Immunopurification and Structural Analysis of a Putative Epithelial Cl⁻ Channel Protein Isolated from Bovine Trachea*

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We have purified to homogeneity a 38-kDa protein (called p38) from bovine tracheal epithelium. This protein, when reconstituted into liposomes, mediates stellbene disulfonate-sensitive ¹²⁵I⁻ conductive uptake. On nonreduced or partially reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis, this protein associates into a doublet of 62–64 kDa. In some experiments a multimer of 140 kDa was also observed. Rabbit polyclonal anti-P38 antibodies have been produced and used to immunopurify the native transporter. Upon reconstitution of the immunofluorophore-labeled protein into liposomes, a 260-fold enhancement of DIDS, 2,2'-disulfonic acid H₂DIDS, dihydroisothiocyanate disulfonic acid uptake was observed as compared to proteoliposomes containing unseparated material. On Western blots of total solubilized tracheal membrane proteins or semipurified fractions, the antibody recognized the 62–64-kDa doublet much better than the original 38-kDa antigen. Similar bands were detected in T84 and CFPAC cells as well. However, if apical membrane proteins were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions, the antibody recognized major bands at 140 and 240 kDa. Upon partial reduction, immunolabeling of these proteins diminishes with the concomitant appearance of the 62–64-kDa doublet. Upon complete reduction, the appearance of 32- and 38-kDa proteins was evident with the disappearance of the 62–64-kDa doublet. We hypothesize that the native Cl⁻ channel is a heteromer containing at least four subunits connected by S-S bridges.

Chloride (Cl⁻) secretion by airway epithelia is indispensable for the formation and proper hydration of the mucous layer that covers the surface of the respiratory tract (1, 2). The overall rate of transepithelial Cl⁻ movement is determined by the activity of Cl⁻ channels located in the apical membrane of airway epithelial cells. These channels are regulated by intracellular levels of Ca²⁺ and cAMP (2, 3–5), and can be irreversibly activated by sustained depolarization in excised membrane patches. The regulation of these Cl⁻ channels is to understand the regulatory features of these important ion channel proteins under normal and diseased conditions. Therefore, it is essential to isolate and purify to homogeneity functionally intact secretory Cl⁻ channels.

Recently, we published a protocol for purification of a 38-kDa protein that mediates voltage- and DIDS⁻-sensitive Cl⁻ conductive activity (7). The protocol includes three steps: separation of total solubilized proteins on a cation-exchange resin, further purification of the enriched fraction on an anionic-exchange resin, and final isolation by SDS-PAGE electrophoresis and electroelution. Two major points concerning the functional and structural properties of the purified 38-kDa protein emerged: 1) SDS-PAGE analysis of the electroleute showed that minor bands (a doublet of 62–64 kDa and a single band of 140 kDa) accompany the major 38-kDa protein. Although we speculated earlier that the high molecular mass proteins must be originally derived from the 38-kDa protein, the relationship between all protein bands in the electroleute remained to be established. One of the approaches that might provide evidence of biochemical identity between these proteins is the generation of polyclonal antibodies against only the 38-kDa protein. 2) Reconstituted proteins of the electroleute had a specific activity of ¹²⁵I⁻ uptake only 5–12-fold higher than that of the unseparated proteins. This value was much lower than expected from a purified Cl⁻ transporter. We suggested that because the purification procedure required low pH and buffers of low osmolarity as well as removal of surrounding phospholipids, the purified protein was partially denatured. We conjectured that a one-step isolation protocol employing relatively mild conditions (i.e. immunopurification) would produce a highly active transporter that would facilitate biochemical characterization of this protein. Thus, rabbit polyclonal antibodies against this 38-kDa protein (anti-P38) were generated to identify all structural components of the native Cl⁻ channel and to improve the functional integrity of the purified transporter.

We present the evidence that immunopurification using immobilized anti-P38 antibodies results in the isolation of a protein that displays a high activity of DIDS-sensitive conductive Cl⁻ transport. The specific activity of immunopurified protein was ~260-fold higher than that of the starting material. The native protein has an apparent molecular mass of 140 kDa under nonreducing conditions, although it may also appear as higher molecular mass bands, probably representing an aggregate of the native transporter. Gradual reduction of 140-kDa protein by increasing the amount of dithiothreitol (DTT) resulted in the appearance of a 62–64-kDa doublet and

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The abbreviations used are: DIDS, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid; H₂DIDS, dihydroisothiocyanate disulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TBS, Tris-buffered saline; TTBS, TWEEN-20 Tris-buffered saline; NC, nitrocellulose; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; Ab, antibody.
a 38-40-kDa single band. Because all of the above proteins are recognized specifically by anti-p38 antibodies, we hypothesize that the native Cl\(^-\) channel is a multimer that consists of at least four subunits cross-linked by S-S bonds.

