient for their retention within the ER. At the permissive temperature, the fusion proteins were correctly delivered to the Golgi complex or plasma membrane, indicating the lumenal epitope of VSVGtsO45 also did not interfere with proper targeting of these molecules. Strikingly, Golgi-localized fusion proteins, but not VSVGtsO45 itself, were found to redistribute back to the ER upon a shift to the nonpermissive temperature, where they misfolded and were retained. This occurred over a time period of 15 min–2 h depending on the chimera, and did not require new protein synthesis. Significantly, recycling did not appear to be induced by misfolding of the chimeras within the Golgi complex. This suggested these proteins normally cycle between the Golgi and ER, and while passing through the ER at 40°C become misfolded and retained. The attachment of the thermosensitive VSVGtsO45 lumenal domain to proteins promises to be a useful tool for studying the molecular mechanisms and specificity of retrograde traffic to the ER.

Newly synthesized proteins in the ER undergo dynamic folding/unfolding reactions as they fold and assemble into functional protein complexes. Such reactions are mediated by ER-specific chaperones and folding enzymes that help prevent nonproductive interactions and irreversible aggregation of proteins (Rothman, 1989; Gething and Sambrook, 1992). Because only correctly folded proteins leave the ER and proceed to the Golgi complex and beyond, whereas incompletely folded, misfolded, or unassembled proteins are retained and/or degraded, the ER serves an important "quality control" function in secretory traffic (Hurtley and Helenius, 1989; Doms et al., 1993). Newly synthesized proteins are thereby kept in contact with the extensive and efficient ER folding machinery until they are conformationally mature, whereas nonfunctional and incomplete protein complexes generally have no access into the secretory pathway.

Given that the ER is the sole compartment that exhibits these quality control functions, mechanisms that retrieve proteins back to the ER could be important for monitoring the fidelity of a wide variety of proteins that have left this compartment. For example, unassembled class I molecules exported from the ER have been found to cycle back to the ER, whereas properly assembled class I molecules are efficiently transported to the cell surface (Hsu et al., 1991). The inventory of other proteins exported from the ER that return to this quality control environment during their lifetime (to undergo possible further modifications and/or degradation) is still incomplete. Many membrane proteins that function at the interface of the ER and Golgi complex, for example, have been found to constitutively cycle between the ER and the Golgi complex. These proteins include: the KDEL receptor (KDELR)[1], which retrieves soluble ER resident proteins that have escaped into the secretory pathway (Semenza et al., 1990; Lewis and Pelham, 1992); vesicle *N*-ethylmaleimide–sensitive factor attachment protein receptors v-SNAREs involved in ER to Golgi transport (Rothman, 1994; Lewis and Pelham, 1996); and putative cargo receptors, such as the mannose-

Address all correspondence to Jennifer Lippincott-Schwartz, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bldg. 18T, Room 101, Bethesda, MD 20892. Tel.: (301) 496-6368. Fax: (301) 496-0078. E-mail: jlippin@helix.nih.gov

1. *Abbreviations used in this paper*: BFA, brefeldin A; COP, coat protein; endo H, endoglycosidase H; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; HA, hemagglutinin; IL2R, interleukin-2 receptor; KDELR, KDEL receptor; KDELRm, KDELR mutant; Mann II, mannosidase II; v-SNARE, vesicle-soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; VSV, vesicular stomatitis virus.

binding protein endoplasmic reticulum–Golgi intermediate compartment (ERGIC)53/p58 (Arar et al., 1995; Itin et al., 1996; Lahtinen et al., 1996), the yeast Emp47p (Schroder et al., 1995), and p24 family members (Schimmoller et al., 1995; Blum et al., 1996). Golgi to ER retrograde trafficking of these proteins could reflect a highly selective process, or alternatively represent a generalized bulk recycling system.

To study the capacity of proteins targeted to the Golgi complex and beyond to return to the folding environment of the ER, we used a well-studied thermoreversible folding mutant of vesicular stomatitis virus (VSV) G protein called tsO45. At 40°C, VSVGtsO45 misfolds, forms noncovalently associated aggregates, and is retained within the ER (Doms et al., 1987, 1988; Hurtley and Helenius, 1989). When the temperature is lowered to 32°C, the aggregates rapidly disassemble, VSVGtsO45 subsequently trimerizes, and then it exits the ER with normal kinetics (Balch et al., 1986; Doms et al., 1987). Importantly, VSVGtsO45 that has exited the ER is no longer thermosensitive (Balch and Keller, 1986; deSilva et al., 1990), whereas VSVGtsO45 delivered back to the ER can reaggregate at 40°C (Doms et al., 1989; de Silva et al., 1990). These properties make VSVGtsO45 a potentially useful tool for studying the mechanisms that underlie retention in and retrieval to the folding/misfolding environment of the ER.

We focus here on fusion proteins upon which the temperature-sensitive lumenal ectodomain of VSVGtsO45 has been appended to different Golgi or plasma membrane targeting proteins/domains, including the KDELR (Hsu et al., 1992), a KDELR mutant (KDELRm; Townsley et al., 1993), TGN38 (Luzio et al., 1990) and the α chain of the interleukin-2 receptor (IL2R; Leonard et al., 1984). Addition of the VSVGtsO45 ectodomain was shown to be sufficient to retain the fusion proteins in the ER at 40°C, where they remained misfolded. At the permissive temperature of 32°C, the fusion proteins correctly localized to the Golgi complex or plasma membrane, indicating that the lumenal epitope of VSVGtsO45 did not interfere with their proper targeting. Remarkably, when cells were shifted from 32° back to 40°C, the Golgi-localized proteins reaccumulated within the ER due to misfolding of the G protein ectodomain. These redistributions did not require new protein synthesis and did not appear to be induced by misfolding of the chimeras within the Golgi complex. Rather, the chimeras exhibited a misfolded phenotype only after their redistribution into the ER. These data suggest that the Golgi-targeted, VSVG fusion proteins undergo constitutive recycling to the ER where they are reexposed to the ER folding machinery. Under conditions when these proteins misfold (i.e., 40°C), they become retained/trapped in the ER, and thereby lose their normal Golgi localization. Additional experiments with VSVGtsO45 that contained a shortened transmembrane domain suggested such Golgi to ER retrograde traffic is not exclusively signal mediated and may be an inherent property of proteins localized to Golgi membranes.

Materials and Methods

Plasmids

For expression in COS cells, constructs were generated in the SV40-based vector pSVGL3, which contained VSVGtsO45 as a reciprocal recombinant called C-D/R-ts (provided by J. Rose, Yale University, New Haven, CT; Gallione and Rose, 1985). Unique Sac I–BamHI restriction sites were used to replace the transmembrane domain and cytoplasmic tail of VSVGtsO45 with the appropriate inserts. VSVG–KDELR wild type was created by subcloning a full-length human homologue of the KDELR, ELP1 (from V. Hsu, Harvard Medical School, Boston, MA; Hsu et al., 1992), into C-D/R-ts. This chimera was made in-frame with tsO45 by digesting with SacI–NotI, blunting the overhanging ends with T4 polymerase, and religating. This generated a spacer region between the lumenal domain of tsO45 and ELP1 containing the amino acids GRFKGTDFLQDF. A myc epitope was attached to the 3′ end of ELP1 (Hsu et al., 1992). All other constructs were generated by subcloning PCR-amplified products using appropriate primers directly into SacI–BamHI digested C-D/R-ts. VSVG–KDELR mutant contained the full-length ELP1 coding sequence with a single aspartic acid to asparagine mutation at position 195 (provided by V. Hsu). This is analogous to the Asp to Asn mutation at position 193 in the human KDELR, hERD2 (Townsley et al., 1993). This construct contained a hemagglutinin (HA) epitope after ELP1. VSVG–TGN38 contained the transmembrane domain and cytoplasmic tail (amino acids 304–357) of TGN38 (provided by J. Bonifacino, National Institutes of Health [NIH] Bethesda, MD; Luzio et al., 1990). VSVG–IL2R contained the transmembrane domain and cytoplasmic tail (amino acids 241–272) of the α chain of the IL2 receptor, or Tac (provided by J. Bonifacino; Leonard et al., 1984), followed by a HA epitope. VSVG–Leu15 contained a transmembrane segment of 15 leucines (following two serines derived from the transmembrane domain of tsO45), followed by two lysines, and then the entire cytoplasmic tail of tsO45. VSVG wild-type–TGN38 was created using the wild-type VSVG template, C-D/ts-R (Gallione and Rose, 1985). For expression in CHO cells, inserts were subcloned into the expression vector pCDM8.1 (Marks et al., 1996). A cDNA coding for mouse α-mannosidase II was provided by K. Moreman (University of Georgia, Athens, GA) and subcloned into the expression vector pCDLSRa. A cDNA for coding for furin in pCDLSRa was provided by N. Wolins (NIH, Bethesda, MD) and J. Bonifacino.

An attempt was made to fuse the lumenal domain of VSVGtsO45 onto the cytoplasmic tail and transmembrane domain of the type II Golgi resident protein mannosidase II. However the resulting construct, mannosidase II (Man II)-VSVGtsO45 misfolded and was retained within the ER at all temperatures.

Cell Lines, Antibodies, and Reagents

COS-7 cells were maintained in DME (Biofluids, Inc., Rockville, MD) supplemented with 10% FBS, 2 mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37°C in a 5% CO_2 incubator. CHO cells were maintained similarly in RPMI 1640 complete medium (as above).

The following antibodies were used: rabbit polyclonal anti-VSV antiserum (provided by C. Machamer, Johns Hopkins University, School of Medicine, Baltimore, MD); conformation-specific mouse monoclonal anti-VSVG antibodies (I1 [8G5F11] and I14 [1E9F9]; provided by D. Lyles, Wake Forest University, Winston-Salem, NC; Lefrancois and Lyles, 1982); rabbit polyclonal antibodies to mannosidase II (provided by K. Moreman); mouse monoclonal anti–mannosidase II antibody (53FC3; provided by B. Burke, University of Calgary, Calgary, Alberta, Canada); rabbit polyclonal anti-ribophorin antiserum (provided by G. Kreibich, New York University Medical Center, New York, NY); polyclonal antiserum versus ER membrane proteins (provided by D. Louvard, Institute Curie, Paris, France); rabbit polyclonal antiserum versus GM130 (provided by G. Warren, Imperial Cancer Research Fund, London, England); rabbit polyclonal antiserum versus furin (provided by E. Dell'Angelica, NIH, Bethesda, MD and J. Bonifacino); rabbit polyclonal anti–β-coat protein (COP) antiserum; and mouse monoclonal antibodies against epitopes in Myc (9E10; provided by J. Humphrey, NIH, Bethesda, MD) and HA (HA.11; from BAbCO, Richmond, CA). FITC and rhodamine-conjugated secondary antibodies specific for mouse or rabbit immunoglobulins were purchased from Southern Biotechnologies (Birmingham, AL).

