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Exposed Thiols Confer Localization in the Endoplasmic Reticulum by Retention Rather than Retrieval*

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The cysteine present in the Ig μ chain tailpiece (μtp) prevents the secretion of unpolymerized IgM intermediates and causes their accumulation in the endoplasmic reticulum (ER). In principle, this can be the consequence of actual retention in this organelle or of retrieval from the Golgi. To determine which of the two mechanisms underlies the cysteine-dependent ER localization, we analyze here the post-translational modifications of suitably engineered cathepsin D (CD) molecules. The glycans of this protease are phosphorylated by post-ER phosphotransferases and further modified in the trans-Golgi to generate a mannose 6-phosphate lysosome targeting signal. Only trace amounts of the μtp-tagged CD (CDMμtpCys) are phosphorylated, unless retention is reversed by exogenous reducing agents or the critical cysteine mutated (CDMμtpSer). In contrast, a KDEL-tagged CD, that is retrieved from the Golgi into the ER, acquires phosphates, though mainly resistant to alkaline phosphatase. Similarly to CDMμtpSer, the few CDMμtpCys molecules that escape retention and acquire phosphates in the cis-Golgi are transported beyond the KDEL retrieval compartment, as indicated by their sensitivity to alkaline phosphatase. These results demonstrate that the thiol-dependent ER localization arises primarily from true retention, without recycling through the Golgi.

Transmembrane and lumenal proteins of the central vacuolar system can achieve their localization in a given organelle either by retention or by retrieval from downstream compartments (reviewed in Ref. 1). While most integral membrane proteins of the Golgi seem to be genuinely retained (2, 3), endoplasmic reticulum (ER)[1] residence largely depends on retrieval mechanisms (4, 5). Thus, resident lumenal proteins marked by a KDEL motif are recognized by a receptor in the Golgi and shuttled back to the ER (5). Retention and retrieval can cooperate in determining ER localization, as in the case of calreticulin (6). However, motifs that mediate ER residence by true retention, *i.e.* independent from retrieval, have not been described so far.

Studies on immunoglobulins (Ig) revealed that the recognition of exposed thiols on unassembled molecules is one of the mechanisms that restrict secretion to structurally mature cargo proteins (7–10). Retention of Ig-λ chains in the ER is mediated by their COOH-terminal cysteine and correlates with the formation of disulfide bonds with numerous resident proteins, including ERp72 and PDI (11). However, whether thiol-dependent quality control mechanisms act by causing recycling from the Golgi or by determining actual retention in the ER is still unclear. To address this issue, we have utilized chimeric human cathepsin D (CD) molecules, in which either the KDEL motif or the carboxyl-terminal 20 amino acids of the IgM heavy chain μ (the μ tailpiece, μtp) had been appended to the Myc-tagged lysosomal protease (7, 12). The μtp is sufficient to cause localization in the ER if the cysteine residue in the penultimate position is present (7, 13). The well known stepwise processing of CD (for review, see Ref. 14) makes this protease a useful reporter to study the interorganelle trafficking. Human CD is synthesized as a diglycosylated precursor of approximately 53 kDa. Upon exit from the ER, mannose groups are phosphorylated by transfer of a *N*-acetylglucosamine 1-phosphate (P-GlcNAc) moiety operated by lysosomal-enzyme phosphotransferases. In the cis-Golgi, and possibly also within the intermediate compartment (for review, see Ref. 15), phosphotransferases recognize high mannose oligosaccharides only when the latter are linked to a certain amino acid context that hallmarks lysosomal enzymes (16, 17). The addition of P-GlcNAc, which is most efficient on the COOH-terminal glycan of pro-CD (18), prevents the formation of complex oligosaccharide chains typical of secretory proteins that traverse the Golgi. Upon further transport, the GlcNAc group is removed by a phosphodiester glycosidase located in the late Golgi stacks and the exposed mannose 6-phosphate mediates lysosomal segregation (19). The efficiency of GlcNAc removal and of delivery to lysosomes may vary depending on the cell type and culture conditions (20), leading to partial secretion (19, 20). When the GlcNAc moiety is removed, the "uncovered" phosphate may be cleaved *in vitro* by treatment with alkaline phosphatase (AP) (21). Hence, at least three phenotypes can be identified that correspond to different subcellular localizations of proCD: (i) nonphosphorylated proteins that have not reached the cis-Golgi, (ii) phosphorylated, AP-resistant forms that are localized in, or have transited through, the cis but not the trans cisternae of the Golgi, and (iii) phosphorylated, AP-sensitive molecules, that are in or beyond the trans-Golgi.

