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The 17 kDa band identified by multiple anti-aquaporin 2 antisera in rat kidney medulla is a histone

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Abstract

The osmotic water permeability of epithelial cells of the inner medullary collecting duct of the kidney is regulated by antidiuretic hormone (ADH). ADH causes the insertion and removal of cytoplasmic vesicles containing the aquaporin (AQP-2) water channel protein which is recognized by multiple rabbit antipeptide antisera raised against amino acid sequences comprising its cytoplasmic carboxyl terminal. Immunoblots of rat kidney membrane fractions as well as human urine have all shown that AQP-2 is expressed exclusively by collecting duct cells and have identified a 29 kDa band (corresponding to the nonglycosylated AQP-2 protein), a broad 35–45 kDa band (corresponding to the mature glycosylated form of AQP-2 protein) and an additional immunoreactive 17 kDa band of unknown origin. We now report that the 17 kDa band identified by these anti-AQP-2 antisera is not an AQP-2 component but rather a denatured histone protein type H2A1. This binding of anti-AQP-2 antisera to denatured H2A1 present in protein samples derived from both kidney inner medulla and human urine is blocked specifically by preincubation of immunoblots with solutions containing the acidic protein gelatin.

Keywords: Papilla; Antidiuretic hormone; Aquaporin 2 water channel; Histone; (Kidney)

1. Introduction

Antidiuretic hormone (ADH) regulates the apical membrane osmotic water permeability in epithelial cells of the collecting duct by the insertion and removal of cytoplasmic vesicles containing water channels [1–3]. A large amount of data including Northern- and Western-blotting, in situ hybridization, immunocytochemistry and oocyte expression studies [4–13], as well as patients with nephrogenic diabetes insipidus possessing mutant aquaporin 2 (AQP-2) proteins [14], have identified the AQP-2 protein as the ADH-elicited water channel. AQP-2 is a member of the AQP family of water channel proteins that is distinguished by several common structural features [15]. A central feature of the AQP proteins is the presence of six transmembrane domains in combination with an extracellular and intracellular loop that is postulated to form a water-selective pore spanning the lipid bilayer of the cell membranes [15,16]. All AQPs including AQP-2 possess a high degree of

Abbreviations: ADH, antidiuretic hormone; AQP, aquaporin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; TFA, trifluoroacetic acid; PKA, cAMP-dependent protein kinase A; PBS, phosphate-buffered saline

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amino acid identity in these channel-forming domains whereas amino acids comprising the cytoplasmic carboxyl terminal domains of various AQPs are highly divergent. Antipeptide antisera raised against the amino acid sequences of AQP carboxyl terminal domains have been used in both immunoblotting and immunolocalization experiments as reagents to distinguish individual members of the AQP family.

Multiple anti-AQP-2 antisera raised by several laboratories have uniformly identified a 29 kDa band (corresponding to the nonglycosylated AQP-2 protein) and a broad 35–45 kDa band (corresponding to the mature glycosylated form of AQP-2 protein) on immunoblots of membranes prepared from kidney medulla and papilla [6–13]. However, reports from analyses of both kidney cell membrane fractions [6–12] and urinary protein fractions [8,13] from both rats [6–12] and humans [8,13] have identified an additional immunoreactive 17 kDa band. Although its actual origin and structure are unknown, this 17 kDa band has been postulated to be a proteolytic fragment of AQP-2 [6,8,12] that may be excreted in the urine [8]. We now report that the 17 kDa band identified by multiple anti-AQP-2 antisera is not a component of the AQP-2 protein but rather denatured histone protein type H2A1, abundantly present in the kidney inner medulla and papilla where it is recognized specifically by some but not all anti-AQP-2 antisera. While anti-AQP-2 antisera exhibit significant binding to denatured H2A1 histone protein on immunoblots, these antisera are not likely to bind to histone H2A1 when it is complexed with DNA in the inner medulla and papilla [5]. This spurious binding of anti-AQP-2 antisera to denatured H2A1 is significantly eliminated by preincubation of immunoblots with blocking solutions containing the acidic protein gelatin.

2. Materials and methods

2.1. Preparation of membrane fractions from rat brain, kidney inner medulla and papilla as well as human urine

Crude membrane fractions were isolated from freshly excised various regions of kidney (cortex, outer medulla, and inner medulla and papilla) or brains of female Sprague-Dawley rats (Charles River Laboratories, Billerica, MA) as described previously [5,6,11]. Briefly, crude homogenates in Buffer A (300 mM mannitol, 12 mM Hepes (pH 7.6) containing 0.1 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin (Sigma Chem. Co., St. Louis, MO)) were centrifuged at $325 \times g$ for 10 min to pellet unbroken cells and nuclei. Crude membranes were then collected by centrifugation of the resulting supernatant at $100\,000 \times g$ for 1 h. These membranes were then solubilized directly in SDS-PAGE solubilization buffer and stored at -80°C prior to use.

To prepare subcellular fractions of rat inner medulla and papilla, crude homogenates were subjected to sequential centrifugation steps and the resulting pellet from each step solubilized directly in SDS-PAGE solubilization buffer. These included: (1) $325 \times g$ for 10 min (unbroken cells and nuclei); (2) $2500 \times g$ for 20 min (large plasma membrane sheets and highly dense organelles); (3) $48\,000 \times g$ for 30 min (mitochondria and assorted organelles); and (4) $200\,000 \times g$ for 60 min (low density microsomal fraction) and (5) cytosol.

