CH28 is a major water transporting protein in erythrocytes and kidney which forms tetramers in membranes (Verhavatza, J. M., Brown, D., Sabolic, I., Valenti, G., Ausiello, D. A., Van Hoek, A. N., Ma, T., and Verkman, A. S. (1993) J. Cell Biol. 123, 605-618). To determine whether CH28 monomers function independently, chimeric cDNA dimers were constructed which contained wild-type CH28 in series with either wild-type CH28, a non-water transporting CH28 mutant (CH189W), or a functional but mercurial-insensitive CH28 mutant (CH189S). Transcribed cRNAs were injected into Xenopus oocytes and plasma membrane expression was assayed by quantitative immunofluorescence. Water channel function was measured by osmotically induced swelling. CH28 homo- and heterodimers were targeted to the oocyte plasma membrane and functioned as water channels. Relative osmotic water permeability (P) values (normalized for plasma membrane expression of monomeric subunits) were: 1.0 (CH28 monomer), 0.0 (CH189W), 1.07 (CH189S), 1.10 (CH28-CH28 dimer) and 0.52 (CH28-CH189S). The increase in oocyte P was linearly related to plasma membrane expression of wild-type CH28 and CH189S subunits. HgCl2 (0.3 mm) inhibited channel-mediated P in oocytes expressing wild-type CH28 monomers and dimers by 85-90%, but did not inhibit P in oocytes expressing CH189S. HgCl2 inhibited P in oocytes expressing CH28-CH189S dimers by 44 ± 10%, consistent with one mercurial-sensitive and one insensitive subunit in the heterodimer. These results indicate that despite their assembly in tetramers, monomeric CH28 subunits function independently as water channels.

Functional Independence of Monomeric CH28 Water Channels Revealed by Expression of Wild-type Mutant Heterodimers

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CH28 is a major water transporting protein in erythrocytes and kidney which forms tetramers in membranes (Verhavatz, J. M., Brown, D., Sabolic, I., Valenti, G., Ausiello, D. A., Van Hoek, A. N., Ma, T., and Verkman, A. S. (1993) J. Cell Biol. 123, 605-618). To determine whether CH28 monomers function independently, chimeric cDNA dimers were constructed which contained wild-type CH28 in series with either wild-type CH28, a non-water transporting CH28 mutant (CH189W), or a functional but mercurial-insensitive CH28 mutant (CH189S). Transcribed cRNAs were injected into Xenopus oocytes and plasma membrane expression was assayed by quantitative immunofluorescence. Water channel function was measured by osmotically induced swelling. CH28 homo- and heterodimers were targeted to the oocyte plasma membrane and functioned as water channels. Relative osmotic water permeability (P) values (normalized for plasma membrane expression of monomeric subunits) were: 1.0 (CH28 monomer), 0.0 (CH189W), 1.07 (CH189S), 1.10 (CH28-CH28 dimer) and 0.52 (CH28-CH189S). The increase in oocyte P was linearly related to plasma membrane expression of wild-type CH28 and CH189S subunits. HgCl2 (0.3 mm) inhibited channel-mediated P in oocytes expressing wild-type CH28 monomers and dimers by 85-90%, but did not inhibit P in oocytes expressing CH189S. HgCl2 inhibited P in oocytes expressing CH28-CH189S dimers by 44 ± 10%, consistent with one mercurial-sensitive and one insensitive subunit in the heterodimer. These results indicate that despite their assembly in tetramers, monomeric CH28 subunits function independently as water channels.
Transcribed cRNA was expressed in Xenopus oocytes. Plasma membrane protein expression was assayed by a novel quantitative immunofluorescence method, and water transport function was measured from the kinetics of oocyte swelling in response to osmotic gradients. The results provided direct evidence that CHIP28 forms tetramers in membranes, the individual monomers function independently.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs—The coding sequence of human CHIP28 was subcloned into a modified SP6 vector containing the Xenopus 5'-untranslated globin enhancer (Fig. 1) to give plasmid pSP64.CHIP28 as described previously (6). The CHIP28 mutants C189S and C189W were prepared by site-directed mutagenesis as described previously (24). To construct dimeric cDNAs, plasmid pSP64.CHIP28 was used as template for PCR amplification (20 cycles) using sense primer 5'-GCCACATG-GCCACCGAGTTCAAGAAG-3' and antisense primer 5'-CTTAGATTCATCAATTGCGGCTTCATCTC-3' encoding Nol and BspHI restriction sites, respectively. This PCR fragment encodes the CHIP28 NH2-terminus translation initiation codon (Ncol site) to nucleotide 269 (AAA) just 5' to the COOH terminus stop codon. The PCR fragment was digested with Ncol and BspHI, gel purified, and ligated into the Ncol-linearized, phosphatase-treated plasmid pSP64.CHIP28. The BspHI restriction site was destroyed following ligation. Insert orientation was confirmed by restriction mapping. This chimeric cDNA (pSP64.CHIP28-CHIP28) encodes a single tandem repeat of two CHIP28 subunits.

