Association of the Epithelial Sodium Channel with Apx and α-Spectrin in A6 Renal Epithelial Cells*

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Recent molecular cloning of the epithelial sodium channel (ENaC) provides the opportunity to identify ENaC-associated proteins that function in regulating its cell surface expression and activity. We have examined whether ENaC is associated with Apx (apical protein Xenopus) and the spectrin-based membrane cytoskeleton in Xenopus A6 renal epithelial cells. We have also addressed whether Apx is required for the expression of amiloride-sensitive Na\(^+\) currents by cloned ENaC. Sucrose density gradient centrifugation of A6 cell detergent extracts showed co-sedimentation of xENaC, α-spectrin, and Apx. Immunoblot analysis of proteins co-immunoprecipitating under high stringency conditions from peak Xenopus ENaC/Apx-containing gradient fractions indicate that ENaC, Apx, and α-spectrin are associated in a macromolecular complex. To examine whether Apx is required for the functional expression of ENaC, αβγ mENaC cRNAs were coinjected into Xenopus oocytes with Apx sense or antisense oligodeoxynucleotides. The two-electrode voltage clamp technique showed there was a marked reduction in amiloride-sensitive current in oocytes co-injected with antisense oligonucleotides when compared with oocytes co-injected with sense oligonucleotides. These studies indicate that ENaC is associated in a macromolecular complex with Apx and α-spectrin in A6 cells and suggest that Apx is required for the functional expression of ENaC in Xenopus epithelia.

Epithelial Na\(^+\) channels mediate entry of Na\(^+\) from the luminal fluid into the cells during the first stage of electrogenic transepithelial Na\(^+\) transport across Na\(^+\)-reabsorbing epithelia (1, 2). Normal function of these channels is critical for processes as diverse as blood volume control and airway fluid homeostasis (2). Although the structure of epithelial Na\(^+\) channels has recently been elucidated at both the biochemical and molecular levels, the interactions of these channels with associated proteins, such as regulatory and cytoskeletal elements, are just beginning to be clarified.

Benoś and co-workers (3–5) have biochemically characterized a renal epithelial Na\(^+\) channel that consists of at least six nonidentical polypeptides and forms amiloride-sensitive, Na\(^+\)-selective channels when incorporated into planar lipid bilayers. Kleyman and collaborators (6) have purified a similar heterooligomeric Na\(^+\) channel complex from Xenopus A6 cells using a monoclonal antibody (RA 6.3) directed against the amiloride-binding component of the sodium channel. A 160-kDa polypeptide expressed in A6 cells, termed Apx (apical protein Xenopus) has been cloned by Staub and co-workers (7). Antibodies directed against Apx cross-react with the biochemically purified Na\(^+\) channels, indicating that Apx is associated with this channel. However, it is unclear whether Apx represents an associated regulatory protein or a subunit of the channel. Although Apx did not reconstitute amiloride-sensitive Na\(^+\) currents when expressed in Xenopus oocytes, coinjection of either Apx antisense mRNA or antisense oligonucleotides with A6 cell total mRNA inhibited the expression of amiloride-sensitive currents, suggesting that Apx has a regulatory function (7). Cantiello and co-workers (8) have presented evidence suggesting that Apx represents the conductive subunit of an epithelial Na\(^+\) channel. Transfection of Apx into a human melanoma cell line lacking amiloride-sensitive Na\(^+\) channels resulted in the expression of a 9 pS, amiloride-sensitive Na\(^+\) channel (8).

The molecular cloning of the highly Na\(^+\)-selective epithelial sodium channel (ENaC) from a variety of epithelial cells, including A6 cells, has revealed that is composed of three homologous subunits, α, β, and γ (2, 9, 10). This channel has a single channel conductance of 4 pS when expressed in Xenopus oocytes and exhibits ion selectivity, gating kinetics, and an amiloride-pharmacological profile similar to that of the 4 pS, highly Na\(^+\)-selective channel expressed in native Na\(^+\)-reabsorbing epithelia (2, 9, 10). Although the relationship of the cloned ENaC to the biochemically purified Na\(^+\) channel has been a point of contention, Rokaw et al. (11) have recently presented data indicating that α, β, and γ ENaC are components of the epithelial Na\(^+\) channel biochemically isolated from A6 cells. In addition, Kleyman and co-workers (12) have revealed that the monoclonal antibody RA 6.3, which was used to biochemically isolate a Na\(^+\) channel complex from A6 cells, recognizes the amiloride-binding site on α ENaC.

There is evidence indicating that both the biochemically

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1 The abbreviations used are: pS, picosiemens; ENaC, epithelial sodium channel; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; xENaC, Xenopus epithelial sodium channel.

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purified epithelial Na\textsuperscript{+} channel and the \(\alpha\) subunit of ENaC are linked to the spectrin-based membrane cytoskeleton. Elements of the spectrin-based membrane cytoskeleton remain associated with the renal epithelial Na\textsuperscript{+} channel during purification from both bovine papillary collecting ducts and A6 cells (13). The SH3 domain of \(\alpha\)-spectrin interacts with proline-rich sequences in the C terminus of \(\alpha\) ENaC in \textit{in vitro} assays and endogenous \(\alpha\)-spectrin binds to \(\alpha\)ENaC in transfected Madin-Darby canine kidney cells overexpressing a \(\alpha\) ENaC (14). Although association of epithelial Na\textsuperscript{+} channels with the membrane cytoskeleton has been implicated in maintaining the polarized distribution of the channels to the apical membrane domain in Na\textsuperscript{+}-reabsorbing epithelial cells and in modulating epithelial Na\textsuperscript{+} channel activity, \textit{in vivo} evidence for this association is lacking.

