# Communication

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## The Mercury-sensitive Residue at Cysteine 189 in the CHIP28 Water Channel*

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**Water channels provide the plasma membranes of red cells and renal proximal tubules with high permeability to water, thereby permitting water to move in the direction of an osmotic gradient. Molecular identification of CHIP28 protein as the membrane water channel was first accomplished by measurement of osmotic swelling of *Xenopus* oocytes injected with CHIP28 RNA (Preston, G. M., Carroll, T. P., Guggino, W. B., and Agre, P. (1992) *Science* 256, 385–387). Since water channels are pharmacologically inhibited by submillimolar concentrations of $Hg^{2+}$, site-directed mutagenesis was undertaken to demonstrate which of the 4 cysteines (87, 102, 152, or 189) is the $Hg^{2+}$-sensitive residue in the CHIP28 molecule. Each cysteine was individually replaced by serine, and oocytes expressing each of the four mutants exhibited osmotic water permeability ($P_f$) equivalent to wild-type CHIP28. After incubation in $HgCl_2$, all were significantly inhibited, except C189S which was not inhibited even at 3 mM $HgCl_2$. CHIP28 exists as a multisubunit complex in the native membrane; however, although oocytes injected with mixed CHIP28 and C189S RNAs exhibited $P_f$ corresponding to the sum of their individual activities, exposure to $Hg^{2+}$ only reduced the $P_f$ to the level of the C189S mutant. Of the six substitutions at residue 189, only the serine and alanine mutants exhibited increased $P_f$ and had glycosylation patterns resembling wild-type CHIP28 on immunoblots. These studies demonstrated: (i) CHIP28 water channel activity is retained despite substitution of individual cysteines with serine; (ii) cysteine 189 is the $Hg^{2+}$-sensitive residue; (iii) the subunits of the CHIP28 complex are individually active water pores; (iv) residue 189 is critical to proper processing of the CHIP28 protein.**

All cell membranes are somewhat permeable to water due to diffusion through the lipid bilayers. In contrast, red blood cells and renal proximal tubules are exceptionally permeable due to the existence of water-selective channels in their plasma membranes (reviewed by Finkelstein (1986)). Unlike diffusional water permeability, channel-mediated transmembrane water movement is osmotically driven, large in magnitude, and characterized by a low Arrhenius activation energy (reviewed by Solomon *et al.* (1983) and Verkman (1989)). Red cell and renal proximal tubule water channels are constitutively active, unlike the vasopressin-regulated water channels of amphibian epithelia and renal collecting ducts (reviewed by Harris *et al.* (1991)). Moreover, water channels are pharmacologically defined by their susceptibility to inhibition by low concentrations of mercurial sulfhydryl reagents which may be reversed with reducing agents (Macey, 1984).

CHIP28 is an integral protein of apparent molecular mass 28 kDa, which is abundant in membranes of red cells and renal proximal tubules (Denker *et al.*, 1988). The cDNA encoding CHIP28 revealed homologies with a family of membrane proteins expressed in diverse species and tissues, including the roots of plants (Preston and Agre, 1991). CHIP28 was first demonstrated to be the molecular water channel by expression in *Xenopus* oocytes, which then displayed $Hg^{2+}$-sensitive osmotic water permeability, $P_f$ (Preston *et al.*, 1992). Water channel function was demonstrated directly by reconstitution of highly purified CHIP28 into liposomes (Zeidel *et al.*, 1992), an observation confirmed by others with partially purified CHIP28 (van Hoek and Verkman, 1992). Therefore CHIP28 has been proposed to be the major water channel in these and other tissues (Agre *et al.*, 1993).

The deduced amino acid sequence of CHIP28 contains 4 cysteines, and the proposed membrane topology includes six probable bilayer-spanning domains (Preston and Agre, 1991). Moreover, the N- and C-terminal halves of the CHIP28 molecule are distantly homologous and may be oriented 180° to each other within the membrane. CHIP28 proteins exist within the membrane as multisubunit complexes, each probably comprised of one glycosylated and three nonglycosylated CHIP28 polypeptides (Smith and Agre, 1991). Apparently no intermolecular disulfides exist, since electrophoretic mobility of CHIP28 is unaffected by reducing agents (Denker *et al.*, 1988). Despite this knowledge, the identity of the $Hg^{2+}$-sensitive residue(s) and the molecular structure of the water pore(s) within the CHIP28 membrane complex remain undefined.

