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Membrane Topology of Aquaporin CHIP

ANALYSIS OF FUNCTIONAL EPITOPE-SCANNING MUTANTS BY VECTORIAL PROTEOLYSIS*

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CHIP is the archetypal member of the aquaporins, a widely expressed family of membrane water channels. The NH_2- and COOH-terminal halves of CHIP are sequence-related, and hydropathy analysis predicted six membrane-spanning domains with five connecting loops (A–E). Here, we determined the membrane topology of CHIP expressed in *Xenopus* oocytes using biologically active recombinant channels. CHIP is glycosylated at Asn-42, indicating loop A is exofacial. An epitope from the coronavirus E1 glycoprotein was inserted into CHIP and localized to the outer or inner leaflet of the membrane by α-chymotrypsin digestion of intact oocytes or inside-out membrane vesicles. The E1 epitope at Thr-120 was protease-sensitive in intact oocytes, indicating that loop C is exofacial. The E1 epitope at Lys-6, Arg-162, or Lys-267 was protease-sensitive in inside-out membrane vesicles, confirming the cytoplasmic location of the NH_2 and COOH termini and loop D. Insertions into loops B and E did not produce active water channels, but their cleavage patterns were consistent with inner (loop B) and outer (loop E) leaflet locations. This study indicates that the functional CHIP molecule is a unique structure with two internal repeats oriented 180° to each other within the membrane.

CHIP is a 28-kDa channel-forming integral membrane protein first identified and purified from red cells (Denker *et al.*, 1988; Smith and Agre, 1991), and the cDNA has been isolated (Preston and Agre, 1991). CHIP functions as a water-selective pore, a discovery first made by expression of the cDNA in *Xenopus* oocytes (Preston *et al.*, 1992) and directly verified by reconstitution of highly purified CHIP protein into proteoliposomes (Zeidel *et al.*, 1992). The known mercury inhibition of water transport (Macey, 1984) is due to a single residue in CHIP, Cys-189 (Preston *et al.*, 1993). These studies were subsequently confirmed by others (van Hoek and Verkman, 1992; Zhang *et al.*, 1993a, 1993b). The related mammalian kidney protein WCH-CD (Fushimi *et al.*, 1993) and the plant tonoplast intrinsic protein γ-TIP (Maurel *et al.*, 1993) were recently shown to form water-selective channels, and these proteins are now referred to as the "aquaporins" (Agre *et al.*, 1993a, 1993b). The aquaporins belong to a larger gene family including MIP, the major intrinsic protein of lens (Gorin *et al.*, 1984), and other proteins expressed in animals, plants, *Drosophila*, yeast, and bacteria (Pao *et al.*, 1991). The functions of most MIP homologs remain undefined, but several may function as pores selective for water or possibly other small uncharged molecules.

CHIP protein is also expressed in the water-permeable epithelia of many mammalian tissues including renal proximal tubules (Denker *et al.*, 1988; Nielsen *et al.*, 1993b; Sabolic *et al.*, 1992) cornea, ciliary body, choroid plexus, biliary ductules, and nonfenestrated capillary and lymphatic endothelia (Nielsen *et al.*, 1993a). CHIP transcripts are expressed in at least three different developmental patterns in fetal rats (Bondy *et al.*, 1993). These CHIP transcripts and proteins are the products of a single gene (Genome Data Base symbol AQP1) that has been isolated and localized to human chromosome 7p14 (Moon *et al.*, 1993).

Red cell CHIP is a homotetramer (Smith and Agre, 1991); however, each monomer may contain a separate water pore (van Hoek *et al.*, 1991; Preston *et al.*, 1993).[1] Biochemical and immunohistochemical studies demonstrated the cytoplasmic locations of the NH_2 and COOH termini (Smith and Agre, 1991; Nielsen *et al.*, 1993b). Hydropathy analysis of the CHIP cDNA predicted six membrane-spanning domains connected by five loops (A–E), and the NH_2- and COOH-terminal halves of the polypeptide are sequence-related internal repeats (Preston and Agre, 1991). The purpose of this study was to define further the structure of CHIP by establishing the membrane topology of functional epitope-tagged CHIP molecules.

EXPERIMENTAL PROCEDURES

Site-directed and Insertional Mutagenesis—The *Bam*HI site in the polylinker of the CHIP expression vector (Preston *et al.*, 1992) was removed by digesting the DNA with *Bam*HI, followed by T4 DNA polymerase to fill in the overhangs and T4 DNA ligase. This construct served as the template for the site-directed and insertional mutagenesis reactions using the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio-Rad). Table I lists the CHIP site-directed and *Bam*HI insertional mutants used in this study. Fig. 1 shows the locations of amino acids targeted for mutagenesis relative to the predicted topology of the CHIP monomer (Preston and Agre, 1991). The 6-base pair *Bam*HI sites were inserted between the second and third base of a codon, resulting in the insertion of the amino acids Asp and Pro. The *Bam*HI insertion at Phe-92 changed this residue to Leu (L92-Bam), which is the naturally occurring amino acid in the rat and mouse CHIP cDNA sequences (Deen *et al.*, 1992; Lanahan *et al.*, 1992). None of the other *Bam*HI insertions changed the coding specificity of the preceding residue. The *Bam*HI insertion between Lys-267 and Pro-268 (K267-Bam) required only a 3-base pair insertion. Mutations were confirmed by enzymatic nucleotide sequencing (United States Biochemical Corp.).

