

- collected by trypsinization, washed once with growth media, once with phosphate-buffered saline, and once with swelling buffer [10 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and protease inhibitors]. Cells were homogenized in swelling buffer (>95% cell lysis). One-quarter of the cell suspension was saved as total cell lysate. The remainder was divided into three portions and layered onto sucrose (25%) dissolved in swelling buffer. Nuclei were collected by centrifugation (2000g for 20 min). One portion of the nuclear preparation was saved. The other two portions were resuspended in 200 μ l of swelling buffer with 0.5% NP-40, 1 mM PMSF, and either 100 mM KCl or 500 mM KCl and incubated at 4°C for 1 hour with gentle shaking. Nuclei were then centrifuged (13,000g for 5 min), and the supernatant was collected. An equal volume of each sample was analyzed by immunoblotting. Quantitation was by densitometric scan of autoradiographs.
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 15. Immunoprecipitation of active cdc2 kinase from mitotic 3T3 cells was as previously described in (4). Seven-eighths of the cdc2 immunoprecipitate was resuspended in kinase buffer (35 μ l) [20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 μ M adenosine triphosphate (ATP), and protease inhibitors] containing GUS (3.5 μ g) and incubated at room temperature for 2 hours with gentle shaking. The reaction mixture was centrifuged, and the supernatant was used for immunoblotting and gel shift assays. The other one-eighth of the cdc2 immunoprecipitate was resuspended in kinase buffer (5 μ l) containing GUS (0.5 μ g) and 1.5 μ M [γ - 32 P]ATP (6000 Ci/mmol, ICN). The 32 P-labeled sample was run on SDS-polyacrylamide gel, dried, and autoradiographed. The labeled GUS band was cut out and counted in a scintillation counter to determine the stoichiometry of phosphorylation. Under identical conditions, GST protein was not phosphorylated by cdc2, thus phosphorylation of GUS was in the US peptide derived from c-Abl.
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Appearance of Water Channels in *Xenopus* Oocytes Expressing Red Cell CHIP28 Protein

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Water rapidly crosses the plasma membrane of red blood cells (RBCs) and renal tubules through specialized channels. Although selective for water, the molecular structure of these channels is unknown. The CHIP28 protein is an abundant integral membrane protein in mammalian RBCs and renal proximal tubules and belongs to a family of membrane proteins with unknown functions. Oocytes from *Xenopus laevis* microinjected with in vitro-transcribed CHIP28 RNA exhibited increased osmotic water permeability; this was reversibly inhibited by mercuric chloride, a known inhibitor of water channels. Therefore it is likely that CHIP28 is a functional unit of membrane water channels.

Water slowly crosses cell membranes by diffusion through the lipid bilayer. However, certain cell types exhibit rapid transmembrane passage of water, probably through specialized water-selective channels. For example, water channels of mammalian RBCs permit cell swelling or shrinkage in response to small changes in extracellular osmolality (1, 2). Water is reabsorbed by channels in mammalian renal proximal convoluted tubules (3) and by vasopressin-regulated water channels in mammalian collecting ducts (4) and amphibian urinary bladder (5). Although water channel physiology has been studied extensively (3, 5),

the molecular structure of the channel is unknown (6, 7). The glucose transporter (8) and band 3, an anion exchanger from RBCs (2), permit small amounts of transmembrane water movement, but neither is a water channel (9, 10).

The 28-kD integral membrane protein CHIP28 is abundant in mammalian RBCs and renal proximal tubules (11). The native CHIP28 protein is a noncovalent assembly of four subunits (12) and is physically similar to certain membrane channels (hence, CHIP28 = channel-forming integral protein of 28 kD). The cDNA encoding CHIP28 has been isolated (13), and the deduced amino acid sequence predicts an integral membrane protein with six bilayer-spanning domains (Fig. 1A). The CHIP28 protein is related to the major intrinsic protein from bovine lens, MIP26, a channel through which lens fiber cells may absorb interstitial fluid (14). CHIP28

and MIP26 are members of a family of bacterial and plant proteins with incompletely defined functions (15). Expression of one family member is induced in the roots and shoots of pea plants by water deprivation (16). Thus we tested whether CHIP28 is a membrane water channel by expressing CHIP28 in oocytes from *Xenopus laevis* and measuring osmotic water permeability.