### EXPERIMENTAL PROCEDURES

*Site-directed Mutagenesis and in Vitro RNA Synthesis*—CHIP28 mutants were made with the Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio-Rad) using the CHIP28 expression vector as template (Preston *et al.*, 1992). Mutations were confirmed by enzymatic nucleotide sequencing (United States Biochemical Corp.). Table I lists the CHIP28 mutants used in this study. Capped RNA transcripts were synthesized *in vitro*, and the RNA was purified as described (Yisraeli and Melton, 1989). RNA was synthesized with T3 RNA polymerase using either *Sma*I- or *Xba*I-digested CHIP28 expression vector DNA, or one of the mutated CHIP28 clones as template.

*Preparation of Oocytes and Measurement of $P_f$*—Stage V and VI oocytes were removed from female *Xenopus laevis* and prepared as described (Lu *et al.*, 1990) with the exception that the amphibia were anesthetized on ice. The day after isolation, oocytes were injected with either 50 nl of water or 1–10 ng of RNA in 50 nl of water. Injected oocytes were maintained for 3 days at 18 °C in modified Barth's buffer prior to osmotic swelling or membrane isolation (Preston *et al.*, 1992).

Osmotic swelling at 22 °C was monitored with a Nikon phase-

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TABLE I
*Site-specific mutations in CHIP28 water channel*

| Mutation | Wild-type | | Mutant | |
|---|---|---|---|---|
| | Amino acid | Codon | Amino acid | Codon |
| C87S | Cys-87 | TGC | Ser-87 | AGC |
| C87M | Cys-87 | TGC | Met-87 | ATG |
| C87W | Cys-87 | TGC | Trp-87 | TGG |
| C87Y | Cys-87 | TGC | Tyr-87 | TAC |
| C102S | Cys-102 | TGC | Ser-102 | AGC |
| C152S | Cys-152 | TGC | Ser-152 | AGC |
| C189S | Cys-189 | TGT | Ser-189 | AGT |
| C189A | Cys-189 | TGT | Ala-189 | GCT |
| C189V | Cys-189 | TGT | Val-189 | GTT |
| C189M | Cys-189 | TGT | Met-189 | ATG |
| C189W | Cys-189 | TGT | Trp-189 | TGG |
| C189Y | Cys-189 | TGT | Tyr-189 | TAC |

contrast microscope equipped with a video camera connected to a computer. Oocytes were transferred from 200 mosM ($osm_{in}$) to 70 mosM ($osm_{out}$) modified Barth's buffer diluted with water. An oocyte image was digitized by computer (Universal Imaging Corporation, West Chester, PA) and stored at 15-s intervals for a total of 5 min or until the time of oocyte rupture. The surface area of the sequential images was calculated using Image-1 computer software (Version 4.01B, Universal Imaging) assuming that the oocytes are spheres without microvilli. The oocyte volume was calculated using the following formula.

$$V = (4/3) \times (area) \times (area/\pi)^{1/2} \quad \text{(Eq. 1)}$$

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 osmotic water permeabilities ($P_f$, cm/s $\times 10^{-4}$) were calculated from osmotic swelling data between 15 and 30 s, initial oocyte volume ($V_0 = 9 \times 10^{-4}$ cm$^3$), initial oocyte surface area ($S = 0.045$ cm$^2$), and the molar ratio of water ($V_w = 18$ cm$^3$/mol) (Zhang *et al.*, 1990) using the following formula.

$$P_f = [V_0 \times d(V/V_0)/dt]/[S \times V_w \times (osm_{in} - osm_{out})] \quad \text{(Eq. 2)}$$

*Oocyte Membrane Isolation and Immunoblot Analysis*—Groups of 5–10 oocytes were transferred with modified Barth's buffer into 1.5-ml microcentrifuge tubes on ice. After chilling for ≥5 min, the buffer was removed and the oocytes were lysed in 0.5–1 ml of ice-cold hypotonic lysis buffer (7.5 mM $Na_2HPO_4$, pH 7.4, 1 mM EDTA buffer containing 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1:2000 diisopropylfluorophosphate) by repeatedly vortexing and pipetting the samples. The yolk and cellular debris were pelleted at 750 × *g* × 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. The membrane pellets were gently washed once with an equal volume of ice-cold hypotonic lysis buffer and were resuspended in 10 μl 1.25% (w/v) SDS/oocyte and electrophoresed into a 12% SDS-polyacrylamide gel (Laemmli, 1970), transferred to nitrocellulose (Davis and Bennett, 1984), incubated with a 1:1000 dilution of affinity-purified anti-CHIP28 (Denker *et al.*, 1988; Smith and Agre, 1991), and visualized using an ECL Western blotting detection system (Amersham Corp.).