A 90-base pair *Bam*HI sequence encoding the E1 tag (Machamer and Rose, 1987) was isolated from pSM224 and ligated into the *Bam*HI sites of the insert constructs. A 123-base pair *Bgl*II sequence encoding the hemagglutinin tag (Tyers *et al.*, 1992) was isolated from pSM492 and ligated into the *Bam*HI sites of the insert constructs. pSM224 and

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[1] J. S. Jung, G. M. Preston, B. L. Smith, W. B. Guggino, and P. Agre, manuscript in preparation.

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Table I
Site-specific and insertional mutations in CHIP

Mutant	Wild-type		Mutant	
	Amino acid	Codon	Amino acid(s)	Codon
N42Q	Asn-42	AAC	Gln-42	CAA
N205Q	Asn-205	AAC	Gln-205	CAA
K6-Bam	Lys-6	AAG	Lys-Asp-Pro	AAggatccG
Q88-Bam	Gln-88	CAG	Gln-Asp-Pro	CAggatccG
L92-Bam	Phe-92	TTC	Leu-Asp-Pro	TTggatccC
T120-Bam	Thr-120	ACT	Thr-Asp-Pro	ACggatccT
R162-Bam	Arg-162	CGT	Arg-Asp-Pro	CGggatccT
V201-Bam	Val-201	GTG	Val-Asp-Pro	GTggatccG
K267-Bam	Lys-267-Pro	AAGCCG	Lys-Asp-Pro	AAGgatCCG

pSM492 were kindly provided by P. Chen, C. Berkower, and S. Michaelis (The Johns Hopkins University School of Medicine, Baltimore). Restriction analysis and enzymatic nucleotide sequencing confirmed the correct orientation and reading frame of all insertions. The resulting amino acid insertions are as follows: E1 tag, DPMFVYAKQSVDTGELESVATGGSSLYTMDP; and HA tag, DLGRIFYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAAQCGPDP.

Preparation of Oocytes and Measurement of P_f[2]—Female *Xenopus laevis* were anesthetized on ice, and stage V and VI oocytes were removed and prepared (Lu *et al.*, 1990). The next day, oocytes were injected with either 50 nl of water or 10–40 ng of cRNA in 50 nl of water. Capped RNA transcripts were synthesized *in vitro* as described (Preston *et al.*, 1993). Injected oocytes were maintained for 2–3 days at 18 °C in modified Barth's solution (MBS) prior to analysis.

Osmotic swelling was performed at 22 °C following transfer of the oocytes from 200 mosM (osm_{in}) to 70 mosM (osm_{out}) MBS diluted with water. Sequential oocyte images were digitized at 15-s intervals for a total of 5 min or until just before the oocyte membrane ruptured, and the volumes of the sequential images were calculated as described (Preston *et al.*, 1993).

The change in relative volume with time, $d(V/V_0)/dt$, up to 5 min (or time of oocyte rupture) was fitted by computer to a quadratic polynomial, and the initial rates of swelling were calculated. The P_f values (centimeters/second × 10^{-4}) were calculated from osmotic swelling data between 15 and 30 s, initial oocyte volume ($V_0 = 9 \times 10^{-4}$ ml), initial oocyte surface area ($S = 0.045$ cm^2), and the molar ratio of water (V_w = 18 ml/mol) (Zhang *et al.*, 1990) using the following formula: $P_f = (V_0 \times d(V/V_0)/dt)/(S \times V_w \times (osm_{in} - osm_{out}))$.

Immunolabeling of Intact Oocytes—Three days after injecting oocytes with 10 ng of cRNA for CHIP, K6-E1, T120-E1, or K267-E1, groups of four to five oocytes were transferred into 15-mm flat-bottom tissue culture dish wells containing 0.5 ml of MBS plus 1 µl of rabbit anti-E1 peptide antibody (Pluta *et al.*, 1992) and shaken at 4 °C overnight. Oocytes were then washed five times with 1 ml of MBS at 4 °C, labeled with 0.5 µCi of ^{125}I-protein A (80 µCi/µg; DuPont NEN) in 0.5 ml of MBS at 4 °C for 1 h, washed five times with 1 ml of MBS at 4 °C, and counted individually. Oocytes labeled with preimmune rabbit serum had significantly lower protein A binding (115–210 dpm) than oocytes labeled with the rabbit anti-E1 peptide antibody.

Oocyte Membrane Isolation—Groups of 2–10 oocytes were transferred with MBS into 1.5-ml microcentrifuge tubes on ice. After chilling, the buffer was removed, and the oocytes were lysed by repeatedly vortexing and pipetting the samples in 100 µl/oocyte ice-cold hypotonic lysis buffer (7.5 mM Na_2HPO_4, pH 7.4, 1 mM EDTA). Protease inhibitors (Sigma) were freshly prepared and used at 1–2 × concentrations (see below). The 1 × concentrations were 20 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml each pepstatin A and leupeptin, and 1:2000 diisopropyl fluorophosphate. For routine membrane isolations, 1 × phenylmethylsulfonyl fluoride and 1 × pepstatin A were added to hypotonic buffer. The yolk and cellular debris were pelleted at 500 × *g* for 5 min at 4 °C. The membranes were then pelleted from the supernatant at 16,000 × *g* for 30 min at 4 °C. The floating yolk was removed from the top of the tubes with a cotton applicator, and the supernatant was removed. Membrane pellets were resuspended in 5–10 µl of 1.5% (w/v) SDS Laemmli sample buffer/oocyte at 60 °C for 10 min and analyzed by SDS-PAGE immunoblotting.