We have exploited immunofluorescence microscopy and biochemical analyses to compare the modes by which a COOH-

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[1] The abbreviations used are: ER, endoplasmic reticulum; AP, alkaline phosphatase; CD, cathepsin D; CDM, Myc-tagged CD; CDMK, CDM + KDEL; CDMμtpCys, CDM + the Ig μ tailpiece (Cys^{575}); CDMμtpSer, CDM + the Ig μ tailpiece (Ser^{575}); Ig, immunoglobulin; 2-ME, 2-mercaptoethanol; Endo-H, endo-β-*N*-acetylglucosaminidase H; PAGE, polyacrylamide gel electrophoresis.

terminal KDEL sequence or an active cysteine in the μtp context lead to the ER localization of chimeric cathepsins. Our results indicate that, unlike KDEL, the μtp does not require recycling between the Golgi and the ER. Hence, thiol-dependent ER residence is due to true retention in this organelle and appears to be proximal with respect to KDEL retrieval.

MATERIALS AND METHODS

Cells, Plasmids, and Transfections—Monkey COS7 fibroblasts (22) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected by calcium phosphate precipitation (23) or lipofection (24) and analyzed between 40 and 60 h after transfection. Plasmids CDMK (12), CDMμtpCys, and CDMμtpSer have been described in detail previously (7). Briefly, the constructs encode a human CD cDNA tagged at the COOH terminus with a c-Myc-derived epitope (*M*) to which the hexapeptide SEKDEL or the μtp (wild-type or with the penultimate cysteine replaced by serine) is appended.

Immunofluorescence—48 h after transfection the cells were trypsinized, seeded at low density on sterile coverslips and cultured overnight. When indicated, cells were incubated for further 4 h with 14.4 mM 2-ME. Cells were then fixed with 3.7% formaldehyde (10 min at room temperature), permeabilized with 0.1% Triton X-100, incubated with the primary antibody (the mouse monoclonal anti-Myc 9E10 or a rabbit anti-calnexin kindly provided by Ari Helenius), followed by the appropriate secondary fluorescein isothiocyanate-conjugated antibodies (Sigma).

Metabolic Labeling, Immunoprecipitation, and Protein Analysis—Metabolic labeling with a [^{35}S]methionine/cysteine mixture (Tran^{35}S-label) or ^{32}P-labeled inorganic phosphate (ICN Biochemicals, Milan, Italy) was performed as described previously (9, 20, 25). Media and cell lysates were immunoprecipitated with a rabbit anti-human CD followed by either protein A-Sepharose (Pharmacia Biotech Inc.) or a goat anti-rabbit IgG conjugated to oxirane beads (26). Alternatively, the chimeric protein was precipitated using the 9E10 monoclonal anti-Myc (27) covalently conjugated to Sepharose. Immunoprecipitates were eluted and analyzed by SDS-PAGE and fluorography (20). When indicated, immunoprecipitates were incubated with 1 milliunit of endo-β-*N*-acetylglucosaminidase H (Endo-H, Boehringer Mannheim) for 24 h at 37 °C (10). The presence of phosphomonoesters on CD molecules was ascertained by treating ^{32}P-labeled immunoprecipitates for 24 h with or without 12.5 units of AP (Boehringer Mannheim), a condition sufficient to remove the phosphates from uncovered phosphomannosyl groups (21). Autoradiograms were scanned by an automated densitometer (Molecular Dynamics).

Western Blot Analysis—In some experiments, ^{32}P- or ^{35}S-labeled immunoprecipitates were resolved by SDS-PAGE and transferred onto nitrocellulose filters. The latter were first exposed to autoradiographic films or to a PhosphorImager (Molecular Dynamics) to detect radiolabeled proteins. Subsequently, to detect both the labeled and unlabeled chimeric proteins, filters were decorated with anti-Myc antibodies followed by peroxidase conjugated anti-mouse K chains and developed by ECL (Amersham Corp.).

