individual minerals would indicate. For example, textural evidence indicates that the chemical reaction that results in garnet growth involves repeated dissolution and reprecipitation of matrix minerals. The concurrent deformation also occurs partly by stress-solution: solution at grain interfaces under high normal compression and reprecipitation at interfaces under low normal compression. These processes repeatedly expose matrix grain interiors to the intergranular medium where diffusivities are higher than the corresponding volume diffusivities. [For more detailed discussion of supporting textural evidence, see J. L. Rosenfeld, in *Preferred Orientation in Deformed Metals and Rocks: An Introduction to Modern Texture Analysis*, H.-R. Wenk, Ed. (Academic Press, Orlando, FL, 1985), pp. 441–461, and D. M. Carmichael, *Contrib. Mineral. Petrol.* **20**, 244 (1969).]
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25. We thank G. Bebout for help in the field and J. DeGrosse for his assistance in cutting and sectioning the garnets used in this study. This work was supported by NSF grant EAR87-07356 to J.L.R. and D.J.D., grant EAR 84-15143 to D.J.D., and grant 1601 to J.L.R. from the Committee on Research of the UCLA Academic Senate. G. Bebout, P. Bird, H. Reiss, and an anonymous reviewer provided critical comments on the manuscript.

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Purification and Reconstitution of Chloride Channels from Kidney and Trachea

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Chloride channels mediate absorption and secretion of fluid in epithelia, and the regulation of these channels is now known to be defective in cystic fibrosis. Indanyloxyacetic acid 94 (IAA-94) is a high-affinity ligand for the chloride channel, and an affinity resin based on that structure was developed. Solubilized proteins from kidney and trachea membranes were applied to the affinity matrix, and four proteins with apparent molecular masses of 97, 64, 40, and 27 kilodaltons were eluted from the column by excess IAA-94. A potential-dependent $^{36}Cl^-$ uptake was observed after reconstituting these proteins into liposomes. Three types of chloride channels with single-channel conductances of 26, 100, and 400 picosiemens were observed after fusion of these liposomes with planar lipid bilayers. Similar types of chloride channels have been observed in epithelia.

CHLORIDE CHANNELS ARE PRESENT in the plasma membrane of most cells. In epithelia these channels act together with other ion transporters, such as the $Na^+,K^+,2Cl^-$cotransporter and the Na^+- and K^+-dependent adenosine triphosphatase, to mediate absorption or secretion of NaCl (*1*). Characterization of the channels has been limited to analysis of electrophysiological properties such as single-channel conductance, voltage dependence, and the effect of second messengers on channel opening. In airway epithelia the opening of a channel with a conductance of 40 to 50 pS and a rectifying current-voltage (*I-V*) relation was stimulated by the adenosine 3′,5′-monophosphate–dependent protein kinase (A-kinase) (*2*). The inability of A-kinase to stimulate channel opening in excised apical membrane patches of secretory cells is thought to reflect the pathogenetic defect in cystic fibrosis (*2*). Similar Cl^- channels are present in fibroblasts and lymphocytes, and here too their opening is defective in cystic fibrosis (*3*).

We have developed high-affinity ligands for the Cl^- channel by screening inhibitors of $^{36}Cl^-$ transport in bovine kidney cortex microsomes (*4*). An indanyloxyacetic acid, IAA-94 (Fig. 1), was the most potent inhibitor with an inhibition constant (K_i) of 1 μ*M*, and [^{3}H]IAA-94 bound to these microsomes with a dissociation constant (K_d) of 0.6 μ*M*. Similar results were seen in apical membranes from bovine trachea. The rank order of potency for inhibition of $^{36}Cl^-$ transport for several inhibitors correlated with that for displacement of [^{3}H]IAA-94, suggesting that IAA-94 binds to the Cl^- channel. We describe here the purification of the solubilized channel by chromatography on an affinity matrix based on the IAA structure. Reconstitution of the purified proteins into planar bilayers revealed the presence of Cl^- channels.