Enzymatic Digestion of Intact Oocytes and Isolated Oocyte Membranes—Four to six oocytes were transferred into 15-mm flat-bottom tissue culture dish wells containing 900 µl of MBS. α-Chymotrypsin (50 units/mg; CooperBiomedical, Inc.) was freshly dissolved in ice-cold MBS at 1 mg/ml and added to the oocytes at 10–100 µg/ml. After 60 min at 32 °C with gradual shaking, the MBS/chymotrypsin solutions was removed, and the oocytes were washed five times with 1 ml of ice-cold MBS containing 2 × protease inhibitors. Oocyte membranes were then isolated in the presence of 2 × protease inhibitors and immediately analyzed by SDS-PAGE immunoblotting.

Membranes for α-chymotrypsin digestion were isolated from oocytes lysed in hypotonic buffer containing 20 µg/ml phenylmethylsulfonyl fluoride and gently washed once in hypotonic buffer without phenylmethylsulfonyl fluoride. The membrane pellets were resuspended in 8 µl of hypotonic buffer/oocyte. α-Chymotrypsin was dissolved at 1 mg/ml in 2 mM HCl and diluted in 2 mM HCl. Reactions containing 40 µl of membranes, 5 µl of 20 mM Tris, pH 8.0, and 5 µl of diluted α-chymotrypsin in 2 mM HCl (or 5 µl of 2 mM HCl) were incubated at 37 °C for 60 min. The reactions were quenched with 450 µl of ice-cold hypotonic buffer containing 2 × protease inhibitors; the membranes were recovered by centrifugation at 16,000 × *g* for 30 min at 4 °C, washed once, and analyzed by SDS-PAGE immunoblotting. Control reactions with membrane pellets resuspended in isotonic MBS and digested with α-chymotrypsin under the same conditions gave identical results (data not shown). Endoglycosidase H digestions of isolated oocyte membranes were performed as described (Preston *et al.*, 1993).

Immunoblot Analysis—Membrane proteins were electrophoresed on 12 or 14% SDS-polyacrylamide slabs (Laemmli, 1970), transferred to nitrocellulose (Bio-Rad) (Davis and Bennett, 1984), incubated with a 1:2000 dilution of affinity-purified anti-CHIP or 1:1000 diluted affinity-purified anti-NH_2-terminal antibodies as described (Denker *et al.*, 1988; Smith and Agre, 1991), and visualized by the ECL Western blotting detection system (Amersham Corp.). Molecular weights were determined relative to the mobility of prestained SDS-PAGE standards (Bio-Rad).

RESULTS

N-Glycosylation Sites—Red cell CHIP is a homotetramer with a complex glycan attached by *N*-linkage to one of the four subunits (Smith and Agre, 1991). When CHIP is expressed in oocytes, complex glycosylated subunits of 35–50 kDa and non-glycosylated core subunits of 28 kDa are detected. Oocytes also express a 30-kDa CHIP polypeptide containing an *N*-linked high mannose oligosaccharide that can be removed by digestion with endoglycosidase H, a characteristic of incomplete glycan maturation. The relative abundance of the core, high mannose, and complex glycosylated CHIP polypeptides varied between experiments; however, mutant forms of CHIP lacking water channel activity exist predominantly as high mannose polypeptides without detectable complex glycosylated subunits (Preston *et al.*, 1993).

The two consensus sites for *N*-glycosylation of CHIP at Asn-42 and Asn-205 were both predicted to be extracellular (Fig. 1); therefore, Asn-42 and Asn-205 were mutated individually or in combination to Gln. CHIP is *N*-glycosylated at Asn-42 in oocytes since mutation of this residue alone eliminated detectable high mannose and complex *N*-glycosylated polypeptides (Fig. 2, *bottom*), confirming the exofacial location of loop A. *N*-Glycosylation at only the first potential site has been demonstrated in other polytopic integral proteins with multiple potential *N*-glycosylation sites.[3] The *N*-glycosylation site mutants had wild-type water channel activities (Fig. 2, *top*), indicating that *N*-glycosylation is not required for channel folding, oligomerization, or cell-surface expression. It remains to be determined whether CHIP is *O*-glycosylated in red cells or oocytes.

BamHI and Epitope Insertions—BamHI sites were inserted at selected locations of the CHIP coding region. The effects of the two amino acid insertions on osmotic water permeability and protein expression were assessed (Fig. 3*A*). Three of the insert mutants (Q88-Bam, T120-Bam, and R162-Bam) had P_f

[2] The abbreviations used are: P_f, osmotic water permeability; MBS, modified Barth's solution; PAGE, polyacrylamide gel electrophoresis.

[3] C. Landolt and R. Reithmeier, personal communication.