RESULTS

CDM-μtpCys, but Not CDM-μtpSer, Colocalizes with CDMK—The chimeric proteins CDMK, CDMμtpCys, and CDMμtpSer have been described previously (Ref. 7, see also Fig. 4). As shown in Fig. 1, both CDMK and CDMμtpCys accumulate in a reticular region, largely overlapping with the distribution of the ER marker calnexin (compare *panels A–D* and *B–E*). Although the intracellular distribution of the two proteins is rather similar, quantitative differences can be observed, the CDMK staining being generally more intense than CDMμtpCys. As confirmed by biochemical data (see below), this reflects differences in intracellular pool sizes, derived from the different turnover rates of CDMμtpCys and CDMK (7). Cells transfected with CDMμtpSer show a perinuclear focal staining (*panel C*) suggestive of intraGolgi accumulation of the chimeric protein. This is consistent with the observation that the cysteine to serine mutation allows secretion of chimeric CD (Ref. 7, see also Fig. 2, *panel B*). To what extent this protein is also transported to lysosomes is not testable with this assay, as the c-Myc tag is rapidly cleaved within these organelles (12).

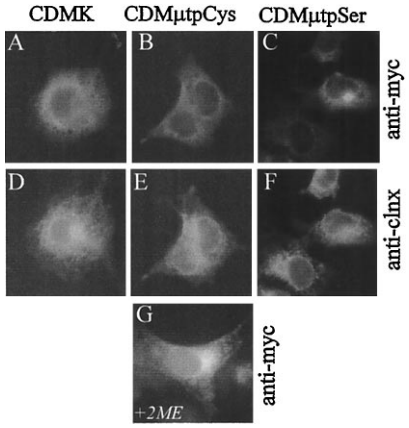

FIG. 1. **Subcellular localization of chimeric CDs.** Fourty-eight hours after transfection with CDMK (*panels A* and *D*), CDMμtpCys (*panels B E*, and *G*) or CDMμtpSer (*panels C* and *F*), COS7 cells were seeded onto sterile slides, cultured for further 16 h, and stained with 9E10 (*panels A–C*, anti-Myc) or anti-calnexin (*panels D–F, anti-clnx*) antibodies. *Panel G* shows the distribution of CDMμtpCys transfectants treated for 4 h with 14.4 mM 2-ME before staining with anti-Myc antibodies.

We have shown previously that thiol-mediated retention mechanisms can be reversed by treating cells with membrane permeant reducing agents, such as 2-ME (9, 28, 29). Also some CDMμtpCys can be mobilized with 2-ME: as shown in *panel G*, the Golgi area of COS7 cells transiently transfected with CDMμtpCys becomes stained by anti-Myc antibodies upon treatment with the reducing agent.

Unlike CDMμtpSer, CDMμpCys Does Not Acquire Phosphates—When cells transfected with CDMμtpCys are pulse labeled with radioactive amino acids, three bands are immunoprecipitated by the anti-human CD antibodies (Fig. 2, *panel A, lane 3*). The two fast migrating bands, which are present in both transfected and untransfected cells (compare *lanes 1* and *3*), likely correspond to different isoforms of the endogenous, monkey proCD (*mP*). The doublet is evident also after digestion with Endo-H (*lanes 2* and *4*), indicating that both these isoforms bear high mannose type oligosaccharides. The upper band present in *lane 3*, but absent from untransfected COS7 cells (*lane 1*), corresponds to the chimeric proCD (*cP*).

Having an easy way to identify the transgene products, we then compared the transport-linked processing of CDMμtpCys and CDMμtpSer by labeling cells in parallel with ^{35}S-labeled amino acids (*panel B*) or with [^{32}P]orthophosphate (*panel C*). The endogenous CD molecules offer an useful internal control of the interorganelle trafficking and intraorganelle processing in the two transfectants. After a 2-h pulse with ^{35}S-labeled amino acids, both the endogenous (*mP*) and the heterologous chimeric CD (*cP*) molecules are detected in the lysates of transfected cells (*panel B*). While some endogenous CD is present in the media of both CDMμtpCys and CDMμtpSer transfectants, only the CDMμtpSer mutant is secreted. Thus, as frequently observed in cultured cells (19, 20), some CD molecules escape lysosomal segregation and are secreted by COS7 cells. After 2 h of chase, the differences between CDMμtpCys and CDMμtpSer are more evident. Little if any CDMμtpCys is secreted (*lane 4*). In contrast, large amounts of CDMμtpSer are present extracellularly, giving yield to a broad band whose heterogeneity likely reflects the processing of the tailpiece glycans (*cP**). As the glycosylation site present in the μtp lacks the consensus sequence for mannose phosphorylation, this sugar undergoes the trimming and processing typical of secretory proteins upon transport through the Golgi (10). Hence, the observation that even after 2 h of chase the CDMμtpCys molecules remain sensitive to Endo-H (compare *lanes 3* and *4* of *panel A*) is

