A 28 kDa sarcolemmal antigen in kidney principal cell basolateral membranes: relationship to orthogonal arrays and MIP26

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SUMMARY

Two recently cloned water channels, CHIP28 and WCH-CD, are homologous to MIP26, an integral membrane channel-forming protein found in lens fiber plasma membranes. CHIP28 is found in basolateral and apical plasma membranes of kidney proximal tubules and thin descending limbs of Henle, whereas WCH-CD is apically located in collecting duct principal cells. So far, the putative water channel that may be responsible for the high constitutive permeability of principal cell basolateral membranes has not been identified. Interestingly, freeze-fracture electron microscopy has shown that characteristic orthogonal arrays of intramembrane particles (OAPs) are found on the basolateral plasma membranes of collecting duct principal cells, and that morphologically identical OAPs present in lens fiber cell plasma membranes contain the protein MIP26. Similar OAPs have also been detected on plasma membranes of other cell types including gastric parietal cells, astroglial cells and skeletal muscle fibers. By indirect immunofluorescence, western blotting and northern blotting, MIP26 was found only in lens fibers. In addition, functional studies on reconstituted and oocyte-expressed MIP26 excluded the possibility that MIP26 might be a basolateral water channel in the kidney. However, a polyclonal antibody raised against skeletal muscle sarcolemmal vesicles, which are enriched in OAPs, produced an intense staining of principal cell basolateral plasma membranes in kidney collecting duct and immunoprecipitated a 28 kDa protein from kidney papilla. The immunoprecipitated protein from papilla was not recognized by anti-CHIP28 or anti-MIP26 antibodies, indicating that principal cell basolateral membranes contain a novel member of the CHIP/MIP family. Because this antibody also stained brain astrocyte end feet, which are enriched in OAPs, it is possible that the 28 kDa protein is related to these structures. We conclude that OAPs probably contain related but distinct proteins that may have different membrane channel functions in different cell types.

Key words: orthogonal array, kidney collecting duct, astrocyte, MIP26, water channel

INTRODUCTION

The plasma membranes of some cell types have a high osmotic water permeability that has been explained by the presence of hydrophilic pores, or water channels, in these membranes. Recently, a water channel protein named CHIP28 has been identified as the erythrocyte plasma membrane water channel (Preston et al., 1992; Van Hoek and Verkman, 1992), and a homologous protein, CHIP28k, has been cloned from the rat kidney (Zhang et al., 1993). Immunocytochemical studies have localized CHIP28 to apical and basolateral plasma membranes of proximal tubules and thin descending limbs of Henle, which are both constitutively permeable to water (Sabolic et al., 1992a; Nielsen et al., 1993). A different water channel protein, WCH-CD, has also been identified in papillary collecting ducts where it is localized to the apical plasma membrane of principal cells (Fushimi et al., 1993). WCH-CD is a strong candidate for the vasopressin-sensitive apical membrane channel in this tubule segment. Functional studies have shown that the basolateral plasma membrane of collecting duct principal cells is also highly permeable to water (Grantham et al., 1969; Woodhall and Tisher, 1973; Strange and Spring, 1987), but the water permeability of this membrane is not vasopressin-sensitive. Neither CHIP28 nor WCH-CD has been detected in the basolateral membrane domain in medullary principal cells, indicating that its constitutively high water permeability might reflect the presence of yet another member of the family of related but distinct water channels.

CHIP28, CHIP28k and WCH-CD are all homologous to the major intrinsic protein from lens, MIP26 (Gorin et al., 1984;
MATERIALS AND METHODS

Freeze-fracture electron microscopy
Freeze-fracture studies were performed on kidney papillary tissue, as well as on purified MIP26 and CHIP28 incorporated into liposomes. Tissues or liposomes were fixed in 2% glutaraldehyde and cryoprotected by immersion in 30% glycerol in PBS for at least 1 hour. Samples were mounted in glycerol on copper freeze-fracture supports, and frozen at −150°C by rapid immersion in Freon 22 cooled by liquid nitrogen. Specimens were fractured at −130°C in a Cressington freeze-fracture apparatus (Cressington Scientific Instruments, Watford, UK), and were shadowed by depositing platinum at an angle of 45°, followed by carbon at 90°. Replicas were cleaned of underly ing tissue by consecutive treatments with sodium hypochlorite, chloroform-methanol and distilled water. Replicas were collected on copper grids, and examined and photographed using a Philips CM10 electron microscope.

Protein purification, reconstitution and functional analysis of MIP26
Purified MIP26 was reconstituted into proteoliposomes containing phosphatidylcholine, phosphatidylinositol and cholesterol (PC:PI:chol molar ratio, 11:1:11) by detergent dilution in 0.2 M EDTA, 10 mM sodium phosphate (pH 7.4) followed by centrifugation. CHIP28 was purified from human erythrocytes and reconstituted into proteoliposomes as described previously (Van Hoek and Verkman, 1992).

