An N-terminal Glycosylation Signal on Cytochrome P450 Is Restricted to the Endoplasmic Reticulum in a Luminal Orientation*

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The mechanism of retention of cytochrome P450 in the endoplasmic reticulum is unknown, and the membrane topology of the N-terminal region remains controversial. To address these problems, a sequence of 25 amino acids containing an internal N-glycosylation site of rabbit cytochrome P450 2C2 was attached to the N terminus of cytochrome P450 2C1. This protein is glycosylated at a single site in a cell-free translation system containing microsomal membranes, as indicated by gel mobility and sensitivity to endoglycosidase H. When expressed in COS1 cells, an immunoreactive species with the same gel mobility as the in vitro synthesized glycosylated product was detected. Treatment with endoglycosidase H changed its mobility to that of an unglycosylated hybrid cytochrome P450 2C1. These results indicate that in intact cells, as in the cell-free system, the N terminus of cytochrome P450 is luminal orientation which is not consistent with a hairpin loop conformation. Sensitivity of the glycosylated protein to endoglycosidase H suggests that the protein does not reach the Golgi compartments. When transfected cells were incubated at low temperatures to inhibit retrograde transport from the intermediate pre-Golgi compartment into the endoplasmic reticulum, localization of cytochrome P450 was not changed, as assayed by subcellular fractionation and immunofluorescence staining. These observations suggest that cytochrome P450 is restricted to the endoplasmic reticulum membrane by a mechanism different from recycling through the intermediate compartment, which is a pathway utilized by soluble endoplasmic reticulum proteins.

Microsomal cytochromes P450 (P450) are integral membrane proteins localized in the endoplasmic reticulum (ER). It is generally accepted that the hydrophobic N-terminal region of these proteins, containing 20–25 amino acids, is a signal recognition particle-dependent signal that directs insertion of P450 into the membrane (Bar-Nun et al., 1980; Sakaguchi et al., 1984). This sequence also functions as a halt-transfer signal, which anchors P450 to the membrane, while the rest of P450 resides on the cytoplasmic side of the membrane (Sakaguchi et al., 1987; Monier et al., 1988; Szczesna-Skorupa et al., 1988; Szczesna-Skorupa and Kemper, 1989). This model is supported by binding studies with site-specific antibodies (Delemos-Chiarandini et al., 1987), accessibility to proteases (Brown and Black, 1988), and membrane insertion of chimeric proteins (Sakaguchi et al., 1987; Szczesna-Skorupa et al., 1988; Monier et al., 1988). However, it remains controversial, whether the N-terminal segment is in a loop configuration or a single membrane-spanning region. Nelson and Strobel (1988) proposed a hairpin model for the N-terminal anchor based on hydrophobicity calculations and studies in which labeling of the N terminus of P450 with membrane-impermeant reagents was consistent with its cytoplasmic orientation (Bernhardt et al., 1983). However, in many P450s, the second segment of the postulated loop has relatively low hydrophobicity and contains multiple prolines and glycines, properties which are more consistent with a β-turn structure than with a membrane-spanning α-helix. Furthermore, recent studies also using membrane-impermeant reagents in both reconstituted and native microsomes have indicated that the N terminus has a luminal orientation (Vergeres et al., 1989, 1991). Recently, the possibility that the second segment of a hairpin loop or another hydrophobic region plays a role in the interaction with the membrane has been raised, based on the observation that P450 2E1, with the N-terminal hydrophobic region of 29 amino acids deleted, is strongly associated with the membranes when expressed in Escherichia coli ( Larson et al., 1991).

A second unresolved question related to the membrane localization of P450 is the mechanism by which it is retained in the ER. Soluble luminal ER proteins contain a C-terminal tetrapeptide, KDEL, which is required for retention (Munro and Pelham, 1987). These proteins appear to be transported from the ER to a “salvage” or intermediate compartment and returned to the ER by interaction with a KDEL receptor (Vaux et al., 1990; Lewis and Pelham, 1990). Several ER membrane proteins located predominantly in the lumen with short C-terminal cytoplasmic tails require at least 2 isoynes at the −3 and −5 position from the C terminus for retention (Jackson et al., 1990; Shin et al., 1991). It has not been shown whether these proteins are also recycled from the salvage pathway. Previous studies have shown that microsomal P450s are restricted to the ER and absent from the Golgi apparatus (Brands et al., 1985; Yamamoto et al., 1985). However, these analyses of steady state levels of P450 would not have detected transient translocation to a salvage pathway. P450s, which are localized mainly in the cytoplasm, contain neither −3, −5 lysine motifs at the C terminus nor the N-terminal membrane insertion signal nor a C-terminal KDEL sequence and must be retained by a different mechanism.