Brefeldin A (BFA) was obtained (Epicentre Technologies, Inc., Madison, WI) and stored as 1-mg/ml stock solutions in methanol. Cycloheximide (Sigma Chemical Co., St. Louis, MO) was stored as 10-mg/ml stock solutions in water. 50 mM 2-deoxy-glucose/0.02% sodium azide was used to deplete cellular ATP levels (Donaldson et al., 1991). AlF_4, an activator of heterotrimeric G proteins, was generated from 30 mM sodium fluoride and 50 μM $AlCl_3$ (Donaldson et al., 1991). Forskolin (Sigma Chemical Co.) is a competitive antagonist of BFA, and was used at 100 μM (Lippin-

cott-Schwartz et al., 1991). *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), is a calmodulin antagonist that has been hypothesized to be an inhibitor of membrane tubulation (used at 10 μM; deFigueiredo and Brown, 1995). Wortmannin, an inhibitor of phosphatidylinositol (PI)-3 kinase has been shown to disrupt trafficking of lysosomal enzymes by interfering with sorting in the TGN (used at 1 μM; Brown et al., 1995). Concanamycin B (used at 25 nM) is an inhibitor of vacuolar proton-ATPases, and has been shown to alter secretory pathway function (Yilla et al., 1993; data not shown). Monensin, a monovalent cation ionophore that blocks transport through the Golgi complex (Tartakoff, 1983), was used at 30 μM. All drugs were purchased from Sigma Chemical Co. Concanamycin B was provided by J. Bonifacino.

Transfection and Immunofluorescence Microscopy

COS-7 cells were transiently transfected by calcium phosphate precipitation for 16 h, washed once in PBS, and then incubated in complete medium for an additional 24 h. Transfections were performed at either 40° or 32°C as indicated. Stable transfectants in CHO cells were generated and selected in 500 μg/ml G418 (Geneticin; GIBCO BRL, Gaithersburg, MD).

Cell fixation and antibody staining was performed according to Cole et al. (1996). Cells were analyzed on a photomicroscope (model III; Carl Zeiss, Inc., Thornwood, NY) using a 63× Planapo lens (NA 1.4), and photography was performed using Tri-X-pan ASA 400 film (Eastman Kodak, Rochester, NY). For analyzing conformation-specific anti-VSVG antibodies by immunofluorescence, cells were processed as above, except that cells were fixed at 32° or at 40°C for 5 min before moving to room temperature.

Confocal images were acquired on a confocal laser scanning microscope (model LSM 410; Carl Zeiss, Inc.), equipped with a triple line Ar/Kr laser with a 100× 1.4 NA Planapochromat oil immersion objective. Each image represents a single averaged (16-line-scan) image collected within the linear range of fluorescence intensity based on the standard table provided with the imaging software. Image overlays represent samples acquired using the sequential mode for double-label collection.

Flow Cytometry

For assaying cell surface expression of VSVGtsO45 chimeras, COS-7 cells were transiently transfected at 40°C with 5 μg of chimeric plasmids into duplicate wells of six-well dishes (Costar Corp., Cambridge, MA). After 36–40 h, 20 mM Hepes, pH 7.4, buffer and 50 μg/ml cycloheximide were added to each well and the dishes were moved to an 18°C water bath for 3 h, and then returned to the 40°C incubator for an additional 2 h. The cells were released from the dishes with 15 mM EDTA/PBS, washed with 10% serum/PBS, gently pelleted, and then placed on ice. For surface staining, samples from one of the duplicate wells were bound to anti-VSVG monoclonal antibodies (a mixture of I1 and I14 antibodies), followed by FITC goat anti–mouse secondary antibodies, both in the absence of saponin. Cells were washed, fixed in 2% formaldehyde/PBS, washed again, and then resuspended in PBS. For analysis of total staining, cells from the second duplicate well were fixed in 2% formaldehyde/PBS for 30 min on ice, washed, and then incubated with primary and secondary antibodies in the presence of 0.2% saponin. Surface and total cellular fluorescence was quantitated by flow cytometry on a FACScan® (Becton and Dickinson Co., Mountain View, CA) using CyQuest software. The analysis shown was gated (M1) for surface expression of cells expressing parental VSVGtsO45 released from 40° to 32°C for 2 h. Kolmogorov–Smirnov statistical analysis was performed to determine that the plots generated were significantly different, at $P < 0.001$ (Young, 1977).

Metabolic Labeling

For metabolic labeling of transiently transfected COS cells, subconfluent monolayers grown in six-well dishes (Costar Corp.) were incubated in suspension in methionine-free DME containing 3% dialyzed FBS for 30 min at 40°C. Cells were pulse labeled with 250–750 μCi/ml Tran^{35}S-label (ICN Biomedicals Inc., Costa Mesa, CA) or EXPRE35SS (NEN Life Science Products, Boston, MA) for the times indicated, washed, and then chased in complete medium containing 15 × excess methionine and cysteine for the times indicated. Aliquots of cells were collected at each time point.

Immunoprecipitation

For pulse-chase analysis in COS cells, metabolically labeled proteins were immunoprecipitated and processed according to Cole et al. (1996). Proteins were denatured by boiling for 5 min in 0.5% SDS/1.0% β-mercaptoethanol, brought to 50 mM sodium citrate, pH 5.5, and then either mock treated or treated with endoglycosidase H (endo H; 30 U/μl; New England Biolabs Inc., Beverly, MA), or a combination of endo H and Neuraminidase (0.2 mU/μl; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 4–16 h at 37°C. Samples were boiled with reducing sample buffer and analyzed by 8% SDS-PAGE. After fixation, gels were treated for fluorography with 1 M salicylate, dried, and then exposed to XAR-5 film (Eastman Kodak). For misfolding analysis in COS cells, cells were processed as above, except that cells were solubilized by adding Triton X-100 (1% final) plus protease inhibitors directly to the samples, and then incubating at 32° or 40°C for 1 min before placing on ice.

Cell Fractionation and In Vitro Misfolding

Briefly, COS 7 cells were grown to 70–80% confluency 2 d after transfection. Cells were scraped and labeled in suspension with 1 mCi/ml Tran^{35}S-label (ICN Biomedicals Inc.) for 75 min, and then chased in unlabeled medium for 75 min at 32°C. All further cell homogenization and fractionation procedures were performed at 4°C, according to Yang et al. (1997). Membrane pellets were analyzed for galactosyltranferase activity (Bole et al., 1986), and then Western blotted with antibodies to ribophorin, to identify fractions containing Golgi and ER membranes, respectively.

Fractions 4–8 (ER) and 13–18 (Golgi) were pooled, split into equal parts, and then pelleted at 125,000 *g* for 1 h. Membrane pellets were resuspended in homogenization buffer plus protease inhibitors, incubated at 32° or 40°C for the indicated times, and then lysed by adding 2 × lysis buffer (homogenization buffer plus 2% Triton X-100, 20 mM NEM) for 2 min at the indicated temperature, before placing on ice. Lysates were immunoprecipitated either with polyclonal anti-VSV antisera or conformation-specific I14 antibodies. Precipitates were analyzed by reducing 8% SDS-PAGE, and then scanned by densitometry (ImageQuant; Molecular Dynamics, Inc., Sunnyvale, CA).

Cycloheximide Treatment

In the recycling assay presented here, cycloheximide was used at concentrations of 50–100 μg/ml in COS cells, and 10 μg/ml in CHO cells. This was sufficient to inhibit protein synthesis ~90%. We found, however, that cycloheximide concentrations that inhibit protein synthesis >95% could effectively prevent retention of the redistributed VSVG chimeras in the ER at 40°C. It appears that subtle changes in protein synthesis can have profound consequences on the ER retention machinery. Although this does not affect the results presented here, this may be the likely explanation for escape of VSVGtsO45 from the ER at 40°C in CHO cells with high levels of viral infection (data not shown; Hammond and Helenius, 1994).

Results

Construction of VSVGtsO45 Fusion Proteins

VSVGtsO45 contains a single amino acid change within its lumenal domain that leads to its misfolding and retention within the ER at 40°, but not at 32°C (Gallione and Rose, 1985). To study quality control features of the secretory pathway such as retention in and retrieval to the ER, we attached the thermosensitive lumenal domain of VSVGtsO45 to proteins and/or targeting domains that normally direct localization to the Golgi complex or plasma membrane (Fig. 1). These included: the KDELR, a Golgi protein that recycles escaped KDEL-containing ligands from the Golgi to the ER (Lewis and Pelham, 1992); KDELR mutant (KDELRm), a recycling-defective form of KDELR (Townsley et al., 1993); the transmembrane domain and cytoplasmic tail of TGN38, a protein characteristic of the TGN (Humphrey et al., 1993); and the transmembrane domain and cytoplasmic tail of the α chain of the IL2R, which functions in IL2 uptake at the cell surface (Leonard et al., 1984). We also replaced the transmembrane domain of VSVGtsO45 with a synthetic stretch of 15 leucine resi-

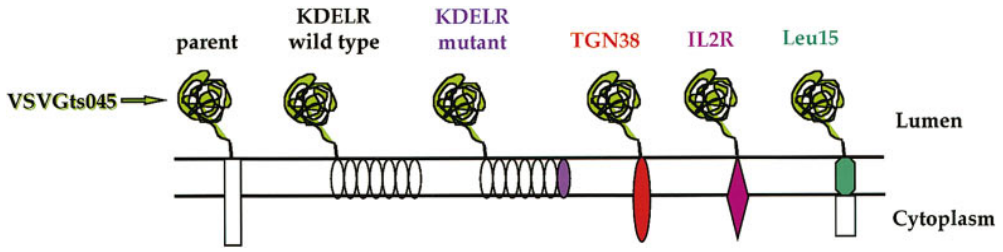

Figure 1. Schematic representation of VSVGtsO45 chimeras. The temperature-sensitive lumenal domain of VSVGtsO45 was fused in-frame with (*left* to *right*): a full-length human homologue of the KDELR (VSVG–KDELR; Hsu et al., 1992); a full-length mutant form of the KDELR, which contains a single amino acid change (Asp to Asn at position 195) that fails to redistribute into the ER upon overexpression of KDEL-containing ligands, and is presumably recycling defective (analogous to Townsley et al., 1993); the transmembrane domain and cytoplasmic tail of TGN38 (Luzio et al., 1990), which contain nonoverlapping domains sufficient to confer steady-state TGN localization (Humphrey et al., 1993; Ponnambalam et al., 1994); the transmembrane domain and cytoplasmic tail of the cell surface protein, the α chain of the IL2R (Leonard et al., 1984); and a construct in which the transmembrane domain of VSVGtsO45 was replaced by a synthetic transmembrane domain composed of 15 leucines. This has been shown to result in accumulation within the Golgi complex of proteins normally destined for the plasma membrane (Munro, 1995). The quaternary structures of these constructs have not been analyzed, yet we assume that VSVG–TGN38, VSVG–IL2R, and VSVG–Leu15 form trimers, since it is the lumenal domain of VSVGtsO45 that is responsible for trimer formation (Doms et al., 1988). The oligomeric nature of KDELR is not known.

dues, which previously has been shown to cause Golgi accumulation of proteins normally destined to the plasma membrane (Munro, 1995).

