Primary Structure of an Apical Protein from *Xenopus laevis* That Participates in Amiloride-sensitive Sodium Channel Activity

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Abstract. High resistance epithelia express on their apical side an amiloride-sensitive sodium channel that controls sodium reabsorption. A cDNA was found to encode a 1,420-amino acid long polypeptide with no signal sequence, a putative transmembrane segment, and three predicted amphipathic α helices. A corresponding 5.2-kb mRNA was detected in *Xenopus laevis* kidney, intestine, and oocytes, with weak expression in stomach and eyes. An antibody directed against a fusion protein containing a COOH-terminus segment of the protein and an antiidiotypic antibody known to recognize the amiloride binding site of the epithelial sodium channel (Kleyman, T. R., J.-P. Kraehenbuhl, and S. A. Ernst. 1991. *J. Biol. Chem.* 266:3907-3915) immunoprecipitated a similar protein complex from [^{35}S]methionine-labeled and from apically radioiodinated *Xenopus laevis* kidney-derived A6 cells. A single ~130-kD protein was recovered from samples reduced with DTT. The antibody also cross-reacted by ELISA with the putative amiloride-sensitive sodium channel isolated from A6 cells (Benos, D. J., G. Saccomani, and S. Sariban-Sohraby. 1987. *J. Biol. Chem.* 262:10613-10618). Although the protein is translated, cRNA injected into oocytes did not reconstitute amiloride-sensitive sodium transport, while antisense RNA or antisense oligodeoxynucleotides specific for two distinct sequences of the cloned cDNA inhibited amiloride-sensitive sodium current induced by injection of A6 cell mRNA. We propose that the cDNA encodes an apical plasma membrane protein that plays a role in the functional expression of the amiloride-sensitive epithelial sodium channel. It may represent a subunit of the *Xenopus laevis* sodium channel or a regulatory protein essential for sodium channel function.

SODIUM transport in tight epithelia requires ion translocation across two structurally and functionally distinct cell surfaces, the apical and basolateral plasma membranes. These membranes are spatially demarcated by tight junctions. In these tight epithelia, an apical amiloride-sensitive sodium channel constitutes the rate-limiting step for sodium reabsorption, while the basolateral Na^+,K^+-ATPase provides the driving force for Na^+ entry by extruding Na^+ out of the cell (for a review see Rossier et al., 1989; Rossier and Palmer, 1992; Smith and Benos, 1991). The epithelial sodium channel differs pharmacologically and electrophysiologically from the well-characterized sodium channel of excitable nerve and muscle cells (Noda et al., 1984). It is inhibited by the diuretic amiloride, but it is insensitive to tetrodotoxin, and it is not voltage gated.

The molecular structure of this important channel has not yet been elucidated. Two groups have purified putative epithelial sodium channels either from an amphibian epithelial cell line (A6 cells) or from mammalian kidney by affinity chromatography using amiloride analogs. Benos et al. (1987) isolated a large 730-kD protein complex consisting of five polypeptides ranging in relative molecular mass from 315 to 55 kD. In contrast, Barbry et al. (1987) purified a 185-kD protein composed of two presumably identical subunits. An antiidiotypic antibody raised against an antiamiloride antibody recognized a 700-kD complex in A6 cell extracts. The complex consisted of several polypeptides ranging from 260 to 70 kD (Kleyman et al., 1991), a pattern reminiscent of the channel purified by Benos et al. (1987). On Western blot under reducing conditions the antiidiotypic antibody recognized an ~140-kD band corresponding to the amiloride-binding protein of the purified channel (Kleyman et al., 1991).

Reconstitution of amiloride-sensitive Na^+ channel activ-

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ity by inserting purified channel proteins into lipid vesicles has been reported (Sariban-Sohraby and Benos, 1986; Barbry et al., 1990*b*), and several groups have expressed amiloride-sensitive sodium channels in *Xenopus laevis* oocytes by injecting poly(A)$^+$ mRNA from different tissues (George et al., 1989; Hinton and Eaton, 1989; Kroll et al., 1989; Palmer et al., 1990). Expression cloning in oocytes has been successful for many monomeric channels and transporters including the Na^+-glucose cotransporter (Hediger et al., 1987), the Na^+-phosphate cotransporter (Werner et al., 1991), and the voltage-gated chloride channel (Jentsch et al., 1990). So far, expression cloning for oligomeric transport proteins has not been reported. Cloning of proteins related to the epithelial sodium channel has been described. Injection of a cDNA encoding a human phenamil-binding protein into *Xenopus laevis* oocytes failed to reconstitute amiloride-sensitive ^{22}Na fluxes (Barbry et al., 1990*a*). In contrast, a preliminary report describes that injection of a cDNA encoding a 55-kD polypeptide from the purified bovine sodium channel reconstituted sodium transport in oocytes, but the activity was not sensitive to amiloride (Cunningham, S. A., M. L. Clements, M. P. Arrate, R. A. Frizzell, and D. J. Benos, unpublished observations).

In this study, we report the cloning of a cDNA from a *Xenopus laevis* A6 cell expression library using an antibody originally raised against the *Bufo marinus* Na^+,K^+-ATPase α subunit. We used this antiserum because it labeled the apical membrane of epithelial cells known to express amiloride-sensitive sodium transport. A full-length cDNA clone isolated from an ovary library, distinct from the Na^+,K^+-ATPase α subunit, encoded an ~160-kD protein associated with the apical surface of A6 cells. The Apx protein is part of a larger complex and participates in amiloride-sensitive sodium channel activity.

Materials and Methods

Cell Culture Conditions

A6 cells (passage 80–90), from the American Type Culture Collection (Rockville, MD), were recloned by limiting dilution, and grown either on plastic dishes or seeded at a density of 1.2×10^6 cells/cm^2 on polycarbonate filters (0.4 μm, 4.5 cm^2, Transwell; Costar Corp., Cambridge, MA) coated with a thin layer of glutaraldehyde cross-linked rat collagen type 1 (Vitrogen; Celtrix Laboratories, Palo Alto, CA). The cells were cultured in amphibian medium supplemented with 5% FBS and were harvested when confluent. The cells were treated for 16 h with 300 nM aldosterone before use for biochemical analysis.

Preparation and Screening of cDNA Libraries

An A6 expression library was prepared and screened as described previously (Verrey et al., 1989). Briefly, a cDNA library was constructed from size-fractionated poly(A)$^+$ mRNA from A6 cells grown on plastic dishes. Size-fractionated inserts (0.5–2.5 kb) were ligated into the pEX 2 expression vector. POP2136 cells were transformed and the expression library was screened with a polyclonal antibody directed against the α subunit of *Bufo marinus* Na^+,K^+-ATPase.

Cloning of Near Full Length cDNA

A λgt11 cDNA library prepared from *Xenopus laevis* ovary mRNA (kindly provided by E. Dworkin, Vienna) was screened under high stringency conditions with randomly primed ^{32}P-labeled 3a cDNA clone.