The molecular cloning of ENaC provides the opportunity to identify proteins associated with the channel that function in regulating its cell surface expression and activity. Here we have examined whether ENaC is associated with Apx and \(\alpha\)-spectrin in A6 renal epithelial cells. In addition, we have addressed whether Apx is required for expression of amiloride-sensitive Na\textsuperscript{+} currents by cloned ENaC in \textit{Xenopus} oocytes. We reveal that ENaC is associated in a macromolecular complex with Apx and \(\alpha\)-spectrin in A6 cells. In agreement with Staub et al. (7), we demonstrate that Apx is required for the expression of amiloride-sensitive Na\textsuperscript{+} currents by \(\alpha\beta\gamma\) ENaC in \textit{Xenopus} oocytes.

**EXPERIMENTAL PROCEDURES**

Cell Culture—A6 renal epithelial cells, derived from the distal nephron of \textit{Xenopus laevis}, were obtained at passage 69 from the American Type Culture Collection (Manassas, VA) and used through passage 84. Cultures—Cultured as described previously (5). A6 cells were subcultured onto either Millipore (Bedford, MA) HAWP filter rings for 84. Cells were cultured as described previously (5). A6 cells were sub- cinated against a 35-mer synthetic peptide corresponding to the C terminus of amiloride-sensitive Na\textsuperscript{+} currents by cloned ENaC (15). Al- though association of epithelial Na\textsuperscript{+} channels with the membrane cytoskeleton has been implicated in maintaining the polarized distribution of the channels to the apical membrane domain in Na\textsuperscript{+}-reabsorbing epithelial cells and in modulating epithelial Na\textsuperscript{+} channel activity, \textit{in vivo} evidence for this association is lacking.

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anti-α-spectrin antibody (1:100 dilution). Following extensive washing monolayers were incubated for 1 h in the corresponding Texas Red-labeled secondary antibody as described above. Monolayers were subsequently incubated in the anti-Apx antibody for 2 h at room temperature. Following extensive washing, monolayers were incubated in the appropriate fluorescein isothiocyanate-conjugated secondary antibody for 1 h at room temperature. Monolayers were then washed and mounted as described above. Single and dual scan confocal images were obtained using a Leica laser scanning confocal microscope. For dual scan confocal images, microscope settings controlling laser intensity and detection sensitivity were standardized to ensure an optimal signal to noise ratio prior to simultaneous detection of the fluorescein isothiocyanate and Texas Red fluorochromes. Digital images in a confocal series were processed in Adobe Photoshop under identical settings.

Expression of ENaC in Xenopus Oocytes and Antisense Inhibition of Apx Expression—α, β, and γ mENaC cDNAs were linearized, treated with proteinase K, phenol-chloroform extracted, and subsequently ethanol precipitated prior to transcription of the capped RNA using the either T7 or T3 RNA polymerase and mMessage mMACHINE in vitro transcription kit (Ambion, Austin, TX). Stage V and VI oocytes for injection with ENaC cRNA and Apx oligonucleotides were isolated from X. laevis females and placed in a solution (CF-SOS) containing 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPS, pH 7.6. Oocytes were defolliculated in CF-SOS with collagenase (mg/ml, type IV, Sigma) at room temperature for 20 min. Oocytes were subsequently washed in CF-SOS supplemented with 1.8 mM CaCl₂ (SOS) and stored at 19 °C in SOS supplemented with 2.5 mM sodium pyruvate and 100 μg/ml gentamicin.

Two pairs of synthetic oligodeoxynucleotides complementary to Apx were synthesized by Life Technologies, Inc. The first pair of oligonucleotides was complementary to nucleotides +455 to +479 of Apx (sense, 5'-GCA TTA AGA ATC GCC CTA ACC AC-3'; antisense, 5'-GGT GTT AGG GCC ATT CTG CTT ATG C-3'). The second pair of oligonucleotides was complementary to nucleotides +30 to +13 of Apx (sense, 5'-CAA TTC AGT TTC AAA GGG-3'; antisense, 5'-GCA TTA AGC AGA TTC CTA ACC AC-3').

Paired oocytes were injected with a 50 nl volume containing: (i) 2 or 10 ng of α, β, and γ mENaC cRNA and 25 ng of Apx antisense oligonucleotide or (ii) 2 or 10 ng of α, β, and γ mENaC cRNA and 25 or 50 ng of Apx sense oligonucleotide. A further control was performed by injecting oocytes with 2–10 ng α, β, and γ mENaC cRNA and 25 nl of diethyl pyrocarbonate-treated H₂O. Injected oocytes were then incubated at 19 °C in modified Barth’s solution for 24–48 h prior to electrophysiological measurements.

Two-electrode Voltage Clamp—Oocytes were perfused with a solution containing 100 mM sodium gluconate, 2 mM KCl, 1.8 mM CaCl₂, 10 mM HEPS, 10 mM tetraethylammonium chloride, and 5 mM BaCl₂, pH 7.2. Whole cell current was measured at a holding potential of −100 mV in the presence or absence of amiloride (10 μM). Amiloride-sensitive current was determined for each oocyte at −100 mV by subtracting the residual current in the presence of amiloride from the base line as described previously (21).