We prepared an expression construct by inserting the CHIP28 coding sequence between the 5' and 3' untranslated sequences of the *Xenopus* β -globin cDNA (17). Defolliculated oocytes were microinjected with water (0.05 μ l) or with up to 10 ng of in vitro-transcribed CHIP28 RNA (18). Expression of CHIP28 was monitored by immunoblot with an antibody to the COOH-terminal cytoplasmic domain of CHIP28 (anti-CHIP) (11, 12). A 28-kD protein was detected with anti-CHIP 24 hours after injection of oocytes with CHIP28 RNA, and it increased in abundance for up to 72 hours. A 35- to 45-kD glycosylated form of CHIP28 was also apparent (Fig. 1B).

We determined the osmotic water permeability after transfer of oocytes from a 200 to a 70 mosM solution by monitoring changes in cell volume with videomicroscopy (19). Control-injected oocytes swelled minimally and failed to rupture even after incubations of >1 hour, whereas oocytes injected with CHIP28 RNA consistently swelled to 1.3 to 1.5 times their initial volume and ruptured within 5 min (Fig. 2). The coefficients of osmotic water permeability (P_f) at 22°C were calculated from the rates of swelling of control-injected oocytes [$P_f = (26.3 \pm 16.5) \times 10^{-4}$ cm/s (\pm SD), $N = 18$] and of CHIP28 RNA-injected oocytes [$P_f = (216 \pm 60) \times 10^{-4}$ cm/s, $N = 24$]. Osmotic water permeability was not increased by injection of oocytes with CHIP28 antisense RNA (10 ng) [$P_f = (18.7 \pm 2.2) \times 10^{-4}$ cm/s, $N = 2$]. When oocyte swelling assays were conducted at 10°C, the osmotic water permeability of the control-injected oocytes was greatly reduced [$P_f = (5.2 \pm 2.8) \times 10^{-4}$ cm/s, $N = 6$], whereas that of CHIP28 RNA-injected oocytes was only modestly reduced [$P_f = (177 \pm 23) \times 10^{-4}$ cm/s, $N = 3$]. The Arrhenius activation energy (E_a) (20) was lower in CHIP28 RNA-injected oocytes ($E_a < 3$ kcal/mol) than in control-injected oocytes ($E_a > 10$ kcal/mol). These values are comparable to those reported for water channels in mammalian RBCs and renal proximal tubules ($P_f = 150$ to 200×10^{-4} cm/s; osmotic > diffusional water permeability; and $E_a < 4$ kcal/mol) (1–3).

Control experiments confirmed the specificity of the effect of CHIP28 expression on osmotic water permeability. Expressed

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sion of γ -aminobutyric acid (GABA) channels in oocytes injected with GABA channel RNA showed an increase in GABA-induced membrane conductance but not osmotic water permeability (21). Thus osmotic water permeability is not a general property of membrane channels. Amphotericin B forms unselective pores in biomembranes (22) and produced large

membrane conductance and increased osmotic water permeability (23). In contrast, when CHIP28 RNA-injected oocytes were compared to control-injected oocytes with a two-electrode voltage clamp, no newly expressed membrane currents were detected in oocytes immersed in Ringer's solution ($N = 8$), in Barth's buffer with urea substituted for NaCl ($N = 2$), or in Barth's buffer diluted with distilled water to 70% tonicity ($N = 3$) (23). Therefore CHIP28 does not appear to function as an ion channel.