*Endoglycosidase H Digestions*—Membranes from 75 μl of human red cells or 15 *Xenopus* oocytes were resuspended to 150 μl at a final concentration of 0.5% (w/v) SDS and 50 mM β-mercaptoethanol. The suspension was solubilized by incubation at 60 °C for 10 min, of which 25 μl was combined with 50 μl of 50 mM sodium phosphate, pH 6.0, and incubated at 37 °C for 18 h in the absence or presence of 50 milliunits of endoglycosidase H (Genzyme, Boston, MA). A control reaction containing 15 μg of ovalbumin confirmed activity of the enzyme. After the incubation, the samples were precipitated for 8 h at −20 °C with 750 μl of ethanol and pelleted at 16,000 × *g* for 30 min at 4 °C. The dried pellets were resuspended to 20 μl with 1.25% (w/v) SDS, and aliquots were electrophoresed through a 12% SDS-polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose for immunoblot analysis (Davis and Bennett, 1984).

## RESULTS AND DISCUSSION

Osmotic water permeability ($P_f$) of *Xenopus* oocytes was measured after injection with 50 nl of water or 50 nl of water containing 1 ng of CHIP28 RNA. Three days after injection, the oocytes were transferred from 200 to 70 mosM modified Barth's buffer, and swelling was measured at 22 °C (see "Experimental Procedures"). Water-injected control oocytes swelled minimally ($P_f = 18$ cm/s $\times 10^{-4}$), whereas CHIP28 RNA-injected oocytes swelled rapidly and ruptured ($P_f = 127$ cm/s $\times 10^{-4}$). This increase in osmotic water permeability was inhibited by incubation in 0.3 mM $HgCl_2$; the inhibition was reversed by subsequent incubation in 5 mM β-mercaptoethanol (Fig. 1).

Site-directed mutagenesis was undertaken to establish the importance of the four cysteines in the CHIP28 molecule and thereby gain insight into the structure and function of the water channel. Although differing in hydrophobicity and ability to form disulfides, serine and cysteine are structurally similar amino acids. Serine was therefore substituted for each of the cysteines in four separate constructs (C87S, C102S, C152S, and C189S). Each of the four mutant RNAs was evaluated with the oocyte swelling assay, and each exhibited increased osmotic water permeability (mean $P_f = 168 \pm 35$ cm/s $\times 10^{-4}$) which was similar to the $P_f$ value of oocytes injected with wild-type CHIP28 RNA (Fig. 2*A*). Although the cysteines at residues 87, 102, and 152 are conserved among other members of the MIP family (Pao *et al.*, 1991), each of the individual cysteines may be replaced without obvious affects upon osmotic water permeability. Cysteine 102 and 152 are located in presumed bilayer spanning domains and may exist as an internal disulfide within the native CHIP28 subunit. If a disulfide exists in the native CHIP28 molecule, it is not critical to osmotic water permeability. Cysteine 189 is not conserved among other members of the MIP family and may also be replaced without significantly affecting water channel function.

Susceptibility of the individual cysteine residues to mercury inhibition was then established by incubating the RNA-injected oocytes in $HgCl_2$. The osmotic water permeabilities of oocytes injected with C87S, C102S, or C152S RNAs or wild-

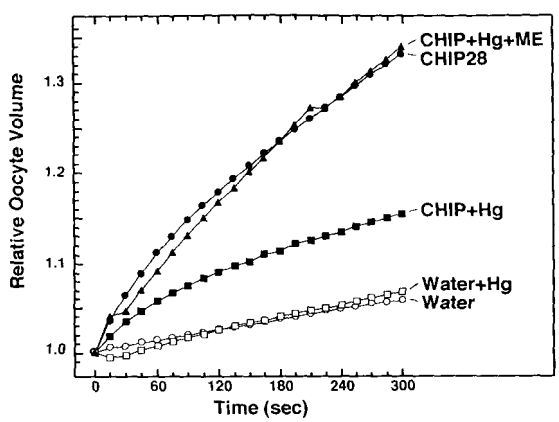


FIG. 1. **Time course and $Hg^{2+}$ inhibition of osmotic swelling of oocytes expressing CHIP28.** Oocytes were injected with 50 nl of water or 50 nl of water containing 1 ng of CHIP28 RNA. After 72 h, the oocytes were transferred from 200 to 70 mosM modified Barth's buffer, and changes in size were measured by videomicroscopy (see "Experimental Procedures"). Where indicated, the oocytes were incubated for 5 min in buffer containing 0.3 mM $HgCl_2$, followed by swelling in the presence of $HgCl_2$ (*Water+Hg* or *CHIP+Hg*). After incubation for 5 min in 0.3 mM $HgCl_2$, other oocytes were then incubated for 15 min in buffer containing 5 mM β-mercaptoethanol and swelling was monitored in the presence of β-mercaptoethanol (*CHIP+Hg+ME*).