N-Glycosylation of proteins has provided a powerful tool...
primer and an NcoI site in the 3' primer. Since P450 2C2 cDNA has two NcoI sites, we chose to place an N-terminal extension on P450 dia and antibiotics were from Gibco Laboratories and fetal bovine to the plasmid pTZC1 which was also cut with these enzymes. pTZCl containing a full-length P450 2C1 cDNA constructed from the original were linearized with BamHI, and plasmids pTZC1 and pTZNCl were of the genomic clone (Zhao et al., 1993).

The amplified fragment was isolated after electrophoresis (phosphate-buffered saline (PBS) containing 10 mg/ml DEAE-Dextran, and 10% of the plates was chased with complete medium at 37 °C and the other at 16 °C in a complete medium supplemented with 10 mM Hepes-KOH, pH 7.3. After 2.5 h, cells from both plates were washed two times with cold PBS, one time with 0.25 M sucrose in 5 mM Hepes-KOH, pH 6.8, and subsequently homogenized with 2 ml of 0.25 M sucrose in 5 mM Hepes-KOH, pH 6.8, by 25 strokes in a Dounce homogenizer (pestle B). Homogenates were loaded on two discontinuous sucrose gradients containing: 1 ml of 2 M sucrose, 3.4 ml of 1.3 M sucrose, 3.4 ml of 1 M sucrose, and 2.75 ml of 0.6 M sucrose (Bole et al., 1986) and spun for 3 h at 39,000 rpm in a Beckman SW 41 rotor. Eleven fractions (about 1 ml each) were collected from the bottom of the tubes, and their content was analyzed by immunoprecipitation. In parallel, cells from one plate which was neither transfected nor labeled were processed in the same way, and the collected fractions were used for markers assays. Cytochrome c reductase activity was measured as described (Omura and Takesue, 1970). Galactosyltransferase was measured using the procedure of Boles et al. (1986).

**Indirect Immunofluorescence**—COS1 cells were grown on coverslips in 35-mm dishes and transfected as described. Forty-eight hours after the transfection, cells were washed with PBS, fixed with 2.5% paraformaldehyde for 25 min, permeabilized with 0.1% Triton X-100, and incubated with primary swine antiserum against P450 2C3. The secondary antibody, fluorescein-conjugated goat anti-swine IgG (Jackson ImmunoResearch Laboratories Inc.). Cells were observed and photographed using a Zeiss photomicroscope III equipped with epi-illumination optics and an HBO 100-watt mercury lamp.

**RESULTS**

**Glycosylation of P450 Mutant NC1 in the Cell-free System**—To examine whether the N termini of P450 2C proteins are oriented to the luminal side of the ER membrane and to provide a potential glycosylation site for monitoring the cellular localization of the protein, a hybrid protein, designated NC1, was constructed by adding a peptide corresponding to amino acids 150 to 178 of P450 2C2 to the N terminus of P450 2C1 (Fig. 1). This peptide contains the sequence Asn-Ala-Ser, a consensual N-glycosylation site, which normally is not glycosylated since this region remains on the cytoplasmic

[2] P. Straub and B. Kemper, unpublished data.
The major translational product of NC1 mRNA translated in reticulocyte lysate has an electrophoretic mobility on SDS gels slower than that of P450 2C1 because of 29 extra amino acids (Fig. 2). Interestingly, a small amount of a protein co-migrating with wild-type P450 2C1 was also present (Fig. 2, lane 2). Although a newly created upstream methionine codon was placed in a context optimized for initiation (purine in a −3 position), some translation apparently starts at the original AUG codon, which does not have a context favorable for initiation (Kozak, 1986).

In the presence of microsomal membranes, translation of NC1 mRNA led to the synthesis of an additional protein with a decreased mobility on the gel (Fig. 2, lane 3), whereas no change was observed for P450 2C1. Treatment with endo H increased the mobility of this protein to that of NC1 made in the absence of membranes (Fig. 2, lane 4). These results show that the N-terminal extension of the NC1 protein undergoes membrane translocation and glycosylation in the in vitro system.