Osmotic water permeability through water channels is inhibited by mercurial sulfhydryl reagents and is restored with reducing agents; diffusion of water through lipid bilayers is unaffected by these reagents (1–3). Incubation of CHIP28 RNA-injected oocytes in 0.3 mM HgCl_2 reduced the osmotic water permeability; more complete

inhibition resulted from incubation in 3 mM HgCl_2 (Table 1). When the CHIP28 RNA-injected oocytes were incubated first with HgCl_2 and then with β -mercaptoethanol, inhibition was reversed, and the oocytes swelled and ruptured. These reagents did not alter osmotic water permeability in control-injected oocytes (Table 1).

We measured the osmotic water permeability in oocytes injected with increasing amounts of in vitro-transcribed CHIP28 RNA. The quantity of RNA injected produced corresponding increases in osmotic water permeability that reflected the quantity of CHIP28 protein expressed (Table 2). However, the relative osmotic water permeability calculated per CHIP28 molecule in normal human RBCs ($P_f = 1.6 \times 10^{-13} \text{ cm}^3/\text{s}$ per molecule of CHIP28) is greater than that in oocytes expressing CHIP28 ($P_f = 10^{-14} \text{ cm}^3/\text{s}$ per molecule of CHIP28) (24). This suggests that either some of the CHIP28 protein expressed in oocytes may be trapped within intracellular compartments, the organization of CHIP28 in oocyte extracellular membranes may be suboptimal, regulatory factors may be required, or the oocyte cytoskeleton may impede more rapid swelling.

The characteristics of CHIP28 are also consistent with other known features of water channels. For example, (i) the total number of CHIP28 monomers in human red cells (2×10^5) (11) is similar to the number of water channels estimated from biophysical analyses of human RBCs (2.7×10^5) (2); (ii) the 28.5-kD molecular size of a CHIP28 monomer (11) is similar to the 30-kD func-

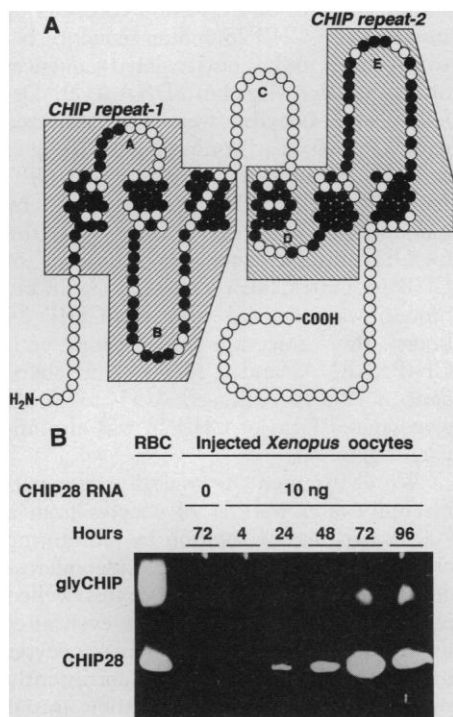


Fig. 1. Potential structure of CHIP28 protein and expression in oocytes. **(A)** Predicted membrane topology of CHIP28 protein illustrating the orientation of tandem repeats. Enclosures represent the first and second repeats within the CHIP28 protein. Black circles each represent an amino acid residue that corresponds to an identical residue in the other repeat; gray circles represent conservative substitutions; white circles represent either amino acids not conserved between the repeats or those outside of the repeats. Intracellular structures are represented at the bottom of the model, and extracellular structures at the top. This model predicts that repeats 1 and 2 are oriented 180° to each other (for example, intracellular loop B corresponds to extracellular loop E). **(B)** Contact print of an immunoblot of human RBC membranes and *Xenopus* oocytes injected with 0 or 10 ng of in vitro-transcribed CHIP28 RNA. After incubation for up to 96 hours, the oocytes were homogenized in 1.25% (w/v) SDS (40 μl), subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose. A contact print of an immunoblot of red cell membranes (0.5 μg of protein) and solubilized oocytes (10 μl) is shown after incubation with a 1:1000 dilution of anti-CHIP28 (11, 12) and visualization with ^{125}I -labeled protein A (27). Positions of CHIP28 and *N*-glycosylated CHIP28 (glyCHIP) are shown at the left.