**EXPERIMENTAL PROCEDURES**

**Materials**—Na\(^{35}S\) was purchased from Du Pont-New England Nuclear. Dowex 1× 8–100, dihydroiothiocyanate diisulfonic acid (H\(_2\)DIDS), valinomycin, Ponceau 5, L-α-phosphatidylcholine, cholesteryl, and complete and incomplete Freund’s adjuvant were obtained from Sigma. The cation-exchange protein assay reagent, and immunoisolated protein A-agarose beads were from Pierce (Rockford, IL). CM-Cellulose, 10,000 < g Centricrons, and 10,000 < g Centripreps were obtained from Amicon (Danvers, MA). Bio-beads SM-2, SDS, acrylamide, biacrylamide, Affi-Gel HZ immunoaffinity kit, molecular weight protein standards, nitrocellulose membrane, and alkaline phosphatase conjugate substrate kit (p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate-toluidine salt) were supplied by Bio-Rad. Donkey alkaline phosphatase-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were obtained from Jackson Immuno Research Lab (Avondale, PA). General laboratory reagents were obtained from analytical grade and were purchased either from Sigma or from Merck (Darmstadt, Federal Republic of Germany).

**Buffers**—The composition of mannitol Tris buffer was (in mM): mannitol (100), Tris-Hepes (5; pH 7.4), and MgCl\(_2\) (0.5). KCl buffer consisted of 100 mM KCl, 5 mM Tris-Hepes (pH 7.4), and 0.5 mM MgCl\(_2\). Protein separation on the cation-exchange resin CM-Cellulose was performed by adsorption of the following buffer: 10 mM MES titrated by 1 M KOH up to pH 6.2, 0.4% Triton X-100 (CM buffer). The running buffer used for SDS-PAGE was 25 mM Tris, 190 mM glycine, 0.1% SDS. The running buffer, when diluted 1:2 with H\(_2\)O, was used as an electrophoresion buffer. When the samples were run on SDS-PAGE for electrophoresion purposes, the composition of the sample buffer was as follows: 80 mM Tris (pH 6.8), 0.02% SDS, and 5% glycerol. Elution buffer for the immunoassay column was 100 mM glucose, pH 2.8. TBS buffer for immunoblotting contained 20 mM Tris (pH 7.5) and 500 mM NaCl. TTBS was TBS buffer containing 0.05% Tween 20.

**Treatments of Tracheal Apical Vesicles Prior to Purification**—Bovine trachea was obtained from a local slaughterline. T84 colonic carcinoma and CFPAC pancreatic adenocarcinoma cell lines were maintained in monolayer culture as previously described (3, 8). Apical membrane vesicles were prepared from bovine tracheal epithelium, T84 cells, and CFPAC cells using a procedure originally described by Smyth et al. (9). Aprotic characterization of apical membranes (10, 11) and extraction of peripheral proteins prior to purification procedures were performed as described previously (7). Protocols for the solubilization, purification, reconstitution, and measurement of a 38-kDa protein were described in detail previously (7). An \(^{35}S\)-uptake assay for estimation of Cl\(^-\) channel activity and calculations of specific activity were performed as published earlier (7).

**Protein Separation on CM-Cellulose Cation Exchanger**—The basic protocol for cation-exchange chromatography has been previously described (7). However, in order to modify this method for large scale protein isolation, several changes were introduced. Presolubilized apical vesicles loaded with mannitol Tris buffer were solubilized with 0.8% Triton X-100 in the presence of 10 mM MES/K\(^+\) (pH 6.2) buffer. After removal of unsoyabilized material, the clear supernatant was mixed with CM-Cellulose beads equilibrated with CM buffer. Maximal binding occurred at a ratio of 1 mg of solubilized protein per 1 ml of resin. The mixture was incubated on ice for 20 min with occasional shaking. Nondenatured proteins were subsequently removed and the resin washed twice with CM buffer. The resin was carefully transferred to a 20-ml column and washed step-wise with 10 mM MES/K\(^+\) buffers of following pH: 7.4; 7.8; 8.0, and 8.2 to elute bound proteins. Na\(^{35}S\) was collected (from 10 ml of resin) were collected.

