

# Functional Activation of Plasma Membrane Anion Exchangers Occurs in a pre-Golgi Compartment

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**Abstract.** Folding and oligomerization of most plasma membrane glycoproteins, including those involved in ion transport, occur in the ER and are frequently required for their exit from this organelle. It is currently unknown, however, where or when in the biosynthetic pathway these proteins become functionally active. AE1 and AE2 are tissue-specific, plasma membrane anion transport proteins. Transient expression of AE2 in a eukaryotic cell line leads to an increase in stilbene inhibitable whole cell  $^{35}\text{SO}_4^{2-}$ -efflux consistent with its function as a plasma membrane anion exchanger. No such increased transport activity was observed in AE1 transfectants, despite the fact that the two proteins were synthesized in roughly equal portions. In contrast, both AE1 and AE2 expression resulted in significant increase in  $\text{Cl}^-/\text{SO}_4^{2-}$  exchange in crude micro-

somes demonstrating that both AE1 and AE2 cDNAs encode functional proteins. Immunofluorescence staining and pulse-chase labeling experiments revealed that while 60% of AE2 is processed to the cell surface of transfectants, AE1 is restricted to an intracellular compartment and never acquires mature oligosaccharides. Crude microsomes from transfected cells were fractionated into plasma membrane and ER-derived vesicles by con A affinity chromatography. All of the AE1 and approximately half of the cellular AE2 was eluted with the ER vesicles, confirming their intracellular localization. Anion transport measurements on these fractions confirmed that the ER-restricted anion exchangers were functional. We conclude that AE1 and AE2 acquire the ability to mediate anion exchange at an early stage of their biosynthesis, before their exit from the ER.

THE secretory pathway is composed of a series of compartments or cisternae in which proteins are posttranslationally modified and sorted to their target destinations (Palade, 1975). These processes require the presence of chemical and ionic gradients across the cisternal membranes. For example, the lumen of secretory vesicles and the distal cisternae of the *trans*-Golgi apparatus are acidic relative to the cytosol and to more proximal compartments (Anderson and Pathak, 1985; Orci et al., 1986; reviewed in Anderson and Orci, 1988). Experimental dissipation of this proton gradient causes morphological changes in the *trans*-Golgi accompanied by secretory defects including missorting and intracellular accumulation of secretory and membrane proteins (reviewed in Mollenhauer et al., 1990). Organelles are acidified by a "vacuolar"  $\text{H}^+$ -ATPase which pumps protons from the cytosol at the expense of metabolic energy (Rudnick, 1986; Al-Awqati, 1986). The magnitude of the transmembrane pH gradient appears to depend on the proton pumping activity (Zhang et al., 1992; Al-Awqati, 1986) and a parallel cAMP-regulated  $\text{Cl}^-$  conductance which shunts the interior-

positive membrane potential created by the proton influx (Bae and Verkman, 1990; Barasch et al., 1988).

Most plasma membrane glycoproteins (including ion transporters, channels, and pumps) exist in their mature conformations as oligomeric complexes (Hurtley and Helenius, 1989). It is well established that protein folding and oligomerization is initiated in the ER; its correct completion is usually a prerequisite for maturation to more distal compartments of the secretory pathway (Hurtley and Helenius, 1989). By contrast, little is known about when and where, during the course of biosynthesis of oligomeric transport proteins, they acquire the functional properties characteristic of their mature forms. Where such functional capacitation occurs, however, is important, since these proteins have the potential to modify the ionic milieu of the compartments through which they pass en route to their intracellular destinations. One possibility is that transporters are maintained in transit in a repressed state and are activated only upon reaching their correct intracellular destination. Signals for activation could include dissociation of transiently associated regulators, covalent modification, subunit assembly, or environmental cues such as lipid composition or membrane potential. Alternatively, proteins could be assembled in the ER and transported through the cisternae of the secretory pathway as catalytically active complexes. Steady-state levels of these "in-transit" proteins could contribute to

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the maintenance of ionic gradients across the membranes of secretory organelles. In support of this is the observation that mutations in a plasma membrane  $\text{Cl}^-$  channel, CFTR, lead to its retention in the ER, and result in secretory abnormalities correlated with defective acidification of the *trans*-cisternae of the Golgi and TGN (Barasch et al., 1991).

Anion exchangers of the AE family are present in the plasma membrane of most mammalian cells where they participate in the regulation of intracellular pH, pCl and volume (reviewed in Alper, 1991; and Kopito, 1990). AE1 and AE2 are two homologous members of this family that are expressed in a cell-type and tissue-specific fashion. AE1 is present in the plasma membrane of mature erythrocytes (Steck, 1978) and the basolateral plasma membrane of intercalated cells of the distal nephron (Drenckhahn et al., 1985; Kopito et al., 1988). AE2 is a broadly distributed anion exchanger found in many different epithelial and non-epithelial cell types (Kopito, 1990). All members of the AE family share a bipartite structural organization consisting of a membrane-associated COOH-terminal  $\sim 400$  amino acid anion exchange domain and an  $\text{NH}_2$ -terminal cytosolic domain (Kopito, 1990). The latter domain of erythrocyte AE1 binds to ankyrin which serves to link the spectrin-actin cytoskeleton to the plasma membrane (reviewed in Low, 1986). The function of the corresponding domain of AE2 is unknown. In this study, we have investigated the biosynthesis and intracellular processing of AE1 and AE2 in transiently transfected human embryonic kidney (HEK) cells. Our data show that these two homologous anion exchangers are processed quite differently through the secretory pathway and demonstrate that both proteins acquire the capacity to catalyze anion exchange while still in an early compartment of the secretory pathway.

## Materials and Methods

### Materials

[ $^{35}\text{S}$ ]sulfate (carrier-free), [ $^{35}\text{S}$ ]methionine ( $>1,000$  Ci/mmol) and [ $^{36}\text{Cl}$ ]chlorine (5–25 mCi/g) were obtained from New England Nuclear (Boston, MA). 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), pyridoxal 5'-phosphate, valinomycin, glucose-6-phosphate, and mannose-6-phosphate were purchased from Sigma (St. Louis, MO). *N*-hydroxy-succinimide biotinate, Affi-Gel Con-A, Bradford protein assay kit, and the Biotin-blot total protein detection kit were obtained from BioRad Laboratories (Richmond, CA). HRP-conjugated streptavidin, HRP-conjugated goat anti-rabbit IgG and the ECL Detection Kit are from Amersham Corp. (Arlington Heights, IL). FITC-conjugated goat anti-rabbit IgG as well as rhodamine-conjugated donkey anti-mouse IgG were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Protein A Sepharose CL-4B was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), Endoglycosidase H (endo H) from Boehringer Mannheim Biochemicals (Indianapolis, IN), and CytoScint and Fluoro-Hance from ICN Bio-medical Inc. (Irvine, CA). All other chemicals were of reagent grade.

### Expression Constructs

Murine AE1 (Kopito and Lodish, 1985) and AE2 cDNA (Alper et al., 1988) and mutants thereof were cloned into the expression vector pRBG4, which contains the human cytomegalovirus immediate early promoter/en-

hancer (Lee et al., 1991). AE2 $\Delta$ 676 contains a deletion from the 5' EcoRI site to the NaeI at position 2205 using an oligonucleotide adaptor (5'AAT-TCAAAGATGGATC) that introduces an in-frame consensus (Kozak, 1981) Met codon. To make the AE2/AE1 chimera, the  $\text{NH}_2$ -terminal domain of mouse AE1 cDNA (Kopito and Lodish, 1985) was excised with EcoRI (5' end) and StuI (1322) and replaced with an AE2 fragment from the 5' EcoRI to NaeI (2205) using an oligonucleotide adaptor (5'GGATCTTTGGAGGCT) that restores both the reading frame and the alignment between the two sequences.

### Transfections and Cell Culture

HEK 293 cells (Graham et al., 1977) were maintained in DME, (GIBCO BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 10% FBS under 5%  $\text{CO}_2$  at 37°C. HEK cells were plated in culture dishes ( $1 \times 10^5/100$ -mm dish or  $\sim 2 \times 10^5/35$ -mm dish, respectively) 12–16 h before transfection and grown in normal DME media supplemented with 100 U of penicillin (GIBCO BRL) per ml and 100 mg of streptomycin (GIBCO BRL) per ml. Transfection was carried out by adding 8 mg per 100 mm or 2 mg per 35-mm culture dish plasmid DNA as a calcium phosphate precipitate (Graham and van der Eb, 1973). After 24 h, cells were routinely washed once with PBS and incubated in normal growth medium for 24–30 h.

