Biogenesis and Transmembrane Topology of the CHIP28 Water Channel at the Endoplasmic Reticulum

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Abstract. CHIP28 is a 28-kD hydrophobic integral membrane protein that functions as a water channel in erythrocytes and renal tubule epithelial cell membranes. We examined the transmembrane topology of CHIP28 in the ER by engineering a reporter of translocation (derived from bovine prolactin) into nine sequential sites in the CHIP28 coding region. The resulting chimeras were expressed in Xenopus oocytes, and the topology of the reporter with respect to the ER membrane was determined by protease sensitivity. We found that although hydropathy analysis predicted up to seven potential transmembrane regions, CHIP28 spanned the membrane only four times. Two putative transmembrane helices, residues 52-68 and 143-157, reside on the lumenal and cytosolic surfaces of the ER membrane, respectively. Topology derived from these chimeric proteins was supported by cell-free translation of five truncated CHIP28 cDNAs, by N-linked glycosylation at an engineered consensus site in native CHIP28 (residue His69), and by epitope tagging of the CHIP28 amino terminus. Defined protein chimeras were used to identify internal sequences that direct events of CHIP28 topogenesis. A signal sequence located within the first 52 residues initiated nascent chain translocation into the ER lumen. A stop transfer sequence located in the hydrophobic region from residues 90-120 terminated ongoing translocation. A second internal signal sequence, residues 155-186, reinitiated translocation of a COOH-terminal domain (residues 186-210) into the ER lumen. Integration of the nascent chain into the ER membrane occurred after synthesis of 107 residues and required the presence of two membrane-spanning regions. From this data, we propose a structural model for CHIP28 at the ER membrane in which four membrane-spanning α-helices form a central aqueous channel through the lipid bilayer and create a pathway for water transport.
types of experiments indicate that the functional unit size of CHIP28 is the monomer: CHIP28 has a target size of 30 kD by radiation inactivation (Van Hoek et al., 1991), and individual CHIP28 subunits in heterodimeric constructs function independently (Shi et al., 1994). Hydrodynamic calculations suggest a CHIP28 aqueous pore size of ~0.2 nm (Zhang et al., 1993a), consistent with the passage of water and exclusion of small solutes. Analysis of CHIP28 secondary structure by circular dichroism and Fourier transform infrared spectroscopy suggests ~45% α-helical content (Van Hoek et al., 1993), consistent with the presence of multiple membrane-spanning regions. Site-directed mutagenesis and inhibitor binding studies indicate that cysteine 189 resides at the external membrane surface and is the site of mercurial inactivation (Van Hoek et al., 1993). Analysis of CHIP28 intrinsic fluorescence indicates that the four tryptophan residues are located in a nonpolar environment within the confines of the bilayer (Farinas et al., 1993). Sequences alignment of CHIP28 with MIP26 family members show several conserved sequences, including two NPA boxes, which may be important for channel function.

Hydropathy analysis of the primary sequence of CHIP28 indicates up to seven potential membrane-spanning domains (Preston and Agre, 1991). Digestion of intact erythrocytes and inside-out erythrocyte-derived vesicles with carboxypeptidase demonstrated that the COOH terminus was cytoplasmic (Smith and Agre, 1991). Primary sequence analysis initially suggested that the NH2 terminus also resided in the cytosol (Preston and Agre, 1991). This cytosolic NH2-terminus topology is supported by recent functional studies using chimeric homo- and heterodimers of CHIP28 arranged in tandem repeats (Shi et al., 1994). Thus, CHIP28 would appear to contain an even number of membrane-spanning regions (0, 2, 4, or 6). Analysis of full-length and a truncated 146-residue amino terminus fragment of CHIP28 in a cell-free expression system indicated that residue Asn 42, located between the first two hydrophobic regions, is the major site of N-linked glycosylation (Zhang et al., 1993a). Therefore, the first hydrophobic region, TM1, likely spans the membrane with its COOH flanking sequences translocated into the lumen of the ER. The topologic orientation of the remaining hydrophobic regions of CHIP28 is unknown. There have been no reports of transmembrane topology for any MIP family proteins.

The purpose of this study was to determine the transmembrane topology and mechanism of biogenesis of the human CHIP28 water channel at the ER. Our approach was, in part, analogous to that pioneered by Beckwith (reviewed in Boyd et al., 1990) for the study of prokaryotic polytopic proteins. This technique uses fusion proteins in which a reporter of translocation is engineered at sequential sites in the coding region of a polytopic protein. Topology of the reporter domain thus reflects topology of the nascent chain at the site of fusion. In the current study, we have developed an analogous technique applicable to eukaryotic polytopic proteins. The reporter domain (P), derived from 142 COOH terminus residues of the secretory protein prolactin (described in Rothman et al., 1988), was fused at nine sequential sites in the CHIP28 coding region to generate CHIP28-P chimeras. These constructs were expressed in cell-free rabbit reticulocyte lysate (RRL) or whole cell Xenopus laevis oocyte expression systems, and topology of the reporter was determined by protection from exogenously added protease. Topology of CHIP28 derived from this translocation-reporter technique was supported by four additional approaches: proteolysis of truncated native CHIP28 protein, characterization of internal sequences (e.g., signal and stop transfer sequences) that direct translocation events in CHIP28 bio genesis, N-linked glycosylation at an engineered glycosylation consensus site (His 69), and localization of the NH2 terminus with a 10-residue epitope flag derived from the protein c-myec.

