Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family

GREGORY M. PRESTON AND PETER AGRE

Departments of Medicine and Cell Biology/Anatomy, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

Communicated by Pedro Cuatrecasas, September 20, 1991 (received for review August 21, 1991)

ABSTRACT CHIP28 is a 28-kDa integral membrane protein with similarities to membrane channels and is found in erythrocytes and renal tubules. A cDNA for CHIP28 was isolated from human fetal liver cDNA template by a three-step polymerase chain reaction (PCR) cloning strategy, starting with degenerate oligonucleotide primers corresponding to the N-terminal amino acid sequence determined from purified CHIP28 protein. Using the third-step PCR product as a probe, we isolated a recombinant from a human bone marrow cDNA library. The combined sequence of the PCR products and bone marrow cDNA contains 38 base pairs of 5' untranslated nucleotide sequence, an 807-bp open reading frame, and ~2 kilobases of 3' untranslated sequence containing a polyadenylation signal. This corresponds to the 3.1-kilobase transcript identified by RNA blot-hybridization analysis. Authenticity of the deduced amino acid sequence of the CHIP28 protein C terminus was confirmed by expression and immunoblotting. Analysis of the deduced amino acid sequence suggests that CHIP28 protein contains six bilayer-spanning domains, two extracellular potential N-glycosylation sites, and intracellular N and C termini. Search of the DNA sequence data base revealed a strong homology with the major intrinsic protein of bovine lens, which is the prototype of an ancient but recently recognized family of membrane channels. These proteins are believed to form channels permeable to water and possibly other small molecules. CHIP28 shares homology with all known members of this channel family, and it is speculated that CHIP28 has a similar function.

The erythrocyte membrane protein has been shown to have an accessible source of new proteins with structural or metabolic functions and a useful model with general relevance to plasma membranes (for reviews, see refs. 1 and 2). During isolation of the 32-kDa Rh polypeptides from human erythrocytes, a 28-kDa integral membrane protein copurified and was considered a breakdown product (3, 4). The 28-kDa protein was later shown to be a unique molecule that is abundant in erythrocytes and renal tubules, and a subpopulation is N-glycosylated (5).

The function of the 28-kDa protein is not yet known, but several observations suggest that it is a membrane channel. The 28-kDa protein behaves as a tetramer when solubilized in Triton X-100, and the N-terminal amino acid sequence of the purified 28-kDa protein (6) is related to that of MIP26, the 26-kDa major intrinsic protein of bovine lens fiber cells (7). When reconstituted into planar lipid bilayers, MIP26 forms tetrameric channels with voltage-regulated conductance (8, 9), and MIP26 appears to function as a channel through which lens fiber cells absorb interstitial fluid (8). MIP26 is the prototype of a family of membrane proteins recently identified in diverse species (10). This report describes isolation of the cDNA* for the 28-kDa protein, which has homology with all known members of the MIP channel family and is referred to as "CHIP28" for channel-like integral membrane protein of 28 kDa.

MATERIALS AND METHODS

Oligonucleotide Primers for Polymerase Chain Reaction (PCR) Cloning. Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer. Primers A–E contained 5' extensions with restriction sites (underlined below) to facilitate cloning the PCR products. Degenerate sense primer A (5'-CTTCTAGAT-TCTGAGGCCCAGT-GTSCCNGA-3') corresponds to amino acids 9–16 (Phe-Arg-Ala-Val-Ala-Glu), and degenerate antisense primer B (5'-TAATCGAT-CCCCATRWSRATRAASACRAA-3') corresponds to amino acids 22–29 (Phe-Val-Ile-Ser-Ile-Gly) determined by microsequencing purified CHIP28 protein (6). Degeneracy is denoted as follows: R = A or G; S = G or C; T = C, A, and G; and W = A or T. Antisense primer C (5'-GCTTCAAGAGGSGTGGCAGAATC-3') and sense primers D (5'-GCTCTAGAGTCTCTTAAAGAAGCTTTCG-3') and E (5'-GAGTTATCTCCCTTGAGGCGACTGTCGTTGCC-3') correspond to the nucleotide sequence identified by the first and second PCR cloning steps (below). Primers λ-L (5'-GTTGGCGGCCGAGCTCCTGGGAGGG-3') and 7-R (5'-TGTACACCA-GACCAACTGTGAATG-3') correspond to sequence on the left and right arms flanking the EcoRI cloning site of Agt11.

