A Single C-terminal Peptide Segment Mediates Both Membrane Association and Localization of Lysyl Hydroxylase in the Endoplasmic Reticulum*

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Endoplasmic reticulum (ER) is a heterologous organelle containing large amounts of newly synthesized polypeptides as well as resident proteins responsible for numerous post-translational modifications including glycosylation, folding, and oligomerization reactions. Because of their abundance, ER-resident proteins must be efficiently segregated from their substrates by specific retention and/or retrieval signals in their primary structure and its association with the ER membranes in vivo have suggested that the enzyme is localized in the ER via a novel retention/retrieval mechanism. We have identified here a 40-amino acid C-terminal peptide segment of LH that is able to convert cathepsin D, normally a soluble lysosomal protease, into a membrane-associated protein. The same segment also markedly slows down the transport of the reporter protein from the ER into post-ER compartments, as assessed by our pulse-chase experiments. The retardation efficiency mediated by this C-terminal peptide segment is comparable with that of the intact LH but lower than that of the KDEL receptor-based retrieval mechanism. Within this 40-amino acid segment, the first 25 amino acids appear to be the most crucial ones in terms of membrane association and ER localization, because the last 15 C-terminal amino acids did not possess substantial retardation activity alone. Our findings thus define a short peptide segment very close to the extreme C terminus of LH as the only necessary determinant both for its membrane association and localization in the ER.

Endoplasmic reticulum (ER) is a heterologous organelle containing large amounts of newly synthesized polypeptides as well as resident proteins responsible for numerous post-translational modifications including glycosylation, folding, and oligomerization reactions. Because of their abundance, ER-resident proteins must be efficiently segregated from their substrates by specific retention and/or retrieval signals in their primary structure.

To date, only two systems, both based on a retrieval mechanism, have been characterized (for reviews see Refs. 1 and 2). The KDEL tetrapeptide at the extreme C terminus is a common signal for a number of luminal chaperones (3). The mechanism is based on the KDEL receptor (erd2), which binds the escaped proteins in the Golgi complex and returns them back to the ER (4, 5). Double lysine and presumably double arginine motifs located in the cytoplasmic domains of several ER membrane proteins also function as retrieval motifs (6, 7). It is known that dilyseine motif-containing proteins bind the complex of cytosolic coat proteins (coatomer), COP 1, and that this interaction mediates the retrieval of these proteins from the Golgi back to the ER (8). Sequences flanking the dilyseine motif also contribute to the steady-state distribution of the proteins between the ER and the Golgi complex (6, 9).

Lysyl hydroxylase (EC 1.14.11.4, procollagen-lysine, 2-oxoglutarate 5-dioxygenase) is an enzyme in the lumen of the ER that catalyzes the hydroxylation of lysyl residues in collagenous sequences (for reviews see Refs. 10 and 11). Unlike most other luminal proteins in the ER, lysyl hydroxylase (LH) does not contain either the KDEL sequence or any of its close homologues in its primary structure (12). In addition, we have previously shown that LH is associated with the ER membranes in vivo and that this interaction involves mainly electrostatic interactions (13). Therefore, it is likely that membrane association is responsible for the localization of LH in the ER. If this were indeed the case, membrane association would represent a potentially novel mechanism by which luminal proteins may be localized in the ER. As a first step to define the mechanism that is responsible for the localization of LH in the ER, we have identified here a C-terminal peptide segment in LH that, when tagged into a reporter protein, is able to mediate both its membrane association and localization in the ER. Thus this peptide segment by itself appears to be sufficient for the correct localization of LH in the ER.

MATERIALS AND METHODS

Plasmids—Plasmids CDM and CDMK encoding human cathepsin D tagged either with a c-Myc-derived epitope (CDM) or c-Myc-SEKDEL (CDMK) were kindly provided by Dr. Hugh Pelham (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK). The CDMK plasmid was used for preparing reporter protein constructs that contained short sequences from LH. First, the SEKDEL sequence of the CDMK was replaced with the sequence encoding the last 40 C-terminal amino acids (CDLH40) of human lysyl hydroxylase isoform 1 (12). The region encoding the 40 C-terminal amino acids was amplified by polymerase chain reaction using the following oligonucleotide primers 5'-CCGAAATTCTAGGACGCCCAGAAGG-3' and 5'-CTAGTCGTAGTTAGGGATCGACGAAGGAGA-3'; EcoRI and HindIII restriction sites. The whole coding region of cathepsin D including the 40 amino acids from LH was further subcloned into the pCDNA3 mammalian expression vector (Invitrogen), containing the cytomegalovirus promoter. CDLH15 construct containing only the last 15 C-terminal amino acids of LH was prepared accordingly, with the exception that the corresponding amino acid region was prepared by annealing two complemen-