A crude membrane fraction from human urine was prepared as described [13] with modification. Briefly, random urine samples were obtained from healthy volunteers after an interval of 12 h of water deprivation. The urine was first centrifuged at $325 \times g$ for 10 min to remove larger debris. A crude urinary membrane fraction was then collected by centrifugation of the resulting supernatant at $100\,000 \times g$ for 1 h and corresponding pellet solubilized directly in SDS-PAGE solubilization buffer that was stored at -80°C prior to use.

2.2. Immunoblotting of kidney and brain membrane proteins

Protein samples containing either crude kidney membranes, brain membranes or purified histone H2A1 (Sigma) were fractionated by SDS-PAGE as described previously [5,6,11] then subjected to Western blot transfer of proteins to nitrocellulose filters (Schleicher and Schuell, Keene, NH) in 192 mM glycine, 25 mM Tris (pH 8.3) at 110 V for 2 h. In selected experiments, histone H2A1 was applied directly to the nitrocellulose membrane by dot blotting. Membranes were then blocked with either 2% bovine serum albumin (BSA) (Sigma) at 25°C for 1 h or 3% gelatin (J.T. Baker, Phillipsburg, NJ) at 37°C for 1 h

in phosphate-buffered saline (PBS) (100 mM NaCl, 50 mM sodium phosphate (pH 7.4)) and 0.3% Tween-20 (Biorad Lab., Richmond, CA) (PBS-Tween).

After rinsing in PBS-Tween, membranes were incubated at 25°C for 1 h with 1:2000 dilution of rabbit antisera raised against various peptides containing sequences corresponding to those reported for the carboxyl terminal domain of AQP-2. After rinsing in PBS-Tween, bound anti-AQP-2 antibody was detected by affinity purified anti-rabbit antisera and enhanced chemiluminescence (ECL) (Amersham Life Sci., Arlington Heights, IL) as described previously [6,11]. Immunoreactivity was quantified by using a Zeineh Soft Laser densitometer as detailed previously [17]. Various rabbit anti-AQP-2 antisera tested included those raised against AQP-2 amino acids: (1) #257–271 (Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Cys-Lys-Ala) in our laboratory [5,6,11], or (2) antisera raised to the identical amino acid sequence (kindly provided by C. Van Os, Nijmegen, The Netherlands), 3) #250–271 (Glu-Val-Arg-Arg-Arg-Gln-Ser-Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Ser-Lys-Ala) (kindly provided by M. Knepper, LKEM, NIH, Bethesda, MD) [5,9,12], or (4) #256–271 [18] (kindly provided by D. Brown, Boston, MA). As additional controls, other rabbit antisera were also tested including anti-$G_{i\alpha 1-2}$ and $G_{i\alpha 3}$ (UBI, Lake Placid, NY).

2.3. Purification of 17 kDa protein recognized by anti-AQP-2 antiserum

Kidney membranes suspended in Buffer A were solubilized by addition of Triton X-100 (BioRad Lab., Richmond, CA) to a final concentration of 1.5%. After incubation at 4°C for 1 h, the resulting Triton X-100-insoluble protein complex was pelleted by centrifugation ($16\,000 \times g$ for 20 min), solubilized in 100% trifluoroacetic acid (TFA) and subjected to SDS-PAGE after neutralization of TFA with solid Tris. After transfer of TFA-solubilized proteins to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) in 10 mM CAPS buffer (pH 11.0) (Sigma) containing 20% methanol and staining with 0.1% amido black, the 17 kDa band was subjected to digestion with trypsin and microsequence analyses by the Rockefeller University Biopolymer Facility using methodology described previously [19]. The identity of the resulting protein sequence was obtained upon search of the GENBANK protein database (National Library of Medicine, Bethesda, MD). A total of three partial sequences were obtained all of which were exact matches to histone H2A1.

2.4. Phosphorylation and immunoprecipitation of AQP-2

Rat kidney inner medullary and papillary endosomes containing abundant AQP-2 protein were purified as described previously [11] and incubated at 37°C in Buffer B (300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM Hepes (pH 7.6)) containing 5% (v/v) glycerol and 0.05% (v/v) 2-mercaptoethanol with 100 μM [γ-^{32}P]ATP (10 Ci/mmol) (New England Nuclear, Boston, MA) and purified catalytic subunit of cAMP-dependent protein kinase A (PKA) (20 U/ml) (Sigma) for 3 min to phosphorylate AQP-2. Labeled endosomes were then solubilized by addition of Triton X-100 to a final concentration of 1.5% and incubated at 4°C for 1 h. Triton X-100-insoluble material was removed by centrifugation ($16\,000 \times g$ for 20 min) and the Triton X-100-soluble supernatant subjected to immunoprecipitation using protein A-purified anti-AQP-2 antiserum linked to Sepharose 4B as described previously [20]. After extensive washing in PBS, bound AQP-2 protein was solubilized by SDS-PAGE solubilization buffer and subjected to SDS-PAGE and autoradiography as described previously [20].

2.5. Assay to detect endogenous proteolysis of AQP-2 protein

Kidney inner medullary and papillary membrane fraction containing abundant AQP-2 was prepared after centrifugation at $48\,000 \times g$ for 30 min during sequential centrifugation steps as described above,except that no protease inhibitors were included in any of the solutions used for suspension of membrane fractions. This membrane fraction was divided into two equal aliquots where one was incubated at 37°C for 1 h while the other was incubated on ice. The incubation was stopped by the addition of SDS-PAGE solubilizing buffer and assessed by immunoblotting using anti-AQP-2 antiserum as described above.

