Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Inefficient Chronic Activation of Parietal Cells in $Ae2_{a,b}^{-/-}$ Mice

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In parietal cells, basolateral $Ae2\ Cl^-/HCO_3^-$ exchanger (Slc4a2) appears to compensate for luminal H^+ pumping while providing Cl^- for apical secretion. In mouse and rat, mRNA variants $Ae2a$, $Ae2b1$, $Ae2b2$, and $Ae2c2$ are all found in most tissues (although the latter at very low levels), whereas $Ae2c1$ is restricted to the stomach. We studied the acid secretory function of gastric mucosa in mice with targeted disruption of $Ae2a$, $Ae2b1$, and $Ae2b2$ (but not $Ae2c$) isoforms. In the oxyntic mucosa of $Ae2_{a,b}^{-/-}$ mice, total $Ae2$ protein was nearly undetectable, indicating low gastric expression of the $Ae2c$ isoforms. In $Ae2_{a,b}^{-/-}$ mice basal acid secretion was normal, whereas carbachol/histamine-stimulated acid secretion was impaired by 70%. These animals showed increased serum gastrin levels and hyperplasia of G cells. Immunohistochemistry and electron microscopy revealed baseline activation of parietal cells with fusion of intracellular H^+/K^+ -ATPase-containing vesicles with the apical membrane and degenerative changes (but not substantial apoptosis) in a subpopulation of these cells. Increased expression of proliferating cell nuclear antigen in the oxyntic glands suggested enhanced $Ae2_{a,b}^{-/-}$ parietal cell turnover. These data reveal a critical role of $Ae2a$ - $Ae2b1$ - $Ae2b2$ isoforms in stimulated gastric acid secretion whereas residual $Ae2c$ isoforms could account to a limited extent for basal acid secretion. (*Am J Pathol* 2006, 169:165–176; DOI: 10.2353/ajpath.2006.051096)

HCl is secreted at the apical membrane of the gastric parietal cell in response to gastrin,¹ histamine, and acetylcholine.^{1–3} Parietal cell stimulation results in insertion of tubulovesicular membranes harboring the H^+/K^+ -ATPase pump in the apical plasma membrane.^{4,5} Cl^- secretion via chloride channels ensures electroneutrality and completes the apical aspect of acid secretion.^{5,6} The massive proton secretion by parietal cells has to be balanced by basolateral import of protons or extrusion of bicarbonate, to maintain a near neutral intracellular pH (pH_i). Na^+ -independent electroneutral Cl^-/HCO_3^- exchange seems to be particularly relevant for bulk transport because it both supplies Cl^- influx for its apical secretion and extrudes HCO_3^- , so as to acidify the cytosol and thereby allow apical proton secretion.^{7–11} Previous studies have offered indirect evidence for a role of the basolateral $Ae2\ Cl^-/HCO_3^-$ exchanger (Slc4a2) in this process,^{12–14} although another basolateral Cl^-/HCO_3^- exchanger, Slc26a7, has been identified in parietal cells, and a major role for this exchanger in gastric secretion was postulated.¹⁵ However, mice with disruption of the $Ae2$ gene were recently reported to have a stomach content with neutral pH on stimulation with histamine, suggesting that gastric acid secretion entirely depends on $Ae2$ function.¹⁶

The $Ae2$ gene, like other members of the AE family, may drive alternative transcription from different promoter sequences. Thus far, five N-terminal variants of the $Ae2$ mRNA (the complete message $Ae2a$ and alternative $Ae2b1$, $Ae2b2$, $Ae2c1$, and $Ae2c2$) have been described to be driven in the mouse stomach.¹⁷ Alternative exons $1b_1$ and $1b_2$ are transcribed from promoter sequences within intron 2, and each one is

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spliced to exon 3 in corresponding 5'-variants Ae2b1 and Ae2b2. The 5' diversity of these messages leads to small changes, the initial 17 amino acids of 1237 residues of Ae2a being replaced by three residues (MTQ) in Ae2b1 and by eight residues (MDFLLRPQ) in Ae2b2. The pattern of alternative transcription from intron 2 is conserved between mouse, rat, and humans,¹⁸ although expression of variants type b in humans seems to be more tissue-specific than in rodent species.^{17,19,20} In mouse and rat, but not in humans,¹⁹ additional alternative exons 1c₁ and 1c₂ are both transcribed from overlapping sequences in intron 5 (with some differences between these species). Exon 1c₁ is spliced to exon 6 in variant Ae2c1, and the more downstream exon 1c₂ proceeds directly with exon 6 in variant Ae2c2.^{14,17,20–22} Expression of variant Ae2c1 is rather stomach-specific,^{20,22} whereas that of Ae2c2 occurs in most tissues (although at very low levels). Recent systematic functional characterization in *Xenopus* oocytes showed that, like the well-characterized Ae2a protein,^{23–25} all variant polypeptides except Ae2c2 can mediate anion exchange. Interestingly, however, Ae2c1 differs from Ae2a, Ae2b1, and Ae2b2 in its alkaline-shifted pH_{o(50)} sensitivity.²⁶ Whether Ae2c transcripts are translated into proteins in the stomach remains to be determined, as no immunocytochemical detection of resultant Ae2c polypeptides has yet been performed in this tissue.

Previously, we reported the generation of mice with a targeted disruption of the more widely distributed variants Ae2a, Ae2b1, and Ae2b2 but not Ae2c1 and Ae2c2 isoforms,²⁷ hereby referred to as Ae2_{a,b}^{-/-} mice. This animal model enables us to assess the relative importance of the c isoforms of Ae2. We show that Ae2a-Ae2b1-Ae2b2 disruption causes near complete absence of all Ae2 protein isoforms from the gastric mucosa, indicating that translation of the Ae2c isoforms in the stomach is very low (less than 3% of total Ae2 protein). Whereas gastric acid secretion is completely abrogated in mice with a disruption of the entire Ae2 gene (Ae2^{-/-} mice),¹⁶ we demonstrate here that gastric acid secretion in Ae2_{a,b}^{-/-} mice is impaired on stimulation. Although under basal conditions Ae2_{a,b}^{-/-} mice have a normal gastric acid secretion they show hypergastrinemia as well as a pattern of chronic activation and increased turnover of parietal cells. Altogether, our findings highlight an important role of Ae2a-Ae2b1-Ae2b2 polypeptides in stimulated gastric acid secretion. Under basal conditions, virtually undetectable levels of Ae2c1 protein in the parietal cells might contribute in some measure to intracellular homeostasis.