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§ The abbreviations used are: ER, endoplasmic reticulum; LH, lysyl hydroxylase; PDI, protein disulfide isomerase; PAGE, polyacrylamide gel electrophoresis.
sary oligonucleotides 5′-AATTTCAGCCACCAAGGGGACCCCGTCA-3′ and 5′-CTAGGGATCCGAAAAGGAGAAAGTCCGATGATAGCTTCGTCG-3′. The annealed fragment was cloned into the CDM plasmid using EcoRI and XbaI restriction sites.

For generating the plasmid that encodes the full-length lysyl hydroxylase tagged with a c-Myc epitope (LHmhc), the coding region of LH was first amplified, using the oligonucleotide primers 5′-GGCGGATCCCATGCGCTCTGAGGCAACCCGCTGTGCTA-3′ and 5′-AGGCCCTCGAGCAAGTTCTCGGAGAGCTGAAAGGA-3′, and then cloned into the PCDNA3 vector using BamHI and EcoRI restriction sites. The c-Myc epitope was inserted by annealing complementary oligonucleotides, 5′-AATTTCAGCCACCAAGGGGACCCCGTCA-3′ and 5′-CTAGGGATCCGAAAAGGAGAAAGTCCGATGATAGCTTCGTCG-3′, followed by cloning of the annealed fragment into the C terminus using EcoRI and XbaI restriction sites to allow visualization with a monoclonal antibody 9E10 (Roche Molecular Biochemicals or Santa Cruz). All amplified sequences were confirmed by automatic sequencing using the ABI PRISM™ AmpliTaq FS dye terminator cycle sequencing Kit (Perkin-Elmer) and an ABI Prism 377 DNA sequencer.

Cell Culture and Transfections—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with Glutamax, 10% fetal bovine serum, and penicillin-streptomycin. Cells were routinely plated on 35-mm Petri dishes 1 day before transfection. Transfections were made using FUGENE® transfection reagent (Roche Molecular Biochemicals). 2 μg of plasmid DNA were used for each transfection, cells were treated for 4 h with the calcium ionophore A23187 (Calbiochem) in serum-free medium and then fixed with 2% para-formaldehyde, 0.1% glutaraldehyde (in phosphate-buffered saline). Fixed cells were processed for immunostaining with the 9E10 antibody and peroxidase-conjugated anti-mouse secondary antibodies using conventional procedures. Stained specimens were examined using a Philips CM 100 transmission electron microscope with an acceleration voltage of 60 kV.

Electron Microscopy—Electron microscopy was performed as described earlier, with slight modifications (14, 15). Briefly, 24 h after transfection, cells were treated for 4 h with the calcium ionophore A23187 (Calbiochem) in serum-free medium and then fixed with 2% para-formaldehyde, 0.1% glutaraldehyde (in phosphate-buffered saline). Fixed cells were processed for immunostaining with the 9E10 antibody and peroxidase-conjugated anti-mouse secondary antibodies using conventional procedures. Stained specimens were examined using a Philips CM 100 transmission electron microscope with an acceleration voltage of 60 kV.

