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Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes

(water transport/aquaporin/kidney collecting duct/airways/brain)

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ABSTRACT **Two water channel homologs were cloned recently from rat kidney, mercurial-insensitive water channel (MIWC) and glycerol intrinsic protein (GLIP). Polyclonal antibodies were raised against synthetic C-terminal peptides and purified by affinity chromatography. MIWC and GLIP antibodies recognized proteins in rat kidney with an apparent molecular mass of 30 and 27 kDa, respectively, and did not cross-react. By immunofluorescence, MIWC and GLIP were expressed together on the basolateral plasma membrane of collecting duct principal cells in kidney. By immunohistochemistry, MIWC and GLIP were expressed on tracheal epithelial cells with greater expression of GLIP on the basal plasma membrane and MIWC on the lateral membrane; only MIWC was expressed in bronchial epithelia. In eye, GLIP was expressed in conjunctival epithelium, whereas MIWC was found in iris, ciliary body, and neural cell layers in retina. MIWC and GLIP colocalized on the basolateral membrane of villus epithelial cells in colon and brain ependymal cells. Expression of MIWC and GLIP was not detected in small intestine, liver, spleen, endothelia, and cells that express water channels CHIP28 or WCH-CD. These studies suggest water/solute transporting roles for MIWC and GLIP in the urinary concentrating mechanism, cerebrospinal fluid absorption, ocular fluid balance, fecal dehydration, and airway humidification. The unexpected membrane colocalization of MIWC and GLIP in several tissues suggests an interaction at the molecular and/or functional levels.**

Several mammalian water-selective transporting proteins (aquaporins) with homology to members of the MIP (major intrinsic protein of lens) protein family (1) have been cloned recently. *Ch*annel-*f*orming *i*ntegral *p*rotein (CHIP28) is expressed in erythrocytes, kidney proximal tubule, thin descending limb of Henle, and epithelial and/or endothelial cells in choroid plexus, alveolus, colonic crypts, ciliary body, and other fluid-transporting tissues (2–7). WCH-CD (AQP2) is the vasopressin-regulated water channel expressed in intracellular vesicles and the apical membrane of principal cells in kidney collecting duct (8, 9). Humans with mutations in CHIP28 have been found recently to have no obvious clinical abnormalities (10), whereas mutations in WCH-CD are associated with hereditary nephrogenic diabetes insipidus (11).

To identify other water channel homologs expressed in mammalian tissues with high water permeability that do not express CHIP28 and WCH-CD, the application of an homology cloning strategy yielded several more MIP family members (12–14). A *m*ercurial *i*nsensitive *w*ater *c*hannel (MIWC) was cloned from a rat lung cDNA library and functioned as a water-selective channel when expressed in *Xenopus* oocytes; *in situ* hybridization indicated MIWC transcript expression in kidney medulla, brain surface, and retina (13). An unusual feature of MIWC expression was the presence of full-length mRNA encoding a functional water channel and a spliced mRNA of unclear significance; the relative expression of full-length transcript to spliced transcript was high in kidney and lung but low in liver and salivary gland. A second homologous protein, *gl*ycerol *i*ntrinsic *p*rotein (GLIP), was cloned from rat kidney independently by Ma *et al.* (14) and Ishibashi *et al.* (AQP3; ref. 15). Both groups reported increased glycerol uptake when GLIP was expressed in *Xenopus* oocytes, whereas Ishibashi *et al.* (15) also reported increased osmotic water permeability. Preliminary immunolocalization studies indicated strong expression of GLIP protein in basolateral membrane of kidney collecting duct, and Northern and PCR Southern blot analyses indicated a wide tissue distribution of the GLIP transcript (14, 15).

The purpose of this study was to determine the tissue distribution and membrane localization of MIWC and GLIP proteins by immunohistochemistry and to determine whether the spliced MIWC transcript was translated. Purified polyclonal antibodies localized MIWC and GLIP in basolateral membrane of principal cells in kidney collecting duct, selected epithelial cells in trachea, colon, and eye, and ependymal cells in brain. Cellular expression of the spliced MIWC transcript was not associated with expression of immunoreactive protein. An interesting result was the colocalization of MIWC and GLIP in certain cell membranes that do not express CHIP28 and WCH-CD.