Materials and Methods

Animal Breeding and Surgical Procedures

Ae2_{a,b}-targeted mice were generated as described.²⁷ Animals were bred in heterozygous couples against FVB/N background. All procedures with animals were approved by the Institutional Animal Care and Use Com-

mittee. Gastric perfusion experiments were performed with 4-month-old female Ae2_{a,b}^{-/-} mice and female Ae2^{+/+} and Ae2_{a,b}^{+/-} littermates as controls, essentially as described.²⁸ Briefly, the stomach was perfused through an inflow catheter with 0.9% NaCl (0.3 ml/minute), and the effluent from an outflow catheter was collected in fractions of 15 minutes. After 60 minutes, carbachol was added to the perfusate (20 μg/ml) and histamine was intravenously injected (23 μg/100 g). pH was measured in effluent fractions, the concentration of H⁺ also being accurately determined by titration with NaOH.

Histopathological Analysis of the Stomach

After sacrifice of the animals, gastric tissue was dissected, fixed, and embedded in paraffin. Identification of cell types in the gastric glands was performed by staining of sections (3 to 4 μm) with the Cook method,²⁹ without the aurantia stain. For immunohistochemistry, deparaffinized sections were washed with distilled water for 5 minutes, placed in citrate buffer (10 mmol/L, pH 6.0), and heated in a microwave oven for antigen retrieval (15 minutes at 750 W and 15 minutes at 375 W). After blocking with goat serum (Dako, Glostrup, Denmark), we used the following antibodies: mouse monoclonal antibodies against H⁺/K⁺-ATPase β-subunit (either clone 2B6 from Research Diagnostic Inc., Flanders, NJ, or clone 2G11 from Sigma, St. Louis, MO), gastrin (CURE/Digestive Diseases Research Center, Los Angeles, CA), and proliferating cell nuclear antigen (PCNA, from Dako), as well as a rabbit polyclonal antibody against the C-terminal sequence of all five Ae2 isoforms (obtained in our laboratory after rabbit immunization with the Ae2 C-terminal peptide 1223-1237; this polyclonal antibody may also cross-react with the C-terminus of Ae1, the major integral transmembrane protein in the erythrocyte plasma membrane). Immunoperoxidase staining was performed by the avidin-biotin-peroxidase complex procedure.³⁰ Sections were washed in Tris-buffered saline for 5 minutes and then in 100 mmol/L of sodium acetate/acetic acid buffer, pH 6.0, for an additional 5 minutes. The peroxidase activity was revealed with 3,3'-diaminobenzidine (Sigma) as described.³¹ For double-immunofluorescence staining, OCT-frozen sections fixed with cold acetone and further hydrated and blocked [with phosphate-buffered saline (PBS)-3% bovine serum albumin] were incubated for 1 hour with the H⁺/K⁺-ATPase antibody from Sigma (diluted 1:50,000). Then, some sections were sequentially incubated with our rabbit Ae2 antiserum (diluted 1:100; 2 hours) followed by incubation with fluorescent secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG (H+L) from Molecular Probes, Eugene, OR, and fluorescein isothiocyanate-labeled goat anti-rabbit IgG from Sigma (reference no. F9887). Alternatively, sections incubated with the H⁺/K⁺-ATPase antibody were further treated with phalloidin-tetramethyl-rhodamine isothiocyanate (TRITC) labeled from Sigma (reference

no. P1951) in PBS supplemented with 0.1% Triton X-100 and 3% bovine serum albumin (20 minutes), followed by incubation with the Alexa-labeled anti-mouse secondary antibody. After washing with PBS, sections were mounted and visualized at specific excitation-emission wavelengths. Terminal dUTP nick-end labeling (TUNEL) assay was performed on OCT-frozen sections with the *In Situ* Cell Death Detection kit, POD (Roche Diagnostics, Penzberg, Germany), according to the manufacturer's instructions. Before mounting the samples for light microscopy visualization, sections were sequentially incubated with anti-fluorescein-POD conjugate and substrate solution.

For electron microscopy, small pieces (~1 mm³) of the gastric corpus were fixed with 4% glutaraldehyde in 100 mmol/L sodium cacodylate buffer, pH 7.3, at 4°C for 3 hours. The material was postfixed in 1% phosphate-buffered osmium tetroxide, pH 7.3, at 4°C for 1 hour. Ultrathin sections (30 nm) were cut, double-stained with uranyl acetate and lead hydroxide, and examined with a Zeiss 10 CR transmission electron microscope.

Relative Quantification of mRNAs in Gastric Mucosa by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Corpus mucosa was extracted as below for Western blot analysis for further isolation of total RNA with the Tri Reagent (T9424; Sigma), aliquots of total RNA being reverse-transcribed. For relative quantitation of mRNA expression levels, resultant cDNAs were subjected to real-time PCR with specific primer pairs, using the iQ SYBR Green Supermix and the iCycler iQ Apparatus (both from Bio-Rad Laboratories, Hercules, CA). Reaction specificities were assessed through detection of expected peaks in the melt curve graphs and electrophoresis of PCR products on agarose gels. To calculate the relative mRNA levels, standard curves were obtained for each primer pair using known dilutions of a control template (a cDNA from wild-type mouse stomach total RNA). The log of relative concentrations in diluted samples (x axis) was plotted versus resultant threshold cycles (y axis), giving the equation of each standard curve. The slope of resultant standard curves reflects the efficiency of each reaction, which may differ depending on the used primer pair. The relative values obtained for each message were normalized with corresponding relative values for GAPDH. Mouse *Ae2* isoform-specific forward primers were 5'-GCTAAGATTGGCCATGAGC-3' for *Ae2a* (in exon 2), 5'-GTGCTGTCAAGCTCCTGCACT-3' for *Ae2b1* (in exon 1b₁), 5'-TTCACCCCTGCCGCGCATGGACTT-3' for *Ae2b2* (in exon 1b₂), 5'-TAGTGTCTCTGAGGGGC-AAAGCA-3' for *Ae2c1* (in exon 1c₁), and 5'-TCCAGGAGTGG-AAGTCAGGT-3' for *Ae2c2* (in exon 1c₂). Moreover, the forward primer 5'-CGGGACACGAAATCTA-GAGC-3' in exon 1 of *Ae2a* was used to assess for a possible mutant transcript in targeted mice due to splicing of exon 1 to exon 3. The common reverse primer 5'-

CGGTGGTATTCAAAGTCTTCC-3' (in exons 3 and 4) was used for *Ae2a*, *Ae2b1*, *Ae2b2*, and mutant transcript. 5'-TCATGAGGTCAAGGTCGGCT-3' was the reverse primer for *Ae2c1* (in exon 6). 5'-CCTCCTCGAATTTTATCCAGC-3' and 5'-CCAACAGCAGCT-CATTCAGC-3' (both in exon 8) as well as 5'-CCAGGCAGGGTCTGCTGATC-3' (in exon 9) were all tested as reverse primers for *Ae2c2*. Forward 5'-GACTACTACAGGAGGGAATC-3' and reverse 5'-AACTCGGATAAGCCCCAACAC-3' were the used primers for *Slc26a7* Cl⁻/HCO₃⁻ exchanger. Forward 5'-CTTC-TAGCTCAGACCCCCGATG-3' and reverse 5'-GTCTCG-GCTGCTAAGTAAGACAG-3' were the primers previously reported for the Na⁺/H⁺ exchanger *Nhe4*,³² whereas forward 5'-ACTGGAAGGACAAGCTCAAC-3' and reverse 5'-TGTGTCTGTTGTAGGACCGC-3' were the primers used for *Nhe1*. The H⁺/K⁺-ATPase β -subunit mRNA was also quantitated by using the forward 5'-GTGCATCTATGTGCTGATGC-3' and the reverse 5'-TGCACACGATGCTGACTTGC-3' primers. GAPDH mRNA was the normalizing control (with forward 5'-CCAAGGTCATCCATGACAA-C-3' and reverse 5'-TGT-CATACCAGGAAATGAGC-3').