Northern Blot Hybridization

Poly(A)$^+$ mRNA from *Xenopus laevis* tissues and A6 cells was isolated as described previously (Geering et al., 1985) and poly(A)$^+$ mRNA from oocytes was extracted according to Probst et al. (1979). Poly(A)$^+$ mRNA was treated with glyoxal for 10 min at 50°C, separated on 0.8% agarose gel, transferred, and UV cross-linked to a nylon membrane (Hybond N, Amersham Corp., Arlington Heights, IL). After prehybridization at 42°C for 6 h, Northern blots were hybridized at 42°C in 50% formamide, 5× SSC, 0.5% SDS, 2× Denhardt's solution, and 200 μg/ml denatured salmon sperm DNA, with 10^6 cpm/ml randomly primed [^{32}P]dATP-labeled cDNA probes. The specific activity of the probe was $1–2 \times 10^9$ cpm/μg. After hybridization, the filters were washed in 2× SSC/0.1% SDS at room temperature followed by 0.1× SSC/0.1% SDS at 65°C for 2 × 30 min with subsequent exposure to X-ray film with an intensifying screen.

Sequence Analysis

Apx cDNA clone was partially digested by EcoRI and the 4.9-kb insert was subcloned into Bluescript M13-SK plasmid (Vector Cloning Systems, San Diego, CA). The Apx clone was sequenced in both directions using the dideoxynucleotide chain termination method (Sanger et al., 1977). Subcloned fragments were sequenced as double-stranded templates and deleted clones were sequenced unidirectionally (Promega Corp., Madison, WI, Erase-a-base system). When necessary, synthetic oligodeoxynucleotides were used as primers.

Antiserum to Fusion Protein

Fusion protein 3a was extracted from *Escherichia coli* and washed with Triton X-100, according to the protocol of Marston et al. (1985) and electroeluted from SDS-PAGE. Rabbits were immunized by injecting 150 μg of electroeluted fusion proteins with complete Freund's adjuvant by injection into popliteal lymph nodes. The rabbits were boosted at 3-wk intervals by subcutaneous injections of 100 μg of antigen in incomplete Freund's adjuvant, and the rabbits were bled 10 d after the last injection.

Selective Cell Surface Radioiodination

Confluent monolayers were rinsed three times with ice-cold amphibian Ringer's solution containing 11 mM glucose and 1 mM Ca^{2+}. Radioiodination was performed following a modification of the procedure of Hubbard and Cohn (1975). Briefly, 1 ml of amphibian Ringer's containing 11 mM glucose and 1 mM Ca^{2+}, 5 μl lactoperoxidase (10 mg/ml), and 1 mCi carrier free [^{125}I]NaI was added to either the apical or the basolateral side of the monolayer. The reaction, started by adding 20 μl of glucose oxidase diluted to 1:100 (type 5; Sigma Chem. Co., St. Louis, MO), was allowed to run for 15 min at 4°C. The reaction was stopped by adding 20 μl of 20% sodium azide and the monolayers were rinsed extensively with ice-cold amphibian Ringer's containing 0.2% sodium azide.

Biosynthetic Labeling of A6 Cells with [^{35}S]Methionine

A6 cells were seeded on collagen-coated polycarbonate filters (4.5 cm^2) and kept in culture for 9 d. The monolayers were then washed three times for 5 min in methionine- and serum-free medium supplemented with 300 nM aldosterone. The filter supports were inverted, and 200 μl of methionine- and serum-free medium supplemented with 300 nM aldosterone and 1 mCi/ml [^{35}S]methionine was added to the basolateral surface of the filter. After a 15-min incubation at 28°C, filters were washed once and placed for up to 6 h (see legend to Fig. 4) in serum-free medium supplemented with 300 nM aldosterone and 10 mM methionine. The monolayers were then washed once at 4°C with amphibian Ringer's solution containing 1 mM $CaCl_2$ and protease inhibitors (1 μM antipain, 1 μM leupeptin, 1 μM pepstatin A [Sigma Chem. Corp.]), and 0.1 mM PMSF (Boehringer Mannheim Corp., Indianapolis, IN).

Immunoprecipitation

Proteins were solubilized in 0.5% deoxycholate (DOC), 1% NP-40, 5 mM EGTA, and 10 mM Tris-HCl, pH 7.4, containing 0.1 mM PMSF, and 5 mg/ml of pepstatin, leupeptin, and antipain. The solubilized material was centrifuged in Eppendorf tubes for 2 min. For denaturation before incubation with antibodies, SDS was added to the supernatant to a final concentration of 0.3%. Immunoprecipitation was performed after diluting the samples with 11 vol of 1% Triton X-100 in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 150 mM NaCl. Antiserum or preimmune serum was added and the mixture incubated at 4°C overnight. For the antiidiotypic antibody an additional step was included. After the incubation with antiidiotypic antibody, 10 μg of an anti-mouse IgG antibody raised in rabbits was added and

incubated for 2 h before adding protein A-Sepharose. This step was not necessary for the anti-Apx antibody. Protein A-Sepharose beads were used as immunoadsorbent (1 h room temperature). The beads were washed three times with 10 mM Tris-HCl pH 7.4, containing 0.1% SDS and 5 mM EDTA. The antigen was eluted by heating for 5 min in sample buffer and reduced. SDS-PAGE and fluorography were performed as described by Geering et al. (1985).

ELISA

ELISA was carried out according to Sorscher et al. (1988). Briefly, round-bottom polyvinyl chloride microtitration plates (96 wells) were coated with 200–300 ng of purified channel protein in 100 μl BBS overnight at 4°C. The next day, a 2-h exposure with 200 μl of a 1% solution of BSA in BBS (75 mM NaCl; 100 mM boric acid; 25 mM sodium borate), to block nonspecific binding sites, was performed. After this blocking step, 100 μl of an appropriate dilution in BSA-BBS of anti-Apx antibody or preimmune serum was placed in the wells for 4 h at room temperature. After this treatment, the plates were exposed for 4 h at room temperature to 100 μl of a 1:4,000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG in 1% BSA-BBS. The reaction was developed by the addition of 100 μl of 1 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine and 0.2 mM $MgCl_2$ (pH 9.8). Control plates were treated identically except that a 1% BSA solution in BBS replaced the antigen. Yellow coloration of the wells indicated a positive reaction that was measured after 10 or 15 min at room temperature by stopping the reaction with 50 μl of 3 N NaOH and reading the absorbance at 410 nm.

Synthetic Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized according to the manufacturer's manual using a DNA synthesizer (Appl. Biosystems Inc., Foster City, CA). The two antisense oligodeoxynucleotides were Apx(−13)-(−30) (5′-CCCTTTGAAACTGAATTG-3′) corresponding to nucleotides −30 to −13, respectively; Apx(+479)-(+455) (5′-GTGGTTAGGGCGATTCTG-CTTATGC-3′) both complementary to Apx cDNA sequence, and the corresponding sense oligodeoxynucleotides Apx(−30)-(−13) (5′-CAATTC-AGTTTCAAAGGG-3′), respectively Apx(+455)-(+479) (5′-GCATAA-GCAGAATCGCCCTAACCAC-3′).