METHODS

Preparation of Antibodies. Oligopeptides were synthesized by the standard solid-phase technique and purified by HPLC. Peptides were synthesized for MIWC (amino acids 287–301, EKGKDSSGEVLSSV) and GLIP (amino acids 270–285, EAENVKLAHMKHKEQI). For immunizations, an N-terminal cysteine was added to each peptide to conjugate with maleimide-activated keyhole limpet hemocyanin (Pierce). MIWC antibodies were raised in rabbits and GLIP antibodies were raised in mice. Antibody titers were assessed serially by dot-blot analysis against specific peptides. Antibodies were affinity-purified by passage of immune serum over peptide columns prepared by covalent reaction of specific peptides with iodoacetyl-crosslinked agarose (Pierce). Antibodies were eluted at pH 2.5 and pH 11.5 followed by rapid titration at pH 8 and dialysis against PBS containing 0.02% sodium azide.

Immunoblot Analysis. Organs from Sprague–Dawley rats were removed and homogenized in 200 mM sucrose/10 mM Tris·HCl, pH 7.4, containing leupeptin (1 μg/ml), pepstatin A (1 μg/ml), and antipain (4 μg/ml). After homogenization in a Potter–Elvehjem apparatus and centrifugation at 3000 × *g* for 10 min, a high-speed pellet was prepared by centrifugation at 100,000 × *g* for 60 min. Membranes were dissolved in SDS

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Abbreviations: MIWC, mercurial-insensitive water channel; GLIP, glycerol intrinsic protein; CHIP, channel-forming integral protein.

loading buffer, heated to 65°C for 5 min, and resolved on a 13% polyacrylamide gel. Proteins were electrotransferred to a poly(vinylidene difluoride) membrane. Membranes were blocked with 2% (wt/vol) bovine serum albumin in PBS for 1 h at room temperature and incubated overnight with immune serum (1:500 dilution). Sites of antigen–antibody reaction were visualized by ^{125}I-labeled protein A (1 μCi/ml; 1 Ci = 37 GBq; ICN Biochemicals).

Immunohistochemistry. Rat tissues were perfused with PBS and then fixed *in situ* with PBS containing 4% (wt/vol) paraformaldehyde. Organs were removed, sliced, and post-fixed for 4 h with the same solution. Sliced tissues were then cryoprotected overnight with PBS containing 30% (wt/vol) sucrose, embedded in OCT compound (Miles), and frozen in liquid N_2. Cryostat sections (4–6 μm) were mounted on Superfrost/Plus microscope slides (Fisher). Slides were incubated for 10 min with PBS containing 1% bovine serum albumin and then with purified anti-MIWC or anti-GLIP antibodies (0.3–0.5 μg/ml) for 1 h at room temperature in PBS containing 1% bovine serum albumin. Control experiments were performed by using the purified antibodies preadsorbed with an excess (>10:1 molar ratio) of specific peptide. Slides were rinsed with 2.7% NaCl to reduce background and then with PBS. For immunofluorescence, slides were incubated for 30 min with sheep anti-rabbit or anti-mouse IgG conjugated with fluorescein or rhodamine (diluted 1:50; BRL), washed in PBS, and mounted by using an antifade solution (Molecular Probes) to retard fluorescence photobleaching. For immunoperoxidase detection, the antibody-incubated slides were incubated for 30 min with peroxidase-conjugated sheep anti-rabbit or mouse F(ab′)$_2$ fragment (diluted 1:100; Amersham). Peroxidase activity was visualized by reaction with diaminobenzidine for 20 min. Slides were photographed on Kodak Gold 100 or Tmax 400 film on a Nikon Optiphot fluorescence microscope or by a cooled charge-coupled device camera using a Leitz-Technical Instruments confocal microscope.

