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Overexpression of AE1 Prague, but not of AE1 SAO, Inhibits Wild-type AE1 Trafficking in *Xenopus* Oocytes

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Abstract. Mutations in the AE1 (band 3) anion exchanger of human erythrocytes have been associated with altered red cell shape and heritable disease.The Southeast Asian Ovalocytosis (SAO) AE1 mutation, a 27 nt deletion producing the Δ400–408 form of AE1, and the AE1 Prague mutation, a 10 nt insertion producing a frameshift after AE1 aa 821 leading to premature termination, are found only in the heterozygous state. We therefore examined accumulation and function of *wt* AE1 polypeptide in *Xenopus* oocytes when coexpressed with AE1 SAO and with AE1 Prague. Our SAO construct lacked the K56E (AE1 Memphis) polymorphism present in the endogenous AE1 SAO protein. Neither mutant AE1 mediated Cl^- uptake into cRNA-injected *Xenopus* oocytes. Coninjection of mutant and *wt* cRNAs led to dose-dependent inhibition of *wt* function by AE1 Prague, but not by SAO. Though in vitro translation of the two mutants revealed little difference in their insertion into microsomal membranes, AE1 Prague accumulated in *Xenopus* oocytes to lower levels than did AE1 SAO or *wt*. Unlike AE1 SAO polypeptide, AE1 Prague polypeptide was not detectable at the oocyte surface. Moreover, overexpression of AE1 Prague, in contrast to AE1 SAO, reduced the accumulation of *wt* AE1, at the oocyte surface. This inhibition occurred in the absence of detectable heteromer formation between the AE1 Prague and *wt* AE1 polypeptides.

Key words: AE1 — Band 3 — Chloride/bicarbonate exchange — *Xenopus* oocytes — Hereditary spherocytosis — Anemia

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Introduction

Mutations in the erythrocyte chloride/bicarbonate exchanger AE1 (band 3), the major intrinsic protein of the erythrocyte membrane, have been associated with heritable alterations in red cell shape of varying clinical consequence. After the initial description of the human AE1 Memphis polymorphism K56E [28], which retards mobility of the 60 kDa AE1 chymotryptic fragment but has no clinical consequence [9], the first AE1 mutation to be studied in depth was Southeast Asian Ovalocytosis (SAO). In SAO, a 27 nt in-frame deletion in the AE1 gene leads to production of a protein lacking amino acids 400–408, residues thought to span the cytoplasmic site of entry of the AE1 polypeptide chain into the inner leaflet of the red cell lipid bilayer. The mutation is found only in the heterozygous state which, though producing ovalocytosis, is not known to have pathological consequences. The AE1 SAO protein is present in the red cell membrane but is nonfunctional as a sulfate/chloride exchanger [8, 15, 24, 27]. The circular dichroism spectra of the holoprotein and the membrane-associated domain are not different from those of *wt* AE1, and the mutant and *wt* proteins are chromatographically inseparable [21, 23]. However, several additional properties suggest altered folding and glycosylation of the mutant protein. In intact red cells, AE1 SAO cannot bind the covalent transport inhibitor ^3H-H_2DIDS [24] or the ectoreactive monoclonal antibody BRIC6. However, solubilized AE1 SAO can be recognized by BRIC6, though AE1 SAO blotted from a sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) to nitrocellulose does not bind the polylactosaminoglycan-specific tomato lectin [3, 23]. SAO red cells contain a higher than normal tetramer/dimer ratio for AE1 [23], and AE1 SAO is more resistant

to extraction by nonionic detergent [21, 23]. This resistance to detergent extraction is associated with markedly decreased rotational mobility of AE1 and with aggregation of AE1-containing intramembranous particles into longitudinal strands [17]. SAO red cells are also more rigid than normal cells, and may offer an increased number of ankyrin binding sites [13, but *see* 23]. The membrane rigidity of SAO red cells is thought to contribute to their increased resistance to invasion by Plasmodium species. SAO red cells incorporate 2–4 times more ^{32}P into AE1 tyrosines than do normal cells [7]. The physiological significance of this observation remains to be determined.

In *Xenopus* oocytes, AE1 SAO is also inactive as an anion transporter, though expressed at the oocyte surface whether in the absence or presence of *wt* AE1 [3]. Moreover, surface expression of recombinant AE1 SAO is potentiated by glycophorin A to the same degree as occurs with *wt* AE1 [4].

The AE1 Prague mutation is a 10 nt insertion leading to a frameshift after amino acid 821, and encoding 70 amino acids of hydrophilic neosequence instead of the putative two last transmembrane domains and the cytoplasmic C-terminus of *wt* AE1 [10]. Like AE1 SAO, AE1 Prague was detected only in the heterozygous state. However, unlike SAO, heterozygous AE1 Prague causes autosomal dominant hereditary spherocytosis accompanied by red cell AE1 deficiency. The AE1 Prague protein appears to be absent from the membrane of the circulating red cell, despite the presence of both mutant and *wt* allele mRNA transcripts in patient reticulocytes. This is consistent with the roughly 40% decrease in red cell *wt* AE1 content, as measured by eosin maleimide fluorescence flow cytometry and by DIDS-sensitive ^{35}S-sulfate uptake.