To prepare cDNAs encoding CHIP28 trimers (and then tetramers), the procedure was repeated using the digested PCR fragment and Ncol-linearized plasmid pSP64.CHIP28-CHIP28. To prepare dimers containing wild-type CHIP28 in series with mutant CHIP28, the C189S and C189W coding sequences were subcloned into pSP64 at Ncol and BamHI restriction sites. The wild-type CHIP28 PCR fragment containing Ncol and BamHI sites was ligated into Ncol-linearized plasmids pSP64.C189S and pSP64.C189W.

To prepare CHIP28-MIP26 dimers, a MIP26 fragment was PCR amplified using sense primer 5'-CCCCCTGCGATGGAACCTGGTCTC-3' and antisense primer 5'-CAGTTCGCATGATCCGTTCGCTGAGCTCGACGACG-3' encoding Ncol and SacI restriction sites, respectively. PCR fragments were digested by Ncol and SacI, gel purified, and ligated into Ncol- and SacI-digested plasmid pSP64. Plasmid pSP64.MIP26 was linearized with Ncol, phosphatase treated, gel purified, and ligated with the wild-type CHIP28 PCR fragment at Ncol and BspHI sites as above.

**RNA Transcription**—Complementary RNA was transcribed in vitro using SP6 polymerase (Bethesda Research Laboratories) and 4 μg of plasmid DNA in a 100-μL volume at 37 °C for 2 h. Digoxigenin triophosphate (Pharmacia Fine Chemicals) was included in the reaction mixture for capping. At the completion of the reaction, plasmid DNA was digested with RNase-free DNase (Invitrogen). After phenol-chloroform-isooctane extraction and precipitation, the tRNA was washed and incubated with immune serum containing a polyclonal antibody to CHIP28 (1:500 dilution) (8) or with preimmune (control) serum in PBS containing 1% bovine serum albumin for 1 h. Sections were rinsed three times with PBS and incubated for 30 min with a fluorescein-conjugated goat anti-rabbit IgG (Boehringer Mannheim) (1:50) in PBS containing 1% bovine serum albumin. Slides were washed three times with PBS and covered with Dabco (diazabicyclo octane) solution to retard fluorescence quenching and a glass coverslip. Fluorescence was quantified using a Leitz inverted epifluorescence microscope equipped with a fluorescein filter set, a 10 x air objective (numerical aperture 0.4), and a cooled charge coupled device camera (Photometrics). The details of the imaging system, instrument calibration, and background-subtraction software were described previously (27). For each set of oocytes, at least 60 images were obtained from multiple areas of oocytes on at least three separate slides. Background-subtracted fluorescence was measured and normalized per unit length of the oocyte plasma membrane.

**Cell-free Translation**—In vitro transcribed cRNA was added to a rabbit reticulocyte lysate (RRL) mixture containing [35S]methionine as described previously (6). Microsomal membranes prepared from dog pancreas were added in some experiments at the start of translation to a final concentration of 8 A260. N-Linked glycosylation in the presence of microsomes was confirmed by translation in the presence of a tripeptide competitive inhibitor of oligosaccharyl-transferase, AsAg-Tyr-Thr. Translation of monomers was performed as described previously (6). Oocyte swelling in response to a 20-fold dilution of the extracellular Barth's buffer with distilled water (26) was included in the translation reaction mixture. Translation of CHIP28 dimeric constructs produced bands of 57, 60, and 63 kDa, representing the non-glycosylated, singly and doubly glycosylated protein, respectively. No monomeric protein was produced. The translated products from the CHIP28-MIP26 (WT-MIP26) chimera underwent only a single glycosylation event as expected. Translation of CHIP28 trimeric and tetrameric constructs generated chains of 75 and 110 kDa, respectively. However, translation was less efficient, and the products were variably glycosylated in the presence of microsomal membranes.