The principal finding was that most chains of CHIP28 span the membrane four times instead of six, as proposed previously. Two hydrophobic regions initially predicted to span the membrane, residues 52–68 and residues 143–157, were located on the luminal and cytosolic surfaces of the ER membrane, respectively. Two internal signal sequences in CHIP28 were identified, which direct nascent chain translocation across the ER membrane. The new model for the predominant CHIP28 topology is consistent with the experimental observations described above and may provide insight into the mechanisms of water transport through CHIP28.

Materials and Methods
cDNA Constructs

To isolate the full-length coding sequence of CHIP28, cDNA was isolated by guanidinium/phenol extraction of RNA from human kidney followed by reverse transcription using an oligo dT primer (Invitrogen cDNA cycle kit; Invitrogen, San Diego, CA) as previously described (Zhang et al., 1993a). CHIP28 cDNA was amplified by the PCR using sense GCCACCATGGC-

CGCGAGTTCAAGGAAGAG and antisense GCCGGATCTCCCTTTAT-

TTGGGTTCACTCTC oligonucleotides encoding Ncol and BamHI restriction sites, respectively. The fragment generated by PCR was digested with Ncol and BamHI and ligated into a modified SP64 vector previously described to give plasmid pSP64.Chip28 (Skach et al., 1993). The CHIP28 open reading frame was sequenced to insure that no errors were introduced during PCR amplification. pSP64.Chip28 was used as a template for PCR amplifications below. Clones 1–9 were generated using a sense oligonucleotide (AGGATCCTGCTAGACACTGAC) corresponding to regions of the plasmid pSP64 (Promega Corp., Madison, WI) and a series of nine antisense oligonucleotides to regions of the CHIP28 coding sequence, which introduce BstEII restriction sites at codons 15, 52, 77, 93, 107, 139, 169, 214, 264 (antisense oligonucleotides: 1, GAAACTGCGTGAC-

CTGCTCCCTCCAG; 2, GCCGGGTTCACTCTCAGTTGCT; 3,

CAATGTTGTCATCGGGTTGAGTG; 4, GTACAGTGTGACCCG-

GAAGCTCGTATCT; 5, GCGGTTGTCATCGGCCCCGACG; 6,

GAGTTGTCATCGGCCCCGCC; 7, CCAGCGTATCGG-

GCCTGAGCCACCAAG; 8, GAATGGGTTCCACCAGAATTACGATG; 9, CTTCATGTGTCACCTCGAGTTGCT). After 30 cycles of PCR, fragments were digested with Ncol and BstEII, gel purified, and ligated into the Ncol/BstEII-digested pSP64.Chip28 (Skach et al., 1993). The chimeric clones thus encode a translocation initiation Met at codon 1 and varying lengths of CHIP28 followed by the P reporter.

Clones S.g.HR2-P, S.g.HR2-3-P, and S.g.HR2-4.P encode an NH2-terminal signal sequence derived from preprolactin (S), a chimpanzee alpha globin domain containing an engineered N-linked glycosylation site (gG), an internal fragment of CHIP28 (HRX), and the P reporter as described (Rothman et al., 1988). CHIP28 fragments were generated by the PCR using a sense oligonucleotide (CCTGCGGGTGACCTCCCCGCCA-

GGCGAACACCCAG) to CHIP28 and antisense oligonucleotide 3, 4, or 6. After 10 cycles of PCR using CHIP28 as template, amplified fragments were digested with BstEII and gel purified. These fragments were ligated into the vector S.g.TM3.P, from which the TM3 sequence was removed (modified from Rothman et al., 1988; Skach and Lingappa, 1994) at the BstEII site between the gG and P domains. The resulting clones contain an S sequence derived from prolactin and the CHIP28-derived fragment in.
served between the globin (gG) and prolactin (P) reporters. Clones CH5.P, CH6.P, and CH5-6.P were constructed using an NcoI/BstEII digested, gel-purified PCR fragment ligated into the NcoI/BstEII digested, phosphatase-treated S.A.L.G.P vector. For construction of CH5.P and CH5-6.P, the sense oligonucleotide GAGATCCATGGCCCGAGATACCTGCT, and anti-sense oligonucleotides were 7 and 8, respectively. For CH6.P, sense oligonucleotide TATGCGCTATGGTATCAGGCGGAGG and anti-sense oligonucleotide TACGTTTGGATTACCAAGACACTCGCGC contains three changes C=A, C=T, and C=T at basepairs 205, 207, and 210 in the CHIP28 coding region, respectively. These result in a single codon change, His=Asn and an adjacent silent mutation creating an SpI restriction site used to screen mutant clones. Mutagenesis was performed as reported previously (Zhang et al., 1993b), and the mutated sequence was confirmed by DNA sequencing.

The clone myc2CH4 was generated using a DNA linker containing CAGT 5' overhangs (sense strand) and CACCATGGAAAGCCACACTCGTG (antisense strand) containing a cloning site in the putative functional sperm domain of CHIP28. The linker was ligated into clone 4 after digestion with NcoI and calf intestinal phosphatase.

**RNA Transcription**

mRNA was transcribed in vitro with SP6 RNA polymerase (New England Biolabs Inc. Beverly, MA) using 2 μg of plasmid DNA in a 10-μl volume at 40°C for 1 h as previously described (Rothman et al., 1988). Aliquots were used immediately or frozen in liquid nitrogen and stored at -70°C.