The absence of homozygous cases of Southeast Asian Ovalocytosis suggests that the homozygous mutation is embryonic lethal [16]. Thus, an understanding of the mechanism by which these mutations exert their physiological effects demands their study in the absence and the presence of *wt* AE1 protein. Here we compare the expression of recombinant AE1 SAO and AE1 Prague in reticulocyte lysate and in *Xenopus* oocytes, and the effects of each mutant on expression of *wt* AE1. We find that in contrast to AE1 SAO, AE1 Prague shows a dose-dependent interference of *wt* AE1 expression in both reticulocyte lysates and in *Xenopus* oocytes. In *Xenopus* oocytes, inhibition of *wt* AE1 function by coexpressed AE1 Prague is explained by reduced *wt* AE1 protein at the oocyte surface.

Materials and Methods

Plasmids

pHB3 encoding human AE1 was a gift of Dr. Sam Lux [18]. The SAO mutation was isolated within a NarI/XbaI fragment (ntl275-1563) of AE1 SAO [8]. This fragment was inserted into complementary NarI/XbaI fragment of pHB3 containing plasmid backbone and the remainder of the wild-type coding region from a non-Memphis individual. A PCR product encoding nt 2268 through 2619 of AE1 Prague [10] was amplified from patient cDNA with the primers 5′-CTTTGGGATGCCCTGGCTCA (sense) and 5′-TAACTGCATGCGCCAGGTCT (antisense). This PCR product was restricted with SphI and BstXI which recognize unique sites within the human AE1 coding region. The resultant internal subfragment was inserted into the appropriately prepared complementary pHB3 plasmid fragment to create pAE1-P encoding the predicted full-length AE1 Prague polypeptide.

PCR amplification was performed with Taq DNA polymerase (Perkin-Elmer/Cetus). Amplification products were sequenced to confirm absence of PCR-induced mutations.

RNA Transcription

cRNA was transcribed from linearized CsCl-purified plasmid DNA using the Ambion (Austin, TX) Megascript Kit according to manufacturer's instructions.

Heterologous AE1 Expression

Female *Xenopus* were anesthetized with 0.17% tricaine. Ovarian segments were exposed via a 1 cm flank incision, excised, minced, and incubated for 1 hr with gentle shaking at room temperature in 2 mg/ml Type A Collagenase (Boehringer Mannheim, Indianapolis) in ND-96 (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $mgCl_2$, and 5 Na HEPES, pH 7.4). After washing, individual oocytes of stage V–VI were manually dissected and defolliculated. On the same or the next day, oocytes were microinjected with 50 nl cRNA solution or water with an automatic nanoliter injector (WPI) or with a manual injector (Drummond Instruments). Oocytes were subsequently incubated in ND-96 at 19°C for 2–3 days until they were used for ion transport assays. The oocyte incubation medium was changed every day following RNA injection.

Isotopic Influx Assays

Experimental conditions were adapted from those previously described [6]. Influx medium prior to addition of radioisotopic chloride consisted of (in mM): 58 Na isethionate, 2 K gluconate, 1.8 Ca gluconate, 1 Mg gluconate, 5 Hepes hemisodium, made from stock solutions and titrated before each use to pH 7.40. Bumetanide (10 μM) was included in all experiments to block endogenous Na/K/2Cl cotransport. All solutions were nominally free of CO_2/bicarbonate.

The influx assay was initiated by transfer of groups of 6–10 water-injected oocytes or 8–12 cRNA-injected oocytes into microtiter wells containing 147 μl of the above influx medium plus 1.76 mCi $Na^{36}Cl$ (ICN, 3 μl of 1.9 M NaCl) to achieve a final NaCl concentration in the influx medium of 38 mM, with a calculated final osmolality of 212 mOsm.

Influx assays were carried out at room temperature for 60 min, a time shown earlier to reside within the linear range of uptake [2, 6]. Influx was terminated by rapid transfer of groups of oocytes through three 25 ml 20°C washes in ND-96 in which gluconate completely replaced chloride. Individual oocytes were transferred to scintillation vials containing 100 μl 1% sodium dodecylsulfate (SDS). Influx values were calculated as nmoles chloride per (oocyte × hr) and expressed as means ± SEM. The significance of transport inhibition by cRNA coinjection compared to *wt* transport was estimated by the two sample *t*-test assuming equal variances. The significance of differences between transport inhibition produced by coninjection with *wt* cRNA of

SAO and Prague cRNAs was tested by the paired two-sample *t*-test for means.