RESULTS

Antibody Characterization—Specificity of the antibody directed against a peptide corresponding to amino acids 107–125 of α xENaC was demonstrated by immunoprecipitation of in vitro translated α xENaC. An ~70-kDa polypeptide, corresponding to the predicted molecular mass of α xENaC, was observed (Fig. 1A). This polypeptide was not immunoprecipitated when the anti-α xENaC antibody was preincubated with excess free immunogenic peptide, nor did this antibody show cross-reactivity with in vitro translated β and γ xENaC (Fig. 1A). The anti-α xENaC antibody specifically recognized ~70-, ~150-, and ~180-kDa polypeptides on immunoblots of A6 cell Triton X-100 extracts (Fig. 1B). Cell surface biotinylation has revealed that the ~180-kDa polypeptide is expressed at the apical cell surface in A6 cell monolayers. To corroborate that the 180-kDa polypeptide recognized by the anti-α xENaC antibody represents α xENaC rather than an unrelated peptide sharing a common epitope with α xENaC, we generated a second anti-α xENaC antibody against the C terminus of α xENaC. An A6 cell apical membrane fraction was immunoprecipitated using the anti-α xENaC antibody and subsequently immunoblotted with a second anti-α xENaC antibody generated against the C terminus. The anti-C terminus antibody specifically recognizes the ~180-kDa α xENaC polypeptide in the immunoprecipitate. D, co-immunoprecipitation of β xENaC with α xENaC from an A6 cell apical membrane fraction. Anti-β xENaC antibody specifically recognizes a ~97-kDa polypeptide corresponding to β xENaC on immunoblots of α xENaC immunoprecipitates (lane 1). This polypeptide was not recognized when the antibody was preincubated with excess free peptide (lane 2). C, an A6 cell apical membrane fraction was immunoprecipitated using the anti-α xENaC antibody and subsequently immunoblotted with a second anti-α xENaC antibody generated against the C terminus. The anti-C terminus antibody specifically recognizes the ~180-kDa α xENaC polypeptide in the immunoprecipitate. D, co-immunoprecipitation of β xENaC with α xENaC from an A6 cell apical membrane fraction. Anti-β xENaC antibody specifically recognizes a ~97-kDa polypeptide corresponding to β xENaC on immunoblots of α xENaC immunoprecipitates (lane 1). This polypeptide was not recognized when the antibody was preincubated with excess free peptide (lane 2).

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The xENaC immunoprecipitate.

The rabbit anti-Apx C-terminal peptide antibody specifically recognized a ~165–175-kDa polypeptide, corresponding to Apx on immunoblots of A6 cell apical membrane proteins (Fig. 2, lane 1). The mouse monoclonal anti-nonerythroid α-spectrin antibody recognized α-spectrin (∼240 kDa) as well prominent ~150- and ~120-kDa proteolytic fragments of α-spectrin (Fig. 2, lane 4). Specificity of antibody binding was determined by preincubation of the anti-peptide antibodies with an excess of free immunogenic peptide (Fig. 2, lane 2) or by substituting nonimmune serum or IgG for the primary antibodies (Fig. 2, lane 4).

Distribution of Apx in A6 Cell Monolayers—Laser scanning confocal microscopy was used to examine the distribution of Apx in A6 cell monolayers. As shown in Figs. 3 and 4, there is heterogeneity in the apical expression of Apx. Optical sectioning of A6 cells revealed that Apx exhibits a punctate staining pattern that is localized to the apical and apico-lateral membrane domains as well as in the apical cytoplasm (Figs. 3 and 4). No specific labeling was observed when the anti-Apx antibody was preincubated with excess free fusion protein (data not shown).

Sucrose Density Gradient Analysis of α xENaC, Apx, and α-Spectrin Extracted from A6 Cells—Much of the ENaC expressed both in vivo (13) and in heterologous systems (22) is insoluble. Because of the difficulty in dissociating protein complexes from the detergent insoluble membrane cytoskeleton, we looked for Triton X-100-soluble complexes containing α xENaC, Apx, and α-spectrin. Nelson and co-workers have previously used this approach to demonstrate an association of Na⁺/K⁺ATPase with the spectrin-based membrane cytoskeleton in Madin-Darby canine kidney cells (18) and retinal pigment epithelial cells (20). To determine whether solubilized α xENaC is in a complex with Apx and α-spectrin, 0.5% Triton X-100 extracts of A6 cell monolayers were separated on 5–20% linear sucrose density gradients. The distributions of α xENaC, Apx, and α-spectrin within the sucrose density gradient fractions were determined by immunoblotting followed by densitometry. Fig. 5 illustrates the results from a representative gradient. Analysis of the sedimentation profile revealed a peak of Apx in fraction 6 that overlapped with the peak of α-spectrin (fractions 4–6) and the 180-kDa polypeptide specifically recognized by the anti-α xENaC antibody (∼10.5 S). The ~70-kDa α xENaC polypeptide sedimented in fractions 6–13, peaking in fraction 8. In contrast, the ~150-kDa polypeptide recognized by the anti-α xENaC antibody peaked in fractions 11–13, which was well separated from the peak distributions of Apx, and α-spectrin. Changing the time of centrifugation resulted in a commensurate shift in the overlapping sedimentation profiles of Apx, α-spectrin, and 180-kDa α xENaC polypeptides (data not shown).