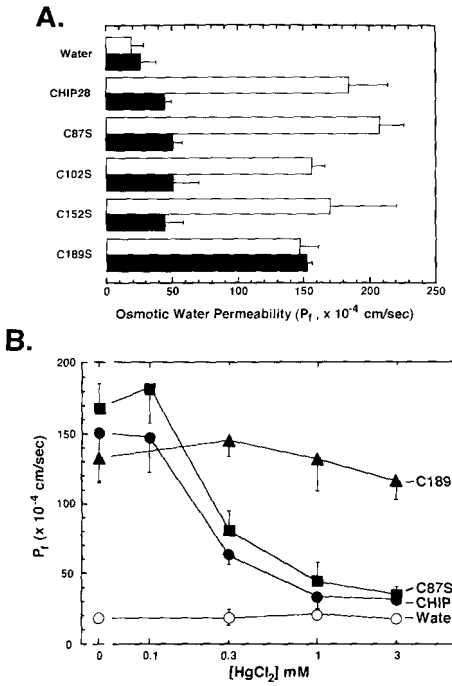


FIG. 2. **Osmotic water permeability and $Hg^{2+}$ inhibition of oocytes expressing CHIP28 or cysteine-to-serine CHIP28 mutants.** *A*, the osmotic water permeability of oocytes expressing CHIP28 or mutants was determined in the absence or presence of 1 mM $HgCl_2$. Oocytes were injected with water or 1 ng of RNA specific for CHIP28 or each of four CHIP28 mutants: C87S, C102S, C152S, or C189S. Osmotic swelling was determined without pretreatment (*open bars*) or after 5 min in buffer containing 1 mM $HgCl_2$, followed by swelling in the presence of $HgCl_2$ (*solid bars*). Shown are the means ± S.D., $n = 4$. Other oocytes pretreated for 5 min in 1 mM $HgCl_2$ followed by 15 min in 5 mM β-mercaptoethanol had $P_f$s equivalent to untreated oocytes (data not shown). *B*, titration of $Hg^{2+}$ inhibition of injected oocytes assessed after 5 min of incubation in buffer containing 0.1–3 mM $HgCl_2$ followed by swelling in the presence of $HgCl_2$. Shown are the means ± S.D., $n = 3$.

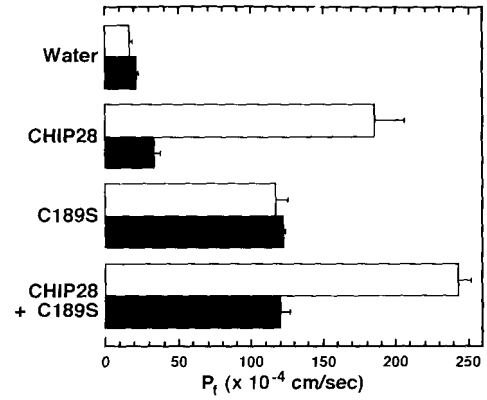


FIG. 3. **Lack of cooperative osmotic water permeability of oocytes co-expressing wild-type CHIP28 and C189S subunits.** Oocytes were injected with water or the following RNAs: 1 ng of CHIP28, 1 ng of C189S, or 1 ng of CHIP28 plus 1 ng of C189S. Standard osmotic water permeability was performed (*open bars*) or after inhibition in 3 mM $HgCl_2$ (*solid bars*). Shown are the means ± S.D., $n = 4$. Oocytes injected with CHIP28 plus C189S RNAs exhibited osmotic water permeability approaching the sum of their independent $P_f$s, but after $Hg^{2+}$ inhibition, the $P_f$ was reduced only to the level of the C189S mutant.

type CHIP28 RNA were significantly inhibited by 1 mM $HgCl_2$ (Fig. 2*A*). This inhibition was fully reversed by subsequent incubation in 5 mM β-mercaptoethanol (not shown). In contrast, the osmotic water permeability of oocytes injected with C189S RNA was not inhibited by 1 mM $HgCl_2$ (Fig. 2*A*), and subsequent incubation in 5 mM β-mercaptoethanol therefore had no effect (not shown).