RESULTS

The C-terminal Peptide Segment of LH Slows the Transport of Cathepsin D out of the ER—The reporter protein constructs used in this work are illustrated in Fig. 1. All constructs are based on the CDM plasmid that we used as a reference plasmid for measuring the normal transport rate of the reporter protein itself. The plasmid encodes human cathepsin D tagged with a 10-amino acid c-Myc epitope that is recognized by the monoclonal antibody 9E10 (17). Cathepsin D is a lysosomal aspartic protease that is secreted into the lysosomes via the mannose-6-phosphate receptor mediated pathway (18). Previous studies have shown that the c-Myc epitope does not alter the intracellular routing of cathepsin D. The epitope appears, however, to be cleaved during the maturation of the cathepsin D in lysosomes (19, 20). The CDM plasmid differs from the CDM plasmid in that the SEKDEL sequence has been added to the C

teins were eluted with Laemmli's sample buffer. Immunoprecipitates were analyzed by SDS-PAGE and, after fixation, the gel was treated with ENHANCE (DuPont) and exposed to x-ray film (Fuji RS). The cell lysate:medium ratios of the different reporter protein constructs were calculated after quantitation of the protein bands with a computer-assisted image analysis program (MCID-M, Imaging Research, St. Catharines, Canada).

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Fractionation of Soluble and Membrane Proteins and Immunoblotting—Transfected cells were washed and scraped into TKM buffer (50 mM Tris, 1 mM MgCl₂, 10 mM KCl, pH 7.4). Cells were disrupted by brief sonication (four times for 5 s) on ice. Cell membranes and supernatants were separated by centrifugation (100,000 × g, 30 min). The membrane fraction was solubilized directly in Laemmli's sample buffer. Soluble proteins were recovered from the supernatant by TCA-precipitation and solubilized in SDS sample buffer. Equivalent amounts of both fractions were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed with a monoclonal antibody against protein disulfide isomerase (16) and with the 9E10 monoclonal antibody, followed by peroxidase-conjugated anti-mouse antibodies (Bioys, Compiegne, France). Immunoreactive proteins were visualized using the ECL system (Amersham Pharmacia Biotech).

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terminus of cathepsin D. Because the SEKDEL motif induces a well known and efficient retrieval of cathepsin D from the Golgi back to the ER (19), this plasmid serves as a control plasmid for a known ER-specific retrieval mechanism.

ER-specific retrieval signals are characteristically found at the C termini of proteins. Therefore, we also focused our first efforts to the C terminus of LH. We constructed two different cathepsin D constructs, one that contains the last 15 C-terminal amino acids (CDLH15) of LH and the other one that contains 25 additional amino acids toward the N terminus of LH (CDLH40), thus consisting altogether of the last 40 C-terminal amino acids of LH (Fig. 1). All protein constructs (CDM, CDMK, CDLH15, and CDLH40) were then transiently expressed in COS-7 cells, and the chimeric proteins were localized with the antibody 9E10. Indirect immunofluorescence showed that both the CDM (Fig. 2A) and the CDLH15 (Fig. 2C) protein constructs were localized predominantly in the perinuclear region, suggestive of their transient accumulation in the Golgi complex during their transport into-post Golgi compartments. Staining of the ER was also evident in some cells, as assessed by double staining with an antibody against protein disulfide isomerase. In contrast, CDMK (Fig. 2B) and CDLH40 (Fig. 2D) protein constructs accumulated mainly in fine reticular structures throughout the cytoplasm. The staining pattern was typical of that of the ER, and this was confirmed by double staining with an anti-PDI antibody (data not shown). In a proportion of cells, the CDLH40 protein was also present in the Golgi region, in addition to the ER.

To evaluate the retardation efficiency of the different protein constructs, we next performed pulse-chase analyses on transfected cells. We labeled the cells with radioactive methionine for 2 h and chased them for 3 h in the presence of ammonium chloride. Ammonium chloride is known to dissipate endogenous targeting of cathepsin D to lysosomes and leads to its secretion into the medium (20, 21). However, when cathepsin D is expressed as a recombinant protein, a nearly equal amount of the protein is secreted into the medium without the drug treatment. To quantify the retardation efficiency of the different cathepsin D protein constructs, we measured their relative amounts in the medium and inside the cells by immunoprecipitation. When the cells were transfected with either CDM or CDLH15 plasmids (Fig. 3), about 80% of the immunoprecipitated proteins were found in the medium. These results indicated that both of these two chimeric proteins were efficiently secreted from the cells, as was also suggested by our immunofluorescence data (Fig. 2).