To purify Cl^- channels from bovine kidney cortex vesicles and from apical membranes of bovine trachea, we first solubilized these preparations in *n*-octyl glucoside. A final detergent concentration of 1.4% solubilized 60% of the protein and 20% of the [^{3}H]IAA-94 binding sites (*5*). The addition of 10% glycerin preserved the number of solubilized binding sites after freezing at −70°C or incubation at 4°C for 24 hours. The concentration of IAA-94 required to displace 50% of the specifically bound ^{3}H-labeled ligand (IC_{50}) from the solubilized proteins was 2 μ*M*, similar to the value obtained for intact vesicles. Potential-driven $^{36}Cl^-$ uptake was observed when the solubi-

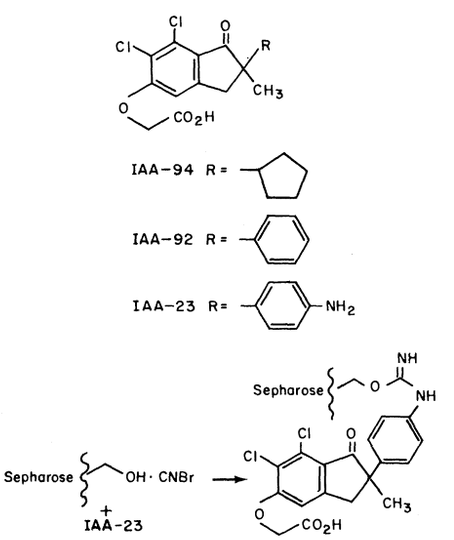

Fig. 1. Structures of IAA and synthesis of the affinity resin. Dry cyanogen bromide (CNBr)–activated Sepharose 4B was reswollen with ice-cold HCl (1 m*M*). After 15 min the resin was washed with a solution containing 0.1*M* $NaHCO_3$ and 0.5*M* NaCl (*p*H 9). The IAA-23 was dissolved in 0.1*M* $NaHCO_3$ and 0.5*M* NaCl (*p*H 9) and added to the swollen resin (1.5 μmol of IAA-23 per milliliter of resin). The mixture was agitated at room temperature for 48 hours. Depletion of the ligand from the supernatant was followed by ultraviolet spectroscopy at a wavelength of 268 nm. The efficiency of depletion averaged 97%. The resin was washed by alternating 0.1*N* tris (*p*H 8.5) and 0.1*N* sodium acetate (*p*H 4.5) and then stored in a solution containing 250 m*M* sucrose, 10 m*M* imidazole (*p*H 7), and 0.02% NaN_3. Before use the resin was washed with a solution containing 250 m*M* sucrose, 10 m*M* imidazole (*p*H 6.0), 10% glycerin, and 1.4% *n*-octyl glucoside.

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lized proteins were reconstituted into liposomes (*6*). This uptake was inhibited by IAA-94 with an IC_{50} of about 1 μ*M*, and inhibition was more effective if the drug was present both inside and outside the liposomes.

We constructed an affinity matrix by coupling IAA-23, a 4′-amino derivative of the potent inhibitor IAA-92, to cyanogen bromide–activated Sepharose 4B (Fig. 1). The affinity resin was incubated with solubilized membranes for 18 hours, after which the supernatant was found to be depleted of 55% of the [^{3}H]IAA-94 renal binding sites and 90% of the tracheal sites. Less than 10% of the total protein was lost from the supernatant in each case. Extensive washing of the resin was necessary to remove nonspecifically bound proteins. Specifically bound proteins were eluted with 100 μ*M* IAA-94. Benzoic acid, which does not interfere with [^{3}H]IAA-94 binding, was present in the wash to minimize any perturbation caused by the presence of IAA-94 in the eluant. Proteins present in the last wash fraction and the specific eluate were precipitated and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The kidney eluate was enriched in 97-, 64-, 40-, and 27-kD proteins (Fig. 2). No other protein bands were generated by eluting with higher concentrations of IAA-94. An abundant protein seen at the top of the gel was present throughout the wash and was not significantly enhanced on elution with IAA-94. Tracheal eluates also contained proteins of the same molecular masses, but the 27-kD protein showed greater enrichment. Other proteins were occasionally eluted, but the four kidney proteins described have been observed in more than 20 preparations. Of the 6 mg of protein applied to the resin, less than 1 μg was eluted (*7*). The presence of seven protease inhibitors during protein solubilization and elution was necessary to obtain these results.