3. Results

3.1. Anti-AQP-2 antiserum specifically recognizes a 17 kDa band in addition to bands of 29 kDa and 35–45 kDa in immunoblots of proteins from rat kidney inner medulla and papilla

We [5,6,11,12] and others [4,7,10,21] have previously employed rabbit antisera raised against the sequence Val-Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Ser-Lys-Ala corresponding to amino acids #257–271 in the carboxyl terminal domain of rat AQP-2 [4] in immunoblotting, immunoprecipitation and immunolocalization experiments in rat kidney inner medulla and papilla. This antiserum identifies three major immunoreactive bands of 17 kDa, 29 kDa and a broad band between 35 and 45 kDa in crude membrane fractions prepared from the medulla and papilla of rat kidney (Fig. 1A). The 17 kDa band is most prominently displayed in lanes from the inner medulla and papilla (Fig. 1A, lane 8) and to a lesser extent in membrane fractions prepared from other regions of the kidney (Fig. 1A, lanes 6 and 7). As shown in lanes 1–5, the 17 kDa band is not visualized in various other rat tissue membranes under these conditions.

The presence of this 17 kDa band is not the result of nonspecific binding of rabbit antiserum to proteins of the kidney inner medulla and papilla since immunoblots using preimmune antiserum do not display binding in this region of the gel (Fig. 1B). As additional controls, we tested other commercial rabbit antisera (anti-$G_{i\alpha 1-2}$ and $G_{i\alpha 3}$ (UBI, Lake Placid, NY)) under identical conditions and observed no 17 kDa band (data not shown). Furthermore, we have demonstrated previously [6] that a pattern of immunoreactive bands identical to that shown in Fig. 1A, lane 8 is observed using affinity purified antisera and that the appearance of all three bands is ablated by preincubation of anti-AQP-2 antisera with excess peptide.

3.2. Experiments investigating the subcellular distribution, enzyme digestion and immunoprecipitations using anti-AQP-2 antiserum suggest the 17 kDa band is not a proteolytic fragment of AQP-2

If the 17 kDa immunoreactive band is a component of the AQP-2 protein, then all AQP-2 bands recognized by anti-AQP-2 antiserum should be enriched in membrane fractions prepared from homogenates of rat kidney inner medulla and papilla. However, as shown in Fig. 2 the 17 kDa band is prominent only in subcellular fractions produced by centrifugation of homogenates at low centrifugal forces (Fig. 2, lanes 2 and 3) and not those produced by high centrifugal forces (Fig. 2, lanes 4 and 5).

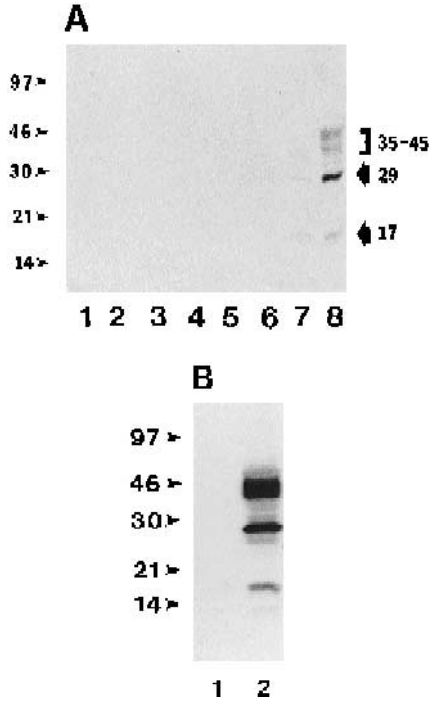

Fig. 1. Immunoblotting analysis of various rat tissues with anti-AQP-2 antisera. (A) Immunoblots containing 40 μg/lane of protein from crude membranes prepared from homogenates of rat brain (lane 1), heart (lane 2), liver (lane 3), lung (lane 4) spleen (lane 5), kidney cortex (lane 6), outer medulla (lane 7) and inner medulla and papilla (lane 8) were blocked using 2% BSA, probed with 1:2000 dilution of rabbit anti-AQP-2 antiserum then developed for the presence of bound antibody using chemiluminescence (see Section 2). Arrows and bracket on the right margin of the blot denote the specific AQP-2 bands of 17 kDa, 29 kDa and 35–45 kDa present in kidney (lanes 6–8) but not other tissues (lanes 1–5). These data from a single experiment are representative of a total of three experiments. (B) Immunoblot strips containing 40 μg/lane of rat kidney inner medulla and papilla membrane protein were probed as described in A with either preimmune (lane 1) or immune (lane 2) anti-AQP-2 antiserum. Specific AQP-2 bands of 17 kDa, 29 kDa and 35–45 kDa are present in lane 2 but not lane 1. These data from a single experiment are representative of a total of five experiments. Small arrowheads denote the relative migration of proteins of known molecular mass ($\cdot 10^{-3}$).

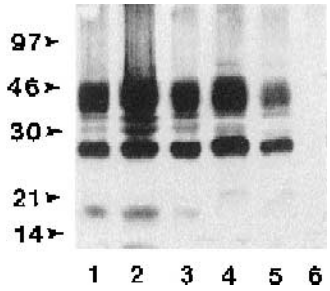

Fig. 2. The 17 kDa immunoreactive band is enriched in subcellular fractions prepared by centrifugation of rat inner medulla homogenates at low centrifugal force. Rat kidney inner medullary and papillary homogenate (lane 1) was subjected to sequential centrifugation steps of increasing magnitude and the resulting membrane pellets subjected to immunoblotting as described in Fig. 1. Crude homogenate (lane 1) was centrifuged sequentially at $325\times g$ for 10 min (lane 2), $2500\times g$ for 20 min (lane 3), $48\,000\times g$ for 30 min (lane 4) and $200\,000\times g$ for 60 min (lane 5). The remaining supernatant was also analyzed (lane 6). A total of 40 μg of protein was loaded in each lane. The small rightward pointing arrowheads denote the position of protein of known molecular mass ($\cdot 10^{-3}$). These data derive from a single experiment representative of a total of six experiments.