### [ $^{35}\text{S}$ ]Sulfate Efflux Measurements

Transfected HEK cells were preloaded for 90 min at 37°C with a buffer containing 140 mM  $\text{K}^+$ , 1 mM  $\text{Mg}^{2+}$ , 2 mM  $\text{PO}_4^{3-}$ , 1 mM  $\text{Ca}^{2+}$ , 92 mM  $\text{SO}_4^{2-}$ , 2 mM gluconate, 10 mM sucrose, 23 mM NMG, 10 mM Hepes/KOH, pH 6.3 (LB[ $\text{SO}_4/6.3$ ]) containing 4  $\mu\text{Ci}$  [ $^{35}\text{S}$ ] $\text{SO}_4^{2-}$  and 10  $\mu\text{M}$  valinomycin added from a 10 mM stock in ethanol. After this incubation time, the cells were scraped, collected, and washed twice with ice cold tracer-free loading buffer. Efflux studies were routinely measured in a disc filter holder (Millipore Continental Water Systems, Bedford, MA) equipped with a modified lid which increases the inside volume of the device to 5 ml and therefore allows the solution to be mixed constantly with a stir bar. In a typical experiment, a 0.2-ml aliquot of the cell suspension was transferred into this filter holder containing a 0.6  $\mu\text{m}$  nitrocellulose filter (type HA; Millipore Continental Water Systems) and diluted 20 times with TB[ $\text{Cl}/7.0$ ] containing 140 mM  $\text{K}^+$ , 1 mM  $\text{Mg}^{2+}$ , 2 mM  $\text{PO}_4^{3-}$ , 1 mM  $\text{Ca}^{2+}$ , 92 mM  $\text{Cl}^-$ , 48 mM gluconate, 10 mM sucrose, 10  $\mu\text{M}$  Hepes/KOH, pH 7.0. This buffer was supplemented with 10  $\mu\text{M}$  valinomycin and pre-equilibrated to 25°C. In control studies, the transport buffer contained either 200  $\mu\text{M}$  DIDS or was replaced with a buffer containing, instead of  $\text{Cl}^-$ , equimolar amounts of gluconate (TB[Glu/7.0]). Fractions (80  $\mu\text{l}$ ) were collected at 30-s intervals through the outlet of the filter holder using a fraction collector. Radioactivity in the sample was determined by scintillation counting. Transport was terminated after 6 min by sucking the remaining suspension through the filter. After washing the filter once with 2 ml cold LB[ $\text{SO}_4/6.3$ ], filter-associated radioactivity was counted. In addition, for each experiment, a time-zero value representing the total amount of preloaded tracer molecules per mg cell protein was determined by filtering an equal aliquot of the ice cold cell suspension through a 0.6  $\mu\text{m}$  filter and counting filter associated radioactivity. Data in Fig. 1 a. are expressed as cell-associated cpm by subtracting the total amount of cpm of the collected fractions from the corresponding time-zero blank.

### Preparation of Crude Microsomes

Transfected HEK cells were harvested from eight 100-mm dishes using a rubber policeman and collected by a low speed centrifugation at 1,500 g for 5 min. After two washes with ice cold PBS cells were resuspended in 1.8 ml of hypotonic lysis buffer (10 mM Tris/HCl, pH 8.0, and 2 mM EDTA) supplemented with 0.1 mM TLCK, 0.1 mM TPCK, and 1 mM PMSF (added from a 200 $\times$  stock in ethanol). The cells were allowed to swell for 15 min on ice and were lysed with 15–20 strokes of an all-glass tightly fitting Dounce homogenizer. Immediately after lysis, 0.2 ml of an ice-cold buffer (300 mM  $\text{Na}^+$ , 20 mM  $\text{Mg}^{2+}$ , 320 mM  $\text{Cl}^-$ , 10 mM Tris/HCl, pH 8.0) was added and the nuclei and whole cells were removed by centrifugation at 1,000 g for 1 min. The supernatant was recentrifuged at 1,500 g for 5 min. Crude microsomes (CM) were collected by sedimentation at 100,000 g for 60 min. (50Ti-Rotor; Beckman Instruments, Inc., Palo Alto, CA). Finally, the resulting pellets containing 400–500  $\mu\text{g}$  of protein were resuspended in 100–200  $\mu\text{l}$  of loading buffer (LB[ $\text{Cl}/7.0$ ): 140 mM  $\text{K}^+$ , 1 mM  $\text{Mg}^{2+}$ , 2 mM  $\text{PO}_4^{3-}$ , 1 mM  $\text{Ca}^{2+}$ , 70 mM  $\text{Cl}^-$ , 72 mM gluconate, 10 mM sucrose, 10 mM Hepes/KOH, pH 7.0) and frozen in liquid nitrogen before use in transport studies within 4 wk.

1. *Abbreviations used in this paper:* CM, crude microsomes; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; ECL, enhanced chemiluminescence; endo H, endoglycosidase H; HEK, human embryonic kidney; M-6-P, mannose-6-phosphate; PLP, pyridoxyl 5'-phosphate; PM, plasma membrane.

## Vesicle Transport Measurement

$^{35}\text{SO}_4^{2-}$  uptake measurements into CM preparations were determined by a rapid filtration technique as described in detail elsewhere (Ruetz et al., 1987). Briefly, CM prepared from transfected cells were quickly thawed in a 37°C water bath and diluted with loading buffer to a protein concentration of 6–9 mg/ml. The CM were revesiculated by 20 passes through a 25-gauge needle and treated with valinomycin (10  $\mu\text{M}$ ) to collapse membrane potential differences. Transport was initiated by diluting an aliquot of 10  $\mu\text{l}$  of the vesicle suspension in 190- $\mu\text{l}$  transport buffer (TB[ $\text{SO}_4/7.0$ ]; 140 mM  $\text{K}^+$ , 1 mM  $\text{Mg}^{2+}$ , 2 mM  $\text{PO}_4^{3-}$ , 1 mM  $\text{Ca}^{2+}$ , 70 mM  $\text{SO}_4^{2-}$ , 23 mM NMG, 25 mM gluconate, 10 mM sucrose, 10 mM Hepes/KOH, pH 7.0) supplemented with 6  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]sulfate. For inhibition studies, this buffer contained 500  $\mu\text{M}$  pyridoxal 5'-phosphate (PLP). At the indicated times, uptake was terminated by addition of 2 ml of an ice-cold stop solution (294 mM sucrose, 10 mM Tris/HCL, pH 7.4, and 5 mM phenylglyoxal). Vesicle-associated ligand was separated from free ligand by immediate filtration through a 0.45- $\mu\text{m}$  nitrocellulose filter (type HA; Millipore Continental Water Systems) preequilibrated in deionized water. After an additional wash with 2 ml of stop solution the filters containing the tracer-loaded vesicles were solubilized in 5 ml of Cytosol and radioactivity was determined by liquid scintillation counting.

## Metabolic Labeling, Immunoprecipitation, and Endoglycosidase Digestion

HEK cells in 60-mm culture dishes, 48 h after transfection, were washed, starved for 30 min in methionine-free DME before metabolic labeling for 15 or 30 min in the same medium containing 50–100  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]Met. Cells were rapidly rinsed and chased in DME with 15 mM methionine supplemented with 10% FCS. After designated chase times cells were washed in PBS, solubilized for 1 h at 0°C in immunoprecipitation buffer (150 mM  $\text{Na}^+$ , 150 mM  $\text{Cl}^-$ , 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 2 mg/ml BSA, and 10 mM Tris/HCL, pH 7.5) supplemented with 0.1 mM TPCK, 0.1 mM TLCK, and 1 mM PMSF. Lysates were precleared by incubation for 1 h at room temperature with pre-immune serum (1:100) and protein A Sepharose followed by centrifugation at 14,000 g at 4°C for 5 min. The supernatant, containing detergent-soluble protein was incubated overnight at 4°C with a 1:100 dilution of anti-AE serum 5297 (Thomas et al., 1989) (which recognizes the COOH terminus of AE2) (Lindsey et al., 1990) together with protein A Sepharose. Immune complexes were recovered by centrifugation and washed once with IPB; once with IPB lacking BSA; once with high salt buffer (150 mM  $\text{Na}^+$ , 150 mM  $\text{Cl}^-$ , 0.1% NP-40, 1 mM EDTA, 10 mM Tris/HCL, pH 7.5); and twice with low salt buffer (2 mM EDTA, 10 mM Tris/HCL, pH 7.5). Immunocomplexes were dissociated by heating for 2 min at 70°C in 10 mM Tris/HCL, pH 6.8, containing 0.2% SDS. The released proteins were incubated for 12–14 h at 37°C with 1 U endoglycosidase H, then heated for 2 min at 70°C in SDS-PAGE sample buffer (Laemmli, 1970). Samples were resolved by SDS PAGE and visualized by fluorography after enhancing the signal with Fluoro-Hance.