Measurements of Serum Gastrin Levels

The serum levels of gastrin were measured in fasting mice by radioimmunoassay with the ¹²⁵I-Gastrin RIA kit (IBL, Hamburg, Germany).

Western Blot Analysis

After decapitation of the animals and stomach dissection, the corpus mucosa was extracted with a razor blade, washed, and briefly centrifuged. Protein extracts were prepared by adding RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L KCl, 1% IGEPAL, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) and a cocktail of protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) followed by sonication. After centrifugation, supernatants were mixed with loading buffer and either boiled (95°C for 5 minutes) or not. Samples run in 0.1% sodium dodecyl sulfate 4 to 20% gradient polyacrylamide gel electrophoresis were electrotransferred to nitrocellulose membranes. After blocking with 10% milk proteins for 1 hour at room temperature, membranes were incubated with our rabbit antibody against *Ae2* C-terminal peptide 1223–1237 (diluted 1:500), followed by incubation with peroxidase-conjugated secondary goat anti-rabbit antibody (Cell Signaling Technology, Beverly, MA) diluted 1:5000 and further visualization of bands with a Western Lightning chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, MA).

Statistical Analysis

Data are given as mean $\pm$ SD. For analysis of a normal distribution of quantitative values we used the KS var Lilliefors and Shapiro Wilks tests. Variables with a normal distribution were analyzed with the one-way analysis of

Figure 1. Quantitative real-time RT-PCR analysis of gastric expression levels of Ae2a, Ae2b1, Ae2b2, and Ae2c1 mRNAs, as well as those for the H⁺/K⁺-ATPase β-subunit, Slc26a7, Nhe4, and Nhe1 in unstimulated Ae2^{+/+}, Ae2_{a,b}^{+/-}, and Ae2_{a,b}^{-/-} mice. For each message, mean values in the wild-type mice are referred to as 100% of corresponding mRNA expression. Relative values obtained for each message were normalized with corresponding relative values for GAPDH. The **top right** diagram shows additional analysis of Ae2a mRNA but using a forward primer for exon 1 (see Materials and Methods) to detect mutant mRNA (exon 1/exon 3 splicing, without exon 2) in Ae2_{a,b}^{+/-} and Ae2_{a,b}^{-/-} samples. The **inset** shows the two products that may result from RT-PCR depending on the genotype: the expected 310-bp band derived from the complete mRNA for Ae2a in Ae2^{+/+} and Ae2_{a,b}^{+/-} gastric samples, and a lower 196-bp band derived from mutant mRNA without exon 2 in Ae2_{a,b}^{+/-} and Ae2_{a,b}^{-/-} samples. Shown bands correspond to amplifications in the linear phase after 30 cycles. RT-PCR for GAPDH mRNA was used as a normalizing control. In the 100-bp ladder to the **left**, the thick upper band is 600-bp long.

variance followed by the Student's *t*-test (number of PCNA-positive cells in the oxyntic gastric glands and serum gastrin levels) or by the Student-Newmann-Keuls (SNK) post hoc test (proton secretion values). A variable with no normal distribution (number of G cells in the antrum) was analyzed with the Mann-Whitney test. All *P* values were two-tailed.

Results

Transcriptional Expression of the Ae2 Gene and Other Acid Secretion-Related Genes in the Stomachs of Targeted Mice

As mentioned in the Introduction, five variants of Ae2 mRNA (Ae2a, Ae2b1, Ae2b2, Ae2c1, and Ae2c2) are

transcribed in mouse stomach from three different promoter sequences of the Ae2 gene.¹⁷ Because our targeting construct disrupted only Ae2a, Ae2b1, and Ae2b2 messages,²⁷ one could expect a possible compensatory increase in the expression of Ae2c transcripts, and more in particular of Ae2c1, which in mouse and rat has been reported to be stomach-specific.^{14,17,20,22,26,33} However, quantitation by real-time PCR revealed no increase in the gastric expression level of type c transcripts in the Ae2_{a,b}^{-/-} mice. In fact, the levels of Ae2c1 variant showed lower values in targeted mice compared with wild-type and heterozygous mice (both *P* < 0.05; see Figure 1). On the other hand, quantitation of Ae2c2 transcript expression gave no detectable signals in several samples of the three genotypes (not shown). This was the case despite trying the highly sensitive procedure of real-time PCR

with three different primer pairs, ie, a common forward primer specific for exon-1c₂ (which together with a reverse primer for Ae2 exon 6 is able to amplify traces of mouse genomic DNA) and three different reverse primers (see Materials and Methods). These results are in agreement with recently published data indicating that expression of Ae2c2 transcript is extremely low in most tissues.²⁶

When we amplified transcripts for Ae2a, Ae2b1, and Ae2b2 variants, PCR products with the expected sizes (235 bp for Ae2a, 207 bp for Ae2b1, and 208 bp for Ae2b2) were obtained in wild-type and heterozygous animals but not in *Ae2_{a,b}^{-/-}* mice (not shown). Quantitative data revealed that the expression levels of these three isoforms in heterozygous mice were each 75 to 80% (instead of 50%) of those in the wild type, suggesting an increased transcription from the single wild-type allele in *Ae2_{a,b}^{+/-}* mice (Figure 1).

In *Ae2_{a,b}^{-/-}* mice, amplification with a particular primer set (see Materials and Methods) could detect a mutant-transcript band (196-bp versus the 310-bp wild-type band) due to exon 1/exon 3 splicing in the absence of exon 2 (Figure 1, inset). In *Ae2_{a,b}^{+/-}* mice both the 196-bp and 310-bp bands could be detected. The efficiency of the exon 1/exon 3 splicing is ~25% of the wild-type splicing, as indicated by densitometry of the two bands detected in heterozygous specimens as well as by the values in real-time PCR quantitation between *Ae2_{a,b}^{-/-}* and *Ae2_{a,b}^{+/+}* samples (ie, in samples with single peaks). Sequence analysis of the mutant band showed that initial ATG codons are not in frame for Ae2 polypeptide, thus precluding Ae2 translation.