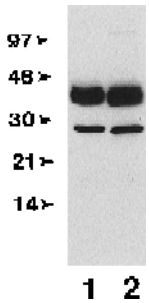

Fig. 3. No 17 kDa band is produced by incubation of membrane fractions containing AQP-2 at 37°C in the absence of proteolytic inhibitors. A kidney membrane fraction containing abundant AQP-2 was prepared from the pellet shown in lane 4 of Fig. 2 during sequential centrifugation steps as described in Fig. 2. For this experiment, all inhibitors of proteolytic enzymatic activity were eliminated from all solutions. Subsequently, aliquots of this high-speed fraction were then subjected to incubation at 37°C for 1 h (lane 2) or incubated on ice for 1 h (lane 1) as a control. Proteolysis of AQP-2 was assessed by Western blotting analysis using anti-AQP-2 antiserum. Despite incubation under conditions that may result in AQP-2 proteolysis, no 17 kDa band is recognized after incubation at 37°C for 1 h (lane 2).

While both the 29 kDa and 35–45 kDa immunoreactive AQP-2 bands are observed in lanes 2–5, the 17 kDa immunoreactive band is observed only faintly (Fig. 2, lane 3) and is not present in membrane fractions shown (Fig. 2, lanes 4 and 5). These data show that the relative abundance of immunoreactivity of the 17 kDa AQP-2 band can be dissociated from that observed by the other AQP-2 bands of larger molecular mass.

It is also possible that the 17 kDa immunoreactive band is a proteolytic fragment of the larger 29 kDa and 35–45 kDa AQP-2 protein generated by endogenous proteases present in kidney inner medullary and papillary membrane fractions. To determine if AQP-2 is proteolyzed during its isolation from cells, we prepared and incubated this kidney membrane fraction containing abundant AQP-2 (prepared as shown in lane 4 of Fig. 3) at 37°C for 1 h in the complete absence of any protease inhibitors. Under these conditions, we observed no 17 kDa protein production as detected by anti-AQP-2 antiserum (Fig. 3, lane 2). In addition, the immunoreactivity of both 29 kDa and broad 35–45 kDa bands was retained completely after the 1 h incubation at 37°C as compared to incubation on ice (Fig. 3, lane 1). These data suggest that the 17 kDa band is not a proteolytic fragment of the larger 29 kDa or 35–45 kDa bands.

As an additional control, we carried out immunoprecipitations using anti-AQP-2 antisera after ^{32}P-labeling of AQP-2 in order to examine the possibility that the 17 kDa immunoreactive band is a fragment of the parent AQP-2 protein. Serine #256 of AQP-2 is phosphorylated by addition of exogenous PKA [21] and only two amino acids separate this PKA phosphorylation site from the amino acid sequence recognized by the anti-AQP-2 antiserum. As a result, any generation of a 17 kDa fragment of AQP-2 recognized by the anti-AQP-2 antiserum would necessitate that proteolytic cleavage of the AQP-2 protein occur in the central portion of the full length AQP-2 sequence. Thus, anti-AQP-2 antiserum should immunoprecipitate a ^{32}P-labeled 17 kDa band of AQP-2 if the 17 kDa protein band is actually a fragment of AQP-2. However, as shown in Fig. 4, only the ^{32}P-labeled 29 kDa and 35–45 kDa bands corresponding to full length nonglycoslylated and glycoslyated forms of AQP-2 protein were recovered in the immunoprecipitate. Taken together, these data displayed in Figs. 2–4 do not support the identity of the 17 kDa immunoreactive band as a carboxyl terminal proteolytic fragment of AQP-2.

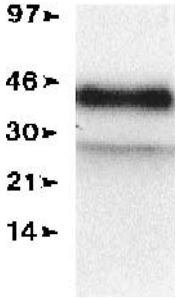

Fig. 4. No ^{32}P-labeled 17 kDa band is recovered in immunoprecipitates of kidney inner medulla and papillary membranes using anti-AQP-2 antisera. After ^{32}P-labeling of inner medullary and papillary membranes by addition of purified catalytic subunit of the cAMP-dependent protein kinase A (PKA) (see Section 2), membranes were solubilized in 1.5% Triton X-100 and the Triton X-100-insoluble portion removed by centrifugation ($16\,000\times g$ for 20 min). The resulting supernatant was subjected to immunoprecipitation using Sepharose conjugated with anti-AQP-2 antiserum. After extensive washing of the Sepharose, bound protein was eluted by addition of SDS-PAGE solubilization buffer, fractionated and the SDS-PAGE gel dried and autoradiographed. Note that only proteins of 29 kDa and 35–45 kDa are visible in the autoradiogram shown. These data from a single experiment are representative of group of 10 experiments.

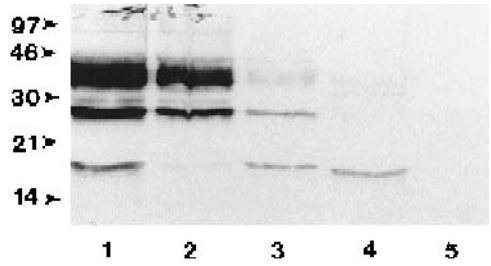

Fig. 5. The 17 kDa immunoreactive protein is recovered in Triton X-100-insoluble and trifluoroacetic acid (TFA)-soluble fractions. Membranes from the inner medulla and papilla were dissolved in 1.5% Triton X-100 as described in Fig. 4. Identical aliquots of 50 μg of protein from starting material (lane 1), Triton X-100-soluble (lane 2) and Triton X-100-insoluble (lane 3) fractions as well as TFA-soluble (lane 4) and TFA-insoluble (lane 5) fractions of the Triton X-100-insoluble fraction were subjected to immunoblotting analysis using anti-AQP-2 antisera as described in Fig. 1. Note that greater than 98% of the 17 kDa immunoreactivity is recovered in the Triton X-100-insoluble fraction that is subsequently soluble in TFA.