**Methanol/Chloroform Protein Precipitation**—In order to concentrate the protein of each sample for SDS-PAGE analysis, the protein in each sample was extracted by methanol/chloroform. To precipitate proteins, 100 μl of sample was mixed at room temperature step-wise with 400 μl of methanol, 100 μl of chloroform, and 300 μl of water. The mixture was vigorously mixed 20 times and the sample was spun at 5000 < g for 10 min and the separation between chloroform and methanol-water solutions was observed. The protein precipitated in the interface between the two phases. The upper phase was carefully removed and 300 μl of methanol (−20 °C) was added. The sample was mixed well and incubated at −20 °C for 20 min. At the end of the incubation, the tube was spun at 4 °C at 10,000 < g for 20 min. All the liquid was removed and the sample was air-dried.

**Rabbit Immunization and IgG Purification**—Immunization of rabbits with 38-kDa protein was performed according to the methods of Nilsson et al. (12) and Krudsen (13) with slight modifications. In order to be certain that the antigen injected into the rabbit did not contain contaminants of higher molecular mass (see Introduction), the following protocol was adopted. Electroeluted proteins from the final step of purification were precipitated by methanol/chloroform, dissolved in 50 μl of fresh sample buffer containing 10 mM DTT, and separated on the gel. Separated proteins were blotted onto nitrocellulose (NC) paper and stained with Ponceau S. The 38-kDa protein (the major band in this electrophoretic picture) was identified, the NC paper was cut, dried in air, and dissolved in 200 μl of dimethyl sulfoxide. Dimethyl sulfoxide-solubilized paper was mixed with an equal volume of complete Freund’s adjuvant for the first injection or with incomplete adjuvant for subsequent injections. It should be noted that NC paper cannot be dissolved in dimethyl sulfoxide if it is not completely dry. Approximately 10 μg of protein was injected six times at 2-week intervals. After the last injection, the rabbit was bled from an ear vein, and the blood (∼50 ml) was collected into the tube containing 50 μl of heparin. Blood was immediately spun at 10,000 < g for 20 min and the clear serum stored at −20 °C. Protein purification from serum was performed by using an immobilized protein A-agarose column. 5–25 μg of purified IgG was obtained from each ml of serum. Immune (anti-p38) or preimmune IgG from the same rabbit (control) was stored at 4 °C until use.

**Monoclonal Antibody Production**—Monoclonal antibodies against 38-kDa protein were prepared according to the methods of Kohler and Milstein (14). Preparation of antigen was performed as follows: electroeluted purified 38-kDa protein was separated on 10% preparative SDS-PAGE and stained with Coomassie Blue. A piece of gel containing this protein band was cut and homogenized in the presence of 5 ml of saline. This solution was mixed (1:1) with complete Freund’s adjuvant and subcutaneously injected into the hindfoot pads, inguinal, and abdominal regions of a female BALB/c mouse. After three injections with interval of 10 days each, the spleen was removed and fused with a nonsecreting myeloma cell line. Tissue culture media from individual clones were screened using Western blots of total solubilized apical membrane proteins reduced by 5 mM DTT. Secondary antibody was anti-mouse IgG-conjugated with alkaline phosphatase. Among 350 clones tested, 23 were positive. The positive clones were recloned to ensure stable production of immunoglobulins. After additional screening, 10 subclones were chosen according to the best reaction on Western blots. Positive subclones were used (see Fig. 8), namely, secreted IgM (lanes a, b, and d) or IgG3 (lanes c).

**Immunopurification**—Purified immune IgG was coupled to agarose beads using Affi-Gel HZ immunoaffinity kit. Conjugation and estimation of IgG binding efficacy were performed as recommended by BioRad. Coupling of IgG was measured mg per each mg of resin.

Affinity resin was washed with 20 ml of cold KCl buffer followed by 20 ml of the same buffer containing 0.4% Triton X-100. Purified presolubilized apical vesicles loaded with KCl buffer (1 mg/ml) were solubilized with 0.8% Triton X-100. Unsolubilized material was removed by ultracentrifugation (35,000 < g, 45 min). Five ml of the solubilized material (~7 mg of protein) were mixed with 1 ml of anti-p38-conjugated agarose beads. After 4-h incubation at 4 °C with permanent mixing, agarose beads were transferred to a 10-ml column, and nonbound proteins were collected and analyzed further (see below). The affinity resin was washed with 30 ml of KCl buffer containing 0.4% Triton X-100 at a flow rate of 15 ml/h. Bound proteins were eluted with 100 mM glycine (pH 2.8). To neutralize pH, 30 μl of 1 M KOH was immediately added to each ml of eluate. In order to estimate the effect of the elution procedure on Cl\(^-\) channel activity, nonbound and starting material were treated as follows: 100 μl of each fraction was titrated by 1 M KOH at 2 min, neutralized to pH 7.4 with 1 M KOH. All the fractions were concentrated to a volume of 100 μl using 10,000 < g concentrators (Amicon). Aliquots of the fractions to be used for the functional assay were mixed with phosphatidylcholine/cholesterol vesicles solubilized in KCl buffer (molar phosphatidylcholine to cholesterol ratio was 1:3), and reconstituted as described previously (7).