## Immunofluorescence Microscopy

Cells, 48 h after transfection on glass coverslips, were washed in PBS fixed in 4% paraformaldehyde or Omnifix II (An-Con Genetics) for 15–30 min at room temperature. Cells were washed in PBS, blocked in PBS containing 5% FBS (PBS/FBS), and incubated with primary antibody 5297 diluted 1:500, together with monoclonal docking protein antibody (generous gift of Dr. David Meyer, University of California, Los Angeles, CA) in PBS/FBS for 1 h at 25°C or 16 h at 4°C. After removing the primary antibody and washing the coverslips in PBS, the cells were incubated for 30–60 min at room temperature with secondary FITC-conjugated goat anti-rabbit IgG (see Fig. 3) together with rhodamine-conjugated goat anti-mouse IgG (see Fig. 4), followed by washing in PBS, distilled water. Nuclei were stained by a 30-s exposure at room temperature to 0.0002% bisbenzamide. Photomicrographs (see Fig. 3) were obtained with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) and photographed with Ektachrome P800 film (Eastman Kodak Co., Rochester, NY). Confocal images (see Fig. 4) were obtained using a MRC600 microscope (BioRad Laboratories) equipped with a krypton/argon laser and a 63 $\times$  1.4 NA objective (Carl Zeiss). Images of anion exchanger (FITC) staining were acquired using a 488-nm excitation. Rhodamine fluorescence was observed using 522-nm excitation. FITC and rhodamine images were acquired consecutively under conditions ensuring complete exclusion of "spill-over" between channels. Images shown in Figs. 3 and 4 represent the average of five consecutive acquisition frames using a Kalman averaging algorithm.

## Con A Chromatography

Crude microsomes were prepared as described above, resuspended in 1.5 ml cold LB[Cl/7.0] buffer supplemented with 2 mM  $\text{MnCl}_2$  (con A buffer) and applied to 2 ml of Affi-Gel con A beads preequilibrated in a 5-ml column with 5–10 bed volumes of cold con A buffer. After incubation at 4°C during 30 min under constant shaking, unbound material was collected by gravity flow, and the column washed three times with 5 ml con A buffer. Specifically bound material was eluted by incubation for 30 min with 1.5 ml con A buffer containing 300 mM  $\alpha$ -methyl mannoside under constant shaking. The eluate was collected and aliquots of the different fractions were directly used for marker enzyme determinations and for SDS-PAGE analysis. For functional transport studies, the fractions were diluted three to four times with cold LB[Cl/7.0] and the vesicles collected by high speed centrifugation as described above. The resulting pellets were resuspended in 50–200  $\mu\text{l}$  cold LB[Cl/7.0] and kept frozen in liquid nitrogen until used.

## Immunoblotting and Cell Surface Labeling

Cells were harvested by scraping with a rubber policeman washed in PBS before lysing in SDS-PAGE sample buffer (Laemmli, 1970) or harvesting CM. Proteins were resolved on 7% SDS-PAGE gels (Laemmli, 1970) and transferred to nitrocellulose as described (Towbin et al., 1979). Protein bands were revealed by enhanced chemiluminescence (ECL; Amersham Corp.). Surface proteins of HEK cells were labeled by incubation for 3 min at 25°C with 40 pmol sulfo-NHS-Biotin (Pierce Chemical Co., Piscataway, NJ) in PBS. Unbound label was removed by washing in ice-cold PBS before cell harvesting and microsome preparation and con A chromatography as described above. Aliquots of the indicated membrane fractions were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose. Surface biotinylated proteins were revealed by developing the blots with HRP-conjugated streptavidin. Total protein was revealed by staining an identical blot with a biotin protein detection kit (BioRad Laboratories) following the manufacturer's directions.

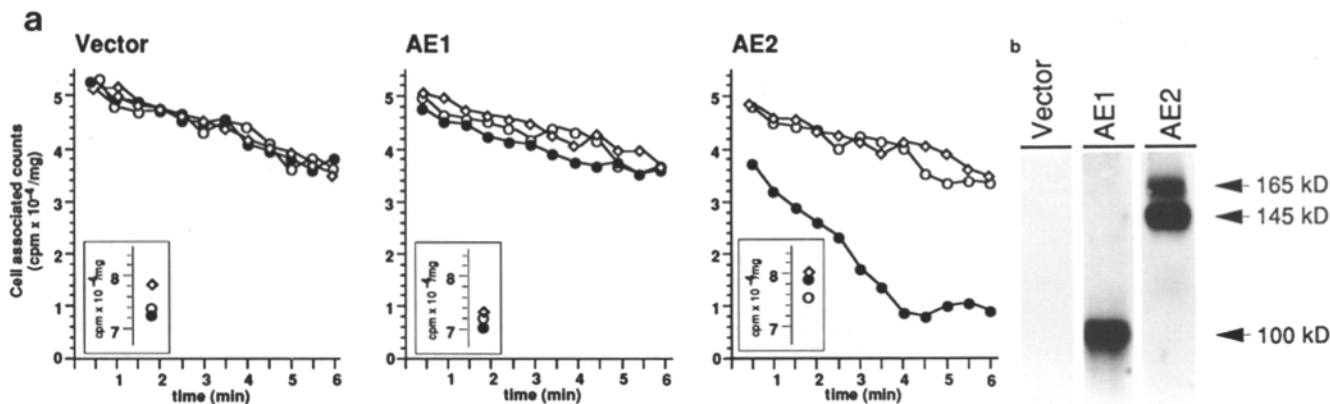
## Enzyme Assays

Enzyme assays were performed under optimal conditions of pH and temperature, and at a protein concentration where the enzyme reaction was in the linear range. 5'-nucleotidase was assayed by the method of Michell et al. (1965) and glucose-6-phosphatase according to De Duve et al. (1955). Inorganic phosphate was determined colorimetrically according to a protocol of Cooper et al. (1977). Protein concentrations were measured by the Bradford procedure (Bradford, 1976) using the BioRad Protein Assay Kit and bovine gamma globulin as the reference standard.

## Results

### Expression of AE2 but Not AE1 Leads to Enhanced Whole-cell [ $^{35}\text{S}$ ]Sulfate Efflux

To evaluate surface expression of functional anion exchangers in transfected cells we measured tracer  $^{35}\text{SO}_4^{2-}$  efflux from bulk populations of AE1 and AE2 transfected HEK cells. The cells were loaded with  $^{35}\text{SO}_4^{2-}$  and  $\text{Cl}^-/\text{SO}_4^{2-}$  exchange was initiated by diluting the suspensions into a  $\text{Cl}^-$  containing medium in the presence of valinomycin to clamp differences in membrane potential (Fig. 1 a). Cells transfected with the vector alone exhibited a slow  $^{35}\text{SO}_4^{2-}$  leak of approximately 3% per min which was unaffected by the presence of the anion exchange inhibitor DIDS (200  $\mu\text{M}$ ) or by the replacement of extracellular  $\text{Cl}^-$  with the impermeant anion, gluconate. This leak was not a consequence of the transfection procedure, since similar efflux rates were observed in untransfected or vector transfected HEK cells (not shown). These findings are consistent with our previous observation that HEK cells express negligible levels of endogenous plasma membrane anion exchange activity (Lee et al., 1991). AE2 transfectants displayed significantly enhanced  $^{35}\text{SO}_4^{2-}$  efflux which was es-



**Figure 1.** Chloride driven  $^{35}\text{SO}_4^{2-}$  efflux from transfected HEK cells. (a) Vector, AE1, and AE2 transfected HEK cells were preloaded with  $\text{LB}[\text{SO}_4/6.3]$  containing  $4 \mu\text{Ci } ^{35}\text{SO}_4^{2-}$  and treated with valinomycin (see Materials and Methods). Efflux was initiated by incubating the cells with  $\text{Cl}^-$ -containing medium ( $\text{TB}[\text{Cl}/7.0]$ ) in the absence (J) or presence (E) of  $200 \mu\text{M}$  DIDS or in  $\text{Cl}^-$ -free medium ( $\text{TB}[\text{Glu}/7.0]$ ) (A). Insets represent the time-zero values for each flux curve. Data are representative of three independent experiments. (b) Immunoblot of transfected cell extract probed with anti-AE antibody.

sentially completed within 4 min. This enhanced efflux was blocked by  $200 \mu\text{M}$  DIDS and by replacement of extracellular  $\text{Cl}^-$  with gluconate. Since AE and vector transfected cells were all initially loaded with  $^{35}\text{SO}_4^{2-}$  to the same extent, represented by the time-zero values (Fig. 1 a, insets), we conclude that the enhanced efflux in populations of AE2 transfectants is due to the presence at the plasma membrane of functional anion exchangers.

In sharp contrast, expression of AE1 in HEK cells never led to a detectable increase in  $^{35}\text{SO}_4^{2-}$  efflux over controls. This difference between the anion exchange capacity of AE1 and AE2 transfected HEK cells could not be attributed to deficient expression of AE1 by HEK cells, since AE1 and AE2 transfectants accumulated comparable steady-state levels of immunodetectable protein (Fig. 1 b). A single  $\sim 100$ -kD electrophoretic species was detected by immunoblotting of extracts from AE1 transfected cells, corresponding to the size of the mature protein in erythrocyte membranes (Steck, 1978). A 145/165 kD doublet, corresponding, respectively, to the core glycosylated and mature forms of the protein (see below), was routinely observed in the AE2 transfectants. These data suggested, therefore, that our AE1 cDNA clone encodes a polypeptide that is defective either in catalyzing anion exchange or in targeting to the plasma membrane.