Expression and Localization of VSVG Fusion Proteins

The cellular distributions and biochemical modifications of VSVGtsO45 and each of the fusion proteins were characterized in transfected COS cells by indirect immunofluorescence microscopy using polyclonal anti-VSV antiserum. At the nonpermissive temperature of 40°C, each of the chimeras, as well as parental VSVGtsO45, showed a reticular ER-like pattern with nuclear envelope staining (Fig. 2, *left*), which coincided with labeling of endogenous ER membrane proteins (data not shown). Metabolic pulse-chase labeling experiments of transfected cells maintained at 40°C revealed that fusion proteins remained sensitive to endo H digestion (Fig. 3, *right*), suggesting they were not transported out of the ER into the secretory pathway at 40°C where their *N*-linked glycans would be modified by Golgi enzymes. Therefore, the VSVGtsO45 lumenal domain appeared to be sufficient for retention of the fusion proteins in the ER at 40°C, consistent with the temperature-induced folding defect demonstrated for VSVGtsO45 (Doms et al., 1987).

Whether the appended VSVGtsO45 ectodomain could properly fold and permit targeting of the chimeras to their predicted destinations was tested in COS cells transfected at 32°C (Fig. 2, *right*). At this temperature, VSVG–KDELR localized predominantly to the Golgi complex with moderate amounts of ER staining. The VSVG–KDELR mutant chimera, by contrast, gave an exclusively Golgi staining pattern when expressed at 32°C, without associated ER staining. Overexpression of KDEL-containing molecules at 32°C induced the redistribution of the KDELR wild-type, but not mutant, chimera into the ER (data not shown). This is consistent with the results of Townsley et al. (1993), and suggests that the G protein ectodomain does not interfere with KDELR function (Lewis and Pelham, 1992). VSVG–TGN38 was similarly targeted to the Golgi complex at 32°C, and could be redistributed to the cell surface by overexpression of other TGN-targeted molecules, similar to results of Humphrey et al. (1993) for TGN38 (data not shown).

The steady-state Golgi distributions of the above chimeras persisted in cells treated with cycloheximide, which blocks new protein synthesis, indicating they were stably maintained within Golgi membranes. This contrasted with VSVGtsO45, VSVG–IL2R, and VSVG–Leu15, which were delivered to the plasma membrane at 32°C, although with different efficiencies. There was a significant accumulation of the Leu15 (and to a lesser extent IL2R) chimera in the Golgi complex (Fig. 2, *right*), but this pool could be completely chased to the plasma membrane with cycloheximide treatment after several hours (data not shown). This suggested that insertion of the Leu15 transmembrane domain slowed transport of VSVG through the Golgi complex, rather than specifically targeting the fusion protein to the Golgi complex (Munro et al., 1995).

In metabolically labeled cells pulsed at 40°, and then chased at 32°C for 2 h, the vast majority of the VSVG–KDELR remained sensitive to endo H (Fig. 3). By contrast, ~50% of the VSVG–KDELRm acquired endo H resistance and neuraminidase sensitivity, suggesting this chimera has access to the *trans*-most processing enzymes of the Golgi complex. VSVG–TGN38 gave a more complex carbohydrate processing pattern with several endo H–resistant bands, including partial sensitivity to neuraminidase. Although the reason for the lack of complete processing of these chimeras is unclear, these results suggest that the KDELRm and TGN38 chimeras become more accessible to distal Golgi processing enzymes than the KDELR wild-type chimera. Both VSVG–Leu15 and VSVG–IL2R became partially endo H resistant and neuraminidase sensitive (Fig. 3), and were fully processed upon longer chase periods (data not shown), similar to parental VSVGtsO45.

Laser scanning confocal microscopy was used to compare the distributions of the VSVG chimeras with other well-characterized Golgi markers (see Nilsson et al., 1993; Shima et al., 1997). Transfected cells were maintained at 32°C, treated with cycloheximide for 3 h, fixed, and labeled with appropriate primary and secondary antibodies, and then optical sections were analyzed. Fig. 4 shows differences in distributions revealed by overlay images from single optical sections (depth of field is ~0.4 μm). The distribution of each of the chimeras extended throughout the Golgi complex, as evidenced by partial colocalizations with

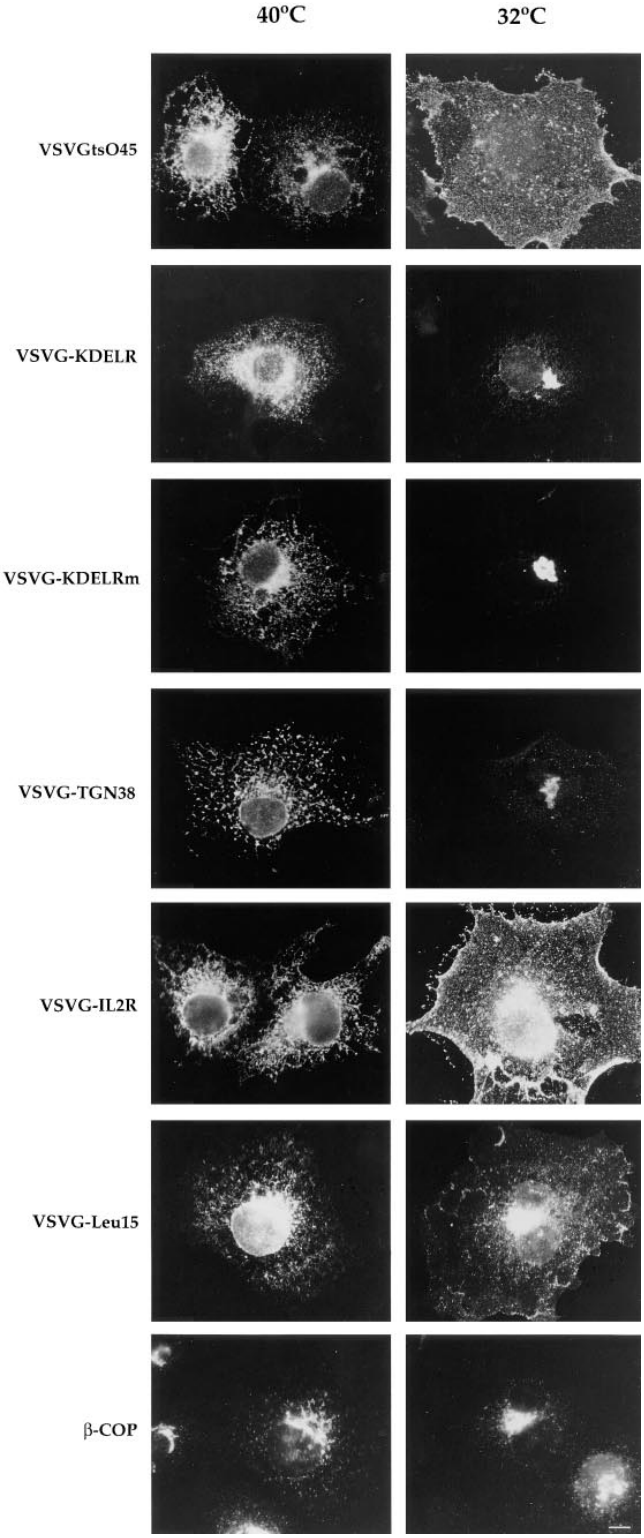

Figure 2. Localization of VSVGtsO45 chimeras at nonpermissive (40°C) and permissive (32°C) temperatures. COS cells were transiently transfected with the indicated constructs and maintained at either 40° or 32°C. After 40 h, cells were fixed, permeabilized, and prepared for indirect immunofluorescence using polyclonal anti-VSV antibodies followed by rhodamine anti–rabbit secondary antibodies. At 40°C, VSVGtsO45, as well as each of the chimeras, were retained in the ER (*left*), whereas at 32°C, the constructs were distributed to the Golgi complex and/or the plasma membrane (*right*). β-COP staining (*bottom*) indicates the distribution of the Golgi complex, and was from cells double labeled for VSVG–Leu15. Bar, 10 μm.