Columns were regenerated by washing with 5 ml of 100 mM citric acid titrated to pH 7.4 with NaOH. The resin was then washed with
50 ml of KCl buffer and stored at 4 °C.

Polyacrylamide Gels and Blots—Protein separation on polyacrylamide gels was performed using the Laemmli system (15). Either 7.5% or 10% polyacrylamide gels were used, as indicated in the figure legends. Standard molecular mass marker proteins (200 to 31 kDa) were simultaneously run on all gels. Unknown molecular masses were calculated from interpolation on log molecular mass versus relative migration from the same standard curves (cf. see Figs. 5–7). Proteins were transferred to nitrocellulose membranes (16) under conditions (100 V for 1.5 h). Blots used either for rabbit immunization or for immunodetection were first stained with Ponceau 5 to verify the transfer. The Ponceau 5-stained blot was washed with TBS buffer for 10 min and then incubated with 2% milk in TBS either for 1 h or overnight. Primary antibodies (12 µg/ml) were dissolved in 1% non-fat dry milk in TTBS and added to the blot for a 2-h incubation. After extensive washing with TTBS, donkey anti-rabbit IgG antibodies (conjugated with alkaline phosphatase) were added at dilution of 1:4000. Development was performed using the p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate-toluidine salt system according to manufacturer's instructions.

Protein Determination—Protein concentration was determined by the Bradford assay (17), using bovine serum albumin as a standard. Protein in detergent-containing samples was estimated by the bicinchoninic acid assay (18).

RESULTS

Specific Detection of Anti-p38 Binding Proteins in Total Triton X-100 Extract—Immune serum was first tested for specificity on blots of total proteins extracted from bovine tracheal apical vesicles (Fig. 1). Vesicles were solubilized by Triton X-100 in the presence of mannitol Tris buffer. The extract was mixed with sample buffer containing 10 mM DTT, separated on a 10% gel, and immunoblotted. Under these conditions immune IgG reacted weakly with the original antigen (38-kDa protein), but showed stronger binding to a doublet of apparent molecular mass 62 and 64 kDa (lane b). Preimmune IgG showed no reactivity to any of the blotted proteins (lane a). In five of the 10 experiments performed, only the upper doublet could be detected. Antibody binding to the 38-kDa protein could not be improved by any modifications of the detection procedure (e.g. 125I-protein A). Thus, it appears that the anti-p38 antibody has specific but low affinity to the 38-kDa protein and higher cross-reactivity to the presumably structurally related doublet of 62–64 kDa.

Immunorecognition of Proteins by Anti-p38 Abs in Fractions Separated by Cation Exchange—Poor protein labeling by immune IgG can be partially explained by low abundance of specific antigen(s) in the total extract. To increase Ab-related proteins in the tested sample, the total Triton X-100 extract was first separated using a cation-exchange resin. Semipurified fractions eluted by pH 7.8–8.5 were shown previously to contain 5–12-fold higher Cl⁻ channel activity than the starting material (7). Western blot analysis of fractions derived from cation-exchange chromatography is shown in Fig. 2. No protein labeling was observed on the blot incubated with the preimmune serum (Fig. 2A), indicating high selectivity of the immune IgG. The same fractions immunoblotted with anti-p38 Abs contained several reactive bands (Fig. 2B). The two first fractions, which contained many proteins and displayed a low specific activity of 125I⁻ uptake, were labeled by antibody in the region of 36–40 kDa. Two major proteins immunodetected in the other fractions (lanes e–g) were of apparent molecular mass 62 and 64 kDa. The 38-kDa protein was detected specifically in the fractions eluted by pH 7.8, 8.0, and 8.2 (Fig. 2B, lanes e–g). The same fractions separated on a reducing gel and visualized by Coomassie Blue showed a major band of 38 kDa and a faintly visible doublet at 62–64 kDa (see Ref. 7, Fig. 8). The appearance of both proteins on the blot correlated with enhanced Cl⁻ channel activity in the cation exchanger-purified fraction, as has been shown in previous studies (7). The results suggested that protein bands of apparent molecular masses 36, 38, 62, and 64 kDa have common antigenic epitopes or, alternatively, represent different forms of the same protein.