To establish that the N-terminal extension placed on P450 2C1 did not affect the cytoplasmic orientation of the protein in the membrane, we analyzed the proteolytic sensitivity of the proteins made in the presence of microsomal membranes. P450 2C1, which is predominantly cytoplasmic, was not protected against proteolytic digestion (Fig. 3, lane 4). In contrast, glycosylated forms of the mutant [Lys2,Arg5]P450 2C2 are resistant to proteolysis (Fig. 3, lane 12) unless the membranes are disrupted by detergent (lane 13), indicating that the P450 was translocated across the membrane, as shown previously (Szczesna-Skorupa and Kemper, 1989). The mutant NC1, like P450 2C1, was not protected against proteolysis (Fig. 3, lane 8) indicating that this protein also remains predominantly on the cytoplasmic side of the membranes and suggesting that the N-terminal extension has not changed the overall membrane topology of P450 2C1.
wild-type P450 2C1 (Fig. 4A, compare lanes 2 and 3).

Endo H treatment of the immunoprecipitated proteins increased the gel mobility of the more slowly migrating NC1 products to that of the unglycosylated NC1 protein, establishing that it was glycosylated (Fig. 4A, lanes 4 and 5). However, glycosylation was relatively inefficient with only about 50% of the NC1 in the glycosylated form (Fig. 4A, lane 3). Subsequent pulse-chase experiments showed that the ratio between glycosylated and unglycosylated product gradually increases with time, and, after 5 h of chase, the majority of the protein is glycosylated (Fig. 4B). This could result either from delayed glycosylation or translocation across the membrane of the N-terminal extension or from increased stability of the glycosylated form relative to the unglycosylated one since the amounts of both decrease during the chase.

These results indicate that in transfected cells, as in the cell-free system, a short peptide preceding the N-terminal membrane insertion sequence of cytochrome P450 2C1 is translocated to the luminal side of the endoplasmic reticulum and is glycosylated. Sensitivity of the glycosylated product to endo H suggests that it is not transported to the Golgi apparatus and remains localized in the ER.

Retention of Cytochrome P450 in ER—Luminal ER proteins are postulated to be transported to an intermediate “recycling” compartment localized between the ER and the cis-Golgi cisternae (Pelham, 1988, 1989; Lippincott-Schwartz et al., 1990). These proteins are subsequently retrieved back to the ER in a temperature- and microtubule-dependent manner. We have performed a series of experiments to examine whether cytochrome P450 2C2, a specific ER membrane-bound protein, undergoes similar recycling.

In addition to endo H, we also examined the sensitivity of the glycosylated NC1 protein to endoglycosidase D (endo D), an enzyme which is active only toward a GlcNAc-Man structure. Normally, this is a transient intermediate detected in the cis-Golgi and is a substrate for the GlcNAc transferase I present in the medial Golgi. Since cis-Golgi-specific oligosaccharide processing enzymes have been detected in the intermediate compartment (Dean and Pelham, 1990), it seemed possible that at least some ER glycoproteins subjected to the recycling might be processed to the GlcNAc-Man intermediate and, as such, become sensitive to endo D. Detectable levels of the GlcNAc-Man structure on some ER-restricted proteins have been found (Rosenfeld et al., 1984; Kabcenc and Atkinson, 1985). In addition, inhibition of the retrieval of recycled proteins to the ER by low temperature or micro-
tubule inhibitors should result in extended exposure of the glycoproteins to the processing enzymes which could increase the level of the endo D-sensitive form.

Glycosylated NC1 is not sensitive to endo D when transfected cells are incubated with radioactive amino acids for 30 min followed by a 2-h chase (Fig. 5, lane 4). To inhibit transport from the intermediate compartment to the ER, transfected cells were also chased at 16 °C for 2 h. Under these conditions, the glycosylated protein remained resistant to endo D (lane 7). Resistance of the glycosylated form of NC1 to endo D was also observed when recycling was inhibited during the chase period by the presence of nocodazole, a microtubule dissociating agent (results not shown). Sensitivity of glycosylated NC1 to endo H remained unchanged (Fig. 5, lanes 3 and 6).