**Xenopus laevis Oocyte Expression**

C57BL/6J females were injected with 100 ng of cRNA per oocyte into the micropipette and injected into stage VI (50 nl/oocyte). After incubation at 18°C for 36 h, the oocytes were isolated and processed for Western analysis and immunoprecipitation. Oocytes were extracted with Buffer A and twice with 0.1 M NaCl, 0.1 M Tris, pH 8.0. Samples were analyzed by SDS-PAGE, ENHANCE (New England Nuclear) fluorography, and autoradiography. Autoradiograms were digitized with an AGFA focus color plus scanner (Adobe Photoshop software) and band intensities were quantified by measuring mean density minus background multiplied by band area.

**Results**

Polytopic integral membrane proteins in eukaryotes that are destined for the cell surface assemble into the ER membrane as the polypeptide chain emerges from the ribosome at the rough ER (Goldman and Blobel, 1981; Braess and Lodish, 1982; Brown and Simoni, 1984; Wessels and Spiess, 1988; reviewed in Skach and Lingappa, 1993). Multiple translocation events required to establish a polytopic topology are directed by internal sequences within the chain. These sequences serve to translocate peptide regions into the ER lumen and/or to integrate the chain into the lipid bilayer (Blobel, 1980; Friedlander and Blobel, 1985; Audigier et al., 1987; Rothman et al., 1988; Wessels and Spiess, 1988; Lipp et al., 1989; Skach et al., 1993). Hydroxylation analysis (Kyte and Doolittle, 1982) was used to identify sequences in CHIP28 that were believed to form hydrophobic membrane-spanning α helices and participate in directing CHIP28 biosynthesis. Seven hydrophobic regions (HRs) are identified in Fig. 1A. However, HR2 and HR5 contain fewer than the 20 contiguous hydrophobic residues that are believed to be required to span a 4-nm wide lipid bilayer in a standard α helical conformation.

A proposed topological model for CHIP28 predicts the presence of six transmembrane helices (Fig. 1B) (Preston and Agre, 1991). Based on this model, we constructed a series of cDNA clones (clones 1–9) containing a COOH terminus reporter of translocation fused to specific sites within the CHIP28 coding region (Fig. 1C). This P reporter encodes a COOH terminus fragment of the secretory protein bovine prolactin that has been shown previously to respond faithfully to upstream signal and stop transfer sequences in a variety of contexts (Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993).

Transmembrane topology of the P domain was determined by its accessibility to PK. This assay is based on evidence that protein domains residing in membrane bound compartments (e.g., the ER lumen) are protected from digestion with exogenously added protease (Perara and Lingappa, 1985). To confirm that protection from PK digestion represented translocation of the reporter into the ER lumen, proteolysis was also performed in the presence of non-denaturing detergents. Under these conditions, membrane integrity is dis-
CHIP28 Spans the Membrane Four Times: Translation of CHIP28 P Chimeras

mRNA from clones 1-9 was transcribed in vitro and transcription products were injected with [35S]methionine into stage VI XO. After 2 h, oocytes were homogenized, digested with PK, and immunoprecipitated with antiprolactin antisera for analysis by SDS-PAGE and autoradiography. Transcripts were also translated in the RRL cell-free translation system in the presence or absence of canine pancreas microsomal membranes, which reconstitutes nascent chain processing at the ER membrane; e.g., protein translocation, N-linked core glycosylation, and signal peptide cleavage.

In Fig. 2, clones derived from the amino terminus half of CHIP28 (clones 1-5) were expressed in vitro and in vivo. Cell-free translation products (labeled RRL) are shown to the left of the corresponding XO immunoprecipitates. Translation of clone 1, containing the first 15 hydrophilic residues of CHIP28 fused to the P reporter (see Fig. 1), yielded a peptide of expected size (17 kD) in both RRL (lanes 1-2) and XO (lanes 3-5). Digestion of oocyte homogenate with PK degraded most P epitope reactivity (lane 4), demonstrating that the P reporter remained in the cytosol. The first 15 residues of CHIP28 therefore lack the signal sequence activity required for chain translocation. In clone 2, the P reporter was engineered 17 residues downstream from HR1 at residue 52 in the CHIP28 coding region. Translation in RRL generated a 25-kD peptide (lane 6) that, in the presence of microsomal membranes, generated a shorter fragment of 22 kD, indicating that the nascent chain underwent a cleavage event (lane 7, upward arrow). Peptides generated in XO comigrated identically with this cleaved fragment, confirming that chain cleavage also occurred in vivo. The 22-kD fragment was translocated into the ER lumen as demonstrated by protection of the prolactin epitope from PK digestion in the absence of detergent (lane 9, upward arrow). Thus, the first 52 residues of CHIP28 contain a signal sequence that directs nascent chain translocation into the ER lumen.

The translocation-associated cleavage of clone 2 likely resulted from unmasking a cryptic cleavage site for signal peptidase near HR1, converting this normally uncleaved CHIP28 signal sequence into a cleaved signal sequence. Unmasking of cryptic signal peptidase cleavage sites has been reported for secretory and bitopic proteins when upstream and/or downstream residues flanking the cleavage site were altered (Lipp and Dobberstein, 1986; Nothwehr and Gordon, 1989, 1990; Nothwehr et al., 1989; Schmid and Spiess, 1988). We observed similar findings when the P reporter was engineered downstream of TM1 and TM3 human P-glycoprotein (Skach and Lingappa, 1994). Flanking residues of internal signal sequences from diverse proteins therefore appear to play an important role in preventing or allowing access of signal peptidase to potential cleavage sites. While the cleavage observed for clone 2 differed from that for native...
it does not alter the key conclusion of these experiments that a signal sequence located within the first 52 residues of CHIP28 directs nascent chain translocation into the ER lumen.