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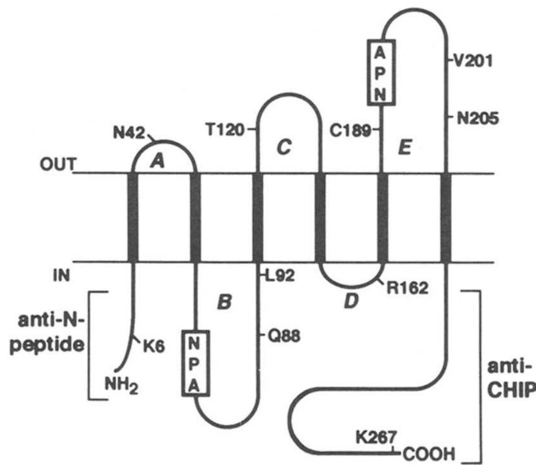

FIG. 1. **Proposed membrane topology of aquaporin CHIP.** Indicated in the model are the regions of CHIP recognized by anti-NH_2-terminal peptide and anti-CHIP antibodies (Smith and Agre, 1991), the proposed locations of amino acids targeted for mutagenesis in this study, the location of the mercury-sensitive residue Cys-189 (*C189*) (Preston *et al.*, 1993), membrane-spanning domains (*heavy dark lines*), exofacial (*OUT*) and cytoplasmic (*IN*) domains, and the highly conserved Asn-Pro-Ala motifs (*NPA*; *boxed*) located in the first and second inverted tandem repeats.

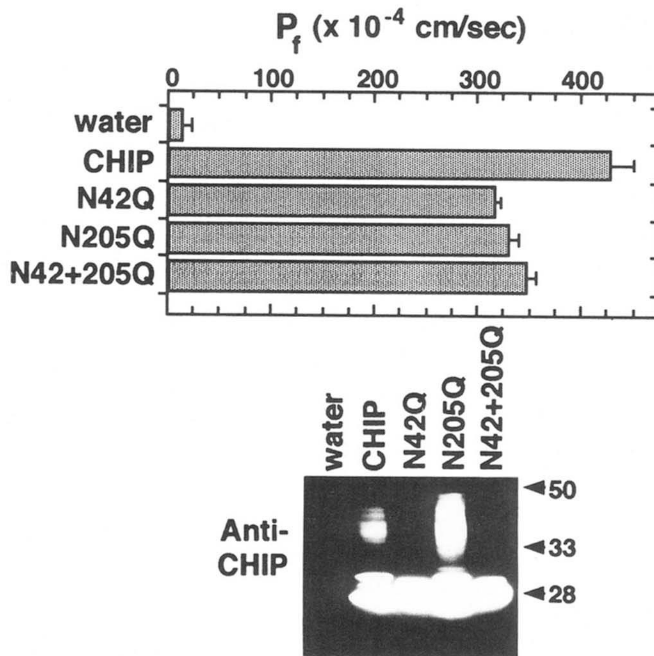

FIG. 2. ***N*-Glycosylation is not required for expression or function of CHIP.** *Top*, P_f values for oocytes injected with 10 ng of CHIP cRNA or the indicated mutant RNA. After 72 h, some of the oocytes were used for osmotic swelling experiments (see "Experimental Procedures"). Shown are the means ± S.D. (n = 3–4). *Bottom*, contact print of an immunoblot of membrane proteins isolated from the remaining oocytes. Each lane contains the equivalent of membranes from 2.5 oocytes run on a 12% SDS-polyacrylamide slab. The blot was incubated with affinity-purified anti-CHIP (see "Experimental Procedures"). Complex *N*-glycosylated CHIP polypeptides migrate as a 35–50-kDa smear (CHIP and N205Q) relative to the mobility of prestained SDS-PAGE standards shown on the right.

values and protein levels resembling oocytes expressing wild-type CHIP. The L92-Bam mutant had reduced protein expression or stability, which abolished CHIP-mediated P_f. The V201-Bam mutant was expressed at wild-type levels with an altered protein glycosylation pattern and lacked CHIP-enhanced P_f.

Two different epitope tags were evaluated at K6-Bam and K267-Bam (see "Experimental Procedures"). The 3.3-kDa E1