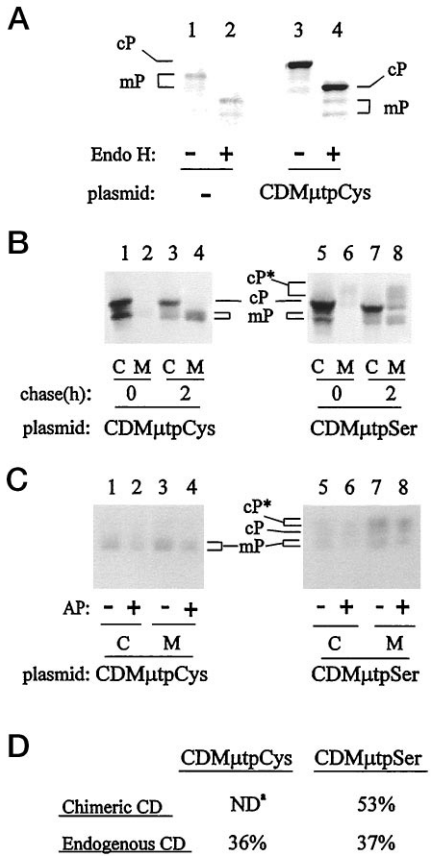

FIG. 2. **Different transport and processing of CDMμtpCys or CDMμtpSer.** CDMμtpCys and CDMμtpSer were transiently expressed in COS7 cells and analyzed after labeling either the protein backbone with ^{35}S-labeled amino acids (*panels A* and *B*) or the phosphomannosyl moieties with [^{32}P]phosphates (*panel C*). In *panel A*, the migration on SDS-PAGE of the endogenous CD (*mP*) was compared with the heterologous CDMμtpCys chimera after pulse labeling for 15 min CDMμtpCys transfectants and untransfected COS7 cells (−) and chasing for 2 h. As in *panels B* and *C*, the different CD forms were immunoprecipitated using polyclonal anti-human CD antibodies, resolved by SDS-PAGE, and revealed by gel fluorography. Samples were treated with or without endoglycosidase H (*Endo H*). *Panel B* shows the immunoprecipitates obtained from cell lysates (*C*) and media (*M*) of cells pulsed for 2 h with ^{35}S-labeled amino acids before or after 2 h of chase. Only CDMμtpSer presents slower migrating forms due to complex-type sugars (*cP**). *Panel C* shows the chimeric or endogenous CD forms observed in the CDMμtpCys or CDMμtpSer transfectants after labeling for 2 h with [^{32}P]phosphates and chasing for further 2 h. It is of note that the ratio between extracellular (*M*) and intracellular (*C*) phosphorylated molecules is higher for CDMμtpSer than for endogenous CD. The nature (covered or uncovered) of the phosphomannosyl group was ascertained on the basis of their sensitivity to alkaline phosphatase (*AP*). *Panel D*, the percentage of AP-resistant CDs (endogenous and chimeric) present in the media of cells chased 2 h was determined by densitometric analyses of the autoradiogram shown in *panel C*. *ND*, not detectable.

