Cytochromes P450 are inserted into and anchored to the endoplasmic reticulum (ER) membrane by a hydrophobic signal sequence at the NH₂ terminus. To determine whether the NH₂-terminal sequence might also have an ER retention signal, the NH₂-terminal 29 amino acids of cytochrome P450 2C1, with and without an additional 29 amino acids containing an N-glycosylation site, were fused either to a soluble cytoplasmic protein, Escherichia coli β-galactosidase, or to a secreted protein, E. coli alkaline phosphatase, and the hybrid proteins were expressed in COS1 cells. Subcellular fractionation indicated that both the β-galactosidase and alkaline phosphatase hybrid proteins cosedimented with marker enzymes for ER membranes, and localization by immunofluorescent staining was consistent with an ER location. Hybrid proteins with the NH₂-terminal glycosylation site were glycosylated in COS1 cells, and the carbohydrate moiety was sensitive to endoglycosidase H digestion, providing further evidence that the proteins were retained in the ER. In vitro studies of membrane insertion of the alkaline phosphatase hybrid indicated that fusion to alkaline phosphatase did not alter the topological properties of the cytochrome P450 NH₂-terminal sequence. In addition, alkaline phosphatase fused to the extracellular and transmembrane domains of epidermal growth factor receptor was transported to the plasma membrane in COS1 cells, which establishes that alkaline phosphatase as a cytoplasmic domain does not prevent transport from the ER. These observations indicate that the large cytoplasmic domain of cytochrome P450 is not required for retention in the ER and suggest that a specific sequence or structure within the NH₂-terminal 29 amino acids functions as an ER retention signal.

Microsomal cytochromes P450 (P450s) are integral membrane proteins localized in the endoplasmic reticulum (ER). P450s characteristically have a hydrophobic amino-terminal region of 20–25 amino acids (Nelson and Strobel, 1988). This sequence functions as both an SRP-dependent ER membrane insertion and a halt-transfer signal when it is fused to heterologous nonmembrane proteins (Sakaguchi et al., 1984; Monier et al., 1988; and Szczesna-Skorupa et al., 1988). Addition of positive charges near the NH₂ terminus converts the insertion signal into a translocation signal for the hybrid proteins or P450 itself (Szczesna-Skorupa et al., 1988; Szczesna-Skorupa and Kemper, 1989). In contrast to the mechanism for insertion of P450 into the ER membrane, little is known about the mechanisms that retain the protein in the ER.

Small peptides which enter the ER lumen are rapidly secreted, which suggests that transport in the secretory pathway occurs by default (Wieland et al., 1987). Consequently, residency of a particular protein in an organelle requires a signal that retains the protein in that location. Soluble luminal ER proteins contain a COOH-terminal tetrapeptide, KDEL, which is required for retention (Muñoz and Pelham, 1987). Deletion of the KDEL sequence results in the secretion of the modified ER proteins, providing further evidence that the default pathway is secretion (Pelham, 1988).

The mechanism for the retention of resident ER transmembrane proteins is less well defined. Analogous to the soluble luminal ER proteins, a retention signal may be required. It has been shown that KDEL appended to the carboxyl terminus of dipeptidyl-peptidase IV, which is a typical surface transmembrane protein, directed the mutant protein to the ER (Tang et al., 1992). This is a potential mechanism for type 2 membrane proteins with luminal COOH-terminal domains. However, none of the integral ER membrane proteins, for which amino acid sequences are known, has such a signal. Mutational analysis of the cytoplasmically exposed tails of several type 1 ER transmembrane proteins suggests that 2 lysines positioned 3 and either 4 or 5 residues from the carboxyl terminus represent the retention motif for these membrane proteins (Jackson et al., 1990; Shin et al., 1991). This motif functions only in the presence of a transmembrane region. The required sequence for their ER retention, therefore, appears to be less exact than that for luminal proteins. P450s, which are type 1 proteins with little or no sequence in the lumen of the ER, do not contain lysine motifs at the COOH terminus or near the NH₂-terminal membrane insertion signal. In addition, they do not contain a COOH-terminal KDEL motif and must be retained by a mechanism different from that for either luminal proteins or the other type 1 membrane proteins with short cytoplasmic tails.