3.3. The purified 17 kDa protein recognized by the anti-AQP-2 antiserum is histone H2A1

As shown in Fig. 5, examination of the fate of the 17 kDa immunoreactive band after Triton X-100 solubilization reveals that greater than 98% of the anti-AQP-2 immunoreactivity is retained in the Triton X-100-insoluble pellet (Fig. 5, lane 3) as compared to the Triton X-100 supernatant (Fig. 5, lane 2). However in contrast to the bulk of the proteins that comprise the Triton X-100-insoluble pellet, the 17 kDa immunoreactive protein can be solubilized by subsequent addition of 100% TFA (Fig. 5, lane 4). After SDS-PAGE fractionation, TFA-solubilized 17 kDa protein was transferred to PVDF membrane and subjected to limited proteolytic digestion and protein sequence analysis. A total of three partial sequences were obtained the longest of which was a total of 14 amino acids (Thr-Arg-Ile-Ile-Pro-Arg-His-Leu-Lys-Leu-Ala-Ile-Arg-Asn). All three sequences were identical to sequences in histone H2A1 [22] with the 14 amino acid sequence corresponding to #79–92 of Histone H2A1. No other matches were recovered in searches of the Genbank protein sequence data base. These data are consistent with enrichment of the 17 kDa band in subcellular fractions of rat kidney medulla and papilla homogenates containing nuclei (Fig. 2, lane 2) as compared to other membrane fractions (Fig. 2, lanes 3–5). Furthermore, the acid solubility of the 17 kDa protein recognized by anti-AQP-2 antiserum corresponds to the well known solubility of the histone H2A1 protein in acid solutions [23].

3.4. Histone H2A1 is recognized by anti-AQP-2 antisera but antibody binding to H2A1 may be eliminated by preincubation with gelatin

To obtain confirmatory data that the 17 kDa protein recognized by anti-AQP-2 antiserum is indeed histone H2A1, immunoblots containing purified H2A1 were probed with anti-AQP-2 antiserum. As shown in Fig. 6, anti-AQP-2 antiserum recognizes a 17 kDa band in lanes containing purified histone H2A1 that co-electrophoreses with the 17 kDa present in crude membrane fractions from rat kidney inner medulla and papilla. However, the apparent degree of anti-AQP-2 immunoreactivity displayed by purified H2A1 is surprisingly low after SDS-PAGE and Western blot transfer (Fig. 6, lanes 2–6). These data result from the poor transfer of histone H2A1 from the gel to the nitrocellulose membrane under

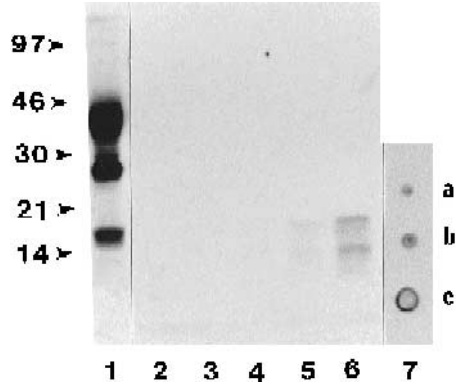

Fig. 6. Anti-AQP-2 antiserum recognizes purified histone H2A1. Individual lanes loaded with either 40 μg of inner medullary and papillary membrane proteins (lane 1) or 0.5 μg (lane 2), 1 μg (lane 3), 2 μg (lane 4), 4 μg (lane 5) and 8 μg (lane 6) of histone H2A1 were subjected to SDS-PAGE and Western blot transfer prior to immunoblotting analysis as described in Fig. 1. Alternatively, lane 7 shows the results of direct application of 0.5 μg (a), 1 μg (b) and 2 μg (c) of H2A1 to the nitrocellulose filter followed by immunoblotting analysis in a fashion identical to lanes 1–6. Note that the anti-AQP-2 antiserum recognizes a 17 kDa band in both kidney membranes as well as histone H2A1.

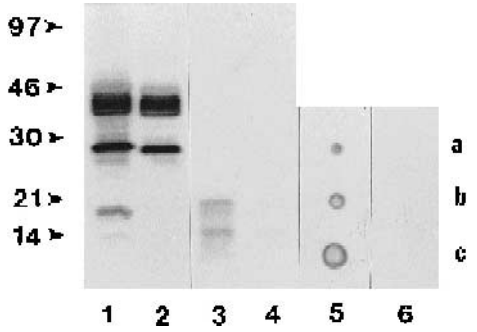

Fig. 7. Preincubation of immunoblots with gelatin ablates AQP-2 antisera reactivity to both the 17 kDa immunoreactive band as well as histone H2A1. Samples containing either 40 μg of kidney membranes (lanes 1 and 2) or purified histone H2A1 (lanes 3–6) were either transferred or applied to a nitrocellulose filter in a fashion identical to that described in Fig. 6. Portions of the filter were preincubated with either 2% BSA (lanes 1, 3 and 5) or 3% gelatin (lanes 2, 4 and 6), then immunoblotted using anti-AQP-2 antiserum as described in Fig. 1. Note that preincubation of kidney membranes (lane 2) as well as histone H2A1 (lanes 4 and 6) with 3% gelatin abolishes antisera binding to both the 17 kDa band as well as H2A1 protein. In contrast, AQP-2 bands of 29 and 35–35 kDa appear unaffected. These data are derived from a single experiment representative of a total of four experiments.

these Western blotting conditions, since application of purified H2A1 protein to the nitrocellulose filter via dot blotting yields anti-AQP-2 immunoreactivity using as little as 500 ng of histone H2A1 (Fig. 6, lane 7).

