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Aquaporin-CHIP-related Protein in Frog Urinary Bladder: Localization by Confocal Microscopy

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Abstract. Aquaporin CHIP, a 28 kDa channel forming protein, has been proposed to function as water channel in both erythrocyte and kidney proximal tubule. Recently, we have reported that in frog urinary bladder, a model of the kidney collecting tubule, polyclonal antibodies against human erythrocyte CHIP recognize and immunoprecipitate a 30 kDa protein from the epithelial cell homogenate. In the present work confocal fluorescence microscopy was used to determine the cellular and subcellular localization of CHIP28-like proteins in the urinary epithelium. A clear labeling of the apical border was found after Triton X-100 permeabilization. The labeling was distributed throughout the apical domain and not restricted to specific domains of the membrane. The staining was also present in the deeper confocal sections where the fluorescence seems to be localized at the cellular contour. No difference in the labeling patterns was observed between resting and ADH-treated bladder.

Specificity of the staining was confirmed by the absence of the labeling pattern when antiserum was preadsorbed on CHIP28 protein immobilized on Immobilon P stripes. Our results suggest that CHIP-like proteins are not proteins inserted in the apical membrane during the antidiuretic response. Moreover, we do not know whether the labeling was due to the presence of CHIP28 itself or an as-yet-unidentified protein sharing immunological analogies with aquaporin CHIP.

Key words: CHIP28 — Aquaporins — Water channels — Vasopressin — Antibodies

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Introduction

The apical plasma membrane of vasopressin (ADH)-sensitive epithelial cells, such as those of the kidney collecting tubule and amphibian urinary bladder, are remarkably impermeable to water in the resting state but, after stimulation with the hormone they become as permeable as most biological membranes [9]. It has been suggested that control mechanisms involve exocytic insertion into the apical membrane of water channel-loaded vesicles located at rest in the intracellular compartments of the granular cells of the amphibian bladder [5, 13] and the principal cells of the mammalian collecting duct [10, 12]. In fact, the rate-limiting barrier for transcellular water transport in these tissues is thought to be the apical plasma membrane facing the urinary compartment and not the basolateral membrane. The water permeability of the basolateral membrane is high and the presence of permanent water channels has been proposed [20]. As far as the molecular identity of the water channels is concerned, the recent discovery of aquaporins [2] has now opened the way for further characterization of water channels. One such water-channel protein described in mammalian tissues is the channel-forming integral membrane protein of 28 kDa (CHIP28), which is the major water-channel protein of erythrocytes [6], renal proximal tubule and descending limb of the loop of Henle [16, 18] and found in several other water-transporting tissues [11, 17]. An additional member of the aquaporin family WCH-CD, 42% identical in amino acid sequence with CHIP28, has been detected in the apical membrane of mammalian kidney collecting tubule and proposed to be the hormone-regulated water channel [8]. Immunolocalization studies performed in rat kidney with polyclonal antisera against WCH-CD [15] have shown that WCH-CD proteins are present in other structures besides the apical plasma membrane of the collect-

ing-duct principal cells and inner medullary collecting duct cells, namely small subapical vesicles and the basolateral membrane in the medulla.

Recently, we reported that an ADH target tissue, the frog urinary bladder, contains proteins possessing immunological analogies with the mammalian erythrocyte CHIP28 [4]. We have now used antibodies to CHIP28 to carry out immunocytochemistry to determine the cellular and subcellular localization of CHIP28-like proteins in the urinary bladder. For this purpose we used confocal fluorescent microscopy for a better approach to the tridimensional structure of the cells, their borders and inner cellular compartments.

Materials and Methods

Materials

All chemicals, if not otherwise indicated, were from Sigma Chemical (St. Louis, MO).