Co-immunoprecipitation of Apx with α xENaC from Individual Sucrose Density Gradient Fractions—We examined whether the co-sedimentation of Apx and α xENaC within fractions 6–8 represents an association between these two proteins by examining if Apx co-immunoprecipitates with α xENaC. α xENaC was immunoprecipitated under high stringency conditions from each sucrose gradient fraction (1–20, bottom to top). Immunoprecipitated proteins were separated on SDS/PAGE gels and transferred to PVDF paper, and the immunoblots were probed with anti-Apx antibody. In agreement with Fig. 5, Apx was detected in the α xENaC immunoprecipitates from fractions 6–8, indicating that the Apx sedimenting
within these fractions was associated with α ENaC (Fig. 6A). Apx was not detected when nonimmune IgG was substituted for the anti-α ENaC antibody in the immunoprecipitation (data not shown). The ~180-kDa polypeptide recognized by the anti-α ENaC antibody was detected in fractions 4–9 (Fig. 6B).

Co-immunoprecipitation of α ENaC, Apx, and α-Spectrin from Peak Apx-containing Sucrose Density Gradient Fractions—To examine whether α ENaC, Apx, and α-spectrin are part of a high molecular mass protein complex, peak α ENaC 180/Apx-containing sucrose density gradient fractions (4–8) were pooled, and α ENaC was immunoprecipitated under high stringency conditions. Immunoprecipitated proteins were separated by SDS-PAGE gels and transferred to PVDF paper, and the immunoblots were probed with anti-Apx antibody (A) or anti-α ENaC antibody (B). A, representative blot of α ENaC immunoprecipitate probed with anti-Apx antibody. In agreement with Fig. 5, Apx (arrowhead) was detected in α ENaC immunoprecipitates from fractions 6–8, indicating that the Apx sedimenting within these fractions is associated with α ENaC. The arrowhead denotes Apx; − indicates a nonspecific band. B, representative blot of α ENaC immunoprecipitate probed with anti-α ENaC antibody illustrating distribution of 180-kDa α ENaC polypeptide. The arrowhead denotes a α ENaC; − indicates a nonspecific band. Data are not quantitative.

To further corroborate an association between α ENaC, Apx, and α-spectrin in A6 renal epithelial cells, we examined whether α ENaC and α-spectrin co-immunoprecipitate with Apx from pooled peak α ENaC 180/Apx-containing sucrose density gradient fractions (4–8) using the anti-Apx peptide antibody. As illustrated in Fig. 8 the α ENaC (~180 kDa) polypeptide was only weakly recognized by the anti-α ENaC antibody in the Apx immunoprecipitate. Polypeptides corresponding to Apx and α-spectrin were detected in the Apx immunoprecipitates. Identical results were obtained when the anti-Apx fusion protein antibody was used to immunoprecipitate Apx (data not shown). These polypeptides were not detected in the immunoprecipitates when nonimmune rabbit IgG was substituted for

FIG. 5. Sucrose density gradient analysis of α ENaC, Apx, and α-spectrin extracted from A6 epithelial cells. A, immunoblots from one representative experiment (SDS-7.5% PAGE). B, distribution of α ENaC, Apx, and α-spectrin in the sucrose gradients. Immunoblots shown in A were quantitated by scanning densitometry. Relative abundance (arbitrary units) of each protein was plotted against the fraction number. The proteolytic fragments of α-spectrin were included in the quantitation of α-spectrin. Analysis of the sedimentation profile revealed a peak of Apx in fraction 6 that overlapped with the peaks of 180-kDa α ENaC polypeptide and α-spectrin. S value marker proteins: apoferritin (17.2 S), peak fraction 3; catalase (11.35 S), peak fraction 7; aldolase (7.35 S), peak fraction 11; bovine serum albumin (4.6 S), peak fraction 15.

FIG. 6. Co-immunoprecipitation of Apx with α ENaC from sucrose density gradient fractions. α ENaC was immunoprecipitated under high stringency conditions from each sucrose gradient fraction (1–20, bottom to top). Immunoprecipitated proteins were separated on 7.5% SDS/PAGE gels and transferred to PVDF paper, and the immunoblots were probed with anti-Apx antibody (A) or anti-α ENaC antibody (B). A, representative blot of α ENaC immunoprecipitate probed with anti-Apx antibody. In agreement with Fig. 5, Apx (arrowhead) was detected in α ENaC immunoprecipitates from fractions 6–8, indicating that the Apx sedimenting within these fractions is associated with α ENaC. The arrowhead denotes Apx; − indicates a nonspecific band. B, representative blot of α ENaC immunoprecipitate probed with anti-α ENaC antibody illustrating distribution of 180-kDa α ENaC polypeptide. The arrowhead denotes a α ENaC; − indicates a nonspecific band. Data are not quantitative.
with Apx, we examined whether there is a co-segregation of Apx with anti-Apx and anti-xENaC antibodies. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE gels and transferred to PVDF paper. Immunoblots were probed with anti-Apx and anti-α-spectrin to determine whether these proteins co-immunoprecipitate with α xENaC. Anti-α xENaC antibody specifically recognized a single polypeptide (~180 kDa) (arrowhead) in the α xENaC immunoprecipitate (lane 1). Apx (~175 kDa) (arrowhead) was recognized by the anti-Apx antibody in the α xENaC immunoprecipitate (lane 3). Anti-α-spectrin antibody recognized α-spectrin (~240 kDa) and its proteolytic fragments (arrowheads) in the α xENaC immunoprecipitate (lane 5). α xENaC, Apx, and α-spectrin were not recognized when the antibodies were preincubated with excess free peptide (lanes 2 and 4) or nonimmune IgG was substituted for immune IgG (lanes 6). Data presented are representative of five independent experiments. – indicates a nonspecific band.