Osmotic water permeability of liposomes containing purified MIP26 or CHIP28 was measured by a stopped-flow light-scattering technique. Liposomes in 50 mM mannitol, 12 mM HEPES-Tris, pH 7.4, were subjected to a 100 mM inwardly directed osmotic gradient in 1 millisecond in a stopped-flow apparatus at 10°C. The time course of 90° scattering light intensity at 520 nm was recorded. Osmotic water permeability (Pf, cm/s) was determined as described previously (Van Hoek and Verkman, 1992). Experiments were performed on at least three different reconstituted vesicle samples, and for each sample, data were averaged from 5-10 consecutive measurements.

Expression of MIP26 in Xenopus oocytes and measurement of water permeability
The full-length coding region of MIP26 was isolated by PCR using cDNA reverse-transcribed from bovine lens mRNA as a template. A pair of primers, (a) 5'-CGAGCTCATGGGGAACGTGCAGAGCTC-3' and (b) 5'-CGGATCCGGCGCTTGCAGTCGTC-3', were designed according to the cloned bovine MIP26 cDNA sequence (Gorin et al., 1984) with introduction of SacI and BamHI restriction sites at the 5' and 3' primer ends, respectively. The PCR fragment was subcloned into a modified pGEM11Zf(+) plasmid that contained the 53 bp 5'-untranslated region of the Xenopus α-globin gene downstream from the T7 promoter (Hasegawa et al., 1992). The construct was confirmed by DNA sequence analysis. For preparation of full-length MIP26 cRNA, the recombinant plasmid was linearized by HindIII and transcribed with T7 polymerase.

Defolliculated Xenopus oocytes were injected with 50 nl of water or cRNA (0.2 mg/ml) in water and incubated at 18°C in Barth’s buffer for 48 hours. Osmotic water permeability was measured by a swelling assay at 10°C as described previously (Zhang et al., 1991). As a positive control, oocytes were injected with 5 ng of cRNA encoding the rat kidney water channel, CHIP28k (Zhang et al., 1993).

Preparation of antibodies
MIP26 was isolated from bovine lens by a modification of the method of Shen et al. (1991). A membrane fraction prepared from lens homogenate was stripped with urea, dissolved in β-octylglucoside and purified by anion exchange and size-exclusion high-performance liquid chromatography (Van Hoek et al., 1993). The purified protein gave a major band at 26 kDa on Coomassie-stained SDS-PAGE without glycosylation. Isolated protein (0.25 mg) was mixed with complete Freund’s adjuvant and injected subcutaneously into New Zealand White rabbits. Thereafter, subcutaneous booster injections of 0.1 mg antigen mixed with incomplete Freund’s adjuvant were given regularly every three weeks. Sera from the bleeds were checked for specific antibody production by immunocytocchemistry on frozen kidney sections and by western blotting against the purified protein.

Antibodies against human red cell CHIP28 were raised in New Zealand White rabbits and anti-CHIP28 IgG was affinity-purified from whole serum by immunoadsorption onto Immobilon-P strips containing CHIP28 as described previously (Sabolic et al., 1992a).

Guinea pig antiserum against skeletal muscle sarcosomal vesicles that are enriched in orthogonal arrays was prepared as described from rabbit skeletal muscle vesicles (Hatton et al., 1987). It is referred to in the text as anti-OAP antiserum. This antiserum recognizes four protein bands in skeletal muscle sarcosomal vesicles, including a protein of around 28-30 kDa apparent molecular mass.

Tissue preparation, immunofluorescence and immunoelectron microscopy
Rats were anesthetized with Nembutal (65 mg/kg body weight, IP). Kidneys were perfused via the abdominal aorta, first with Hanks’ balanced salt solution for 1-2 minutes and then with a fixative containing 2% paraformaldehyde, 10 mM sodium periodate and 75 mM...
Fig. 1. Freeze-fracture electron micrographs of kidney collecting duct principal cell basolateral membrane E-face (a) and P-face (b). The orthogonal arrays of intramembrane particles on the P-face (arrows) leave complementary imprints on the E-face (circles). Inset shows details of P-face intramembrane particle assembly in orthogonal arrays ($\times 200,000$). Adjacent rows of intramembrane particles are shown by arrows. The lens fiber cell plasma membrane E-face (c), shows similar arrays of imprints (arrows). The imprint spacing in lens fiber plasma membranes E-face (d) is the same as in collecting duct orthogonal arrays (arrows). Bar, 75 nm.
lysine (PLP) for 10 minutes. Organs were removed, sliced, and kept overnight in the same fixative at 4°C, followed by washing (three times) with phosphate-buffered saline (PBS: 0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4). The tissue slices were then kept in PBS containing 0.02% NaN₃ at 4°C until further use. When required, the cortex, outer and inner stripes of the outer medulla, and inner medulla were dissected manually. Lens was dissected from the eye, cut into smaller pieces, and fixed by immersion in PLP overnight. Other tissues including stomach, brain and muscle were also removed for examination.