In contrast, the KDEL-tagged cathepsin D (CDMK plasmid) was efficiently retained inside the cells, and only negligible amounts of the protein were found in the medium (Fig. 3). Similarly, the CDLH40 protein (Fig. 3) was also mostly retained inside the transfected cells (in the ER; Fig. 2), and about 75% of the protein was generally recovered from the cells, the rest being found in the medium. Similar results were also obtained in the absence of ammonium chloride. Both of these protein constructs (CDMK and CDLH40) were also found to undergo autocatalysis under low pH conditions (22) and to bind to immobilized pepstatin A (an active site inhibitor for cathepsin D), indicating that their retardation was not due to their incorrect folding and association with the quality control machinery. Collectively, the above results show that the C terminus of LH indeed contains substantial retardation activity. This activity, however, does not seem to reside in the very extreme C terminus of LH, because the CDLH15 construct was efficiently secreted from the transfected cells.

The Full-length LH Is Also Partially Secreted from the Transfected Cells—For comparative reasons, we next transfected cells with the LHMyc plasmid that encodes the full-length LH. For comparative reasons, we next transfected cells with the LHMyc plasmid that encodes the full-length LH. Western blotting of total cell lysates from transfected cells with the 9E10 antibody confirmed the expression of a double band of the expected size of both the nonglycosylated (80 kDa) and glycosylated (87 kDa) forms of LH (Fig. 4B). The expressed full-length LH protein was also found to be enzymatically active, as judged by the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (23).

Pulse-chase experiments (Fig. 4C) performed as above (at
40 h after the transfection) showed that most (85–90%) of the expressed c-Myc-tagged LH protein was recovered from the cells. Nevertheless, 10–15% of the full-length LH was also secreted into the medium during the 3-h chase. Thus, the retardation efficiencies of the full-length LH protein and the CDLH40 protein constructs were rather comparable with each other. The CDLH40 construct thus appears to contain most of the information needed for the correct localization of the full-length LH in the ER. The retardation efficiency of the KDEL-tagged cathepsin D was clearly highest of the protein constructs tested.

**CDLH40 Protein Associates with the ER Membranes**—Previously, we have shown that the full-length LH is associated in vivo with the ER membranes (13). To test whether the 40-amino acid segment of the LH can also convert cathepsin D into a membrane-associated protein, we fractionated transfected cells into membrane and soluble fractions and followed the distribution of the chimeric proteins in the two fractions by SDS-PAGE and immunoblotting. Both the full-length LH (LH-Myc) and CDLH40 protein construct were found to co-sediment almost exclusively with the cellular membranes (Fig. 5A). Only negligible amounts of either protein were recovered from the membrane-depleted fractions. Thus, the 40-amino acid peptide segment of LH is, indeed, able to mediate the association of cathepsin D with the ER membranes. In contrast, endogenous PDI (a KDEL-containing protein) was recovered mainly in the soluble fractions (~70%). The CDMK protein, which we used as an additional control protein, was equally distributed between

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**Fig. 3. Secretion of different reporter protein constructs in transfected cells.** A, transfected cells were labeled with [35S]Met/Cys for 2 h and subsequently chased for 3 h in medium containing 10 mM NH₄Cl. Proteins were recovered from the cells (lanes C) or from the medium (lanes M) by immunoprecipitation with agarose-conjugated 9E10 antibody. Bound proteins were resolved by SDS-PAGE and analyzed by autoradiography. Minor bands below the major protein product probably represent degradation products, which were not included for the calculation of the retardation efficiencies. B, quantitation of the proportions of intracellular and secreted proteins in transfected cells. Note that in experiments with CDM or CDLH15, most of the reporter protein was detected in the medium, whereas in cells transfected with CDMK or CDLH40, the reporter protein was retained intracellularly.

**Fig. 4. Expression of the full-length lysyl hydroxylase in COS cells.** A, localization of the expressed LH-Myc protein after staining with 9E10 antibody. Note the accumulation of LH-Myc protein in the ER. B, Western blot analysis using 9E10 antibody of proteins from cells expressing LH-Myc. According to their size, the 87- and 80-kDa bands detected by the antibody probably represent glycosylated and unglycosylated forms of LH, respectively. C, pulse-chase experiments of the LH-Myc shows that the majority of the LH-Myc protein is found in the cell lysate (lane C), but a substantial portion (both the 87- and 80-kDa bands) is also secreted into the medium (lane M).
**C Terminus of LH Is Sufficient for ER Localization**