If CHIP28 inserts sequentially and cotranslationally into the ER membrane, as has been shown for other eukaryotic polytopic proteins (Braell and Lodish, 1982; Brown and Simoni, 1984; Wessels and Spiess, 1988), then a downstream sequence (e.g., HR2, HR3, or HR4) should terminate ongoing chain translocation, span the membrane, and re-establish the P domain in a cytosolic orientation. When the P reporter was engineered nine residues downstream of HR2 (clone 3) the P reporter was again translocated into the ER lumen. 77% of P-reactive chains were protected from PK digestion in the absence of detergent (Fig. 2, lanes 13-15, upward arrows). Based on the size of the protected fragment, HR2 appeared to translocate into the ER lumen rather than span the membrane. Signal cleavage was also observed for clone 3 because the in vivo products (lane 13) comigrated with the cleaved 27-kD fragment generated in RRL in the presence of microsomes (lane 12).

Similar experiments were performed with clones 4 and 5 to determine at what position chain translocation terminated during CHIP28 biogenesis. When the P domain followed HR3 (clone 4), it was again translocated into the ER lumen as demonstrated by protease protection of P-reactive chains (lanes 18-20, upward arrows). However, when the P reporter was placed 14 residues beyond the start of HR4 (clone 5), it became accessible to PK digestion (lane 24). Therefore, the P reporter in clone 5 was oriented in the cytosol. Since upstream residues were in the ER lumen, a second membrane-spanning region must exist between residues 80-107. In clones 4 and 5, there was no shift in size of the peptide chain upon addition of microsomal membranes (lanes 16 vs. 17 and 21 vs. 22). The presence of additional downstream residues from CHIP28 in clones 4 and 5 therefore prevented the translocation-associated cleavage event at HR 1 observed with clones 2 and 3. However, an additional fragment of ~16-17 kD was observed for clone 4 that was reactive with antiprolactin antisera. This fragment was translocated into the ER lumen (e.g., protease protected) and therefore probably represents less efficient cleavage of the reporter during translocation at a site significantly downstream of HRI. Glycosylation at Asn42, the sole consensus site for N-linked glycosylation in these clones, generated additional protein species in vitro (lanes 17 and 22, downward arrows) and in vivo (lanes 18 and 23). Differences in efficiency of glycosylation of clone 4 in RRL and XO probably reflect functional differences in oligosaccharyltransferase activity in these systems.

The transmembrane topology of CHIP28 predicted by these data is shown schematically beneath the autoradiograms. We conclude that for most chains the amino terminus of CHIP28 through residue 107 spans the ER membrane twice rather than three times as originally proposed. This topology is directed by signal sequence activity encoded within the first 52 residues that encompasses the first HR. A second sequence that likely includes portions of the third and fourth HRs terminates chain translocation and forms the second membrane-spanning region. Thus, the second and possibly part of the third HRs do not span the bilayer, but are instead located at the ER luminal side of the membrane.

The topology of the COOH terminal half of CHIP28 was determined next using clones 6-9. Fig. 3 shows the results obtained by expressing clones 6-9 in XO and assaying topology of the P reporter by PK sensitivity. Only the XO data are presented because no amino terminus cleavage events were observed for any of these clones in RRL (data not shown). Translation of clones 6-9 generated both glycosylated and nonglycosylated chains, as demonstrated by multiple bands present in the autoradiograms; e.g., lane 1. Clones 6 and 7, in which the P reporter follows the fourth and fifth HRs, respectively, generated chains with the P reporter accessible to PK digestion and, hence, oriented in the cytosol. Topology of these chains is illustrated beneath the

Figure 2. Autoradiogram of translation products generated in RRL (lanes 1 and 2, 6 and 7, 11 and 12, 16 and 17, 21 and 22) or XO (lanes 3-5, 8-10, 13-15, 18-20, 23-25) from clones 1-5. Canine pancreas microsomes were added to cell-free translation mixtures as indicated (lanes 2, 7, 12, and 17, and 22). XO were immunoprecipitated with antiprolactin antisera without PK digestion (lanes 3, 8, 13, 18, and 23), with PK digestion (lanes 4, 9, 14, 19 and 24) or with PK in the presence of detergent (lanes 5, 10, 15, 20, and 15) (see Materials and Methods). Single upward arrows indicate cleaved chains generated in RRL with addition of microsomes. Double upward arrows indicate prolactin reactive chains protected from PK digestion in the absence, but not in the presence of detergent. Downward arrows show glycosylated chains. The percent of prolactin reactivity protected from digestion was quantified and is provided beneath the autoradiogram. Schematic representation of chain topology for each clone based on proteolysis results is also shown at the bottom. Diagrams do not imply association of the chain with membrane lipids.
When the P reporter was at the extreme COOH end of CHIP28 (clone 9), it was also digested by PK (lane 11) and, therefore, located in the cytosol. Only clone 8, in which the P reporter followed HR6, demonstrated protease protection and translocation of the reporter into the ER lumen. After PK digestion of clone 8, several glycosylated, full-length chains were observed, as well as a minor 30-kD band (lane 8, upward arrow). This smaller fragment was believed to represent a prolactin-reactive cleavage product generated by partial PK digestion of full-length chains at accessible cytosolic residues. A doubly glycosylated band (lane 8, downward arrow) resulted from glycosylation at both potential N-linked consensus sites, Asn42 and Asn205. The presence of the P reporter in clone 8 therefore increased accessibility of Asn205 to oligosaccharyltransferase because Asn205 was not glycosylated in native CHIP28 (Zhang et al., 1993a; Van Hock, A. N., and A. S. Verkman, unpublished observations). Since N-linked carbohydrate addition is restricted to the ER lumen, glycosylation of Asn205 provides independent evidence for translocation of this domain along with the P reporter into the ER lumen.

