The Cytoplasmic and N-terminal Transmembrane Domains of Cytochrome P450 Contain Independent Signals for Retention in the Endoplasmic Reticulum*

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Microsomal cytochrome P450 is inserted into the membrane of the endoplasmic reticulum (ER) by its N-terminal signal-anchor sequence which also functions as an ER retention signal. To analyze further potential retention signals of cytochrome P450, topological domains of cytochrome P450 2C1 or 2C2, epidermal growth factor receptor, a plasma membrane protein, and bacterial alkaline phosphatase, a secreted protein were exchanged. The N-terminal signal-anchor of cytochrome P450 2C1 functioned as an ER retention signal when placed at the N terminus of several reporter proteins but not when fused at the N terminus of the extracellular domain of epidermal growth factor receptor, with or without a heterologous cytoplasmic domain. Chimeric proteins in which the cytoplasmic domain of cytochrome P450 2C2 was substituted for that of epidermal growth factor receptor were retained in the ER indicating that an independent retention signal is present in the cytoplasmic part of cytochrome P450 2C2. These chimeras were enzymatically active which argues against misfolding as the primary cause of retention. The ER retention signal of the cytoplasmic domain could not be localized to a single amino acid segment by deletion analysis. These results show that cytochrome P450 2C2 contains redundant, complex ER retention signals in its cytoplasmic and N-terminal hydrophobic domains and that the function of the N-terminal signal is context-dependent.

In eukaryotic cells, the endoplasmic reticulum (ER) is the first compartment encountered by proteins destined for localization in membranous organelles or secretion. In order to maintain the unique compartmental composition of the organelles in this pathway, it is important that proteins with different destinations be efficiently sorted. It is widely accepted that proteins are transported by a bulk flow of vesicles, and their final localization is specified by organelle-specific retention signals (1). Thus, ER-specific proteins must be sequestered from proteins destined for secretion or other organelles.

In general, ER proteins can achieve their specific localization in two ways: by direct retention or by retrieval from distal compartments in the pathway. Best studied is the mechanism of retention of soluble luminal ER proteins, for which a C-terminal KDEL (HDEL for yeast proteins) sequence has been shown to function as an ER retention signal (2). Since the receptor recognizing this signal is located in the intermediate compartment and Golgi, it is believed that ER retention of KDEL-containing proteins is achieved by their retrieval from the early Golgi (3, 4). New studies suggest that, at least for some proteins, the KDEL sequence is not sufficient to ensure ER retention and additional structural motifs may play a role in ER localization (5–7).

Much less information is available about the mechanism of ER retention of integral membrane proteins. For some of the ER membrane proteins, a C-terminal sequence of KXXX or KXXXX has been shown to serve as an ER retention signal in a manner similar to the KDEL signal, i.e. it also functions in retrieval of these proteins from the early Golgi compartment (6, 8, 9). However, in some proteins this sequence seems to be a non-essential, redundant signal, and primary ER retention is achieved by a different mechanism (10).

It is not known how ER retention of some of its most crucial membrane components is achieved. Typical ER membrane proteins, such as ribophorins, cytochromes P450 (P450) and their reductase, and cytochrome b_{5} do not contain KXXX-like signals. Their retention also could be mediated either by constant retrieval from the early Golgi or by primary retention, by being excluded from the transport vesicles. Some of these proteins, including P450, seem to be restricted to the ER and do not undergo recycling through the early Golgi (10–12). It is not known whether this retention results from the exclusion of an ER-restricted protein from the vesicle budding area or its structural incompatibility with packaging in vesicles. It has been suggested that some ER membrane proteins may form a network of interacting supramolecular complexes that would prevent their incorporation into vesicles (10, 12).

The hydrophobic N-terminal region of P450 is thought to be inserted in the ER membrane whereas the remaining part of the protein is on the cytoplasmic side of the membrane (13–16). In addition to the N terminus, some other sequences of the protein may also interact with, but not span, the membrane (17, 18). The 29 N-terminal amino acids of P450 2C1 and 2C2 can induce ER retention when fused to the N terminus of reporter proteins that normally are cytoplasmic or secreted (19). Similar conclusions, mapping the ER retention signal to the N terminus of P450 have been reached by Murakami et al. (20). These studies with chimeric proteins indicated that the...
cytoplasmic domains are not required for ER retention but did not test whether this domain might also contribute to ER retention. P450 is probably restricted to the ER in a manner that excludes this protein from the recycling pathway and its large, bulky cytoplasmic domain was suggested as a possible reason for exclusion from the transport vesicles (11, 20). It was, therefore, of interest to further analyze whether an ER cytoplasmic domain, in the absence of the N-terminal ER retention signal, can be transported out of the ER. We now report that both the N-terminal and the cytoplasmic domains of P450 2C1 and 2C2 contain independent redundant signals for retention in the ER.