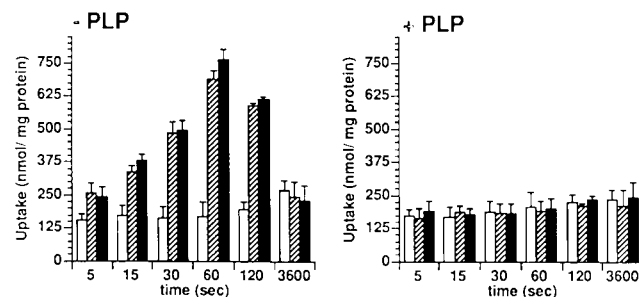
#### Anion Exchange Activity in Crude Microsomes from AE1 and AE2 Transfected HEK Cells

To discriminate between these possibilities, we measured anion exchange as a function of  $^{35}\text{SO}_4^{2-}$  uptake into  $\text{Cl}^-$ -loaded crude microsomal vesicles prepared from transfected HEK cells (Fig. 2). Crude microsomes from AE1 and AE2 transfected cells accumulated tracer  $^{35}\text{SO}_4^{2-}$  to a maximum value greater than three times equilibrium within 60 s. Tracer accumulation was dependent on the presence of an outward gradient of transportable anion (not shown) and decreased to background levels as the counter-anion gradient dissipated. No such uptake was observed in crude microsomes from vector transfectants. The gradient-driven  $^{35}\text{SO}_4^{2-}$  uptakes observed with AE1 and AE2 were similar in magnitude and, in both cases, were completely inhibited by the addition

of the anion exchange inhibitor pyridoxal 5'-phosphate (Cabantchik et al., 1975). In a separate study (S. Ruetz and R. Kopito, manuscript in preparation) we have demonstrated that both AE1 and AE2, when assayed in crude microsomes, share extensive functional similarities, including cation independence, stilbene sensitivity and a strong dependence on preloaded transportable anions. These data indicate that both AE1 and AE2 cDNA clones encode functional proteins which, when assayed in crude microsomes from transiently transfected HEK cells, function as anion exchangers with comparable relative activities. The discrepancy between AE1 and AE2 activity when measured in intact cells must therefore reflect differences in the targeting, stability or regulation of the two proteins at the plasma membrane.

#### Intracellular Processing of AE1 and AE2 in Transfected HEK Cells

Laser scanning confocal microscopy was used to evaluate the expression and intracellular distribution of AE1 and AE2 an-



**Figure 2.** Gradient driven  $\text{Cl}^-/\text{SO}_4^{2-}$  exchange in crude microsomes. Crude microsomes from vector ( $\square$ ), AE1 ( $\blacksquare$ ), and AE2 ( $\square$ ) transfected HEK cells were preloaded with  $\text{LB}[\text{Cl}]$  containing  $70 \text{ mM}$   $\text{Cl}^-$ . Tracer  $^{35}\text{SO}_4^{2-}$  uptake was initiated by diluting the vesicle suspension in a 20-fold excess of  $\text{TB}[\text{SO}_4/6.3]$  containing  $6 \mu\text{Ci/ml}$   $^{35}\text{SO}_4^{2-}$ . (Right) Identical experiment in the presence of  $500 \mu\text{M}$  pyridoxal 5'-phosphate. Data are the mean  $\pm$  SD of triplicate determinations from three separate CM preparations.

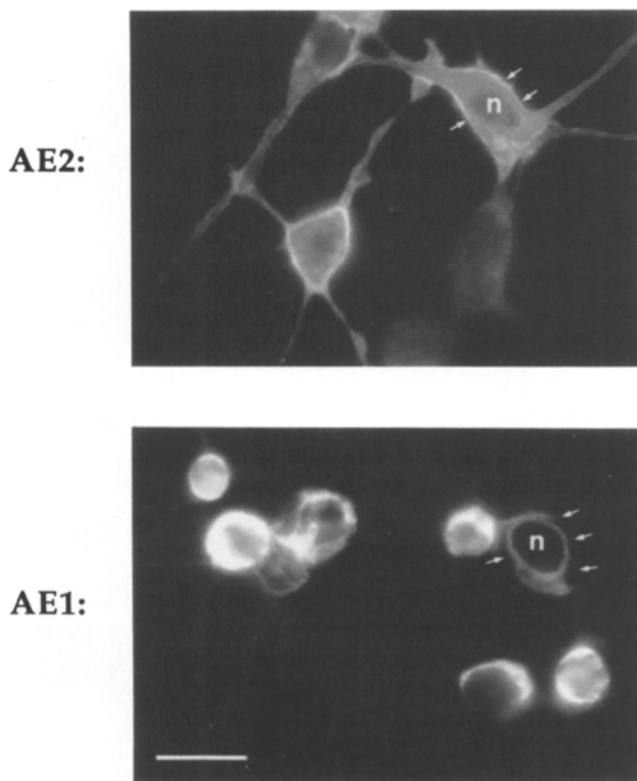


Figure 3. Immunofluorescence staining of AE-transfected HEK cells. Arrows denote cell periphery; n, nucleus. Bar, 20  $\mu$ m.

ion exchangers expressed in HEK cells (Figs. 3 and 4). Antipeptide antibody 5297, against the extreme COOH terminus of AE1, reacted strongly with both AE1 and AE2 in fixed transfected HEK cells; immunoreactivity in both cases was blocked by the presence of excess immunogen peptide (not shown). Immunostaining of AE2 transfected cells was most intense at the cell periphery surrounding both the "body" of the cell (Fig. 3, *arrows*), and along the fine processes that normally elaborate from HEK cells (Fig. 4 *A*, *arrowheads*). Intracellular staining was also observed, most prominently in a reticular pattern, often surrounding the nucleus. Nuclei were identified by simultaneously staining with Hoechst dye and observing the cells under the appropriate optics (not shown). In marked contrast, AE1 transfected cells differed in both overall morphology and distribution of AE1 immunoreactivity (Fig. 3). AE1-expressing cells were typically rounded in appearance, lacked processes and detached readily from the coverglass (Figs. 3 *A* and 4 *C*). Intense AE1 immunoreactivity was observed in a perinuclear, and occasionally juxtannuclear, reticular pattern (Fig. 4 *C*, *asterisk*). Double immunofluorescence was used to assess the distribution of anion exchangers in transfected cells. A strong correlation was observed between the intracellular staining pattern observed for both AE1 and AE2 transfectants with anion exchanger antibody (Fig. 4, *A* and *C*) and with a mAb against docking protein, a marker for the RER (Fig. 4, *B* and *D*). By contrast, no correlation was observed between the anion exchanger immunofluorescence and staining with antibodies to the Golgi apparatus ( $\beta$ -COP) or lysosomes