\[\text{Fig. 7. Co-immunoprecipitation of Apx and } \alpha \text{xENaC from sucrose density gradient fractions 4–8. Sucrose density gradient fractions 4–8 were pooled, and } \alpha \text{xENaC was immunoprecipitated under high stringency conditions using the anti-α xENaC antibody. Immunoprecipitated proteins were separated on 7.5% SDS/PAGE gels and transferred to PVDF paper. Immunoblots were probed with anti-Apx and anti-α-spectrin to determine whether these proteins co-immunoprecipitate with α xENaC. Anti-α xENaC antibody specifically recognized a single polypeptide (~180 kDa) (arrowhead) in the α xENaC immunoprecipitate (lane 1). Apx (~175 kDa) (arrowhead) was recognized by the anti-Apx antibody in the α xENaC immunoprecipitate (lane 3). Anti-α-spectrin antibody recognized α-spectrin (~240 kDa) and its proteolytic fragments (arrowheads) in the α xENaC immunoprecipitate (lane 5). α xENaC, Apx, and α-spectrin were not recognized when the antibodies were preincubated with excess free peptide (lanes 2 and 4) or nonimmune IgG was substituted for immune IgG (lanes 6). Data presented are representative of five independent experiments. – indicates a nonspecific band.}\]

FIG. 7. Co-immunoprecipitation of Apx and α-spectrin with α xENaC from sucrose density gradient fractions 4–8. Sucrose density gradient fractions 4–8 were pooled, and α xENaC was immunoprecipitated under high stringency conditions using the anti-α xENaC antibody. Immunoprecipitated proteins were separated on 7.5% SDS/PAGE gels and transferred to PVDF paper. Immunoblots were probed with anti-Apx and anti-α-spectrin to determine whether these proteins co-immunoprecipitate with α xENaC. Anti-α xENaC antibody specifically recognized a single polypeptide (~180 kDa) (arrowhead) in the α xENaC immunoprecipitate (lane 1). Apx (~175 kDa) (arrowhead) was recognized by the anti-Apx antibody in the α xENaC immunoprecipitate (lane 3). Anti-α-spectrin antibody recognized α-spectrin (~240 kDa) and its proteolytic fragments (arrowheads) in the α xENaC immunoprecipitate (lane 5). α xENaC, Apx, and α-spectrin were not recognized when the antibodies were preincubated with excess free peptide (lanes 2 and 4) or nonimmune IgG was substituted for immune IgG (lanes 6). Data presented are representative of five independent experiments. – indicates a nonspecific band.

Inhibition of ENaC Expression by Apx Antisense Oligonucleotides—Previous work by Staub and co-workers (7) has indicated that Apx is required for the expression of amiloride-sensitive Na⁺ currents in Xenopus oocytes injected with A6 cell poly(A⁺) RNA. To corroborate our biochemical data indicating an association of ENaC with Apx, we similarly employed an antisense approach to examine whether Apx examined is also required for the expression of amiloride-sensitive Na⁺ currents by ENaC in Xenopus oocytes. To first determine whether Apx is constitutively expressed in Xenopus oocytes, immunoblots of a microsomal fraction prepared from oocytes were probed with the anti-Apx peptide antibody. As shown in Fig. 10A, a ~175-kDa polypeptide was specifically recognized by the anti-Apx antibody, indicating that Apx is expressed in Xenopus oocytes.

Co-injection of Xenopus oocytes with αβγ mENaC cRNA and sense oligonucleotide complementary to nucleotides +455 to +479 of Apx showed an amiloride-sensitive current of 1657 ± 365 nA (Fig. 10B) similar to oocytes injected with mENaC cRNA alone (data not shown). However, a marked reduction in amiloride-sensitive current was seen in oocytes co-injected with the corresponding antisense oligonucleotide, 70 ± 15.2 nA, p < 0.001. In order to confirm the specificity of the effect of Apx inhibition on amiloride-sensitive current, the experiment was repeated with sense and antisense oligonucleotides, corresponding to the 5′-untranslated region of Apx (~30 to −13).
shown in Fig. 10C, a marked reduction in amiloride-sensitive current was similarly seen in oocytes co-injected with αβγ mENaC and antisense oligonucleotides when compared with sense co-injected controls (45 ± 15 nA versus 2601 ± 1090 nA, p < .05). To corroborate that antisense oligonucleotides reduced expression of Apx in *Xenopus* oocytes, microsomal fractions were prepared from *Xenopus* oocytes co-injected with mENaC RNA and either antisense or sense oligonucleotides. The microsomal fractions were separated by SDS/PAGE, transferred to PVDF paper, and immunoblotted with anti-Apx antibodies. As shown in Fig. 10D, there was a marked reduction in the 175-kDa polypeptide, corresponding to Apx, in oocytes co-injected with antisense oligonucleotides when compared with oocytes co-injected with sense oligonucleotides.