EXPERIMENTAL PROCEDURES

Materials—Tran35S-label was from ICN Radiochemicals, endoglycosidase H from Boehringer Mannheim, and protein A-Sepharose from Pharmacia (Uppsala, Sweden). Cell culture media and antibiotics were from Life Technologies, calf serum from Sigma. Monoclonal antibody against the external domain of human epidermal growth factor receptor was from Upstate Biotechnology Inc. (Lake Placid, NY), FITC-conjugated goat anti-mouse IgG was from Tago (Burlingame, CA), polyclonal antiserum against alkaline phosphatase was from Dr. Robert Gennis (University of Illinois, Urbana, IL).

Plasmid Constructions—Construction of plasmids pTZC2, pTZC1, pTZEGFR, pCMVC2, pCMVEGFR, pEGFRPHOA, and pC1PHOA has been described (11, 15, 19). The remaining plasmids, encoding chimeric proteins, were constructed as shown in Fig. 1. Respective DNA fragments were either obtained by restriction digestion or PCR amplification.

Chimeric proteins are designated with three letters, which refer to the luminal (extracellular), transmembrane, and cytoplasmic domains, respectively. E, C, P, and O refer to EGFR, P450, alkaline phosphatase, and no domain, respectively.

Chimera ECC contains the extracellular domain of EGFR, the transmembrane domain, and the cytoplasmic domain of P450 2C2, as shown in Fig. 1, to create ECC.

To create chimera ECC, the extracellular and transmembrane domain of P450 was attached to the cytoplasmic domain of P450 2C2, beginning with residue 29. Since the presence of P450 2C2 amino acids 21–28 seems to be important for its enzymatic activity, we constructed chimera ECC', which includes these residues (Fig. 1). The chimera OEC also contains residues 21–28.

Following are the pairs of oligonucleotide primers used for PCR in various constructions: pCMV-EOE, KpnTM, 5'-GAAGAAGCTTCTTCTCCCAAGGTCTCTCTTTTCTGATAGGAGGGCATTCCC-3' (Fig. 2), 5'-GCCAGCTGAGAACAGGAAAAGTGT-3' (Fig. 4), and c2END; and pCMV-EECI2, which encodes a chimera with P450 2C2 residues 208–348 deleted, 5'-GCAGGTACCATCATGGTGGAGGAGCTGA-3' and c2END; and pCMV-EECN1, in which both, N- and C-terminal sequences of P450 at the N terminus is retained in the ER, as indicated by immunofluorescent staining of reticul intracellular membrane proteins (Fig. 3A) while the analogous protein in which the luminal domain of EGFR was deleted (OEE) was present in the plasma membrane. Interestingly, OEC, a chimera with only the cytoplasmic domain of P450 fused to the transmembrane domain of EGFR also exhibited a reticular pattern by immunofluorescence staining suggesting retention in the ER. These results were confirmed by cellular fractionation studies (Fig. 3B). After a 30-min pulse and 4 h of chase, OEC was restricted to the ER fraction while substantial amounts of OEE were in the plasma membrane fraction. OEC was also restricted primarily to fractions containing ER membranes with no protein present in the plasma membrane. These results suggest that the cytoplasmic domain as well as the N-terminal sequence have ER retention properties. The reason for the differences in the fractionation pattern of OCE and OEC is not clear but may reflect differential distribution into rough or smooth ER, the latter being the normal site of P450 in the liver.

To further examine whether the cytoplasmic domain of P450 has ER retention properties, chimeras which contain the glycosylated extracellular domain of EGFR were studied (Fig. 4). Transfected COS1 cells were pulse-labeled with Tran35S-label for 30 min and then chased for 4 h. During a chase of this length, all the endogenous EGFR, which is slightly larger than the chimeric constructs, becomes resistant to endo H (Fig. 4A). ECC, in which the extracellular domain of EGFR is followed by a full-length P450, was retained in the ER, as shown by its complete sensitivity to digestion with endo H (Fig. 4A). Since ECC contains the P450 N-terminal membrane insertion sequence which functions as an ER retention signal, this result was expected. Surprisingly, EEC, which does not contain the P450 N-terminal sequences, also remained sensitive to endo H digestion even after a prolonged chase time, indicating that it was retained in the ER as well (Fig. 4A).