In Vitro Transcribed RNA

Capped RNA was transcribed from linearized cDNA clones either with T7- or SP6-RNA polymerase according to Melton et al. (1984). Transcribed RNA was extracted twice with chloropane, twice with chloroform/iso-amylalcohol (24:1), and precipitated. Antisense RNA covered nucleotides −43 to 1032, and sense Apx cRNA nucleotides −43 to 4675.

Microinjection into Oocytes

Oocytes (Dumont stages V-VI) were obtained from *Xenopus laevis* females and maintained at 19°C as described (Palmer et al., 1990). Briefly, oocytes were removed from an anesthetized frog and incubated in modified Barth's saline (MBS) containing 85 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)$, 0.4 mM $CaCl_2$, and 12 mM Hepes, buffered to 7.2 with NaOH, plus penicillin (10 mg/ml) and streptomycin (5 mg/ml). After removal, the oocytes were defoliculated with 0.25% collagenase (type 1A; Sigma Chem. Corp.) in MBS without Ca^{2+}. After overnight incubation at 19°C, oocytes were injected with 50 nl nanopure H_2O containing either 50 ng A6 poly(A)$^+$ mRNA or 10 ng Na^+,K^+-ATPase α- and 5 ng β-subunit cRNA. When indicated the RNA was supplemented with 50 ng of oligodeoxynucleotide. Alternatively, 1.25 μg of A6 mRNA was solubilized in 1.25 μl of 10 mM NaCl, supplemented with 5 ng antisense RNA, or with 5 ng antisense RNA and 100 ng of Apx cRNA. 50 nl/oocyte was injected.

Biosynthetic Labeling of Injected Oocytes with [^{35}S]Methionine

20 ng of Apx cRNA (nucleotides −43 to 4675) was injected into *Xenopus laevis* oocytes. Water-injected oocytes served as control. Oocytes were then incubated for 24 h in MBS containing 1 mCi/ml [^{35}S]methionine at 19°C. Oocytes were then washed three times with 5 ml of MBS and their quality checked. Healthy oocytes were transferred into an Eppendorf Inc. (New York, NY) tube and 20 μl/oocyte homogenization buffer (0.1 mM NaCl, 1% Triton X-100, 20 mM Tris/HCl, pH 7.6, 1 μM PMSF, 1 μM antipain, 1 μM pepstatin, and 1 μM leupeptin) was added. The oocytes were dispersed with a blue tip (Eppendorf Inc.), the homogenate centrifuged at 12,000 rpm in a microcentrifuge at 4°C. The supernatant was saved, SDS was added to a final concentration of 3.7%, and immunoprecipitation with the anti-Apx antibody was performed.

Electrophysiology

After incubation in MBS for 2 d at 19°C, amiloride-sensitive current was measured as described (Palmer et al., 1990), and the activity of Na^+,K^+-ATPase was determined according to Horisberger et al. (1991).

Results

Cloning of an Apical Protein from Kidney-derived Epithelial Cells

A polyclonal antibody directed against the α subunit of *Bufo marinus* Na^+,K^+-ATPase labeled both cell surface membranes of amphibian kidney and colon epithelia as well as *Xenopus laevis* kidney-derived A6 cells (Fig. 1). This apical labeling seen in tight epithelia, known to express amiloride-sensitive sodium transport, prompted us to use the antibody to screen an expression library constructed from A6 kidney cell mRNA in order to clone the cDNA corresponding to the apical antigen. Indeed, the antibody identified a distinct 572-bp clone (3a) in addition to the expected Na^+,K^+-ATPase α subunit clone (Verrey et al., 1989). This cDNA hybridized to a 5.2-kb transcript from A6 cells and oocytes. The presence of the Apx mRNA in oocytes encouraged us to use this 572-bp clone to screen a *Xenopus laevis* ovary λgt 11 cDNA library. The longest insert (4.9 kb) was further analyzed and shown to contain a 4,260-nt open reading frame encoding a protein that we called Apx for apical protein *Xenopus*.

Nucleotide and Deduced Amino Acid Sequence

The nucleotide sequence and the predicted amino acid se-

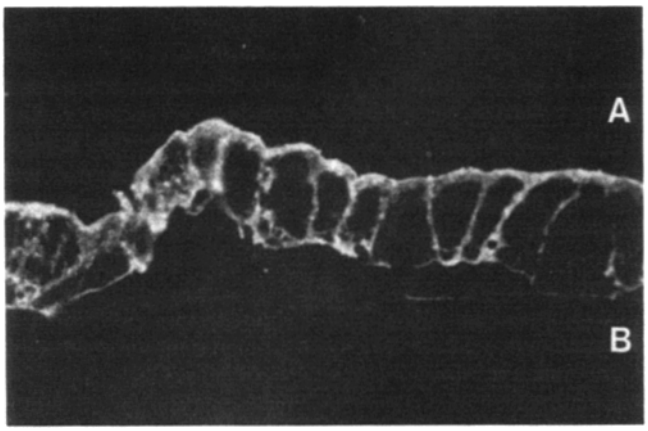

Figure 1. Labeling of *Xenopus laevis* kidney by an anti-Na^+,K^+-ATPase α-subunit antibody. Immunofluorescence micrograph was performed on A6 cells grown to confluency on collagen-coated polycarbonate filters. The cells were fixed in paraformaldehyde-lysine-periodate, and 0.5-μm-thick frozen sections were prepared and stained with a rabbit anti-α-subunit antibody, a biotinylated donkey anti-rabbit IgG antibody, and phycoerythrin-streptavidin. The antibody labels all cell surface domains (*A*, apical membrane; *B*, basolateral membrane). Similar observations were made using similar histological methods on tissue sections of the *Bufo marinus* kidney and colon (Rossier et al., 1989). A preimmune serum served as control and no labeling was detected.