The purified proteins were reconstituted into phospholipid vesicles by detergent dialysis. Dialysis removed both the detergent and IAA-94 present in the elution buffer. Several distinctive Cl^- channels were seen after fusion of these proteoliposomes with planar lipid bilayers. No cation-selective or nonselective channels were found. Although incorporation events were rare, we observed Cl^- channels in each of the 15 separate preparations tested. Initial conductance jumps were observed and were often due to the simultaneous insertion of several identical channels into the planar membrane, suggesting that the channels aggregated at some point before their incorporation into the vesicles.

A Cl^- channel with a conductance of 26 pS in 150 m*M* KCl and a linear *I-V* relation was present in the kidney proteins (Fig. 3A). The channel had a reversal potential of 26 mV in a 350 to 100 m*M* KCl gradient, implying a Cl^- to K^+ selectivity ratio of

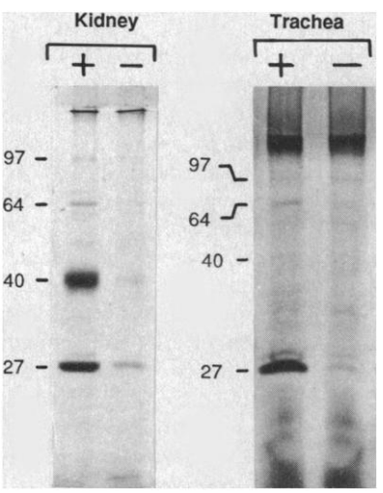

Fig. 2. Electrophoresis of purified Cl^- channel proteins from kidney and trachea. Kidney or trachea membranes were extracted with 1*M* sodium thiocyanate and solubilized at a concentration of 6 mg of protein per milliliter in a solution containing 250 m*M* sucrose, 10 m*M* imidazole (*p*H 7.0), 10% glycerin, and 1.4% *n*-octyl glucoside. After 30 min the mixture was centrifuged at 100,000*g* for 1 hour, and the supernatant was collected. The IAA-23 affinity resin (1 ml) was mixed with 1 ml of solubilized membranes (6 mg of protein) and agitated for 18 hours at 4°C. The mixture was then transferred to a column, and the resin was washed at a rate of 0.3 ml/min with 35 ml of a solution containing 250 m*M* sucrose, 10 m*M* imidazole (*p*H 6.0), 10% glycerin, 0.6% *n*-ocytl glucoside, 100 μ*M* benzoic acid, and 1% ethanol. Bound material was eluted with 7 ml of the wash solution containing 100 μ*M* IAA-94 in place of 100 μM benzoic acid. The eluate (+) and last wash fraction (−) (7 ml) were precipitated in acetone and chromatographed on SDS-PAGE. Proteins were detected by silver staining of the gel.