P450s might be retained by a specific retention motif or by interactions of P450 with other proteins to form a large network. It has been proposed that the resident ER proteins may form large networks which prevent their transport out of the ER (Andersson et al., 1985; Burgert and Kvist, 1985; and Iwasa et al., 1992). P450s are known to form aggregates in the membrane with other P450s (Alston et al., 1991) and must also interact with P450 reductase (Gut et al., 1983; Ohta et al., 1992) which would be consistent with the networking hypothesis. If a specific retention signal is present, it may be
in or near the NH2-terminal transmembrane domain. In support of this idea, Golgi membrane protein retention signals linked to the transmembrane domain (Machamer and Rose, 1987; Munro, 1991; Teasdale et al., 1992; and Aoki et al., 1992). To determine whether ER retention determinants are present in the NH2-terminal transmembrane domain, we have constructed hybrid cDNAs in which fragments of a cDNA encoding the NH2-terminal 29 amino acids of the P450 are fused with cDNAs encoding two different reporter proteins. In transfected cells, hybrid proteins with both reporters are retained in the ER, establishing that the 461-amino acid cytoplasmic domain of P450 is not required for ER retention and suggesting that a specific sequence or structure within the NH2-terminal region of P450 is responsible.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal antibody directed against *Escherichia coli* β-galactosidase was purchased from Promega Biotec. Monoclonal antibody against the external domain of human EGF receptor (EGF-R) was from UBI (Lake Placid, NY). Fluorescein-conjugated goat-anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG were from Tago (Burlingame, CA). TranS-αS-labeled was from ICN Radiochemicals (Irvine, CA), UDP-[3H]Gal was from Du Pont-New England Nuclear, and endoglycosidase H (endo H) was from Boehringer Mannheim. Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Cell culture media and antibiotics were from Life Technologies, Inc. and fetal bovine serum was from Intergen (Purchase, NY).

**Plasmid Constructions**—Starting plasmids, with pertinent restriction sites, for the hybrid constructions are shown in Fig. 1A. For P450-β-galactosidase hybrids, plasmid pCH110 was cleaved with ClaI, filled in using E. coli DNA polymerase I, Klenow fragment, digested with Bsu36I, and the 219-bp fragment was isolated. Separately, the 3231-bp Bsu36I-BamHI fragment of pCH110 was isolated. These two purified fragments were ligated and the resulting CiaI (blunt-ended)-BamHI fragment encoding β-galactosidase was subcloned into the XbaI (blunt-ended with DNA polymerase I, Klenow fragment) and BamHI site of pCMV5 to produce the plasmid pCMV5GAL. The plasmid pC1GAL or pC1GAL was constructed by inserting KpnI-HindIII (blunted with Klenow) fragments of pTZCl or pTZNCl (Szczesna-Skorupa and Kemper, 1993), respectively, into the XbaI-HindIII sites of pCMVGAL. The plasmid pTZCl encodes a hybrid protein in which the NH2-terminal 1-29 amino acids of P450 2C1 are fused to the NH2-terminal 29 amino acids containing an N-glycosylation site. The plasmid pTZNCl contains cDNA encoding P450 2C1 with a 29-amino acid extension present in the NC1 construction are shown. In the GAL constructions the fusion proteins encoded by plasmids pClGAL, pClPHOA, pNClGAL, and pNC1PHOA are the EGF-R NH2-terminal extracellular domain (E.C.), the membrane spanning domain (T.M.), and the membrane integration assay at high pH were performed as described previously (Szczesna-Skorupa and Kemper, 1993).

**Expression in COS1 Cells**—Transfection of COS1 cells, metabolic...
Subcellular Fractionation—To fractionate cells expressing the β-galactosidase fusion protein, transfected COS-1 cells were homogenized with a Teflon-glass homogenizer, followed by 10-20 bursts of sonification, to completely break the cells in buffer (0.25 M sucrose, 5 mM Hepes-KOH, pH 6.8) with or without 1 mM EDTA. The homogenate was fractionated by differential centrifugation (Clark and Waterman, 1991). The distribution of protein in each subcellular fraction was quantitated by measuring β-galactosidase activity.