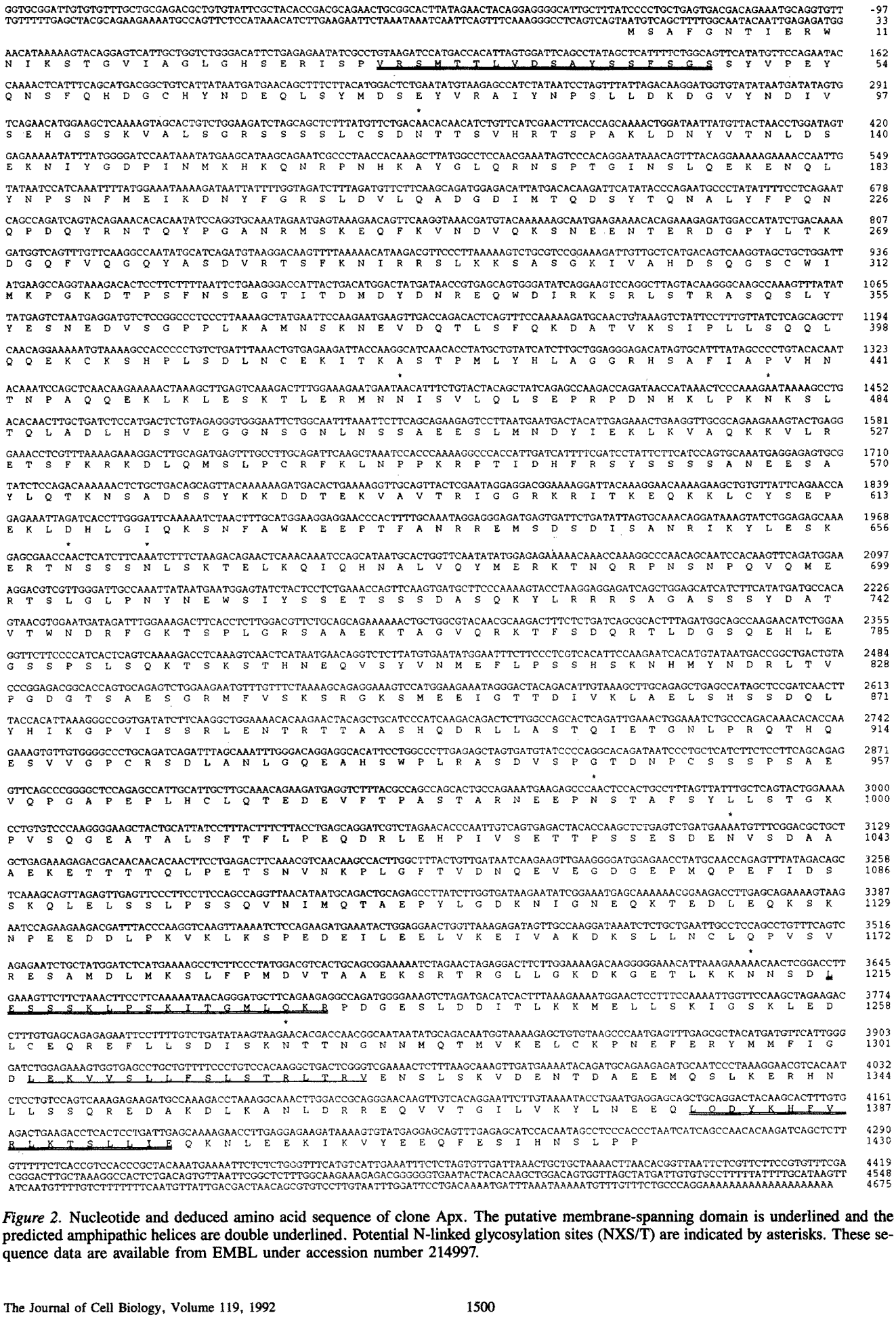

Figure 2. Nucleotide and deduced amino acid sequence of clone Apx. The putative membrane-spanning domain is underlined and the predicted amphipathic helices are double underlined. Potential N-linked glycosylation sites (NXS/T) are indicated by asterisks. These sequence data are available from EMBL under accession number 214997.

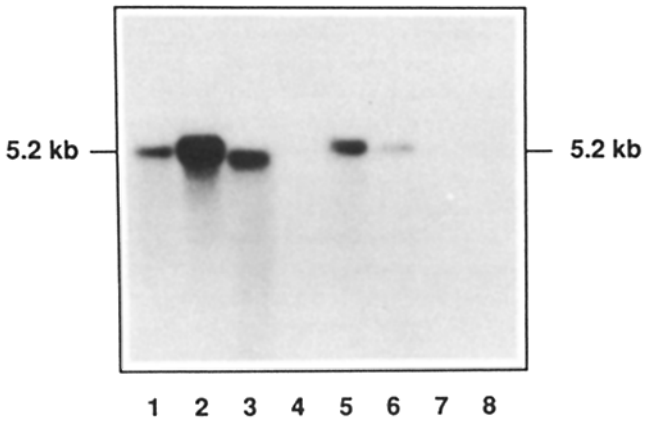

Figure 3. Distribution of mRNA for Apx in *Xenopus laevis* tissues. Poly(A)$^+$ mRNA was prepared from oocytes stage III/IV (lane *1*), A6 (lane *2*), kidney (lane *3*), distal (lane *4*) and proximal (lane *5*) intestines, stomach (lane *6*), heart (lane *7*), and retina (lane *8*) as described (Geering et al., 1985). Northern blot analysis was performed using ^{32}P-labeled Apx cDNA. Each line contains 2 μm of poly(A)$^+$ mRNA fractionated on a 0.8% denaturing glyoxal agarose gel. Apx mRNA in distal intestine and retina are visible on longer exposure time. Apx mRNA from different tissues migrated at slightly different molecular weights; this may represent a problem of migration, as Na^+,K^+-ATPase α- and β-subunit mRNA showed similar small differences on the same blot (data not shown).

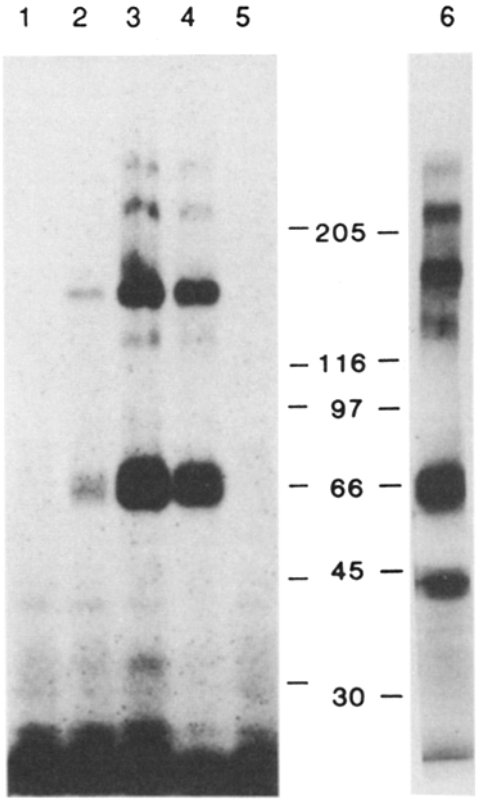

Figure 4. Immunoprecipitation of A6 cell proteins labeled with [^{35}S]methionine. A6 cells were grown on collage-coated filters, and were incubated with [^{35}S]methionine for 15 min (lane *1*) or pulsed with [^{35}S]methionine, and subsequently chased in media supplemented with 10 mM unlabeled methionine for 30 min (lane *2*), 1 h (lanes *3* and *6*), or 6 h (lane *4*) as described in Materials and Methods. Labeled proteins were immunoprecipitated with anti-Apx antibody (lanes *1–4*), or with preimmune serum (lane *5*), or with an antiidiotypic antibody recognizing the amiloride binding site (lane *6*) and detected by 5–13% SDS-PAGE under reducing conditions followed by autofluorography. Migration of various molecular mass is indicated (note that the scale is slightly different for lanes *1–5* vs. lane *6*). A preimmune serum was used as control and no labeled proteins were recovered. The protein at 40–41 kD observed in lane *6* is not always immunoprecipated with this antibody. There is indication that it may represent a G protein, which does copurify with the putative amiloride-sensitive sodium channel (Ausiello et al., 1992).