**DISCUSSION**

Na⁺-reabsorbing epithelial cells are polarized with their plasma membranes divided into two structurally and biochemically distinct domains: the apical, which faces the luminal compartment, and the basolateral, which rests on the basement membrane and is in contact with the interstitial compartment. Transepithelial Na⁺ transport requires the spatial localization of ENaC to the apical membrane and Na⁺/K⁺ ATPase to the basolateral membrane. Immunocytochemical studies have demonstrated that ENaCs are restricted to the microvillar domain of the apical membrane in Na⁺-reabsorbing epithelial cells (10, 23), and patch clamp electrophysiological studies indicate that ENaCs are clustered within these microdomains (1). This is analogous to the clustering of ion channels at neuronal synapses and the acetylcholine receptors at the neuromuscular junction (24). Although the physiological significance of sequestering ENaC to the microvillar domain is unclear, it may be a mechanism whereby ENaC and associated regulatory proteins are compartmentalized for the dynamic regulation of ENaC.

The recent molecular cloning of ENaC facilitates the identification of cytoskeletal and regulatory proteins that are associated with ENaC within these microdomains. Indeed Rotin and co-workers (14) have identified a conserved proline-rich region within the C terminus of α ENaC that mediates binding of α ENaC to the SH3 domain of α-spectrin. Microinjection of a C-terminal α ENaC fusion protein into rat alveolar cells, which express apically restricted α-spectrin, resulted in apical localization of the fusion protein. Based upon these data it was concluded that the interaction of α ENaC with α-spectrin is involved in determining the apical distribution of ENaC (14).

Rotin and associates (25) have also shown that the C terminus of each ENaC subunit contains a tyrosine-based internalization motif, PPXY. This motif in β and γ ENaC interacts with the ubiquitin ligase Nedd4. Nedd4-mediated ubiquination results in the targeting of the assembled αβγ ENaC to lysosomes for degradation (26, 27). Emerging evidence indicates that internalization of the channels via endocytosis (28) and their subsequent targeting to lysosomes may function to regulate the cell surface expression of ENaC. Although these elegant studies have provided novel insight into ENaC interacting proteins that are critical for regulating the cell surface expression of ENaC, we have limited knowledge concerning proteins that are associated with ENaC in native epithelia. In this report we present data that support an *in vivo* association of ENaC with Apx and α-spectrin in A6 renal epithelial cells.
αxENaC—In this study we have used an antibody generated against amino acids 107–125 of the extracellular loop of αxENaC to identify ENaC. This antibody specifically recognizes in vitro translated αxENaC as well as a ~70-kDa polypeptide on immunoblots of A6 cell apical microsomes, which corresponds to the molecular mass of αxENaC in A6 cells reported by May et al. (29). This is consistent with the predicted molecular mass of nonglycosylated αxENaC. In addition to the ~70-kDa polypeptide, this antibody specifically recognizes polypeptides of ~150 and ~180 kDa on immunoblots of an apical membrane fraction prepared from A6 cell monolayers. Using cell surface biotinylation we have been able to demonstrate that the ~180-kDa polypeptide recognized by the anti-αxENaC antibody is expressed at the apical surface of A6 cell monolayers. The migration of αxENaC at 150- and 180-kDa polypeptides is in agreement with the recent data from Rokaw et al. (11) who demonstrated that an antibody generated against a synthetic peptide corresponding to the C terminus of αxENaC, similarly recognized a 150–180-kDa polypeptide in A6 cells. These data suggest that αxENaC may exist as a homo- or heterodimer in A6 cells. The presence of α ENaC as a homodimer is in keeping with stoichiometric studies indicating that the channel consists of two α subunits (21, 30). Furthermore they agree with recent findings from heterologous expression systems indicating that ENaC subunits can tightly associate to form homo- and hetero-meric complexes (31). Interestingly, the molecular mass of the polypeptides recognized by the αxENaC antibody is comparable to the molecular mass reported for the amiloride-binding subunit of the biochemically characterized A6 cell epithelial Na+ channel (4, 6).

The resistance of the putative αxENaC-containing dimers to dissociate into monomers by heating and dithiothreitol (this paper) and 8 M urea is surprising, particularly in light of the fact that we have observed αENaC migrating at a molecular mass of ~100 kDa on immunoblots of mouse M-1 CCD cells and human airway epithelial cells. However, there are examples in the literature of oligomeric integral membrane proteins, including ion channels, which are resistant to disruption by conventional methods used to dissociate protein oligomers. Glycophorin A, the major integral membrane protein of the erythrocyte, is a dimer that is resistant to disruption by heat and SDS (32), whereas bacterial outer membrane porins exist as trimers that are stable in SDS and 8 M urea but break down into monomers when heated (33). The K+ channel (SKC1) of Streptomyces lividans is a tetramer that is stable in β-mercaptoethanol and SDS (34). Although the majority of the SKC1 oligomers will dissociate into monomers by boiling, oligomers can still be detected by immunoblotting following boiling and SDS/PAGE (35). In each of these examples, interactions between the secondary structures of the proteins (α helices or β-sheets) have been proposed to confer stability of the multimer. It thus conceivable that secondary structure interactions between αxENaC monomers or between αxENaC and βxENaC or γxENaC monomers confer stability to the 150–180-kDa αxENaC immunoreactive polypeptide.