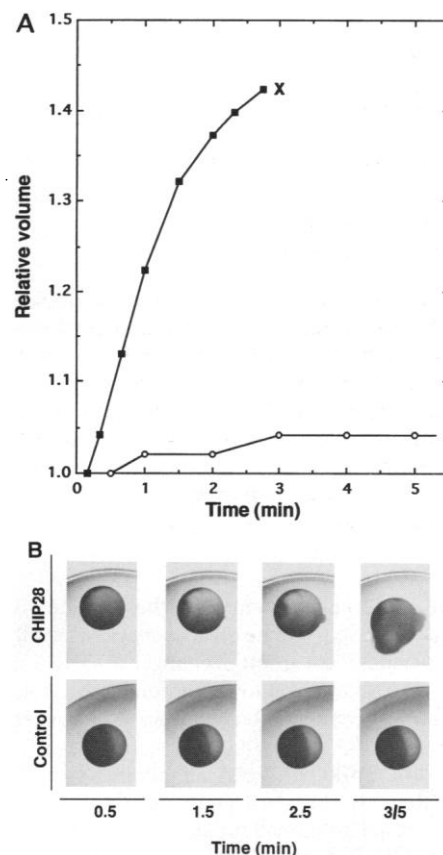


Fig. 2. Increased osmotic water permeability of CHIP28 RNA-injected *Xenopus* oocytes. After 72 hours, control-injected and CHIP28 RNA-injected (10 ng) oocytes were transferred from 200 mosM to 70 mosM modified Barth's buffer, and changes in size were observed by videomicroscopy (19). **(A)** Osmotic swelling of representative control-injected (open circles) and CHIP28 RNA-injected (filled squares) oocytes. Time of rupture is denoted (X). **(B)** Photos of injected oocytes at indicated times. Oocytes injected with CHIP28 RNA (3 min) or control (5 min) are denoted 3/5.

Table 1. Inhibition of osmotic water permeability of CHIP28 RNA-injected oocytes with HgCl_2 , and restoration with β -mercaptoethanol (ME). After 72 hours, control- or CHIP28 RNA-injected (10 ng) oocytes were treated as indicated. Some oocytes were left in standard Barth's buffer prior to measurement of osmotic swelling at 22°C . Other oocytes were incubated 5 min in Barth's buffer containing 0.3 mM or 3 mM HgCl_2 prior to osmotic swelling in the presence of HgCl_2 . Still other oocytes were incubated with HgCl_2 as described, then removed and incubated for 15 min in 5 mM ME prior to osmotic swelling in the presence of ME.

RNA injected	Oocyte treatment (mM)		P_f	SD*	N†
	HgCl_2	ME			
None	0	0	27.9	18.8	8
	0.3	0	20.3	9.2	2
	0.3	5	25.4	2.2	2
CHIP28	0	0	210	40.7	10
	0.3	0	80.7	3.7	3
	3.0	0	34.5	11.2	3
	0.3	5	188	50.8	3

*SD, standard deviation.

†N, number of experiments.

Table 2. Comparisons between the amount of CHIP28 RNA injected, osmotic water permeability, and number of copies of CHIP28 protein expressed in *Xenopus* oocytes. After 72 hours, control-injected oocytes and oocytes injected with 0.1 to 10 ng of CHIP28 RNA were studied for osmotic swelling at 22°C. The amount of CHIP28 protein was estimated by dilutional immunoblotting (11, 27).