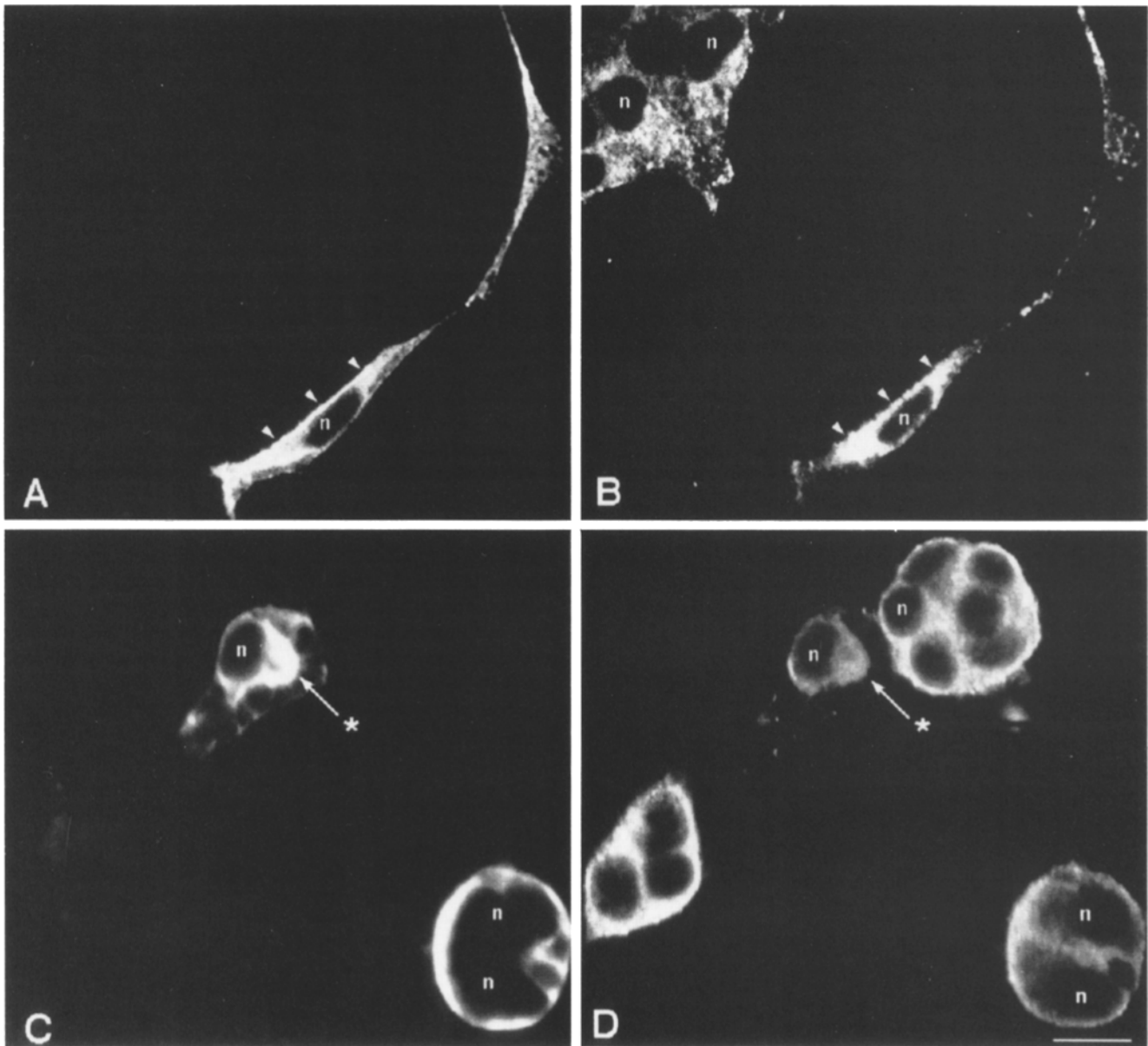
(mannose-phosphate receptor) (data not shown). These results indicated significant differences between the intracellular distributions of AE1 and AE2 in HEK cells. AE2 was detected both at the cell periphery and in an intracellular compartment, probably the ER. The absence of immunodetectable AE1 from the cell surface suggest that AE1 is either poorly targeted to, or unstable at, the plasma membrane.

To further characterize the intracellular processing of transfected anion exchangers, we studied the kinetics of conversion of Asn-linked oligosaccharides from high-mannose to complex-type forms as a measure of transit of the proteins through the secretory pathway. HEK cells expressing AE1 or AE2 were labeled for 30 min with [ $^{35}$ S]Met and chased for various times as indicated (Fig. 5). The AE1 and AE2 polypeptides were then immunoprecipitated with 5297 antibody and subjected to digestion with endo H. The loss of endo H-sensitivity marks the conversion of Asn-linked oligosaccharides to complex forms by a process which occurs in the medial Golgi stacks. Processing of AE2 in transfected HEK cells (Fig. 5) from a 145-kD endo H-sensitive form to a 165-kD endo H-resistant form was detectable by 30 min of chase and was nearly complete by 5 h. AE1 gave rise to a characteristically broad 100-kD band which, by contrast to AE2, was never processed to a slower mobility form and never acquired endo H resistance during the 5-h chase. Both proteins were comparably stable ( $t_{1/2} \sim 3$  h) throughout the chase. We did not observe labeled polypeptides in the 60–80-kD range in immunoprecipitates of AE-transfected cells, suggesting that neither AE1 nor AE2 forms a stable complex with HSP70 proteins such as BiP (Haas and Wabl, 1984).

Taken together, these data indicate that in transfected HEK cells, AE2 is processed to an endo H-resistant compartment where it accumulates at steady state at the plasma membrane. Immunoblots of AE2-transfected HEK cells indicated that, at steady state,  $\sim 60\%$  of the protein was associated with the lower mobility band, corresponding to the endo H-resistant form (see Figs. 1 *b*, 7 *c*). In striking contrast, AE1 never acquired endo H-resistant oligosaccharides and failed to accumulate in a higher molecular weight form, suggesting that the protein is retained in a pre-Golgi compartment. Identical results were obtained with HEK cells expressing an independently isolated cDNA clone encoding the full-length human AE1 protein (Lux et al., 1989), suggesting that the processing defect reported above is an intrinsic property of AE1, not an idiosyncrasy of a particular cDNA clone.

#### ***Surface-exposed Anion Exchangers Can Be Blocked by a Membrane-impermeant Reagent***

To independently assess differences in cell surface expression of functional AE1 and AE2 anion exchangers, we exploited the ability of the membrane-impermeant inhibitor DIDS to irreversibly modify and inactivate surface-exposed anion exchangers. AE1 and AE2 transfected cells were incubated with 2 mM extracellular DIDS, a concentration 1,000 to 10,000 times the  $K_i$  (Funder et al., 1978; Lee et al., 1991), conditions which should result in the irreversible inhibition of all surface-exposed anion exchangers. Cells were washed free of unbound inhibitor and crude microsomes were prepared and assayed for  $\text{Cl}^-/\text{SO}_4^{2-}$  exchange as

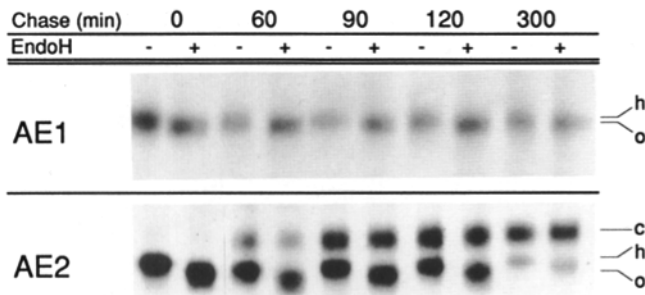


**Figure 4.** Confocal localization of anion exchangers and docking protein in transfected HEK cells. Confocal images of AE2 (*A* and *B*) and AE1 (*C* and *D*) transfected HEK cells. (*A* and *C*) Anion exchanger immunofluorescence. (*B* and *D*) Docking protein immunofluorescence. Bar, 20  $\mu\text{m}$ .

described above. DIDS treatment inhibited <10% of total transport activity in crude microsomes from AE1 transfected cells (Fig. 6), indicating that few AE1 molecules were accessible to the reagent. By contrast, nearly 60% of the total anion exchange activity in crude microsomes from AE2 transfectants was blocked by extracellular DIDS. Anion exchange activity in CM from both AE1 and AE2 was completely blocked when DIDS was present during the microsome preparation (allowing it to react with both the inside and outside of the vesicles), confirming that the conditions used were sufficient to block all surface accessible exchangers. Thus, the difference we observed in intracellular processing of AE1 and AE2 is reflected in the proportion of functional anion exchangers exposed at the cell surface.

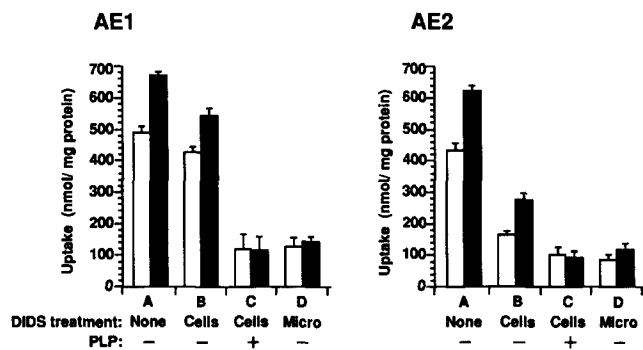
#### ***Fractionation of Crude Microsomes into Plasma Membrane and ER-derived Vesicles***

To further characterize the intracellular compartment to which functional AE1 and AE2 are restricted, we used lectin affinity chromatography to separate plasma membrane (PM) from ER-derived vesicles. PM-derived microsomes typically are "rightside-out", i.e., the oligosaccharide-bearing domains of glycoproteins face the medium and should therefore bind lectin, while ER-microsomes have the opposite orientation (Arion et al., 1976; Bell et al., 1981) and should not bind. CM prepared from AE1 and AE2 transfectants were fractionated by con A Sepharose affinity chromatography into a flow-through fraction and an  $\alpha$ -methyl mannoside