RESULTS

Fig. 1*A* shows that the MIWC antibody recognized a single protein in rat kidney medulla with an apparent molecular mass of 30 kDa, whereas the GLIP antibody recognized a 27 kDa protein and a faint diffuse band at higher molecular mass probably representing glycosylated GLIP; immunoblots with preadsorbed antibodies were negative. The sizes of 30 and 27 kDa were the same as those detected in cell-free translation studies of cDNAs encoding MIWC (13) and GLIP (data not shown). There was no cross-reactivity of MIWC and GLIP antibodies based on the recognition of different proteins on the immunoblot and no cross-staining in *Xenopus* oocytes expressing MIWC and GLIP complementary RNAs (data not shown). In addition, cross-reactivity would not be expected because the peptides used to generate the polyclonal antibodies were not homologous.

Immunofluorescence with MIWC antibody showed strong staining in collecting duct in kidney medulla (Fig. 1*C*) with a negative preadsorbed antibody control (Fig. 1*B*). By confocal microscopy at high magnification, the fluorescein-labeled anti-rabbit secondary antibody was localized to the basolateral membrane of principal cells in collecting duct (Fig. 1*D*); intercalated cells (arrow) were not stained. Similar staining was observed along the full length of the cortical and medullary collecting duct. Identification of principal cells was confirmed by counterstaining the apical membrane with an antibody against the WCH-CD protein (data not shown). Fig. 1*E* shows an identical distribution of GLIP protein as demonstrated by colocalization of a rhodamine-labeled anti-mouse secondary antibody. MIWC and GLIP proteins were not found elsewhere in kidney.

Fig. 2 shows MIWC and GLIP expression in airways and lung by immunoperoxidase. In trachea, GLIP was localized primarily to the basolateral membrane of epithelial cells (Fig. 2*D*; for control, see Fig. 2*C*). MIWC colocalized to the basolateral membrane of tracheal epithelial cells but consistently showed a more lateral distribution (Fig. 2*B*; for control, see Fig. 2*A*). Mucous cells appear to be unstained. GLIP was not detected in smaller airways and alveoli (data not shown);

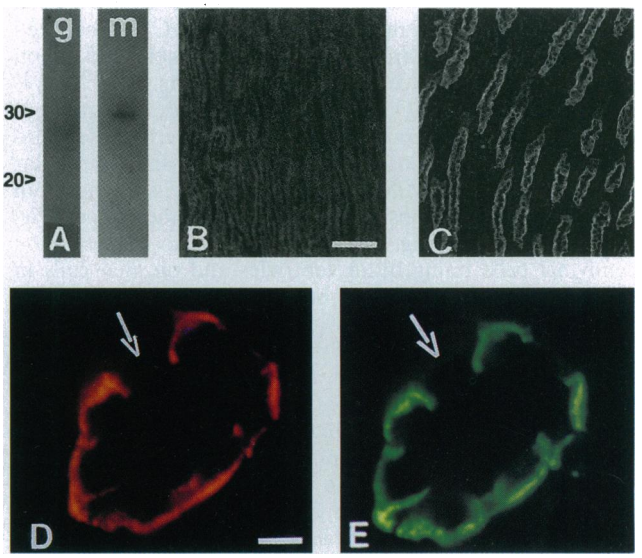

FIG. 1. Immunoblot and immunofluorescence of rat kidney medulla by using purified MIWC and GLIP polyclonal antibodies. (*A*) Immunoblot of medulla homogenate showing recognition of 30-kDa (lane m, MIWC antibody) and 27-kDa (lane g, GLIP antibody) proteins. (*B* and *C*) Low-magnification immunofluorescence of renal medulla stained by purified MIWC antibody (*C*) and preadsorbed control (*B*). (Bar = 250 μm.) (*D* and *E*) High-magnification confocal microscopy showing double immunostaining of basolateral membrane of kidney collecting duct by MIWC (*D*) and GLIP (*E*) antibodies. Arrow points to unstained intercalated cell. (Bar = 15 μm.)