In vitro AE1 Biosynthesis and Immunoprecipitation

cRNAs were expressed in the rabbit reticulocyte lysate system with canine pancreatic microsomes (Promega) as recommended by the supplier. Metabolic labeling was with ^{35}S-TransLabel (ICN, 24 μCi in a 25 μl reaction). Protein biosynthesis was terminated and microsomes solubilized by addition of 80 μl immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM ethylenediamine tetra-acetate, sodium salt (EDTA), 10 mM Tris HCl, pH 7.5, 1% NP-40, 0.5% deoxycholate, and 2 mg/ml bovine serum albumin (BSA). AE1 polypeptides were immunoprecipitated by addition of 1 ml of monoclonal antihuman AE1 directed to an undisclosed epitope of the cytoplasmic domain (Sigma, clone B-136) or by addition of 5 μl of crude rabbit polyclonal antihuman antiserum. This serum was raised against human AE1 C-terminal amino acid residues 900–911 linked via an added N-terminal Cys to maleimidobenzylsuccinimido (MBSS)-derivatized keyhole limpet hemocyanin (KLH). The antibody incubations were 1 hr at 4°C, followed by addition of 20 μl Protein A Sepharose (Sigma, 50% v/v), resuspension in IP buffer for 1 hr. Immunoprecipitates were then washed 4–5 times in IP buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with protein size standards from BRL-Gibco.

Microsomes pretreated with or without 0.1 M NaOH for 30 min at 0°C were purified from the cell-free translation mix by centrifugation for 30 min at 45,000 rpm in a Beckman TLA-45 rotor through a 300 μl cushion of (mM) 250 sucrose, 500 KCl, 5 dithiothreitol, 50 lysine, 3 $MgCl_2$, 50 Hepes hemipotassium salt, pH 7.5. The microsomal pellets were resuspended in 140 mM NaCl, 20 mM Na phosphate, pH 7.5 and washed 3 times for 10 min at 45,000 rpm in the same rotor prior to analysis by SDS-PAGE.

Immunoprecipitation of heterologous AE1 from *Xenopus* Oocytes

Groups of 15–30 cRNA-injected or water-injected oocytes were incubated for 48 hr in 200 μl ND-96 containing 1 mCi/ml of ^{35}S-methionine (30 μM). All subsequent steps were carried out at 4°C. Metabolically labeled oocytes were homogenized in 100 μl oocyte IP buffer containing (in mM): 50 Tris-HCl, pH 8.0, 1 EDTA, 1 phenylmethylsulfonyl chloride (PMSF), and 0.04 each of leupeptin, pepstatin, and antipain. The homogenate was incubated with shaking for 30 min, then centrifuged in a microfuge for 10 min. The resultant supernatants were brought to 500 mM NaCl and precleared with 5% normal rabbit serum. Preadsorbed supernatants were then incubated with either of the above described antihuman AE1 antibodies for 1 hr, followed by protein A-Sepharose precipitation. The sepharose pellets were washed six times in 1 ml oocyte IP buffer containing 500 mM NaCl, twice more with IP buffer containing 250 mM NaCl, then analyzed by SDS-PAGE fluorography.

Detection of AE1 at the Oocyte Surface

Groups of 10–30 intact ^{35}S-labeled oocytes were washed in modified ND-96 in which 48 mM NaCl was replaced with 100 mM sucrose and pH adjusted to 8.0 with NaOH. Oocytes were then incubated for 3 hr in the same buffer in the presence or absence of 5 mg/ml papain, pronase, or chymotrypsin (Sigma). Proteolyzed oocytes were washed 3 times in 10 ml ND-96 containing 2 mM PMSF, 1 mg/ml BSA and once more in ND-96 containing 2 mM each of PMSF and iodoacetamide (Sigma) before immunoprecipitation was performed as described above. The proportion of AE1 cleaved by proteolysis was determined by scanning densitometry of the resultant fluorographs and quantitation of intact AE1 and its proteolytic P60 fragment by ScanAnalysis v. 2.1 (Biosoft, Cambridge, UK). No correction was made for possible aggregation of ^{35}S-labeled AE1 or P60, and so the reported proportions of AE1 polypeptides at the oocyte surface represent maximum estimates.