quence of the Apx clone are shown in Fig. 2. The cDNA contains a 4,260-nt open reading frame coding for 1,420 amino acids corresponding to a 159,467-M_r protein. According to the scanning model and the Kozak consensus sequence (Kozak, 1989) for translation initiation, the first potential start codon 225 nt downstream of the 5′ end represents a strong initiation site (CAGTAATG). Several potential start sites, however, are present further downstream (for example, Met 73, Met 151, Met 190, and Met 211). The deduced amino acid sequence shows no leader sequence, eight potential N-glycosylation sites, a single putative transmembrane segment at the COOH terminus (residues 1,303 to 1,320) (Fig. 2, *underlined*), and three predicted amphipathic α helices (residues 31 to 48; 1,215 to 1,232; 1,380 to 1,396) (Fig. 2, *double underlined*). There is no similarity with other sequenced proteins from the EMBL/Genbank data bases, including the voltage-gated sodium channel (Noda et al., 1984), the human phenamil-binding protein (Barbry et al., 1990*a*), and the α subunit of *Xenopus laevis* Na^+,K^+-ATPase (Verrey et al., 1989). Sequences of the partial 572-bp 3a clone and the Apx clone (nucleotides 3,571–4,142) are identical. A 180-bp cDNA probe derived from the 5′-end of the Apx clone hybridized to the same 5.2-kb transcript in A6 cells and *Xenopus laevis* oocytes, and primer extension on mRNA of both tissues using a 5′-end oligodeoxynucleotide showed extended products of identical length. Taken together, these results indicate that A6 cells and oocytes synthesize the same transcript.

Tissue Distribution of Apx mRNA in Xenopus laevis

The Apx cDNA hybridized to a 5.2-kb transcript on Northern blots containing poly(A)$^+$ mRNA isolated from *Xenopus laevis* kidney, proximal intestine, oocytes, and A6 cells, and to a lesser extent to mRNA from distal intestine, stomach, and eye (Fig. 3). No signal was detected in heart and other tissues were not studied.

The Apx Protein Forms a Multimeric Complex during Biosynthesis

A chimeric protein containing an amino acid sequence corresponding to the 572-bp 3a clone was produced in *E. coli* using a prokaryote heat-inducible expression vector (pEX) (Stanley and Luzio, 1984). A polyclonal antibody raised against the fusion protein was used to immunoprecipitate the Apx protein from A6 cells after it had been biosynthetically labeled with a 15-min pulse of [^{35}S]methionine and followed by a chase of up to 6 h. Immediately after completion of the pulse, no labeled protein was recovered from the cell lysate (Fig. 4, lane *1*). With increasing chase time, a complex

protein pattern consisting of ~270; 230-(extrapolated values), 160-, 130-, and 70-kD polypeptides was immunoprecipitated (Fig. 4, lanes *2–4*). Similarly, an antiidiotypic antibody which recognizes the amiloride binding site of the epithelial sodium channel failed to immunoprecipitate the newly synthesized subunit after a 15-min pulse (Kleyman et al., 1991); a complex pattern comparable to that recovered with the antifusion protein antibody was seen during 1 h of chase (Fig. 4, lane *6*). No labeled proteins were immunoprecipitated with a preimmune serum (Fig. 4, lane *5*).

The Apx Protein Is Restricted to the Apical Membrane of A6 Cells

To determine whether the Apx protein was membrane associated and restricted to the apical cell surface, A6 cells were grown to confluency on collagen-coated Transwell® filters, and the membrane surface was radioiodinated either from the apical or the basolateral side. Several polypeptides were immunoprecipitated with the anti-Apx antibody from apically but not basolaterally radioiodinated cells (Fig. 5, lanes *1* and *2*), and the protein complex pattern was similar to that seen with [^{35}S]methionine biosynthetically labeled cells. After reduction, a single ~130-kD polypeptide was recovered from apically radioiodinated membranes (Fig. 5, lane *3*). Since the Apx protein is accessible to cell surface radioiodination using nonpermeant reagents (Hubbard and Cohn, 1975), it is likely that the protein is membrane associated with at least some sequences exposed to the exterior. Interestingly, the relative molecular mass of the Apx protein corresponds to that of an apical membrane polypeptide recognized by an antiamiloride antibody in A6 cell membranes photolabeled with an amiloride analog (NMBA) (Kleyman et al., 1989). Whether the Apx protein corresponds to an amiloride-binding protein awaits further biochemical confirmation.

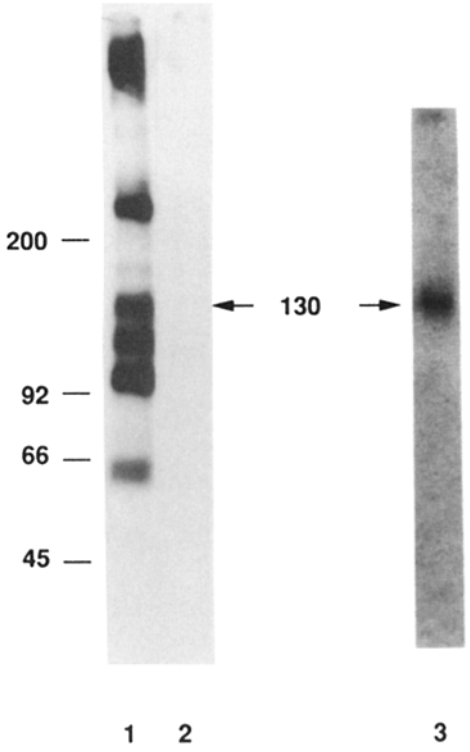

Figure 5. Apx cell surface expression assessed by immunoprecipitation of radioiodinated A6 cell proteins. A6 cells were grown on collagen-coated filters for 7 d. Apical (lane *1*) or basolateral membranes (lane *2*) were selectively iodinated (as described in Materials and Methods). In one case, microsomes were prepared from apically iodinated A6 cells (lane *3*). Immunoprecipitations were performed either without prior reduction (lanes *1* and *2*), or after reduction with DDT (lane *3*) with anti-Apx antibody. Reduction and alkylation does not affect the apparent molecular weight of the Apx protein. Molecular weight standards are indicated for lanes *1* and *2*. A preimmune serum was used as control and no labeled proteins were recovered.