Analysis of the distribution of the polypeptides recognized by the anti-αxENaC antibody in with the 5–20% sucrose sucrose density gradients revealed that the ~150-kDa polypeptide had a different sedimentation pattern than that of the ~70- and ~180-kDa polypeptides, peaking in fractions 11–13. In light of recent data indicating (i) that the majority of the ENaC synthesized remains as in its soluble, unglycosylated form (36) and (ii) unglycosylated α ENaC will assemble into oligomers (31), the ~150-kDa polypeptides may represent unglycosylated α ENaC-containing dimers that have not assembled into a macromolecular complex with Apx and α-spectrin. However, it is equally plausible the 150-kDa polypeptide represents another αxENaC-containing species that exhibits a different sedimentation profile, such as a xENaC complexed with another protein. The ~70-kDa polypeptide may represent nonglycosylated, monomeric αxENaC that resulted from Triton X-100 induced disruption of subunit interactions. Using an in vitro expression system, Cheng et al. (31) have presented data indicating that solubilization of ENaC complexes with either CHAPS or Triton X-100 can result in dissociation of subunits from the assembled ENaC complex.

Alternatively, the ~70-kDa polypeptide represent may represent unglycosylated αxENaC that accumulated in the cytosol. Recent studies (36) have indicated that ENaC assembly and maturation is inefficient in Xenopus oocytes and unassembled ENaC subunits accumulate in the cytosol. The data presented in this paper suggest that inefficient assembly of ENaC may also occur in A6 cells as the 70- and 150-kDa polypeptides (and not the 180-kDa cell surface, associated polypeptide) were the predominant forms of αxENaC recognized by the anti-α xENaC antibody.

Apx Is Associated with ENaC and Is Required for the Function Expression of ENaC—The cDNA for Apx was initially isolated by Staub and co-workers (7) from an A6 cell cDNA library. Antibodies directed against Apx specifically recognized a 160–180-kDa polypeptide in the biochemically isolated renal Na+ channel, suggesting that Apx is associated with the Na+ channel (7). To further corroborate a relationship of Apx to the epithelial Na+ channel, Staub et al. (7) addressed whether Apx directly participated in amiloride-sensitive Na+ channel activity. Although coinjection of Apx cRNA did not reconstitute amiloride-sensitive Na+ transport, coinjection of either Apx antisense oligonucleotides or antisense RNA together with A6 cell poly(A+) selected mRNA inhibited the expression of amiloride-sensitive Na+ currents in oocytes (7). The conclusion from these data was that Apx is either a channel subunit or an associated regulatory protein. However, because this work was performed prior to the cloning of ENaC, the relationship of Apx to ENaC and its role in ENaC function was heretofore unclear.

More recently, Cantillo and colleagues (8) have expressed Apx in a human melanoma cell line. Apx transfection resulted in the expression of a 9 pS amiloride-sensitive Na+ current that resembled the 9 pS amiloride-sensitive Na+ channel expressed in A6 cells grown on nonpermeable supports. Based upon these data they concluded that Apx encoded the 9 pS Na+ channel expressed in A6 cells (8).

Our immunocytochemical data indicate that Apx is associated with both the apical microvilli and the apicolateral membrane domain in filter grown A6 cells. In addition there is a apical intracellular pool of Apx. Taken together, these data suggest that Apx is more abundant in A6 cells than would be predicted if Apx were to function as an epithelial Na+ channel. Indeed, immunolocalization of ENaC subunits in nontransfected cells has proven problematic, possibly due to the low level of ENaC expression. To provide data corroborating an association of ENaC with Apx we examined if ENaC and Apx co-immunoprecipitated from detergent extracts of A6 cell monolayers following their fractionation in sucrose density gradients. A population of αxENaC was found to routinely co-sediment with Apx in fractions 4–8 of the sucrose density gradient. From these fractions we were able to specifically co-immunoprecipitate Apx and ENaC under high stringency conditions. We interpret the co-immunoprecipitation of Apx and 180-kDa αxENaC from sucrose gradient fractions to indicate that a population of the 180-kDa αxENaC is associated with another αxENaC-containing species that exhibits a different sedimentation profile, such as a xENaC complexed with another protein.
with Apx. The fact that the αxENaC was weakly detected in the Apx immunoprecipitates indicates that there is a population of Apx that is not associated with ENaC. This is keeping with our immunocytochemical data, which, as discussed above, suggests that Apx is more abundant in A6 cells than ENaC.

In the present study we employed an antisense approach to examine whether Apx is required for the expression of amiloride-sensitive Na⁺ currents by cloned ENaC. We show by immunoblotting that Apx is constitutively expressed in *Xenopus* oocytes. Injection of *Xenopus* oocytes with αβγmENaC cRNA resulted in amiloride-sensitive highly Na⁺-selective currents that were inhibited by co-injection of the cRNA with antisense oligodeoxynucleotides, which were targeted against two distinct regions of Apx, -30 to -13 and +455 to +479. These are the same regions of the Apx sequence that were targeted by Staub et al. (7), and they encompass both the initiation site and an upstream AUG codon. Our data, taken together with that of Staub and colleagues (7), indicate that Apx plays a critical role in the expression of ENaC in *Xenopus* epithelia; however, they do not support Apx functioning as a Na⁺ channel (8).