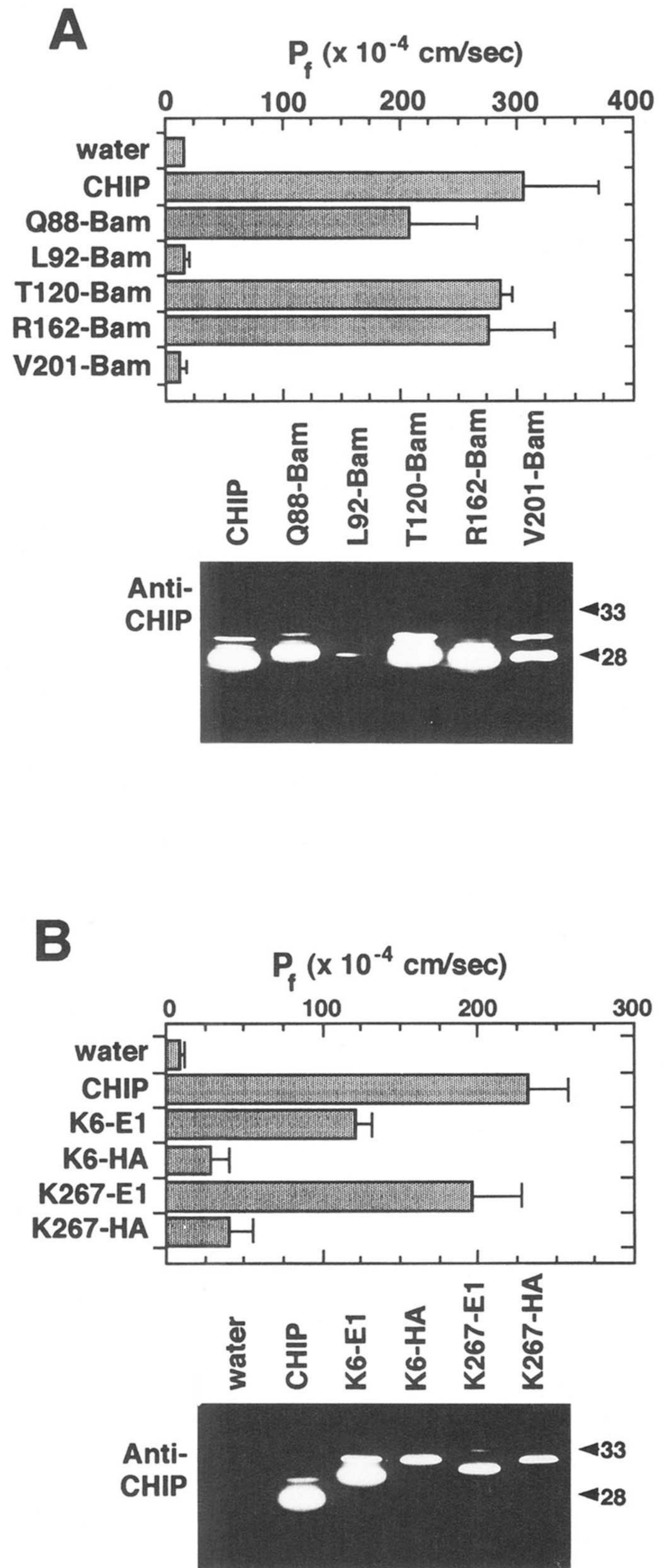

FIG. 3. **Introduction of Asp-Pro (*Bam*HI sites) and viral protein epitopes into CHIP.** *Bam*HI site insertional mutagenesis was performed at selected points within CHIP (*A*); E1 and HA epitopes were then inserted at K6-Bam and K267-Bam sites (*B*). The P_f (*top*) was measured, and levels of CHIP protein (*bottom*) were determined. In each experiment, oocytes were injected with 10 ng of the indicated cRNA. After 48 h, osmotic swelling experiments (mean ± S.D., n = 3–4) and immunoblot analysis were performed as described for Fig. 2 and under "Experimental Procedures."

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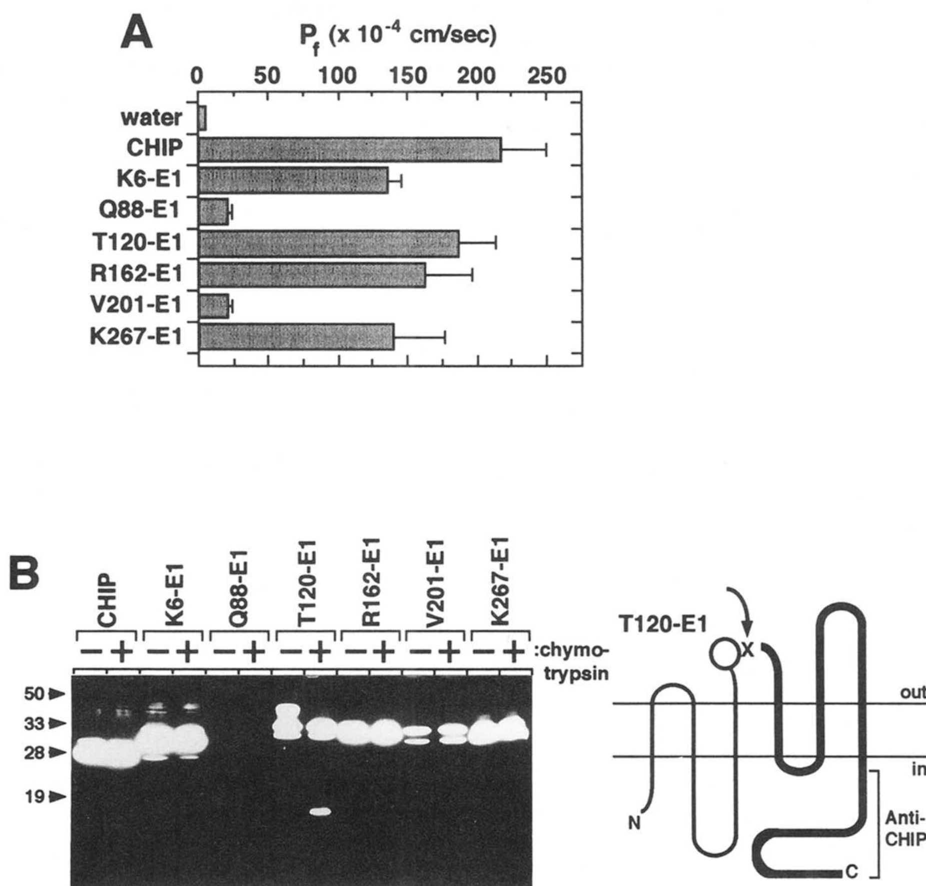