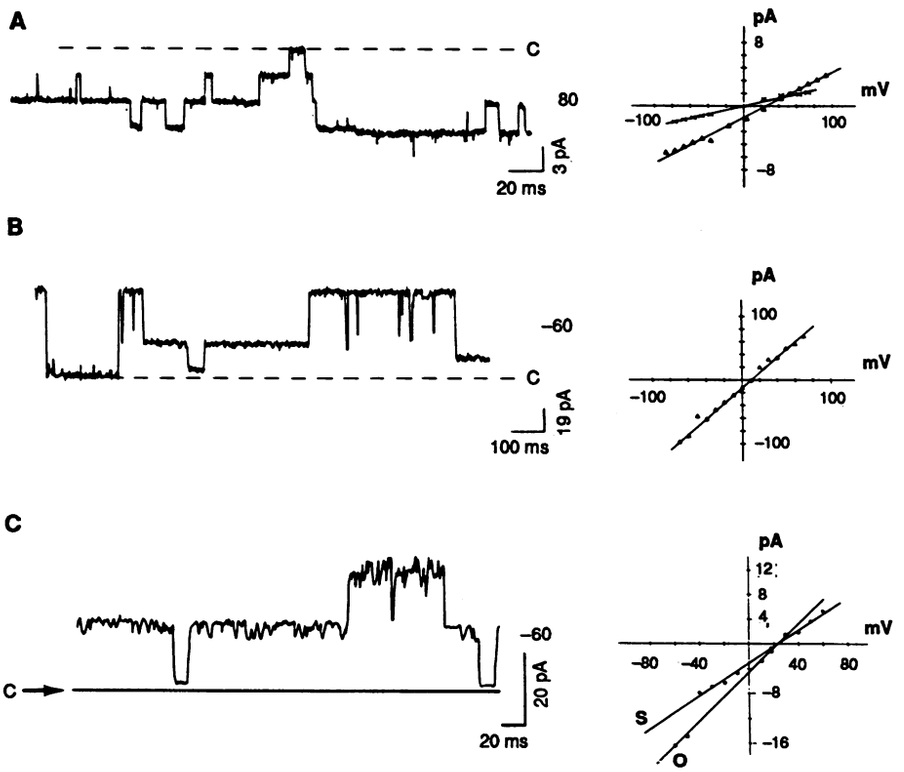

Fig. 3. Single-channel recordings (left) and *I-V* relations (right) of purified Cl^- channels reconstituted into planar lipid bilayers (*14*). (**A**) Channel purified from bovine kidney with a single-channel conductance of 26 pS. The trace shows the channel in 350 m*M* KCl where the single channel conductance is 70 pS. A downward deflection represents channel opening. C represents closed state. There are three identical channels in the record. Holding potential (in millivolts) is indicated to the right of the trace. *I-V* relation for this channel was obtained in the presence of a 150 to 10 m*M* KCl gradient (triangles) or with symmetrical 150 m*M* KCl (indicated by x's). (**B**) A large conductance anion channel (400 pS) with multiple substates purified from bovine kidney. An upward deflection represents channel opening. The *I-V* relation for the fully opened state was obtained in the presence of a 150 to 10 m*M* KCl gradient. (**C**) Channel purified from bovine trachea with a single-channel conductance of 100 pS. There are two identical channels in this bilayer. In the open state the channel rapidly flickers between the fully open state and a subconductance state. Flicker noise and amplitude increase during the interval when two channels are open simultaneously. An upward deflection represents channel opening. The *I-V* plot was obtained in a 350 to 100 m*M* KCl gradient. The two lines indicate the subconductance state (S) and the fully open state (O). Both have identical reversal potentials.

greater than 20:1, and the open channel probability showed slight voltage dependence.

A second type of channel had a conductance of 400 ± 50 pS in 150 m*M* KCl and showed a low Cl^- to K^+ selectivity ratio varying between 2:1 and 5:1 (Fig. 3B). This channel showed multiple subconductance states, and the probability of populating these subconductance states seemed to be a complex function of voltage and time. The open probability of this channel was near 1.0 at all voltages, unlike similar channels in a variety of tissues (*8*). This type of channel was present in both kidney and trachea.

A third type of channel had a conductance of 240 pS in symmetrical 350 m*M* KCl (Fig. 3C); if we assume there is a linear relation between conductance and ionic activity, this implies a conductance of about 100 pS in 150 m*M* KCl. The channel had a linear *I-V* relation and exhibited at least one conductance substate that was about 75% of the main open-state conductance. The reversal potential of both conductance states was 24 mV in the presence of a 350 to 100 m*M* KCl gradient, implying a Cl^- to K^+ selectivity ratio of 13:1. The kinetics of the channel were not markedly voltage-dependent. This channel was also seen in both kidney and trachea.

All three types of Cl^- channels were unaffected by the inhibitors IAA-94 (100 μ*M*), stilbene isothiocyanates (500 μ*M*), or potent anthranilic acid analogs. Inhibitors were added to both cis and trans chambers. As all of the channels bound to an inhibitor column, the purification or reconstitution procedure may induce a conformational change in the channel that uncouples ligand binding from channel closure or, alternatively, the ligand binding site may be lost, proteolysed, or denatured after elution.

An estimate of the degree of channel purification achieved was obtained by comparing the extent of $^{36}Cl^-$ uptake into proteoliposomes containing the purified proteins with that into liposomes containing solubilized kidney cortex microsomes. The Cl^- channels were reconstituted together with bacteriorhodopsin, a light-activated, electrogenic proton pump, so that light induced intravesicular acidification and generated an inside-positive membrane potential (*9*). This potential can be used to drive the transport of anions if the vesicles also contain the appropriate channel. Light induced an uptake of $^{36}Cl^-$ into liposomes containing solubilized kidney cortex microsomes and bacteriorhodopsin, and there was an efflux of the isotope when the light was turned off. The proton electrochemical gradient generated by bacteriorhodopsin is composed of a *p*H gradient and a membrane potential. At any given electrochemical gradient, a reduction in the *p*H gradient should increase the membrane potential and vice versa. Increasing the buffer concentration inside the liposomes resulted in an increased rate of $^{36}Cl^-$ uptake (Fig. 4A). Addition of valinomycin, to collapse the membrane potential, completely prevented the light-induced $^{36}Cl^-$ uptake.