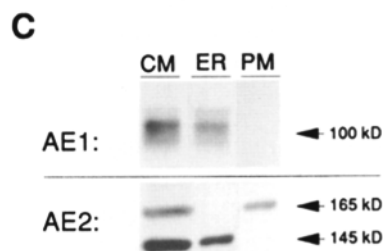
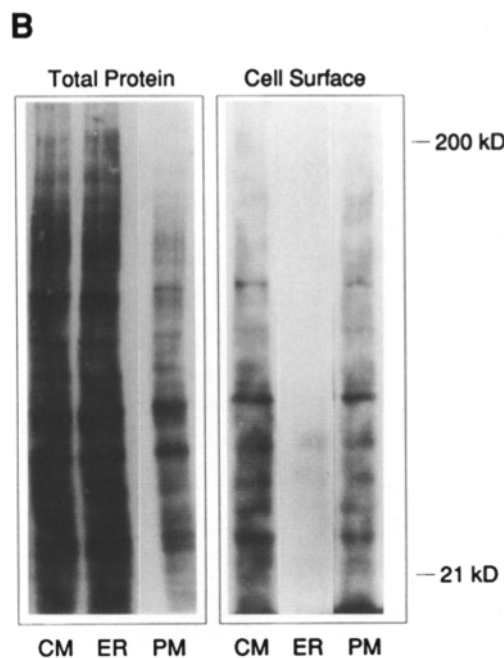
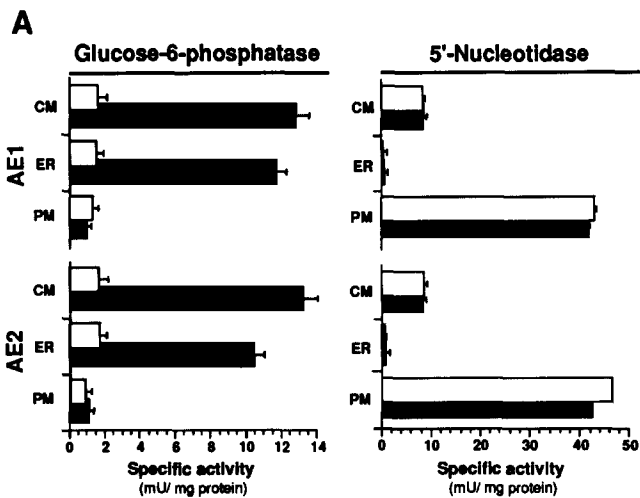


**Figure 5.** Endo H sensitivity of newly synthesized anion exchangers. AE transfected cells were pulse labeled with [<sup>35</sup>S]Met and chased for the indicated times. Immunoprecipitates from solubilized cells were treated (+) with endoglycosidase H (*endo H*) and resolved by 7.5% SDS-PAGE and fluorography. The mobilities of bands corresponding protein containing no (*o*), high mannose (*h*), or complex (*c*) Asn-linked oligosaccharides are indicated.

eluate. The activities of the marker enzymes glucose-6-phosphatase and 5' nucleotidase were used to assess the degree of enrichment of the fractions in ER and PM markers, respectively (Fig. 7 *a*). Identical results were obtained for AE1 and AE2 transfectants, indicating that anion exchanger expression does not influence the fractionation. Most of the glucose-6-phosphatase activity was recovered in the flow-through fraction, indicating a nearly quantitative recovery of ER vesicles from the starting crude microsomes. Moreover, activity of this luminal ER enzyme for the membrane-impermeant substrate, mannose-6-phosphate (M-6-P), was observed only in the presence of detergent, demonstrating that the vesicles are tightly sealed and that the "normal" orientation of the ER is preserved. In contrast, the eluate fraction, which contained <5% of the total glucose-6-phosphatase activity, was highly enriched in the PM marker 5' nucleotidase. Since the activity of this enzyme, which is normally present at the cell surface (Michell and Hawthorne, 1965; Bell et al., 1981), was independent of detergent, we conclude that the flow-through contains PM-derived vesicle in the "normal", i.e., rightside-out orientation.



**Figure 6.** Inhibition of cell surface anion exchangers. Gradient driven Cl<sup>-</sup>/SO<sub>4</sub><sup>2-</sup> exchange was determined in crude microsomes from AE1 (*left*) and AE2 (*right*) transfected HEK cells as described in Fig. 2. Fluxes were determined for 60 (□) and 120 (■) s time points. In *B* and *C*, CM were derived from cells treated with 2 mM DIDS. CM in *D* were prepared in the presence of 2 mM DIDS. Data are the mean ± SD of quadruplicates of three separate experiments.



**Figure 7.** Characterization of subcellular fractions isolated by con A affinity chromatography. (*a*) Specific activity and detergent lability of PM (5' nucleotidase) and ER (glucose-6-phosphatase) marker enzymes in con A separated fractions. Enzyme activities were determined in the absence (□) or presence (■) of 0.05% deoxycholate. (*b*) Blot of surface labeled (*right*) and total (*left*) proteins from surface biotinylated HEK cells. (*c*) Immunoblot of AE1 and AE2 polypeptides in subcellular fractions from transfected cells.



The small amount (<5%) of cross-contamination of enzymatic markers in the PM and ER fractions described above could arise either from incomplete binding of rightside-out vesicles to lectin Sepharose or from contamination with ER and PM vesicles in inverted orientations. Such inverted vesicles may result from vigorous homogenization during the preparation of CM. To discriminate between these possibilities, HEK cells were surface labeled with NHS-biotin before preparation of microsomes (Sargiacomo et al., 1989). Separation of these vesicles by con A chromatography revealed surface-biotinylated polypeptides exclusively in the eluate (PM) fraction (Fig. 7 b). No cell-surface proteins were detected in the ER fraction, despite the fact that approximately equal amounts of total protein were loaded on the gel (Fig. 7 b, left).

These results strongly suggest that all vesicles containing exofacial oligosaccharide bind to the column. Thus, the minor cross-contamination of the enzymatic markers in the column fractions must be due to ER- or plasma membrane-derived vesicles that have assumed an inverted orientation. Taken together, these data demonstrate that crude microsomes derived from transfected HEK cells can be resolved by con A chromatography into two distinct vesicle populations that are enriched in ER and plasma membrane markers. Although some contamination of the ER fraction with Golgi-derived membranes cannot be excluded by these studies, our data demonstrate that both vesicle fractions are tightly sealed and preserve the sidedness of their parental membranes.

Immunoblots of microsomes from transfected HEK cells were used to assess the distribution of anion exchanger polypeptides in the different fractions (Fig. 7 c). The 100-kD band, corresponding to immature (endo H-sensitive) AE1, was detected in immunoblots of CM from AE1-transfected cells. Recovery of this band exclusively in the ER fraction further supports our conclusion that AE1 does not mature to a post-ER compartment. In contrast, AE2 transfectants express two immunoreactive bands, of 145 and 165 kD, corresponding precisely to the endo H-sensitive and -resistant species, respectively, seen in the pulse-chase experiment (Fig. 5). These two bands were quantitatively resolved into ER and PM fractions by con A chromatography (Fig. 7 c), further confirming their identities. We conclude that con A affinity chromatography can be used to efficiently separate CM vesicles from transfected HEK cells into pre-Golgi (ER) and post-Golgi (PM) fractions. Both fractions contain sealed membrane vesicles that are suitable for evaluating anion exchanger activity.

#### The ER Forms of AE1 and AE2 Are Functional Anion Exchangers

To determine when, during the course of AE biogenesis, the proteins acquire the ability to catalyze anion exchange, we evaluated  $\text{Cl}^-/^{35}\text{SO}_4^{2-}$  activity in the individual microsomal sub-fractions (Fig. 8). As expected from the distribution of AE1 polypeptide in these fractions (Fig. 7 c), AE1-mediated anion exchange activity was recovered exclusively in the ER fraction. The small amount of anion exchange activity present in the PM fraction from AE1 transfectants is similar to the background flux (CM vesicles in the presence of PLP), consistent with the absence of immunodetectable AE1 in the PM fraction (Fig. 7 c). By contrast, significant anion ex-

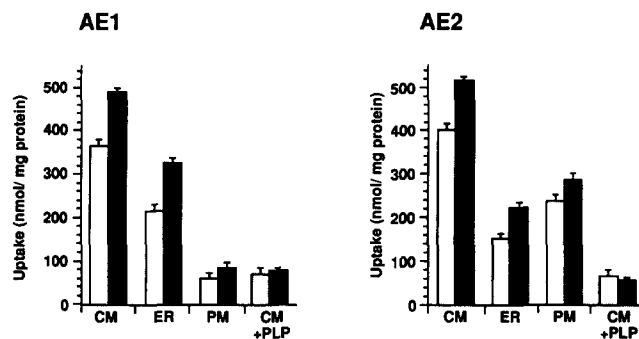


Figure 8. Anion exchange activity in subcellular fractions from AE1 and AE2 transfected HEK cells. Gradient driven  $\text{Cl}^-/\text{SO}_4^{2-}$  exchange was determined at 60 (□) and 120 (■) s time points on subcellular fractions as described in the legend to Fig. 2. Data are the mean  $\pm$  SD of quadruplicates from three independent experiments.