Preparation of Erythrocyte Plasma Membranes

Human erythrocytes were obtained from blood bank units and stored at 4°C. Hemoglobin-free erythrocyte ghosts were prepared essentially according to the methods described by Fairbanks, Steck and Wallach [7]. Ghosts from washed frog erythrocytes were obtained by hemolysis in 40 volumes of 5 mM phosphate buffer, pH 7.4, containing 2 mM $MgCl_2$ to prevent disintegration of nuclei. Membranes were prepared by sonication of a suspension of ghosts for 30 sec followed by a 10 min centrifugation of nuclei and nondisrupted erythrocyte membranes at $750 \times g$ in a Sorvall SS 34 rotor. The supernatant was centrifuged at $48{,}000 \times g$ for 20 min and the resultant pellet was used as the membranes.

Isolation of the 28 kDa Protein and Production of Polyclonal Antibodies

Electrophoresis of proteins from unsealed human ghosts was performed on preparative SDS gels containing 13% acrylamide according to Laemmli [14]. Gels were then stained with Coomassie blue and the regions of 28 kDa, even if weakly stained, were sliced out and destained. The 28 kDa band was eluted by shaking the gel pieces for 48 hr at 22°C in a buffer containing 0.2% (w/v) SDS, 10 mM phosphate buffer pH 7.4, 1 mM NaN_3 [Merck] [19]. Eluted peptides were concentrated and dialyzed extensively against 0.15 M NaCl, 10 mM phosphate buffer, pH 7.4 (PBS).

For the immunization, after the collection of preimmune serum, approximately 100 μg of dialyzed 28 kDa peptides were mixed with an equal volume of complete Freund's adjuvant and injected intravenously to young rabbits. Booster injections were performed in the same way with incomplete Freund's adjuvant after 21 days and then subsequently every 10 days. Some rabbits developed strong responses after the sixth injection, as determined by immunoblots on both ghost proteins and on SDS-eluted 28 kDa peptides. Sera obtained from immunized animals were carefully decanted and stored in small aliquots at −20°C in the presence of 0.1% (w/v) NaN_3.

Affinity Purification of Anti-28 kDa Peptide Antibodies

Proteins from human erythrocyte ghosts were separated in 13% SDS-PAGE slab gels and then electrophoretically transferred to Immobilon (Millipore, Bedford MA). A horizontal strip of membrane including the 28 kDa band (whose localization had been ascertained by Western blot of a vertical cut strip) was excised, washed six times with PBS containing 5% nonfat dry milk (blocking buffer), three times with PBS only and saturated with blocking buffer for 60 min. The Immobilon strips were then incubated for 4–5 hr at room temperature with the anti-28 kDa peptide immune serum diluted 1:4 with blocking buffer. After this step, the strips were rinsed with several changes of PBS and adsorbed anti-28 kDa peptide antibodies were released by incubation in 0.2 M glycine-HCl pH 2.8 twice for 5 min under vigorous shaking. The solution containing the eluted antibodies was immediately neutralized with 1 N NaOH, diluted to a final volume of 15 ml with PBS and concentrated in a microconcentrator tube (cutoff 10 kDa, Amicon, MA). The concentrated antibody solution was used at a dilution of up to 1:20 for Western blot and immunofluorescence studies.

Preparation of Frog Urinary Bladder and Kidney Cortex Homogenates

Double pithed frogs were perfused via the heart with a Ringer solution to remove blood from the bladder vessels. Bladders were isolated, filled with Ca^{2+}-free EGTA-Ringer (in mM, 112 NaCl, 5 KCl, 2.5 $NaHCO_3$, 1 EGTA, pH 8.1) and immersed in the same solution for 20 min at 4°C. The urinary epithelial cells were removed by gentle massage of the bladder, washed two times by a centrifugation at $600 \times g$ for 10 min with Ringer solution and resuspended in a small volume of a hypotonic buffer containing (in mM) 7.5 HEPES, 7.5 KCl, 0.1 EDTA, 1 PMSF and 0.75% (w/v) sucrose, pH 8.0. The cell suspension was homogenized on ice by 200 strokes in a Dounce B and undisrupted cells and nuclei were removed by centrifugation at $4{,}600 \times g$ for 10 min. Proteins were assayed according to the method described by Bradford [3]. The homogenate was stored in small aliquots at −20°C until used for immunoblotting or immunoprecipitation studies.