Light also induced $^{36}Cl^-$ uptake into liposomes containing bacteriorhodopsin and purified Cl^- channel proteins (Fig. 4B). The extent of $^{36}Cl^-$ uptake was dependent on the amount of Cl^- channel protein incorporated. In the presence of valinomycin, uptake was reduced to the extent seen with liposomes reconstituted with bacteriorhodopsin alone (*10*). Comparison of the magnitude of $^{36}Cl^-$ uptake observed with unpurified and purified channels gave a purification factor of at least 1000, if we assume that there was about 1 μg of reconstituted purified protein (*7, 11*). The $^{36}Cl^-$ uptake assay, unlike single-channel recording, is a "macroscopic" assay that requires a large number of molecules to give a measurable uptake. Thus the proteins detected in the eluate of the affinity column (Fig. 2) are probably mediating the observed uptake of $^{36}Cl^-$.

Chloride channels with similar properties as those reported here (the 26- and 400-pS channels in particular) have been detected in patch-clamp studies of epithelia (*1, 2, 8, 12*), suggesting that these purified channels are derived from the plasma membrane. It is not known why the purified and reconstituted channels were insensitive to channel blockers, but similar results were found after the purification and reconstitution of the Ca^{2+} release channel from sarcoplasmic reticulum; in this case, channel proteins purified on an immunoaffinity column were no longer inhibited by ryanodine (*13*). Finally, it remains to be seen whether the gene for any of the affinity-purified Cl^- channel proteins resides in the cystic fibrosis locus.

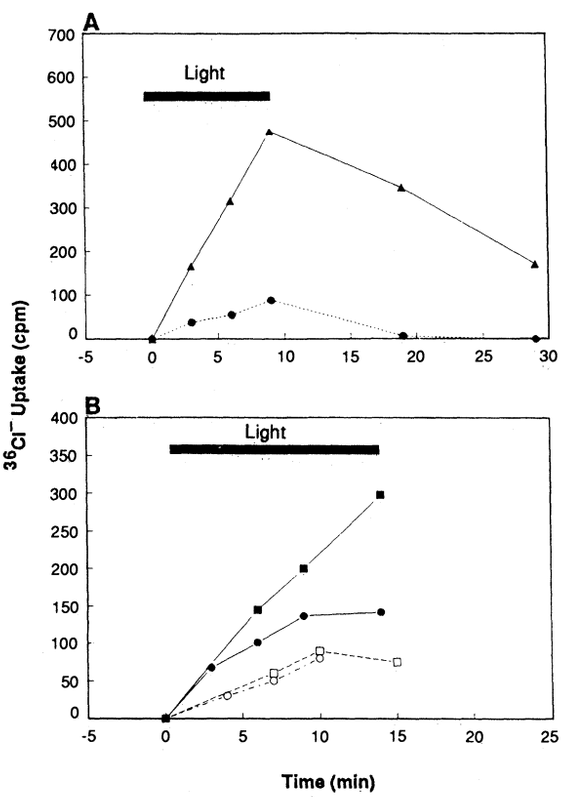

Fig. 4. Reconstitution of Cl^- channels with bacteriorhodopsin. (**A**) Crude vesicles (6 mg of protein) were solubilized and reconstituted either with a 10 m*M* tris solution (circles) or with 100 m*M* Pipes-tris (triangles). Results are averages of three separate preparations (*14, 15*). A mixture of 0.8 ml of solubilized kidney cortex vesicles (6 mg of protein per milliliter), 0.2 ml of bacteriorhodopsin (1 mg/ml), and 10 mg of asolectin, all in a final concentration of 1.5% *n*-octyl glucoside, was dialyzed against 1 liter of a solution containing 100 m*M* KCl and 10 m*M* tris (*p*H 8.0) for 1 day with one change of solution and for a further day against either 10 m*M* potassium gluconate and 10 m*M* tris (*p*H 7.0) or 100 m*M* Pipes-tris (*p*H 7.0). The proteoliposomes were then frozen at −80°C until use. Immediately before use the vesicles were thawed to room temperature and sonicated for 25 s in a Branson 2200 bath sonicator. (**B**) Less than 1 μg of purified protein (open or closed circles) or a tenfold higher amount (open or closed squares) was reconstituted as described in (A), except that Pipes-KOH (*p*H 7) was added to a concentration of 50 m*M* before freezing. Valinomycin (10 nmol per milligram of lipid) was added before the light was turned on (open symbols). Results are an average of seven separate preparations. Channels were purified as described (see legend to Fig. 2).