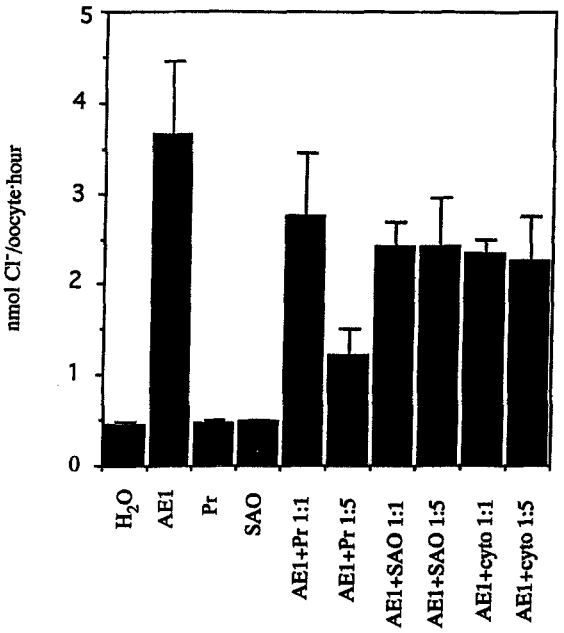

Fig. 1. AE1 Prague coexpression inhibits *wt* AE1-mediated $^{36}Cl^-$ influx. Forty-eight hr after cRNA injection of oocytes, 60-min influxes were measured in isotonic ND96 containing 38 mM Cl^-. *wt* AE1 cRNA (2.5 ng) was injected as noted with either 2.5 or 12.5 ng cRNA encoding AE1 SAO or AE1 Prague, or with 0.8 or 4.0 ng cRNA encoding the AE1 1–422 (cyto). These values represent *wt*:mutant mole ratios of 1:1 and 1:5. Flux values are means ± SEM of 4 identical experiments, each with 8–12 oocytes per individual group. Identical results were noted in 96 mM Cl^- medium. AE1 Prague, but not AE1 SAO, displayed a dose-dependent increase in inhibition of *wt* AE1 function.

Results

AE1 Mutant Function in *Xenopus* Oocytes

Figure 1 shows that whereas *wt* AE1 cRNA conferred on oocytes a 7-fold increase in $^{36}Cl^-$ influx, the Prague and the SAO mutants were both inactive as chloride transporters in oocytes. Coinjection of AE1 Prague and *wt* cRNAs at a 1:1 molar ratio led to 31% inhibition of AE1-mediated $^{36}Cl^-$ influx. Further increase in the mutant-to-*wt* molar ratio to 5:1 produced 79% inhibition of AE1-mediated $^{36}Cl^-$ influx. In contrast, coinjection of AE1 SAO and *wt* cRNA produced 43 and 42% inhibition of *wt* levels of transport at 1:1 and at 5:1 molar ratios, respectively. This inhibition was no different from that produced by coinjection of control cRNA encoding AE1

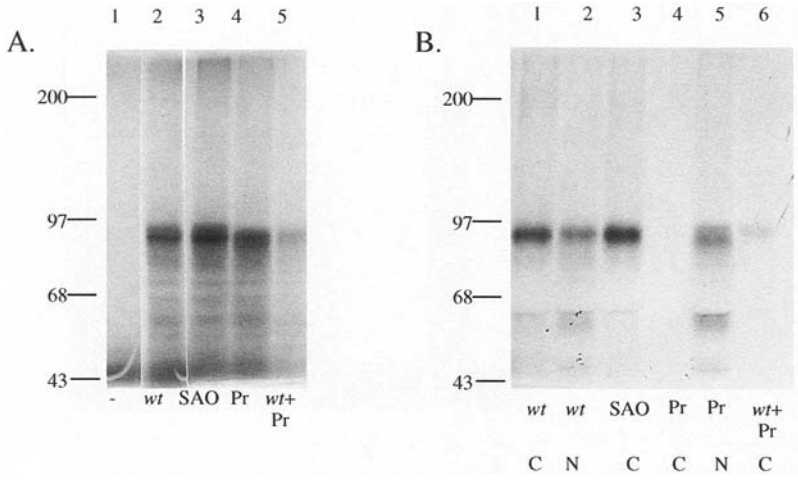

Fig. 2. Cotranslation of AE1 Prague with *wt* AE1 inhibits *wt* polypeptide accumulation without evidence of hetero-oligomer formation. (*A*) In vitro translations from 1 μg cRNA in the presence of dog pancreatic microsomes and ^{35}S-Translabel were loaded (3 μl from 25 μl reactions) directly onto a 10% polyacrylamide gel. (*B*) Alternatively, aliquots of microsomes from the same reactions were isolated on a sucrose cushion, extracted with Triton X100, and immunoprecipitated with antibody to human AE1 C-terminal peptide (lanes 1,3,4,6) or to an anonymous epitope within the N-terminal cytoplasmic domain (lanes 2,5). Cotranslations in both panels *A* and *B* were performed with 1 μg each of *wt* and Prague cRNAs. Although when separately expressed, *wt* and each mutant AE1 polypeptide accumulated to similar levels, coexpression of *wt* and Prague led to decreased polypeptide accumulation, and *wt*:Prague hetero-oligomers were not detected.

1–422 (lacking the transmembrane domain of AE1), 45 and 48% inhibition at 1:1 and 5:1 molar ratios, respectively. Similar degrees of inhibition at 1:1 and 1:5 molar ratios were produced by cRNA encoding the unrelated plasmalemmal transport protein NCE1, the Na^+/Ca^{2+} exchanger [14, *not shown*]. Thus, only AE1 Prague showed a dose-dependent increase in inhibition of coexpressed *wt* AE1 in *Xenopus* oocytes ($P < 0.01$ for the comparison between 1:5 molar ratio of *wt*:SAO and *wt*:Prague, n = 4 experiments with oocytes from 4 frogs).