To establish whether the lack of $Hg^{2+}$ inhibition of the C189S mutant reflects absolute or relative resistance, oocytes injected with CHIP28 RNA or mutant RNAs were incubated in $HgCl_2$ concentrations ranging from 0.1 to 3 mM. Oocytes expressing CHIP28 or C87S exhibited negligible inhibition by 0.1 mM $HgCl_2$, complete inhibition by 3 mM $HgCl_2$, and partial inhibition at intermediate concentrations (Fig. 2*B*). In contrast, oocytes expressing C189S exhibited no significant inhibition even at 3 mM HgCl; still higher concentrations of $HgCl_2$ could not be accurately evaluated due to oocyte toxicity. Therefore cysteine 189 is the mercury-sensitive residue within the CHIP28 molecule.

CHIP28 protein exists as a membrane complex probably comprised of four CHIP28 subunits (Smith and Agre, 1991), but it remains uncertain whether the tetrameric complex forms a single water pore or whether individual subunits each contain a pore. The physiologically active but $Hg^{2+}$-resistant C189S mutant was therefore used to test for potential cooperativity between CHIP28 subunits. RNAs corresponding to CHIP28 and C189S were mixed and injected into oocytes, and the osmotic water permeability corresponded to nearly the sum of $P_f$s determined for the individual RNAs (Fig. 3). When osmotic water permeability of the coinjected oocytes was determined after incubation in 3 mM $HgCl_2$, it was reduced to a level identical to that of oocytes injected only with C189S RNA (Fig. 3). Although not proven, it is likely that the CHIP28 and C189S subunits exist together within the same oligomeric complexes, however, the sensitivity of wild-type CHIP28 and the resistance of C189S to $Hg^{2+}$ were each fully and individually retained. This supports the hypothesis that each CHIP28 subunit contains a single pore, thereby resembling the porin or gramicidin channels (reviewed by Jap and Walian (1990)) rather than the $K^+$ channels in which four subunits create a single channel (reviewed by Jan and Jan (1989)). This hypothesis is further supported by radiation inactivation studies, which demonstrated that the functional water channel has a target size of 30 kDa (van Hoek *et al.*, 1991), a value corresponding to the mass of the individual CHIP28 subunit.

Despite determination of its primary amino acid sequence, the molecular structure of the water pore within the CHIP28 protein is not obvious (Preston and Agre, 1991). The results presented here suggest that water molecules may permeate CHIP28 through a single pore in each subunit which contains a narrow region near residue 189. Therefore $HgCl_2$ inhibition may simply represent occlusion of the water pore by covalent attachment of a $Hg^{2+}$ to the free sulfhydryl at cysteine 189. Consistent with this hypothesis, cysteine 189 is located in a region of the CHIP28 molecule predicted by Chou-Fasman and Garnier-Robinson algorithms to be a turn separating regions of β structure. Like other members of the MIP family (Wistow *et al.*, 1991), the CHIP28 protein contains an internal homology between the N- and C-terminal halves of the protein, which is most striking when residues 14–113 are aligned with residues 140–231. Cysteine 87 and cysteine 189 lie within hydrophobic loops of the CHIP28 protein, which are thought to be mirror-image repeats existing on opposite sides of the lipid bilayer (Preston and Agre, 1991; Preston *et al.*, 1992). Although each loop contains a cysteine, their locations within the homologous polypeptides are not identical. Thus if $Hg^{2+}$ reacts with cysteine 87, this apparently does not result in occlusion of the water pore as it does at cysteine 189.

To test this hypothesis, a series of CHIP28 mutants was constructed in which cysteine 87 or cysteine 189 were individually replaced by amino acids of other dimensions, and the

osmotic water permeability was measured after expression in oocytes (Fig. 4*A*). Alanine has a side chain smaller than cysteine and serine, and oocytes expressing the alanine mutant (C189A) exhibited osmotic water permeability similar to wild-type CHIP28. The side chain of valine is slightly larger than cysteine or serine, and oocytes expressing the valine mutant (C189V) exhibited markedly reduced osmotic water permeability. Mutants with methionine, tryptophan, or tyrosine at residues 87 or 189 were also constructed, since each of these amino acids contains a side chain even larger than valine. The osmotic water permeabilities of oocytes injected with mutant RNAs containing these substitutions at residue 87 (C87M, C87W, and C87Y) exhibited osmotic water permeabilities similar to oocytes expressing wild-type CHIP28 (Fig. 4*A*). In contrast, oocytes injected with mutant RNAs containing these substitutions at residue 189 (C189M, C189W, and C189Y) exhibited negligible osmotic water permeability, which was equivalent to water-injected control oocytes (Fig. 4*A*).