The subcellular fractionation was performed as described by Bole et al. (1986) with the following modifications. The cells were homogenized in 2 ml of the buffer (0.25 M sucrose, 5 mM Hepes-KOH, pH 6.8, 1 mM EDTA) by 25 strokes in a Dounce homogenizer (pestle type B). The whole homogenates were layered over discontinuous sucrose gradients. After centrifugation, 1 ml fractions were collected from the bottom of the tubes, and protein in each fraction was assayed by measuring β-galactosidase activity or analyzed by immunoprecipitation. In parallel, fractions from cells which were neither transfected nor labeled were assayed for enzyme markers for subcellular organelles.

Assay for β-Galactosidase and Marker Enzymes—β-Galactosidase activity was measured according to Miller (1972). Protein concentration was determined using the Bradford procedure (1976). NADPH-cytochrome c reductase was assayed by a modification of the method of Omura and Takegaki (1970) by measuring the initial rate of reduction of cytochrome c from a reaction of 500 μl containing 0.25 mM NADPH, 0.1% Triton X-100 in PBS for 20 min at room temperature. Cells were permeabilized by treatment with 0.1% Triton X-100 in PBS for 5 min followed by washing with 0.1% gelatin in PBS. Coverslips were incubated with primary antibody for 1 h at room temperature, washed with 0.1% gelatin in PBS, incubated with secondary antibody for 1 h at room temperature, and washed for 30 min with 0.1% gelatin in PBS. Cell surface staining of EGF-R and EGF-R was obtained with a primary antibody specific for the extracellular domain of EGF-R, followed by secondary antibody in cubation. Coverslips were mounted on glass slides and photographed using a Zeiss photomicroscope III equipped with epi-illumination optics and an HBO 100 watt mercury lamp.

RESULTS

Localization of P450-β-Galactosidase Hybrid Proteins—Almost all of the P450 molecule is thought to be localized on the cytoplasmic side of the ER membrane with the exception of the NH2-terminal membrane anchor (De Lemos-Chiarandini et al., 1987; Sakaguchi et al., 1987; and Szczesna-Skorupa and Kemper, 1989). To determine whether this NH2-terminal sequence is also responsible for retaining P450 in the ER, we initially replaced the P450 cytoplasmic domain in P450 2C1 with E. coli β-galactosidase. β-Galactosidase is a cytoplasmic protein and should have no specific signals for retention in the ER. Since there is no β-galactosidase activity in mammalian cells, the location of the hybrid protein can be easily determined by enzymatic assay.

Previous studies of hybrid proteins in cell-free systems had shown that the first 29 amino acids of P450 2C2 were sufficient for insertion into microsomal membranes (Szczesna-Skorupa et al., 1988), but the mechanism of insertion and stability of the membrane proteins in whole cells has not been studied. In the presence of Mg2+, nearly all of the C1GAL hybrid protein expressed in COS1 cells was recovered in particulate fractions after differential centrifugation of cellular homogenates (Fig. 2). However, over half of the C1GAL was present in the nuclear fraction, consistent with the distribution observed for P450 17α expressed in COS1 cells (Clark and Waterman, 1991). Interestingly, substantial amounts of native β-galactosidase, which is a soluble cytoplasmic protein, were also recovered in the nuclear fraction. In contrast, in the presence of EDTA, the amount of activity in the nuclear pellet was substantially reduced and 76% of C1GAL was present in the microsomal fraction, as expected for an ER protein, and 84% of native β-galactosidase was present in the supernatant. These crude fractionation experiments demonstrate that C1GAL expressed in COS1 cells is bound to membranes that pellet with the microsomal fraction.

To better define the membrane compartment containing C1GAL, whole cell homogenates were subjected to discontinuous sucrose gradient centrifugation to separate the membrane organelles. Activities of marker enzymes for membranes of the ER, Golgi apparatus, and plasma membranes were well separated (Fig. 3, lower panel). The distribution of β-galactosidase activity for the C1GAL fusion protein was similar to that of the ER marker, NADPH-cytochrome c reductase activity, but clearly different from patterns for Golgi
and plasma membrane markers (Fig. 3, upper panel). These results establish that the C1GAL protein was associated with the ER membrane.