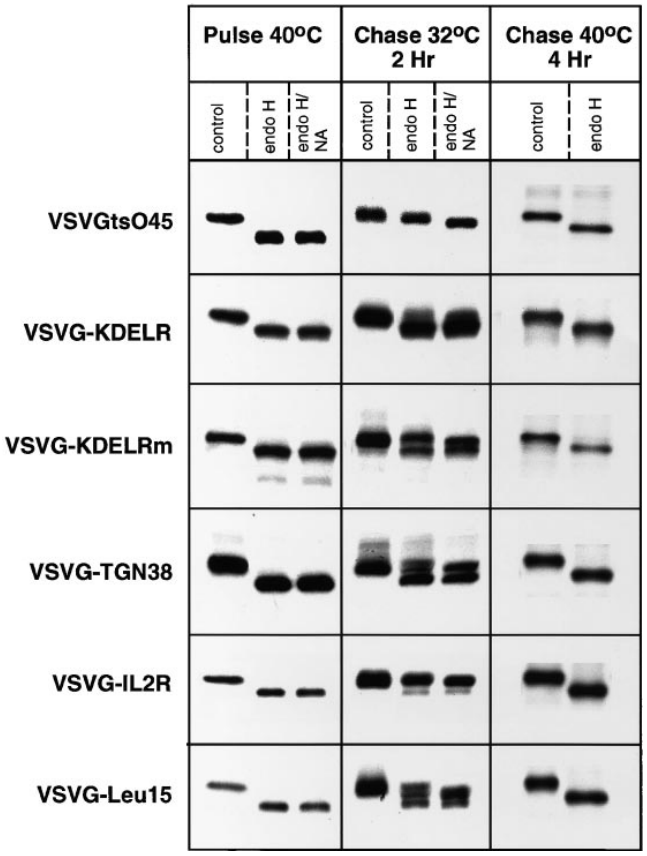

Figure 3. Processing of VSVG chimeras at nonpermissive and permissive temperatures. COS cells were pulse labeled with [^{35}S]methionine for 20 min at 40°C, and then chased at 32°C in unlabeled medium for 2 h, or maintained at 40°C for 4 h. The cells were detergent solubilized and immunoprecipitated with anti-VSVG antibodies. Immunoprecipitates were treated with or without endo H or a combination of endo H and neuraminidase (endo H/NA) before analysis by SDS-PAGE under reducing conditions. Note the increase in mobility for tsO45 and several of the chimeras when treated with endo H/neuraminidase. Complete processing for the Leu15 and IL2R chimeras occurred during longer chase periods.

the *cis*-Golgi membrane protein GM130 (Nakamura et al., 1995), the medial Golgi enzyme mannosidase II (Rabouille et al., 1995), and the TGN marker, furin (Bosshart et al., 1994). However, clear gradations of distribution existed, even in transiently expressing cells. VSVG–KDELR showed the greatest degree of overlap with GM130 and mannosidase II, although it can be seen to extend into the TGN, whereas VSVG–KDELRm and VSVG–TGN38 were significantly concentrated within the TGN (see overlay with furin), with less accumulation in the proximal regions of the Golgi (GM130 and mannosidase II).

Taken together, these results show that addition of even full-length proteins to the ectodomain of VSVGtsO45 permitted not only the proper thermoreversibility of the G protein ectodomain, but the proper targeting specificity dictated by the attached molecules and/or domains.

Retrograde Transport of Golgi-localized VSVG Fusion Proteins

To study whether VSVG fusion proteins ever return to the

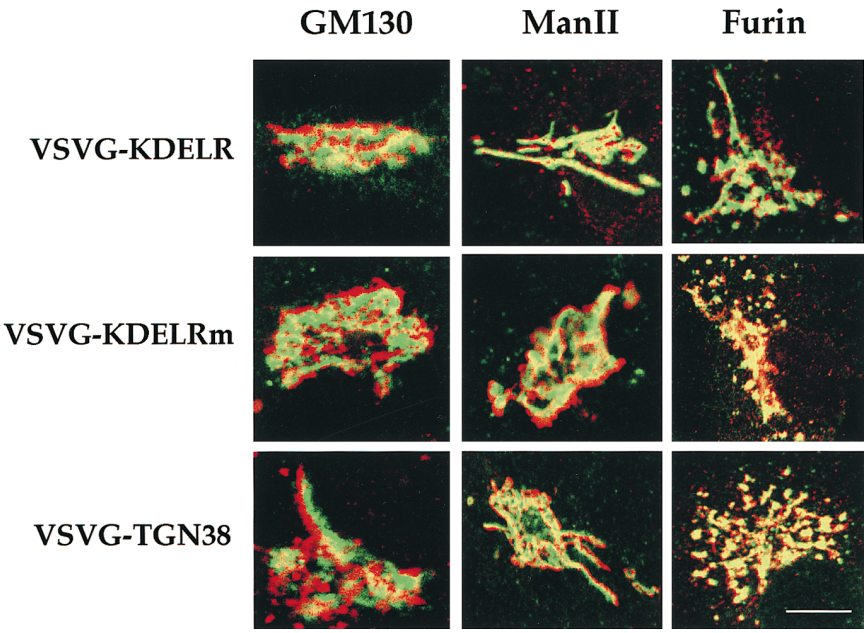

Figure 4. Colocalization of VSVG chimeras with Golgi markers by immunofluorescence microscopy. COS cells transfected at 32°C with the indicated chimeras and indicated Golgi marker proteins (*GM130* shows endogenous protein) were treated with cycloheximide for 3 h, fixed, and then double labeled with antibodies to VSVG (*left*) and Golgi proteins (*top*) followed by FITC-coupled (*VSVG*) and rhodamine-coupled (Golgi marker) secondary antibodies. Each panel shows an overlay image, with yellow indicating the region of overlap. Bar, 5 μm.

folding environment of the ER once transported into the Golgi complex, we examined the effect of a temperature shift to 40°C on the distribution of Golgi-localized chimeras. Assuming the ER is the only compartment where misfolding of proteins leads to their retention (Hurtley and Helenius, 1989), any of the Golgi-localized fusion proteins that recycled to the ER would be predicted to become misfolded and trapped there over time in cells shifted to 40°C.

Cells transfected with VSVGtsO45 or the various chimeras at 40°C were shifted to 32°C for 2 h (for VSVG–KDELR, VSVG–KDELRm, and VSVG–TGN38), or to 18°C for 3 h (for VSVGtsO45, VSVG–IL2R, and VSVG–Leu15) to accumulate these proteins within the Golgi complex (Fig. 5, *left*). Cells were then shifted to 40°C for 2 h, fixed, and then examined by immunofluorescence microscopy. As shown in Fig. 5 (*middle*), whereas VSVGtsO45 and VSVG–IL2R were primarily transported to the plasma membrane after a shift to 40°C, VSVG–KDELR, VSVG–KDELRm, and VSVG–TGN38 redistributed into the ER. VSVG–Leu15 showed an intermediate ER/plasma membrane distribution. Double labeling with antibodies to mannosidase II (Fig. 5, *bottom row*), or to the peripheral Golgi coat protein, β-COP (data not shown) showed that redistribution of the fusion proteins into the ER at 40°C did not affect the distribution of other Golgi proteins or disrupt the organization of the Golgi complex. In addition, a wild-type VSVG–TGN38 chimera (whose lumenal domain was not thermosensitive for misfolding) showed no change in its Golgi localization in cells shifted to 40°C (data not shown), indicating the redistributions were specific for fusion proteins containing the tsO45 mutation.

Several observations suggested that the accumulation of the fusion proteins within the ER reflected their retrograde transport into the ER, rather than degradation of the Golgi-localized proteins and ER retention of newly synthesized pools at 40°C. First, redistribution of the fusion proteins into the ER occurred in the absence of new protein synthesis, since transfected cells treated with 50–100 μg/ml cycloheximide (sufficient to inhibit protein synthesis in these cells by 85–90%) gave the same pattern and kinetics of redistribution as untreated cells (data not shown). Second, there was no evidence for a significant loss of material during the temperature shift as revealed from pulse-chase/immunoprecipitation experiments (see Fig. 9). Finally, in biochemical assays with organelles that had been separated by gradient fractionation, the percentage of metabolically labeled VSVG–TGN38 in Golgi membranes decreased in cells shifted from 32° to 40°C for 2 h, with a corresponding increase in ER-enriched fractions (data not shown).

Cells expressing the KDELR, KDELRm, and TGN38 chimeras that were shifted to 40°C showed normal Golgi staining patterns when subsequently returned to 32°C (Fig. 5, *right*). Thus, the ER accumulation phenotype of these proteins is fully reversible. These results indicate that it is possible to trap and accumulate within the ER at 40°C tsO45-containing proteins that have been recycled from all Golgi compartments.

Kinetics and Properties of Retrograde Transport

To compare the kinetics of redistribution into the ER for the different Golgi resident chimeras, cells maintained at 32°C were shifted to 40°C for various lengths of time, fixed, and then examined by immunofluorescence microscopy. The length of time at 40°C necessary to redistribute each of the chimeras completely back into the ER was then determined and was used to estimate recycling kinetics. VSVG–KDELR redistributed into the ER with a $t_{1/2}$ of ~20 min, whereas VSVG–TGN38 and VSVG–KDELRm redistributed more slowly ($t_{1/2}$, ~60 and ~75 min, respectively). Thus, each of the fusion proteins exhibited a distinct rate of transport to the ER, which appeared to be fastest for proteins localized in the *cis*-most regions of the Golgi and slower for proteins localized in the *trans*-Golgi/TGN.

Several drug treatments were found to inhibit redistribution of the VSVGtsO45 fusion proteins into the ER upon a temperature shift from 32° to 40°C. These included: ATP