FIG. 4. **Effects of E1 insertional mutagenesis on osmotic water permeability and α-chymotrypsin sensitivity of CHIP in intact oocytes.** *A*, the P_f of oocytes injected with 10 ng of the indicated cRNA was assessed 72 h after injection. Shown are the means ± S.D. (n = 4–5). *B*, oocytes injected with 10 ng of the indicated cRNA were maintained for 72 h. Groups of four oocytes were incubated for 60 min at 32 °C in the absence (–) or presence (+) of 10 µg/ml (data not shown) or 100 µg/ml α-chymotrypsin. The oocytes were then washed, and their membranes were isolated and subjected to immunoblot analysis following electrophoresis of two oocyte membrane equivalents on a 14% SDS-polyacrylamide slab. The relative mobility of prestained SDS-PAGE standards is shown on the left of the contact print. At the right is a diagram representing the digestion of T120-E1 with α-chymotrypsin at an exofacial domain. Indicated in the model are the site of α-chymotrypsin digestion (*arrow* pointing to *X*), the location of the E1 tag (*circle*), and the CHIP fragment recognized on the blot by the anti-CHIP antibody following digestion (*thick black line*).

epitope tag encodes 25 amino acids from the COOH terminus of the avian coronavirus E1 glycoprotein, which is not involved in either glycosylation or membrane binding (Machamer and Rose, 1987). The 4.8-kDa HA epitope tag from the influenza hemagglutinin HA1 peptide encodes 9 amino acids in a triple tandem cassette (Tyers *et al.*, 1992). Introduction of the E1 epitope at either site did not affect CHIP protein expression or P_f (Figs. 3*B* and 4). In contrast, insertion of the HA epitope at either site significantly reduced protein expression and P_f (Fig. 3*B*).

The E1 epitope was then inserted into the *Bam*HI sites at Gln-88, Thr-120, Arg-162, and Val-201. Oocytes expressing T120-E1 or R162-E1 had P_f values and protein expression levels similar to those of wild-type CHIP (Fig. 4). V201-E1 exhibited a low P_f, although the protein was expressed at nearly wild-type levels. Insertion of the E1 epitope at Gln-88 resulted in greatly reduced levels of protein expression and a low P_f.

Identification of Exofacial Domains of CHIP in Intact Oocytes —The orientation of the E1 epitope in the plasma membranes was assessed with anti-E1 peptide antiserum (Pluta *et al.*, 1992). Control oocytes, oocytes expressing wild-type CHIP, and oocytes expressing the functional mutant K6-E1, T120-E1, or K267-E1 were incubated with rabbit anti-E1, followed by ^{125}I-protein A. Similar protein A binding was obtained with control oocytes (3100 ± 125 dpm, mean ± S.D., n = 4) and with oocytes expressing wild-type CHIP (3125 ± 210 dpm), K6-E1 (3375 ± 355 dpm), or K267-E1 (3010 ± 410 dpm). In contrast, oocytes expressing T120-E1 had 60% more ^{125}I-protein A binding than control oocytes (5010 ± 1145 dpm), suggesting an exofacial location for Thr-120.

The resistance of CHIP in intact red cells to protease digestion (Smith and Agre, 1991) and the presence of protease-sensitive residues in the E1 epitope were exploited to identify more definitively exofacial domains of CHIP. Intact oocytes expressing wild-type CHIP or one of the six E1 epitope-tagged CHIP proteins were digested with α-chymotrypsin, washed, and studied by immunoblot with the COOH-terminal specific anti-CHIP antibody (Fig. 4*B*). As expected, wild-type CHIP was resistant to protease digestion in intact oocytes.

Four of the E1 epitope-tagged CHIP proteins formed functional water channels (Fig. 4*A*). K6-E1, R162-E1, and K267-E1 contain the epitope at sites expected to be cytoplasmic (Fig. 1), and all three of these proteins were resistant to protease digestion in the intact oocytes (Fig. 4*B*). In contrast, loop C was predicted to be exofacial. Consequently, T120-E1 was partially cleaved by α-chymotrypsin digestion to a 17-kDa polypeptide (Fig. 4*B*). The >35-kDa complex glycosylated polypeptides were almost completely digested, whereas the 34-kDa high mannose and 32-kDa core polypeptides were relatively resistant. Membrane channel proteins overexpressed in *Xenopus* oocytes are known to have only a small percentage of the expressed protein in the plasma membrane (Nishimura *et al.*, 1993). Thus, these results suggest that most complex glycosylated CHIP is transported to the plasma membrane. Similar results were obtained with trypsin and with lower concentrations of α-chymotrypsin; higher concentrations of protease were deleterious to the oocytes. Surprisingly, the same P_f values were obtained for undigested and chymotrypsin-digested T120-E1-expressing oocytes, suggesting that cleavage of the protein at this location does not destroy function (data not shown).