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5. For measurements of [^{3}H]IAA-94 binding, kidney vesicles were solubilized as described (legend to Fig. 2). Solubilized proteins were incubated at 4°C for

30 min with 0.1 μM [^{3}H]IAA-94 (12 Ci/mmol). Bound ligand was separated from free by rapid gel filtration through a 0.5 cm by 3 cm G-50 Sephadex column, and the radioactivity present in the eluate was determined in a liquid scintillation counter to provide a measure of total binding. Nonspecific binding was defined as that occurring in the presence of 100 μM IAA-94, and the difference between total and nonspecific binding yielded specific [^{3}H]IAA-94 binding.

6. Chloride channels were reconstituted by a detergent dilution method [M. Kasahara and P. C. Hinkle, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 396 (1976); W. P. Dubinsky and L. B. Monti, *Am. J. Physiol.* **251**, C713 (1986)]. Solubilized proteins (see legend to Fig. 2) were diluted tenfold into excess asolectin (12 mg/ml) and frozen in an acetone-dry ice bath. The mixture was thawed at room temperature and sonicated in a bath-type sonicator for 25 s; external Cl^- was removed from the vesicles by passing them through an anion exchange column. Uptake of $^{36}Cl^-$ into the liposomes was measured at a final Cl^- concentration of 5 mM (2 μCi/ml), and uptake was stopped by passing the vesicles through a second anion exchange resin.
7. The concentration of eluted proteins was below the resolution of standard protein determination assays. On the basis of silver staining of known quantities of protein standards in the same gels, we estimated that for each preparation less than 1 μg of protein was present in the column eluate. Although silver staining is poorly quantitative, this estimate was supported by NH_2-terminal amino acid analysis of the 27-kD protein, which showed the presence of about 100 ng of protein.
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14. The purified channels were reconstituted into phospholipid vesicles by a detergent dialysis procedure. The vesicles were formed by concentrating 7 ml of the affinity column eluate to 1 ml with a PM-10 filter (Amicon, Danvers, MA) that had been coated with a 1% fibrinogen solution. The concentrated material was added to 10 mg of asolectin [Y. Kagawa and E. Racker, *J. Biol. Chem.* **246**, 5477 (1971)] and 9 mg of *n*-octyl glucoside, vortexed, placed in a dialysis tube (Spectra/por, Spectrum Medical Industries, Los Angeles, CA; 14-kD cutoff), and dialyzed for 15 hours against 1 liter of a solution containing 10 mM KCl, 700 mM sucrose, and 10 mM Hepes (*p*H 7.0). The dialyzate was changed and dialysis was continued for an additional 3 hours. Vesicles were stored on ice until use. Planar bilayers (4% asolectin in decane) were formed by the brush technique in a 100-μm hole [A. Finkelstein, *Methods Enzymol.* **32**, 489 (1974)] in a Teflon partition. Vesicles were squirted at the membrane through a micropipette positioned 20 to 50 μm from the planar bilayer [M. H. Akabas, F. S. Cohen, A. Finkelstein, *J. Cell Biol.* **98**, 1063 (1984); W. Niles and F. S. Cohen, *J. Gen. Physiol.* **90**, 703 (1987)]. The cis (vesicle-containing) chamber contained either 350 mM KCl, 10 mM $CaCl_2$, and 10 mM Hepes (*p*H 7.0) or 150 mM KCl, 400 mM urea, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0). The trans side contained either 100 mM KCl, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0) or 10 mM KCl, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0) but without urea. The single-channel currents were amplified with a current-to-voltage converter and recorded on a PCM video tape recorder (Indec Systems, Sunnyvale, CA). The data were digitized and analyzed with the use of interactive programs on a laboratory computer system (Indec Systems). Records were filtered at 300 Hz before digitization through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Potentials given are those in the cis chamber relative to virtual ground in the trans chamber. Selectivity ratios were calculated by means of the Goldman-Hodgkin-Katz equation with activity coefficient corrections.
15. Light-induced $^{36}Cl^-$ uptake was measured by adding 6 mM $^{36}Cl^-$ (2.3 μCi/ml) to thawed and sonicated liposomes in a glass fluorometric cuvette. The vesicles were exposed to light from a Kodak 650H slide projector. Samples (80 to 120 μl) were removed at the indicated times, applied to an 8-cm anion exchange column, and eluted with 1 ml of a solution containing 250 mM sucrose and 10 mM imidazole (*p*H 7.0) to remove extravesicular $^{36}Cl^-$. Uptake in the dark was measured from identical samples of each preparation and was found to be linear with time, and this uptake was subtracted from the light-dependent flux. The individual values were within 10% of the reported averages.
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Persistence of Abnormal Chloride Conductance Regulation in Transformed Cystic Fibrosis Epithelia