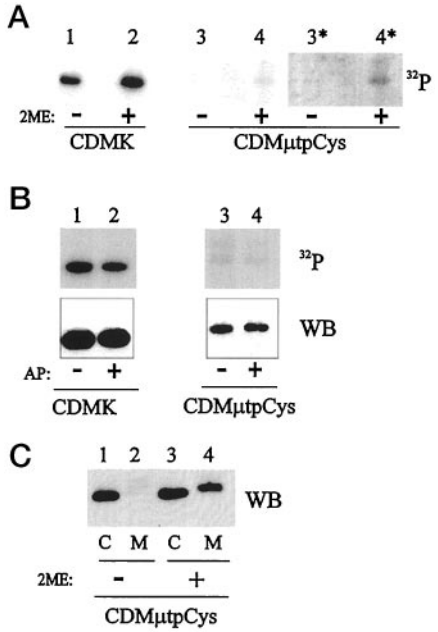

FIG. 3. **Comparison of CDMK and CDMμtpCys phosphorylation in the absence or presence of 2-ME.** *Panel A,* CDMμtpCys or CDMK transfectants were labeled for 4 h with [^{32}P]orthophosphate, in the presence (−) or absence (+) of 14.4 mM 2-ME. Cell lysates were immunoprecipitated with 9E10 anti-Myc antibodies and resolved by SDS-PAGE under nonreducing conditions. *Lanes 3** and *4** show an overexposure of the autoradiogram. *Panel B*, the anti-Myc immunoprecipitates obtained from 2-ME-treated cells were incubated with or without alkaline phosphatase (*AP*), resolved by SDS-PAGE under reducing conditions, and transferred to nitrocellulose. Filters were first exposed to PhosphorImager screens (^{32}P) and then developed by ECL with anti-Myc followed by peroxidase-conjugated anti-mouse Ig K chains (*WB*). The relevant bands were quantitated by densitometry. *Panel C*, the anti-Myc immunoprecipitates from the cell lysates (*C*) and media (*M*) of CDMμtpCys transfectants, treated for 2 h with or without 2ME, were transferred onto nitrocellulose filters and developed by ECL as above (*WB*).

consistent with their ER localization.

As expected, in both transfectants the endogenous proCD molecules acquire phosphates and are secreted, indicating transport throughout the secretory pathway (20, 30). Likewise, CDMμtpSer molecules are able to traverse the Golgi stacks, as demonstrated by the presence of phosphorylated, AP-sensitive molecules in both the cell lysates and media (*panel C, lanes 5–8*). In contrast, phosphorylated CDMμtpCys are not detected, suggesting that an active μ tailpiece is sufficient to prevent transport to the phosphotransferase compartments. Endogenous CD and CDMμtpSer show partial sensitivity to AP (*panel C*), likely due to inefficient uncovering. About one-third of the endogenous and half of the chimeric molecules bear AP-resistant phosphates (*panel D*).

Exogenous Reducing Agents Induce Phosphorylation of CDMμtpCys—The ER localization of proteins bearing the KDEL signal is ensured through a continuous retrieval from the early Golgi stacks (for review, see Ref. 31). Consistently, CDMK acquires phosphates (Fig. 3, *panel A, lane 1*), an observation previously made by Pelham (12). In contrast, phosphorylated CDMμtpCys are barely detected (*lane 3*) even after prolonged exposures of the gel (*lane 3**) unless 2ME is added (see below). In this experiment, samples were normalized with respect to the rate of transgene synthesis, determined by parallel short ^{35}S pulse labeling experiments (not shown). Owing to their different half-lives in the ER, the steady state pool of CDMK is generally higher than that of CDMμtpCys. However, even taking this into account, we find that CDMK bears at least 10 times more phosphates than CDMμtpCys. Indeed, the ^{32}P/WB densitometric ratios (see legend to Fig. 3 for details) were 0.6 and 0.05 for CDMK and CDMμtpCys, respectively (0.5 and 0.15 after 2-ME treatment).

As shown in Fig. 1, *panel G*, part of CDMμtpCys is mobilized by 2-ME. Consistent with this, 2-ME also induces the secretion (Fig. 3, *panel C*) and phosphorylation (Fig. 3 *panel A, lanes 4, and 4**) of CDMμtpCys. Like CDMμtpSer, the phosphorylated CDMμtpCys display partial sensitivity to AP (Fig. 3, *panel B, lanes 3* and *4*), while the vast majority of CDMK are resistant (*lanes 1* and *2*). These results indicate that the lack of phosphorylation of CDMμtpCys is not due to alterations in the folding of the chimeric molecule, but rather to its actual retention in a compartment proximal to the site in which phospho-