The cortex from rat kidney was dissected, cut in small pieces and homogenized in a glass-Teflon homogenizer (Braun). Aliquots of homogenate were centrifuged at $4{,}600 \times g$ for 10 min and the pellet was discarded. The supernatant was spun at $47{,}000 \times g$ for 30 min and the pellet resuspended in PBS and utilized for biochemical analysis.

Immunoblots

Proteins, 100 μg per lane, from several samples were separated by electrophoresis on 13% SDS-PAGE slab gels and transferred to Immobilon membranes. The membranes were briefly stained with Coomassie blue to visualize the transferred protein bands. The Immobilon sheets were destained and incubated for 60 min in blocking buffer comprising 5% nonfat dry milk, 150 mM NaCl, 1% Triton X-100 and 10 mM phosphate buffer, pH 7.4 (solution 1), followed by an incubation with either preimmune or immune serum (diluted 1:700 in solution 1) or affinity-purified antibodies (diluted 1:20 in solution 1) at room temperature for 4 hr. The blots were then washed by several changes of solution 1, incubated for 60 min with a 1:3,000 dilution in solution 1 of peroxidase-conjugated goat anti-rabbit antibody, washed again and peroxidase activity revealed by enhanced chemioluminescence (ECL, Amersham) in a dark room.

Preparation of 28 kDa Peptide Antibody-depleted Serum

The antiserum against 28 kDa peptide was affinity-purified. Affinity-purified antibodies were mixed with an excess of 28 kDa peptide immobilized on Immobilon P stripes. After 4 hr incubation, the stripes containing antibodies specifically directed against 28 kDa peptide were removed and the remaining serum was further processed for the immunocytochemistry.

Immunocytochemistry

The immunolabeling was performed on frog bladders isolated and mounted between two Lucite multichannel chambers so that appropriate controls could be run on the same bladder. After 30 min preincubation in Ringer solution on both apical and serosal sides, some fragments of the same bladder were stimulated with 100 mU/ml ADH for 15 min; all samples were then fixed with 4% paraformaldehyde (in PBS) for 30 min. To reduce the interference of free aldehyde groups, the preparations were exposed to 50 mM NH_4Cl (in PBS) for 20 min. Afterward, bladders were permeabilized with Triton X-100 0.2% in PBS, for 20 min and then exposed apically, two times for 15 min, to sheep preimmune serum. Finally, the bladders were incubated for 4 hr with affinity-purified antibodies (diluted 1:4 in PBS). After six washes of 20 min, fluoresceine-conjugated goat anti-rabbit immunoglobulin (Gar-FITC, Biosys), diluted 1:400 was apically incubated for 1 hr. The bladders were washed four times. The apical surface was labeled with a fluorescent marker as Texas Red WGA (wheat germ agglutinin). Finally, samples were cut, mounted on glass slides with PBS-glycerol (1:1) containing 1% *n*-propylgallate. Preparations were examined and photographed with a Leitz confocal scanning laser microscope. No labeling was observed in control bladders. These controls used (a) preimmune sera (b) omission primary or secondary antibody (c) antiserum adsorbed with excess of 28 kDa peptide.

Results

Immunoblot Studies

Figure 1 shows a representative immunoblot of frog erythrocyte ghosts (lane *a*) and of membrane proteins isolated from frog urinary bladder homogenate, probed with 28 kDa peptide affinity-purified antibodies. Results obtained confirm that both possess proteins immunologically related to mammalian erythrocyte CHIP28. In particular, a 30 kDa protein together with a component at higher molecular weight is constantly revealed. This last component may contain, as we previously suggested [4], the glycosylated and/or the polymeric form of CHIP-like 30 kDa protein. When immunoblot experiments were performed with 28 kDa peptide antibodies previously adsorbed with an excess of 28 kDa peptide, no more proteic bands were revealed in both cases.