Since EEC is expressed in COS1 cells, the endogenous EGFR might interact with the chimeric proteins forming unnatural dimers that were retained in the ER. To exclude this possibility, we also expressed these chimeras in CHO cells that do not express detectable amounts of EGFR. In these cells, both ECC and EEC were sensitive to endo H digestion after a 30-min labeling pulse and remained sensitive after a chase of 4 h.
EGFR expressed in these cells became completely resistant to endo H after a 4-h chase (not shown).

In contrast, analogous chimeras which did not contain the cytoplasmic domain of P450 were not retained in the ER. Both ECO and EEO, which have the cytoplasmic domain deleted, became resistant to endo H (Fig. 4A), and we have shown that a chimera with a substitution of alkaline phosphatase for the EGFR cytoplasmic domain (EEP) is also transported to the plasma membrane (19). These data demonstrate that the cytoplasmic domain of P450 is responsible for the retention of EEC in the ER which suggests that an efficient ER retention function exists in the cytoplasmic part of P450 that is independent of the N-terminal transmembrane domain.

Enzymatic Activity of the Chimeric Proteins Retained in the ER

(Fig. 4B). EGFR expressed in these cells became completely resistant to endo H after a 4-h chase (not shown).

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ER—Since ER retention can result from the misfolding of some proteins, it was important to establish whether the cytoplasmic domain of P450 present in the various chimeric constructions was able to achieve its proper conformation. To test proper conformation, the enzymatic activities of chimeric proteins were assayed. Cytochrome P450 2C2 has a lauric acid hydroxylase activity that can be easily detected in transfected COS1 cells (23). Since EEC, as originally made, did not include P450 2C2 amino acids 21–28 which are important for its activity, we subsequently included these residues in chimeras EEC9 and OEC. The presence of this sequence has no effect on the ER retention (data not shown). The chimeras ECC9, EEC9, and OEC all have activities very similar to that of native P450 when expressed in COS1 cells. Relative to the activity of P450 2C2 (100%), the means and standard errors of the activities for the chimeric proteins in three experiments were: 109 ± 2.7% for ECC9, 119 ± 8.7% for OEC, and 111 ± 10.8% for EEC9. Immunoprecipitation of radiolabeled proteins showed that the proteins were expressed at similar levels (data not shown). The cytoplasmic domain of P450 in ECC9 and OEC must be properly folded and correctly inserted in the ER membrane since it retains full enzymatic activity, thus, retention of these chimeras in the ER is not an artifact of misfolding.

Mapping the ER Retention Signal in the Cytoplasmic Domain of P450—In order to map the localization of the potential ER retention signal, we constructed a series of deletions in chimera EEC in the cytoplasmic domain of cytochrome P450 which are illustrated in Fig. 5A. Mutant EECN1 with a deletion of 163 amino acids from the N terminus of P450 2C2 remained sensitive to endo H digestion while the larger endogenous EGFR became resistant (Fig. 5B). However, further deletion of 35 more residues (EECN2) resulted in a protein with complete resistance to endo H digestion, consistent with its transport out of the ER. Since this additional 35 amino acid deletion altered localization of the chimera, we tested whether its deletion would be sufficient for elimination of ER retention function. As is evident from endo H sensitivity, the chimera EECl1 was retained in the ER and, thus, deletion of only 35 internal residues, encoding amino acids 164–198, does not affect ER retention function. We also tested whether the 35 amino acid peptide encoding amino acids 164–198 can induce the ER retention when fused to the truncated EGFR. This hybrid was transported from the ER with the same kinetics as...
EGFR, indicating lack of ER retention activity in the analyzed peptide (results not shown).

More extensive deletion of the internal sequences of the cytoplasmic part of P450, EECI2, also did not affect sensitivity to endo H digestion. On the other hand, deletion of the C-terminal 152 amino acids, as in chimera EECI1, caused partial release of ER retention as indicated by partial resistance to endo H. However, a significant fraction of this mutant is efficiently retained in the ER, since endo H sensitivity could be observed even after prolonged chase (not shown). Since in this mutant the deleted region contains the Cys which is the fifth ligand for the heme, it is clearly inactive enzymatically. The loss of ER retention function as a result of C-terminal deletions was more dramatic if combined with an N-terminal deletion. Deletion of only 78 C-terminal amino acids from EECN1, which was sensitive to endo H, resulted in a chimera (EECN1) completely resistant to endo H. These results indicate that the ER retention mediated by the cytoplasmic domain of P450 is not encoded by a single primary sequence and may be the result of either a specific three-dimensional structure of the folded protein or interaction of specific segments localized in the N-terminal and C-terminal part of this domain.

