Lysyl Hydroxylase, a Collagen Processing Enzyme, Exemplifies a Novel Class of Luminally-oriented Peripheral Membrane Proteins in the Endoplasmic Reticulum*

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Lysyl hydroxylase (LH), an enzyme required early during collagen biosynthesis, appears to be exceptional among proteins that are thought to be residents of the endoplasmic reticulum (ER). It is a homodimer and does not contain either of the two previously characterized ER-specific retention motifs (KDEL or the double lysine motif) in its primary structure. We now show that LH, nevertheless, resides in the lumen of the ER. In immunofluorescence experiments, LH co-localizes with a KDEL-containing protein, protein disulfide isomerase (PDI), and also co-sediments with it after fractionation of subcellular organelles by sucrose density gradient centrifugation. In addition, LH seems to be stress-inducible. In one respect, however, LH differs from PDI and other known luminal proteins in the organelle. It is found in situ only in association with the ER membranes. Our cell fractionation and Triton X-114 phase separation experiments suggest that it binds to the membranes via weak electrostatic interactions. LH can thus be regarded as a first luminally-oriented "peripheral membrane" protein which has been characterized in the ER. The results suggest a novel possibility by which ER lumen can acquire its specific protein components from the bulk flow.

Maintenance of subcellular organelles requires continuous synthesis of both soluble and membrane proteins. In the exocytic pathway, newly synthesized proteins first enter the endoplasmic reticulum (ER), where their synthesis is completed. Evidence that KDEL-independent retention mechanisms also exist has been demonstrated by studying the retention of s-cyclophilin in the ER (Arber et al., 1992). s-Cyclophilin is a member of a family of cytoplasmic proteins whose exact function is unknown. Unlike other members of this protein family, s-cyclophilin contains a cleavable signal sequence which targets it into the exocytic pathway. It, however, does not possess the KDEL retention sequence at its COOH terminus. The signal responsible for the positioning of s-cyclophilin was demonstrated to be buried in the COOH-terminal sequence of the protein (Arber et al., 1992). The proprotein, however, did not co-localize with the ER marker proteins, but rather with calreticulin, suggesting that it resides in a specific ER subcompartment, probably the calciosome. This finding raises the possibility that each ER subcompartment (Sitia and Meldolesi, 1992) may require its own retention signal. Thus, the mechanisms responsible for the retention of ER-resident proteins seem to be more complex than previously thought.

Two distinct retention signals have thus far been identified for ER-resident proteins. A COOH-terminal tetrapeptide, Lys-Asp-Glu-Leu (KDEL) or its close variant (Manro and Pelham, 1987; Pelham, 1990), has been shown to be both necessary and sufficient for the retrieval of a number of soluble proteins in the lumen of the ER. Proteins that carry this signal include BiP (binding protein), PDI (protein disulfide isomerase), glucose-regulated protein (endoplasm), and calreticulin, collectively termed as reticuloplasmins (Koch, 1987). The retention of ER-resident membrane proteins appear also to depend on a short COOH-terminal sequence. This signal contains the crucial double-lysine motif (KKXX or KXKX) instead of KDEL (Jackson et al., 1990). It was originally identified using an adenoavirus membrane glycoprotein (E9/19K) as a model protein (Pasero et al., 1987), but it appears to be responsible for the retention of some endogenous proteins as well (Jackson et al., 1993). Evidence favoring the idea that both of the KDEL- and the double-lysine peptide motifs function as retrieval rather than true retention signals has been presented (Pelham, 1990; Jackson et al., 1993).

Additional possibilities, or at least variations to these basic themes, to retain proteins in the ER exist. Recently, an ER-resident integral membrane protein has been identified in yeast, which contains the KDEL retention sequence at its extreme COOH terminus (Sweet and Pelham, 1992). Thus, the KDEL sequence is not strictly restricted only to soluble proteins in the lumen of the ER. Evidence that KDEL-independent retention mechanisms also exist has been demonstrated by studying the retention of s-cyclophilin in the ER (Arber et al., 1992). s-Cyclophilin is a member of a family of cytoplasmic proteins whose exact function is unknown. Unlike other members of this protein family, s-cyclophilin contains a cleavable signal sequence which targets it into the exocytic pathway. It, however, does not possess the KDEL retention sequence at its COOH terminus. The signal responsible for the positioning of s-cyclophilin was demonstrated to be buried in the COOH-terminal sequence of the protein (Arber et al., 1992). The protein, however, did not co-localize with the ER marker proteins, but rather with calreticulin, suggesting that it resides in a specific ER subcompartment, probably the calciosome. This finding raises the possibility that each ER subcompartment (Sitia and Meldolesi, 1992) may require its own retention signal. Thus, the mechanisms responsible for the retention of ER-resident proteins seem to be more complex than previously thought.