Plasmid Constructions, Fetal Liver PCR Clones. Standard molecular biological methods were routinely employed (11). The cloning of CHIP28 from fetal liver was performed by a three-step PCR strategy. The template for each step was 2–10 ng of DNA from a λgt11 human fetal liver cDNA library (provided by Bernard Forget, Yale University). Control reactions contained nonrecombinant phage λ DNA. In step 1, PCR amplification was made with degenerate primers A and B to determine the 19-bp sequence between their sites on the cDNA (1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; 25 cycles). The PCR products of the expected size [70–90 base pairs (bp)] were eluted from an acrylamide gel, cloned into pBluescript II (Stratagene), and transformed into bacteria. Plasmid DNA was sequenced from five colonies, and one (pPCR-1) contained a 19-bp insert corresponding to the intervening amino acid sequence (Phe-Leu-Ala-Thr-Thr-Leu) from which antisense primer C was designed.

In step 2, PCR amplifications were designed to determine the nucleotide sequence of the 5' end of the CHIP28 cDNA. First antisense primer B and primer λ-L were used to enrich the template mixture (1 min at 94°C, 1 min at 54°C, and 1 min at 72°C; 30 cycles). An aliquot was then amplified with antisense primer C and primer λ-L (30 cycles). The PCR products were cloned into pBluescript II and transformed into bacteria, and colony lifts on Colony/Plaque Screen

Abbreviations: CHIP28, channel-like integral membrane protein of 28 kDa; MIP26, major intrinsic protein of bovine lens (ref. 7).

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M77829).
membranes (DuPont) were probed with $^{32}$P-end-labeled primer A. DNA sequencing was performed on plasmid DNA isolated from seven colonies that hybridized strongly with primer A, and one insert of 110 bp corresponded to the 3' end of CHIP28 cDNA (pPCR-2, Fig. 1). Partially overlapping sense primers D and E were derived from this sequence.

In step 3, PCR amplifications were designed to determine the nucleotide sequence of the 3' end of the CHIP28 cDNA. First, sense primer D and primer λ-R were used to enrich the template mixture (1 min at 94°C, 1 min at 65°C, and 3.5 min at 72°C; 35 cycles). DNA molecules >500 bp were purified by using the GeneClean II kit (Bio 101, La Jolla, CA) and amplified with sense primer E and primer λ-R (3 cycles). An aliquot of the final reaction was analyzed by Southern analysis with $^{32}$P-end-labeled primer C as probe. A single 850-bp product that hybridized with primer C was cloned into pBluescript II. The plasmid DNA was completely sequenced on both strands (pPCR-3). This clone contained sequences corresponding to the known N terminus and the distal amino acid sequence of CHIP28 protein (see Results and Discussion).

Isolation of a CHIP28 cDNA from Human Bone Marrow. The insert from pPCR-3 was gel-purified, labeled with [α-32P]dCTP (6,000 Ci/mmol, Amersham; 1 Ci = 37 GBq) by using a random priming kit (Pharmacia LKB), and used to probe 2.5 × 10⁶ plaques of an adult human bone marrow cDNA library in Agt10 (Clontech) on Colony/Plaque Screen membranes. A single positive plaque was isolated, and the 2.9-kb insert was subcloned into pBluescript II (pCHIP, Fig. 1).

**Fig. 1.** Organization of CHIP28 recombinants and their nucleotide and deduced amino acid sequences. (Upper) Diagram representing the second-step fetal liver PCR product (pPCR-2), the 2.9-kilobase (kb) insert isolated from the human bone marrow library (pCHIP), and the restriction and exonuclease fragments from which nucleotide sequences were obtained (arrows). The translational initiation, termination, and message polyadenylation sites are indicated. (Lower) The 5' untranslated nucleotide sequence, the open reading frame, the first 500 bp of 3' untranslated sequence, and the amino acid sequence of CHIP28 deduced from pPCR-2 and pCHIP, and the terminal 3' untranslated nucleotide sequence with the major polyadenylation signal $\AA\AA\AA\AA\AA$. The stop codon is represented by "...". Amino acid residues determined by microsequencing of the purified CHIP28 protein are underlined (6). The nucleotide sequence $\AA\AA\AA\AA\AA\AA\AA\AA$ probably corresponds to the polyadenylation signal of the 1.1-kb minor mRNA species.
Preparation of pCHIP Deletion and Expression Constructs. Approximately 1.6 kb was removed from the 3' end of pCHIP with EcoRI–HindIII. The resulting 1.3-kb EcoRI–HindIII fragment containing the 5' end of CHIP28 cDNA was used to make a series of exonuclease III deletion constructs for DNA sequencing (12).