To date a mammalian homolog of Apx that associates with ENaC has not been identified. However, a human homolog of Apx, Apxl (Apx-like) has been cloned from a human retina cDNA library (37). In addition to the retina, Northern analysis has revealed that Apxl is expressed in placenta, lung, pancreas, and kidney. Although absent from Apx, a single PDZ domain, which shows homology to the PDZ domain of the syntrophin isoforms, is situated near the N terminus of Apxl (37, 38). PDZ domain-containing proteins are localized to submembranous microdomains and have been implicated in intracellular signaling and the clustering of receptors and ion channels (26, 39). For example, the PDZ domain of syntrophin binds to the C termini of SkM1 and SkM2 voltage-gated Na⁺ channels, thereby clustering the channels and linking them to the dystrophin-based membrane cytoskeleton (39, 40). Although speculative, the homology between Apx and the PDZ domain-containing protein Apxl suggests that Apx may function in the clustering and/or stabilizing of ENaC within microdomains of the cell surface by linking ENaC to the spectrin-based membrane cytoskeleton, either through a direct interaction with an ENaC subunit or through interactions with additional ENaC-associated proteins.

**Association of ENaC and Apx with the Membrane Cytoskeleton**—In this report we present evidence supporting the association of ENaC, Apx, and α-spectrin in a macromolecular complex in A6 cells. The identification of a macromolecular complex containing ENaC, Apx, and α-spectrin is in agreement with our earlier data demonstrating an association of the spectrin-based membrane cytoskeleton with the epithelial Na⁺ channel biochemically isolated from bovine papillary collecting ducts and A6 cells (13). It also agrees with the data of Rotin et al. (14) demonstrating a direct interaction between αENaC and α-spectrin.

Following sucrose density gradient centrifugation of Triton X-100 extracts of A6 cell monolayers that were labeled apically, the homology between Apx and the PDZ domain-containing proteins are localized to submembranous microdomains and have been implicated in intracellular signaling and the clustering of receptors and ion channels (37). For example, the PDZ domain of syntrophin binds to the C termini of SkM1 and SkM2 voltage-gated Na⁺ channels, thereby clustering the channels and linking them to the dystrophin-based membrane cytoskeleton (39, 40). Although speculative, the homology between Apx and the PDZ domain-containing protein Apxl suggests that Apx may function in the clustering and/or stabilizing of ENaC within microdomains of the cell surface by linking ENaC to the spectrin-based membrane cytoskeleton, either through a direct interaction with an ENaC subunit or through interactions with additional ENaC-associated proteins.

**Association of ENaC and Apx with the Membrane Cytoskeleton**—In this report we present evidence supporting the association of ENaC, Apx, and α-spectrin in a macromolecular complex in A6 cells. The identification of a macromolecular complex containing ENaC, Apx, and α-spectrin is in agreement with our earlier data demonstrating an association of the spectrin-based membrane cytoskeleton with the epithelial Na⁺ channel biochemically isolated from bovine papillary collecting ducts and A6 cells (13). It also agrees with the data of Rotin et al. (14) demonstrating a direct interaction between αENaC and α-spectrin.

Following sucrose density gradient centrifugation of Triton X-100 extracts of A6 cell monolayers that were labeled apically with biotin, we have been able to detect biotinylated 180-kDa ENaC in fractions 4–8. This suggests that a population of the ENaC-Apx α, and α-spectrin-containing macromolecular complexes that we have identified in A6 cells is expressed at the cell surface. The association of ENaC with Apx and α-spectrin may be a mechanism whereby ENaC is clustered and retained within the microvillar domain of the apical membrane in Na⁺-reabsorbing epithelia, thereby preventing its rapid turnover by endocytosis. This would be analogous to the interactions between H⁺/K⁺-ATPase, whose β subunit contains an internalization motif, and the membrane cytoskeleton in gastric pari-etal cells (41). Interactions between the membrane cytoskeleton and H⁺/K⁺-ATPase stabilize the pump in the apical membrane of acid secreting parietal cells, thereby preventing its endocytotic removal (41). Furthermore, association of ENaC with the spectrin-based membrane cytoskeleton may either play a role in regulating ENaC function (42, 43) or it may function as a scaffolding for the compartmentalization of regulatory proteins, such as kinases and G proteins.

It is conceivable that the macromolecular complexes that we have identified also represent ENaC-containing vesicles that are linked to the spectrin-based cytoskeleton. Spectrin is associated with intracellular vesicles containing the vasopressin responsive water channel aquaporin 2 in renal cells (44) and the glucose transporter GLUT4 in skeletal muscle cells (45). Both of these transport proteins translocate to the cell surface in response to physiologic stimuli. It has been proposed that spectrin plays a role in the regulation of their intracellular trafficking by attaching the transporter vesicles to the cytoplasmic actin network (44, 45). The spectrin-based membrane cytoskeleton may play a similar role in ENaC trafficking during the recruitment of ENaC to the apical membrane from an intracellular pool in response to physiologic stimuli, such as vasopressin (46).

In summary, our present data demonstrating that ENaC occurs in a macromolecular complex with Apx and α-spectrin in A6 epithelial cells. In addition we provide evidence that Apx is required for ENaC expression. We propose that the association of ENaC with the Apx and spectrin-based membrane cytoskeleton functions in sequestering and stabilizing ENaC within microdomains of the apical membrane. Furthermore, this association may function in the compartmentalization of proteins that regulate ENaC. This would be analogous to the sequestering of ion channels, kinases, and other signaling proteins at the post synaptic density through their interactions with membrane cytoskeletal and PDZ domain-containing proteins (47).

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