To obtain additional data linking the 17 kDa immunoreactive protein of rat kidney inner medulla and papilla to the properties of histone H2A1, immunoblots were blocked by preincubation with either 2% BSA or 3% gelatin. In contrast to BSA, gelatin is an acidic protein that readily binds to basic histones [23]. As shown in Fig. 7, preincubation of lanes with 3% gelatin (Fig. 7, lanes 2, 4 and 6) as compared to 2% BSA (Fig. 7, lanes 1, 3 and 5) ablated the binding of anti-AQP-2 antisera to the 17 kDa band present in both kidney membranes (Fig. 7, lanes 1 and 2) as well as purified H2A1 histone (Fig. 7, lanes 3–6). Under these conditions, no significant alteration in the immunoreactivity of the 29 kDa or 35–45 kDa AQP-2 were observed (Fig. 7, lanes 1 and 2). These data suggest that the H2A1 epitope recognized by anti-AQP-2 is masked by the binding of gelatin to the denatured histone protein.

To demonstrate that histone H2A1 possesses actual apparent crossreactivity with denatured AQP-2 pro-

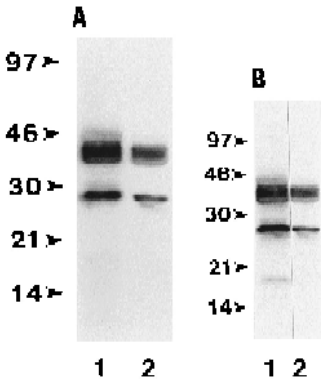

Fig. 8. Preabsorption of anti-AQP-2 antiserum with histone H2A1 reduces the AQP-2 immunoreactivity and eliminates the appearance of 17 kDa band in blots of inner medulla membranes blocked with 2% BSA. Identical quantities of anti-AQP-2 antiserum were preabsorbed with Sepharose cross-linked with either BSA (lane 1) or H2A1 (lane 2). These were then used to immunoblot 40 μg/lane of rat kidney inner medullary and papillary membranes blocked with either 3% gelatin (A) or 2% BSA (B) as described in Fig. 7 and Fig. 1, respectively. As shown in A, preabsorption of anti-AQP-2 antiserum with H2A1 (lane 2) reduced its reactivity by $71 \pm 17\%$ ($n = 5$) as compared to control (lane 1). Panel B demonstrates that preabsorption of anti-AQP-2 antiserum with H2A1 protein eliminates the appearance of the 17 kDa band when immunoblots are blocked with BSA ($n = 3$).

tein, anti-AQP-2 antiserum was preabsorbed with Sepharose conjugated with either BSA or H2A1 protein. The absorbed anti-AQP-2 antiserum was then utilized to probe immunoblots of rat kidney membranes blocked with 3% gelatin (Fig. 8A). As compared to the BSA control (Fig. 8A, lane 1), preincubation with histone H2A1 (Fig. 8A, lane 2) reduced the immunoreactivity of anti-AQP-2 antisera to both 29 kDa and 35–45 kDa bands by $71 \pm 17\%$ ($P < 0.05$, $n = 5$). In addition, as shown in Panel B of Fig. 8, preabsorption of anti-AQP-2 antisera with H2A1 (lane 2) but not BSA (lane 1) resulted in a disappearance of the 17 kDa band from immunoblots of kidney membranes blocked with 2% BSA. Taken together, these data displayed in Figs. 6–8 suggest that histone H2A1 shares epitopes present on denatured AQP-2 protein present in immunoblots.

3.5. Rabbit antisera from multiple laboratories raised against carboxyl terminal sequences of AQP-2 contain immunoreactivity to both the 17 kDa band in rat kidney membranes as well as purified histone H2A1

To determine whether the immunoreactivity of the anti-AQP-2 antiserum raised in our laboratory is unique in its recognition of the 17 kDa and histone H2A1, we tested seven other anti-AQP-2 antisera each raised independently in individual animals by