The absence of SAO function in *Xenopus* oocytes reproduced the results of Groves et al. [3]. The partial inhibition of *wt* AE1 by SAO was also briefly noted by Groves et al. in a single experiment performed in the presence of glycophorin A [3, 4]. Though AE1 Prague appears to be absent from the red cell membrane, the increasing degree of inhibition by AE1 Prague of *wt* AE1 function in the oocyte might have been exerted at the oocyte surface. The example of CFTR Δ508 shows that mutations which prevent normal trafficking to the mammalian cell plasma membrane at 37°C may permit trafficking to the oocyte surface at 19–21°C, the temperature of oocyte experiments [1]. Alternatively, AE1 Prague might interfere with the cotranslational insertion or postinsertional stabilization of AE1 in the endoplasmic reticulum, or with subsequent trafficking of AE1 to the oocyte surface. Any of these effects could be mediated by direct hetero-oligomer formation with *wt* AE1 or by indirect mechanisms. Therefore, recombinant *wt* and mutant AE1 polypeptides were studied in reticulocyte lysates and in *Xenopus* oocytes to examine these possibilities.

In vitro Translation of *wt* and Mutant AE1 Polypeptides

Figure 2*A* shows that *wt* and SAO AE1 polypeptides migrate indistinguishably in SDS-PAGE, whereas AE1 Prague polypeptide migrates detectably faster. *wt* AE1, AE1 SAO and AE1 Prague polypeptides accumulate to comparable levels when translated by reticulocyte lysate in the presence of dog pancreas microsomes. However, cotranslation of *wt* AE1 and AE1 Prague leads to reduced accumulation of the combined AE1 polypeptides (lane 5). Cotranslation of *wt* AE1 and AE1 SAO did not produce similar inhibition (*not shown*).

When translated individually in the presence of dog pancreas microsomes, *wt* AE1, AE1 SAO, and AE1 Prague are each deglycosylated completely by peptidyl N-glycosidase F in the presence of Triton X100 (*not shown*).

AE1 Prague and *wt* AE1 do not Form Detectable Hetero-oligomers

This reduction in synthesis of *wt* AE1 in the presence of AE1 Prague might have been accompanied by hetero-oligomer formation in the microsomal membrane or following detergent solubilization. The possibilities were tested by coimmunoprecipitation from solubilized microsomes. Figure 2*B* shows that *wt* AE1 was precipitated by either polyclonal antibody to the C-terminus (lane 1, C) or by monoclonal antibody to a cytoplasmic epitope (lane 2, N). AE1 SAO was also recognized by both antibodies (lane 3 and *not shown*). However, AE1 Prague was recognized only by antibody to the cytoplasmic epitope (lane 5, N), and not by antibody to the C-terminal epitope (lane 4, C), consistent with the presence of a mutant C-terminal neosequence in AE1 Prague.

AE1 exists as stable dimers and tetramers in detergent-solubilized red cell plasma membrane [22]. Oligomerization is thought to occur in the endoplasmic reticulum, though this remains to be proven. If in vitro translated *wt* and Prague AE1 polypeptides were to form hetero-oligomers in dog pancreas microsomes, then the

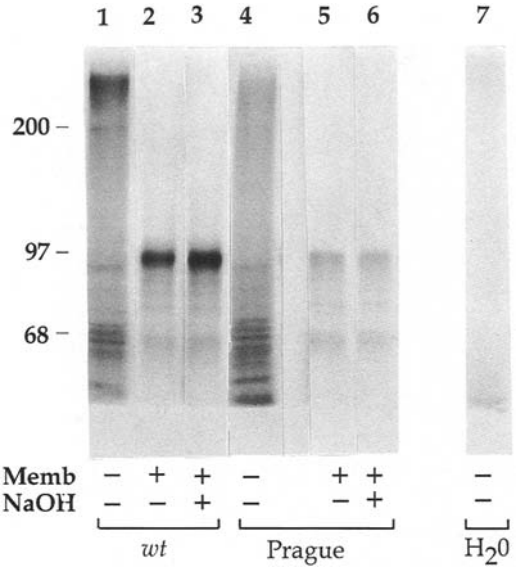

Fig. 3. In vitro translation of *wt* AE1 (lanes 1–3) and AE1 Prague (lanes (4–6) in the absence (lanes 1,4) or presence of microsomes (lanes 2,3,5,6). Lane 7 was from a reaction with cRNA omitted. Three µl were applied from a 25 µl reaction. Both *wt* and Prague polypeptides were inserted into microsomal membranes to render them resistant to NaOH stripping.