Cross-reactivity with Fractionated (CM-Cellulose) Membrane Proteins of T84 Cells—One of the first questions raised by these studies was whether the antibodies directed against this bovine tracheal Cl⁻ channel may recognize similar protein(s) on other cells exhibiting Cl⁻ conductance properties. Two types of cells were chosen for this purpose: T84 (a human colon cancer epithelial cell line) and CFPAC (a pancreatic adenocarcinoma cell line). The Cl⁻ channel properties of both cell lines are well characterized electrophysiologically (3, 8, 19), and are similar to those observed in tracheal epithelia (5, 20, 21). Therefore, it was reasonable to assume that they contained proteins structurally related to the 38-kDa polypeptide. In initial experiments, total solubilized proteins extracted from purified plasma membranes were exposed to anti-p38 Abs. None of these proteins reacted either with

![Fig. 1. Specificity of anti-p38 antibodies](image1)

![Fig. 2. Detection of anti-p38 binding proteins in fractions separated on the cation-exchange resin CM-Cellulose.](image2)
immune or preimmune IgG. Because the similar experiment with bovine tracheal apical vesicles also resulted in weak binding of Abs (Fig. 1), immunoblots were repeated using CM-Cellulose-separated fractions instead of total protein. The results of one of these separation experiments are shown in Fig. 3. The upper panel (A) demonstrates Coomassie Blue-stained gel of fractions derived from the cation-exchange separation. The lower panel (B) shows a corresponding Western blot of identical samples. Lanes a and b in both A and B represent non-bound proteins and the pH 6.3 fraction, respectively. These fractions contained protein bands that were stained by Coomassie Blue, but none of these proteins reacted with the antibodies. This finding confirmed the high binding selectivity of anti-p38 Abs. Lanes c-e are proteins eluted from CM-Cellulose resin by buffers of pH 7.8, 8.0, and 8.2. Similarly eluted proteins derived from bovine tracheal apical membrane vesicles usually contained enriched Cl-channel activity (Ref. 7, Fig. 7) that correlated with the enhanced appearance of the 38-kDa protein (Ref. 7, Fig. 8). T84 plasma membrane proteins eluted by high pH (8.2) demonstrated a similar pattern of protein distribution. In these fractions antibodies specifically labeled a doublet of proteins of apparent molecular mass 62-64 kDa. Identical results were obtained using CFPAC separated proteins (data not shown). It should be noted that in a few (3 of 10) experiments (using either bovine trachea, T84 or CFPAC plasma membranes) a single band of 60 kDa appeared instead of a doublet. Establishment of Structural Relationships between 62-64-kDa Doublet and 38-kDa Protein—The consistent recognition of 60-66-kDa proteins by anti-p38 antibodies may indicate one of the following: 1) the high molecular mass proteins are contaminants that were not eliminated during the preparation of antigen; 2) the upper doublet and a 38-kDa protein share common antigenic sites; and/or 3) the protein bands in the range of 60 kDa represent a dimerized form of previously purified 38 kDa. In order to test the first possibility, the 38-kDa electroeluted protein was subjected to an additional cycle of gel separation and electroelution. The aim of this experiment was to test whether the high molecular mass components diminish or disappear with subsequent separation. Fig. 4 shows SDS-PAGE of unseparated proteins, cation-exchange-enriched proteins, and extracted proteins after the first and second electroelutions (lanes a--d, respectively). It is evident and not surprising that the major protein band on lanes b--d is at 38 kDa. However, both electroeluates contained a weakly stained doublet at 62-64 kDa. The presence of this doublet in the CM-Cellulose-enriched fraction (lane b) cannot be demonstrated by Coomassie staining due to interference of irrelevant proteins of similar molecular mass. However, as shown in Figs. 2 and 3, anti-p38 Abs not only specifically detected 62-64-kDa doublet in the above fraction, but also indicated its relative enrichment in comparison to starting material. These results exclude the possibility of irrelevant contamination and strengthen the hypothesis of a direct relationship between all observed proteins in the 38-kDa electroeluate. Characterization of Protein Immunopurified by Anti-p38 Antibodies—A major goal of these studies was to purify native protein from bovine tracheal apical vesicles using anti-p38 antibodies. Immunopurification of this protein has a clear advantage over our previously reported protocol (7) being a mild and short-term procedure. Isolation of anti-p38 binding proteins was performed as described under “Experimental Procedures.” Eluted material was analyzed by SDS-PAGE and Western blots for protein composition, and by reconstitution for Cl-channel activity. In order to compare the activity with non-bound and unseparated proteins, the latter were exposed to the same treatments as proteins eluted from immunoaffinity columns (see “Experimental Procedures”). Structural Analysis of Immunopurified Protein—Fig. 5 demonstrates a Western blot of unseparated and immunoelectroeluted proteins resuspended in KCl buffer. No DTT was present during separation. Under these conditions anti-p38 Abs recognized two bands: a major one of 140-150 kDa and a minor band of approximately 240 kDa. Both bands were strongly and selectively labeled by antibodies in either total extract or immunoelectroeluates (Fig. 5). The data illustrated in this figure were surprising because none of the high molecular mass proteins have been previously observed. We considered the possibility that the massive appearance of these proteins on the blot occurred due to two factors: (a) presence of KCl buffer during immunopurification instead of mannitol Tris or MES/K+ buffers used earlier; and (b) absence of reducing agents during gel separation. The immunoelectroeluate also contained few minor

