Phosphorylation of ezrin on threonine 567 produces a change in secretory phenotype and repolarizes the gastric parietal cell

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Summary

Phosphorylation of the membrane-cytoskeleton linker protein ezrin has been functionally linked to acid secretion and vesicle recruitment to the apical secretory membrane in gastric parietal cells. Phosphorylation of the conserved T567 residue of ezrin has been shown to alter the N/C oligomerization of ezrin and promote the formation of actin-rich surface projections in other cells. To test the importance of T567 as a regulatory site for ezrin in parietal cell activation, we incorporated wild-type (WT) and mutant forms of ezrin, including the nonphosphorylatable T567A mutation and a mutant mimicking permanent phosphorylation, T567D. All ezrin constructs included C-terminal cyan-fluorescent protein (CFP) and were incorporated into adenoviral constructs for efficient introduction into cultured parietal cells from rabbit stomach. Fluorescence microscopy was used to localize CFP-ezrin and monitor morphological responses. Accumulation of a weak base (aminopyrine) was used to monitor receptor-mediated acid secretory response of the cultured cells. Similar to endogenous ezrin, WT and T567A CFP-ezrin localized heavily to apical membrane vacuoles with considerably lower levels associated with the surrounding basolateral membrane. Interestingly, H,K-ATPase within cytoplasmic tubulovesicles was incorporated into the apical vacuoles along with WT and T567A mutant ezrin. In these parietal cells secretagogue stimulation produced a striking vacuolar expansion associated with HCl secretion and the secretory phenotype. Expression of T567D CFP-ezrin was quite different, being rarely associated with apical vacuoles. T567D was more typically localized to the basolateral membrane, often associated with long spikes and fingerlike projections. Moreover, the cells did not display secretagogue-dependent morphological changes and, to our surprise, H,K-ATPase was recruited to the T567D CFP-ezrin-enriched basolateral projections. We conclude that T567 phosphorylation, which is probably regulated through Rho signaling pathway, may direct ezrin to membrane-cytoskeletal activity at the basolateral membrane and away from apical secretory activity. The large basolateral expansion is predicted to recruit membranes from sources not normally targeted to that surface.

Key words: ERM proteins, Acid secretion, Cytoskeleton, Rho, Rho kinase

Introduction

Ezrin is a member of the ERM (ezrin/radixin/moesin) family of proteins that is implicated in linking functional activities of the plasma membrane to the actin cytoskeleton. The linkage and regulatory functions of ezrin are thought to occur via phosphorylation of one or several of the potential sites along its 300+ amino acid length. It may well be that some of the phosphorylation sites are specific to the cell type, or more appropriately the signaling pathway, that might ultimately lead to different cellular responses. In almost all cases the functional activity of ezrin is manifest at the cell membrane where it participates in the formation of a variety of surface projections, including microspikes, microvilli, filopodia, folds and lamellipodia.

Ezrin has been shown to be phosphorylated on tyrosine residues, particularly on Tyr145 and Tyr353, upon growth factor stimulation (Chen et al., 1994; Gould et al., 1986; Simons et al., 1998). However, prevention of Tyr phosphorylation by point mutation does not alter ezrin localization to microvilli, nor does it alter cell morphology (Crepaldi et al., 1997).

Phosphorylations on serine and/or threonine have been shown to have direct effects on the functional activity of ezrin. In A-431 cells stimulated by epidermal growth factor (EGF), Bretscher (Bretscher, 1989) observed increased ^{32}P incorporation into serine, as well as tyrosine, that correlated with surface folds and microspike formation. Identification of ezrin in gastric parietal cells was first made on the basis of the increased level of ^{32}P incorporated into an 80 kDa protein (later called ezrin) when the cells were stimulated to secrete acid via the cAMP pathway (Urushidani et al., 1987). Subsequent studies showed that apically polarized ezrin is a necessary component for HCl secretion by parietal cells, which also depends on a massive membrane recruitment of proton pumps into the apical membrane (Yao and Forte, 2003; Yao et al., 1993). Phosphoamino analysis of ezrin from stimulated

parietal cells revealed primary incorporation of ^{32}P into phosphoserine, with virtually no detectable levels of phoshothreonine or phosphotyrosine (Urushidani et al., 1989). Indeed, subsequent studies in primary parietal cell cultures indicated that Ser66 may play a positive supporting role in the apical membrane expansion that was associated with cell stimulation and secretion (Zhou et al., 2003a).