The cDNA constructs encoding wild-type CHIP28 monomers and trimers were sequenced and no frameshifts were observed. The cRNA was expressed in Xenopus oocytes. Fig. 3A shows representative time courses of oocyte swelling in response to dilution of the extracellular buffer with distilled water. Water permeability in oocytes expressing CHIP28 monomers and dimers was strongly increased, whereas little functional expression was observed for the trimers and tetramers. Subsequent experiments were therefore carried out with monomers and dimers where expres-

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1 The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline; WT, wild-type; RRL, rabbit reticulocyte lysate.
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Fig. 1. cDNA constructs for expression of CHIP28 dimers. A, schematic of vectors showing SP6 promoter, 5'-untranslated sequence of the *Xenopus* globin gene and CHIP28 insert(s). See text for details. B, schematic of dimeric protein containing two CHIP28 units in series. The nucleotide and amino acid sequence at the fusion site is shown.

Expression levels were adequate for quantitative analysis of protein expression and function. Fig. 3B shows the relationship between the amount of cRNA injected and oocyte osmotic water permeability (Pₖ) measured at 10 °C. Pₖ increased linearly with the cRNA amount to 0.25 ng for the CHIP28 monomer and to 0.5 ng for the dimer. The decreased "efficiency" for expression of dimers compared with monomers indicates differences in plasma membrane protein expression and/or single channel water permeabilities.

To relate oocyte water permeability to CHIP28 expression, a quantitative immunofluorescence approach was developed to determine the relative amount of CHIP28 expressed at the oocyte plasma membrane. Oocytes expressing various constructs were fixed, sectioned and immunostained as described under "Experimental Procedures." All samples were processed in parallel and at least three separate tissue blocks (each containing 10–20 oocytes) were prepared for each sample to minimize day-to-day and oocyte-to-oocyte variability in sectioning and immunostaining. Oocyte sections were examined as shown in Fig. 4A. A measuring area surrounding a 50–100 μm length of membrane was selected, and the background-subtracted integrated pixel intensity was quantified and expressed per unit length of oocyte membrane. All measurements were made in membranes of the oocyte "animal" pole. Fig. 4 shows strong plasma membrane staining in oocytes expressing CHIP28 (A) and C189W (D) monomers, and WT-WT (B), WT-C189W (E), and WT-C189S (F) dimers. The low background staining observed in the water-injected (control) oocytes (C) was not different from that observed in CHIP28-expressing oocytes using preimmune serum (not shown).

The relative water permeability of wild-type and mutant CHIP28 constructs was compared in the next series of studies. Fig. 5A shows representative swelling data. Oocytes expressing the WT-C189W dimer had significant, although less water permeability than oocytes expressing the WT-WT dimer. Oocytes expressing the C189W monomer had the same water permeability as water-injected oocytes even though the C189W protein was trafficked normally to the oocyte plasma membrane. These results indicate that a non-functional C189W monomer does not inactivate WT CHIP28 in the WT-C189W heterodimer. Thus, the monomeric CHIP28 subunit is likely able to function independently as a water channel.

Quantitative analysis of the functional data for a series of oocytes is provided in Fig. 5B. The ordinate is the increase in oocyte Pₖ (∆Pₖ) calculated from the difference between Pₖ in oocytes expressing the indicated cRNA and Pₖ in water-injected oocytes. The abscissa is the relative plasma membrane expression of CHIP28 (or mutant) subunits determined by quantitative immunofluorescence. Abscissa values have been normalized per unit length of oocyte plasma membrane. Background signal (generally <5% of total signal) obtained from parallel measurements in water-injected oocytes (as in Fig. 4C) has been subtracted. A value of unity has been assigned to oocytes expressing 0.5 ng of wild-type CHIP28 monomer. Fig. 5B shows a linear relationship between ∆Pₖ and plasma membrane expression for oocytes injected with cRNA encoding WT CHIP28 and C189S monomers and WT-WT and WT-C189S dimers. These results indicate similar single channel water permeabilities for the WT and C189S monomers when expressed in monomeric or dimeric constructs. In contrast, C189W monomers were expressed at the oocyte plasma membrane but exhibited no increase in Pₖ. In two separate sets of studies, oocytes expressing the WT-C189W heterodimer had 47 ± 5% (∆Pₖ/plasma membrane expression) compared with that of the WT-WT homodimer. This values does not differ significantly from 50%. Therefore, a non-functional subunit in a CHIP28 dimeric chimera does not affect the function of the wild-type subunit.