The exonuclease III construct containing nucleotides 594–826 (encoding the last 70 amino acids of the pCHIP reading frame, the stop codon, and 14 bp of 3' untranslated sequence) was used to prepare the β-galactosidase/CHIP28 expression constructs. The 232-bp Sma I–Sma I fragment was cloned into the Sma I site of pBS (Stratagene), and plasmids with a single Sma I insert were sequenced. CHIP28-3' inserts were obtained in frame with β-galactosidase or in the reverse orientation.

DNA Sequencing and Analysis. Double-stranded DNA sequencing was performed by using Sequenase 2.0 (United States Biochemical) and [α-35S]dATP (1,000 Ci/mmol; Amersham). The amino acid sequence of CHIP28 was used to search for homologies in the GenBank DNA data base with the TFASTA program.

RNA Isolation and Northern Blot Analysis. RNA was isolated from human bone marrow, mouse kidney, and mouse spleen cells as described (13) and was enriched for poly(A)+ RNA. Anemic mouse spleen RNA was provided by Terry Bishop (Johns Hopkins University). This RNA was isolated from colony-forming units erythroid (CFU-E) progenitor- enriched mouse spleens 3.5 days into recovery from thiamphenicol-induced erythroid aplasia (14).

RNA was electrophoresed on 1% agarose gels in 20 mM Mops (3-[N-morpholino]propanesulfonic acid), pH 7.0/8 mM sodium acetate/1 mM Na2EDTA, pH 8.0/2.2 M formaldehyde. Denatured RNA was transferred to nitrocellulose filters (Bio-Rad) by capillary elution. Filters were prehybridized for 2–6 hr and hybridized overnight at 42°C in 50% formamide containing 5× SSPE (0.75 M NaCl/40 mM Na2HPO4/6 mM Na2EDTA, pH 7.4), 5× Denhardt's reagent (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 0.2% sodium dodecyl sulfate with 5–10× 104 dpm of denatured probe per ml made by gel-purifying the inserts from vectors and labeling as described above.

RESULTS AND DISCUSSION

Isolation of the cDNA Encoding CHIP28. The probe used to isolate the CHIP28 cDNA was prepared by a three-step PCR amplification of human fetal liver DNA template (see Materials and Methods). Degenerate primers were designed corresponding to proximal and distal segments of the N-terminal amino acid sequence of purified CHIP28 protein (6). Nondegernate primers were used with λ phage primers in steps 2 and 3 to amplify the 5' and 3' sequence. The fetal liver PCR products were subcloned into Bluescript vectors (pPCR-1, pPCR-2, and pPCR-3). pPCR-3 was used as a probe to isolate a single positive plaque from 250,000 phage in a λgt11 human bone marrow cDNA library. The 2.9-kb bone marrow insert was subcloned (Fig. 1, pCHIP).

Analysis of the CHIP28 cDNA. The sequences of pPCR-2 and pCHIP partially overlap and together contain the sequence of the entire 269 amino acid CHIP28 protein (Fig. 1). The sequence of pPCR-2 contains a strong homology with the consensus for translational initiation (15), followed by the nucleotide sequence encoding the first 22 amino acids of the purified CHIP28 protein that lacks the N-terminal methionine. The complete sequences of both strands of the first 1287 bp of pCHIP were determined by double-stranded DNA sequencing exonuclease III deletion constructs made from an EcoRI–HindIII restriction fragment (Fig. 1 Upper). pCHIP begins at nucleotide +15, with respect to the initiating ATG, and contains an open reading frame with a stop codon at +808. Multiple stop codons occurred within the first +400 nucleotides in the other two reading frames. The nucleotide sequences obtained from human fetal liver DNA (pPCR-3) and from adult human bone marrow (pCHIP) are identical up to nucleotide +631. From this point, the sequence of pPCR-3 corresponds to the consensus for a splice donor site, suggesting that it contains an unspliced intron (16). pCHIP contains two polyadenylation signal sequences, a weak polyadenylation signal sequence AACAAA at nucleotide +1068 followed by +2 kb of 3' untranslated sequence ending in the standard polyadenylation signal sequence AAATAAA (Fig. 1 Lower).

Several lines of evidence indicate that the deduced amino acid sequence corresponds to that of the native red cell CHIP28 protein. The size of the deduced protein (Fig. 1 Lower) and the native protein (6) are both 28.5 kDa, and the 34 N-terminal amino acids are identical. The amino acid content of the deduced protein and the native protein are also nearly identical (not shown). Confirmation that the deduced amino acid sequence of the C-terminus of CHIP28 is authentic was achieved by expression in bacteria of the exonuclease fragment +594–826 (encoding the 70 C-terminal amino acids) in frame with β-galactosidase. An antibody to the C-terminal domain of CHIP28 protein (6) reacted strongly with a 12-kDa fusion protein in extracts from bacteria transformed with the β-galactosidase/CHIP28 fusion construct (Fig. 2A, lane 3). Extracts from control cells failed to react with the antibody.