Small tissue blocks were infiltrated with 2.3 M sucrose overnight, frozen in liquid nitrogen, and sectioned at 1 μm thickness on a Reichert FC4D ultracryomicrotome. Sections were placed on Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburg, PA), kept in PBS for 10 minutes, preincubated for 15 minutes with 1% BSA in PBS, and then incubated at room temperature for 90 minutes with either preimmune or immune anti-CHIP28 or anti-MIP26 antiserum (centrifuged at 16,000 g for 10 minutes, and diluted up to 1:1600 with PBS), affinity-purified anti-CHIP28 antibody (diluted 1:4 with PBS), or anti-OAP antiserum diluted 1:100. Sections were washed two times for 5 minutes in PBS containing 2.7% NaCl (high-salt PBS), plus two times in regular PBS. Washing with high-salt buffer decreases non-specific binding of antibodies. The sections were then incubated for 60 minutes with fluorescein-coupled goat anti-rabbit IgG (10 μg/ml in PBS) (Calbiochem), followed by washing two times for 5 minutes in high-salt PBS, and two times in regular PBS. For double-staining, some sections were also incubated for 60 minutes with a monoclonal antibody against the 31 kDa subunit of the vacuolar H⁺-ATPase (provided by Dr. Steven Gluck, Washington University, St Louis) followed by rhodamine-conjugated goat anti-mouse IgG (15 μg/ml; Calbiochem). Sections were finally mounted in 50% glycerol in 0.2 M Tris-HCl, pH 8.0, containing 2% n-propyl gallate to retard quenching of the FITC fluorescence signal. Sections were examined with a Nikon FXA photomicroscope equipped for epifluorescence and photographed using Kodak T-Max 400 film push-processed to 1600 ASA.

Rat brain was fixed by perfusion with 4% paraformaldehyde and OAP antigenic sites in brain were revealed by pre-embedding labeling using the peroxidase/anti-peroxidase technique as previously described (Ariyasu et al., 1985). Pre-immune serum was used as a control. Immunostained tissue was embedded in epoxy resin and thin sections were examined with and without subsequent heavy metal staining at 60 and 80 keV using a JEOL JEM 100CX electron microscope.

**Preparation of kidney homogenates and isolation of membrane vesicles**

All preparations were made from kidneys of Sprague-Dawley rats. Cortical brush border membrane vesicles were isolated by the Mg/EGTA-aggregation method of Biber et al. (1981). Cortical basolateral membrane vesicles and endocytic vesicles were isolated by the differential and Percoll density gradient centrifugation methods of Scalera et al. (1981) and Sabolic and Burckhardt (1990), respectively. Endocytic vesicles from renal papilla were isolated by the method of Sabolic et al. (1992b). The isolated vesicles were used immediately or were kept in liquid nitrogen until use.

**Immunoprecipitation from kidney papilla**

Protein A-Sepharose CL4B was used according to the instructions given by the manufacturer (Pharmacia, Uppsala, Sweden). For each sample to be analyzed 8 μl polyclonal rabbit antiserum was coupled to 200 μl hydrated Protein A-Sepharose. The mixture was incubated for 2 hours at 4°C with mild agitation. The antibody-bead complexes were pelleted by centrifugation and washed with PBS containing 0.05% glutaraldehyde (to enhance coupling) for 5 minutes. The mixture was washed in PBS followed by PBS containing 2% glycine, with a Nikon FXA photomicroscope equipped for epifluorescence and photographed using Kodak T-Max 400 film push-processed to 1600 ASA.

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and washed again in PBS prior to incubation with a membrane preparation from rat kidney papilla homogenate.

The kidney papilla was removed from 6 rats, cut into small pieces and homogenized in ice-cold buffer (300 mM mannitol, 12 mM HEPES/Tris, pH 7.4). All subsequent steps were performed at 4°C. The suspension was spun at 2,500 g for 15 minutes and the pellet containing nuclei and unbroken cells was discarded. The supernatant was spun at 47,000 g for 60 minutes; the pellet was resuspended in PBS and the protein content was determined by the Bradford (1976) assay.

Samples of the membrane preparation from papilla (~250 µg) were diluted 1:10 in incubation buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM MgCl2, 1 mM CaCl2, 2% Triton X-100, 0.5% NP-40 containing 1 mM PMSF and 1 µg/ml aprotinin) and kept on ice for at least 20 minutes. Preclearing of each sample was accomplished by adding 4 µl of preimmune serum to the suspension and mixing for 1 hour on a rocking platform. Then, 100 µl of hydrated Protein A-Sepharose CL4B was added and incubated for 1 hour. The suspension was then centrifuged and the pellet was discarded. The supernatant was combined with the pellet of Protein A-Sepharose complexed with specific antibodies. Each sample was incubated for 2-3 hours on a rocking platform, then pelleted by centrifugation. The supernatant was removed and the pellet was dispersed in washing buffer (same as the incubation buffer with 1/5 the concentration of detergents) briefly vortexed, then centrifuged. The washing step was repeated 4 times. As a control, immunoprecipitation was performed without addition of primary antibody to the Protein A-Sepharose.