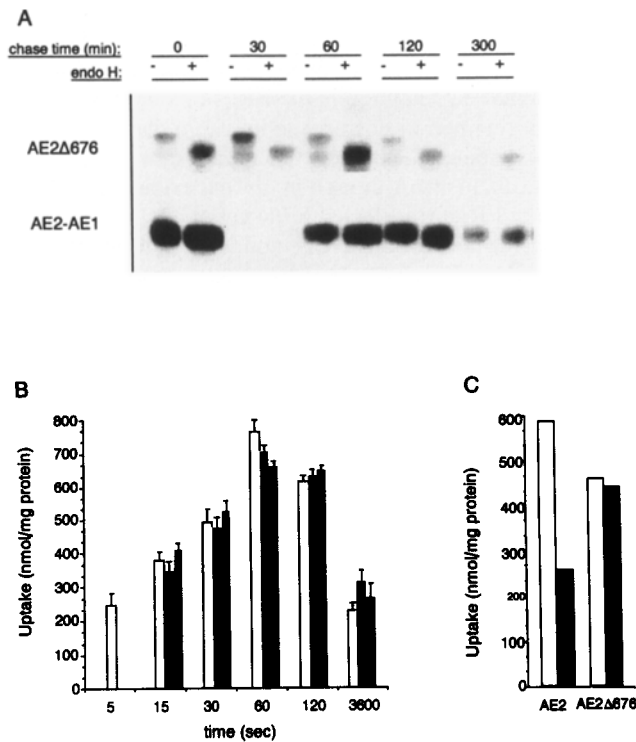
change activity in AE2 transfectants was recovered in both ER and PM fractions. Further, the proportion of activity in the ER (40%) and PM (60%) fractions correlated well with the distribution of the 145- and 165-kD forms of the protein in the ER and PM fractions, respectively (Fig. 7 c). Since we can recover nearly all of the anion exchange activity of crude microsomes in the two con A fractions, we conclude that both AE1 and AE2 are functional as anion exchangers in their immature, high-mannose ER forms.

#### The NH<sub>2</sub>-terminal Domain of AE2 Is Necessary, but Not Sufficient, for Exit from the ER

The above data show that, despite the high overall sequence and structural similarity between AE1 and AE2, and the fact that both proteins are present at the surface of cells where they are endogenously expressed, the two proteins undergo very different intracellular fates when expressed transiently in HEK cells. Unlike AE2, which matures to the cell surface of HEK cells, AE1 is retained, in a catalytically functional state, in the endoplasmic reticulum. Since the cytoplasmic NH<sub>2</sub>-terminal domain of AE1 is known to associate with several tissue-specific cytoplasmic and cytoskeletal proteins and is the site of greatest sequence divergence with AE2 (Kopito, 1990), we constructed a mutant (AE2Δ676) lacking the NH<sub>2</sub>-terminal 676 amino acid residues that constitute the cytoplasmic domain. Like AE1, this mutant protein accumulated in an intracellular perinuclear compartment in transfected HEK and COS cells and resulted in similar alterations in cell morphology (data not shown). Pulse-chase labeling (Fig. 9 a) revealed that, like AE1, the AE2 mutant was not processed to an endo H-resistant lower mobility form. Although this truncated AE2 mutant retained the ability to mediate anion exchange when measured in crude microsomal vesicles (Fig. 9 b), no significant fraction of this activity was accessible to extracellular DIDS treatment (Fig. 9 c), arguing that, like AE1, AE2Δ676 is retained in a functional state within the ER. These data suggested that the NH<sub>2</sub>-terminal cytoplasmic domain of AE2 is required to permit its exit from the ER.

To test whether the NH<sub>2</sub>-terminal cytoplasmic domain of AE2 allows export from the ER via interaction with a puta-





**Figure 9.** Functional expression of mutant anion exchanger in HEK cells. (A) Endo H sensitivity of newly synthesized anion exchanger mutants as described in legend to Fig. 5. (B) Anion exchange activity in CM from HEK cells transfected with wild-type AE2 (□), AE2-AE1 (▨), and AE2Δ676 (■). (C) Effect of extracellular DIDS treatment on gradient-driven  $^{35}\text{SO}_4^{2-}$  uptake. Fluxes were determined as described in the legend to Fig. 6, at 60 s timepoints on CM prepared from untreated (□) cells or cells exposed to 2 mM DIDS (■) for 1 h at 37°C.

tive cytoplasmic factor, we constructed a chimeric anion exchanger (AE2-AE1) in which the 678 amino acid  $\text{NH}_2$ -terminal domain of AE2 was fused to the membrane-associated COOH-terminal domain of AE1. The site of fusion between these proteins was chosen to be within a highly conserved sequence 19 amino acids upstream of the predicted first transmembrane spanning segment, in order to maximize conformational similarity between the chimera and the two parents. Expression of the AE2-AE1 chimera in HEK cells resulted in the synthesis of a 135-kD polypeptide that was immunoprecipitated with antibody 5297 (Fig. 9 a). The mobility of this polypeptide corresponds well to the predicted molecular weight of the chimera. The smaller increase in mobility of AE2-AE1 compared to AE2 following treatment with endo H is consistent with the fact that AE2 has two additional sites of N-linked glycosylation compared to the single site present in AE1. Significantly, like AE1 and AE2Δ676, the AE2-AE1 chimera failed to acquire resistance to endo H during the course of the 5-h chase, suggesting that the chimeric polypeptide also failed to be exported from the ER. This chimeric protein also gave rise to a catalytically active anion exchanger when assayed in crude microsomes from transfected cells (Fig. 9 b). We conclude that the  $\text{NH}_2$ -terminal domain of AE2, although necessary for intracellular transport of AE2, is not sufficient to “rescue” the

corresponding and homologous domain of AE1 from retention in the ER. Differences in the intracellular processing of AE1 and AE2 cannot, therefore, arise simply from differences in the  $\text{NH}_2$ -terminal domains of these two homologous proteins.

## Discussion

In this paper we have shown that two highly homologous anion exchangers, AE1 and AE2, are processed quite differently by the secretory pathway of HEK cells. Five lines of evidence support the conclusion that a significant fraction of AE2 protein is targeted to the HEK cell plasma membrane where it catalyzes anion exchange. First, expression of AE2 leads to significantly increased whole cell anion exchange as measured by  $^{35}\text{SO}_4^{2-}$  efflux (Fig. 1). Second, immunofluorescent staining of AE2 transfected cells reveals extensive localization of the protein to the cell periphery (Figs. 3 and 4). Third, pulse-chase studies indicate that AE2 is posttranslationally processed to a form bearing complex-type Asn-linked oligosaccharides (Fig. 5). Fourth, ~60% of the total functional anion exchangers in AE2 transfectants are accessible to extracellular treatment with the membrane-impermeant inhibitor DIDS (Fig. 6). Fifth, a similar fraction (60%) of total microsomal immunoreactive AE2 protein (Fig. 7 c) and activity (Fig. 8) was recovered in a plasma membrane fraction isolated by lectin affinity chromatography.

In marked contrast to AE2, our data show that the vast majority of AE1 synthesized in HEK cells fails to mature to the cell surface. We observed no significant enhancement of whole-cell anion flux in the AE1 transfectants, despite the fact that transfected HEK cells accumulated comparable steady-state levels of AE1 and AE2. The observation of similar levels of anion exchange activity in crude microsome fractions from both AE1 and AE2 transfected cells (Fig. 2) demonstrates that both clones encode functional proteins. Therefore the difference between AE1 and AE2 in the whole cell experiments reflects a relative deficiency in the cell-surface expression of AE1. This conclusion is supported by the absence of plasma membrane immunofluorescence staining of AE1 transfectants (Fig. 3) and colocalization of AE1 immunofluorescence with that of a marker for the rough ER (Fig. 4), by the failure of extracellular DIDS to significantly diminish total microsomal anion exchange activity and by the near-quantitative recovery of AE1 protein (Fig. 7 c) and activity (Fig. 8) in a highly enriched ER fraction. We observe an identical pattern of subcellular localization, and similar kinetics of oligosaccharide processing of both AE1 and AE2 expressed in COS cells (data not shown), suggesting that our data reflect an intrinsic difference between these two anion exchangers and not an idiosyncrasy of HEK cells. We conclude that AE1 and AE2 are processed differently through the secretory pathway in HEK cells and that unlike AE2, AE1 is retained in an enzymatically active state in a pre-Golgi compartment, most probably the ER.

The AE2 gene is expressed endogenously in several different epithelial and non-epithelial cells (Kopito, 1990; Alper, 1991), but the AE2 polypeptide has been identified only in the choroid plexus epithelium, where it is localized to the basolateral plasma membrane (Kopito et al., 1989). In the

present study we show that AE2 expressed in HEK cells acquires complex-type (endo H-resistant) oligosaccharides and is processed to the plasma membrane in an electrophoretic form (165 kD) indistinguishable from that observed endogenously in the choroid plexus (Lindsey et al., 1990). This observation implies that HEK cells possess all of the components necessary for the correct biosynthesis, posttranslational processing and plasma membrane targeting of AE2. However, the presence of a significant fraction (40–60%) of the total steady-state AE2 in an endo H-resistant compartment in transfected HEK cells suggests that exit from the ER is rate-limiting for the processing of AE2 in these cells, a conclusion supported by the double immunofluorescence staining with the ER marker.