In a variety of cells and tissues an important phosphorylation site was identified in the C-terminal region of moesin (Nakamura et al., 1995), which was later shown to be conserved and phosphorylated among ERM proteins: Thr558 in moesin; Thr567 in ezrin; and Thr564 in radixin (Hayashi et al., 1999; Matsui et al., 1998). Many investigators agree that phosphorylation at this site is regulated through the Rho small GTPase signaling pathway, but there seems to be a great deal of variance in specifying possible downstream effectors and regulators, depending on the system studied, and whether the experiments were made in vivo or in vitro (Fukata et al., 1998; Hishiya et al., 1999; Maeda et al., 2002; Matsui et al., 1998; Matsui et al., 1999; Oshiro et al., 1998; Pietromonaco et al., 1998). T564 phosphorylation of the recombinant COOH-terminal half of radixin did not affect its ability to bind to actin filaments in vitro, but significantly suppressed its direct interaction with the NH2-terminal half of full-length radixin, suggesting an impairment of N/C-ERMAD interaction (Matsui et al., 1998). These observations are supported by studies on the crystal structure of moesin (Pearson et al., 2000). Collectively these data indicate that Rho-kinase-dependent phosphorylation interferes with the intramolecular and/or intermolecular head-to-tail association of ERM proteins.

The expression of mutant forms of moesin or ezrin in which T558 or T567, respectively, was mutated to Ala (preventing phosphorylation) or to Asp (simulating permanent phosphorylation) has prominent effects on the induction of surface membrane elaborations in several systems. When COS cells expressing wild-type or moesin T558A mutant were cultured under serum-depleted conditions, there was a depletion of microvilli-like structures, whereas microvilli remained in cells expressing the moesin T558D mutant (Oshiro et al., 1998). In addition, the expression of moesin T558A prevented RhoA-induced formation of microvilli, suggesting that Rho-kinase regulates moesin phosphorylation downstream of Rho. Thus, phosphorylation at T558 plays a crucial role in the formation of microvilli. In LLC-PK1 epithelial cells stably transfected with the T567D mutant form of ezrin, there was an observed shift from inactive ezrin oligomers to active monomers, which correlated with the formation of surface lamellipodia, membrane ruffles and tufts of microvilli (Gautreau et al., 2000). Thus, phosphorylation of ERM proteins at threonine in the C-terminus is an important step in their activation and cytoskeleton-membrane interaction.

The purpose of the present study was to examine the role of phosphorylation of ezrin T567 on the structural and functional activity of gastric acid secretory cells. The approach was to use adenoviral constructs of wild-type (WT) and mutant forms of ezrin (T567A and T567D) to introduce expression of ezrin protein including a cyan fluorescent protein (CFP) marker into primary cultures of parietal cells. The experiments were designed (1) to discriminate the cytolocalization of the labeled ezrin markers in the basal, nonsecreting state of parietal cells, (2) to examine the functional and morphological responses of the cells when stimulated to secrete acid, and (3) to determine the influence that the various ezrin forms have on the distribution of important structural proteins (e.g. actin) and cargo proteins (e.g. H,K-ATPase) involved in regulated membrane translocation and recruitment. We found that phosphorylation of ezrin on T567 was not essential for either the normal targeting of ezrin to the apical microvillar surface or the phenotypic acid secretory response of parietal cells. On the contrary, introduction of the T567D mutant, simulating permanent phosphorylation at that site, produced striking changes to parietal cell polarity and phenotype. The cells were devoid of a secretory response, T567D ezrin was almost entirely expressed at the basolateral membrane, usually in the form of dense, long, microvillar projections, and this change in cell polarity involved the redistribution of membrane and cargo protein (e.g. H,K-ATPase) from the rest of the cell.

Materials and Methods

Primary culture of gastric parietal cells from rabbit

All procedures and treatments for handling animals were reviewed and approved by the Berkeley Animal Care and Use Committee. New Zealand White rabbits were sedated with a subcutaneous cocktail of 100 mg/ml ketamine and 20 mg/ml xylazine. Pentobarbital sodium (Nembutal; Abbott) was administered intravenously to achieve surgical anesthesia, and a midline abdominal incision was made. The aorta was then exposed and clamped, and a catheter was inserted for perfusion with PBS (in mM: 150 NaCl, 3.6 phosphate, 1 $CaCl_2$, 1 $MgSO_4$, pH 7.4) at high pressure. After the stomach was cleared of blood, it was removed from the animal, opened, and washed several times with PBS. The animal was killed by a lethal dose of Nembutal. The gastric mucosa was scraped from the stomach muscle layer and finely minced. The minced mucosa was washed two times with PBS and another two times with MEM containing 20 mM HEPES, pH 7.4 (MEM-HEPES).