Fusion of the N-terminal Signal of P450 to the EGFR Luminal Domain Suppresses the ER Retention Function—Chimeric protein ECO was found to be resistant to endo H digestion (Fig. 3), indicating that it was not retained in the ER despite the presence of the P450 N-terminal ER retention signal. In contrast, other chimeric proteins, P450/b-galactosidase, P450/alkaline phosphatase, and P450/EGFR (OCE) were retained in the ER by the P450 N-terminal signal. Since the most obvious difference between these retained hybrids and ECO is the location of the P450-derived signal, it raised the possibility that the ER retention signal of the P450 N terminus functions only when located at the N terminus of the protein and/or when it is followed by additional protein sequence.

This possible position effect was further analyzed using chimera ECE, EEP, and ECP. Chimera ECE is EGFR with its transmembrane segment replaced by the P450 N-terminal 29 amino acids and ECP is similar except that alkaline phosphatase is substituted for the EGFR intracellular domain. EEP, which is transported to the plasma membrane (19), was used as a control. The chimeras and EGFR all were resistant to endo H digestion, consistent with their transport out of the ER (Fig. 6). Since the antibody used cross-reacts with endogenous EGFR in COS1 cells, which is the same size as ECE, ECE was also expressed in CHO cells, which are devoid of an endogenous EGFR. Immunofluorescent staining of the surface of non-permeabilized cells transfected with chimera ECE provides addi-

**Fig. 4.** Endoglycosidase H sensitivity of chimeric proteins synthesized in COS1 (panel A) and CHO (panel B) cells. Cells transfected with plasmids encoding chimeras ECC, EEC, ECO, and EEO were labeled with Tran35S-label for 30 min and chased in complete medium for 4 h (panel A). CHO cells (panel B) were pulse-labeled for 30 min only (lanes 1, 2, 5, and 6) or additionally chased for 4 h (lanes 3, 4, and 7-10). The expressed proteins were immunoprecipitated with the antibody against the extracellular domain of EGFR. This antibody cross-reacts with endogenous EGFR of COS1 cells, indicated with a dot. Immunoprecipitated proteins were digested with endo H for 18 h and analyzed by SDS-polyacrylamide gel electrophoresis.

**Fig. 5.** Endoglycosidase H sensitivity of deletion mutants. A, schematic localization of deletions introduced into the cytoplasmic domain of P450. The single line represents the deleted fragment, whereas the bar stands for the P450 fragment(s) remaining in the chimera EEC. The top bar represents full-length P450 (490 amino acids) in which first 28 amino acids (membrane-binding domain), that are deleted in chimera EEC, are shown by a single line. B, COS1 cells, transfected with the corresponding deletion mutants, were processed as described in legend to Fig. 2. The expressed proteins were immunoprecipitated with the antibody against P450 2C3 (for EECI1 and EECI2) or the extracellular domain of EGFR (for all remaining chimeras) and digested with endo H.
Retention of P450 in the ER

Studies on the subcellular localization of P450 2C2 indicate that it is directly retained in the ER and does not undergo recycling from the intermediate or Golgi compartment (11). The hydrophobic N-terminal signal/anchor sequence of P450 has been shown to be sufficient to restrict localization of several reporter proteins to the ER membrane (19, 20). Murakami et al. (20) have shown that the ER retention of a chimera mediated by the P450 (M1) N-terminal signal also does not involve recycling. Thus, it would appear that the exclusion of P450 from the recycling pathway is mediated by its N-terminal ER retention signal. The location of this signal in the N-terminal transmembrane domain is consistent with the transmembrane location of targeting signals for Golgi specific proteins (25–27). The double lysine ER retention motif of some type 1 ER membrane proteins is also located close to the transmembrane region (8). Membrane properties or proteins specific to an organelle may mediate the retention function of such membrane-localized signals. The binding of membrane-associated coat proteins to a double-lysine motif may be an example of such an interaction (28, 29).