Among a vast number of the luminal proteins of the ER, LH protein has two unique features, namely the absence of known ER-specific retention/reticulum signals and its association in vivo with the ER membranes. In this report, we have localized a short peptide segment in the C terminus of LH that alone was sufficient for the localization of cathepsin D in the ER and for its association with the ER membranes. The 40-amino acid C-terminal peptide segment of LH therefore appears to possess all the necessary information needed to localize endogenous LH in the ER membranes. The retardation did not result from aberrant folding of the reporter protein and its putative association with the ER quality control machinery, because the enzyme retained its autocatalytic properties in vitro and also bound to its active site inhibitor, pepstatin A.

By using two overlapping constructs (CDLH40 and CDLH15) in which the last 15 C-terminal amino acids are common, we were able to show that only the CDLH40 protein was efficiently retained in the ER in the transfected cells, whereas the CDLH15 construct (cathepsin D tagged with the last 15 C-terminal amino acids) was readily transported out of the ER and could be recovered from the medium in pulse-chase experiments. The most crucial portion of this 40-amino acid segment both for the membrane association and ER localization appears therefore to be buried within the first 25 amino acids (amino acids 688–712 of LH1 isoform) of this 40-amino acid segment. This portion contains few positively charged amino acids, which may directly mediate the association of LH with the membranes (14). According to the sequence data, this region and the whole 40-amino acid segment are also nearly identical between different species and also very homologous (~90%) to recently cloned other two human and mouse LH isoforms (24–27). Conservation of this C-terminal segment suggests that other LH isoforms may use the same region for their localization in the ER. Our data, however, do not exclude the possibility that the last 15 amino acids may also contribute to the retardation efficiency of the CDLH40 construct, although alone (CDLH15) they did not have substantial retardation activity.

Our findings, showing that membrane association and ER localization are strictly coupled phenomena, provide the first direct evidence for the view (13) that the membrane association is responsible for the localization of the enzyme in the ER. Membrane association therefore raises important questions about the identity of the membrane counterpart(s) with which LH and the identified peptide segment associates in vivo. One possibility is that LH could associate with the ER membranes by interacting directly with membrane phospholipids. For example, a number of cytoplasmic proteins that possess pleckstrin homology domains associate with the membranes by binding to phosphatidylinositol phosphates (28). However, according to sequence analyses (SMART version 3.0 or E-MOTIF), no such a motif is present in the full-length LH protein sequence or in the 40-amino acid C-terminal segment. Another possibility for the membrane association of LH is a carbohydrate-mediated retention of incompletely folded proteins with the quality control machinery (29). Although the full-length LH is N-glycosylated, the C-terminal peptide segment does not contain any potential N-linked glycosylation sites, suggesting that its membrane association does not involve asparagine-linked carbohydrate structures.

The most likely possibility, therefore, is that LH associates with the ER membranes via specific protein-protein interactions. This is also supported by our preliminary molecular sizing experiments, in which we have found that about half of the full-length, c-Myc-tagged LH is solubilized from the cells as a high molecular weight complex. The large size of the complex excludes putative interactions with the ER membrane phospholipids. In this respect, it is interesting to note that prolyl-4-hydroxylase, a heterotetrameric (α2β2) collagen processing enzyme, is localized in the ER via its β-subunit, protein disulfide isomerase (30), which is a well known KDEL-containing protein. However, because this protein complex is not membrane-associated (Fig. 5 and Ref. 13), a different kind of interaction appears to be responsible for the membrane association and localization of LH in the ER. Other known proteins, which

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**DISCUSSION**

The two fractions (Fig. 5A). The reason for the membrane association of the CDMK protein is not known, but it may be due to its interaction with the KDEL receptor, which has been shown to redistribute into the ER when KDEL-containing proteins are overexpressed (5).

We next processed transfected cells for immunoelectron microscopy after staining the chimeric protein with the 9E10 antibody followed by peroxidase-conjugated secondary antibody. To allow better visualization of the expressed protein within the ER lumen, a calcium ionophore, A23187, was used to induce the dilatation of the ER cisternae before fixation of the cells (15). Electron microscopy (Fig. 5B) confirmed the expected association of the CDLH40 protein with the ER membranes. Thus, both our biochemical and immunohistochemical data indicate that the 40-amino acid C-terminal segment alone is able to mediate the association of the reporter protein with the ER membranes.