The topology determined for each of these chimeras is shown schematically beneath the autoradiograms. The second and fifth HRs are shown outside the membrane because the data indicated that they did not span the lipid bilayer. These experiments did not distinguish whether these segments interact with the lipid bilayer (e.g., perhaps as a β strand), or if they interact with corresponding regions of other monomers in the CHIP28 tetramer.

**The Peptide Region between HR1 and HR4 is Translocated into the ER Lumen: Cell-free Translocation of Truncated CHIP28**

The topological model for CHIP28 derived from studies using the P reporter in XO contains four membrane-spanning regions with two peptide loops translocated into the ER lumen. This topological model predicts a translocated peptide region (residues 15–115) that should be glycosylated and protected from protease digestion in clones 5–9. Such a fragment was not observed in Figs. 1 and 2 because these oocyte translation products were immunoprecipitated with antiprolactin antisera.

To test the prediction that residues 15–115 are translocated into the ER lumen, clones 5–9 were truncated at BstEII (P reporter fusion site) and translated in the RRL translation system in the presence or absence of microsomal membranes. Nascent chains were released from the ribosome with puromycin, and total translation products were analyzed before and after digestion with PK (Fig. 4). Nontruncated clones 5–9 containing the P reporter were tested in parallel and gave similar results (data not shown). In all cases, PK digestion of these chains generated a protease-protected 16-kD fragment. Glycosylation of this fragment was confirmed by translation and proteolysis in the presence of a tripeptide competitive inhibitor of oligosaccharyltransferase (Ac-Asn-Tyr-Thr). This 16-kD protected fragment was first observed in clone 5 (lane 3, downward arrow), where nearly the entire chain was predicted to be translocated across the ER membrane (diagrammed beneath the autoradiogram). Glycosylated chains from this clone (lane 2, upward arrow) were therefore inaccessible to PK digestion.

Glycosylated chains from truncated clones 6–9 (Fig. 4, lanes 6, 10, 14, and 18, upward arrows) also generated the 16-kD fragment (lanes 7, 11, 15, and 19, downward arrows) as a result of PK digestion at the cytosolically exposed region following HR4 (indicated beneath the autoradiogram). Only two methionine residues are present in these chains (Met 1 and Met 96). Therefore, the 16-kD protease-protected fragment must have originated from the same amino terminus region in all clones. The translocated COOH terminus peptide loop predicted to be translocated in clone 9 (residues 162–229) was not visualized because it lacked methionine residues.

These experiments revealed several additional features of CHIP28 topology. Chains generated from truncated clone 8 (lane 13) underwent glycosylation at one (lane 14, upward arrow) or two (lane 14, horizontal arrow) sites, Asn 42 and Asn205. Thus removal of HR7 increased accessibility of oligosaccharyltransferase at Asn205 as was observed in XO. Because the majority of chains were cleaved by PK (generat-
previous observations that the P reporter faithfully follows topological information encoded in the nascent chain and, therefore, accurately reflects events of CHIP28 biogenesis.

These results support the topology derived from in vivo expression of P reporter chimeras in XO. Visualization of total translation products from the cell-free translation system allowed analysis of CHIP28 topology in the absence of the P reporter, confirming that the region from HR1 to HR4 was protected from PK digestion, and thus spanned the membrane and/or was located in the ER lumen. Because this fragment was unchanged in size for successively larger clones, topology of this region remained stable as subsequent downstream regions of CHIP28 were synthesized. In addition, this 16-kD fragment migrated identically with proteolysis products of full-length CHIP28 (data not shown) and comigrated with the previously published proteolysis fragment of full-length CHIP28 (Zhang et al., 1993a). The concordance between these results using different expression systems and CHIP28 clones, with and without the P reporter, supports previous observations that the P reporter faithfully follows topological information encoded in the nascent chain and, therefore, accurately reflects events of CHIP28 biogenesis.

HR1 Spans the Membrane with the Amino Terminus Located in the Cytosol

Analysis of clones 1–9 in XO and truncated clones 5–9 in RRL provide strong evidence that CHIP28 spans the ER membrane four rather than six times. These results, as well as other indirect studies (Shi et al., 1994), are consistent with a cytosolic location of the CHIP28 amino terminus. However, the amino terminus of chains generated from clones 4 and 5 appears to be inaccessible to PK digestion; e.g., no shift in size or loss of methionines was observed after PK digestion. This finding could result from (a) the short length of the cytosolic region (only 12–15 residues) and/or its proximity to the membrane; (b) secondary structure or tertiary folding that do not allow access to the PK active site; or (c) the alternate possibility that the amino terminus actually resides on the ER luminal surface of the membrane.