The washed pieces of minced mucosa were subjected to collagenase digestion in Minimal Essential Medium (MEM; Gibco BRL, Gaithersburg, MD), supplemented with 20 mM *N*-2-hydroxyethylpiperazine (HEPES), pH 7.4, containing 0.125 mg/ml collagenase (Sigma, St Louis, MO) and 0.25 mg/ml bovine serum albumin (BSA). The resulting suspension included individual cells, large and small gastric glands, and large debris particles. Large elements of debris were removed by straining the suspension through a 40-μm mesh. Because of their large size, intact gastric glands and large conglomerates of cells settled out in 10-15 minutes, leaving a dense suspension of individual cells. Intact cells were recovered by centrifuging the suspension three times at 200 ***g*** for 5 minutes, followed by resuspension in fresh HEPES-MEM. These procedures resulted in a cell suspension that was typically 70-75% parietal cells. Cells were plated onto Matrigel (Collaborative Biomedical, Stony Brook, NY) -coated coverslips as described by Chew (Chew, 1994; Chew et al., 1989) in 12-well plates and incubated at 37°C in culture medium A, which consisted of DMEM/F-12 (Gibco BRL), supplemented with 20 mM HEPES, 0.2% BSA, 10 mM glucose, 8 nM epidermal growth factor, 1X SITE medium (Sigma), 1 mM glutamine, 100 U/ml penicillin/streptomycin, 400 g/ml gentamicin sulfate, and 15 g/l geneticin or 20 g/ml novobiocin, pH 7.4.

Generation of recombinant adenovirus (rAD)

Recombinant adenoviruses expressing CFP-tagged ezrin (wild-type, T567A mutant and T567D mutant) were generated using the AdMax system (Microbix Biosystems, Canada). Generation of rAD/EzWT-CFP was described previously (Zhu et al., 2005). Briefly, ezrin cDNA was inserted into pECFP/N1 to fuse with the CFP sequence. From the

resultant pECFP-N1/ezrin, ezrin-CFP sequence was amplified by PCR and inserted into an AdMax shuttle vector pDC311, resulting in pDC311/Ez-C. By cotransfection of human embryonic kidney HEK293 cells with pDC311/Ez-C and pBHGloxDE1,3Cre (Microbix Biosystems, containing modified adenovirus type-5 genome) using the CellPhect Transfection kit (Amersham Biosciences, UK), recombination between these two plasmids led to infectious virus production. A single viral colony was isolated, amplified and titrated. Aliquots of virus were stored at –80°C.

Site-directed mutation was done with the QuikChange®II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the mega-primer method (Ke and Madison, 1997). To make the T567D mutant, mega primers were made by PCR with pDC311/EzWT-CFP as template and with the following primer pair: sense primer, gccgggacaagtacaag*gat*ctgcggcagatccgg (containing Thr (ACG)→Asp(GAT) mutation); anti-sense primer, cgtcgccgtccagctcgaccag (CFP antisense sequence). The mega primers carrying the T D mutation were then used to amplify the whole plasmid pDC311/EzWT-CFP. The resultant pDC311/EzT567D was confirmed by DNA sequencing of the whole open-reading frame. Recombinant AD expressing EzT567D-CFP was similarly generated by co-transfection of HEK293 as described above.

Recombinant AD/EzT567A-CFP was obtained following a similar procedure for rAD/EzT567D-CFP except that mega primers were made with the following primer pair: sense primer gccgggacaagtacaa-g*gcg*ctgcggcagatccgg (containing Thr (ACG)→Ala(GCG) mutation); anti-sense primer, cgtcgccgtccagctcgaccag (CFP antisense sequence).

Adenoviral infection of gastric parietal cells

Infections were performed 5 hours post-plating. Cells were infected with the control virus alone or with recombinant adenoviral constructs incorporating CFP-ezrin WT, T567A and T567D. Infection was executed by 3×10^6 particles/ml of different viruses to the medium A surrounding the cultured cells. Cultures were incubated at 37°C for 12 hours and then changed to fresh medium without viruses. We chose our experimental conditions based on the level of expression of GFP and CFP-ezrin WT, T567A and T567D as determined by intensity of fluorescence measured with a Spex fluorometer and the general appearance of the cells. Direct observation of GFP/CFP, and subsequent immunostaining, indicated that more than 80% of positively identified parietal cells were expressing the constructs.