These results suggest that P450 does not undergo recycling through the salvage pathway, at least based on its oligosaccharide structure. However, since all the enzymatic activities present in this compartment have not been completely iden-
that a protein enters the recycling pathway. Therefore, we transfected cells with P450 2C2 in the pCMV5 expression vector with ["S"]methionine for 90 min and subsequently chased for 2.5 h either at 37 °C or 16 °C. After sucrose gradient centrifugation, fractions were analyzed for the presence of P450 2C2 by immunoprecipitation and polyacrylamide gel electrophoresis (Fig. 6). The majority of the immunoprecipitated protein was detected in the first two fractions (panel A) coincident with NADPH cytochrome c reductase activity, a marker for the ER (panel B). A small amount of the protein, as well as the reductase activity, was detected in fractions 5 and 6, which may represent smooth ER membranes. Most of the P450 was also associated with the ER when transfected cells were chased at 16 °C (Fig. 6, panel A) or 15 °C (not shown). At low temperatures, which block retrieval, some membrane-bound ER proteins have been shown to shift to the intermediate or cis-Golgi compartment (Hua et al., 1991). Some immunoreactive material was detected in fractions containing the Golgi apparatus, especially in the sample incubated at 16 °C. Most of these proteins had low molecular weights and may represent intermediates in P450 2C2 degradation which occurs in autolysosomes (Masaki et al., 1987). At 16 °C, proteolysis of P450 may be slower, resulting in the accumulation of peptide fragments. The persistence of most of the P450 2C2 in the ER fraction under conditions shown to inhibit retrograde transport also supports the hypothesis that P450s do not undergo recycling to the intermediate compartment.

Immunofluorescent Analysis of Cytochrome P450 2C Localization—We have also compared subcellular distribution of P450 2C2 by immunofluorescence in transfected cells grown at 37 °C or 16 °C. The immunostaining pattern of P450 2C2 expressed in COS1 cells is consistent with its localization in the ER (Fig. 7A). The same pattern of staining was observed when transfected cells were incubated for 3 h at 16 °C before fixing and staining (Fig. 7B), or when incubation was carried out in the presence of nocodazole (not shown). Similar results were obtained with NC1. Therefore, restriction of the retrieval of proteins from the recycling compartment does not influence the localization of cytochrome P450 2C2. This result, in agreement with the biochemical data, also indicates that cytochrome P450 2C2 does not undergo recycling through the salvage pathway.

**DISCUSSION**

Localization of the N-terminal amino acid of microsomal P450s has important implications for its membrane topology. Disposition of the N-terminal region of P450 in a loop configuration, as suggested (Nelson and Strobel, 1988), would require a cytoplasmic localization of the N-terminal residue and it would assign a region between amino acids 30 and 60 as a second membrane-spanning segment. In previous cell-free studies, a short peptide from opsin was translocated to the luminal side of the membrane when attached to the N terminus of P450 suggesting a luminal disposition of the N terminus (Monier et al., 1988). However, since this peptide is normally localized at the N terminus of opsin and translocated into the lumen, it may have properties that facilitate its translocation and, possibly, affect the topology of the P450 N terminus. Furthermore, analysis in a cell-free system may not always be an accurate model for a process such as the membrane insertion of P450. In order to examine this problem, we have attached to the N terminus of P450 2C1 a short peptide derived from the closely related P450 2C2, which contains a potential glycosylation site. In its natural context, this peptide does not cross the membrane and contains a balance of negative and positive charges so it should have minimal influence on membrane topology. If there is any effect, the addition of the extra N-terminal amino acids should reduce the tendency of the N-terminal end of the signal sequence to translocate into the ER lumen. However, in a cell-free system, this peptide is translocated to the luminal side of the membrane and glycosylated confirming the cell-free studies with the opsin peptide. In transfected COS1 cells, NC1 was also glycosylated, providing evidence that the results in the cell-free studies accurately reflect the topology of P450 in whole cells. Interestingly, estrogen aromatase, P450 19, purified from the human placenta, was recently shown to be a glycoprotein with the glycosylation most likely at its N terminus (Sethumadhavan et al., 1991). Thus, in this native P450, as in the hybrid P450s, the N-terminal sequence probably has a luminal orientation. Apparently, translocation of the N terminus is not blocked by an N-terminal extension with a balanced charge. These results with the hybrid proteins and P450 19 represent strong evidence for the luminal localization of the N-terminal methionine of microsomal P450. Thus, this evidence, combined with studies using antibody binding (DeLemos-Chiarandini et al., 1987) or N-terminal labeling with membrane-impermeable reagents (Vergere et al., 1989, 1991), supports a model of P450 membrane topology with one N-terminal membrane-spanning segment, at least for the family 2 P450s which have been studied in most detail. It is possible that in some P450s an N-terminal loop structure is formed since there is considerable variation in the N-terminal sequence of P450s in different families. Other hydrophobic regions of the protein may also form important contacts with the membrane which might explain the tight binding to membranes of mammalian P450s with the N-terminal anchor deleted that are expressed in E. coli (Larson et al., 1991).