Real-time PCR quantitation of the levels of mRNAs for other proteins that are (or might be) involved in acid secretion such as the H⁺/K⁺-ATPase β -subunit, Slc26a7 (a Cl⁻/HCO₃⁻ exchanger previously localized at the basolateral membrane of parietal cells),¹⁵ and Nhe4 (a Na⁺/H⁺ exchanger proposed as possibly coupled with Ae2 in the parietal cells)³² as well as Nhe1, was also performed. As shown in Figure 1, expression levels of these messages showed no significant differences between the three genotypes. *Ae2_{a,b}^{-/-}* mice had a tendency to higher mean values than those observed in the wild-type animals (although with no statistical significance). This tendency in *Ae2_{a,b}^{-/-}* mice is slightly in contrast with the relative decreases (40 to 70%) in the expression levels of H⁺/K⁺-ATPase β -subunit and Slc26a7 mRNAs reported in the complete Ae2-null mice.¹⁶

Assessment of Ae2 Variant Proteins in the Stomachs of Targeted Mice

To analyze whether any Ae2 polypeptide is translated from the remaining Ae2c1 transcript in the *Ae2_{a,b}^{-/-}* mice, we performed Western blot analysis and immunofluorescence using a polyclonal rabbit antibody against the C-terminal sequence 1223 to 1237 conserved in all Ae2 isoforms. In gastric tissue preparations from both

Figure 2. Western blot analysis of corpus mucosa extracts from unstimulated mice. **A:** Immunoblot of boiled extracts from *Ae2^{+/+}*, *Ae2_{a,b}^{+/-}*, and *Ae2_{a,b}^{-/-}* mice with a rabbit polyclonal antibody against an Ae2 C-terminal peptide 1223-1237. **B:** Immunoblot of nonboiled extracts from *Ae2^{+/+}* and *Ae2_{a,b}^{-/-}* mice visualized with the Ae2 antibody. **C:** Immunoblot of non-boiled extracts from *Ae2^{+/+}* and *Ae2_{a,b}^{-/-}* mice using a monoclonal antibody against the H⁺/K⁺-ATPase β -subunit (Research Diagnostic Inc., Flanders, NJ).

wild-type and heterozygous mice boiled in sodium dodecyl sulfate sample buffer, a major band (>300 kD) was detected; this band was virtually absent in *Ae2_{a,b}^{-/-}* boiled preparations, with no additional bands of different size (Figure 2A). Nonboiled wild-type samples showed a much lower amount of the 300-kD molecular weight band, and instead two bands of ~165 kD and 150 kD appeared, which again were virtually absent in stomach preparations from *Ae2_{a,b}^{-/-}* mice (Figure 2B). Densitometric analysis of blots from either procedure revealed that the total residual amount of protein in *Ae2_{a,b}^{-/-}* mice represented at the most 3% of the amount observed in wild-type stomachs.

The 300-kD band detected with our Ae2 antibody has also been observed with different Ae2 antibodies by other investigators, who speculated that this most likely reflects Ae2 dimers.^{22,34,35} One hundred sixty-five kD is in turn the predicted size for glycosylated monomeric variants Ae2a, Ae2b1, and Ae2b2, whereas 150 kD is slightly greater than the size of 140 kD estimated for glycosylated monomeric Ae2c isoforms.³⁵ It is unlikely that the 150-kD band exclusively corresponds to Ae2c proteins because both the 150-kD and 160-kD bands are basically absent in the *Ae2_{a,b}^{-/-}* mice. We thus hypothesize that the 150-kD band in wild-type samples primarily represents a proteolytic breakdown product or differently glycosylated Ae2a-Ae2b1-Ae2b2 polypeptides. Still, we cannot exclude completely that this band may also contain a very small amount of the Ae2c1 polypeptide resulting from translation of residual Ae2c1 variant transcript. We therefore estimate that translation of Ae2 protein from this transcript should amount to 3% or less of the total Ae2 protein. In line with our quantitative data on the expression of H⁺/K⁺-ATPase β -subunit mRNA, Western blot analysis with an antibody against H⁺/K⁺-ATPase showed no differences at the protein level between mice of the three genotypes (Figure 2B).

Figure 3. Double-immunofluorescence staining of the parietal cells in the gastric glands of the oxyntic mucosa from unstimulated wild-type and $Ae2_{a,b}^{-/-}$ mice, by using a monoclonal antibody against the H^+/K^+ -ATPase β -subunit (visualized in green; Sigma, St. Louis, MO) and a rabbit polyclonal antibody against the Ae2 C-terminal peptide 1223-1237 (visualized in red). Intracellular immunostaining for H^+/K^+ -ATPase is observed in both wild-type and $Ae2_{a,b}^{-/-}$ parietal cells. However, the Ae2 antibody stains the basolateral membrane of the parietal cells in the wild-type parietal cells (arrowheads in corresponding merged images), but not the parietal cells of the $Ae2_{a,b}^{-/-}$ gastric glands. In both wild-type and $Ae2_{a,b}^{-/-}$ specimens, the Ae2 antibody can also recognize the trapped red blood cells (arrows in middle images) because of its cross-reactivity with Ae1. Original magnifications: $\times 200$ in the top rows for each genotype and $\times 1000$ in corresponding bottom rows.

Double-labeling immunofluorescence of wild-type specimens with our polyclonal antibody to the C-terminal Ae2 peptide and the H^+/K^+ -ATPase antibody localized Ae2 at the basolateral plasma membrane of the parietal cells (Figure 3), which concurs with a former report.¹⁵ Parietal cells from $Ae2_{a,b}^{-/-}$ mice showed labeling of H^+/K^+ -ATPase, but there was no detectable Ae2 immunoreactivity (Figure 3). In $Ae2_{a,b}^{-/-}$ mucosa our Ae2 antibody only stained a few erythrocytes, because it can also recognize the C-terminus in erythrocyte Ae1 due to its high homology with the Ae2 C-terminus. The different staining pattern of $Ae2_{a,b}^{+/+}$ and $Ae2_{a,b}^{-/-}$ samples authenticates that the Ae2 antibody recognizes Ae2 and confirms that if there is any expression of Ae2c isoforms in the $Ae2_{a,b}^{-/-}$ oxyntic

mucosa it occurs at very low and undetectable levels in immunofluorescence.

Gastric Acid Secretion in $Ae2_{a,b}^{-/-}$ Mice

To assess parietal cell function we performed gastric perfusion of wild-type, heterozygous, and $Ae2_{a,b}^{-/-}$ mice under basal conditions and on stimulation with histamine and carbachol. Titration of the effluent showed similar basal H^+ secretion rate in all genotypes ($\sim 0.6 \mu\text{Eq } H^+/15 \text{ minutes}$; Figure 4). Histamine/carbachol stimulation resulted in a sixfold increase in H^+ secretion in wild-type mice whereas a significantly lower increase (2.5-fold) was found in $Ae2_{a,b}^{-/-}$ animals (Figure 4, left); the stimulation pattern in heterozygotes was similar to that in wild-type mice (Figure 4, right). The differences in stimulated proton secretion rates were highly significant ($P < 0.001$ for $Ae2_{a,b}^{-/-}$ versus $Ae2^{+/+}$ and $Ae2_{a,b}^{+/-}$). Finally, time-course analysis of intragastric pH after histamine/carbachol stimulation showed that $Ae2_{a,b}^{-/-}$ mice took much longer (60 minutes after stimulation) to reach their minimal pH of 4.0 compared with wild-type and heterozygous mice, which reached pH 3.5 at 30 minutes.