To further delineate the subcellular distribution of C1GAL, we have also compared the intracellular distribution of P450 2C1 and C1GAL fusion proteins using indirect immunofluorescence. Similar to P450 2C2 (Szczech-Skorupa and Kemper, 1993), P450 2C1 expressed in COS1 cells exhibits a typical ER staining pattern around the nuclear envelope as well as a fine reticular network extending throughout the cytoplasm (Fig. 4A). A similar pattern of staining was observed in cells transfected with cDNA encoding the C1GAL fusion protein (Fig. 4B). However, some of the immunoreactive C1GAL was also found in large clumps that surrounded the nucleus of the cell. No staining was seen when an equivalent concentration of preimmune serum was used (data not shown). The nature of the clumps of β-galactosidase is not known, although they resemble aggregation of other expressed proteins in subcompartments of the ER (Vertel et al., 1989) or Russell bodies (Valetti et al., 1991). This clumping may be a mechanism for the cell to deal with abnormal or overexpressed proteins in the ER membrane. Nevertheless the underlying reticular staining pattern seen with the fusion protein is highly suggestive of an association with the ER.

To test independently whether the hybrid β-galactosidase proteins are restricted to the ER, cells were transfected with NC1GAL which contains an NH2-terminal extension with an N-glycosylation site. P450 2C1 modified with this extension is glycosylated at a single site and is sensitive to digestion by endo H (Szczech-Skorupa and Kemper, 1993). After incubation of transfected cells for 30 min with [35S]methionine, two radiolabeled species were precipitated with antibodies to β-galactosidase (Fig. 5, lane 3). Treatment with endo H resulted in the elimination of the upper band (lane 2) demonstrating that some of the NC1GAL had been N-glycosylated. The crucial observation is that even after 20 h of chase the glycosylated protein remains sensitive to digestion by endo H. Since modifications to N-glycosylated proteins in the Golgi confer resistance to endo H, these studies are consistent with ER retention of the hybrid protein.

Localization of EGFR-Alkaline Phosphatase Hybrid Proteins—Although these data strongly suggest that the first 29 amino acids of P450 may be sufficient to retain the protein in the ER, it is possible that the large size of β-galactosidase (1,023 amino acids) interferes with transport out of the ER. In addition to the large size, β-galactosidase is also known to aggregate (Fowler and Zabin, 1983). To address this problem, we constructed hybrid proteins in which the NH2-terminal regions of P450 2C1 and NC1 were fused to another reporter protein, bacterial alkaline phosphatase. This reporter protein is about the same size as P450 and has been used extensively in studies of membrane topology (Manoil and Beckwith, 1986).

To establish that alkaline phosphatase itself as a cytoplasmic domain of a membrane protein does not prevent transport out of the ER, the cytoplasmic domain of the glycosylated plasma membrane protein, human EGF-R, was replaced with alkaline phosphatase. After labeling the proteins for 30 min with [35S]methionine, EGFRphoA was immunoprecipitated with anti-phoA antibodies and appeared as
the times indicated. Cells were then detergent solubilized, and the expressed NC1GAL proteins were immunoprecipitated with anti-β-galactosidase antibodies. Immunoprecipitates were divided in half and treated with (+) or without (−) endo H before analysis by SDS-PAGE. The positions of NC1GAL and its glycosylated form as indicated at right were determined using prestained high molecular mass markers (Bio-Rad) as reference.

![Fig. 5. Endoglycosidase H sensitivity of NC1GAL synthesized in COS1 cells.](image)

**FIG. 5. Endoglycosidase H sensitivity of NC1GAL synthesized in COS1 cells.** Cells transfected with pNC1GAL were labeled with Tran35S-label for 30 min and chased in complete medium for the times indicated. Cells were then detergent solubilized, and the expressed NC1GAL proteins were immunoprecipitated with anti-β-galactosidase antibodies. Immunoprecipitates were divided in half and treated with (+) or without (−) endo H before analysis by SDS-PAGE. The positions of NC1GAL and its glycosylated form are marked and the position of C1GAL protein is shown at right as a control.

![Fig. 6. Kinetics of EGFRphoA biosynthesis in COS1 cells.](image)

**FIG. 6. Kinetics of EGFRphoA biosynthesis in COS1 cells.** COS1 cells transfected with pEGFRphoA or pEGF were processed as described in the legend to Fig. 5. The expressed proteins were immunoprecipitated with anti-PhoA antibodies for EGFRphoA or with the antibody against the extracellular domain of EGF-R. Approximate sizes of the proteins indicated at the right was determined using prestained high molecular mass markers (Bio-Rad) as reference.