Immunolocalization by Confocal Microscopy

For these experiments, bladders were fixed with paraformaldehyde, permeabilized or not with Triton X-100 and apically incubated with anti-28 kDa peptide affinity-purified antibodies. Immunocomplexes were visualized by incubation with a second anti-rabbit antibody conjugated with FITC (Fig. 2). The apical surface was labeled with a fluorescent marker, Texas Red-WGA. Some bladders were stimulated with ADH before fixation. The preparations were examined with a confocal scanning laser microscope to obtain xy optical sections as steps ranging from 0.5 to 0.8 μm or xz section in the apical to basal vertical axis. The red and green areas correspond, respectively, to the fluorescence of Texas Red WGA and of FITC-labeled anti-28 kDa peptide antibodies. The yellow color indicates the colocalization of the two fluorophores. Results obtained (Fig. 2*a*) indicate that the apical border of the epithelial cells is clearly labeled by the two markers. Some cells are more strongly immunolabeled than others. The concomitant absence of Texas Red-WGA labeling indicates that they are also very poor in *n*-acetylglucosamine. CHIP28 staining is present in the deeper confocal sections (Fig. 2*b*) where the fluorescence seems to be localized at the cellular contour. The nucleus itself remains negative. In the same preparation the staining is variable from a field to the other. The xz optical section of the same preparation (Fig. 2*c*) shows both an apical and a cytoplasmic staining of the epithelial cells. The pattern observed is the same either at rest or after ADH stimulation (*not shown*).

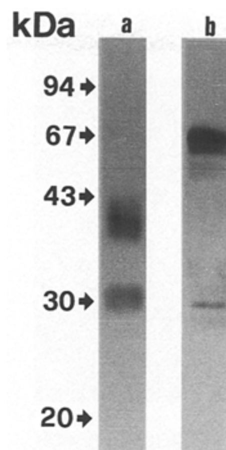

Fig. 1. Immunoblot showing the reactivity of affinity-purified anti-28 kDa peptide antibodies on frog erythrocyte membranes (lane *a*) and on whole homogenate of epithelial cells from frog bladders (lane *b*). Membrane proteins (60 μg) were analyzed in each lane. On the left the migration of molecular weight markers is indicated.

When the bladder was not permeabilized before the treatment with the affinity-purified antibodies either at rest or after ADH challenge, no staining was observed by anti-28 kDa peptide affinity-purified antibodies (*not shown*). When confocal analysis was carried out on bladders from salt-acclimated frogs, a condition in which