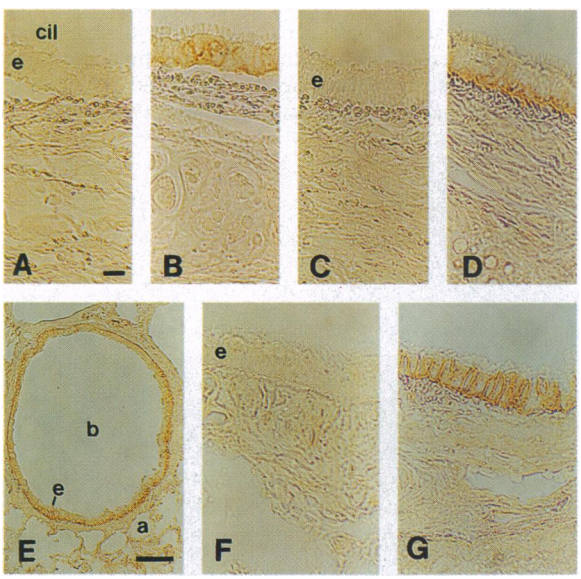

FIG. 2. Immunoperoxidase localization of MIWC and GLIP in airways and lung. (*A–D*) Expression of MIWC (*B*) and its control (*A*) and GLIP (*D*) and its control (*C*) in the basolateral membrane of rat tracheal epithelium. (Bar = 15 μm.) (*E*) Low-magnification micrograph showing MIWC expression in bronchus in rat lung. (Bar = 100 μm.) (*F* and *G*) High-magnification micrograph showing MIWC expression on basolateral membrane of bronchus (*G*) and its control (*F*). cil, Cilia; e, epithelium; b, bronchus; a, alveolus.

however, MIWC was expressed strongly in medium-sized bronchi (Fig. 2*E*). At high magnification, MIWC was localized to the basolateral membrane of bronchial epithelia and not on alveolar epithelia (Fig. 2*G*; for control, see Fig. 2*F*).

We have reported (14) that GLIP was expressed on meningeal cells lining the brain surface. Immunoperoxidase staining of rat brain by using a MIWC antibody showed similar strong expression of MIWC on meningeal cells at the brain surface (pia mater) opposite the subarachnoid membrane (Fig. 3*B*, for control, see Fig. 3*A*). MIWC was also expressed on ependymal cells lining brain ventricles and on glial cells in brain matter (data not shown) but not in choroid plexus (Fig. 3*D*, for control, see Fig. 3*C*).

Whereas MIWC and GLIP colocalized in the same cell membranes in kidney, trachea, and brain, their distributions differed in eye. Fig. 4*B* (for control, see Fig. 4*A*) shows GLIP expression in conjunctiva; at higher magnification (Fig. 4*C*), staining was observed on the plasma membranes of conjunctival epithelial cells. Expression of GLIP was not observed in mucous cells and MIWC was not found in conjunctiva. In contrast, MIWC was expressed in iris and ciliary body (Fig. 4*E*, for control, see Fig. 4*D*). At higher magnification (Fig. 4*F*), MIWC was localized to the basolateral membranes of nonpigmented epithelial cells in ciliary body and in the contiguous pigmented cells in iris. GLIP was absent in iris and ciliary body. In retina, only MIWC was expressed (Fig. 4*H*, for control, see Fig. 4*G*). At high magnification, MIWC was seen in layers corresponding to the inner and outer nuclear layers and the ganglion cell layer. MIWC and GLIP were not expressed in cornea, lens, and other structures in the eye.

In distal colon, both GLIP and MIWC were weakly expressed in the basolateral membrane of villus epithelial cells but not in goblet cells (Fig. 5 *B* and *D*; for control, see Fig. 5 *A* and *C*). No staining was observed in crypt epithelial cells. Expression of MIWC and GLIP protein was not detected in small intestine, liver, pancreas, spleen, cardiac muscle, and testis.