CHIP28 RNA injected (ng)	P_i	SD	N	CHIP28 ex- pressed*
	cm/s $\times 10^{-4}$			
0	13.7	3.3	3	0
0.1	50.0	10.1	3	<0.1
0.5	112	29.2	4	0.4
2.0	175	38.4	4	1.6
10.0	221	14.8	2	10

*Copies per oocyte $\times 10^{-11}$.

tional unit of proximal renal tubule water channels as revealed by radiation inactivation studies (25); (iii) the major 3-kb CHIP28 transcript in mouse RBC progenitors and kidneys (13) corresponds to the 2.5-kb fraction of reticulocyte and kidney mRNA encoding the greatest water channel activity (9); (iv) CHIP28 proteins in intact RBCs are impervious to proteolytic digestion (11, 12), as are water channels (7, 26); and (v) RBC membranes and the apical and basolateral faces of renal proximal convoluted tubules are the major sites of immunoreactive CHIP28 (11) and correspond to the known sites of constitutively active water channels but not to the vasopressin-regulated water channels of the distal nephron.

The possibility exists that CHIP28 may function as a water channel regulator rather than as the water channel itself. The testing of this possibility will require reconstitution of purified CHIP28 protein into liposomes and direct measurement of osmotic water permeability. However, taken together, our observations strongly suggest that CHIP28 is the functional unit of the constitutively active water channels of RBCs and proximal renal tubules and raise the possibility that other members of this protein family from diverse organisms may also function to facilitate water permeability.

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- Construction of the vector from which synthetic CHIP28 RNA was transcribed in vitro. DNA that contained the entire coding sequence of CHIP28 protein was formed by three-way ligation of (i) a 60-bp Eco RI-Xmn I DNA fragment containing nucleotides -38 to +22 (pPCR-2) and (ii) an 800-bp Xmn I-Sma I DNA fragment containing nucleotides +23 to +822 (pCHIP) of CHIP28 cDNA (13) with (iii) Eco RI-Sma I-digested pBLUESCRIPT II (Stratagene). The blunt-end 860-bp Eco RI-Sma I DNA fragment was ligated into the Bgl II site of pSP64T [P. A. Krieg and D. A. Melton, *Nucleic Acids Res.* **12**, 7057 (1984)]. A 1.1-kb Hind III-Pst I DNA that contained the CHIP28 cDNA coding sequence in frame with the 5' and 3' untranslated sequences of the *Xenopus* adult β -globin gene was ligated into pBLUESCRIPT II. Conventional molecular genetic techniques were used [J. Sambrook, E. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)] with commercially available restriction endonucleases and DNA ligase (Gibco BRL). Confirmation of the recombinants was made by enzymatic nucleotide sequencing (U.S. Biochemical). Capped RNA transcripts were synthesized in vitro, and the RNA was purified as described [J. K. Yisraeli and D. A. Melton, *Methods Enzymol.* **180**, 42 (1989)]. Sense CHIP28 RNA was synthesized with T3 RNA polymerase and Sma I-digested CHIP28 expression vector, and antisense CHIP28 RNA was made with T7 polymerase from Hind III-digested vector.
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$$V/V_0 = (D1 \times D2)^{3/2} / (D1_0 \times D2_0)^{3/2}$$
The change in relative volume with time, $d(V/V_0)/dt$, up to 8 min (or time of oocyte rupture) was fitted by computer to a quadratic polynomial, and initial rates of swelling were calculated. Osmotic water permeability (P_i , in cm/s) was calculated from osmotic swelling data, initial oocyte volume ($V_0 = 9 \times 10^{-4}$ cm³), initial oocyte surface area ($S = 0.045$ cm²), and the molar volume of water ($V_w = 18$ cm³/mol) (6):
$$P_i = [V_0 \times d(V/V_0)/dt] / [S \times V_w \times (\text{osm}_{in} - \text{osm}_{out})]$$
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- Calculated from $P_i \times S \times N^{-1}$, where human RBC $P_i = 200 \times 10^{-4}$ cm/s (1), the surface area ($S = 1.6 \times 10^{-6}$ cm²) and $N = 2 \times 10^5$ CHIP28 proteins per human red cell (11).
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