Hypergastrinemia and G-Cell Hyperplasia in $Ae2_{a,b}^{-/-}$ Mice

Serum gastrin levels in $Ae2_{a,b}^{-/-}$ were significantly higher than in wild types ($144 \pm 48 \text{ pmol/L}$ in $Ae2_{a,b}^{-/-}$ mice versus $89 \pm 52 \text{ pmol/L}$ in wild-type mice; $P < 0.01$). Gastrin levels of heterozygotes were not different from those in wild-type animals ($68 \pm 47 \text{ pmol/L}$). Staining of the antral gastric mucosa for gastrin revealed a threefold increased number of G cells in $Ae2_{a,b}^{-/-}$ mice compared to wild-type animals (an average of 35 ± 7.6 $Ae2_{a,b}^{-/-}$ cells/field versus 12.7 ± 3.2 wild-type cells/field, $P = 0.0011$; Figure 5). The number of G cells in heterozygous mice was similar to that in wild-type animals (not shown).

Morphological Alterations of Parietal Cells

The observation of hypergastrinemia and hyperplasia of G cells in $Ae2_{a,b}^{-/-}$ mice suggested that the gastric tissue in $Ae2_{a,b}^{-/-}$ mice is in an increased state of activation as an attempt to compensate for impaired acid secretion. Immunohistochemical staining with antibodies against H^+/K^+ -ATPase, the proton pump directly responsible for the gastric acid secretion, confirmed this (Figures 6 and 7). Staining of parietal cells in $Ae2_{a,b}^{-/-}$ mice indicated that there is a fusion of intracellular H^+/K^+ -ATPase-containing vesicles with the apical membrane of the parietal cells both with and without histamine/carbachol stimulation, whereas in wild-type mice this fusion was only observed on stimulation (Figure 6). The occurrence of such a fusion in unstimulated $Ae2_{a,b}^{-/-}$ parietal cells was further confirmed through co-labeling with H^+/K^+ -ATPase anti-

Figure 4. Gastric proton secretion rate in $Ae2^{+/+}$, $Ae2_{a,b}^{+/-}$, and $Ae2_{a,b}^{-/-}$ mice. **Left:** Wild-type mice (filled circles) and $Ae2_{a,b}^{-/-}$ mice (open circles). **Right:** Wild-type mice (filled circles) and $Ae2_{a,b}^{+/-}$ mice (open triangles). Fractions of the gastric fluid were collected during 15 minutes and titrated to neutrality to determine proton content. Both histamine and carbachol were administered after 60 minutes from the initiation of the study. Proton secretion rates (y-axis) are expressed as $\mu\text{Eq. H}^+$ /15 minutes; asterisks, $P < 0.001$; statistical differences between $Ae2_{a,b}^{-/-}$ and $Ae2_{a,b}^{+/-}$ (not shown) were like those obtained when comparing $Ae2_{a,b}^{-/-}$ versus $Ae2_{a,b}^{+/+}$ (left).

body and TRITC-conjugated phalloidin (a toxin that binds essentially to actin microfilaments, ie, to F-actin or polymerized actin,³⁶ but not to the depolymerized or globular G-actin). F-actin is associated with the membranes of the secretory canaliculus but not with the resting tubulovesicles.^{37,38} Thus, unstimulated normal parietal cells have no polymerized F-actin, but rather depolymerized G-actin, and are expected not to bind labeled phalloidin. Certainly, this was the case in our unstimulated wild-type parietal cells (Figure 7). However, unstimulated $Ae2_{a,b}^{-/-}$ parietal cells showed apical immunofluorescence signals from TRITC-conjugated phalloidin, which co-localized with the H^+/K^+ -ATPase immunofluorescence, characteristic of parietal cell activation (Figure 7). In addition to these features in the unstimulated $Ae2_{a,b}^{-/-}$ mice, immunoperoxidase staining for H^+/K^+ -ATPase suggested that a fraction of

their parietal cells undergo degenerative processes (Figure 6D). Because $Ae2$ is thought to function in pH_i correction during apical proton secretion, it is possible that, in the absence of this function, parietal cells are damaged because of deficient pH_i homeostasis during apical proton secretion.

Electron microscopy of gastric mucosa (Figure 8) provided a striking difference in morphology between $Ae2_{a,b}^{-/-}$ and wild-type parietal cells. Unstimulated $Ae2_{a,b}^{-/-}$ parietal cells showed well-developed tubulovesicular system and collapsed canaliculi with no lumen (Figure 8A), that resemble recycling early secretory elements³⁹; moreover, mitochondria appeared concentrated at the cell periphery in these unstimulated $Ae2_{a,b}^{-/-}$ parietal cells (Figure 8A). On stimulation, $Ae2_{a,b}^{-/-}$ parietal cells showed alterations of the tubulovesicular system, which varied in severity from collapsed canaliculi and decreased number of tubulovesicles (Figure 8B) to highly distorted canaliculi (Figure 8C). Also, stimulated $Ae2_{a,b}^{-/-}$ parietal cells showed a lower electron density, the periphery of the cell becoming rather free from organelles (see Figure 8, B and C, for stimulated targeted cell and compare with Figure 8E for stimulated wild-type cell). Additionally, mitochondria in stimulated $Ae2_{a,b}^{-/-}$ parietal cells appeared more dispersed than in basal conditions, and some showed intramitochondrial holes and distorted cristae (Figure 8G). On the other hand, both unstimulated and stimulated $Ae2_{a,b}^{-/-}$ parietal cells contained a higher number of dense lysosomes than the wild-type controls.

H^+/K^+ -ATPase immunohistochemistry indicated that the number and localization of parietal cells within the glands in the oxyntic mucosa were rather similar in $Ae2_{a,b}^{-/-}$ and wild-type mice (not shown). Although there were some parietal cells undergoing degenerative processes in the $Ae2_{a,b}^{-/-}$ mucosa (Figure 6D), a TUNEL assay did not display increased apoptosis (not shown). In any case, the maintenance of a normal number of parietal cells despite increased degenerative changes suggested an increased turnover of these cells in the $Ae2_{a,b}^{-/-}$ mice. We therefore stained gastric mucosa for PCNA. In wild-type oxyntic glands only

Figure 5. Antral mucosa of the stomach from unstimulated wild-type (A) and $Ae2_{a,b}^{-/-}$ (B) mice were stained with an antibody to gastrin. For each animal, cell count was performed in five serial sections of the antrum; five to seven fields of the antral mucosa, in which the pyloric glands are viewed longitudinally, were selected and the average number of gastrin-positive cells (G-cells) per field was estimated. A significant increase in the number of G cells was found in the $Ae2_{a,b}^{-/-}$ antral mucosa. Original magnifications, $\times 200$.