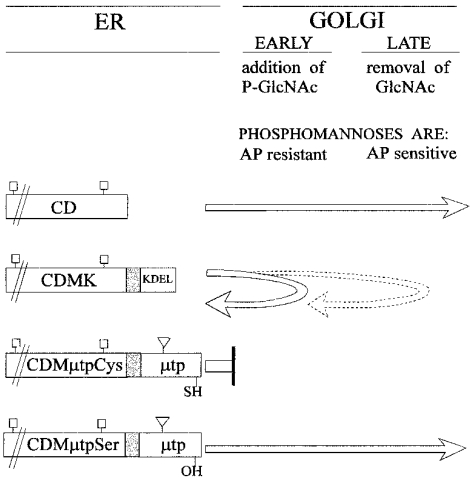

FIG. 4. **Retention and retrieval: different mechanisms of ER localization.** Scheme illustrating the proposed fates of endogenous and chimeric cathepsins, as revealed by their post-translational modifications. The *gray box* represents the 9E10 Myc tag, inserted between the CD encoding sequence and the different carboxyl-terminal extensions. The two oligosaccharides indicated as *small squares* lie within the amino acid context typical of lysosomal enzymes, while the *triangle* indicates glycans that can undergo mannose trimming and processing.

transferases are located.

DISCUSSION

The main conclusion that we draw from these studies is that the thiol-dependent ER localization of proteins is due primarily to actual retention in this organelle without retrieval from the Golgi. The key finding is that, in COS transfectants, CDMμtpCys molecules do not acquire phosphates unless transport is induced by reducing agents or by mutating the critical cysteine residue (Figs. 2 and 3). In contrast, CDMK molecules, which show a similar subcellular distribution by immunofluorescence (Fig. 1), are intensely phosphorylated (Fig. 3). The differences in trafficking and processing detected in our chimeras cannot be ascribed to alterations in the cellular transport machinery, as the endogenous monkey proCD acquires phosphates and is transported similarly in all transfectants. Thus, the addition of an exposed thiol is sufficient (i) to confer ER residence and (ii) to prevent post-ER modifications on a protein not naturally resident in the ER. The results are summarized in the scheme depicted in Fig. 4. Proteins with exposed thiols are effectively retained (and degraded) before the compartment where lysosomal enzymes are phosphorylated. The precise localization of the phosphotransferases within the cis-Golgi network (32), and in particular their presence within the intermediate compartment remains to be determined (15, 33, 34). The possibility that thiol exposing proteins transit rapidly through the phosphorylation compartment and are then immediately degraded cannot be formally ruled out. However, several observations argue against this model. The few CDMμtpCys molecules that reach the cis-Golgi and are phosphorylated seem to proceed undisturbed to the trans-Golgi stacks, thus escaping irreversibly the cysteine-mediated quality control. This is in agreement with our previous findings that thiol-mediated retention is not operative in or beyond the Golgi (29). Moreover, the degradation of CDMμtpCys is not impaired by brefeldin A (7), suggesting that, as shown in other systems (35), its proteolysis does not require vesicular transport.

During B cell differentiation, the μtp cysteine mediates the different fates of unpolymerized IgM, retention and degradation in B cells, polymerization and secretion in plasma cells (13). Likewise, for unknown reasons, the fate of CDMμtpCys varies depending on the cell lines in which it is expressed: in transient COS7 transfectants, retention (and degradation) prevail over the μtp-dependent dimerization, while in stable CHO transfectants some CDMμtpCys molecules escape retention, mostly through formation of covalent homodimers (7).[2] Hence, depending on the host cell line, the μtp can be exploited as a portable ER dimerization (or retention) module.

While the evidence for a retrieving function of the KDEL receptor is overwhelming, it seems clear that additional mechanisms contribute in determining the localization of ER resident proteins. If the latter had unrestricted access to the Golgi, the KDEL receptor would be easily saturated. Moreover, BiP, PDI, ERp72, and calreticulin mutants lacking the KDEL (or KDEL like) motif are secreted at different rates and in general very slowly (36). The KDEL motif, as originally proposed by Munro and Pelham (37), acts then as a salvage mechanism to return proteins which left the ER. Exit from this organelle is thought to occur by a default pathway, limited by retention (38). However, it can be also accelerated by receptor-mediated transport (39), possibly by concentration in ER subregions connected with the forward transport machinery (40–42). It would seem conceivable that thiol-mediated retention and other mechanisms that restrict the exit of cargo proteins from the ER reduce also the movement of resident proteins. Indeed, unassembled Ig-λ chains form reversible disulfide bonds with numerous proteins in the ER (11). Disulfide interchange reactions can be one of the mechanisms that contribute to the formation of an ER protein matrix that functions to exclude macromolecules from vesicular transport (11, 42). Thus, with hydrophobic, lectin-sugar and other types of dynamic interactions (43, 44), thiol reactivity would contribute in regulating the export of proteins to the Golgi.

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