MIP26 is homologous to CHIP28 but does not function as a water channel (17). Pₖ in oocytes expressing MIP26 monomers was not different from that in water-injected oocytes. Pₖ in oocytes expressing CHIP28-MIP26 heterodimers was 14 ± 4 × 10⁻⁴ cm/s (n = 45), significantly greater than 7.8 ± 0.8 × 10⁻⁴ cm/s (n = 38) in control oocytes (p < 0.02). Thus CHIP28 is functional even when fused with a very different non-water
Expression of the CHIP28-MIP26 heterodimer was too low for quantitative analysis of plasma membrane expression.

Studies of mercurial inhibition of a heterodimer containing a mercurial-sensitive monomer (WT CHIP28) and a mercurial-insensitive monomer (C189S) were carried out. As shown in Fig. 6A, addition of HgCl₂ during the swelling assay strongly inhibited water permeability in oocytes expressing the WT-WT homodimer but had little effect in oocytes expressing the C189S mutant. Intermediate inhibition was observed in oocytes expressing the WT-C189S heterodimer in which only the WT subunit is sensitive to HgCl₂. Percentage inhibition was determined from the initial slope of the swelling curve (before HgCl₂ addition) and the slope measured at 1–2 min after HgCl₂ addition. The data obtained for a series of oocytes is summarized in Fig. 6B. The increase in \( P_f \) over that observed in water-injected oocytes \( \Delta P_f \) was strongly inhibited (85–90%) by 0.3 mM HgCl₂ in oocytes expressing WT CHIP28, WT-MIP, and WT-C189W proteins but was not inhibited significantly in oocytes expressing C189S. \( \Delta P_f \) in oocytes expressing WT-C189S protein was inhibited by 44 ± 7%, not significantly different from 42.5% (the average between 0 and 85%). This finding strongly supports the conclusion that CHIP28 monomers function independently.

### DISCUSSION

The purpose of this study was to determine whether CHIP28 monomers functioned independently as water channels. We reported recently that CHIP28 was assembled as tetramers in biological membranes and reconstituted proteoliposomes (20). The strategy adopted in the present study was to examine the function of heterodimers consisting of wild-type and mutant CHIP28 monomers. The association of wild-type and mutant CHIP28 monomers was forced by expression of cDNA chimeric dimers encoding wild-type CHIP28 in series with wild-type or mutant CHIP28. Previous studies in which oocytes were injected with cRNA encoding wild-type and mutant CHIP28 monomers (23, 24) could not define the functional water transporting unit size because association of wild-type and mutant CHIP28 monomers could not be assured. Further, a linear dependence of water permeability on the amount of CHIP28 expression gives no information about the functional water transporting unit size if all monomers are associated as tetramers. We find here that monomeric subunits in wild-type mutant CHIP28 heterodimers functioned independently based on two experimental approaches: measurement of single channel water permeability for heterodimers containing CHIP28 and non-functional (C189W) subunit, and HgCl₂ inhibition of water permeability in a heterodimer containing CHIP28 and C189S. The results provide strong evidence that CHIP28 monomers function independently and suggest that each CHIP28 subunit contains a separate aqueous pore and mercurial inhibition site.