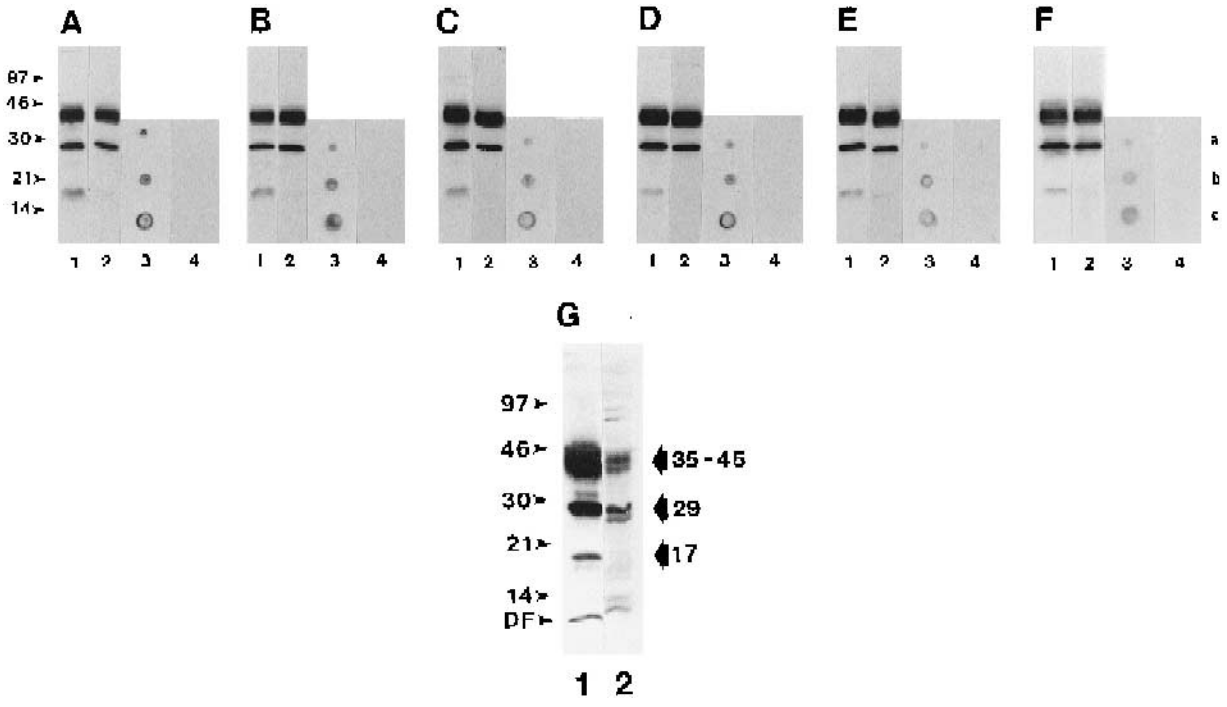

Fig. 9. Rabbit antisera from multiple laboratories contain immunoreactivity to both a 17 kDa band in rat kidney membranes and purified histone H2A1. Nitrocellulose strips containing either 40 μgs of inner medullary and papillary membranes (lanes 1 and 2) or 0.5 (a), 1 (b) or 2 (c) μg of histone H2A1 (lanes 3 and 4) were blocked with either 2% BSA (lanes 1 and 3) or 3% gelatin (lanes 2 and 4), then incubated with individual lots of rabbit antiserum raised against various carboxyl terminal AQP-2 sequences (panels A–F) (see Section 2). (A, B) Antiserum #1 and #2 raised to 22mer peptide AQP-2 amino acid #250–271 in separate rabbits (provided by M. Knepper, LKEM, NIH, Bethesda, MD); (C–F) Antisera #3–6 all raised to 15mer peptide AQP-2 amino acid #257–271 in separate rabbits (provided by our laboratory or C. Van Os, Nijmegen, The Netherlands). Note that all six antisera identify a 17 kDa immunoreactive band in kidney membranes as well as histone H2A1 when strips are blocked with 2% BSA. In contrast, blocking with 3% gelatin ablates or greatly diminishes antisera reactivity to 17 kDa band and H2A1. (G) Nitrocellulose strips prepared as described in panel A were immunoblotted in a paired experiment with either anti-AQP-2 antiserum (shown in Fig. 1, lane 1) or another anti-AQP-2 antiserum [18] provided by D. Brown Boston, MA (lane 2) after blocking strips in 2% BSA. Note that in contrast to the other seven anti-AQP-2 antisera tested, this anti-AQP-2 antiserum does not exhibit specific reactivity to the 17 kDa band. This is a single experiment representative of a total of four.

our own and other laboratories. As shown in Fig. 9A–G, six of the seven exhibited identical characteristics to that displayed in Fig. 7 using antiserum raised by our laboratory, while one (panel G) exhibited no reactivity toward proteins present in the 17 kDa region of kidney membrane immunoblots. All six anti-AQP-2 antisera recognized a 17 kDa band in rat kidney membranes (Fig. 9, lane 1) and displayed immunoreactivity to purified histone (lane 3) that were both ablated by preincubation with 3% gelatin (Fig. 9, lanes 2 and 4). These data demonstrate that recognition of a histone H2A1 of apparent molecular mass of 17 kDa present in either rat kidney inner medullary and papillary membrane fractions or purified form is not limited to a single rabbit anti-AQP-2 antiserum. Instead, a total of six antisera raised independently to peptides containing AQP-2 carboxyl terminal sequences all exhibit identical properties including recognition of a 17 kDa band while another displays no such reactivity.

Immunoblotting analysis of proteins present in a crude membrane fraction derived from human urine fractions has also been reported to show a 17 kDa immunoreactive band which the authors suggested might be the result of proteolytic fragments of AQP-2 [7]. To determine if the 17 kDa band recognized by rabbit anti-AQP-2 antiserum in membrane fractions of rat kidney is also present in human urine samples, immunoblotting analysis was performed on human urinary proteins as described in Fig. 7 after blocking nitrocellulose strips in either 2% BSA (Fig. 10, lane 1) or 3% gelatin (Fig. 10, lane 2). In a manner identical to that displayed in samples of rat kidney protein, this antiserum also identified a 17 kDa immunoreactive band that was absent in samples blocked with 3% gelatin. Since crude fractions derived from human urine contain abundant fragments of renal epithelial cells shed from renal tubules, the presence of fragments of nuclei of renal epithelial cell as well as membranes provides a likely explanation for the presence of the 17 kDa band in human urine.