Immunoblotting procedure
Samples of homogenates, isolated membranes or purified protein were solubilized in sample buffer (1% SDS, 30 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 12% (v/v) glycerol) and heated to 80°C for 10 minutes. Proteins were subjected to 10% to 15% acrylamide gradient SDS-PAGE, and western blotting was performed as previously described following transfer of proteins to Immobilon-P (Sabolic et al., 1992a). Peroxidase conjugated to the secondary antibodies was detected by the enhanced chemiluminescence technique (ECL, Amersham, UK).

Northern blot analysis
A 100 µg sample of mRNA from rat kidney cortex, rat kidney medulla and bovine lens was resolved on a 1.2% formaldehyde-agarose gel and blotted onto a nitrocellulose membrane. Full-length MIP26 cDNA was labeled with [α-32P]dCTP by random priming. After standard prehybridization, the membrane was hybridized in 50% formamide, 5x SSC, 5x Denhardt’s solution, 2% SDS and 0.1% SDS at 65°C, and autoradiographed at ~80°C with double intensifying screens for 24 hours.

RESULTS
Freeze-fracture electron microscopy
As described previously, the basolateral plasma membranes of kidney collecting duct principal cells (Fig. 1a,b) and lens fiber plasma membranes (Fig. 1c,d) contain large numbers of so-called orthogonal or square arrays of particles, or OAPS (Humbert et al., 1975; Kistler and Bullivant, 1980; Orci et al., 1981; Dunia et al., 1987; Zampighi et al., 1989). Arrays of pits are observed on the complementary plasma membrane exoplasmic face (E-face) of principal cells (Fig. 1a, circles) and lens fibers (Fig. 1c, arrows). OAPS (Fig. 1b) are formed of large square particles, 13.0±1.0 nm (mean ± s.d., 24 measurements) in diameter, which themselves are composed of smaller intramembrane particles, as seen under favorable shadowing conditions (Fig. 1b, inset). The subunit structure of the particles, as well as the geometric arrangement of the intramembrane particles, can be best appreciated on the complementary E-face of the plasma membrane. On this membrane leaflet, the impressions left by the P-face particles produce highly ordered, orthogonally aligned rows of small pits, with a center-to-center spacing of ~7 nm (Fig. 1d, arrows). Four pits on the E-face, or four intramembrane particles on the P-face, occupy the area of one large square particle on the P-face, indicating clearly that the P-face square particles have a tetrameric subunit structure. In addition, purified MIP26 incorporated into liposomes produced similar orthogonal arrays (Fig. 2a), with complementary imprints (Fig. 2b), indicating that this assembly into higher-order structures is a property of the MIP26 proteoliposomes (Humbert et al., 1975; Kistler and Bullivant, 1980; Orci et al., 1981; Dunia et al., 1987; Zampighi et al., 1989).

Table 1. Spacing of intramembrane particles in orthogonal arrays

<table>
<thead>
<tr>
<th>Orthogonal arrays</th>
<th>Spacing (nm)</th>
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<tbody>
<tr>
<td>MIP26 proteoliposomes</td>
<td>6.8±0.2 (14)</td>
</tr>
<tr>
<td>Lens fiber plasma membranes</td>
<td>6.9±0.1 (34)</td>
</tr>
<tr>
<td>Principal cell basolateral membranes</td>
<td>6.8±0.1 (49)</td>
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Data are means ± s.d., number of measurements in parenthesis. Measurements were made on the distinct imprints found on the E-face of cell plasma membranes (Fig. 1a,c,d), or the corresponding liposome membrane leaflet (Fig. 2b).

Fig. 3. Analysis of MIP26 water permeability. (A) Expression in *Xenopus* oocytes. Oocytes were injected with water, 10 ng of cRNA encoding CHIP28 or 50 ng of cRNA encoding MIP26. A representative time-course of oocyte swelling is shown at the left in response to a 5-fold dilution of the extracellular buffer at 10°C (see Materials and Methods). Average data (mean ± s.e.) for 8 to 12 oocytes is shown on the right. (B) Reconstitution of purified MIP26 in proteoliposomes. Osmotic water permeability was measured by stopped-flow light-scattering in proteoliposomes reconstituted with MIP26 or CHIP28. Where indicated, 0.3 mM HgCl2 was added. The data are shown on 3 contiguous scales.
proteins, and is not related to interaction with cytoskeletal elements or other cellular components.