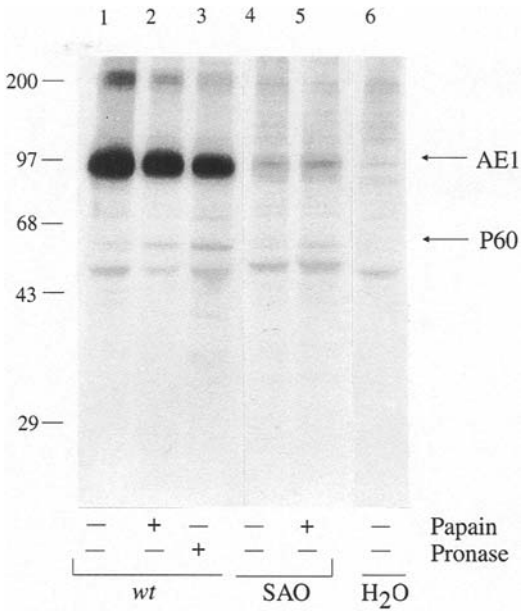

Fig. 4. *wt* AE1 cRNA-injected (lanes 1–3) and AE1 SAO cRNA-injected oocytes (lanes 4,5) were metabolically labeled as described in Materials and Methods. The intact oocytes were incubated with papain (lanes 2,5), pronase (lane 3) or without protease (lanes 1,4). Then, membranes were prepared, solubilized, and subjected to immunoprecipitation with anticytoplasmic domain MoAb. Solubilized protein from 10 oocytes was loaded in each lane. Lane 6 pictures water-injected oocytes. The experiment shown was representative of 4 similar experiments. Note that AE1 SAO and *wt* AE1 polypeptides were detected at the oocyte surface.

amount of AE1 Prague detectable in a C-terminal antibody immunoprecipitation should reflect the amount of AE1 hetero-oligomer solubilized from the microsomes. Lane 6 of Fig. 2*B* shows that C-terminal antibody precipitated only *wt* AE1 from a cotranslation reaction, whereas anticytoplasmic domain epitope coprecipitated both polypeptides (*not shown,* but *see* Fig. 6). Identical results were obtained when Triton X100 extracts of membranes containing only *wt* AE1 and only AE1 Prague were mixed and incubated for 30 min at 4°C prior to coimmunoprecipitation. Thus no AE1 *wt*/Prague hetero-oligomers were detected by this coimmunoprecipitation assay. These experiments were performed under conditions in which hetero-oligomerization was demonstrable between *wt* and mutant murine AE1 polypeptides (*not shown*).

AE1 Prague is Inserted into Microsomal Membranes

The lack of transport activity of AE1 Prague in *Xenopus* oocytes might have been secondary to impaired cotranslational insertion into endoplasmic reticulum. Thus, the possibility was tested that the accumulation of AE1 Prague in Fig. 2 represented association with microsomes in the absence of insertion. As shown in Fig. 3, both *wt* AE1 (lane 1) and AE1 Prague (lane 4) polypeptide showed a tendency to produce SDS-resistant aggregates when translated in the absence of pancreatic microsomes compared to monomeric polypeptide in SDS produced in the presence of microsomes (lanes 2 and 5). Alkali stripping of the microsomal membranes failed to diminish the quantity of membrane-associated *wt* AE1 polypeptide (lane 3) or AE1 Prague polypeptide (lane 6). The corresponding alkali supernatants after neutralization lacked detectable AE1. Thus, both *wt* and mutant AE1 polypeptides were integrally incorporated into the lipid bilayer of the microsomal membranes.

AE1 Prague is not Detectable at the Surface of cRNA-injected *Xenopus* Oocytes

Recombinant AE1 has been detected at the oocyte surface by virtue of its susceptibility to chymotrypsin digestion by Groves et al. [3]. Digestion of intact oocytes with extracellular papain or pronase (Fig. 4, lanes 2 and 3) produced more reliably detectable 60 kDa N-terminal AE1 fragments (P60) than did chymotrypsin, when evaluated using our antibodies. In the experiment shown, 9% and 23% of total *wt* AE1 (at maximum) were accessible to operationally defined surface digestion by papain or pronase, respectively (the bulk of AE1 methionine is absent from the P60 fragment). Treatment of water-injected oocytes with neither papain, pronase, nor chy-

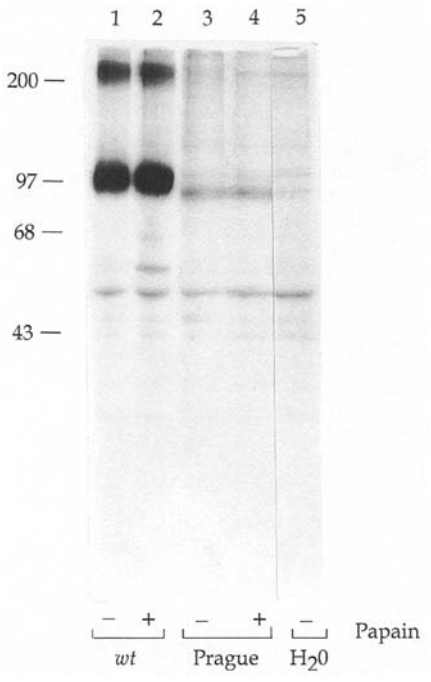

Fig. 5. Fifteen *wt* AE1 cRNA-injected oocytes (lanes 1,2) and 30 AE1 Prague cRNA-injected oocytes (lanes 3,4) were metabolically labeled, incubated in the absence (lanes 1,3) or presence of papain (lanes 2,4), then subjected to immunoprecipitation with anticytoplasmic domain MoAb. Protein from 15 water-injected oocytes was loaded in lane 5. The experiment shown was representative of 4 similar experiments. Note that AE1 Prague polypeptide was not detected at the oocyte surface.