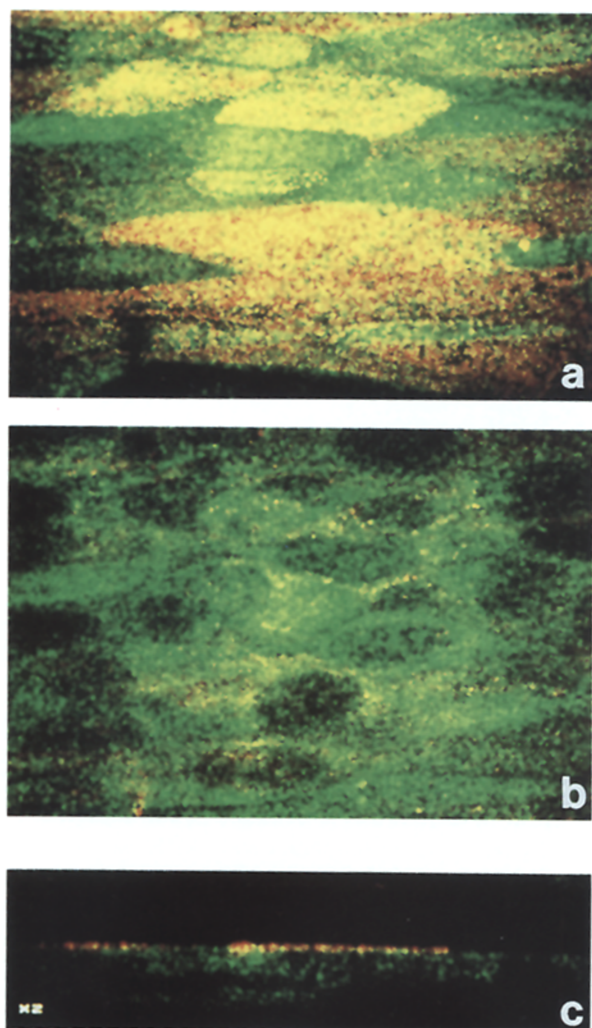

Fig. 2. Immunolocalization of the CHIP28-like proteins using a double labeling technique. Anti-28 kDa peptide affinity-purified antibodies were detected by a second FITC-coupled anti-rabbit antibody. The cell coat was labeled with Texas Red-coupled WGA. Confocal views were obtained using a 63× objective (n.a. = 1.43; Field 79 × 79 μm). The red and green area correspond, respectively, to the fluorescence of Texas Red WGA and FITC-labeled anti-28 kDa peptide antibodies. The yellow color indicates the colocalization of the two fluorophores. (*a*) Confocal slice of the apical frontier demonstrating a clear labeling of the apical border by the two markers. (*b*) Deeper confocal section of the same field. (*c*) xz optical section of the same field. The apical surface is visualized by the red fluorescence.

the water transport is increased [21] and the 30 kDa protein is maximally expressed [4], an intense immunolabeling of the epithelium was observed. No significant difference was observed between resting and ADH-treated bladders (*not shown*). When 28 kDa peptide antibodies were previously absorbed with an excess of 28 kDa peptide, no more stain was detected (*not shown*).

Discussion

Immunoblot with affinity-purified 28 kDa peptide antibodies demonstrate that these antibodies recognize a 30 kDa protein of frog red blood cells as well as a broader band that could correspond to a glycosylated form of CHIP28. In frog urinary bladder, anti-CHIP28 antibodies detect a 30 kDa protein together with a higher molecular weight component. Control immunoblot carried out with 28 kDa peptide antibody-depleted serum confirm the specificity of the revealed proteins. We thus tried, using confocal laser microscopy, to localize the precise binding sites of anti-28 kDa peptide antibodies in the epithelial cells of the frog urinary bladder. Confocal images confirmed an important labeling of the epithelium, by the anti-28 kDa peptide antibodies that was not observed in control hemibladders in which one of the antibodies had been omitted. The specificity of the staining was supported by the absence of the staining when 28 kDa peptide antibody-depleted serum was used for the staining. Also, no staining was observed when the bladder was not permeabilized by Triton X-100 before the treatment by the affinity-purified antibodies, an observation in line with the reports [19] that the intracytoplasmic COOH-terminal domain of CHIP28 contains most of the epitopes recognized by the anti-28 kDa peptide antibodies.

The distribution of the staining in the epithelium, however, was surprising. Confocal images showed an intense labeling of the apical membrane, which is the limiting barrier for transepithelial water transport and is also the site where vasopressin is generally believed to regulate the epithelial permeability to water. In this membrane the labeling was distributed throughout the apical domain and not restricted to specific domains of the membrane. Interestingly, an important immunostaining was also observed in the subapical and in the perinuclear cytoplasm. On the whole, thus, there appeared to exist several pools of binding sites in the cell with a distribution the location of which is in line with the shuttle hypothesis [22] proposed for the ADH-induced water particle aggregates.

Other data suggest, however, that the CHIP28-like proteins are not the proteins newly inserted in the apical membrane during the antidiuretic response. First, the apical membrane labeling distribution was much broader than that of the ADH-induced intramembrane particle aggregates. Also, there was no clear difference in the immunofluorescence labeling patterns of resting and ADH-challenged bladders. Further observations will be required to settle whether the observed labeling is entirely due to active forms of CHIP28 aquaporins or alternatively to inactive channels or also to as-yet-unidentified proteins that may share structural similarity with the CHIP28. It remains also to establish if the

CHIP28-like protein reported by us is or is not the frog aquaporin CHIP recently sequenced [1].

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