Figure 6. Immunoperoxidase staining of mouse parietal cells for H^+/K^+ -ATPase. **A:** Oxyntic gastric glands from unstimulated $Ae2_{a,b}^{+/+}$ mice show dispersed intracellular immunostaining of parietal cells. **B:** After histamine/carbachol stimulation in the wild-type mice, H^+/K^+ -ATPase localizes to the secretory canaliculi in oxyntic glands as expected because of fusion of intracellular H^+/K^+ -ATPase-containing vesicles with the apical membrane. **C:** In unstimulated $Ae2_{a,b}^{-/-}$ oxyntic glands, H^+/K^+ -ATPase immunostaining reveals a pattern that is very similar to that observed in stimulated wild-type glands (arrowheads in **B** and **C**), as it localizes to the secretory canaliculi rather than spread in the cytoplasm of the parietal cells. Such a staining pattern in unstimulated $Ae2_{a,b}^{-/-}$ oxyntic glands suggests an activation state of targeted parietal cells even under basal conditions. **D:** In stimulated $Ae2_{a,b}^{-/-}$ oxyntic glands there is strong H^+/K^+ -ATPase labeling at the secretory canaliculi; arrows indicate parietal cells undergoing degenerative processes (eg, vacuolation and lysis). **E** and **F:** Oxyntic glands from unstimulated and stimulated $Ae2_{a,b}^{-/-}$ mice, respectively, which further illustrates the degenerative changes in the parietal cells (arrows). Original magnifications: $\times 400$ (**A–D**); $\times 600$ (**E** and **F**).

a few PCNA-expressing cells were present in the neck of each gland (Figure 9), in accordance with the reported localization of stem cells.⁴⁰ The number of PCNA-positive cells was more than threefold increased in $Ae2_{a,b}^{-/-}$ oxyntic glands (Figure 9), which supports that cell turnover might be increased in gastric glands of the $Ae2_{a,b}^{-/-}$ mice.

Discussion

According to the current mechanistic models, HCl secretion across the apical membrane of the parietal cell requires basolateral Cl^-/HCO_3^- exchange activity.^{7–11} This prevents intracellular alkalization and drives vectorial Cl^- transport. We show that Ae2 fulfils this function, particularly in the stimulated state. We found that in the

$Ae2_{a,b}^{-/-}$ mice, gastric acid secretion is severely impaired under stimulated conditions but normal under basal conditions. These results are at variance with an earlier report of Gawenis and colleagues.¹⁶ In mice with a complete disruption of the entire Ae2 gene, they observed that gastric contents after histamine stimulation had a neutral pH, suggesting that acid secretion is completely abrogated in the absence of all Ae2 isoforms. The information from this study in combination with our present results cannot exclude that residual expression of Ae2c1 isoform in our targeted animals ($<3\%$ of the total Ae2 protein in the wild-type mice, and undetectable by immunofluorescence) might contribute to support the low acid secretion in the basal state. It must be borne in mind, however, that additional factors most probably contribute to these different results. Firstly, we have measured acid

Figure 7. Assessment of abnormal activation state in unstimulated $Ae2_{a,b}^{-/-}$ parietal cells by co-localizing H^{+}/K^{+} -ATPase (visualized in green) and polymerized F-actin (visualized in red) to the secretory canaliculi (**top**). H^{+}/K^{+} -ATPase was labeled by using the antibody against H^{+}/K^{+} -ATPase β -subunit (Sigma) while polymerized F-actin was detected with TRITC-conjugated phalloidin (Sigma). Phalloidin only binds actin when it is polymerized in actin microfilaments (F-actin) but not when it is dissociated into actin globular subunits (G-actin).³⁶ Thus, in unstimulated normal parietal cells no phalloidin staining should be observed because actin is normally depolymerized in G-actin under basal conditions; this was the case in the control unstimulated wild-type sample in which no TRITC-conjugated phalloidin red immunofluorescence was observed while there occurred intracellular green immunofluorescence due to H^{+}/K^{+} -ATPase immunostaining (**bottom**). Original magnifications, $\times 400$.

secretion in empty *in situ* perfused stomachs, both under basal and histamine/carbachol stimulated conditions. Gawenis and colleagues¹⁶ only measured the steady state pH of gastric contents *ex vivo* 15 minutes after subcutaneous injection of histamine. With our method actual proton secretion rates are measured, which is more sensitive to alterations than measuring the steady state pH of gastric contents. Secondly, the genetic background of the two knockout models was different: our animals were bred against FVB/N background whereas the mice of Gawenis and colleagues¹⁶ were of mixed 129S6/SvEv and Black Swiss background. Absence of Ae2 may have generated a more severe phenotype against the latter background than in our model. Indeed, Gawenis and colleagues¹⁶ reported that at the time gastric pH was measured (18 days of age) the $Ae2^{-/-}$ mice had stopped feeding well, exhibited severe growth retardation, became emaciated, and showed evidence of dehydration (skin tenting). In fact, $Ae2^{-/-}$ mice exhibited high morbidity and mortality after 15 days of age, with the oldest $Ae2^{-/-}$ mouse surviving to only 40 days of age.¹⁶ In our $Ae2_{a,b}^{-/-}$ mice there is some antenatal and perinatal death.²⁷ However, the surviving animals do not display the severe symptoms described in the complete knockout¹⁶ and have a normal life span, although our $Ae2_{a,b}^{-/-}$ show deafness and defective bone development (unpublished observations) similarly to what was reported for $Ae2^{-/-}$ mice.¹⁶ It is unlikely that the observed difference in severity of phenotype is caused by the expression of the Ae2c isoforms because expression

of Ae2c1 is limited to the stomach, and in addition to its extremely low widespread expression, Ae2c2 appears to be inactive.²⁶ Several knockout models exist with complete abrogation of gastric acid secretion and those animals are quite healthy.²

The fact that acid secretion is only partly impaired in our targeted animals raises the question of which other mechanisms of intracellular acidification may be active. A new Na^{+} -independent electroneutral Cl^{-}/HCO_3^{-} exchanger protein named Slc26a7 has been localized at the basolateral membrane of the parietal cell,¹⁵ and it could account for the partial acid secretion capacity observed in $Ae2_{a,b}^{-/-}$ mice in their early adulthood. Transcriptional expression of *Slc26a7* was found to be conserved in the corpus mucosa from our $Ae2_{a,b}^{-/-}$ mice (Figure 1B). It could be that this exchanger contributes to maintenance of a neutral pH_i under basal conditions but fails to do so during high flux conditions, in which Ae2 is the principal exchanger. It is known that the activity of Ae2 is highly dependent on the cytosolic pH (it steeply increases at pH greater than 7),²³ whereas Slc26a7 activity appears to tolerate a wider spectrum of pH_i .¹⁵ However, the observed achlorhydria in the complete Ae2 knockout mice¹⁶ does not support the possible subsidiary role for Slc26a7. It must be mentioned though that the mRNA levels for this Cl^{-}/HCO_3^{-} exchanger in those severely distressed $Ae2^{-/-}$ mice were reduced to $\sim 40\%$ of wild-type levels.¹⁶