The construction of cDNA chimeras encoding tandem repeat homo- and heterodimers to determine functional subunit size in a membrane-associated multimer was recently introduced for studies of K⁺ channel function. Tytsga and Hess (25) examined the gating of heterotetramers composed of two or four RCK1 K⁺ channels with different gating properties on a single cDNA. Like CHIP28, both the amino and carboxyl termini of the RCK1 channel were cytoplasmically oriented. They reported cooperative gating in RCK1 channels in which the individual subunits interacted at a functional level. From similar studies on heterotetramers consisting of TEA sensitive and insensitive RBK1 K⁺ channels, Kavanaugh et al. (30) concluded that bound TEA interacts simultaneously on all subunits. In contrast, our results showed that a mutant CHIP28 subunit affects neither the water transporting function nor the mercurial sensitivity of adjacent wild-type CHIP28. However, our data provide no information about whether the membrane assembly of CHIP28 in tetramers is required for water transporting function and whether isolated CHIP28 monomers exist and are functional. The non-covalent assembly of CHIP28 monomers in tetramers probably provides the lowest thermodynamic energy state in membranes.

An important methodological development reported here is the application of quantitative immunofluorescence to measure CHIP28 plasma membrane expression. Oocytes expressing...
FIG. 3. Expression of wild-type CHIP28 constructs in Xenopus oocytes. A, time course of oocyte swelling at 10°C in response to a 20-fold dilution of the extracellular Barth’s buffer with distilled water. Oocytes were microinjected with 1 ng of cRNA encoding CHIP28 monomers or multimers. Relative oocyte volume was determined by a real-time imaging method (26). B, oocyte 

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P_r(P, in oocyte P_r (S.E., 10–12 oocytes in each group) as a function of the amount of injected cRNA encoding CHIP28 monomers and dimers.

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\text{Relative oocyte volume} = \frac{1.0}{1.2}
\]

\[
\text{INJECTED cRNA (ng)}
\]

various CHIP28 constructs were fixed, sectioned, and immunostained in parallel, and plasma membrane fluorescence was quantified by image analysis. A cooled CCD camera was utilized for its excellent sensitivity and linear response characteristics. Based on a previous quantitative imaging study of endosomal acidification (26), custom C-language software was written to determine the background-subtracted pixel intensity/unit length of oocyte membrane. The linearity between incremental \( P_r \) and plasma membrane immunofluorescence in WT CHIP28-injected oocytes supported the utility of the approach. However, there were several sources of variability in the imaging assay, including variations in: protein expression in different oocytes and in different areas of each oocyte, tissue section thickness and geometry, and antibody staining efficiency. For this reason all samples were processed in parallel, measurements were made only from the animal pole in oocytes sectioned near their greatest diameters, and >50 measurements were carried out for each sample using multiple oocytes on separate slides. Notwithstanding these technical concerns, quantitative immunofluorescence provides a practical approach to quantifying plasma membrane expression of a foreign protein in Xenopus oocytes for determination of single channel properties.

There were several assumptions made in the analysis that we believe are justified, yet they cannot be subjected to direct experimental validation. It was assumed that the anti-CHIP28 antibody stained with equal efficiency wild-type CHIP28, and the C189S and C189W mutants. We believe that this assumption is justified because the antibody is primarily directed against the CHIP28 carboxyl terminus (8); the carboxyl terminus is not required for function and is located on the opposite side of the membrane from residue C189. It was assumed that the wild-type mutant heterodimers participate in the formation of membrane-associated tetramers. If, however, the het-
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REFERENCES


**Fig. 6. Mercurial inhibition of CHIP28 homo- and heterodimers.** A, time course of swelling in oocytes injected with cRNA encoding WT-WT (1 ng), C189S (0.5 ng), or WT-C189S (1 ng). At the arrow, HgCl₂ (final concentration 0.3 mM) was added. B, summary of data (S.E., n = 20-30) for a series of oocytes. Percentage inhibition of incremental ΔPᵢ (ΔΔPᵢ) was calculated from fitted swelling curve slopes before and 1-2 min after HgCl₂ addition.

We conclude that CHIP28 monomers function independently even though they associate in membranes as tetramers. Each monomer might therefore contain a separate water transport channel, and CHIP28 monomers remained isolated, then the principal conclusion of this study, that CHIP28 monomers functioned independently, would remain valid. It is possible that CHIP28 heterodimers might form large octameric complexes in which wild-type CHIP28 monomers were centrally associated with the mutant subunits at the periphery; however, steric constraints would likely prevent the formation of such large complexes. Even if a multimeric complex did form, the HgCl₂ inhibition studies of water permeability in oocytes expressing the WT-C189S heterodimer indicate that monomeric subunits function independently.

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