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An airway epithelial cell line (CF/T43) was developed by infecting cultured airway epithelial cells from patients with cystic fibrosis (CF) with the pZIPneoSV(X)1/SV40T retrovirus and selecting for G418 resistance and ion transport properties. The distinctive chloride secretory phenotypes of the CF cell line CF/T43 and a normal cell line (NL/T4) were not perturbed by SV40T-induced cell transformation. Epithelial cell lines generated from CF cells with the SV40T gene can be used to test candidate CF genes and to evaluate the molecular mechanisms responsible for the CF phenotype.

CYSTIC FIBROSIS (CF) IS AN AUTOSOMAL recessive disease that affects epithelia of the airways, sweat glands, pancreas, and other organs. Abnormal regulation of transport proteins in the apical cell membrane, including the conductive Cl^- channel, is the direct effect of the abnormal gene (*1*). These abnormalities are exhibited in primary cultures of CF airway epithelial cells, but the limited availability of these cells currently restricts CF research. The gene encoding for the SV40 large T (SV40T)

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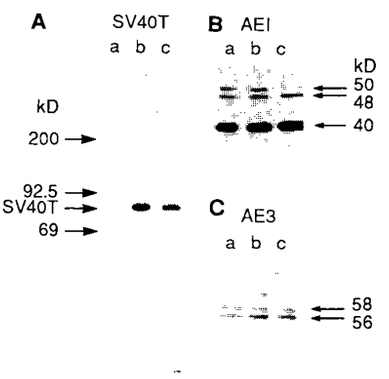

Fig. 1. (**A**) SV40T expression in CF/T43 and NL/T4 cell lines. Lane a, primary culture of normal airway epithelial cells; lane b, CF/T43; lane c, NL/T4. Cells were labeled with [^{35}S]-methionine (100 μCi/ml; >1000 mCi/mmol) (Amersham) in methionine-free Ham's F12 medium for 1 hour. The labeled cells were washed in phosphate buffered saline (PBS) and solubilized in lysis buffer (154 mM NaCl, 10 mM Na_2HPO_4, *p*H 7.2, 1% Triton X-100, 0.5% deoxycholate, and 1 mM NaF). The homogenate was centrifuged at 50,000*g* for 1 hour, and the supernatant (>10^7 tricarboxylic acid–precipitable counts per minute) immunoprecipitated with mouse monoclonal antibody 416 (Oncogene Science) to SV40T antigen (Ab-2a) according to the manufacturer's protocol (*16*). Immunoprecipitated antigen was analyzed by 8% SDS–polyacrylamide gel electrophoresis (*17*) followed by autoradiography. (**B**) Expression of AE1 cytokeratin and (**C**) expression of AE3 cytokeratin; the lanes are as in (A). Cells were collected by trypsinization and solubilized in sample buffer (62.5 mM tris-HCl, *p*H 6.8, 1% SDS, 2 mM phenylmethylsulfonylfluoride, 1 mM EDTA, 2.5 μg of leupeptin per milliliter, 2.5 μg of aprotinin per millilter, 5% glycerol, 50 mM dithiothreitol, and 0.001% bromophenol blue, *p*H 6.8). Proteins were separated by 8% SDS–polyacrylamide gel electrophoresis (*17*), and immunoblot analysis was performed (*18*). The reactivity of keratins with monoclonal antibodies AE1 and AE3 (*19*) was visualized by immunogold silver staining with Auro Probe BL plus streptovidin (Janssen Biotech, Olen, Belgium) according to the manufacturer's protocol.