The AE1 gene is expressed in a more limited number of cell types than is AE2 (Kopito, 1990). Like AE2 however, the AE1 polypeptide is found in the plasma membranes of cells where it is endogenously expressed. Erythroid precursor cells from anemic mouse spleen actively synthesize high levels of AE1 which is cotranslationally inserted into the ER membrane and glycosylated at a single Asn-linked site (Braell and Lodish, 1981). The polypeptide is processed to an endo H-resistant form and becomes sensitive to extracellular protease 30–45 min after synthesis (Braell and Lodish, 1981). Thus, in a cell type that endogenously expresses erythrocyte proteins, AE1 matures through the Golgi apparatus and is targeted to the plasma membrane with kinetics comparable to those observed for AE2 in HEK cells. By contrast to AE2, AE1 expressed in HEK cells fails to acquire endo H-resistant N-linked oligosaccharides and appears to be quantitatively retained in the ER. Thus, although both AE1 and AE2 are normally residents of the plasma membrane in the cells where they are endogenously expressed, they are targeted quite differently by the HEK cell secretory pathway.

Membrane traffic through the secretory pathway is thought to proceed via a “bulk flow” process involving sequential fission and fusion of vesicular intermediates with the major compartments (Rothman and Orci, 1992). According to this hypothesis, integral membrane and secretory proteins are transported by “default” to the plasma membrane unless they possess specific signals for retention in or cycling between the compartments of the pathway. Two classes of proteins are retained in the ER, suggesting the existence of two quite different types of retention signals. Soluble “resident” components of the ER lumen contain a specific COOH-terminal tetrapeptide signal that is both necessary and sufficient for their retention (Munro and Pelham, 1987). Sequences involved in retaining integral membrane protein residents of the ER appear to be more diverse, perhaps reflecting their greater diversity in topology and quaternary structure. Mutational analysis indicates that ER retention signals for some type I membrane proteins are present within their cytoplasmic COOH-terminal tails (Shin et al., 1991; Gabathuler and Kvist, 1990). None of these features has been shown to participate in the ER retention of polytopic integral membrane proteins such as anion exchangers; we can identify no apparent similarities or homologies between AE1 or AE2 and known ER residents.

The ER is the site of entry of membrane proteins into the secretory pathway; it is also the organelle in which they acquire secondary, tertiary and often quaternary structure. Exit

from the ER can be viewed as a “quality control” point (reviewed in Hurtley and Helenius, 1989); protein folding (or misfolding), therefore, can be regarded as a second type of specific signal for retention in the ER. Misfolded proteins are generally retained in this organelle where they are often found tightly associated with BiP and are degraded by a non-lysosomal mechanism. Although in our experiments AE1 is retained in the ER of transfected HEK cells, it is no less stable than plasma membrane AE2, and does not appear to form a stable complex with BiP or other members of the HSP70 family. It is also unlikely that AE1 retention is the consequence of a mutation in our AE1 clone, since we observed the same ER-localized phenotype in cells transfected with a different AE1 cDNA clone independently isolated from a different species. Finally, the demonstration that HEK cell AE1 is enzymatically active argues that its retention in the ER cannot be a consequence of gross misfolding.

Protein oligomerization also frequently occurs in the ER and is usually a prerequisite for export into the distal compartments of the secretory pathway (Hurtley and Helenius, 1989). Like misfolded proteins, unassembled subunits of oligomeric proteins accumulate in the ER. Unlike misfolded proteins, however, these subunits are frequently not degraded and fail to form stable associations with BiP. In most cases, the subunits required for export of integral membrane proteins from the ER are stoichiometrically present in stable complexes of the mature protein in its native membrane. On the other hand, the *ninA* gene product, which is required for correct biosynthesis and maturation of the Rh1 and Rh2 rhodopsins through the secretory pathway of *Drosophila* photoreceptor cells, does not appear to be associated with the mature protein in rhabdomere membranes (Colley et al., 1991). This gene, which encodes an ER membrane protein homologous to a family of peptidyl-prolyl *cis-trans* isomerases, is thought to transiently associate with a specific subset of *Drosophila* rhodopsins, and catalyze their correct folding.

In the erythrocyte plasma membrane AE1 probably exists as a mixture of homodimers and homotetramers (reviewed in Casey and Reithmeier, 1991). Mature AE1 also associates stoichiometrically with several cytoplasmic proteins including glycolytic enzymes, hemoglobin and the membrane-cytoskeletal proteins ankyrin and band 4.1 (reviewed in Low, 1986). An association between AE1 and the integral membrane protein glycophorin A, which is present in the erythrocyte membrane at similar copy number, has also been suggested on the basis of biophysical measurements (Nigg et al., 1980) and co-immunoprecipitation studies (Wainwright et al., 1989). All of these proteins can therefore be considered potential participants in an AE1 heterooligomeric complex. It is possible that assembly of such a complex may be a prerequisite for maturation of AE1 from the ER and that the inability of HEK cells to process AE1 reflects the absence in these cells of one or more components of this complex. Presumably, HEK cells possess machinery capable of facilitating the folding of the more widely expressed AE2 protein, although the presence of almost half of steady-state AE2 in the ER suggests that such machinery may be rate limiting. Our observation that removal of the NH<sub>2</sub>-terminal domain of AE2 results in arrest of the mutant polypeptide in the ER is consistent with a role for that domain in the assembly of such a complex. However, our inability to “rescue” ER-arrested AE1 by fusing the NH<sub>2</sub>-terminal cytoplasmic do-

main of AE2 argues against a simple model, and suggests the potential existence of an interaction between the two principal structural domains of anion exchangers during their biosynthesis.

In contrast to the wealth of data on the kinetics of protein assembly and folding in the secretory pathway, little is known about where and when integral plasma membrane proteins become functionally activated. Such proteins, including ion channels and transporters, must transit the secretory pathway and undergo a sequence of posttranslational structural modifications before arriving at the plasma membrane. These covalent modifications may be necessary to activate the ion transport capacity of channels and pumps. Other modifications or interactions may act to maintain them in an inactive or silent state until they reach their correct destination. Wonderlin and French (1991) fused axoplasmic vesicles from squid giant axon with planar bilayers to establish that voltage gated Na<sup>+</sup> and K<sup>+</sup> channels, normally associated with the plasma membrane, are functionally active within an intracellular compartment. Since these vesicles are derived from a relatively late, post-Golgi stage of biosynthesis, it was not possible to deduce how early in the secretory pathway activation occurs. An alternative approach has been to determine the kinetics with which transporters acquire high-affinity ligand binding. The nicotinic acetylcholine receptor (Smith et al., 1987; Gu et al., 1989), a ligand-gated cation channel, and the Na<sup>+</sup>,K<sup>+</sup>-ATPase (Caplan et al., 1990) acquire the capacity for high affinity binding to their respective toxins early after synthesis, probably in the ER. However, the capacity of these early biosynthetic intermediates to form functional ion transporters has not been investigated. Ligand-gated transporters like the nicotinic acetylcholine receptor can be effectively silenced during intracellular transit by the absence of ligand. By contrast, the majority of transporters, including facilitated diffusion carriers, ion exchangers and pumps, must either be subject to regulation by endogenous factors or contribute to the steady-state ionic composition of the lumen of secretory pathway cisternae. Conceivably, transient association of ion transport subunits with intracellular "chaperones" may be one means to preserve them in an inactive state during biosynthesis.

Our data show that in highly enriched populations of ER-derived vesicles, biosynthetic intermediates of AE1 and AE2 from transfected HEK cells are catalytically active. Although we cannot exclude the possibility that the exchangers were activated in the process of ER vesicle isolation, they suggest that modification of the AE polypeptides in post-ER compartments is not necessary for their functional activation. Further studies will be necessary to determine whether—in cells where they are endogenously expressed—AE1 or AE2 could contribute to the maintenance of steady-state anion and proton gradients across the cisternae of secretory organelles.

If en passant transporters destined for the plasma membrane do contribute to the ionic composition of the cisternae of the secretory compartments through which they pass, then maintenance ionic homeostasis in these compartments must depend on the relative activities of the constellation of transporters present at steady-state. In such a case, overexpression of a single class of transporter could result in ionic imbalance, and lead to catastrophic consequences for the cell. Difficulties in obtaining overexpression of plasma membrane

ion transporters and ion channels in mammalian cells are common, although not frequently reported in the literature. This suggests an alternative to the hypothesis that retention of AE1 in the ER is due to misfolding or misoligomerization due to the lack in our cells of the appropriate chaperone or subunit. Perhaps overexpression of functional AE1 anion exchanger in HEK cells results in anionic or proton imbalance across the membrane of one of the compartments of the secretory pathway. Such abnormal ionic gradients could interfere with the function of the secretory pathway, resulting in the further accumulation of correctly folded AE1 protein, and possibly, leading to the morphologic alterations that we observe. A prediction of this hypothesis would be that AE1 overexpression would interfere with the processing of unrelated polypeptides that share the same secretory pathway. Such experiments are currently underway.

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