Several lines of evidence indicate that the deduced amino acid sequence corresponds to that of the native red cell CHIP28 protein. The size of the deduced protein (Fig. 1 Lower) and the native protein (6) are both 28.5 kDa, and the 34 N-terminal amino acids are identical. The amino acid content of the deduced protein and the native protein are also nearly identical (not shown). Confirmation that the deduced amino acid sequence of the C-terminus of CHIP28 is authentic was achieved by expression in bacteria of the exonuclease fragment +594–826 (encoding the 70 C-terminal amino acids) in frame with β-galactosidase. An antibody to the C-terminal domain of CHIP28 protein (6) reacted strongly with a 12-kDa fusion protein in extracts from bacteria transformed with the β-galactosidase/CHIP28 fusion construct (Fig. 2A, lane 3). Extracts from control cells failed to react with the antibody.

Fig. 2. Immunoblot of native and expressed CHIP28 protein and Northern blot analyses of CHIP28 mRNA. (A) Autoradiograph of an immunoblot containing 40 μg of protein from human erythrocyte membranes (lane 1) or extracts of bacteria transformed with Bluescript containing the CHIP28 nucleotide fragment from 594 to 829 cloned in the reverse orientation (lane 2) or in frame with β-galactosidase (lane 3). The proteins were electrophoresed into a 15% polyacrylamide/sodium dodecyl sulfate gel (17), transferred to a nitrocellulose blot (18), and incubated with affinity-purified anti-28-kDa fragment followed with 125I-labeled protein A (6). The mobility of molecular weight standards in kDa is noted at the left. (B) Lanes: 1–3, Northern blot of human bone marrow RNA: 5 μg of poly(A)+ RNA (lane 1), 30 μg of poly(A)+ RNA (lane 2), and 15 μg of total RNA (lane 3); 4–7, total RNA from mouse tissues: 10 μg of anemic spleen RNA (lanes 4 and 6), 20 μg of kidney RNA (lane 5), and 5 μg of normal spleen RNA (lane 7). The blots were hybridized with 32P-labeled inserts from pPCR-3 (containing 850 bp of fetal liver CHIP28 PCR product) (B Upper blots) or pA1 (beta-actin probe) (B Lower blots). The size of RNA markers in kb is shown at the left. The major and minor signals at 3.1 and 1.1 kb are identified by arrows.
when the same insert was in the reverse orientation relative
to the $\beta$-galactosidase open reading frame (Fig. 2A, lane 2).

Northern analysis of human bone marrow mRNA showed
a single transcript of 3.1 kb (Fig. 2B), indicating that nearly
the entire CHIP28 cDNA had been isolated. RNA isolated
from mouse kidney contained a similar transcript, suggesting
that the red cell and renal tubule CHIP28 proteins may be
encoded by the same gene. A CHIP28 transcript was not
detected in normal mouse spleen but was dramatically in-
duced in anemic mouse spleen, a rich erythroid tissue. Even
prolonged autoradiographic exposure of the blot failed to
demonstrate a CHIP28 transcript in RNA from the normal
spleen, whereas a second transcript of 1.1 kb became weakly
visible in RNA from anemic mouse spleens. RNA from both
tissues hybridized with a probe for actin.

Analysis of CHIP28 Structure. The deduced amino acid
sequence and previous biochemical studies have provided
insight into the possible structure of the CHIP28 protein.
Computer analysis of the deduced amino acid sequence using
the algorithm of Kyte and Doolittle (19) demonstrated six
strongly hydrophobic regions that most likely correspond to
bilayer spanning domains (Fig. 3 Upper). The lack of a
cleavable leader sequence and charge distribution (20) sug-
gests that the N terminus is cytoplasmic. Biochemical studies
showed that the C terminus of CHIP28 protein is also cyto-
plasmic (6) and corresponds to the hydrophilic 37-amino acid
C-terminal domain predicted by the hydrophobicity analysis.

A potential model of membrane topology was designed
(Fig. 3 Lower). A subpopulation of CHIP28 is known to be
N-glycosylated (5), and two potential N-glycosylation sites
are located on exofacial loops A and E. Endofacial loop B and
exofacial loop E are unusually hydrophobic, suggesting that
they may associate with the lipid bilayer. The fourth bilayer
spanning domain contains a glutamate, and the first, second,
and fifth may also contain charged residues. Triton X-100-
solubilized CHIP28 protein behaves like a tetramer (6) and
therefore may contain a total of 24 bilayer-spanning domains,
a characteristic of membrane channels (21).