We have observed that fasting $Ae2_{a,b}^{-/-}$ mice have hyperplasia of G cells (Figure 5) and hypergastrinemia. High circulating gastrin levels are often encountered in

Figure 8. Electron microscopy of the parietal cells in the oxyntic mucosa from unstimulated *Ae2_{a,b}^{-/-}* mice (A), stimulated *Ae2_{a,b}^{-/-}* mice (B–C), unstimulated wild-type mice (D), and stimulated wild-type mice (E). Unstimulated *Ae2_{a,b}^{-/-}* parietal cells (A) show a well-developed tubulovesicular system (TV) but also collapsed canaliculi with no lumen (arrows) and mitochondria located at the periphery of the cell (MT). In stimulated *Ae2_{a,b}^{-/-}* mice, there are parietal cells (B) with collapsed canaliculi (arrows), decreased tubulovesicles, and peripheral space somewhat free from organelles (arrowheads). There are also parietal cells in stimulated *Ae2_{a,b}^{-/-}* oxyntic glands (C) in which canaliculi are broken and rather disorganized (dotted circles). The dotted circle in E indicates a well-organized canaliculi in a stimulated wild-type parietal cell. F and G: Higher magnification of stimulated wild-type and *Ae2_{a,b}^{-/-}* parietal cells, respectively. In the latter, some mitochondria (MT) with intramitochondrial holes and distorted cristae are shown. Original magnifications, $\times 3350$.

animal models with low gastric acid secretion,² which may represent a compensatory mechanism. It seems that the hypergastrinemia observed in our targeted mice causes parietal cell stimulation even in the basal state.

Figure 9. PCNA staining of gastric oxyntic glands from unstimulated wild-type (A) and *Ae2_{a,b}^{-/-}* (B) mice. For each animal, cell count was performed in five serial sections of the corpus of the stomach; five to seven fields of the oxyntic mucosa, in which the oxyntic glands are viewed longitudinally, were selected and the average number of PCNA-positive cells per field was estimated. The number of PCNA-positive cells was more than threefold increased in *Ae2_{a,b}^{-/-}* oxyntic glands, indicating increased cell division and proliferation in these glands. Original magnifications, $\times 200$.

Immunoperoxidase labeling for H^+/K^+ -ATPase, the proton pump directly responsible for the gastric acid secretion, as well as immunofluorescent co-labeling for H^+/K^+ -ATPase and microfilaments of polymerized actin both revealed that unstimulated *Ae2_{a,b}^{-/-}* parietal cells exhibit a fusion of the tubulovesicular system and pericanalicular redistribution of H^+/K^+ -ATPase-containing vesicles (Figures 6 and 7). In the wild-type parietal cell, it has been described that these changes only occur on stimulation and constitute a characteristic pattern of the secreting status.^{37,41} Electron microscopy confirmed the activation-like pattern in the unstimulated *Ae2_{a,b}^{-/-}* parietal cell, but with a well-developed tubulovesicular system and collapsed canaliculi (Figure 8A) that resemble recycling early secretory elements.³⁹ These changes were accompanied by mitochondrial alterations with intramitochondrial holes and distorted cristae and increased numbers of lysosomes. Subcellular alterations in the *Ae2_{a,b}^{-/-}* parietal cell worsened with secretory stimulation. These alterations together with the previously mentioned degenerative features observed in targeted parietal cells by immunoperoxidase staining for H^+/K^+ -ATPase (but with no evidence for substantial apoptosis) pointed toward severe damage and death of a fraction of parietal cells by necrosis. Despite that, the total number

of parietal cells seemed to be maintained. Most probably this could be achieved by a compensatory proliferation of parietal cells, as suggested by increased PCNA staining of the $Ae2_{a,b}^{-/-}$ oxyntic gastric glands.

In conclusion, our data show that Ae2 is relevant for pH homeostasis under conditions of increased proton flux in parietal cells and that under stimulated conditions this function is primarily achieved by the Ae2a and Ae2b isoforms, whereas minute levels of the Ae2c1 isoform and maintained expression of the Slc26a7 Cl^-/HCO_3^- exchanger both might be partially sufficient to counterbalance pH alkalization under basal conditions. The shortage in the cytosolic acidification system may lead to the enhanced parietal cell degeneration that might be compensated by enhanced parietal cell proliferation.