**FIG. 3.** SDS-PAGE and immunoblot analyses of fractionated T84 plasma membrane proteins. Purified plasma membranes of T84 cells were separated by cation exchange as described under “Experimental Procedures.” Fractions of non-bound proteins and those eluted by pH 6.3, 7.8, 8.0, and 8.2 (lanes a--e on panels A and B, respectively) were subjected to gel analysis (A, Coomassie Blue staining) and Western blot using anti-p38 Abs (B). Samples were reduced by 10 mM DTT and boiled for 2 min. Protein loading was: 50 µg per lanes a and b; 15 µg per lanes c and d; and 25 µg per lane e.

**FIG. 4.** SDS-PAGE of electroeluted material after double gel separation. Cationic-enriched fraction (b), electroeluted (c), and double-electroeluted (d) proteins were obtained as described under “Experimental Procedures” and “Results.” Total extract from bovine tracheal apical membranes (a) and fractions plotted above (b--d) were separated on a 10% gel under reducing conditions (10 mM DTT) and stained by Coomassie Blue. Lanes a--d represent 75, 25, 10, and 7.5 µg of proteins, respectively.
under "Experimental Procedures." To detect the specific bands, 75 pg of total solubilized vesicles in KCl buffer or ~3 pg of immunoeluted proteins were separated on a 10% nonreduced gel and transferred to nitrocellulose. Immunoblot was developed as described under “Experimental Procedures.”

bands that migrated at molecular masses of 40, 64, and 97 kDa. These protein bands were evident on many blots and gels (Fig. 1–4). This observation supported our hypothesis that the protein composition of both immuno- and electrophoresis differ quantitatively but not qualitatively.

In order to test the effect of KCl (100 mM) on the extraction of high molecular mass proteins, apical vesicles were solubilized either in KCl or mannitol Tris buffer, separated under nonreduced conditions, transferred to nitrocellulose, and exposed to anti-p38 Abs. The results shown in Fig. 6 demonstrate that only KCl-solubilized proteins contained the 140-kDa and ~240-kDa protein band (Fig. 6). The mannitol Tris extract was completely negative. This finding is in accord with the previously obtained data (Fig. 1) that indicated low immunoreactivity toward the proteins solubilized in the presence of mannitol Tris buffer.

The findings presented in Figs. 5 and 6 suggested that the previously purified 38-kDa protein as well as the 62–64-kDa doublet represent smaller parts of a higher molecular mass protein. Considering the facts that the 140-kDa and higher proteins appeared only on nonreduced gels and smaller molecular mass proteins were observed only after sample reduction, it is reasonable to conclude that assembly of the native protein occurs via disulfide bridges. The next experiment was therefore designed to test the nature of the relationships existing between the 140-kDa protein and lower molecular mass bands. Total solubilized proteins (KCl buffer) were treated with 0, 2, or 10 mM DTT prior to separation. Anti-p38 Abs immunolabeled on the nonreduced sample (Fig. 7, lane a) the same proteins shown in Figs. 5 and 6 (140 kDa, 240 kDa, and a few weakly recognized bands in the range of 38–36 kDa). Exposure of the sample to 2 mM DTT led to the disappearance of 240 kDa and gradual degradation of 140 kDa. Several protein bands of apparent molecular mass ~40, 62, 64, 97, and 100 kDa became evident on the blot (Fig. 7, lane b). Elevation of the DTT concentration to 10 mM led to complete dissociation of a 140-kDa protein and the intermediate components (97–100 kDa) to two major products of 62 and 64 kDa. The labeling intensity of the 38-kDa protein did not change after reduction, possibly indicating low affinity of the antibodies to this separated component. This observation is in accord with all the blots shown above, where the strongest affinity was observed towards higher molecular mass bands and gradually diminished with disaggregation of the protein to lower molecular mass subunits.