DISCUSSION

MIWC and GLIP were colocalized on the basolateral membrane of principal cells in kidney collecting duct. This membrane is believed to be constitutively water permeable (16, 17), whereas water permeability in the corresponding apical membrane is regulated by the vasopressin-induced trafficking of vesicles containing WCH-CD water channels (18). MIWC and GLIP were not expressed in vasa recta or in the proximal tubule and thin descending limb of Henle where water permeability is high and CHIP28 is expressed (3, 4, 19). These results suggest but do not prove a role for MIWC and GLIP in

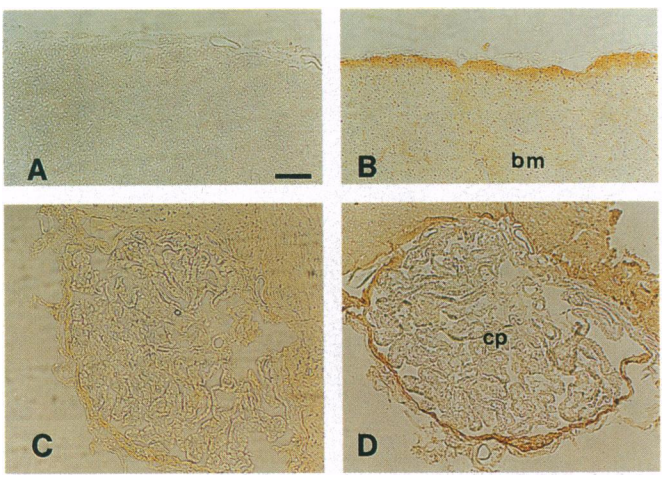

FIG. 3. Immunoperoxidase localization of MIWC in rat brain. (*A* and *B*) Localization of MIWC to meningeal cells at the brain surface at low magnification (*B*) and its control (*A*). (Bar = 100 μm.) (*C* and *D*) Localization of MIWC to ependymal cells lining ventricles without staining of choroid plexus (*D*) and its control (*C*). bm, Brain mater; cp, choroid plexus.

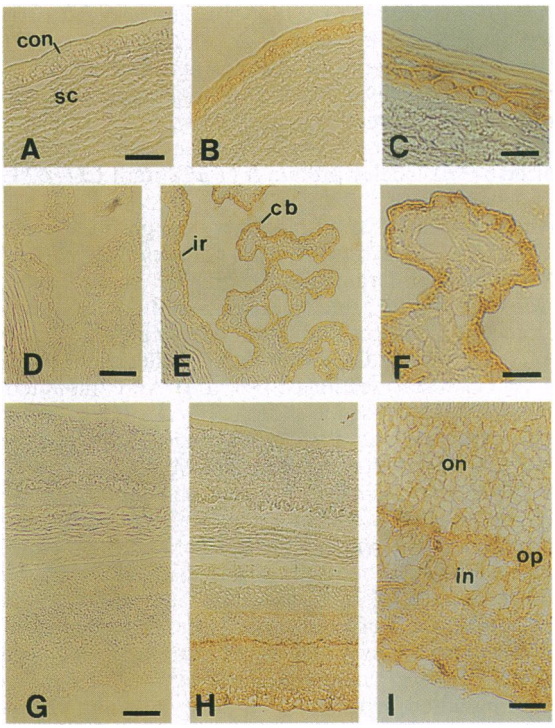

FIG. 4. Localization of MIWC and GLIP protein in eye. (*A–C*) Staining of conjunctival epithelium (*B* and *C*) and its control (*A*) with GLIP antibody. (Bars: *A* and *B*, 100 μm; *C*, 25 μm.) (*D–F*) Staining of epithelium of ciliary body and iris (*E* and *F*) and its control (*D*) with MIWC antibody. (*G–I*) Staining of retina with MIWC antibody. (Bars: *D*, *E*, *G*, and *H*, 100 μm; *F* and *I*, 25 μm.) conj, Conjunctiva; sc, sclera; ir, iris; cb, ciliary body; in, inner nuclear layer; on, outer nuclear layer; op, outer plexiform layer.