The two functionally related but structurally divergent collagen processing enzymes, prolyl-4-hydroxylase and lysyl hydroxylase (LH) (Kivirikko et al., 1992) also seem to belong to the family of luminal ER-resident proteins. However, only in the case of prolyl-4-hydroxylase, morphological data are available to support its presence in the ER (Olsen et al., 1973). While prolyl-4-hydroxylase is an α2β2 heterotetramer, which is now

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† The abbreviations used are: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; LH, lysyl hydroxylase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
known to be retrieved "passively" in the ER via its KDEL-containing β-subunit (protein disulfide isomerase, PDI) (Vuori et al., 1992), lysyl hydroxylase together with some other proteins in the ER does not contain the KDEL retention motif in its primary structure (Bishoff et al., 1990; Mylläry et al., 1991; Arber et al., 1992; Hautala et al., 1992a; for a review, see Pelham (1990)), and therefore does not seem to be retained by a similar mechanism. To prove this, we have now carried out detailed immunolocalization analyses and solubility tests in comparison to protein disulfide isomerase (PDI), a well-known ER marker protein. Our results indicate that LH, unlike PDI and other previously characterized proteins of the ER lumen, is a luminally oriented peripheral membrane protein that does not associate with the KDEL-containing proteins or with their receptor, but rather with some as yet unidentified constituent of the ER membranes via weak electrostatic interactions. It is likely that this novel type of interaction is responsible for the retention of LH in the ER lumen.

Discontinuous Sucrose Density Gradient Centrifugation—Cellular contents were fractionated by discontinuous sucrose velocity gradient centrifugation according to a modified protocol of Bole et al. (1986). Cells were scraped into PBS, collected by centrifugation, and resuspended in TKM buffer (50 mM Tris, pH 7.4, 10 mM KC1, 1 mM MgCl2) containing 1 mM sodium azide, 20 mM benzamidine, 100 µg/ml leupeptin (100 µg/ml) as protease inhibitors. Cells were then disrupted by homogenization (Dounce, tight fitting pestle, 20–30 strokes), and ice-cold sucrose was added immediately after cell lysis to a final concentration of 250 mM. The suspension was layered on top of the discontinuous sucrose gradient, consisting of 2 ml of 0.5, 1.0, 1.25, 1.5, 1.75, and 2.0 sucrose solutions (in the above homogenization buffer). The samples were centrifuged at 100,000 × g for 3 h at 4 °C before collection of 1-ml fractions from each sucrose interphase. Aliquots of the fractions were subjected to SDS-PAGE and immunoblotting, and the intensity of the stained protein bands (LH, 88 kDa; PDI, 57 kDa) in each fraction was quantitated by using a computer-assisted image analysis program (MCID-M1, Imaging Research Inc., St. Catharines, Canada).

Lysyl Hydroxylase Associates with the ER Membranes

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**RESULTS**

*LH Is a Resident of the ER*—Since no morphological data are available to support the presence of LH in the ER, we first used indirect immunofluorescence and affinity-purified peptide-specific antibodies against LH to verify its presence in the organelle. We compared the subcellular distribution of LH with that of the PDI, a KDEL-containing ER marker protein, which was visualized simultaneously in the same cells with a monoclonal anti-PDI antibody (Höyhtyä et al., 1984). In human skin fibroblasts (Fig. 1A), the anti-PDI antibody stained reticular structures, which concentrated around the nucleus, and included the nuclear membrane itself. In human cells (Fig. 1B), but not in chicken fibroblasts (data not shown) in which the peptide sequence is divergent (Myllylä et al., 1991), the same reticular structures were also stained with the anti-LH antibodies, indicating almost identical co-localization of the two proteins in the ER. The Golgi area (Fig. 1D) identified with a Golgi-specific antiserum near the nucleus was not stained with the anti-PDI antibody (Fig. 1C). Similarly, the negatively stained Golgi area can also be seen in Fig. 1, A, the visualization of bound antibodies on thin cryosections. Examination of the sections confirmed that PDI (data not shown) was distributed evenly within the lumen of the ER, whereas LH was localized evenly within the entire lumen of the ER (data not shown). Both staining methods thus gave identical results. The finding that LH associates with the ER membranes, we used Triton X-114 phase-separation method of Bordier (Bordier, 1981). This method provides a convenient way to separate soluble proteins from integral membrane proteins. The presence of LH and PDI in the phase-separated fractions was determined after SDS-PAGE by immunoblotting. Both PDI and LH partitioned into the aqueous phase (Fig. 4), if phase separation was carried out under conventional conditions (150 mM NaCl). Thus, in contrast to our immunocytochemical observations (Fig. 3), both enzymes were recovered as soluble proteins in the phase-separated fractions. Identical results were obtained using different buffers (e.g., PBS instead of Tris-150 mM NaCl), or if the cellular contents were separated by centrifugation into soluble and insoluble fractions.}

*Fractionation Experiments*—To demonstrate biochemically that LH associates with the ER membranes, we used Triton X-114 phase-separation method of Bordier (Bordier, 1981). This method provides a convenient way to separate soluble proteins from integral membrane proteins. The presence of LH and PDI in the phase-separated fractions was determined after SDS-PAGE by immunoblotting. Both PDI and LH partitioned into the aqueous phase (Fig. 4), if phase separation was carried out under conventional conditions (150 mM NaCl). Thus, in contrast to our immunocytochemical observations (Fig. 3), both enzymes were recovered as soluble proteins in the phase-separated fractions. Identical results were obtained using different buffers (e.g., PBS instead of Tris-150 mM NaCl), or if the cellular contents were separated by centrifugation into soluble and insoluble fractions.
membrane proteins under the same ionic conditions. Half-maximal release was obtained at 60–65 mM KCl concentrations. These results suggest that LH associates with the ER membrane most probably via weak ionic bonds. In contrast, the partitioning of PDI was found to be independent of the ionic conditions, and over 95% of PDI was always recovered as a soluble form (Figs. 4 and 5).