To resolve these ambiguities, we confirmed that HR1 spanned the membrane in a C-Trans (type II) topology by engineering a 10-residue epitope flag at the amino terminus of clone 4 to generate clone myc2CH4. RRL translation of this clone is shown in Fig. 5. In the presence of microsomal membranes, clone 4 and myc2CH4 underwent N-linked glycosylation (lanes 2 and 4, respectively), demonstrating that addition of the myc epitope did not abolish translocation activity of the signal sequence. Full-length chains generated from clone 4 were protected from PK digestion (lanes 5 and 6), as was observed in XO (Fig. 2). Glycosylated chains generated from clone myc2CH4 were also protected from PK digestion but underwent a shift in size from 32 kD to generate a doublet at 31–31.5 kD, demonstrating that a small region of the chain remained cytosolic and accessible to PK (lanes 8 and 9). Topology of chains from myc2CH4 was confirmed by immunoprecipitation of chains with either antiprolactin antisera against the COOH terminus (lanes 8–10) or monoclonal antibody Myc1-9E10 (Evans et al., 1985) against the c-myc-derived amino terminus flag (lanes 11–13). Full-length chains reacted against both antisera before PK digestion. However, no protected
chains were recovered after immunoprecipitation with antibody Myc-9E10, indicating the myc epitope was digested by PK treatment (compare lanes 9 and 12).

We chose to analyze topology of clone 4 because it was shortest of the chimeric clones that did not undergo cleavage by signal peptidase during translocation, and thus should exhibit a transmembrane topology. Given that the amino terminus of CHIP28 before HR1 is highly charged, it would likely pose a considerable energy barrier to locate this entire region in the lipid bilayer. These results thus confirm that HR1 (possibly with some of its flanking sequences) does indeed span the membrane. Furthermore, we noted that PK-digested chains from clone myc2CH4 comigrated with full-length protected chains from clone 4 (lanes 6 and 9, downward arrows). Therefore, despite their C-Trans transmembrane topology, PK remained unable to completely digest the extreme amino terminus and cytosolically exposed residues. Thus, the amino terminus of CHIP28 appears to be intrinsically resistant to PK digestion, supporting our interpretation for lack of digestion of this region in intact or truncated clones 4-9.

**Membrane Integration of CHIP28 Requires Two Membrane-spanning Regions Encoded within the First 107 Residues**

At pH 11.5, peripheral and secretory proteins are extracted from membranes vesicles, whereas integral membrane proteins remain associated with the lipid bilayer (Fujiki et al., 1982). To determine at what position during chain growth CHIP28 becomes fully integrated into the ER membrane, XO-expressing clones 1-9 were homogenized, and aliquots were incubated in either sodium carbonate (pH 11.5) or Tris (pH 7.0) solutions. Samples were centrifuged, and proteins recovered in the membrane pellet (P) or supernatant (S) were immunoprecipitated and identified by SDS-PAGE and autoradiography. Aliquots of homogenates from XO expressing known secretory and transmembrane control proteins (bovine prolactin and S.L/St.gG.F, respectively) were added before treatment.

When expressed in XO, clones 1-9 all generated chains that were associated with the membrane pellet at neutral pH (Fig. 6, lanes 1, 2, 3, 5, 9, 10, 13, 14, 17, 18, 21, 22, 25, 26, 29, 30, 33, and 34). Chains from clones 1-3 were extracted quantitatively from membranes at pH 11.5 (lanes 3, 4, 7, 8, II, and 12) and were recovered in the supernatant. Because these chains lack some or all of HR1, they would not be expected to integrate into the ER membrane. However, chains from clone 4, which were not cleaved during translocation and therefore contain an intact amino terminus, were also extracted (70%) from membranes at pH 11.5 (lanes 15 and 16). The presence of a single transmembrane region alone therefore failed to efficiently integrate the nascent CHIP28 chain into the membrane. This behavior contrasts with previous studies on bovine rhodopsin (Friedlander and Blobel, 1985). Addition of 14 residues from HR4 (clone 5) resulted in an abrupt change in membrane integration status. 74% of chains were integrated into the membrane. This change in membrane integration observed with clone 5 occurred when the P reporter was fused to residue 107 of CHIP28, precisely at the same site in which the P reporter was found to be in the cytosol as shown in Fig. 2, lanes 23-25. Therefore the presence of two membrane-spanning regions is required for membrane integration of CHIP28. With addition of the remaining residues from the HR4 (clone 6), chains were quantitatively resistant to extraction by sodium carbonate. Once chain integration occurred, all peptides containing additional regions of CHIP28 (clones 6-9) remained fully integrated and were resistant to extraction at pH 11.5, as expected for a bona fide integral membrane protein. The percentage of chains integrated into the membrane is shown graphically in Fig. 6 B.