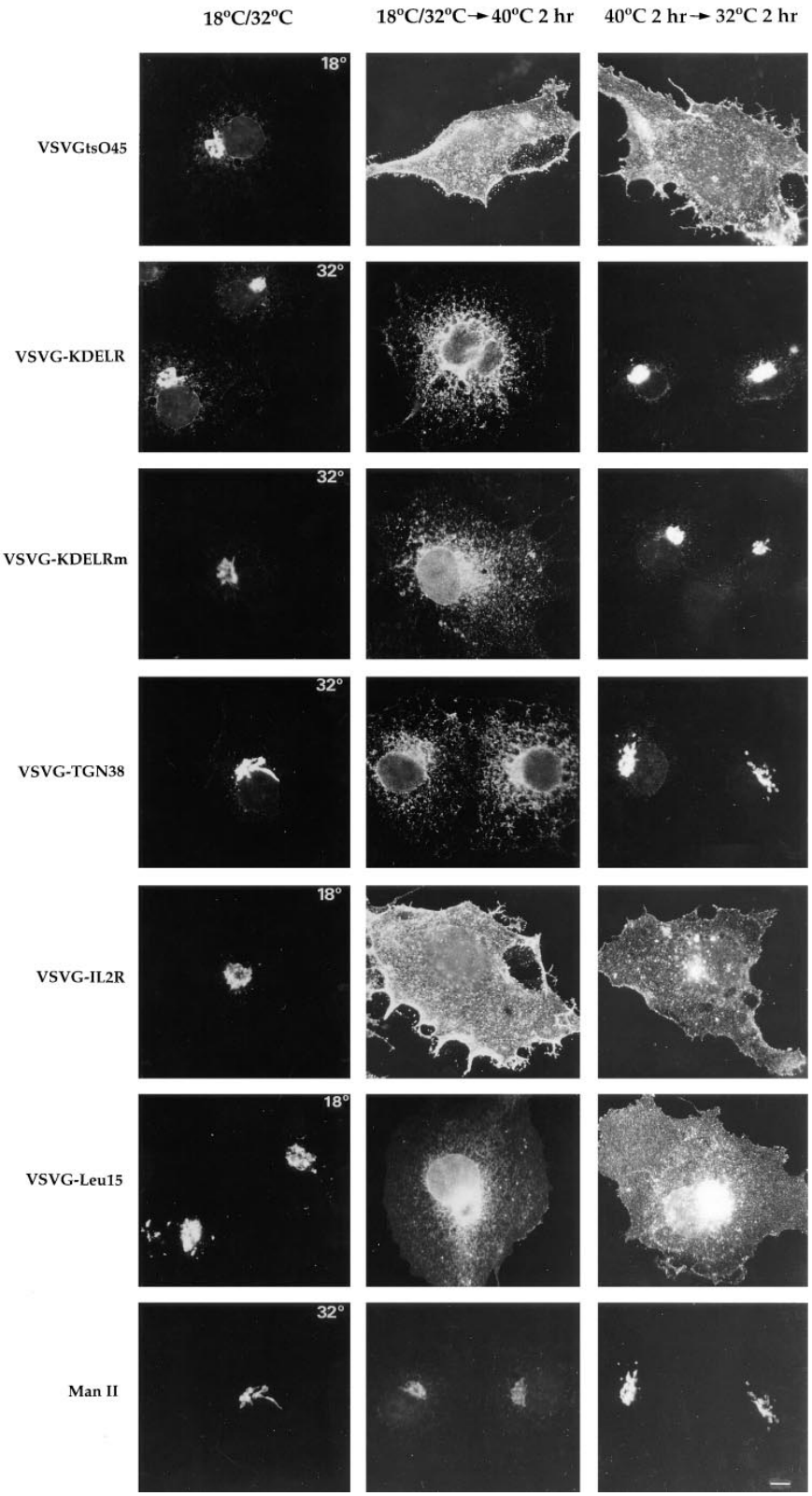

Figure 5. Membrane cycling dynamics of VSVG chimeras. COS cells transfected at 40°C were shifted to either 18°C for 3 h (*VSVGtsO45*, *IL2R*, and *LEU15* chimeras), or to 32°C for 2 h (*KDELR* wild type, *KDELRm*, and *TGN 38* chimeras; *left*), and then shifted to 40°C for 2 h (*middle column*), before shifting back to 32°C for an additional 2 h (*right*). Cells were fixed, permeabilized, and prepared for immunofluorescence microscopy using anti-VSV antibodies. The Golgi complex was visualized with antibodies to cotransfected α-mannosidase II (*Man II*), followed by FITC-labeled anti–mouse secondary antibodies (*bottom*). The panels showing mannosidase II staining correspond to cells double labeled for VSVG–TGN38. Whereas VSVGtsO45, VSVG-IL2R and some VSVG-Leu15 were delivered to the plasma membrane upon a shift to 40°C (*middle column*), the KDELR wild type, KDELRm, and TGN38 chimeras had redistributed into the ER. Mannosidase II staining was generally unaffected by the temperature shift, although a slight increase in ER staining can often be seen. Notice the presence of Golgi staining with the Leu15 and IL2R chimeras, but not VSVGtsO45, after a return to 32°C (*right*). Bar, 10 μm.

depletion with 2-deoxy-glucose/sodium azide; AlF_4, an activator of heterotrimeric G proteins; forskolin, an antagonist of BFA; W7, a calmodulin antagonist; wortmannin, a phosphatidylinositol-specific kinase inhibitor; concanamycin B, an inhibitor of vacuolar proton-ATPases; and the Na^+ ionophore, monensin (Table I; refer to Materials and Methods). CHO cells stably expressing VSVG–TGN38 were incubated at 32°C to accumulate the chimera in the Golgi complex, and again for an additional 10 min with the indicated drugs at 32°C before shifting to 40°C for 2 h. Cells

Table I. Inhibition of Retrograde Membrane Traffic

Treatment	32° to 40°C	BFA
Control	—	—
−ATP	++	++
AlF_4	+*	+*
Forskolin	++	++
W7	+	+
Wortmannin	+	+‡
ConcanamycinB	+	++
Monensin	—	++

CHO cells stably expressing VSVG–TGN38 were incubated with the indicated drugs or pharmacological treatments for 10 min at 32°C before shifting either to 40°C for 2 h, or adding 1 μg/ml BFA and incubating at 32°C for 30 min. Refer to Materials and Methods for concentrations and descriptions of the individual treatments. Cells were fixed and double labeled with anti-VSVG antibodies and antiserum to the Golgi resident enzyme, mannosidase II. The redistribution of VSVG–TGN38 into the ER at 40°C, and VSVG–TGN38 and mannosidase II into the ER with BFA, is shown as (—) for control cells. Full and partial inhibition of these redistributions are indicated by (++) and (+), respectively. *Indicates VSVG–TGN38 becomes vesiculated in the Golgi region; ‡indicates the redistribution of VSVG–TGN38 into the ER is partially inhibited, whereas redistribution of mannosidase II is unaffected.

were fixed and then examined by immunofluorescence microscopy. All of the above treatments, excluding the ionophore monensin, effectively prevented redistribution of VSVG–TGN38 into the ER at 40°C without changing its Golgi distribution. This was compared with the redistribution of Golgi membranes into the ER by BFA (Doms et al., 1989; Lippincott-Schwartz et al., 1989). Interestingly, all of the above treatments, including monensin, also inhibited retrograde transport of VSVG–TGN38 into the ER with BFA (Table I). Although the molecular mechanisms through which these compounds prevent retrograde traffic are unclear, these results suggest that membrane pathway(s) from the Golgi to the ER, followed by the fusion proteins during temperature shifts and BFA treatment, share similar, but not identical, properties.

Recycling of Itinerant VSVG Fusion Proteins

Although VSVGtsO45 was delivered exclusively to the cell surface when shifted to 40°C after first being accumulated in the Golgi complex (Fig. 5, *middle*), a significant proportion of VSVG–Leu15 and VSVG–IL2R, most noticeably VSVG–Leu15, had indeed redistributed back to the ER. Staining of unfixed nonpermeabilized cells with anti-VSVG antibodies showed reduced levels of surface staining for the chimeras as compared with the parent molecule (data not shown). Quantitation of surface expression by flow cytometry (Fig. 6) revealed that, although the percentage of cells expressing detectable levels of surface staining were similar (∼7–9%), VSVG–Leu15 exhibited mean fluorescence intensity levels significantly lower than VSVGtsO45 (60% of tsO45). VSVG–IL2R showed an intermediate level of cell surface expression (75% of tsO45). Since total expression levels were equivalent when cells were stained in the presence of detergent (data not shown), this result indicates that significantly less VSVG–Leu15 (and VSVG–IL2R) had been delivered to the plasma membrane than parental VSVGtsO45. The remainder (∼40% of VSVG–Leu15 and ∼25% of VSVG–IL2R) had presumably redistributed to the ER (Fig 5, *middle*), since Golgi staining was evident for the Leu15 (and IL2R) chimeras, but not the parent G protein when the temperature

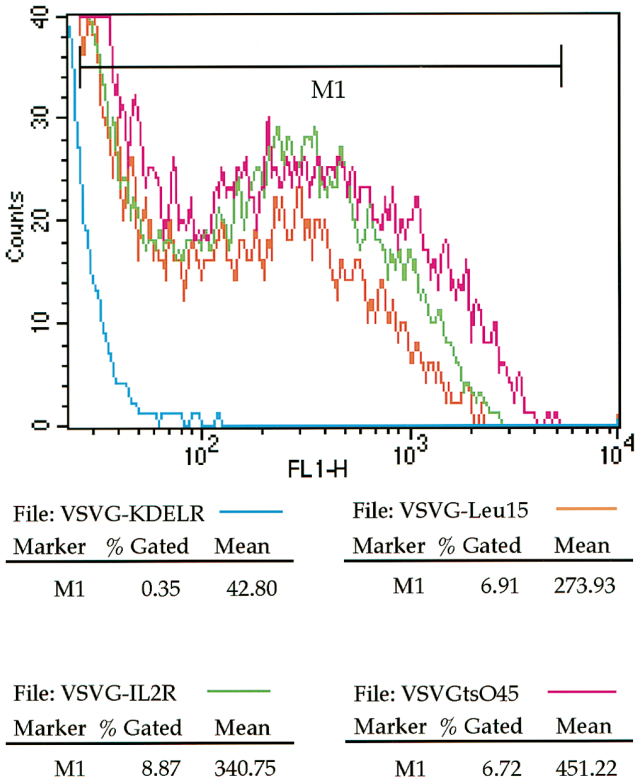

Figure 6. Surface staining of VSVG chimeras by flow cytometry. COS cells were transiently transfected with 5 μg VSVGtsO45, or the LEU15, IL2R, or KDELR wild-type chimeric plasmids, and then maintained at 40°C. At 40 h posttransfection, cycloheximide was added, and cells were placed at 18°C for 3 h, and then returned to 40°C for an additional 2 h. Intact cells were harvested and stained on ice with anti-VSVG monoclonal antibodies, and then with secondary FITC-conjugated anti–mouse IgG. Shown is the surface-staining intensity (x-axis) measured for cells gated for positive VSVGtsO45 surface expression (*M1*). Cell number is plotted on the y-axis. Statistical analysis indicates significantly higher mean fluorescence intensity for VSVGtsO45 (mean value ∼451) than for the Leu15 (∼274) and IL2R (∼341) chimeras, with the KDELR wild-type chimera (∼43) serving as a negative cell surface control. Parallel samples analyzed in the presence of 0.2% saponin determined total cellular staining and showed equivalent expression levels for each construct.

was subsequently lowered from 40° to 32°C (Fig. 5, *right*). As these experiments were performed in the presence of cycloheximide, this material must have originated from previously redistributed Golgi pools.

The observation that VSVGtsO45, –IL2R, and –Leu15 were delivered from the ER to the Golgi complex upon a shift from 40° to 32°C (or 18°C) at approximately equivalent rates (data not shown; Fig. 5, *left*) implies that it is their transport through the Golgi complex, rather than exit from the ER, that is slowed when compared with VSVGtsO45. When combined with the recycling and biochemical processing data, these results suggest that whereas proteins rapidly transported through the Golgi complex (VSVGtsO45) continue to the cell surface after the temperature shift, proteins that are either stably retained (VSVG–KDELR, –KDELRm, and –TGN38) or slowly

transported through the Golgi (VSVG–Leu15 and IL2R) have the capacity to be retrieved to the ER.