Although evidence has been presented indicating that the 140-, 60-, and 36–38-kDa bands are different structural forms of the same protein, direct proof of this hypothesis can be achieved only by amino acid sequencing or probing with monoclonal antibodies directed against single epitopes. The antigen used to generate these monoclonal antibodies was the electroeluted p38. Fig. 8 shows several monoclonal antibodies subclones tested on the total solubilized vesicular proteins that were reduced by 5 mM DTT. Subclone A detected the 38-kDa protein only (Fig. 8, lane a). Subclones B and C bound to the 38-kDa and 60-kDa proteins (lanes b and c), while subclone D (lane d) recognized 38-kDa, 60-kDa, and high molecular mass forms. Polyclonal mouse immune IgG de-
Antibodies against Epithelial Cl\(^{-}\) Channel

We recently developed a purification protocol for a 38-kDa protein mediating conductive Cl\(^{-}\) channel activity (7). This protein had an apparent molecular mass of 38 kDa under disulfide reducing conditions. We showed earlier that additional minor bands of higher molecular mass copurified with this 38-kDa protein, and that these components may represent different parts of a native Cl\(^{-}\) channel complex. In the present study we generated polyclonal and monoclonal antibodies against this 38-kDa protein. These antibodies were used to verify the structural similarity between the higher molecular mass proteins and the 38-kDa Cl\(^{-}\) channel protein. Additional use of the polyclonal antibodies included immunopurification of the native transporter.

**DISCUSSION**

We recently developed a purification protocol for a 38-kDa protein mediating conductive Cl\(^{-}\) channel activity (7). This protocol involves only a single step instead of three; 2) delipidation of the eluted proteins occurs only in the final washing step; this step is immediately followed by replacement of native phospholipids with phosphatidylincholine/cholesterol; and 3) optimal extraction of protein is provided by using KCl buffer (Fig. 6) instead of the low osmolarity and KCl solution (immunopurification) or in an immobilized state (Table I, Figs. 5-7). Reduction of high molecular mass proteins and the 38-kDa Cl\(^{-}\) channel protein.

**TABLE I**

**Demonstration of Cl\(^{-}\) channel activity mediated by anti-p38 binding proteins**

| Fraction | 125I uptake | %Inhibition by H\(_2\)DIDS | %Inhibition by valinomycin | -Fold enrichment |
|----------|-------------|-----------------------------|-----------------------------|-----------------|
| Total soluble proteins | 0.22 ± 0.03 | 55 | 72 | 1 |
| Total soluble proteins + pH 2.5 | 0.08 ± 0.01 | 49 | 70 | 0.36 |
| Non-bound proteins | 0.13 ± 0.01 | 25 | 71 | 0.59 |
| Non-bound proteins + pH 2.5 | 0.04 ± 0.005 | 74 | 66 | 0.38 |
| Eluted proteins | 20.85 ± 1.8 | 89 | 95 | 94.77 |

The presented data are the average of seven individual immunopurification experiments, based on four different preparations of bovine membrane proteins or those treated by low pH (95- and 260-fold enrichment, respectively). 125I uptake was expressed as nmoles/mg of protein/5 min. The last observation is in accord with previously reported data indicating enhanced solubilization of certain membrane proteins by non-ionic detergents in the presence of salt (31-33) and/or buffers of high pH (35).

It is likely that the differences between the two purification protocols are responsible for the 24-fold higher rate of 125I uptake by the immunopurified protein as compared to the previously isolated 38 kDa protein (~95 versus ~4 nmoles of 125I/mg of protein/5 min) (7). One potentially protein-destructive step during the immunopurification procedure is the exposure to buffer of pH 2.8 for 2 min to dissociate the antibody-antigen complex. As can be seen in Table I, exposure of reconstituted protein to the low pH elution buffer for 2 min produced a 3-fold decrease in 125I uptake. Nonetheless, the relatively mild isolation conditions and minimal reduction in functional transport capacity makes immunopurification the favored purification method.