Nonpermeabilized transfected cells were labeled with antibodies against the extracellular domain of EGF-R and then with fluorescein-conjugated goat anti-mouse IgG. A and C are phase contrast micrographs and B and D are fluorescence micrographs of the same fields, respectively.

![Fig. 7. Immunofluorescence localization of EGFRphoA.](image)

**FIG. 7. Immunofluorescence localization of EGFRphoA.** Nonpermeabilized transfected cells were labeled with antibodies against the extracellular domain of EGF-R and then with fluorescein-conjugated goat anti-mouse IgG. A and C are phase contrast micrographs and B and D are fluorescence micrographs of the same fields, respectively.

![Fig. 8. Subcellular fractionation of EGFRphoA and C1phoA.](image)

**FIG. 8. Subcellular fractionation of EGFRphoA and C1phoA.** Cells transfected with pEGFRphoA or pC1phoA were labeled for 1 h and subsequently chased for 5 h. Cellular homogenates were centrifuged through a discontinuous sucrose gradient, and 12 fractions were collected from the bottom of the tube as described in the legend to Fig. 3. Each fraction was immunoprecipitated and analyzed by SDS-PAGE.

EGFRphoA proteins, thus, are transported to the plasma membrane.

The transport of EGFRphoA to the plasma membrane was further confirmed by subcellular fractionation. Subcellular fractionation of cells labeled with [35S]methionine for 30 min followed by a 2-h chase revealed that a substantial amount of EGFRphoA was still present in the ER, some was in Golgi, and little was in the plasma membrane fractions (not shown). After a longer chase time of 5 h, a fraction of the EGFRphoA could be recovered in the plasma membrane fractions (Fig. 8A, lanes 11 and 12), suggesting a relatively slow transport out of the ER which is consistent with the 5 h required to develop endo H resistance. These results show that alkaline phosphatase as a cytoplasmic domain of a membrane protein does not prevent transport from the ER to the plasma membrane and is a useful reporter for cellular localization studies of P450.

**Localization of P450-Alkaline Phosphatase Hybrid Proteins**—We first examined whether fusion of phoA to the NH2 terminus of NC1 changes the topological properties of the NH2-terminal signal sequence by analysis in a cell-free translation system. Translation of the hybrid mRNA produced a major radioactive protein with the expected size (Fig. 9A, lane 2) and, in the presence of microsomal membranes, additional...
slower migrating bands were observed (lane 3). Treatment with endo H eliminated the more abundant slower migrating band, indicating that the NH2-terminal extension of NC1 had been modified by N-glycosylation (not shown). The NC1phoA was completely digested with proteinase K, indicating that the phoA domain was on the cytoplasmic side of the membrane as expected (lanes 4 and 5). In contrast, glycosylated forms of the secreted protein, prepro-α-factor, which have been translocated to the ER lumen, were protected from protease treatment unless the membranes were destroyed with detergent (lanes 8 and 9).

To further establish that the hybrid proteins were integral membrane proteins, microsomes containing in vitro translated products were isolated and extracted with 0.1 M Na2CO3. As controls, most of P450 2C1, a membrane protein, was not extracted from the membrane pellet by the alkaline buffer (Fig. 9B, lane 3), whereas the glycosylated prepro-α-factor, translocated into the ER lumen, was extracted into the supernatant (lane 6). Over 50% of NC1phoA was found in the pellet fraction (lane 1), which indicates the fusion protein is membrane-bound. The phoA may reduce the affinity of binding to the membranes since a higher amount of the hybrid protein, including the glycosylated form, was extracted compared with P450 2C1. These in vitro results demonstrate that the NH2-terminal region of P450 2C1 in the hybrid protein continues to function as a combination insertion, halt-transfer, and anchor sequence as it does in native P450 (Sakaguchi et al., 1987; Szczesna-Skorupa et al., 1988).

In transfected COS1 cells expressing C1phoA, we first examined the subcellular localization of fusion proteins by gradient fractionation. After a chase of 5 h, most of the C1phoA was present in the ER containing fractions and very little of the C1phoA was present in the plasma membrane fractions (Fig. 8B) in contrast to the results with EGFR-phoA (Fig. 8A). These results indicate that the NH2-terminal P450 sequence is sufficient to retain alkaline phosphatase in the ER.