**FIG. 5. Membrane association of the CDLH40 protein.** A, cells expressing CDLH40, CDMK, or LHMyC protein were fractionated into membrane (lanes M) and soluble (lanes S) fractions. The distribution of the reporter proteins and PDI between the two fractions was analyzed using 9E10 and anti-PDI antibodies. Both the CDLH40 and LHMyC protein constructs were found almost exclusively in the membrane fraction, in contrast to PDI, which was recovered mostly in the soluble fraction. CDMK protein was distributed equally to both fractions. B, an electron micrograph showing A23187-treated cells expressing the CDLH40 protein. Note the peroxidase-precipitate along with the ER membrane. For comparison, endogenous PDI and procollagen are localized throughout the ER lumen in the drug-treated fibroblasts (15). er, endoplasmic reticulum; m, mitochondria; n, nucleus.
associate with the ER membranes, include e.g. inositol 1,4,5-trisphosphate 3-kinase (31), and tyrosine phosphatase PTP-1B (32). The association of the former also likely involves specific protein-protein interactions (31), whereas the association type of the latter is not known. Both proteins, however, are found on the cytoplasmic side of the ER membranes.

The retardation efficiency mediated by the C-terminal peptide segment (CDLH40) and of the full-length LH itself (LHMyc) were shown to be lower than that of the KDEL-containing CDMK construct under similar experimental conditions. Because both the LHMyc and the CDLH40 protein constructs were secreted from the cells to some extent (10–15% and 20–25%, respectively), the retention machinery responsible for the localization of the LH protein constructs seems to be more easily saturable than that of the KDEL-receptor mediated retrieval mechanism. This suggestion is consistent with the known expression levels of the endogenous proteins in vivo. The KDEL-containing chaperones and folding enzymes are generally known as the most abundant proteins in the ER lumen, whereas LH is expressed at a much lower level in tissues and cells studied thus far (33). Thus, there is no need for a highly efficient retention/retrieval system for LH in the ER lumen. The suggestion is also supported by the observed differences in the amount of the secreted, but not intracellular, CDLH40 protein with transfection time, being 15, 25, and 50% at 24, 40, and 48 h after the transfection, respectively.2 The difference in the rate of secretion between the two LH constructs (LHMyc and CDLH40) may also result from their differential expression levels in the transfected cells. For some unknown reason, the LHMyc construct appears to be expressed consistently at lower levels in COS-7 cells during transient transfections. The difference in the secretion could also be due to small conformational differences that may exist within the C-terminal peptide segment between the Myc-tagged full-length LH and the CDLH40 construct. We did not attempt to increase the retardation efficiency of the CDLH40 protein, e.g. by extending the length of the C-terminal segment toward the N terminus of LH because of the presence of nearby cysteine residues, which have been shown to act as signals for the quality control machinery (20, 34).

As a conclusion, we report here the identification of a 40-amino acid peptide segment in the C terminus of LH that alone is sufficient to convert otherwise a soluble lysosomal protein, cathepsin D, into an ER membrane-associated protein. Within this peptide segment, the first 25 amino acids appeared to be the most crucial ones in terms of ER localization. The retardation efficiency mediated by this 40-amino acid segment was comparable with that of the c-Myc-tagged full-length LH, especially if their expression levels in the transfected cells are taken into account. The 40-amino acid segment appears therefore to contain all the necessary information for the correct localization of the endogenous LH in the ER as well. The maximal retardation efficiencies of the LH protein constructs were, however, lower than that of the KDEL-receptor mediated retrieval mechanism. Thus, both the steady-state membrane association and low capacity/saturability appear to be the hallmark features of this novel type of retention/retrieval mechanism.