**The Second and Third Hydrophobic Regions of CHIP28 Fail to Stop Translocation of a Nascent Chain**

Data from the CHIP28.P chimeras (clones 1-9) suggest that the second and part of the third HRs were translocated into the ER lumen by the signal sequence encompassing TM1. Thus, it is predicted that these hydrophobic regions should not terminate chain translocation across the ER membrane. This hypothesis was tested by determining whether HR2, HR2-3, or HR2-4 could terminate translocation of an otherwise secretory protein when engineered into a defined context. We placed regions of CHIP28 cDNA into a defined chimeric cassette, S.gG-X-P. This cassette encodes the signal sequence of prolactin (S), gG and the P domain. X represents sequences derived from CHIP28 engineered between the globin and prolactin domains. Translocation of this polypeptide is initiated by the cleaved, NH2-terminal signal sequence and continues until either the entire peptide translocates into the ER lumen or until a stop transfer sequence terminates translocation. When X comprises a stop transfer sequence, the polypeptide is an integral membrane protein in which the globin domain resides in the ER lumen and the P domain in the cytosol; e.g., type 1 or N-Trans protein topology (Rothman et al., 1988).
Three clones containing the second, second and third, or second through fourth HRs of CHIP28 engineered between the gG and P domains were constructed, S.gG.HR2.P, S.gG.HR2-3.P, and S.gG.HR2-4.P, respectively (diagrammed in Fig. 7 A). These clones were expressed in XO, and the resulting polypeptides were digested with PK before immunoprecipitation with either antiprolactin or antiglobin antisera. The data shown in Fig. 7 B demonstrate that clones containing HR2 or HR2 and HR3 failed to terminate translocation effectively (70% of chains translocated completely into the ER lumen). Full-length secretory chains remained protected from PK digestion and reactive to both globin and prolactin antisera. When HRs 2–4 were included (clone S.gG.HR2-4.P), chains were transmembrane. PK degraded nearly all full-length chains (92% degraded), yielding only a 29-kD globin-reactive fragment protected from protease (lane 14, upward arrow). Glycosylation was confirmed by translation in the presence of a competitive inhibitor (AcAsn-Tyr-Thr) of oligosaccharyltransferase (lanes 3 and 5). The amino terminus flanking region of HR2 was translo-

The Peptide Sequence after HR2 is Translocated and Undergoes N-linked Glycosylation at an Engineered Consensus Site

The previously proposed structural model of CHIP28 predicts that the peptide region between HR2 and HR3 resides in the cytosol (Fig. 1 B). In contrast, the current study demonstrates that this region is translocated into the ER lumen. To further confirm CHIP28 topology predicted by these experiments, we created a new N-linked glycosylation site immediately beyond HR2 by engineering a single residue mutation at His69 (Fig. 8). A consensus site translocated into the ER lumen may be glycosylated efficiently, poorly, or not at all, depending on its precise context (Pollack and Atkinson, 1983). Therefore, lack of glycosylation provides no information of lumenal vs cytosolic location. However, glycosylation of a fraction of chains at residue 69 would provide additional evidence in favor of the topology derived from expression of truncated CHIP28 chains and chimeric reporters.

The clone CHIP28 (H69N) was translated in the RRL translation system (Fig. 8). In the presence of microsomal membranes, wild-type CHIP28 underwent glycosylation at a single site (Asn42), as shown by a 3-kD shift in migration (lane 2, horizontal arrow). The CHIP28 mutant, H69N, showed an additional glycosylated species in the presence of microsomes, demonstrating that some chains were also glycosylated at this second site (lane 4, horizontal arrow). Glycosylation was confirmed by translation in the presence of a competitive inhibitor (AcAsn-Tyr-Thr) of oligosaccharyltransferase (lanes 3 and 5). The amino terminus flanking region of HR2 was translo-
CHIP28 Contains a Second Internal Signal Sequence that Reinitiates Nascent Chain Translocation

As shown in Figs. 3 and 4, the peptide sequence located between the HR6 and HR7 of CHIP28 (residues 186–210) resides in the ER lumen. If CHIP28 assembles into the membrane cotranslationally, a signal sequence should be located upstream of residue 186, which reinitiates this translocation event. To identify such a signal sequence, we constructed three clones in which regions of CHIP28 cDNA were isolated and engineered at an amino terminus location, upstream of the P reporter. These clones containing residues 125–169, 155–214, and 125–214 were designated HR5.P, HR6.P, and HR5-6.P, respectively, and they are diagrammed in Fig. 9A.

These clones were expressed in the RRL system in the presence and absence of microsomal membranes. As shown in Fig. 9B, chains generated by HR5.P remained cytosolic and PK sensitive. Thus HR5 lacks signal sequence activity required to translocate a carboxy terminus reporter. In contrast, chains generated by clone HR6.P were both glycosylated (lanes 5 and 6, downward arrow) and protease protected (lane 7). HR6, therefore, encodes an internal signal sequence capable of initiating nascent chain translocation into the ER lumen. Similarly, chains from clone HR5-6.P (lanes 9–12) were translocated into the ER lumen based on glycosylation and protease protection. Translocation of the P domain in this heterologous context confirms the presence of the signal sequence predicted by expression of intact and truncated clones 5–9 in XO and RRL.

Discussion

We have used protein chimeras to determine the topology of CHIP28 at the ER membrane. Fig. 10A shows the transmembrane topology of CHIP28 determined by these studies. There are four membrane-spanning helices. Two peptide domains reside in the ER lumen, residues 36–90 and residues 186–210.
Figure 9. Clones HR5.P, HR6.P, and HR5-6.P were translated in RRL in the presence or absence of microsomal membranes as indicated. N-linked glycosylation of HR6.P and HR5-6.P resulted in a slower migrating band in the presence of microsomes (lanes 5–6 and 9–10). Digestion of chains with PK in the absence of detergent degraded chains from HR5.P, but not HR6.P and HR5-6.P, indicating that the P reporter was translocated into the ER lumen for these chains. Translation products were immunoprecipitated with antiprolactin antisera before SDS-PAGE.