The Apx Protein Is Part of a Putative Amiloride-sensitive Sodium Channel Complex

As it has been shown that the antiidiotypic antibody recognizing the amiloride-binding site cross-reacts with a protein complex isolated from A6 cells and proposed to be an amiloride-sensitive sodium channel (Benos et al., 1987), we tested if the anti-Apx antibody cross-reacts with this purified protein complex. ELISAs were performed either with protein isolated from A6 cells or from bovine kidney papilla cells (Fig. 6). The anti-Apx antibody reacted with the material from either source, but the reactivity to the A6 channel protein was higher than to that of the bovine complex. There was no signal with the preimmune serum. On Western blot, the antifusion protein antibody reacted with a single ~160-kD protein from the biochemically purified homogenous channel protein isolated from both A6 cells and bovine renal papillae (data not shown). The biochemically purified sodium channel has recently been shown to form amiloride-sensitive (K_i = 50–100 nM), sodium selective (P_{Na}/P_K = 8:1) ion channels when reconstituted in liposomes (Saraban-Sohraby et al., 1992). Taken together, these data suggest that

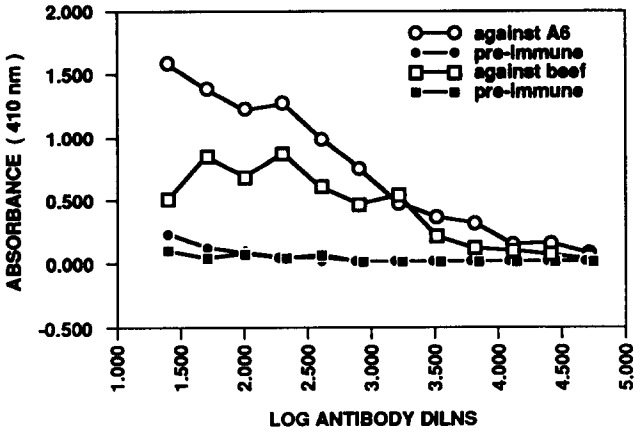

Figure 6. Apx protein is part of the putative sodium channel purified either from A6 cells and bovine kidney cells. The sodium channel purified from A6 cells and bovine kidney served as antigen to coat 96-well plates. The antigen was revealed with the rabbit anti-Apx protein serum and with a sheep IgG directed against rabbit IgG and coupled to peroxidase. Titration curves (absorbance vs. log antibody dilution) are shown for the A6 cell (○) and the bovine kidney channels (□). Preimmune serum (●,■) served as control.

the Apx protein is part of the multimeric epithelial sodium channel.

Antisense Oligodeoxynucleotides or Antisense RNA Complementary to the Apx Sequence Inhibit Expression of Amiloride-sensitive Sodium Current in mRNA-injected Xenopus Oocytes

Our biochemical studies suggested a relationship between the Apx protein and the amiloride-binding subunit of the epithelial sodium channel. The two proteins were restricted to the apical cell surface, shared a similar relative molecular mass, required maturation for recognition by their respective antibodies, and formed similar multimeric complexes. To determine whether this structural similarity is associated with a functional relationship, we performed studies in *Xenopus laevis* oocytes. To find out whether the Apx cDNA encodes a sodium pore-forming unit or a subunit required for sodium channel activity, we synthesized the corresponding cRNA and expressed it in oocytes. No detectable amiloride-sensitive sodium current (Palmer et al., 1990) was measured in the injected oocytes, although the protein Apx encoded by the injected cRNA was translated (Fig. 7), indicating that the cRNA did not encode by itself a complete amiloride-sensitive pore-forming unit. To determine whether the protein, however, was involved in sodium channel activity, A6 poly(A)$^+$ mRNA and oligodeoxynucleotides complementary to the coding strand of Apx cDNA were coinjected into oocytes. The amount of amiloride-sensitive current following A6 poly(A)$^+$ mRNA injection varied from 3 to 100 nA depending both upon the origin of the oocytes (variation among females) or upon the batch of A6 poly(A)$^+$ mRNA. In all experiments, reversibility of amiloride's effect on oocyte currents was demonstrated by changing the bath to amiloride-free media. No amiloride-sensitive sodium current was detected in water-injected oocytes (data not shown). Two different antisense oligodeoxynucleotides produced an 80% inhibition of amiloride-sensitive sodium current, while the corresponding sense oligodeoxynucleotides had no effect (Fig. 8 *A*, *black bars*). As an additional control, the effect of the same oligodeoxynucleotides on Na$^+$,K$^+$-ATPase activity was tested following the injection of *Xenopus laevis* α- and β-subunit Na$^+$,K$^+$-ATPase cRNA (Fig. 8 *A*, *striped bars*) (Horisberger et al., 1991). Na$^+$,K$^+$-ATPase current, monitored in the presence of 10 mM K$^+$ after loading the oocytes with sodium, ranged between 100 and 400 nA. No significant inhibition by Apx antisense or sense oligodeoxynucleotides was observed. Furthermore, the four oligodeoxynucleotides tested did not alter general protein synthesis ([^{35}S]methionine incorporation; water injected: 1.65 $\times$ 10^7 cpm/mg total protein; antisense oligonucleotide: 1.79 $\times$ 10^7 cpm/mg: sense oligonucleotide: 1.63 $\times$ 10^7 cpm/mg) or endogenous Na$^+$,K$^+$-ATPase activity (data not shown). Northern blot analysis of mRNA recovered from coinjected oocytes revealed that >90% of Apx mRNA was degraded by endogenous RNase H in the presence of antisense, but not sense oligodeoxynucleotides, whereas Na$^+$,K$^+$-ATPase mRNA remained intact (not shown). When antisense cRNA covering the first 1,000 bp was coinjected with A6 poly(A)$^+$ mRNA, amiloride-sensitive sodium channel activity was significantly reduced, and this inhibition was overcome by coinjecting excess of Apx cRNA (Fig. 8 *B*). When Apx cRNA was coinjected with A6 poly(A)$^+$ mRNA, no change in amiloride-sensitive sodium currents was observed. Our results are consistent with the involvement of the Apx protein in amiloride-sensitive sodium channel activity and they rule out that the protein acts per se as the complete amiloride-sensitive sodium-conducting pore.

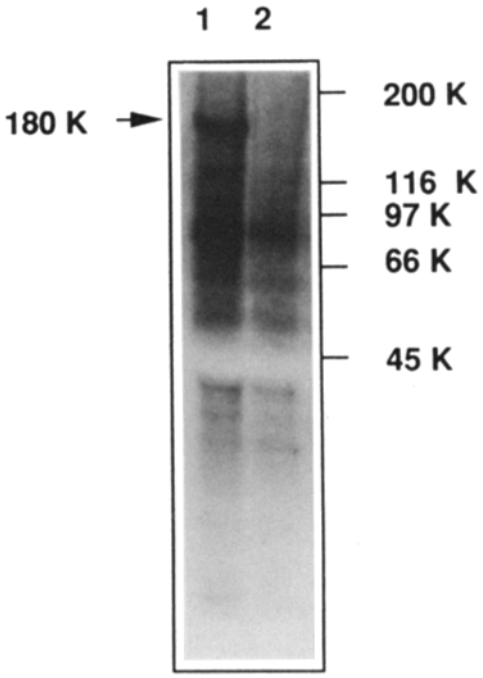

Figure 7. Expression of Apx cDNA in *Xenopus laevis* oocytes. Oocytes were injected either with Apx 4.9 cRNA (lane *1*) or with water (lane 2), pulsed for 24 h with [^{35}S]methionine, and lysed; and Apx protein was immunoprecipitated with anti-Apx antibody. The proteins were analyzed by SDS-PAGE and autoradiography. Several bands are detected both in the immunoprecipitates from cRNA and control injected oocytes indicating that they nonspecifically reacted with the antifusion protein antibody. In contrast, the 180-kD polypeptide (*arrow*), which corresponds to the protein encoded by the Apx cRNA, is detected only in the cRNA-injected oocytes.