The localization of alkaline phosphatase fusion proteins in the ER was confirmed by immunofluorescent staining of permeabilized cells. Cells transfected with pC1PHOA yielded a reticular pattern of staining similar to that seen with the cells expressing P450 2C1 (Fig. 4, A and C). As a control, the immunofluorescent patterns of transfected cells expressing phoA without its signal sequence were visualized. With this “soluble” phoA, uniform staining throughout the cells was observed (Fig. 4D), as expected for a cytoplasmic protein. For reasons that are not clear, the glycosylation of NC1phoA was much less efficient than that of NC1 (Szczesna-Skorupa and Kemper, 1993) or NC1GAL in COS1 cells or NC1phoA in the in vitro translation system. We also consistently observed two strong bands, even after a short pulse. Both proteins were insensitive to endo H digestion, regardless of the chase time, so it is not likely that there was a product-precursor relationship between them. The lower band probably corresponds to a product resulting from downstream initiation at Met60, the natural initiator of P450 2C1, as a start codon. Initiation at this site was observed for NC1 (Szczesna-Skorupa and Kemper, 1993). Nevertheless, sufficient glycosylation could be detected to permit analysis of endo H sensitivity. The glycosylated fusion protein remained endo H-sensitive throughout the labeling and chase periods up to 9 h (Fig. 10). All of these experiments with the phoA reporter are consistent with those using the β-galactosidase reporter and indicate that the NH2-terminal 29 amino acids of P450 are sufficient to retain proteins in the ER.

**DISCUSSION**

The mechanism by which proteins with large cytoplasmic domains and little or no luminal sequence are retained in the ER is not known. Misfolded or unassembled multisubunit proteins may be retained in the lumen by interaction with a

**FIG. 9.** A protease sensitivity of NC1PhoA proteins in the in vitro system. The mRNAs for NC1PhoA and prepro-α-factor were translated in the wheat germ cell-free system in the absence or presence of microsomal membranes. Aliquots of the translation mixture were subsequently digested with proteasine K (0.3 mg/ml) with or without the addition of Triton X-100. The same equivalent of reaction mixtures were loaded on an SDS-polyacrylamide gel. The positions of the prepro-α-factor band, indicating that the NH2-terminal extension of NC1 had been modified by N-glycosylation (not shown). The NC1phoA and its glycosylated form are indicated on the left and the positions of prepro-α-factor (PRECURSOR) and its proteolytically cleaved and glycosylated form (PROCESSED) are shown on the right. B, insertion of fusion proteins synthesized in vitro into microsomal membranes. The mRNAs of NC1phoA, P4502C1, and prepro-α-factor were translated in a wheat germ cell-free system in the presence of rough microsomes. After translation was completed, the mixtures were treated with Na2CO3, pH 11.5, and then separated into the membrane (P) and supernatant (S) fractions by centrifugation. Equivalent amounts of reaction mixture in the supernatant and pellet fractions were analyzed by SDS-PAGE, followed by fluorography. PROT, protein; MEMB, membrane; TX-100, Triton X-100.

**FIG. 10.** Endoglycosidase H sensitivity of NC1phoA synthesized in COS1 cells. COS1 cells were transfected with pNC1PHOA and processed as described in the legend to Fig. 5 legend except that polyclonal anti-PhoA antibodies were used for immunoprecipitation.
chaperon such as BiP (Bole et al., 1986; Gething et al., 1986). P450 has the COOH-terminal domain on the cytoplasmic side of the membrane, leaving essentially no luminal sequence to interact with BiP, and no sequences comparable with the retention signals known in other ER proteins. Therefore, P450 must be retained in the ER by a different mechanism. One possibility that has been proposed for other ER resident proteins (Andersson et al., 1985; Burgert and Kvist, 1985; and Ivesa et al., 1992) is that their retention in the ER may result from assembly into a network in the ER membrane which prevents access to the transport vesicles. It is possible that P450 and the two reporter proteins used in this study, β-galactosidase and alkaline phosphatase, attached to the P450 2C1 NH2 terminus could also form cytoplasmic networks that would cause retention in the ER. β-Galactosidase, in particular, is a large protein that has been shown to form aggregates (Fowler and Zabin, 1983) and the punctate patterns of immunofluorescence staining for some of the C1GAL suggest an abnormal ER distribution and aggregation. However, alkaline phosphatase as a cytoplasmic domain of a membrane protein does not inherently inhibit movement out of the ER as shown by transport of the EGFR-alkaline phosphatase chimera to the plasma membrane. Retention in the ER when alkaline phosphatase is substituted for the large cytoplasmic domain of P450 demonstrates that specific interactions mediated by the catalytic domain of P450 with other P450s, with P450 reductase, or other ER proteins, which potentially could form a large network, are not required for P450 ER retention. Consistent with this conclusion are studies in which the cytoplasmic domain of a microsomal P450, e17, was replaced with that of a mitochondrial P450, e27 (Sakai et al., 1992). This hybrid protein was localized in microsomal membranes when it was expressed in yeast, indicating that retention in the ER was not dependent on specific signals in the microsomal P450 catalytic domain. The data, therefore, strongly suggest that the NH2-terminal region of P450 2C1 is sufficient for retention of the protein in the ER.