**Structural Analysis of Immunopurified Cl\(^{-}\) Channel**—Under nonreduced conditions both mouse and rabbit anti-p38 polyclonal antibodies recognize proteins of 140 and 240 kDa. Specific antibody binding to these proteins occurred either in solution (immunopurification) or in an immobilized state (blot) (Figs. 5-7). Reduction of high molecular mass proteins by DTT yielded several products. The major reduction components of the 140- and 240-kDa proteins had molecular...
masses of 62, 64, and 38 kDa. Intermediate products after incomplete reduction (2 mM DTT) appeared at 97–100 kDa (Fig. 7, lane b), and probably represent mixed aggregates of lower molecular mass bands. The protein bands in the range of 60 kDa were also detected by anti-p38 rabbit polyclonal antibodies on Western blots of semipurified fractions from T84 cells (Fig. 3). This observation might indicate that proteins, functionally similar to bovine tracheal Cl⁻ channel, exist in T84 epithelial cells. Interestingly, both rabbit and mouse polyclonal antibodies recognize the original 38-kDa polypeptide antigen very weakly. The detection of several protein bands with mild DTT treatment (2 mM) and the observation that the proteins in the range of 60 kDa appeared variably as a singlet, doublet, or triplet suggests that this 140-kDa protein is comprised of at least four nonidentical components. Fig. 8 demonstrates that at least one of the monoclonal antibodies was capable of detecting a single antigenic epitope that is common for 38-, 60-, and 140-kDa bands. Most of the other subclones detected either the 38- or 58- and 60-kDa bands. One possible explanation for these observations is that many of the epitopes recognized by different monoclonal antibodies may be obscured in the total vesicle preparation by the presence of an excess of other unrelated proteins. Alternatively, conformational differences between the above protein forms may either bury or expose antigenic sites.

Thus, using either monoclonal or polyclonal antibodies, we were able to demonstrate the structural identity of all the aforementioned protein components. Moreover, two of our experimental approaches showed that these components represent different forms of the same protein: (a) electroeluted 38-kDa polypeptide, even after the second cycle of gel separation, contains 62–64-kDa and 140-kDa bands as well (Fig. 4); (b) immunopurified protein of 140 kDa in the absence of reducing conditions can be converted to low molecular mass subunits by the addition of DTT (Figs. 7 and 8). Our current results confirmed that the activity of this Cl⁻ channel is extremely sensitive to modifications of thiol-disulfide status of the native protein.

Comparison with Previous Studies—Few attempts have been made to purify and to identify Cl⁻ channel(s) from different tissues. Finn et al. (36) reported the inhibition of Cl⁻ conductance by monoclonal antibodies raised against Necturus gallbladder cells. The antibodies recognized 219- and 69-kDa proteins on reduced gels. Breuer (31), using the radioactive anion blocker [3H]DIDS, identified proteins of 65 and 31 kDa (reduced gel) in renal thick ascending loop of Henle cells. Although only the 31-kDa band has been proposed to be a part of the Cl⁻ channel, the participation of the 65-kDa protein could not be excluded. The extraction of Cl⁻ channel activity from these membranes was optimal in the presence of 200 mM KCl and greatly diminished in buffers of lower ionic strength, similar to the results reported here. Landry et al. (37), published the purification of a Cl⁻ channel from kidney and trachea membranes using IAA-94 (indanyloxyacetic acid) affinity resin. The major proteins eluted from the column had molecular masses of 97, 64, 40, and 27 kDa (reduced gel). However, later it was found that immunoprecipitation of only 64 kDa correlated with the removal of Cl⁻ channel from the total extract (38). This protein (64 kDa) had an unusual property to appear variably either as a doublet of 62–64 kDa or as a single band (38). Recently, Preston et al. (39) demonstrated that cytosolic vesicles from bovine trachea contain Cl⁻ channel activity comparable to that observed in the apical vesicles. SDS-PAGE analysis of these membranes revealed three major bands of 200, 67, and 40 kDa. The minor copurified bands included 97- and 30–35-kDa proteins (39). Thines and co-workers (40) reported on the purification and electrophysiological characterization of voltage-dependent anion channel. This protein (porin 31HL) has a molecular mass of 31 kDa, and it is found in the plasmalemma of B lymphocytes (40, 41). The purification protocol for porin 31HL is remarkably similar to the described procedure for our 38-kDa protein (double ion-exchange chromatography which includes CM- and DEAE-cellulose steps followed by gel filtration in SDS on sephacryl S-300) (41). Porin 31HL, reconstituted in planar lipid bilayer membranes, displays properties characteristic of a DID5-sensitive Cl⁻ channel (40). These data, taken together with findings presented here, may indicate that the homologous Cl⁻ channels have been identified by different approaches in different tissues. Ultimately, the amino acid sequence of individually purified proteins will ascertain the identity of these proteins.

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