As shown in Table 1, the E-face imprint spacing was found to be identical in lens, in proteoliposomes and in principal cell basolateral membranes (6.8 to 6.9 nm). In contrast to MIP26, purified CHIP28 incorporated into liposomes did not produce orthogonal arrays. However, in appropriate shadowing conditions, intramembrane particles formed by CHIP28 could be resolved into 4 distinct subunits (Verbavatz et al., 1993), forming a tetramer of ~7.2 nm greatest diameter after correction for the thickness of the platinum coating.

**Water permeability measurements on reconstituted and oocyte-expressed MIP26**

To determine whether MIP26 can function as a transmembrane water channel, oocyte expression and liposome reconstitution experiments were performed. Previous work has shown that these methods are suitable for detecting water channel activity of CHIP28 (Zhang et al., 1991; Van Hoek and Verkman, 1992; Zeidel et al., 1992) and WCH-CD (Fushimi et al., 1993).

cDNA encoding MIP26 was prepared from reverse-transcribed bovine lens mRNA by PCR amplification as described in Materials and Methods. In vitro transcribed cDNA was expressed in *Xenopus* oocytes for functional assay of water permeability. Fig. 3A shows the time course of oocyte swelling after diluting the extracellular buffer 5-fold with distilled water. Whereas injection of 10 ng of CHIP28 mRNA gave a very large increase in $P_f$ compared to the $P_f$ in the water-injected oocytes, injection of 50 ng of MIP26 mRNA did not significantly increase oocyte $P_f$. Fig. 3B shows stopped-flow light-scattering measurements of osmotic water permeability in proteoliposomes reconstituted with similar quantities of MIP26 and CHIP28. The downward deflection indicates liposome shrinkage in response to the osmotic gradient. While shrinkage was very fast in the CHIP28-reconstituted proteoliposomes, the liposomes containing MIP26 showed slow shrinkage similar to

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**Fig. 4.** Cryostat sections of rat kidney papilla (a,b) showing immunofluorescent staining of collecting duct (CD) basolateral plasma membranes with anti-OAP antiserum (a) and thin descending limbs of Henle (TDL) with anti-CHIP28 antiserum (b). The anti-OAP antiserum shows only a very weak staining of thin descending limbs, and conversely, the anti-CHIP28 antiserum does not stain collecting duct plasma membranes. Cryostat section of the outer stripe of the outer medulla (c,d) showing a collecting duct and adjacent proximal tubules. The section is double-stained with anti-OAP antiserum (c) and a monoclonal antibody against the 31 kDa subunit of the proton pumping ATPase (d). The cells that are not stained with anti-OAP antiserum in (c) are all stained with the anti-31 kDa monoclonal antibody, identifying them as intercalated cells. In addition, both A-type intercalated cells with exclusively apical proton pumps (arrows) and B-type intercalated cells with a bipolar distribution of proton pumps (arrowhead) are unstained with anti-OAP antiserum. Bars: 25 μm (a,b) and 15 μm (c,d).
that in the CHIP28 liposomes after inhibition by HgCl\textsubscript{2}. HgCl\textsubscript{2} had no effect on water permeability in the MIP26 liposomes.

**Immunofluorescence microscopy of kidney**

Anti-MIP26 antiserum gave no detectable staining of any structure in the kidney (not shown). However, the anti-OAP antiserum produced a strong staining of basolateral plasma membranes in the collecting duct. This basolateral staining was strongest in the inner medulla (Fig. 4a). The intensity of fluorescence was greater on the basal membrane, and was less intense on the lateral aspects of principal cells. In some incubations, thin limbs of Henle showed a weak and variable staining that may reflect some cross-reactivity of the anti-OAP antiserum with CHIP28, which is extremely abundant in descending thin limbs. Alternatively, this observation may reflect the presence of a novel antigen recognized by anti-OAP antiserum in these thin limb cells. In contrast, anti-CHIP28 antiserum produced an intense staining of thin limbs, but no detectable collecting duct staining (Fig. 4b).

**Double labeling with anti-OAP and anti-H\textsuperscript{+},ATPase antibodies**

In the initial part of the inner medullary collecting duct, as well as in outer medullary and cortical collecting ducts, a mosaic pattern of positive and negative epithelial cell staining was observed. Double labeling demonstrated that the cells with basolateral OAP staining (Fig. 4c) did not stain with a monoclonal antibody against the 31 kDa subunit of the proton-pumping ATPase (Fig. 4d). Our previous studies have demonstrated that proton-transporting intercalated cells of the collecting duct are strongly stained with anti-proton pump antibodies (Brown et al., 1987, 1988); the OAP-negative cells are, therefore, intercalated cells and the positive cells are principal cells.