Discussion

In this paper we have described the cloning and sequencing of a cDNA encoding a protein (Apx) that is associated with the apical membrane of the amphibian kidney-derived A6 cells. During its synthesis, intracellular transport and membrane assembly, the Apx protein interacts with other polypeptides and becomes part of a large complex. The Apx protein is essential for amiloride-sensitive sodium channel activity, although it does not constitute by itself the amiloride-sensitive sodium-conducting pore.

The Apx Protein Is Part of a Multimeric Complex Associated with the Apical Plasma Membrane of Sodium-transporting Epithelial Cells

The Apx cDNA was cloned using an antiserum directed against the α subunit of *Bufo marinus* Na$^+$,K$^+$-ATPase. The rationale to use such an antibody for screening an expression library was based on the unexpected labeling by the antibody of the apical plasma membrane of cells known to accumulate

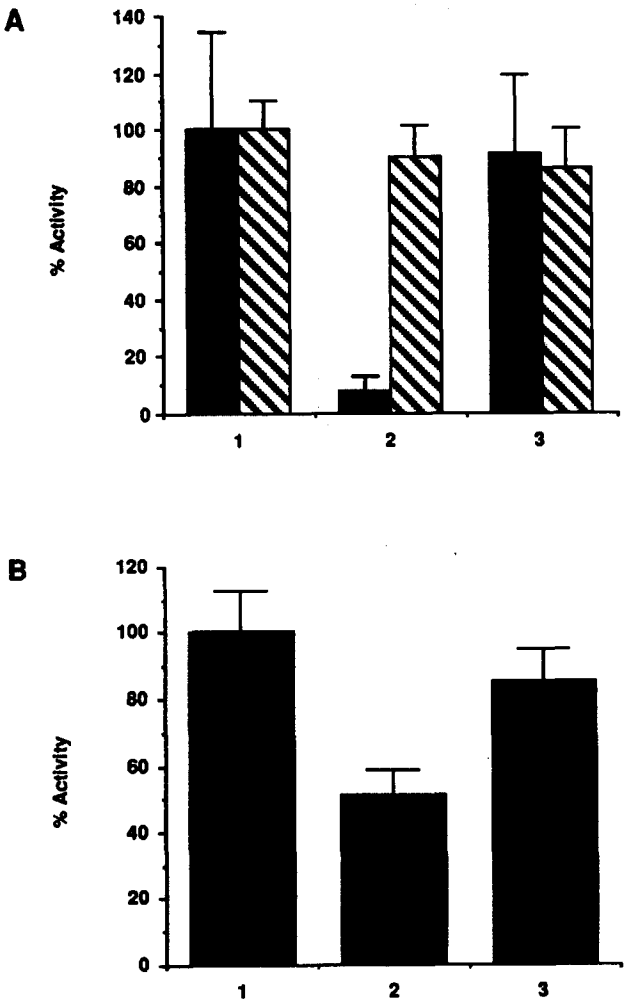

Figure 8. Inhibition of A6 poly(A)$^+$ mRNA induced amiloride-sensitive sodium current in *Xenopus laevis* oocytes by antisense oligodeoxynucleotides (*A*) or antisense cRNA (*B*) complementary to the Apx sequence. (*A*) Oocytes were coinjected with A6 poly(A)$^+$ mRNA (*black bars*) or Na^+,K^+-ATPase α- and β-subunit cRNA (*striped bars*) and (*1*) water, (2) antisense, or (*3*) sense oligodeoxynucleotides. Injected oocytes were incubated at 19°C for 2 d. Amiloride-sensitive sodium current was measured as described (Palmer et al., 1990), (*black bars*). Six experiments (*n* females: 6) have been performed using the oligodeoxynucleotides (antisense or sense) described in Materials and Methods and yielded always similar results. Shown is one representative experiment using oligodeoxynucleotide Apx (+479)-(+455). The mean (±SEM) amiloride-sensitive current for A6 mRNA-injected oocytes (arbitrarily chosen as 100% of activity) was 11.0 ± 3.8 nA (n = 7 individual oocytes). The addition of antisense oligodeoxynucleotide to the injectate reduced amiloride-sensitive Na^+ current by 91.4% compared with the corresponding sense oligodeoxynucleotide (p = 0.013; paired t test). Mean total current of the oocytes was 119 ± 9.8 nA and was not significantly different between the different pools of oocytes. Sodium pump current was measured in the presence of 10 mM K^+ after the oocytes were loaded with sodium (Horisberger et al., 1991). The mean current for α- and β-mRNA injected oocytes (striped bar 2; chosen as 100% of activity) was 188 ± 27 nA (*n* oocytes: 17; *n* females: 2) and 0% of activity corresponds to the endogenous Na^+,K^+-ATPase activity (60 nA). This suggests that Apx sense and antisense oligodeoxynucleotides do not produce a nonspecific inhibition of cell function. Oligonucleotides directed against another region (nucleotides −30 to −3) influenced the expression of amiloride-sensitive sodium current in the same fashion. In this case 88% inhibition was produced by the antisense compared with the sense oligonucleotide (amiloride-sensitive current of control oocytes: 56.2 ± 13.2 nA), whereas the expression of exogenous Na^+,K^+-ATPase activity was not significantly influenced. (*B*) Oocytes were coinjected with 50 ng of A6 poly(A)$^+$ mRNA suspended in 50 nl of 10 mM NaCl complemented with either (*1*) no cRNA, (*2*) 0.2 ng of antisense cRNA covering ~1,000 bp of the 5′ end, or (*3*) 0.2 ng of antisense cRNA and 4 ng of Apx full-length cRNA. Injected oocytes were incubated as above and amiloride-sensitive currents were measured after 2 d. The mean (±SEM) amiloride-sensitive sodium current (lane *1*) was 56.7 ± 7.15 nA (*n* oocytes: 17; *n* females: 2).

Na^+,K^+-ATPase in their basolateral membrane and to express apical membrane-associated amiloride-sensitive sodium channels. We postulated that the antiserum recognized two distinct antigens, the Na^+,K^+-ATPase α subunit and a distinct apical membrane protein. Indeed, two different cDNA clones were isolated, one encoding Na^+,K^+-ATPase α subunit and the second a protein with no sequence homology to other known proteins, including the Na^+,K^+-ATPase α subunit (Verrey et al., 1989). The absence of sequence similarity between the α subunit and the Apx protein suggested that the two proteins shared a common conformational epitope not observed by inspecting and comparing their primary structures, or alternatively that the anti-α-subunit serum antibody contained a contaminating antibody specific for the Apx protein.