Expression of two E1 epitope-tagged CHIP proteins resulted in only a small increase in P_f relative to buffer controls (Fig. 4*A*). Oocytes expressing V201-E1 were expected to have a low P_f since insertion of the *Bam*HI site alone at this location reduced the P_f. V201-E1 was expressed at nearly wild-type levels, but no protease sensitivity was detected in intact oocytes (Fig. 4*B*). It is likely that these insertions at Val-201 perturb protein folding, resulting in significantly reduced cell-surface expres-

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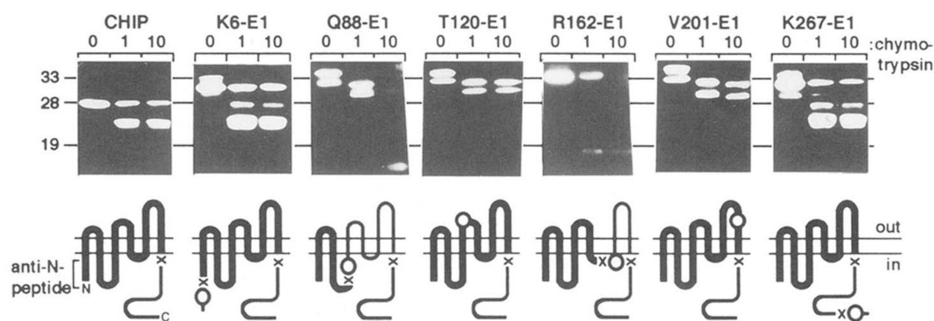

FIG. 5. **Effects of E1 insertional mutagenesis on α-chymotrypsin sensitivity of CHIP in oocyte membrane vesicles.** Oocytes injected with 10 ng of the indicated cRNA (40 ng for Q88-E1) were maintained for 72 h prior to membrane isolation. Membranes were incubated at 37 °C for 60 min in the absence (*0 lanes*) or presence of 1 (*1 lanes*) or 10 (*10 lanes*) μg/ml α-chymotrypsin. The membranes were recovered by centrifugation, washed, and subjected to immunoblot analysis with the anti-NH_2-terminal peptide antibody following electrophoresis of three oocyte membrane equivalents on a 14% SDS-polyacrylamide slab. The relative mobility of prestained SDS-PAGE standards is shown. Exposure times for the immunoblots varied from 2 to 60 min. Shown below the contact prints are models of CHIP and the different E1 insertional proteins. Indicated are the regions recognized by the anti-NH_2-terminal peptide antibody (left of CHIP model), E1 tags (*circles*), locations of α-chymotrypsin digestion in inside-out membrane vesicles (*X*), and the CHIP fragments recognized by the antibody after digestion (*thick black lines*).

sion. Insertion of the E1 epitope at Q88-Bam markedly reduced total CHIP protein levels, reflecting reduced protein synthesis or stability (Fig. 4*B*). Longer exposure of the immunoblot demonstrated that Q88-E1 was expressed, but there was no evidence of protease sensitivity. Other experiments with larger amounts of injected Q88-E1 cRNA supported these findings (data not shown).

Identification of Cytoplasmic Domains of CHIP in Intracellular Membrane Vesicles—The ~4-kDa COOH-terminal cytoplasmic tail of CHIP is removed when red cell inside-out membrane vesicles are digested with α-chymotrypsin, but the NH_2 terminus and the rest of the molecule remain embedded in the lipid bilayer and may be detected on immunoblots with an anti-NH_2-terminal peptide antibody (Smith and Agre, 1991). Cellular membranes isolated from osmotically lysed oocytes consist primarily of intracellular microsomes with protein orientations inverted with respect to the cell surface. Most CHIP molecules expressed in oocytes are located within these intracellular vesicles; therefore, digestion of isolated oocyte membranes resembled the digestion of red cell inside-out membrane vesicles (Fig. 5).

Membranes were isolated from oocytes expressing CHIP or one of the E1 epitope-tagged CHIP molecules. The membranes were digested with α-chymotrypsin and analyzed by immunoblot with the anti-NH_2-terminal peptide. Wild-type CHIP was digested from 28 to 24 kDa (Fig. 5), corresponding to proteolytic digestion at Trp-245, Leu-238, or Leu-231. These results verify that the membranes are primarily inside-out as expected and will permit evaluation of cytoplasmic domains of the recombinant molecules. α-Chymotrypsin digestion of K6-E1 and K267-E1 cleaved the E1 epitopes and COOH-terminal domains, leaving a fragment of 24 kDa (Fig. 5). Digestion of both K6-E1 and K267-E1 also produced 27-kDa fragments derived from high mannose modified CHIP polypeptides, which were eliminated by endoglycosidase H digestion (data not shown). In this experiment, a small amount of undigested CHIP, K6-E1, and K267-E1 protein was observed. Complete proteolytic digestion to the expected fragment could be achieved by increasing the amount of α-chymotrypsin or by decreasing the amount of membranes in the reactions.