186–210, consistent with the extracytoplasmic locations for N-linked glycosylation sites Asn42 and Asn205, and the critical cysteine at position 189 (Preston et al., 1993; Zhang et al., 1993b). Our studies suggest that HR2 and HR5 do not span the ER membrane, but instead, reside on the luminal (external) and cytoplasmic surfaces, respectively. Proteolysis of translation products from clone 9 (Fig. 3, lane 10–12) is in good agreement with the cytosolic location of the COOH terminus previously proposed (Preston and Agre, 1991; Smith and Agre, 1991). The cytosolic location of the amino terminus was confirmed using an NH2-terminal epitope flag.

COOH reporters have been successfully used to map topology of a wide variety of prokaryotic polytopic proteins (reviewed in Boyd et al., 1990), as well as several eukaryotic polytopic proteins (Fridlander and Blobel, 1985; Chavez and Hall, 1991; Silve et al., 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993). The P reporter used here was chosen because it lacks intrinsic translocation activity and responds faithfully to signal and stop transfer sequences in a wide variety of heterologous contexts (Andrews et al., 1988; Rothman et al., 1988; Chavez and Hall, 1991; Skach et al., 1993).
The presence of N-linked glycosylation provides powerful evidence for lumenal localization of acceptor sites at least at some time during protein biogenesis (Pollack and Atkinson, 1983; Lipp et al., 1989). Nascent chain glycosylation of CHIP28 at the engineered Asn69 therefore plays an important role in corroborating a key conclusion from the protease-protection studies. Because not all acceptor sites in membrane insertion, the reporter technique may produce erroneous results because secondary structure required for membrane insertion may require synthesis of residues downstream from the reporter fusion site. Posttranslational membrane insertion has been observed for certain proteins with extensive β sheet structure, such as porins (Pfanner et al., 1990; Mannella, 1990; Mayer et al., 1993; Sugawara and Nikaido, 1992) and α-helical proteins, such as annexins (Brisson et al., 1991; Lewit-Bentley et al., 1992; Concha et al., 1993). Integration of the amino half of CHIP28 (residues 1-107), independent of the carboxy terminus, and identification of internal sequences within CHIP28, which direct biogenesis at the ER membrane, strongly support a cotranslational model of CHIP28 biogenesis, as has been previously shown for other polytopic eukaryotic proteins (BraeU and Lodish, 1982; Brown and Simoni, 1984; Wessels and Spiess, 1988; Audigier et al., 1987; Skach et al., 1993). Furthermore, newly synthesized CHIP28 failed to integrate into microsomal membranes when membranes were added to the RRL reaction mixture after completion of translation, confirming a cotranslational mechanism for membrane integration (unpublished observations).

From our data, we propose a model for CHIP28 biogenesis in which the first signal sequence of CHIP28 (residues 1-52) targets the nascent chain-ribosome complex to the ER membrane and initiates translocation of COOH flanking residues. Translocation is terminated by a subsequent stop transfer sequence contained within HR4 (possibly together with HR3), which forms the second membrane-spanning helix. A second internal signal sequence (residues 155-186) reinitiates translocation and forms the third membrane spanning helix. Stop transfer activity of HR7 terminates translocation and establishes the fourth and final membrane-spanning helix.

It is possible that during or shortly after these events of CHIP28 biogenesis, subsequent regions of CHIP28 fold and associate with membrane lipids and/or the channel opening. Generalized hydropathy analysis of HR2 and HR5 is consistent with the presence of amphipathic β strand (Van Hoek et al., 1993), suggesting that these segments might be partially embedded at the membrane surface. Such a structure might be similar to the proposed antiparallel β strands of the P region of voltage-gated ion channels (Miller, 1992a, 1992b). Alternatively, they may participate in monomer-monomer interactions between CHIP28 subunits. For reasons stated above, we feel that it is unlikely HR2 and/or HR5 span the ER membrane in a traditional manner as α helices or as a β sheet structure.

Based on data supporting the functional independence of CHIP28 monomers (see introduction), it is predicted that a single aqueous pore should span each monomer. Based on the topology shown here in the ER, Fig. 10 B shows a simple arrangement of four membrane-spanning α helices (each of ~1 nm diameter), which produce a central channel that can accommodate water molecules but not larger solutes. Hydropathy indices were calculated along the helical surface (collapsed to two dimensions), as described in the figure legend. Remarkably, although the overall hydrophobicity of each helix was high (large positive values), the helices could be rotated so that the polar side chains lined a central pore. Similar analysis performed for HRs 2, 3, and 5 did not reveal unique polar regions of the helix. Note that the relative positions of HRs 1, 4, 6, and 7 are arbitrary and are not determined by our data. The hydrophobicity analysis is consistent with the passage of water through a polar channel bounded by the four helical domains predicted from our experiments.

Note added in proof. Preston et al. (1994, J. Biol. Chem. 269:1668-1673) recently reported a topology for CHIP28 significantly different from that determined by this study. Experiments are in progress to determine whether these differences are accounted for by multiple isoforms, changes in topology during intracellular trafficking, or other explanations.

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References


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