This result contrasts with studies on P450s with the NH2-terminal region deleted which showed that a fraction of the protein remains membrane bound when expressed in yeast, E. coli, or COS1 cells (Yabusaki et al., 1988; Larson et al., 1991; and Clark and Waterman, 1991), suggesting that the NH2-terminal is not critical for ER insertion and retention. Although such bacterially expressed P450 is active when reconstituted, the bacterial and COS1 membrane forms are not active. The mechanism of membrane insertion of these proteins is, therefore, presumably different from the physiological mechanism. This idea is reinforced by the observation that substitution of heterogeneous NH2-terminal regions, even from non-P450 ER proteins, restores activity to a P450 with its NH2-terminal region deleted (Clark and Waterman, 1992).

An alternative hypothesis to a NH2-terminal retention signal in P450 is the default pathway for membrane proteins with large cytoplasmic domains is retention in the ER. The mechanism of transfer of proteins from the ER to the Golgi is thought to be mediated by small transport vesicles (Rothman and Orci, 1992) which are formed by invagination of the membrane into the cytoplasm and then fission of the membrane to form a vesicle. In this process, the invaginations become “coated” with a protein complex, termed a coatomer, on the cytoplasmic side which may be essential for the formation of the vesicle (Orci et al., 1993). It seems reasonable that the membrane proteins which contain large cytoplasmic domains might interfere with coatomer formation on the membrane or be excluded by the coatomers so that they cannot be transported. Type 1 membrane proteins with short cytoplasmic tails interact with tubulin in vitro (Dahlhoff et al., 1991) and that interaction of these proteins with microtubules and other cytoplasmic proteins might provide them with the equivalent of a large cytoplasmic domain that precludes transport. On the other hand, there are examples of proteins with large cytoplasmic domains that are transported out of the ER. One example is EGF-R which has a 542-amino acid cytoplasmic domain (Yarden and Ullrich, 1988) similar in size to that of P450. In these cases, either the cytoplasmic domain would have to be compatible with coatomer formation or a positive signal for transport in the luminal or transmembrane domain would have to be present. There is, however, no precedent for such a positive transport signal. In contrast, several studies have demonstrated that retention of membrane proteins in the Golgi complex is dependent on sequences in the transmembrane domain (Nilsson et al., 1991; Swift and Machamer, 1991; Aoki et al., 1992; Russo et al., 1992; and Teasdale et al., 1992) and the charged residues flanking the transmembrane domain (Munro, 1991; Nilsson et al., 1991; Colley et al., 1992; and Burke et al., 1992). These last studies suggest that the transmembrane domain of P450 is that the default pathway for membrane proteins toward the plasma membrane. Retention in the ER when alkaline phosphatase and BiP (Bole et al., 1986) are not required for P450 ER retention.

**Acknowledgments**—We thank Dr. Florian Rücker for T773-19UphoA, Dr. R. J. Davis for the human EGF-R cDNA, Dr. R. B. Gennis for anti-phoA antibodies and Dr. E. F. Johnson for P450 2C3 antiserum. We are also grateful to Debbie Bieler for excellent technical help and to Dr. G. Lepar and T. M. Pazdera for critical reading of the manuscript.

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ER Retention of P450 by the NH2-terminal Signal Sequence