**Immunoelectron microscopy**

Immunogold labeling of ultrathin frozen sections with anti-OAP antiserum demonstrated a marked labeling of the basolateral plasma membrane of collecting duct principal cells (Fig. 5a). Most of the gold particles were located on the external side of the membrane (Fig. 5a, inset), indicating that the anti-OAP antiserum recognizes an externally oriented epitope on this membrane domain. Thin descending limbs of Henle showed only a weak labeling (Fig. 5b), compared to the extensive labeling produced in this cell type by anti-CHIP28 antibodies (Sabolic et al., 1992a). No immunogold labeling of collecting duct principal cells was found with anti-CHIP28 antibodies (not shown).

**Immunofluorescence staining of lens, brain and other tissues**

**Lens**

A strong staining of lens fiber cell plasma membranes, was seen with anti-MIP26 serum (Fig. 6a), whereas pre-immune serum did not stain the lens. Anti-CHIP28 antibodies did not stain rat lens fibers but, interestingly, they produced an intense labeling of the surface epithelial cells of the lens (Fig. 6b). These epithelial cells did not stain with anti-MIP26 antibodies (Fig. 6a). The anti-OAP antiserum produced a weak but specific staining of lens fibers as well as lens epithelial cells (Fig. 6c,d), indicating either a possible weak cross-reactivity with MIP26, which is abundant in the fibers, and with CHIP28, which is abundant in the surface epithelium, or the presence in both of these locations of a novel antigen recognized by anti-OAP antiserum.

**Brain**

The distribution of orthogonal arrays has been well documented in the brain (Landis and Reese, 1974; Anders and Brightman,
These structures are most abundant along pericapillary surfaces of astrocytic endfeet, which ensheathe brain capillaries, and they are also associated with the processes of these same cells at the pial-glial limitans. To investigate further the supposition that the anti-OAP antiserum recognizes OAP structures in tissues other than skeletal muscle (the source of the antigens), we examined the immunoreactivity in rat cerebral cortex. We found that the distribution of immunoreactivity matched the previously chronicled distribution of OAPs seen by freeze-fracture. Fig. 7 shows a brain capillary surrounded by astrocytic processes that are heavily stained by the peroxidase-anti-peroxidase reaction. Diffusion of the peroxidase reaction products limits the resolution afforded by this technique, so that the entire cell cytoplasm appears stained. The inset shows a detail of an immunoreactive astrocytic end-foot process at higher magnification. Adjacent endothelial cells are not stained with the anti-OAP antiserum.

The pial-glial limitans was also stained by this antibody preparation (not shown).

Because orthogonal arrays of intramembrane particles resembling those in the kidney, lens fibers and astrocytes have also been described in gastric parietal cells (Bordi et al., 1986), we tested anti-MIP26, anti-CHIP28, and anti-OAP antibodies against sections from gastric tissue. None of the antibodies stained gastric parietal epithelial cells of the stomach (not shown). Intestinal epithelium, where the orthogonal arrays were originally described (Staehelin, 1972), was also negative (not shown).

**Western and northern blot analysis**

Because immunocytochemical studies showed that anti-MIP26 antibodies did not stain kidney sections, we performed immunoblotting and immunoprecipitation studies to characterize further the nature of the protein(s) recognized by the anti-OAP serum in principal cells. First, we confirmed that MIP26 is absent from kidney. Fig. 8A shows that whereas anti-MIP26 antiserum recognizes MIP26 purified from bovine lens (lane 1), no reactivity is seen against purified CHIP28 (lane 2), kidney papillary homogenate (lanes 3), or cortical basolateral plasma membranes (lane 4). In addition, no MIP26 staining was found in cortical brush border membrane vesicles or in cortical and papillary endosomes (not shown). The absence of MIP26 from the kidney was confirmed by northern blot analysis (Fig. 8D). There was intense hybridization of full-length MIP26 cDNA probe to a 1.5 kb sized mRNA from bovine lens, but no detectable hybridization to mRNA from kidney cortex or medulla. A full-length CHIP28k probe hybridized strongly to an mRNA of 2.8 kb in kidney cortex and papilla (Zhang et al., 1993), but not to mRNA from bovine lens (not shown).

Fig. 8B shows that anti-CHIP28 antibodies did not recognize MIP26 (lane 1), but strongly stained both non-glycosylated (28 kDa) and glycosylated (40-70 kDa) forms of CHIP28 (lanes 2-4), as previously reported (Sabolice et al., 1992a; Nielsen et al., 1993).

Because these data suggest that the basolateral OAPs of principal cells contain a protein that is antigenically distinct from MIP26, we used the anti-OAP antiserum to immunoprecipitate material from rat kidney papilla in order to characterize further the recognized protein. Fig. 8C (lane 1) shows that the OAP antiserum immunoprecipitated a major band at 28 kDa from papillary homogenates, together with a less distinct band at slightly higher molecular mass. Bands at other molecular masses were not detectable in the kidney papillary preparation. Anti-OAP antiserum did not cross-react with CHIP28, shown by western blotting (Fig. 8C, lane 2), and the immunoprecipitated 28 kDa band was not stained with anti-CHIP28 or anti-MIP26 antibodies (not shown).