Folding Properties of VSVG Fusion Proteins in the ER and Golgi Complex

The redistribution of VSVG chimeras into the ER after a temperature shift to 40°C is presumed to be due to misfolding/retention of the G protein ectodomain within the ER as these molecules recycle from the Golgi complex. This interpretation was tested using the conformation-specific VSVG antibody, I14 (Lefrancois and Lyles; 1982), which has previously been used to detect folded, but not misfolded, VSVG molecules (Doms et al., 1988, 1989; Machamer et al., 1990). As shown in Fig. 7 (*a*), I14 staining of VSVG–TGN38 in stably expressing CHO cells gave a primarily Golgi staining pattern at 32°C. As in COS cells, this staining coincided with β-COP (data not shown) and the Golgi enzyme mannosidase II (Fig. 7 *b*). After short periods at 40°C, before VSVG–TGN38 had redistributed into the ER (data not shown), or under conditions where its redistribution was inhibited by ATP depletion (Fig. 7 *e*), VSVG–TGN38 staining by I14 antibodies was still detected in the Golgi complex. This indicated that VSVG–TGN38 did not significantly misfold when it was in the Golgi complex at 40°C, even after several hours during which recycling was inhibited (e.g., with ATP depletion).

Under conditions where VSVG–TGN38 was transported into the ER (i.e., upon longer periods at 40°C), specific staining for VSVG–TGN38 by I14 was lost (Fig. 7 *c*), but reappeared when cells were shifted from 40° back to 32°C for 10 min under conditions where export from the ER was blocked (e.g., ATP depletion; Fig. 7 *g*). Loss of I14 staining of VSVG–TGN38 in the ER at 40°, but not at 32°C, was also observed in cells with Golgi proteins redistributed into the ER with BFA (Doms et al., 1989; Lippincott-Schwartz et al., 1989; Fig. 7, compare *i* with *k*). These results are consistent with the idea that the mechanism for the accumulation of the VSVG chimeras within the ER in cells transfected at 40°C and during the 40°C temperature shift is due to misfolding of the G protein ectodomain in this compartment. Moreover, changes in conformation of VSVG–TGN38 leading to loss of labeling by I14 antibodies appeared to occur only after the protein had redistributed into the ER.

Biochemical analysis provided further evidence that misfolding of the chimeras at 40°C occurred only after the proteins had redistributed into the ER. A cell fractionation procedure to separate ER from Golgi membranes was developed to analyze misfolding in vitro. COS cells expressing VSVG–TGN38 were metabolically labeled with [^{35}S]methionine at the permissive temperature to generate labeled proteins in both the ER and Golgi complex. Cells were gently scraped, pelleted, and then a postnuclear supernatant was layered over a continuous 0–26% OptiPrep gradient (refer to Materials and Methods). Fractions were collected and analyzed for the distribution of ER (ribophorin) and Golgi (galactosyltransferase) membrane markers (Fig. 8 *a*). Separate ER and Golgi fractions were pooled and aliquots were incubated at either 32° or 40°C for 60 min to assess the capacity of the individual membrane compartments to misfold VSVG–TGN38. After sol-

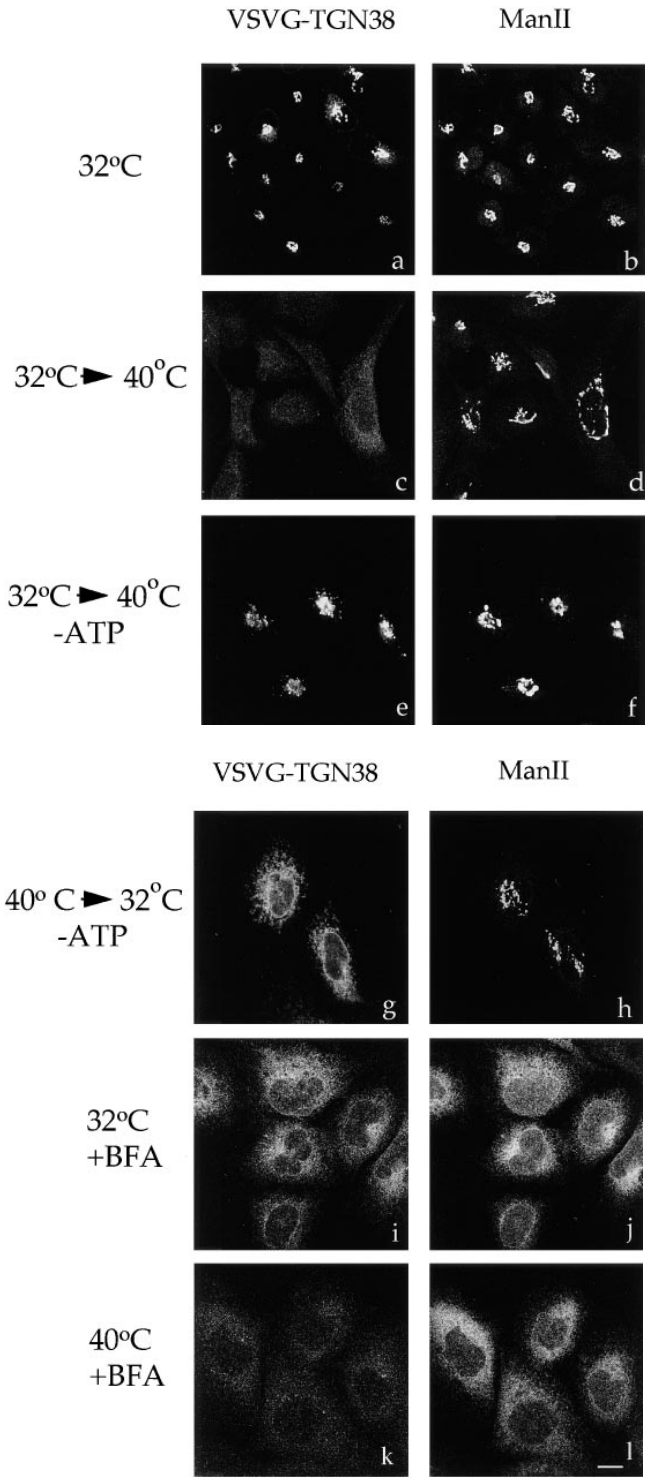

Figure 7. Analysis of cycling using conformation-sensitive antibodies. CHO cells stably expressing VSVG–TGN38 were treated as indicated and stained with conformation-sensitive I14 antibodies to VSVG (*a*, *c*, *e*, *g*, *i*, and *k*), and antibodies to the Golgi enzyme, mannosidase II (*b*, *d*, *f*, *h*, *j*, and *l*). Images were acquired on a Zeiss laser scanning confocal microscope. Notice the complete lack of I14 staining in cells treated at 40°C (*e*), but not when recycling was inhibited (*c*). The brightness of the cells in *e* and *k* was artificially enhanced to show the cell outline. Bar, 10 μm.

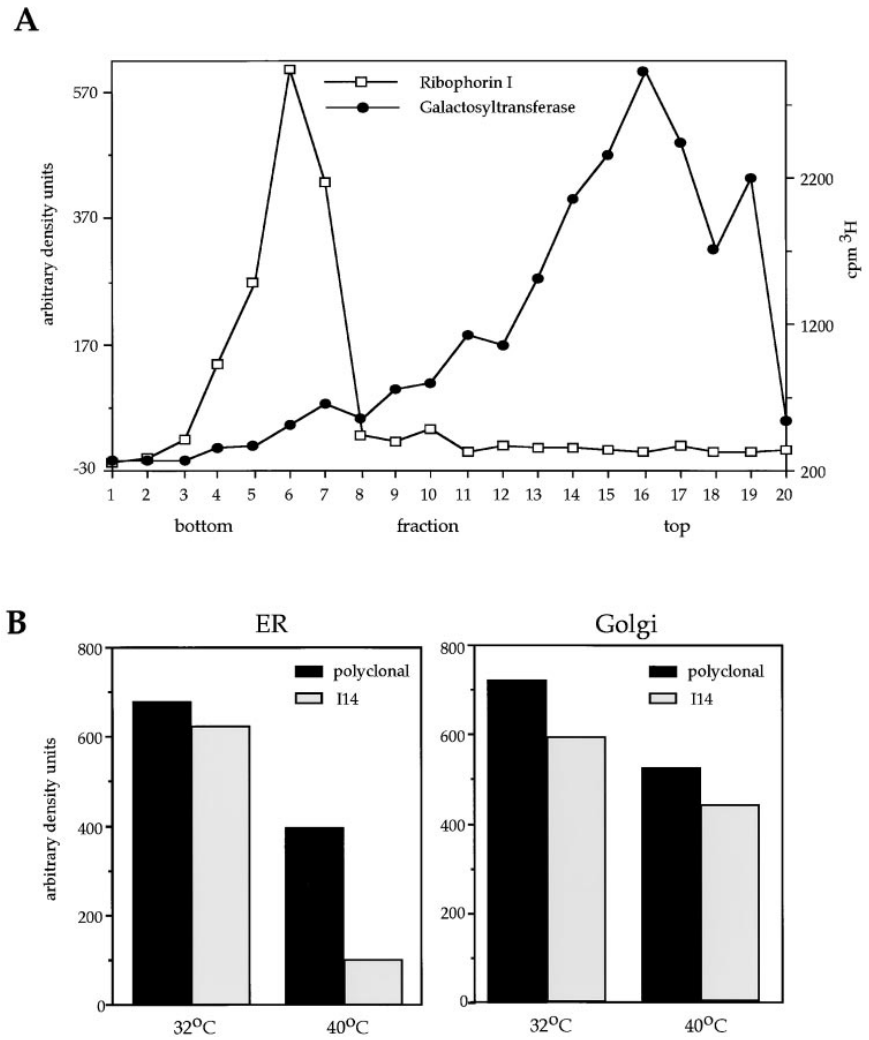

Figure 8. Fractionation of ER and Golgi membranes and misfolding in vitro. (*A*) COS cells transfected with VSVG–TGN38 were labeled with [^{35}S]methionine, chased to generate labeled molecules in both the ER and Golgi complex, and then homogenized and fractionated on a 0–26% Optiprep gradient, as described in Materials and Methods. Fractions were analyzed for the presence of specific markers; ribophorin (*ER*), and galactosyltransferase activity (*Golgi*). (*B*) Pooled ER and Golgi fractions were aliquoted into equal volumes and incubated at either 32° or 40°C for 60 min. Membranes were lysed at the indicated temperatures, placed on ice, and immunoprecipitated with polyclonal anti-VSV antiserum or conformation-sensitive anti-VSVG I14 antibodies. Precipitates were analyzed by SDS-PAGE and scanning densitometry. Notice the relative ability of I14 antibodies to recognize VSVG–TGN38 from Golgi, but not from ER, fractions at 40°C.

ubilization at the indicated temperatures, VSVG–TGN38 was immunoprecipitated with either polyclonal antibodies that recognize both folded and misfolded G protein, or with the conformation-sensitive I14 antibody.