T120-E1 contains the epitope in exofacial loop C (see above and Fig. 1). α-Chymotrypsin digestion of T120-E1 membrane vesicles resulted in cleavage of the 32-kDa polypeptide to a 29-kDa fragment still recognized by the anti-NH_2-terminal peptide (Fig. 5). V201-E1 gave the same digestion pattern in membrane vesicles as T120-E1 (Fig. 5). Both T120-E1 and V201-E1 existed in oocytes in two predominant forms: 32-kDa core and 34-kDa high mannose polypeptides, the latter of which was endoglycosidase H-sensitive (data not shown). Complex glycosylated polypeptides could be detected on immunoblots using the COOH-terminal specific antibody with T120-E1 and the other recombinant CHIP molecules exhibiting wild-type P_f values (Fig. 4*B*; also see Preston *et al.* (1993)). However, complex glycosylated polypeptides were not readily detected with the anti-NH_2-terminal peptide antibody (Fig. 5).

R162-E1 is a functional water channel containing the E1 epitope in membrane-connecting loop D, which is predicted to be cytoplasmic (Fig. 1). Surprisingly, R162-E1 was exceptionally sensitive to α-chymotrypsin digestion in membrane vesicle preparations, producing an unstable 18-kDa fragment corresponding to protease digestion near Arg-162, but with an intensity significantly lower than that of the undigested 32-kDa polypeptide. Likewise, Q88-E1 was ultimately digested to an unstable fragment of <14 kDa, but only after digestion of the COOH-terminal domain (Fig. 5). This two-step protease digestion pattern suggests that the COOH-terminal domain is more accessible to the protease than the epitope in Q88-E1.

DISCUSSION

CHIP is a widely expressed integral membrane protein and the archetypal member of the aquaporins (Agre *et al.*, 1993a, 1993b), a family of water-selective pores also found in mammalian renal collecting ducts (Fushimi *et al.*, 1993) and in plant tonoplasts (Maurel *et al.*, 1993). The sequences of the aquaporins are related to the major intrinsic protein of lens, MIP, a membrane channel with undefined specificity (Gorin *et al.*, 1984), and to several other proteins from widely divergent species (Pao *et al.*, 1991). The aquaporins are freely permeated by water, but fail to pass protons, other ions, or uncharged solutes. The explanation for water-selective transport is unknown since only limited structural information exists. Although MIP and CHIP are homotetramers (Aerst *et al.*, 1990; Smith and Agre, 1991), radiation inactivation studies (van Hoek *et al.*, 1991) and pharmacological analyses of coexpressed CHIP and the mercury-resistant CHIP mutant C189S (Preston *et al.*, 1993) suggest that each individual subunit contains an aqueous pore. Near- and far-UV circular dichroism of MIP revealed ≈50% α-helix and ≈20% β-structure (Horwitz and Bok, 1987). Hydropathy analysis of the deduced amino acid sequences of CHIP and the other MIP homologs predicted six bilayer-spanning domains (Preston and Agre, 1991; Gorin *et al.*, 1984), and the proteins contain two sequence-related tandem repeats (Pao *et al.*, 1991; Wistow *et al.*, 1991).

The goal of this study was to establish experimentally the

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membrane topology of CHIP by mutating *N*-glycosylation sites or inserting E1 viral protein epitope tags with protease cleavage sites (Fig. 1). The CHIP mutants were expressed in *Xenopus* oocytes, and the ability of each to transport water was determined since preservation of this function is necessary to confirm the relevance of the deduced topology to that of the native CHIP molecule. This study demonstrated exofacial locations of Asn-42 (loop A) and Thr-120 (loop C) and cytoplasmic locations of Lys-6 (NH_2 terminus), Arg-162 (loop D), and Lys-267 (COOH terminus) in water-transporting CHIP mutants. This study also suggests that Gln-88 (loop B) is cytoplasmic and Val-201 (loop E) is exofacial, although these mutants exhibited only marginally increased P_f values. Previous studies have documented the existence of the single mercury-sensitive residue at Cys-189 (loop E) (Preston *et al.*, 1993), which is thought to lie near the exofacial leaflet of the lipid bilayer (Macey, 1984). These structural determinations are most likely relevant to the structures of the other aquaporins and MIP homologs. For example, WCH-CD contains a single potential *N*-glycosylation site that is likely to be exofacial since the location corresponds to loop C. The studies reported here are all consistent with the topology model of CHIP (Fig. 1). Therefore, the two internal tandem repeats, corresponding to the NH_2- and COOH-terminal halves of the molecule (CHIP-1 = residues 14–113 and CHIP-2 = residues 140–231), are oriented 180° to each other as originally proposed (Preston *et al.*, 1992).

This study has advanced our understanding of the structure of CHIP subunits and provides a hypothesis for further investigating the structure of CHIP and other aquaporins. Mutations in loops B and E were less well tolerated than elsewhere in the CHIP molecule (Figs. 3*A* and 4), and preliminary analysis of a series of CHIP mutants containing single amino acid substitutions throughout loops B and E revealed that most mutants exhibited markedly reduced water channel activities.[1] Loops B and E contain the Asn-Pro-Ala motifs that are conserved in the aquaporins and in all mammalian and plant homologs of MIP (Pao *et al.*, 1991; Wistow *et al.*, 1991). Moreover, loops B and E are both very hydrophobic and may extend into the lipid bilayer. Loops B and E are therefore candidate structures of the pore-forming domains for CHIP and the other aquaporins. Such a structure is consistent with the recognized reciprocal transport of water into and out of cells containing water channels. Further studies will concentrate upon loops B and E to define further the structure and function of these domains.

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