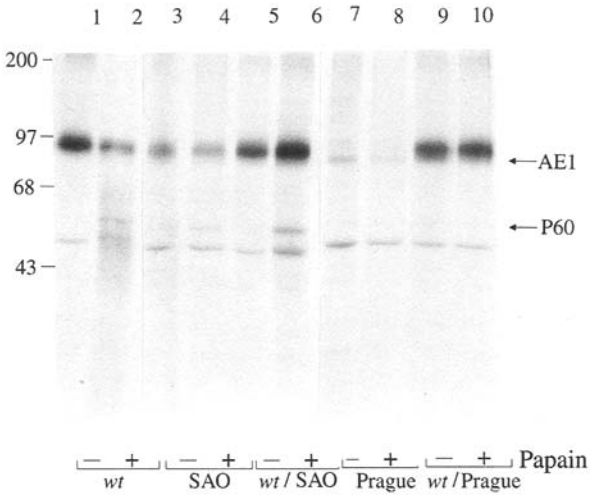

Fig. 6. Fifteen metabolically labeled, cRNA-injected oocytes were incubated in the absence (odd lanes) or presence of papain (even lanes). Membrane fractions were solubilized and immunoprecipitated with anticytoplasmic domain MoAb. The experiment shown was representative of 3 similar experiments. Note that AE1 Prague, but not AE1 SAO, decreased *wt* AE1 accumulation at the oocyte surface.

motrypsin produced detectably increased $^{36}Cl^-$ influx (*not shown*). However, whereas pronase occasionally produced oocyte softening, the oocytes tolerated papain treatment without a change in appearance or evident fragility. Therefore, papain was used for subsequent experiments. In 6 similar experiments, 12.7 ± 0.8% (SEM) of *wt* AE1 (at maximum) was located at the oocyte surface as judged by papain digestion. In contrast, in this experiment and in 3 additional similar experiments, 26.4 ± 1.6% of AE1 SAO (at maximum) was detected at the oocyte surface. This increased surface expression of AE1 SAO in the absence of the Memphis polymorphism compared to *wt* AE1 confirmed the data of Groves et al. using chymotrypsin to cleave AE1 SAO containing the Memphis polymorphism [3].

Figure 5 shows that total accumulation of AE1 Prague in oocytes was greatly reduced compared to *wt* AE1. In three papain digestion experiments in which membranes from 30 metabolically labeled oocytes were solubilized and subjected to immunoprecipitation, no 60 kDa papain fragment indicative of surface expression was detected. Thus, within the limits of detection of this assay, AE1 Prague was not processed to the surface of *Xenopus* oocytes, and so differed from AE1 SAO in this characteristic. This difference correlated with the presence of AE1 SAO in SAO red cells and the apparent absence of AE1 Prague in Prague spherocytotic red cells. The AE1 Prague mutation did not show the temperature-sensitive putative folding phenotype exhibited in *Xenopus* oocytes by the prevalent CFTR mutation Δ508 [1].

AE1 Prague Coexpression Interferes with Accumulation of *wt* AE1 at the Oocyte Surface

Figure 6 shows that SAO coexpression in *Xenopus* oocytes with *wt* AE1 did not reduce the accumulation of the 60 kDa papain fragment at the oocyte surface. In contrast, coexpression of AE1 Prague, which itself was not detectable at the oocyte surface, with *wt* AE1 nearly abolished generation of the 60 kDa papain fragment. In this experiment, and two additional similar experiments, the average % *wt* AE1 expressed at the oocyte surface was reduced by AE1 Prague coexpression from 10.6% to nearly undetectable levels. This result correlated reasonably with the degree of inhibition of chloride transport by AE1 Prague coexpression (Fig. 1).

Discussion

Most human mutations in AE1 are present only in the heterozygous form. Reticulocytes from individuals heterozygous for either AE1 SAO or for AE1 Prague appear to express similar levels of mutant and *wt* mRNA. Thus, an integral part of the characterization of these mutants in heterologous systems must be in the context of coexpression with *wt* AE1. AE1 Prague red cells [10] and SAO red cells [24] both display about 60% of normal

AE1 function. SAO red cells have normal total AE1 levels with near-equal amounts of mutant and wild-type protein, whereas AE1 Prague red cells have 60% of normal AE1 levels. All of the AE1 protein in AE1 Prague red cells is *wt*. The AE1 Prague polypeptide has been thus far undetectable in the red cell membrane. In the current study, we compared the interactions with *wt* AE1 of AE1 Prague and of Memphis-negative AE1 SAO in the *Xenopus* oocyte expression system.

In the current study, as in the previous study of Groves et al. [3], AE1 SAO was delivered to the oocyte surface (Fig. 4), but was inactive there (Fig. 1). Its presence at the surface did not diminish *wt* AE1 function any more than did the products of other coinjected cRNAs, whether encoding soluble or transmembrane proteins (Fig. 1). The AE1 Memphis polymorphism E56K which cosegregates with AE1 SAO is clinically silent, and has not been associated with altered sulfate transport measured under a limited range of experimental conditions. However, AE1 Memphis red cells display a 20% reduction in transport rate for phosphoenolpyruvate despite unimpaired phosphate transport [11]. The current results demonstrate formally that the SAO phenotype of loss-of-function without impaired trafficking to the cell surface does not require the presence in *cis* of the Memphis polymorphism.