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References

- Dockray GJ, Varro A, Dimaline R, Wang T: The gastrins: their production and biological activities. *Annu Rev Physiol* 2001, 63:119–139
- Samuelson LC, Hinkle KL: Insights into the regulation of gastric acid secretion through analysis of genetically engineered mice. *Annu Rev Physiol* 2003, 65:383–400
- Schmidt WE, Schmitz F: Genetic dissection of the secretory machinery in the stomach. *Gastroenterology* 2004, 126:606–609
- Agnew BJ, Duman JG, Watson CL, Coling DE, Forte JG: Cytological transformations associated with parietal cell stimulation: critical steps in the activation cascade. *J Cell Sci* 1999, 112:2639–2646
- Yao X, Forte JG: Cell biology of acid secretion by the parietal cell. *Annu Rev Physiol* 2003, 65:103–131
- Sherry AM, Malinowska DH, Morris RE, Ciruolo GM, Cuppoletti J: Localization of ClC-2 Cl^- channels in rabbit gastric mucosa. *Am J Physiol* 2001, 280:C1599–C1606
- Muallem S, Blissard D, Cragoe Jr EJ, Sachs G: Activation of the Na^+/H^+ and Cl^-/HCO_3^- exchange by stimulation of acid secretion in the parietal cell. *J Biol Chem* 1988, 263:14703–14711
- Seidler U, Carter K, Ito S, Silen W: Effect of CO_2 on pH_i in rabbit parietal, chief, and surface cells. *Am J Physiol* 1989, 256:G466–G475
- Paradiso AM, Townsley MC, Wenzl E, Machen TE: Regulation of intracellular pH_i in resting and in stimulated parietal cells. *Am J Physiol* 1989, 257:C554–C561
- Thomas HA, Machen TE: Regulation of Cl^-/HCO_3^- exchange in gastric parietal cells. *Cell Regul* 1991, 2:727–737
- Seidler U, Hubner M, Roithmaier S, Classen M: pH_i and HCO_3^- dependence of proton extrusion and Cl^- -base exchange rates in isolated rabbit parietal cells. *Am J Physiol* 1994, 266:G759–G766
- Stuart-Tilley A, Sardet C, Pouyssegur J, Schwartz MA, Brown D, Alper SL: Immunolocalization of anion exchanger AE2 and cation exchanger NHE-1 in distinct adjacent cells of gastric mucosa. *Am J Physiol* 1994, 266:C559–C568
- Jons T, Warrings B, Jons A, Drenckhahn D: Basolateral localization of anion exchanger 2 (AE2) and actin in acid-secreting (parietal) cells of the human stomach. *Histochemistry* 1994, 102:255–263
- Rossmann H, Alper SL, Nader M, Wang Z, Gregor M, Seidler U: Three 5'-variant mRNAs of anion exchanger AE2 in stomach and intestine of mouse, rabbit, and rat. *Ann NY Acad Sci* 2000, 915:81–91
- Petrovic S, Ju X, Barone S, Seidler U, Alper SL, Lohi H, Kere J, Soleimani M: Identification of a basolateral Cl^-/HCO_3^- exchanger specific to gastric parietal cells. *Am J Physiol* 2003, 284:G1093–G1103
- Gawenis LR, Ledoussal C, Judd LM, Prasad V, Alper SL, Stuart-Tilley A, Woo AL, Grisham C, Sanford LP, Doetschman T, Miller ML, Shull GE: Mice with a targeted disruption of the AE2 Cl^-/HCO_3^- exchanger are achlorhydric. *J Biol Chem* 2004, 279:30531–30539
- Lecanda J, Urtasun R, Medina JF: Molecular cloning and genomic organization of the mouse AE2 anion exchanger gene. *Biochem Biophys Res Commun* 2000, 276:117–124
- Aranda V, Martinez I, Melero S, Lecanda J, Banales JM, Prieto J, Medina JF: Shared apical sorting of anion exchanger isoforms AE2a, AE2b₁, and AE2b₂ in primary hepatocytes. *Biochem Biophys Res Commun* 2004, 319:1040–1046
- Medina JF, Lecanda J, Acín A, Ciesielczyk P, Prieto J: Tissue-specific N-terminal isoforms from overlapping alternate promoters of the human AE2 anion exchanger gene. *Biochem Biophys Res Commun* 2000, 267:228–235
- Wang Z, Schultheis PJ, Shull GE: Three N-terminal variants of the AE2 Cl^-/HCO_3^- exchanger are encoded by mRNAs transcribed from alternative promoters. *J Biol Chem* 1996, 271:7835–7843
- Stuart-Tilley AK, Shmukler BE, Brown D, Alper SL: Immunolocalization and tissue-specific splicing of AE2 anion exchanger in mouse kidney. *J Am Soc Nephrol* 1998, 9:946–959
- Alper SL, Rossmann H, Wilhelm S, Stuart-Tilley AK, Shmukler BE, Seidler U: Expression of AE2 anion exchanger in mouse intestine. *Am J Physiol* 1999, 277:G321–G332
- Stewart AK, Chernova MN, Shmukler BE, Wilhelm S, Alper SL: Regulation of AE2-mediated Cl^- transport by intracellular or by extracellular pH requires highly conserved amino acid residues of the AE2 NH_2 -terminal cytoplasmic domain. *J Gen Physiol* 2002, 120:707–722
- Chernova MN, Stewart AK, Jiang L, Friedman DJ, Kunes YZ, Alper SL: Structure-function relationships of AE2 regulation by Ca^{2+} -sensitive stimulators NH_4^+ and hypertonicity. *Am J Physiol* 2003, 284:C1235–C1246
- Stewart AK, Kerr N, Chernova MN, Alper SL, Vaughan-Jones RD: Acute pH-dependent regulation of AE2-mediated anion exchange involves discrete local surfaces of the NH_2 -terminal cytoplasmic domain. *J Biol Chem* 2004, 279:52664–52676
- Kurschat CE, Shmukler BE, Jiang L, Wilhelm S, Kim EH, Chernova MN, Kinne RK, Stewart AK, Alper SL: Alkaline-shifted pH_o sensitivity of AE2c1-mediated anion exchange reveals novel regulatory determinants in the AE2 N-terminal cytoplasmic domain. *J Biol Chem* 2006, 281:1885–1896
- Medina JF, Recalde S, Prieto J, Lecanda J, Saez E, Funk CD, Vecino P, van Roon MA, Ottenhoff R, Bosma PJ, Bakker CT, Oude Elferink RPJ: Anion exchanger 2 is essential for spermiogenesis in mice. *Proc Natl Acad Sci USA* 2003, 100:15847–15852
- Wang KS, Komar AR, Ma T, Filiz J, McLeroy J, Hoda K, Verkman AS, Bastidas JA: Gastric acid secretion in aquaporin-4 knockout mice. *Am J Physiol* 2000, 279:G448–G453
- Cook HC: Periodic acid-Schiff-toluidine blue-aurantia: a stain for the gland cells of the stomach. *Stain Technol* 1962, 37:317–319
- Hsu SM, Raine L, Fanger H: The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase techniques. *Am J Clin Pathol* 1981, 75:816–821
- Shu SY, Ju G, Fan LZ: The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988, 85:169–171
- Gawenis LR, Greeb JM, Prasad V, Grisham C, Sanford LP, Doetschman T, Andringa A, Miller ML, Shull GE: Impaired gastric acid secretion in mice with a targeted disruption of the NHE4 Na^+/H^+ exchanger. *J Biol Chem* 2005, 280:12781–12789
- Chen D, Zhao CM, Hakanson R, Rehfeld JF: Gastric phenotypic abnormality in cholecystokinin 2 receptor null mice. *Pharmacol Toxicol* 2002, 91:375–381
- Zolotarev AS, Shmukler BE, Alper SL: AE2 anion exchanger polypeptide is a homooligomer in pig gastric membranes: a chemical cross-linking study. *Biochemistry* 1999, 38:8521–8531
- Frische S, Zolotarev AS, Kim YH, Praetorius J, Alper S, Nielsen S, Wall SM: AE2 isoforms in rat kidney: immunohistochemical localization and regulation in response to chronic NH_4Cl loading. *Am J Physiol* 2004, 286:F1163–F1170
- Cooper JA: Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 1987, 105:1473–1478
- Mercier F, Reggio H, Devilliers G, Bataille D, Mangeat P: Membrane-

- cytoskeleton dynamics in rat parietal cells: mobilization of actin and spectrin upon stimulation of gastric acid secretion. *J Cell Biol* 1989, 108:441–453
38. Nguyen NV, Gleeson PA, Courtois-Coutry N, Caplan MJ, Van Driel IR: Gastric parietal cell acid secretion in mice can be regulated independently of H/K ATPase endocytosis. *Gastroenterology* 2004, 127:145–154
39. Forte JG, Black JA, Forte TM, Machen TE, Wolosin JM: Ultrastructural changes related to functional activity in gastric oxyntic cells. *Am J Physiol* 1981, 241:G349–G358
40. Karam SM: Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci* 1999, 4:D286–D296
41. Forte JG, Hanzel DK, Urushidani T, Wolosin JM: Pumps and pathways for gastric HCl secretion. *Ann NY Acad Sci* 1989, 574:145–158