As shown in Fig. 8 (*b*), polyclonal anti-VSV recognized VSVG–TGN38 in both ER and Golgi fractions regardless of the incubation temperature. I14 was also able to precipitate VSVG–TGN38 from fractions containing Golgi and ER membranes at 32°C. However, incubation at 40°C dramatically reduced the ability of I14 to recognize VSVG–TGN38 from ER fractions, whereas immunoprecipitation from Golgi fractions was only slightly affected. Therefore, consistent with de Silva et al. (1990), only the ER appears to contain specific factors capable of misfolding tsO45-containing chimeras at the nonpermissive temperature.

We next determined whether we could observe the recycling/misfolding process in intact cells biochemically. COS cells expressing VSVG–TGN38 were metabolically labeled at 40°C, chased in unlabeled medium at 32°C for either 5 min, where the majority of newly synthesized molecules should still be in the ER, or for 2 h to accumulate the labeled chimera in the Golgi complex, before shifting back to 40°C for an additional period of time. After solubilization at the indicated temperatures, VSVG–TGN38 was immunoprecipitated with either polyclonal or conformation-sensitive I14 antibodies. As shown in Fig. 9, whereas VSVG–TGN38 could fold completely after a 5-min chase at 32°C, only 25% could be immunoprecipitated with I14 after a subsequent shift to 40°C. Thus, folded VSVG–TGN38 can rapidly misfold when it is contained within the environment of the ER. However, when VSVG–TGN38 had been chased for 2 h at 32°C and then shifted to 40°C, >80% remained properly folded after a 10-min incubation at 40°C. It is likely that the remaining misfolded fraction represents a population of VSVG–TGN38 molecules that has not left the ER. Therefore, as with the parent G protein (de Silva et al., 1990), and consistent with the in vitro data (Fig. 8), the chimera becomes resistant to misfolding when it is delivered to the Golgi complex. If, after a 2-h chase at 32°C, labeled cells were shifted to 40°C for increasing periods of time, the amount of I14 precipitable material decreased, whereas material recognized by polyclonal anti-VSV remained unchanged (Fig. 9). The temporal loss of I14 immunoreactivity, therefore, correlated with recycling and subsequent misfolding of VSVG–TGN38 as it redistributed into the ER. This misfolding could be reversed by a subsequent short incubation at 32°C (Fig. 9). Thus, the fusion proteins do not appear to misfold in the Golgi com-

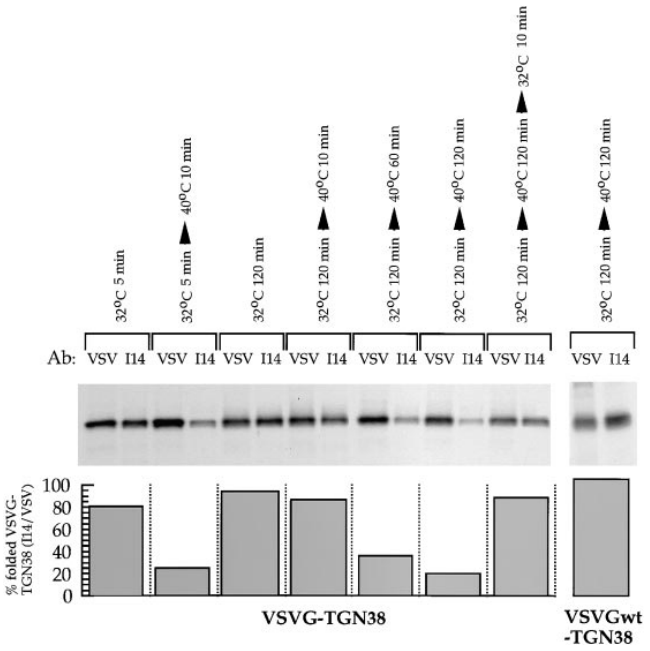

Figure 9. The relationship between misfolding and recycling in intact cells. COS cells expressing VSVG–TGN38 were pulse labeled at 40°C, and then chased in unlabeled medium at 32°C for either 5 min or 2 h. After each chase point, equal aliquots were shifted back to 40°C for an additional 10 min. Cells were solubilized and immunoprecipitated with polyclonal anti-VSV or monoclonal I14 antibodies. Whereas labeled material within the ER is sensitive to misfolding, material chased into the Golgi becomes resistant. Continued incubation at 40°C leads to a decrease in the percentage of folded but not total VSVG–TGN38. A subsequent shift back to 32°C permits refolding of VSVG–TGN38. Notice that a wild-type VSVG–TGN38 chimera is totally resistant to misfolding at 40°C. Quantitation reflects the average of two independent experiments.

plex upon a shift to 40°C, but only once they have recycled into the ER.

Discussion

An Assay for Retrograde Traffic in Mammalian Cells

Retrograde transport of proteins and lipids from the Golgi complex back to the ER is widely recognized as crucial for proper functioning of the secretory pathway. Such retrograde flow maintains the surface area of the ER in the face of extensive membrane outflow into the secretory pathway (Wieland et al., 1987; Hoffman and Pagano, 1993), ensures the return of escaped ER resident proteins (Jackson et al., 1993; Letourneur et al., 1994; Pelham, 1995), and recycles membrane machinery involved in ER to Golgi traffic (Lewis and Pelham, 1996; Rothman, 1996). The experiments reported here suggest an additional role of retrograde traffic: to periodically return Golgi-localized proteins to the folding environment of the ER.

To examine whether proteins that have left the ER ever return to its folding environment, we used a retrograde transport assay based on the capacity of the ER to retain misfolded proteins. We found that we could append the entire temperature-sensitive ectodomain (~60 kD) of the VSVGtsO45 to several Golgi proteins or targeting domains without disrupting the thermoreversible characteristics of VSVGtsO45, or the ability of the modified proteins to target and function properly. We reasoned that if these Golgi-localized chimeras were recycled through the ER, they should become trapped and accumulate within the ER over time at the nonpermissive temperature. This would be due to the unique capacity of the ER to retain misfolded VSVGtsO45 (Doms et al., 1987; de Silva et al., 1990). The rate and extent such proteins accumulate in the ER, losing their normal localization, should reflect their capacity for recycling.

Cycling Dynamics of VSVG Fusion Proteins

Addition of the VSVGtsO45 ectodomain to KDELR, KDELRm, and domains of TGN38 and IL2R conferred the thermosensitive folding characteristics of VSVGtsO45 to these proteins/targeting domains, resulting in their misfolding and retention in the ER after biosynthesis at 40°C. It had no effect, however, on proper folding and targeting of these proteins at the permissive temperature. At 32°C, VSVG–KDELR gave a mixed Golgi/ER distribution and remained endo H sensitive, consistent with the idea that the KDELR cycles between the Golgi and ER (Lewis and Pelham, 1992; Tang et al., 1993), and is primarily localized to the proximal regions of the Golgi complex (Griffiths et al., 1994). In contrast, the VSVG–KDELRm chimera showed little ER staining, had sugar modifications characteristic of the *trans*-Golgi/TGN, and showed extensive colocalization with the TGN marker protein, furin. Thus, the single amino acid change (Asp to Asn at position 193) within KDELR is sufficient to alter the distribution of this molecule within the Golgi complex. VSVG–TGN38 localized to the Golgi complex at 32°C and could be redistributed to the cell surface by overexpression of additional molecules that contained TGN-targeting signals (data not shown). This is consistent with the results of Humphrey et al. (1993), and is likely due to saturation of the sorting machinery required to maintain TGN localization (Marks et al., 1996). Thus, VSVG–TGN38 was likely targeted to the TGN.

In cells in which the VSVGtsO45 fusion proteins or parental VSVGtsO45 were localized to the Golgi complex, a temperature shift to 40°C for 2 h resulted in the redistribution of VSVG–KDELR, VSVG–KDELRm, and VSVG–TGN38 into the ER. For VSVG–KDELR, this occurred with a $t_{1/2}$ of 20 min, whereas for VSVG–TGN38 and VSVG–KDELRm, it was ~60 and 75 min, respectively. VSVGtsO45, by contrast, did not redistribute to the ER, but was delivered to the cell surface. The changes in distribution of the chimeras did not require new protein synthesis, indicating that their accumulation within the ER was not due to newly synthesized material, but represented a preexisting population that had recycled from the Golgi complex.

Recycling Does Not Appear to Be Induced by Misfolded Proteins

The extensive redistribution of VSVG–KDELR, VSVG–KDELRm, and VSVG–TGN38 into the ER after 2 h of shift to the nonpermissive temperature was surprising. Although KDELR is thought to constitutively cycle between the ER and Golgi complex (Lewis and Pelham, 1992), and,

therefore would be predicted to redistribute to the ER in this assay, there is no evidence for recycling of KDELRm and TGN38, which are believed to be relatively stable residents of the Golgi complex. This raises the question, therefore, whether the observed retrograde transport of these molecules reflects constitutive recycling (independent of the VSVG lumenal tag), or is artificially induced by attachment of the lumenal domain of VSVGtsO45. Misfolding of this domain at 40°C in the Golgi complex could be recognized for retrieval by a chaperone-like molecule such as BiP that has leaked out of the ER (Hammond and Helenius, 1994). Several lines of evidence, however, suggest that retrieval was not activated within the Golgi complex, and that misfolding occurred within ER and not Golgi membranes. First, parental VSVGtsO45 that had accumulated within the Golgi complex was not retrieved to the ER upon a shift to 40°C, but was delivered solely to the cell surface. Since it contains the thermosensitive ectodomain similar to all fusion proteins, partial delivery back to the ER upon a temperature shift should have been expected under a chaperone-retrieval model. Second, our data indicated that misfolding occurred within ER and not Golgi membranes, as assessed with conformation-sensitive antibodies, in both isolated membrane fractions (Fig. 8) and intact cells (Fig. 9). Third, inhibiting redistribution into the ER by ATP depletion, etc. did not reduce the ability to stain for the chimeras in the Golgi complex with conformation-specific antibodies even after 2 h at 40°C, whereas staining in the ER was completely eliminated (Fig. 7). Finally, proteins involved in the assisted folding of newly synthesized secretory and membrane proteins, such as BiP, protein disulfide isomerase, and calnexin, are almost exclusively localized to the ER, with little or no escape into the Golgi complex (Akagi et al., 1989; Bole et al., 1989; Rajagopalan et al., 1994). It is unlikely that under normal conditions escaped chaperones could have retrieved the entire Golgi-localized pools of VSVG–KDELR, –KDELRm, and –TGN38 within 2 h after a shift to the nonpermissive temperature (for VSVG–KDELR, complete redistribution occurred within 20–40 min).

