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 these 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 (CDµtpCys) are phosphorylated, unless retention is reversed by exogenous reducing agents or the critical cysteine mutated (CDµtpSer). 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 CDµtpSer, the few CDµtpCys 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 luminal 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 luminal 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-acetylgalactosamine 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-
terminal KDEL sequence or an active cysteine in the \( \mu t \) context lead to the ER localization of chimeric cathepsins. Our results indicate that, unlike KDEL, the \( \mu t \) 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), CDM\( \mu t \)pCys, and CDM\( \mu t \)pSer 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 \( \mu t \)p (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 m\( \text{M} \) 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 \( ^{35} \text{S} \)-methionine/cysteine mixture (Tran^35S-label) or \( ^{32} \text{P} \)-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 million units of endo-\( \mu \)-N-acetylgalactosaminidase B (Endo-H, Boehringer Mannheim) for 24 h at 37°C (10). The presence of phosphomonoesters on CD molecules was ascertained by treating \( ^{32} \text{P} \)-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, \( ^{32} \text{P} \)- or \( ^{35} \text{S} \)-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 radioactivity. When indicated, immunoprecipitates were incubated with 1 million units of endo-\( \mu \)-N-acetylgalactosaminidase B (Endo-H, Boehringer Mannheim) for 24 h at 37°C (10). The presence of phosphomonoesters on CD molecules was ascertained by treating \( ^{32} \text{P} \)-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).

RESULTS

CDM-\( \mu t \)pCys, but Not CDM-\( \mu t \)pSer, Colocalizes with CDMK—The chimeric proteins CDMK, CDM\( \mu t \)pCys, and CDM\( \mu t \)pSer have been described previously (Ref. 7, see also Fig. 4). As shown in Fig. 1, both CDMK and CDM\( \mu t \)pCys 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 CDM\( \mu t \)pCys. As confirmed by biochemical data (see below), this reflects differences in intracellular pool sizes, derived from the different turnover rates of CDM\( \mu t \)pCys and CDMK (7). Cells transfected with CDM\( \mu t \)pSer show a perinuclear focal staining (panel C) suggestive of intra-Golgi 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).
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, CDM\textsubscript{tpSer} 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 CDM\textsubscript{tpCys} are not detected, suggesting that an active μ tailpiece is sufficient to prevent transport to the phosphotransferase compartments. Endogenous CD and CDM\textsubscript{tpSer} 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).

**Fig. 3.** Comparison of CDM\textsubscript{K} and CDM\textsubscript{tpCys} phosphorylation in the absence or presence of 2-ME. Panel A, CDM\textsubscript{tpCys} or CDM\textsubscript{tpSer} transfectants were labeled for 4 h with \[^{32}\text{P}\]orthophosphate, in the presence (−) or absence (+) of 14.4 mM 2-ME. Cell lysates were immunoprecipitated with SE10 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 (26) and then developed by ECL with anti-Myc followed by peroxidase-conjugated anti-mouse IgG chains (WB). The relevant bands were quantitated by densitometry. Panel C, the anti-Myc immunoprecipitates from the cell lysates (C) and media (M) of CDM\textsubscript{tpCys} or CDM\textsubscript{tpSer} transfectants, treated for 2 h with or without 2ME, were transferred onto nitrocellulose filters and developed by ECL as above (WB).

Exogenous Reducing Agents Induce Phosphorylation of CDM\textsubscript{tpCys}—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, CDM\textsubscript{K} acquires phosphates (Fig. 3, panel A, lane 1), an observation previously made by Pelham (12). In contrast, phosphorylated CDM\textsubscript{tpCys} 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 \[^{35}\text{S}\]pulse labeling experiments (not shown). Owing to their different half-lives in the ER, the steady state pool of CDM\textsubscript{K} is generally higher than that of CDM\textsubscript{tpCys}. However, even taking this into account, we find that CDM\textsubscript{K} bears at least 10 times more phosphates than CDM\textsubscript{tpCys}. Indeed, the \[^{32}\text{P}/\text{WB}\]densitometric ratios (see legend to Fig. 3 for details) were 0.6 and 0.05 for CDM\textsubscript{K} and CDM\textsubscript{tpCys}, respectively (0.5 and 0.15 after 2-ME treatment).

As shown in Fig. 1, panel G, part of CDM\textsubscript{tpCys} 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 CDM\textsubscript{tpCys}. Like CDM\textsubscript{tpSer}, the phosphorylated CDM\textsubscript{tpCys} display partial sensitivity to AP (Fig. 3, panel B, lanes 3 and 4), while the vast majority of CDM\textsubscript{K} are resistant (lanes 1 and 2). These results indicate that the lack of phosphorylation of CDM\textsubscript{tpCys} 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-
transient COS7 transfectants, retention (and degradation) prevail over the µp-dependent dimerization, while in stable CHO transfectants some CDµtpCys molecules escape retention, mostly through formation of covalent homodimers (7, 8). Hence, depending on the host cell line, the µp 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 also be 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-α 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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ER Localization by True Retention

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