Previously, we proposed that the N-terminal region of P450 inserts in a "head-in" orientation into the membrane (Szczesna-Skorupa et al., 1988). Monier et al. (1988) proposed that the P450 N terminus inserts initially as a loop which then reorients to a single membrane-spanning head-in orientation. Partial cleavage of the P450 signal sequence when it was fused to growth hormone provided support for this idea since cleavage could only occur if the C-terminal end of the
sequence was at least transiently exposed to the luminal signal peptide (Monier et al., 1988). The luminal orientation of the opsin and P450 2C2 glycosylation peptides fused to the P450 N termini support an initial loop formation. Since the N-terminal extensions are neither hydrophobic nor topologically active, the hydrophobic signal sequence must interact with the membrane prior to translocation of the N-terminal sequences. A model for native P450 in which the hydrophobic core of the N-terminal signal initially inserts into the membrane as a loop and subsequently reorients in a way that the N terminus is on the luminal side of the membrane is more consistent with these studies than a head-in insertion model.

The ultimate topology and function of the N-terminal region may depend on a balance between hydrophobic forces pulling the N terminus to the luminal side and N-terminal hydrophilic forces resisting membrane insertion. This idea is supported by studies showing that either decreasing the length of the hydrophobic core or increasing N-terminal positive charges convert the P450 “stop-transfer” signal to a translocation signal (Szczezna-Skorupa et al., 1988; Sato et al., 1990; Sakaguchi et al., 1992). Decreasing the hydrophobicity of the core may decrease the force tending to pull the N terminus into the membrane. Charged groups at the N terminus, particularly positive charges that could interact with the negatively charged phospholipids at the membrane surface, would retard entry of the N terminus into the membrane. This idea would also be consistent with the bias toward positive charges on the cytoplasmic side of membrane-spanning helices. The ultimate orientation of the membrane-spanning helix might then determine the function of the signal. Reorientation of an initial N-terminal loop may be a necessary step in expressing a stop-transfer function.

Recently, it has been shown that some luminal ER proteins are transported into the intermediate compartment (cis-Golgi network), or salvage compartment, and subsequently retrieved back into the ER (Pelham, 1988). It appears that recognition of a C-terminal KDEL retention signal by a receptor is responsible for retrieval (Vaux et al., 1990; Lewis and Pelham, 1990). The only membrane-bound proteins shown to recycle between ER and Golgi are unassembled major histocompatibility complex class I molecules, which fail to reach the cell surface. Hsu et al. (1991) demonstrated an altered cellular distribution of these proteins if cells were incubated at low temperature or treated with a microtubule-dissociating drug to prevent retrograde transport. In contrast, we were unable to detect a similar dramatic change in the distribution of P450 2C2 protein when cells were incubated at low temperatures (15 °C or 16 °C) and subsequently analyzed using immunofluorescent staining or immunoprecipitation of subcellular fractions. In addition, the sensitivity of the carbohydrate moiety on NC1 to cleavage by endo H or endo D did not change, which is consistent with a lack of processing by the Golgi associated enzymes. These studies indicate that P450 2C2 is retained in the ER membrane without recycling through the salvage pathway.

The mechanism of retention of cytochromes P450 in the membrane of the ER is not known. P450s contain neither C-terminal basic residues nor a KDEL sequence which represent retention signals for type I membrane and luminal ER proteins, respectively. It is possible that a different specific sequence in the P450 may interact with a receptor to prevent transport from the ER. Alternatively, P450 molecules are known to aggregate and to form complexes with other ER membrane proteins (Ingelman-Sundberg, 1986). Formation of such a network could prevent incorporation of P450 molecules into the vesicles budding from the ER for transport to the Golgi or intermediate compartment and be responsible for ER retention. Recently, a similar mechanism has been postulated for ER retention of ribophorins, which form a supramolecular complex with other proteins and are not transported to the intermediate compartment (Ivessa et al., 1992).

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