A hydropathy plot of the Apx protein revealed one putative COOH-terminus hydrophobic transmembrane segment and three putative amphipathic α helices. These may mediate membrane insertion as reported for complement protein C9 or perforin (Peitsch et al., 1990). The NH_2 terminus lacks the characteristic features of a cleavable signal sequence, as it is the case for the α subunit of Na^+,K^+-ATPase (Verrey et al., 1989), or the voltage-gated sodium channel from excitable cells (Noda et al., 1984). Apical membrane association of the Apx protein is demonstrated by the cell surface radioiodination experiments (Fig. 5). Sequences of the Apx protein must be exposed on the membrane for them to be readily available for labeling by reagents which are unable to permeate the intact plasma membrane. The precise membrane topology of the protein, however, has not been established. The Apx protein is associated with other polypeptides and forms a multimeric complex in the apical, but not the basolateral, cell surface of A6 cells (Fig. 5). Immunoprecipitation of the newly synthesized Apx protein requires its association with these additional polypeptides, a property shared with the polypeptide that carries the amiloride binding site (Fig. 4) (Kleyman et al., 1991). This suggests that the Apx protein and the protein carrying the amiloride binding site undergo conformational changes during membrane assembly and/or maturation before their epitopes can be recognized by their respective antibody. These results are consistent with previous work which demonstrated that other transport proteins change their conformation once properly assembled in the membrane (Geering, 1990).

The Apx Protein Participates in Amiloride-sensitive Sodium Channel Activity

Structural and functional evidence from our study suggests that the Apx protein participates in amiloride-sensitive so-

dium channel activity. We have shown that the Apx protein shares many features with the amiloride-binding subunit of the epithelial sodium channel. Under nonreducing conditions, the antiidiotypic antibody directed against the amiloride binding site of the epithelial sodium channel (Kleyman et al., 1991) and the antibody specific for the Apx protein immunoprecipitate similar protein complexes from biosynthetically labeled or cell surface–radioiodinated A6 cells. Each antibody recognizes an ~130-kD polypeptide under reducing conditions and reacts with the putative sodium channel purified from A6 cells or bovine kidney cells (Kleyman et al., 1991; Fig. 6) which contains a similarly sized protein. This polypeptide binds amiloride (Benos et al., 1987) and its relative molecular mass varies between 130 and 180 kD. A similar variation in relative molecular mass has been observed for the Apx protein. In oocytes, the expressed Apx protein is ~180 kD (Fig. 7), whereas the protein recovered from the apical membrane of A6 cells is an ~130-kD polypeptide. The upper value is consistent with the 159,467-M_r value calculated from the deduced amino acid sequence of the Apx clone. The reasons for such variations are not known and differences in the degree of glycosylation are unlikely to account for such large divergences. Proteolytic cleavage during isolation or purification could explain the observed decrease in size. Proteolysis of Na^+ channel–related proteins may, however, play a physiological role. In the rabbit urinary bladder proteolysis of an amiloride-sensitive sodium channel has been correlated with its sensitivity to amiloride and its relative selectivity to Na^+ and K^+ (Lewis and Clausen, 1991). A similar mechanism may be present in A6 cells as different types of amiloride-sensitive sodium channels are measurable (Hamilton and Eaton, 1986). Alternatively, translation initiation at AUG codons situated further downstream may explain the observed size variance.

In functional studies we demonstrated that the Apx protein is required for the expression of amiloride-sensitive sodium channel activity. *Xenopus laevis* oocytes injected with poly(A)$^+$ mRNA from A6 cells expressed amiloride-sensitive sodium currents that were markedly reduced by Apx-specific antisense oligodeoxynucleotides or antisense cRNA (Fig. 6). Antisense strategies including oligodeoxynucleotides (Shuttleworth and Colman, 1988) or antisense cRNA (Melton, 1985) have been widely used to analyze the function of cloned genes or cDNAs (for review see Colman, 1990). Antisense oligodeoxynucleotides promote specific cleavage of mRNAs by oocyte endogenous RNase H, whereas antisense cRNA which hybridizes to the translation initiation site will efficiently block translation. Both of these antisense approaches affected amiloride-sensitive sodium channel activity, which indicated that the Apx protein is required for the expression of a channel activity. The Apx protein per se, however, does not constitute a complete amiloride-sensitive sodium-conductive pore because injection of a sense cRNA covering the entire Apx coding region into oocytes failed to reconstitute amiloride-sensitive sodium currents, although the protein Apx is translated. Together these results suggested that the Apx protein is required for sodium channel activity. It may be only one portion of the pore that is either essential for the assembly of a multimeric complex or it indirectly modulates channel activity. These speculations are consistent with the model proposed by Benos et al. (1987) in which the renal epithelial sodium channel consists of several subunits including an amiloride binding protein (130 to 180 kD) that may correspond to the Apx protein and a pore-forming unit controlling the sodium conductance. This view is further strengthened by our observation that the anti–Apx antibody does cross-react with the purified putative sodium channel from A6 cells (Fig. 6), indicating that the protein Apx is part of the purified putative sodium channel. This model differs significantly from the model proposed by Barbry et al. (1987, 1990*b*), who purified a 180-kD protein dimer taking advantage of its affinity to the amiloride analog phenamil. Although their protein induced amiloride-sensitive sodium transport after incorporation into lipid vesicles, the injection of the corresponding cRNA into oocytes did not produce amiloride-sensitive ^{22}Na fluxes (Barbry et al., 1990*a*). This hydrophilic phenamil-binding polypeptide which does not have a putative transmembrane segment, does not demonstrate any similarity with the Apx protein. Whether this discrepancy reflects species or tissue-specific differences or channel heterogeneity (Garty and Benos, 1988) remains to be established and will require the cloning of the various subunits in different species.

The tissue distribution of the Apx protein in *Xenopus laevis* is in large part consistent with the known expression of amiloride-sensitive sodium channel activity in distal kidney nephron (Palmer and Frindt, 1986), distal colon (Zeiske et al., 1982) and in the embryo during early development (Biggers et al., 1988). The lower abundance of Apx mRNA in kidney tissue compared to A6 cells, a cell line which shares features with tubular cells from the distal nephron, suggests that only some kidney cells, probably the distal tubular cells, express the Apx protein. This agrees with the generally accepted concept that amiloride-sensitive sodium channels are restricted to the distal part of the nephron. Although Na^+ channels in the *Xenopus laevis* colon have been reported to be calcium sensitive but amiloride insensitive (Krattenmacher et al., 1990), our mRNA expression data are compatible with the observation that amiloride-sensitive Na^+ channels are present in the mammalian colon (Zeiske et al., 1982). We detected Apx mRNA in stomach and, although it is unknown whether amphibia have Na^+ channel in this tissue, Machen et al. (1978) documented amiloride-sensitive sodium transport in the gastric mucosa of newborn pigs and rabbits. We also observed Apx mRNA in large amounts in oocytes, although no Apx protein is detectable (Fig. 7), and during *Xenopus laevis* early embryonic development (Burgener-Kairuz et al., 1990); these observations are consistent with the idea of expression of an amiloride-sensitive sodium channel during blastulation and neurulation (for review see Biggers et al., 1988).

In conclusion, we have cloned and sequenced a novel protein that is associated with the apical surface of the amiloride-sensitive sodium transporting amphibian A6 cell line. The protein, which is part of a large molecular complex, shares properties with the protein carrying the amiloride binding site. The Apx protein is required for amiloride-sensitive sodium channel activity in *Xenopus laevis*.

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