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REFERENCES

1. Teasdale, R. D., and Jackson, M. R. (1996) Annu. Rev. Cell Dev. Biol. 12, 27–54
2. Pelham, H. R. (1991) Curr. Opin. Cell Biol. 4, 585–591
3. Munro, S., and Pelham, H. R. (1987) Cell 48, 899–907
4. Lewis, M. J., and Pelham, H. R. (1996) Nature 384, 162–163
5. Lewis, M. J., and Pelham, H. R. (1992) Cell 68, 353–364
6. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1993) J. Cell Biol. 121, 1177–1333
7. Letourneau, F., Gaynor, E. C., Hennecke, S., De-Molliere, C., Duden, R., Emr, S. D., Riezman, H., and Cosson, P. (1994) Cell 79, 1199–1207
8. Schutze, M. P., Peterson, P. A., and Jackson, M. R. (1994) EMBO J. 13, 1696–1705
9. Itin, C., Schindler, C., and Hauri, H. P. (1995) J. Cell Biol. 131, 57–67
10. Kirikov, K. I., Myllyla, R., and Pihlajaniemi, T. (1992) in Post-translational Modifications of Proteins (Harding, J. J., and Crabbe, M. J. B., eds) pp. 1–51, CRC Press, Boca Raton, FL.
11. Procko, D. J., and Kirikov, K. I. (1995) Annu. Rev. Biochem. 64, 433–434
12. Hautala, T., Byers, M. G., Eddy, R. L., Shows, T. B., Kirikov, K. I., and Myllyla, R. (1992) Genomics 13, 62–69
13. Kellokumpu, S., Sormunen, R., Heikkinen, J., and Myllyla, R. (1994) J. Biol. Chem. 269, 30524–30529
14. Kellokumpu, S., Neff, L., Janssa-Kellokumpu, S., Kopito, R., and Baron, R. (1998) Science 243, 1308–1311
15. Kellokumpu, S., Suokas, M., Risteli, L., and Myllyla, R. (1997) J. Biol. Chem. 272, 2770–2777
16. Hoyhtya, M., Myllyla, R., Piirva, J., Kirikoviko, K. I., and Tryggvason, K. (1984) Eur. J. Biochem. 141, 477–482
17. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
18. Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330
19. Pelham, H. R. (1988) EMBO J. 7, 913–918
20. Fra, A. M., Fagioli, C., Finazzi, D., Sitia, R., and Alberini, C. (1993) EMBO J. 12, 4755–4761
21. Braulke, T., Gerze, H. J., Slot, J. W., Hasilik, A., and von Figura, K. (1987) Eur. J. Cell Biol. 43, 316–321
22. Zhu, Y., and Conner, G. E. (1994) J. Biol. Chem. 269, 3846–3851
23. Kirikoviko, K. I., and Myllyla, R. (1982) Methods Enzymol. 82, 245–304
24. Valtavaara, M., Papponen, H., Pirttila, A.-M., Hiltunen, K., Keler, H., Helder, H., and Myllyla, R. (1997) J. Biol. Chem. 272, 6831–6834
25. Valtavaara, M., Sipaner, C., Sipaner, J., and Myllyla, R. (1998) J. Biol. Chem. 273, 12981–12986
26. Pasoja, O., Kauhtuvisto, A., Ala-Kokko, L., Kosonen, T., and Kirikoviko, K. I. (1998) Proc. Natl. Acad. Sci. USA, 95, 10482–10486
27. Ruotsalainen, H., Sipil, L., Kerkela, E., Pospiiech, H., and Myllyla, R. (1999) Matrix Biol. 18, 325–329
28. Shaw, G. (1996) BioEssays 1, 35–46
29. Parodi, A. J. (1999) Biochim. Biophys. Acta 1426, 287–295
30. Venter, K., Pihlajaniemi, T., Myllyla, R., and Kirikoviko, K. I. (1992) J. Biol. Chem. 267, 4213–4217
31. Soriano, S. Thomas, S. High, S., Griffiths, G., D'Santos, C., Cullen, P., and Burton, G. (1997) Biochem J. 324, 579–589
32. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992) Cell 68, 545–560
33. Heikkinen, J., Hautala, T., Kirikoviko, K. I., and Myllyla, R. (1994) Genomics 24, 464–471
34. Isidoro, C., Maggioni, C., Demoz, M., Pizzagalli, A., Fra, A. M., and Siaita, R. (1996) J. Biol. Chem. 271, 26138–26142

2 M. Suokas, R. Myllyla, and S. Kellokumpu, unpublished observations.