Thus, the redistribution into the ER observed at 40°C did not likely occur by activating retrieval via misfolding of the VSVG chimeras within the Golgi complex. However, we cannot rule out the possibility that subtle conformational changes, such as transient unfolding or temporary aggregation, may influence the trafficking of these molecules. Furthermore, since the epitope recognized by the I14 antibody used in this study is one that forms late in the VSVG folding process (Doms et al., 1988), it is unlikely that significant conformational changes lead to unfolding of the chimeras within the Golgi complex at 40°C. Based on the above results, the simplest interpretation for redistribution of the VSVG chimeras into the ER upon shift to the nonpermissive temperature is that it reflects a constitutive recycling pathway followed by these molecules. At 40°C, the chimeras become trapped in the ER due to misfolding of the VSVG ectodomain in the unique lumenal environment of the ER.

Retrograde Traffic May Not Require Specific Signals

If the VSVG chimeras are indeed constitutively recycling from the Golgi to the ER, an important question is whether their retrieval is mediated by specific retrieval signals located on the attached proteins or targeting domains, or is due to nonselective or bulk-flow transport. Many membrane proteins known to cycle between the ER and Golgi complex contain dibasic signals within their cytoplasmic domains (Jackson et al., 1993; Gaynor et al., 1994; Schutze et al., 1994; Itin et al., 1995) that are thought to be recognized by a sorting machinery that mediates their retrieval from the Golgi complex (Letourneur et al., 1994). The finding that VSVG–TGN38, which lacks known Golgi to ER retrieval signals within its cytoplasmic tail (Luzio et al., 1990), and VSVG–KDELRm, which is defective for KDEL-mediated retrieval (Townsley et al., 1993), undergo retrograde transport to the ER, raises the question of whether membrane cycling between the Golgi complex and ER is an inherent feature of Golgi-localized proteins that does not require specific retrieval motifs.

It has been noted that many Golgi resident proteins have shorter transmembrane domains than molecules targeted to the cell surface (Bretscher and Munro, 1993). A general lipid sorting model was proposed for Golgi protein localization involving partitioning into thinner regions of the Golgi bilayer and exclusion from forward moving transport intermediates (Bretscher and Munro, 1993; Munro, 1995). To test whether Golgi retention of proteins with shortened transmembrane domains correlates with Golgi to ER recycling activity, we replaced the 23–amino acid transmembrane domain of VSVGtsO45 with a synthetic domain of 15 leucines (Munro, 1995). Cells expressing VSVG–Leu15 at 32°C showed a significant accumulation within the Golgi complex as the protein was slowly transported through the Golgi to the plasma membrane. When these cells were shifted to 40°C, a significant proportion of VSVG–Leu15 redistributed to the ER rather than to the plasma membrane. In contrast, under the same conditions, VSVGtsO45 was delivered solely to the plasma membrane. This suggests that itinerant proteins moving rapidly through the Golgi complex (VSVGtsO45) are less available for recycling than those that move more slowly (VSVG–IL2R > VSVG–Leu15), and raises the interesting possibility that at least for proteins en route to the cell surface, anterograde and retrograde transport coexist within the Golgi complex as mutually competing processes.

Since replacing the transmembrane domain of VSVGtsO45 with a shorter hydrophobic domain would be expected to primarily influence the lipid environment with which this protein interacts, rather than generate a novel retrieval motif, these results support a relationship between lipid partitioning and retrograde traffic. The Golgi complex is known to be the site of major lipid transitions between the ER and plasma membrane (Orci et al., 1981; van Meer, 1989; Munro, 1995). Changes in lipid composition across the Golgi stacks (Bretscher and Munro, 1993) may establish a gradient of recycling activity, such that *cis*-Golgi proteins would become partitioned within membranes with high levels of recycling activity. This activity would then decrease across the Golgi stack by distilling back to earlier cisternae or to the ER (Rothman and Wieland, 1996). *trans*-Golgi proteins would tend to partition into membranes with less recycling activity, and recycle to the ER more slowly.

An increasing body of evidence suggests that Golgi resident proteins cycle within the Golgi complex (Johnston et al., 1994; Hoe et al., 1995; Harris and Waters, 1996). Since Golgi enzymes contain short cytosolic domains lacking conventional Golgi to ER retrieval signals suggests that alternative mechanisms for recycling of these proteins exist. If such cycling were mediated by a lipid-partitioning mechanism, it may extend not only within the Golgi complex, but all the way back to the ER, a view that is consistent with our results. Specific retrieval determinants (e.g., KDEL and KKXX; Letourneur et al., 1994; Pelham, 1995) may increase recycling efficiency of selected proteins, but the overall extent of retrograde transport within the Golgi complex and back to the ER would be a constitutive process that operates independently of these types of signals. A recent study by Johannes et al. (1997) supports this thinking and raises questions regarding the role of putative retrieval signals. Shiga toxin B fragments carrying either wild-type or mutated KDEL signals were found to be delivered from the cell surface through the Golgi complex to the ER with equal kinetics. This indicates that the KDEL signal itself is not likely to be important for retrograde transport per se, but rather for retention within the ER (Johannes et al., 1997).

Implications

An important function of Golgi protein recycling to the ER may be to allow Golgi proteins to be periodically exposed to the folding machinery in the ER where they could undergo further modification/repair and/or degradation. The ER is known to be unique in its content of folding enzymes and chaperones that catalyze dynamic folding/unfolding reactions (Gething and Sambrook, 1992). Such reactions are necessary for folding and assembly of newly synthesized proteins into functional, transport-competent complexes, and ensure that nonfunctional and incomplete protein complexes do not leave the ER (Hurtely and Helenius, 1989; Doms et al., 1993). Given that the ER is the major compartment where such misfolding and retention occurs, recycling to the ER could be important for monitoring the fidelity of proteins that spend most of their lifetime in distal secretory compartments. The fact that properly folded VSVGtsO45 chimeras recycled back to the ER and became quickly misfolded and trapped at nonpermissive temperatures supports this hypothesis.

A major consequence of constitutive cycling of Golgi proteins (and lipids) would be its contribution to the dynamic nature of the Golgi complex. If the bulk of Golgi membrane components constitutively recycle to the ER, then the integrity of the Golgi complex will be critically dependent upon (and likely regulated by) the return of such components from the ER. Consistent with this thinking, virtually all conditions that interfere with delivery of proteins from the ER into the Golgi complex, including treatments with BFA, ilimaquinone, okadaic acid, and overexpression of particular rab mutants, result in dispersal of Golgi membranes (Lippincott-Schwartz et al., 1989; Lucocq, 1992; Takizawa et al., 1993; Wilson et al., 1994; Martinez et al., 1997). One well-studied example is microtubule disruption by nocodazole (Cole et al., 1996), which blocks translocation of pre-Golgi structures into the centrosomal region where the Golgi complex normally resides (Saraste and Svensson, 1991; Presley et al., 1997). Golgi enzymes in nocodazole-treated cells redistribute gradually over several hours to ER exit sites, giving rise to Golgi fragmentation (Cole et al., 1996). Since ERGIC-53, a protein which rapidly and constitutively cycles between the ER and Golgi (Itin et al., 1995), redistributes within 15 min from the Golgi to these sites after first moving through the ER, the slower accumulation of Golgi resident components to the same ER exit sites in nocodazole-treated cells has been proposed to reflect constitutive bulk cycling of Golgi components back to the ER (Cole et al., 1996).

The idea that all Golgi components, including Golgi enzymes, are suggested to recycle periodically through the ER does not imply that the ER/Golgi system is a uniform mixing compartment. Differences in recycling rates, as well as sorting mechanisms that effectively segregate Golgi enzymes, may prevent full spatial and temporal contact of Golgi enzymes with potential substrates. Thus, all Golgi resident proteins may not become uniformly modified. It is evident, however, that significant mixing does occur within the Golgi. Many proximal Golgi proteins possess distal Golgi modifications (Moreman and Touster, 1985; Johnson et al., 1994; Harris and Waters, 1995), and distal Golgi enzymes can recycle to early Golgi compartments (Hoe et al., 1995). Evidence of biochemical modification does not verify the location where the processing event occurs, however; only that enzyme and substrate (and sugar nucleotide donor, for example) associate within a permissive processing environment. Therefore, it cannot be ruled out that evidence of a particular Golgi modification actually reflects recycling of Golgi enzymes to earlier Golgi compartments, rather than transport of substrate forward into more distal Golgi compartments. Based on the work presented here, it is not unreasonable to extend this thinking to suggest that certain ER proteins may acquire Golgi modifications via recycling of Golgi enzymes, even if they never actually leave the ER.

In summary, we have developed a novel assay for monitoring Golgi to ER retrograde transport in living cells that is capable of addressing the extent, specificity, and biochemical requirements of protein transport through this pathway. Results with this assay suggest entry into the retrograde pathway is not exclusively signal-mediated, and may be an inherent property of proteins distributed within Golgi membranes. If correct, the overall magnitude of Golgi to ER retrograde traffic would be much greater than previously expected, with important implications for our understanding of Golgi maintenance and biogenesis.

We wish to thank J. Bonifacino, C.E. Ooi, J. Donaldson, H. Radhakrishna, and K. Hirschberg (all from NIH, Bethesda, MD) for valuable comments on the manuscript. In addition, we wish to thank T. LaVaute and J. Sloan-Lancaster for help with the FACS® analysis.

Received for publication 18 August 1997 and in revised form 3 November 1997.

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