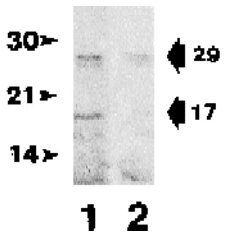

Fig. 10. Anti-aquaporin 2 immunoblots of human urine display a 17 kDa band that is ablated by blocking with 3% gelatin. A crude membrane preparation was prepared by ultracentrifugation of human urine from antidiuretic normal human volunteers and 40-μg aliquots were subjected to immunoblotting analyses identical to that described in lanes 1 and 2 of Fig. 7. Note that blocking of immunoblotting strip with 2% BSA results in appearance of 28 kDa and 17 kDa bands (lane 1). In contrast, after blocking the strips with 3% gelatin, the 28 kDa band remains while the 17 kDa band is ablated. These data are from a single representative experiment performed a total of four times on separate urine samples.

4. Discussion

Considerable data demonstrate that rabbit antisera raised to peptides containing amino acid sequences present in the C-terminal of AQP-2 specifically identify the AQP-2 protein in immunolocalization and immunoblotting experiments [4–13]. These data show that AQP-2 protein is expressed exclusively by ADH responsive epithelial cells in kidney collecting duct. These cells are most abundant in kidney inner medulla and papilla but are also constituents of the collecting ducts of kidney cortex and outer medulla. The presence of prominent 29 kDa and 35–45 kDa bands on immunoblots that correspond to the predicted sizes of nonglycosylated and glycosylated AQP-2 proteins respectively together with specific prominent labeling of the apical plasma membrane and adjacent vesicles in ADH responsive epithelial cells in rat kidney inner medulla and papilla has led to a general acceptance of these antisera as being specific for the AQP-2 protein. However, multiple reports of the absence [18] and presence of a 17 kDa immunoreactive band [6,8,11,12] together with many other publications where this region of immunoblots is not shown [7,10], have raised questions as to whether this 17 kDa band is structurally related to AQP-2 or is another distinct protein.

These data displayed in Figs. 1–10 demonstrate that seven separate anti-AQP-2 antisera recognized this 17 kDa immunoreactive band on immunoblots of membrane fractions prepared from the rat kidney. In contrast, one antiserum displayed no reactivity to a 17 kDa band when assayed under identical conditions. No significant immunoreactivity of 17 kDa

band is present in other rat tissues such as brain, heart, liver, lung and spleen. Moreover, the degree of total immunoreactivity of 17 kDa band in various tissue regions of kidney is directly proportional to the abundance of both 29 kDa and 35–45 kDa bands. To determine the nature of this 17 kDa band, we performed experiments to distinguish whether this immunoreactive band is a proteolytic fragment of the larger 29 kDa or 35–45 kDa AQP-2 protein or another unrelated immunoreactive protein.

Our purification and limited protein sequencing data show that the 17 kDa band is not a structurally-related AQP-2 protein but rather a denatured histone H2A1. Purification of the 17 kDa band took advantage of the acid solubility of histone proteins while the bulk of the other proteins present in the TX-100-insoluble pellet remained in an insoluble protein mass. Purified histone H2A1 coelectrophoreses with the 17 kDa band from rat inner medulla and papilla and both bind anti-AQP-2 antisera. This spurious labeling of purified histone H2A1 and the 17 kDa band in kidney medulla and papilla exhibited by anti-AQP-2 antisera can be eliminated from immunoblots containing histone, rat kidney protein and human urine samples by preincubation of blots in 3% gelatin. True crossreactivity between epitopes present in histone H2A1 and AQP-2 is demonstrated by the preabsorption of anti-AQP-2 antiserum with histone H2A1 protein which results in a $71 \pm 17\%$ reduction in anti-AQP-2 binding to both the 29 kDa and 35-45 kDa bands of AQP-2 protein as well as ablation of the 17 kDa band.

Identification of the exact epitope(s) recognized by anti-AQP-2 antisera is not readily apparent from analyses of the sequence of the histone H2A1 protein. Comparison of the published H2A1 protein sequence to that of the AQP-2 peptide Glu-Leu-His-Ser-Pro-Gln-Ser-Leu-Pro-Arg-Gly-Ser-Lys-Ala reveals no identical full length matches. However, such analyses are complicated by the fact that multiple forms of H2A1 protein are expressed in various vertebrate tissues and the sequence of relative few H2A1s have been determined completely [22,23].

At the present time, the reason why significant immunoreactivity of 17 kDa band is present only in kidney inner medulla and papilla but not other rat tissues is interesting, but yet to be fully understood. One possible reason is the presence of a specific histone isoform H2A1 in only kidney preparations and/or a larger recovery of nuclear fractions containing histone H2A1 during subcellular fractionation from unknown factors such as size or density of kidney nuclei.

The elimination of anti-AQP-2 antisera binding to denatured histone H2A1 on immunoblots by preincubation with gelatin provides a possible explanation for both the lack of labeling of nuclei in immunocytochemistry sections as well as reports that do not apparently detect significant amounts of 17 kDa protein on immunoblots using anti-AQP-2 antisera. Since histones remain in close contact with nuclear DNA in immunocytochemistry sections [5], epitope(s) recognized by anti-AQP-2 antisera may not be exposed and binding of anti-AQP-2 antisera is prevented. In a similar fashion, permeabilization or solubilization of cellular proteins with nondenaturing detergents such as Triton X-100 that preserve histone binding to DNA also would be expected to eliminate or minimize AQP-2 antisera binding to histone H2A1. In contrast, solubilization of crude cellular fractions [5–12] or concentrated urinary protein containing cellular debris including nuclei [8] with the denaturing detergent SDS when applied directly to SDS-PAGE gels likely results in exposure of these H2A1 epitopes on immunoblots subsequently blocked with BSA.

In conclusion, the immunoreactive 17 kDa protein present in the kidney inner medulla and papilla is not a component of AQP-2, and care should be exercised in any immunoassay using many existing anti-AQP-2 antisera to prevent spurious binding to this histone H2A1 protein expressed in kidney membranes.

Acknowledgements

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