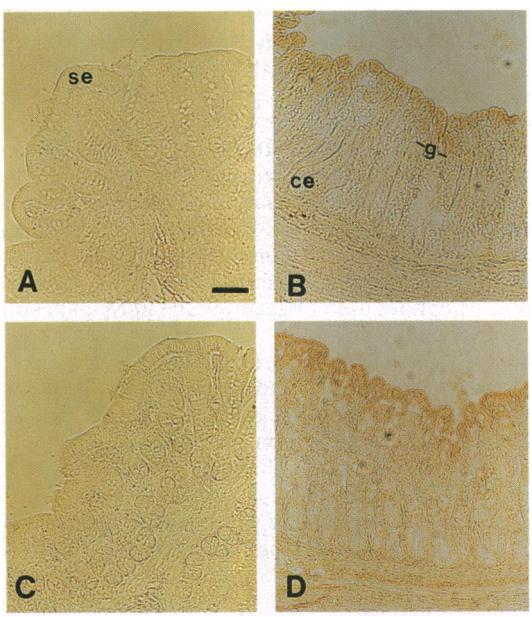

FIG. 5. Coexpression of MIWC and GLIP in surface epithelial cells in rat colon. (*A* and *B*) Immunoperoxidase staining showing GLIP expression on the basolateral membrane of surface epithelial cells in descending colon (*B*) and its control (*A*). (*C* and *D*) Similar pattern of MIWC staining (*D*) and its control (*C*). (Bar = 100 μm.) se, Surface epithelium; ce, crypt epithelium; g, goblet cell.

the urinary concentrating mechanism. Determination of the relative importance of these proteins in basolateral membrane solute transport will require quantitative analysis of protein expression levels and single-channel permeabilities and/or studies on mammals lacking functional MIWC or GLIP protein.

It was reported that CHIP28 was expressed in pulmonary capillary endothelium and to a lesser extent in the alveolar epithelium (6, 20) and that transalveolar airspace-to-capillary water movement was mediated by mercury-sensitive water channels (20). The localization of MIWC to the airway suggests its involvement in transbronchial fluid movement for airspace hydration. The lack of expression of known water channels in the apical membrane of tracheal and bronchial epithelia suggests that other as yet unidentified water channels may exist in lung.

In eye, MIWC was expressed in ciliary body and iris epithelia, the same cells expressing CHIP28 and known to be involved in secretion of aqueous humor (5–7). The expression of MIWC in neural cell layers in retina was unexpected because fluid transport has not been thought to have a role in visual signal processing. None of the water channels identified to date is expressed on the retinal pigmented epithelium, a tissue believed to participate in ion and water transport (21). The strong expression of GLIP on conjunctival epithelium is also of unclear significance since the conjunctiva has been thought to play a passive protective role in the eye.

In the gastrointestinal tract, MIWC and GLIP were found only in surface epithelial cells of colon, which plays the primary role in fecal dehydration (22). Previous immunolocalization and *in situ* hybridization showed expression of CHIP28 in crypt epithelium in rat colon (5, 7). The lack of water-channel expression in stomach and small intestine is consistent with functional data in vesicles derived from these tissues showing low plasma membrane water permeability (23, 24). The absence of MIWC immunostaining in liver, a tissue in which the spliced MIWC transcript is primarily expressed (13), suggests that the MIWC spliced transcript is not translated.

The colocalization of MIWC and GLIP in plasma membranes of several cell types was a surprising finding. Because MIWC functions as a water channel (13) and GLIP functions as a glycerol transporter (14, 15) and possibly as a water channel (15), their coexpression might simply represent the need for certain cell membranes to carry out the transport of both water and small solutes. Alternatively, their coexpression might indicate the existence of independent regulatory mechanisms or the redundant expression of proteins that carry out an essential physiological function.

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