**DISCUSSION**

The purpose of this study was to test the hypothesis that the lens fiber membrane protein MIP26 or a similar protein might be located in the basolateral plasma membrane of collecting duct principal cells, where it would form orthogonal arrays of particles and could function as a water channel. MIP26 (Gorin...
et al., 1984) has 42% identity to the CHIP28 water channel (Preston and Agre, 1991), and a recent report has shown that it has 60% identity to WCH-CD, a second water channel that is expressed in the kidney collecting duct apical membrane (Fushimi et al., 1993). In addition, the first six amino acids of the NH₂ terminus of WCH-CD are identical to those of MIP26. The WCH-CD protein is a candidate for the regulated water channel that is recycled to and from the principal cell apical membrane during vasopressin stimulation, and which plays a key role in formation of hypertonic urine by the collecting duct. The presence of distinct water channels in basolateral membranes of collecting duct principal cells (Humbert et al., 1975; Orci et al., 1981; Nakamura and Nagano, 1985). MIP26 also forms OAPs on lens fiber plasma membranes (Kistler and Bullivant, 1980; Dunia et al., 1987; Zampighi et al., 1989). Similar OAPs have been reported in a variety of other plasma membranes from widely different cell types, including gastric parietal cells (Bordi et al., 1986), brain oligodendrocytes (Dermietzel, 1974; Hatton and Ellisman, 1981), intestinal epithelial cells (Staehelin, 1972; Staehelin, 1973), skeletal muscle cells (Rash et al., 1974; Ellisman et al., 1976; Hatton et al., 1987; Jimi and Wakayama, 1990; Neuhaus et al., 1990), pneumocytes (Bartels and Miragall, 1986), non-pigmented cells of rat ciliary epithelium (Hirsch et al., 1988), and tracheal epithelial cells (Widdicombe et al., 1987). The organization of intramembrane particles into strikingly similar orthogonal arrays could indicate that an identical or closely related protein is present in the arrays from these cell types.

Fig. 7. Astrocyte staining with anti-OAP antibodies. The intermediate magnification micrograph in (A) depicts a peroxidase-stained astrocytic end-foot process (AS) surrounding a brain capillary. Astrocytic end-foot processes as well as other areas of the brain in which OAPs are seen by freeze-fracture are heavily stained by this antibody preparation raised against the skeletal muscle OAP preparation. Diffusion of the DAB reaction product within the cell makes it difficult to determine the precise location of the antigenic sites using this technique. This electron micrograph is from rat cerebral cortex. EN, endothelium. Bar, 1 μm. (B) is a micrograph of a similar end-foot process (AS) at higher magnification. Foci of PAP-associated densification (arrows) are seen along the surface of the astrocyte plasma membrane where it is applied to the basal lamina, that surrounds the brain capillary. This is the site where OAPs are most abundant in similar preparations observed in freeze-fracture replicas. EN, endothelium. Bar, 0.25 μm.
In addition to the description of OAPs in several mammalian tissues, we have previously reported that both vasopressin and isoproterenol induce the appearance of similar OAPs on the apical plasma membrane of the responsive cell type in amphibian epidermis (Brown et al., 1983). Both agents increase intracellular cyclic AMP and concomitantly increase transepithelial water permeability in this tissue. While vasopressin treatment also induces intramembrane particle aggregates to appear in apical plasma membranes of amphibian urinary bladder granular cells and collecting duct principal cells, only in the toad skin do the induced structures resemble orthogonal arrays (Brown, 1991). The structural similarity of these arrays to those formed by MIP26, together with their relationship to water permeability in toad epidermis, suggest that the square arrays may indeed be involved in plasma membrane water permeability in collecting duct and perhaps in other cell types. It has been reported that the basolateral membrane surface area occupied by OAPs in principal cells of the rat kidney is increased by dehydration of the animals (Nakamura and Nagano, 1985), indicating that these arrays may play a role in water transport. Nevertheless, the arrays still occupied 8% of the basolateral membrane even in vasopressin-deficient homozygous Brattleboro rats that do not concentrate their urine. They are also abundant on basolateral membranes of mice with severe nephrogenic diabetes insipidus (Brown and Orci, 1988), indicating that they are not vasopressin-regulated like the apical water channel in principal cells.