**LH Is Released from the Membranes as an Enzyme Dimer**

The solubilized enzyme, i.e. LH which partitioned into the aqueous phase in the presence of 150 mM NaCl, was enzymatically active, and had a molecular mass of about 150 kDa, as assessed by size exclusion chromatography (Fig. 6). Previous studies (Turpeenniemi-Hujanen et al., 1980) have indicated that the purified enzyme is a homodimer composed of two identical subunits, which are noncovalently linked to each other. Thus, the partitioning of the 88-kDa protein into the aqueous phase did not result from the dissociation of the enzyme subunits, one of which might still have remained membrane-bound. Rather, it resulted from the destruction of the bonds that keep the dimeric enzyme bound to its membrane counterpart(s).

**DISCUSSION**

The experiments described above were designed to examine the subcellular distribution and solubility of LH in comparison to that of PDI. The rationale for attempting these experiments was our recent finding that LH, a putative ER-resident enzyme, does not possess any of the ER-specific retention motifs in its primary structure (Myllylä et al., 1991; Hautala et al., 1992a). Yet, it has been putatively placed in the ER by using cell fractionation methods (Guzman et al., 1976; Peterkofsky and Assad, 1979). Its residency in the ER lumen is also supported by the findings that it has a cleavable signal sequence (Myllylä et al., 1991), and that it contains endo H-sensitive oligosaccharide side chains (Myllylä et al., 1988). This is also compatible with its functional role in the synthesis and formation of collagen triple helices (Kivirikko et al., 1992b). Our results obtained from indirect immunolabeling experiments are fully compatible with these observations. They demonstrated that LH co-localizes almost identically with PDI, a known resident of the ER. Therefore, LH seems to reside in the same subcompartment where the KDEL-containing proteins reside.

The most likely explanation for the similar distribution of these two enzymes in the ER would be that LH associates,
analogsly to the α-subunit of prolyl-4-hydroxylase (Vuori et al., 1992), to the KDEL-containing proteins such as PDI. However, although the KDEL-containing proteins can transiently associate with their receptor in a post-ER/early Golgi compartment where the free receptor has been shown to reside (Lewis and Pelham, 1990; Tang et al., 1993), they are characteristicaly soluble proteins in the ER lumen (Bole et al., 1986; Koch, 1987). This also holds true for PDI and for proteins such as prolyl-4-hydroxylase that form hetero-oligomers with PDI (Olsen et al., 1973; Vuori et al., 1992). We also demonstrated here that PDI distributed evenly within the ER lumen (Fig. 3A), and that over 90% of it partitioned into the aequous phase during our phase separation experiments (Fig. 4).

In contrast, we observed that LH was associated in situ only with the ER membranes (Fig. 3B and C). The association of LH with the membranes could also be demonstrated in vitro by showing that LH, in contrast to PDI, partitioned almost exclusively into the detergent phase if the phase separation experiments were carried out under low ionic strength conditions (Fig. 4). The partitioning of LH was, however, dependent on the ionic conditions used. It partitioned into the aequous phase under isotonic conditions (Fig. 4, see also below) and thus behaved as a soluble protein in these experiments. In accordance with this, recent cloning data (Myllylä et al., 1991; Hautala et al., 1992a) have also failed to demonstrate any putative membrane-spanning domains in its primary structure. On the basis of the above results, LH can thus be regarded as a peripherally associated membrane protein in the lumen of the ER. Therefore, it apparently cannot be complexed with the soluble KDEL-containing proteins such as PDI and be retained along them by the KDEL-receptor-dependent mechanism. Rather, it appears to be a true resident of the ER lumen. Unlike other known proteins of the ER lumen, however, LH can be regarded as a peripheral membrane protein that associates with some as yet unidentified constituent of the ER membranes via weak electrostatic interactions. To the best of our knowledge, no other protein with this distribution and type of association has previously been identified in the ER. The results thus suggest a novel possibility by which LH and probably also some other ER-resident proteins as well can be retained in the organelle.

Collectively, our results provide both morphological and biochemical evidence that LH, in spite of the lack of the known ER-specific retention motifs in its primary structure, seems to be a true resident of the ER lumen. Unlike other known proteins of the ER lumen, however, LH can be regarded as a peripheral membrane protein that associates with some as yet unidentified constituent of the ER membranes via weak electrostatic interactions. To the best of our knowledge, no other protein with this distribution and type of association has previously been identified in the ER. The results thus suggest a novel possibility by which LH and probably also some other ER-resident proteins as well can be retained in the organelle.

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**REFERENCES**


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