In contrast, AE1 Prague accumulated in oocytes at low levels, was not detectable at the oocyte surface (Fig. 5), and interfered with the surface accumulation of *wt* AE1 (Fig. 6). These properties were consistent both with the lack of anion transport by AE1 Prague in oocytes, and with its inhibition of *wt* AE1 transport function (Fig. 1). The transport-negative phenotype of AE1 Prague was not due to substantially reduced insertion into the endoplasmic reticulum membrane (Fig. 3). Unlike CFTR, expression of AE1 Prague polypeptide at 19°C did not permit detectable accumulation at the oocyte surface. Hetero-oligomer formation between AE1 Prague and *wt* AE1 polypeptides was not detected, and so could not serve as an explanation for functional impairment by AE1 Prague of coexpressed *wt* AE1 (Fig. 2). Either *wt*-Prague hetero-oligomers did not form in appreciable amount, or they were insufficiently stable to survive immunoprecipitation. In contrast, AE1 SAO and *wt* AE1 exist as stable heterooligomers in SAO red cell membranes [12].

If indeed, AE1 Prague polypeptide were to form hetero-oligomers with *wt* AE1 polypeptide leading to rapid proteolytic degradation, then AE1 Prague would act as a type of dominant negative mutation. Such a phenotype might have consequences during erythropoiesis, while allowing stable incorporation of *wt* AE1 homo-oligomers into the plasma membrane of the erythroblast. The simplest model of dominant negative hetero-oligomer formation in *Xenopus* oocyte expression experiments assumes a binomial distribution of possible mutant and wild-type protomers. Such a model further assumes that the presence of one inactive protomer in a complex completely inhibits the complex or leads to its degradation, and that cRNA translation, protomeric protein trafficking and stability, and interprotomeric interactions are equivalent for all combinations of *wt* and mutant proteins [*see,* for example, 19]. Under these assumptions, if 1 mutant subunit of a heterodimer confers complete loss-of-function, then coinjection of the two cRNAs in a 1:1 ratio predicts 75% inhibition of activity. If 1, 2, or 3 mutant subunits within a heterotetramer are required to confer loss-of-function, then the expected inhibitions produced by a 1:1 cRNA ratio are 94%, 69%, and 31%.

Coinjection of *wt* AE1 with AE1 Prague in a 1:1 ratio inhibited AE1-mediated $^{36}Cl^-$ influx by 42%, but this inhibition was "nonspecific," (that is, did not increase with increasing dose of mutant and did not exceed the inhibition produced by "nonspecific" cRNAs). However, a 5-fold excess of AE1 Prague but of no other cDNA tested incrementally inhibited AE1-mediated $^{36}Cl^-$ influx. The net inhibition of 79% represents 59% inhibition of the residual flux remaining after subtraction of the above-defined "nonspecific" component of inhibition.

Under the simplifying assumptions discussed above, this degree of inhibition is inconsistent with the formation of heterodimers, but possibly consistent with heterotetramers which require two or three mutant protomers to inactivate tetramer activity. However, AE1 Prague/*wt* hetero-oligomers were not detectable by coimmunoprecipitation either from reticulocyte lysates or from *Xenopus* oocytes. Moreover, if tetramers containing one or two AE1 Prague protomers were functionally active, then some AE1 Prague should have been detectable at the oocyte surface unless present below the threshold of detection, or if AE1 Prague has lost sensitivity to protease cleavage due to conformational changes in the exofacial loop between transmembrane spans 5 and 6.

The ability of soluble fragments of AE1 and of NCE1 holoprotein to partially inhibit *wt* AE1-mediated $^{36}Cl^-$ uptake into oocytes is reminiscent of similar "nonspecific" inhibition of the skeletal muscle chloride channel ClC-1 by the cystic fibrosis transmembrane regulator protein CFTR [26], and complicates analysis of inhibition. It may be that the oocyte is limiting in a factor required for optimal translocation and processing of AE1 (and perhaps also ClC-1 [26]). The synthesis or presence of AE1 Prague may lead to saturation or consumption of this limiting factor. Alternatively, it remains possible that AE1 Prague cannot be cleaved by papain, pronase, or chymotrypsin secondary to conformational changes in the ecto-loop between TM5 and TM6.

The AE1 Prague polypeptide appears to be absent from the mature red cell membrane of heterozygotes

with hereditary spherocytosis. For this reason the physiological significance of inhibition of *wt* AE1 function by coexpressed AE1 Prague remains unclear. However, this functional interaction may be important in the differentiation of the red cell lineage in AE1 Prague patients. Our results from *Xenopus* oocytes allow the speculation that AE1 Prague never reaches the plasma membrane of erythroblasts, and retards and alters the developmental accumulation and stabilization of plasmalemmal *wt* AE1 and the membrane-associated cytoskeleton in this form of hereditary spherocytosis. Experiments to test this hypothesis in erythroblasts are currently underway.

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