Despite the high degree of morphological similarity described in this report, the orthogonal arrays in the kidney (and in all tissues other than lens) do not contain MIP26. MIP26 could not be detected in any region of the kidney by immunoblotting, immunocytochemistry or northern analysis. However, an antibody raised against skeletal muscle sarcosomal vesicles that are enriched in OAPs (anti-OAP antiserum) strongly labeled the basolateral plasma membrane of collecting duct principal cells and brain astrocytes. The immunoreactivity appeared most concentrated on the basal (rather than the lateral) aspect of the principal cell plasma membrane, and the astrocyte end-feet adjacent to capillaries, precisely where quantitative studies have revealed the highest number of orthogonal arrays. (Ellisman et al., 1976; Hatton and Eltisman, 1981; Orci et al., 1981). Furthermore, the anti-OAP antiserum immunoprecipitated a 28 kDa protein from rat papillary homogenates. This protein was not recognized by either anti-CHIP28 or anti-MIP26 antibodies by western blotting. In addition, the anti-OAP serum did not react either with purified CHIP28 or with a red blood cell plasma membrane preparation that is rich in CHIP28. In an earlier study, we reported a faint reactivity of our original anti-CHIP28 antiserum with both apical and basolateral plasma membranes of cortical collecting ducts, and suggested that a protein similar to CHIP28 was present in both of these membrane domains (Sabolic et al., 1992a). Presumably, the apical protein is the CHIP28 homologue, WCH-CD, which is exclusively present in the collecting duct, and has been localized at the apical pole of principal cells using anti-peptide antibodies that do not cross-react with CHIP28 (Fushimi et al., 1993). Despite the fact that the anti-OAP antiserum was originally reported to recognize other protein bands in sarcolemmal vesicles (Hatton et al., 1987), only the 28 kDa protein was immunoprecipitated from kidney papilla. Because principal cells from the papilla contain OAPs, and because these arrays are present in the original preparation used to prepare this antiserum, it is probable that the 28 kDa protein is common to the OAPs in both tissues. However, label fracture studies will be required to prove that this protein is indeed present in OAPs rather than in other domains of the basolateral membrane. Recently, we have raised a new anti-CHIP28 antibody against whole red cell CHIP28 that produces an intense staining of principal cell basolateral membranes, in addition to staining cell types known to contain CHIP28 (Valenti et al., unpublished observations). We do not know if this antiserum and the anti-OAP antiserum recognize the same or distinct proteins in principal cell basolateral membranes. So far, we have been
unable to demonstrate clearly that the new CHIP antiserum cross-reacts with the 28 kDa protein immunoprecipitated from rat papilla with anti-OAP antiserum.

What is the role of the OAPs found in tissues other than lens, and which do not contain the MIP26 protein? It has been pointed out that they are often concentrated on plasma membrane regions that are adjacent to capillaries (Ellisman et al., 1976; Hatton and Ellisman, 1981; Orci et al., 1981). A few observations reporting changes in the membrane density of OAPs have been made in different tissues, including in the kidney during dehydration, as discussed above. In the rat stomach, exposed to pentagastrin, acid secretion by parietal cells is increased along with a marked increase in the number of OAPs on basolateral plasma membranes (Bordi et al., 1986). OAPs can be induced in slow-twitch skeletal muscle by reinnervation with the nerve from a fast-twitch muscle (Ellisman et al., 1978). In patients with Duchenne muscular dystrophy, the number of OAPs is reduced on the muscle cell plasma membrane (Wakeyama et al., 1989). In astrocytes, OAP density is reduced rapidly following ischemia (Suzuki et al., 1984). A marked redistribution of OAPs occurs in astroglia in alumina-induced epileptic foci in rats (Hatton and Ellisman, 1984). Although these observations have not helped to elucidate their function, they are consistent with the hypothesis that the OAPs are involved in membrane transport or permeability (Orci et al., 1981). While our present data indicate that MIP26 is probably not a transmembrane water channel, electrophysiological measurements on purified, reconstituted MIP26 have demonstrated that the protein does have ion channel activity (Shen et al., 1991).

The anti-OAP antiserum did not cross-react with epithelial cells from the intestine or stomach, indicating that morphologically similar OAPs may be composed of a family of related, but separate, proteins, perhaps with distinct functions. Nevertheless, several lines of evidence suggest a possible relationship between the OAPs found in the basolateral plasma membrane of collecting duct principal cells and water transport: (1) their freeze-fracture appearance is identical to that of OAPs in toad skin that are induced by treatments that increase membrane water permeability; (2) their basolateral localization is consistent with the high water permeability of this membrane; (3) the area of basolateral plasma membrane occupied by OAPs is increased by dehydration of rats.

Despite this circumstantial connection, there is still no direct evidence relating OAPs to membrane water permeability. Our data show that OAPs formed by MIP26 do not contain functional water channels, at least under our conditions of assay. It is possible that morphologically similar OAPs contain related but distinct proteins that may confer different functions on these characteristic structures in different cell types. To determine if the 28 kDa protein that is recognized by the anti-OAP antiserum in principal cell basolateral plasma membranes is a transmembrane water channel, or whether it has a distinct function unrelated to water channel activity, will require its cloning and expression.

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