Oocyte membranes were analyzed by immunoblot to determine if the inactive mutant proteins were expressed. Wild-type CHIP28 and the mutant proteins were found in similar quantities; however, only oocytes containing mutant proteins which exhibited increased osmotic water permeability had glycosylation patterns resembling that of oocytes expressing wild-type CHIP28 proteins (Fig. 4*B*). Membranes from oocytes injected with mutant RNAs encoding inactive water channels all lacked the high molecular weight glycosylated subunits and contained a prominent band with electrophoretic mobility slightly above the 28-kDa subunit (Fig. 4*B*, *single arrow*). This band was also noted in very small quantities in oocytes expressing wild-type CHIP28 protein or functional CHIP28 mutants. Other studies demonstrated that this new band represents mutant CHIP28 polypeptide chains with *N*-linked carbohydrate containing high mannose oligosaccharides, which were removed by digestion with *N*-glycanase (not shown) or endoglycosidase H (Fig. 4*B*). The abnormal electrophoretic mobility of this new band is therefore apparently due to incomplete trimming and maturation of the *N*-linked glycan rather than due to defective core glycosylation. Although it is very likely that the defective glycosylation of CHIP28 proteins results from defective protein folding, it cannot presently be resolved whether these mutant CHIP28 polypeptides are functionally inactive water channels, since their membrane distribution is likely to be abnormal because of disrupted intracellular membrane targeting (reviewed by Pelham (1989)).

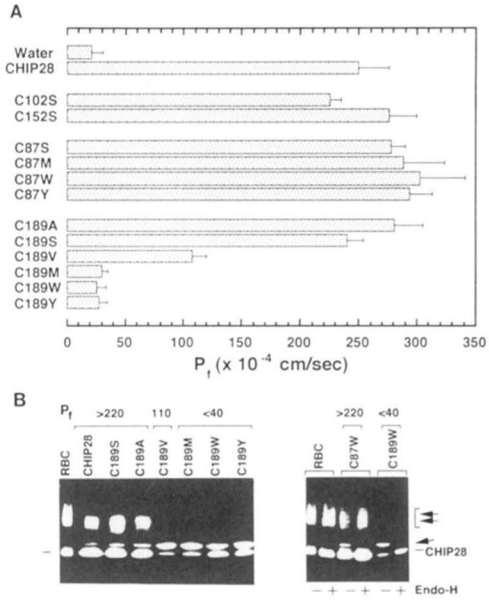


FIG. 4. **Osmotic water permeability and immunoblot analysis of CHIP28 and selected mutant forms of CHIP28.** *A*, oocytes were injected with water or 10 ng of the indicated RNA and osmotic water permeability was measured. Shown are the means ± S.D., $n$ = 3. *B*, contact print of an immunoblot of membranes prepared from human red cells or oocytes injected with wild-type CHIP28 or the mutant CHIP28 RNAs. Each *lane* contains membranes prepared from approximately $3 \times 10^7$ red cells or the equivalent of one oocyte. As indicated, some membranes were solubilized and digested with endoglycosidase H (see "Experimental Procedures"). The *double arrow* on the *right* identifies the *N*-glycosylated CHIP28 protein containing a high molecular weight glycan resembling red cells, and the *single arrow* identifies *N*-glycosylated CHIP28 protein containing a high mannose oligosaccharide found only in oocytes.

The studies reported here demonstrate that residue 189 is critical to the structure, function, and processing of the CHIP28 water channel. Permeability of the water pore is blocked by reaction of $Hg^{2+}$ with cysteine 189 in the native molecule. Moreover, residue 189 is apparently critical to the native folding of the CHIP28 protein, as identified by abnormal glycan biosynthesis at other sites of the molecule (asparagine 42 or 205). Future studies employing site-directed mutagenesis may establish the size of this critical domain in the water pore and the possible existence of other domains potentially critical to the function of the CHIP28 water channel. Information derived from studies of CHIP28 will also very likely provide molecular insight into the structure and functions of the other members of this newly recognized protein family.

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