In spite of the experimental evidence for a retention signal in the N-terminal region of P450 and these mechanistic considerations, the data presented indicate that P450 contains two segments of high hydrophobicity within P450 so that peripheral interactions with the membrane may be important in the retention. This view is consistent with evidence that regions in the cytoplasmic domain of P450s are associated with the membrane, in addition to the N-terminal transmembrane domain (17, 18). A second mechanism for retention of ER proteins may be the formation of networks that are excluded from the transport vesicles (10, 12). Oligomerization as a mechanism for retention has been suggested for some Golgi proteins (31, 32) and for p63, a protein localized in cis-Golgi network (33). P450 is known to interact with other ER membrane proteins, such as P450 reductase, as part of its enzymatic activity, and it has been suggested that microsomal P450s form oligomeric complexes, possibly hexamers (34, 35). The retention of EECN1 and EEC1 which are presumably inactive molecules, missing 163 N-terminal amino acids and the Cys that is the fifth ligand for heme, respectively, eliminates normal interactions in the enzymatic process as part of the retention mechanism. Self-aggregation or interaction with other membrane or cytoplasmic proteins to form a network or a receptor-mediated process remains possible.

The ER retention function of the N terminus of P450 seems to be dependent on its context in the chimeras. When at the N terminus of the protein, which is its normal position in P450 2C1, it causes the retention of several reporter proteins (19). However, when the EGFR luminal domain is fused to the beginning of the N-terminal signal sequence, as in ECE, ECP,
or ECO, the chimeric proteins are transported from the ER to the plasma membrane. In contrast, a relatively short sequence of 29 amino acids containing a glycosylation site preceding this signal did not interfere with retention function (19). The N-terminal retention signal of P450 (M1) was also functional in retention when placed at the C terminus of a chimera (20). These results suggest that properties of the protein fused at the N terminus of the retention signal may determine whether it is functional or not. One possibility is that the EGFR extracellular domain has positive signals for transport out of the ER which override the retention signal in the P450 N-terminal region. This seems unlikely since EEC and ECC are retained in the ER, although if such a positive signal was dominant to the N-terminal but not cytoplasmic retention signals it would be possible. Further studies will be required to determine definitively the reason for the different effects of protein sequence fused at the beginning of the N-terminal retention signal on its function.

The context-dependent nature of the N-terminal signal also suggests that the length of the hydrophobic core of the transmembrane domain is not critical to its retention function. It has been postulated that the main difference between transmembrane sequences of the ER, Golgi, and plasma membrane proteins is the length of the hydrophobic core, which is related to the difference between these membranes’ content of cholesterol and resulting thickness of respective bilayers (36). According to this model, the hydrophobic transmembrane segment of P450, which is shorter than the membrane-spanning peptide of EGFR, would retain a reporter protein in the ER, whereas the transmembrane segment of EGFR would permit transport of the same reporter to the plasma membrane. Consistent with this prediction, OCP (19) and OCE are retained in the ER, and OEE is transported to the plasma membrane. Therefore, a transmembrane domain derived from a plasma membrane protein (EGFR) allows the chimera to reach the plasma membrane whereas chimera inserted into the membrane by a P450 ER-specific signal is retained in the ER. However, contrary to these predictions, chimeras ECO and EEO, anchored in the membrane by C terminally located transmembrane domains, are both transported to the plasma membrane as are EEP and ECP that have a cytoplasmic domain of alkaline phosphatase. Thus, the localization of a protein cannot be predicted simply from the origin and the length of a transmembrane sequence. Clearly, other properties of such a sequence and its context in the protein mediate the retention in a specific compartment.

Redundant signals for organelle targeting and retention may be a general phenomenon. Although the KDEL signal and retrieval mechanism has been clearly established, it has been shown that deletion of the KDEL signal from calreticulin does not release this protein from the ER (5). Two independent mechanisms seem to be operating: KDEL-based retrieval and direct retention mediated by a calcium-binding domain. Similarly, a subunit of the N-oligosaccharyl transferase complex, an ER membrane protein, is not released from the ER when its C-terminal double-lysin retrieval signal is mutagenized, also pointing to the existence in its structure of an additional, independent retention signal (10). The presence of two distinct retention signals organized similarly to that of P450 2C2 has recently been shown for a cis-Golgi network-specific protein, Sed5 (37). This protein’s targeting and retention is mediated by its N-terminal transmembrane domain and its cytoplasmic domain, in which no discrete signal could be mapped. This and other studies (33, 38) suggest that the organelle-specific retention of a protein may involve the action of more than one signal.

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