Homology with the MIP Channel Family. Computer search
of the GenBank DNA data base identified a strong homology

![Hydropathy and proposed membrane topology of CHIP28. (Upper) Deduced amino acid sequence of CHIP28 computer-analyzed for hydrophobicity by using the algorithm of Kyte and Doolittle (19) with a seven-residue window. (Lower) Proposed topology of CHIP28 within the erythrocyte membrane (see text). Loops A, C, and E are exofacial; loops B and D and the N and C termini are endofacial. Two potential glycosylation sites are marked by an asterisk. A black background with white letters denotes residues identical in CHIP28 and MIP26 proteins (7).](image-url)
Table 1. Percent identity of amino acid sequences in CHIP28 and members of the MIP channel family

<table>
<thead>
<tr>
<th>Protein</th>
<th>CHIP-1 (14-113) (Repeat 1)</th>
<th>CHIP-2 (140-231) (Repeat 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP</td>
<td>47</td>
<td>56</td>
</tr>
<tr>
<td>Bib*</td>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>GipF†</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>Nod‡</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>TIP§</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td>TUR³</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>TobRB7⁷</td>
<td>37</td>
<td>47</td>
</tr>
</tbody>
</table>

Sequences were compared as described (22) by using GenBank BESTFIT program with gap weight 3 and length weight 0.1.

*Big Brain, Drosophila neurogene product (24).
†Glycerol facilitator from the inner membrane of Escherichia coli (25, 26).
‡Peribacteroid membrane protein from soybean/Rhizobium root nodules (27).
§Tonoplast intrinsic protein from seeds of many higher plants (28).
³Pea shoot turgor responsive gene (29).
⁴Tobacco root membrane protein (30).

with MIP26, the major intrinsic protein of bovine lens (7). When the N-termini are aligned, the deduced amino acid sequences of the two proteins are 42% identical overall (Fig. 3 Lower). However, while the C-terminal cytoplasmic domains of the two proteins are of nearly identical size, only 4 of 35 C-terminal residues are shared.

The sequences of endofacial loop B and exofacial loop E are clearly related. BESTFIT computer program analysis of the CHIP28 amino acid sequence demonstrated that the protein is comprised of two homologous domains that are 20% identical and correspond approximately to the first half (residues 14–113) and the second half of the molecule (residues 140–231). Similar domains in MIP26 also have been shown to be related, and these internal tandem repeats may have resulted from duplication of an ancestral progenitor gene (22, 23). New members of the MIP channel family recently have been identified in Drosophila and bacteria, and several have been identified in plants (10). All known members of the MIP channel family contain the internal tandem repeats (22, 23). The deduced amino acid sequences of the tandem repeats for CHIP28 and the repeats in seven members of the MIP channel family were compared (Table 1), and CHIP28 is related to all. The homologies between members of the MIP channel family are stronger than the homology between the first and second repeats within each member of the MIP channel family, indicating that evolutionary appearance of the tandem repeats preceded divergence of the organisms.

Despite the homology with the MIP channel family, the function of the CHIP28 protein is not known. The function of MIP26 has been debated, and recent evidence indicates that MIP26 proteins form voltage-regulated channels (9) through which lens fiber cells may absorb interstitial fluid (8). The predicted structure of the internal tandem repeats is noteworthy. The three bilayer-spanning domains of the first repeat are oriented 180° to the bilayer-spanning domains of the second repeat, suggesting a bidirectionally active channel. The other members of the MIP channel family are also thought to be membrane channels needed for permeability of water and small molecules (22), and interestingly, water deprivation induces expression of TUR, a MIP homolog expressed in the roots of pea shoots (29).

CHIP28 exists in membranes of erythrocytes and renal tubules and therefore may be related to the protein channels through which facilitated permeability of water occurs (31, 32). If CHIP28 is a water channel, it is logical to speculate that it may be clinically important in settings such as erythrocyte dehydration in sickle cell disease and in the perturbations of water and electrolyte balance found in various renal diseases. These hypotheses may now be tested by expression of CHIP28 and by evaluation of CHIP28 function in various clinical states.

We thank Barbara Smith, Terry Bishop, Eric Fearon, Andrew Yeager, and Ana Chepelnisky for samples, reagents, and valuable discussions. This work was supported in part by Grant HL33991 from the National Institutes of Health. G.M.P. is the recipient of Postdoctoral Fellowship T32 HL07525 (1990–1991) from the National Institutes of Health and a Fellowship from the American Heart Association Maryland Affiliate (1991–1992). P.A. is an established investigator of the American Heart Association.