[^{14}C]AP uptake assays

Stimulation of parietal cells was quantified using the aminopyrine (AP) uptake assay. The AP uptake assay measures the accumulation of AP in acidic spaces caused by the proton-pumping enzyme H,K-ATPase. In the neutral state, AP freely equilibrates across biological membranes, but protonation of this weak base in acidic spaces gives it a positive charge and traps it. [^{14}C]AP (about 40 nCi/ml) was added to the medium surrounding cells plated onto each coverslip. Cells were either held in a resting state with the H_2-receptor blocker cimetidine at a final concentration of 100 μM, or stimulated with histamine and isobutylmethyl xanthine (IBMX) to final concentrations of 100 and 50 μM, respectively, or stimulated with histamine and IBMX in the presence of SCH28080, a proton pump inhibitor. Cultures were gently shaken for 25 minutes at 37°C. After the incubation, the coverslips were removed from the medium A and quickly dipped in PBS to remove external counts, and then transferred to 2× sample buffer (125 mM Tris·HCl, 4% SDS, 20% glycerol and 10% β-mercaptoethanol, pH 6.8), where they remained for 45 minutes at room temperature.

Western blotting of cell lysates

A portion of the infected cells scrapings were used for western blots as an assay for the expression of the CFP-ezrin WT, T567A and T567D. Equal amounts of protein were loaded onto 10% SDS-PAGE gels. After running, proteins were transferred to nitrocellulose membranes by a wet-transfer apparatus (Idea Scientific), and the blot was blocked in 5% milk in PBS for at least 1 hour. Blots were probed with mouse monoclonal anti-ezrin (4A5) at 1:5000 dilution, mouse monoclonal anti-β-H,K-ATPase (2G11) at 1:5000 dilution, and affinity purified rabbit anti-GFP (Immunology Consultants Lab) at 1:1000 dilution, respectively. Blots were then probed with horseradish peroxidase-tagged goat anti-mouse IgG or horseradish peroxidase-tagged goat anti-rabbit IgG accordingly. Bands were detected using the Western Lightning Chemiluminescence Regent Plus.

Immunofluorescence and confocal microscopy

For cytolocalization of exogenously expressed GFP-ezrin, cultured parietal cells were infected with recombinant adenoviral constructs incorporating CFP-ezrin WT, T567A and T567D, and maintained in medium A for 36-40 hours. Some cultures were then treated with the secretory stimulants histamine and IBMX to final concentrations of 100 and 50 μM, respectively, with or without SCH28080, a proton pump inhibitor. Treated cells were then fixed with 4% formaldehyde for 10 minutes and washed three times with PBS followed by permeabilization in 0.25% Triton X-100 for 10 minutes. Before application of primary antibody, the fixed and permeabilized cells were blocked with 2% bovine serum albumin in phosphate-buffered saline followed by incubation of primary antibodies against ezrin (4A5, Covance, Berkeley, CA), GFP (Immunology Consultants, Newberg, OR) or H,K-ATPase (2G11, Affinity Bioreagents, Boulder, CO). In the case of ezrin and GFP the primary antibodies were then visualized by fluorescent labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). In the case of H,K-ATPase, the primary antibody was either pre-labeled with the Zenon Mouse IgG Labeling Kit (Molecular Probes, Eugene, OR), or visualized by a fluorescent labeled secondary antibody. Coverslips were mounted in Vectorshield (Vector). Images were collected on three different microscopes using 63× or 40× objectives. Some images were collected using a conventional epifluorescence microscope, Nikon Microphot-FXA. Confocal images were collected either on a Zeiss 510 fluorescence microscope using Zeiss software, or on a Nikon TE2000-U microscope equipped with Solamere (Salt Lake City, UT) spinning disc laser confocal technology for live cell imaging. Figures were constructed using Adobe Photoshop.

Scanning electron microscopy

Preparation for scanning electron microscopy (SEM) was conducted using untreated or infected parietal cells cultured on silicon wafer chips. The cells were fixed using 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least one hour, followed by four washes in 0.1 M cacodylate buffer. Samples were treated with 1% osmium tetroxide in 0.1 M cacodylate buffer for post-fixation; the cells were then washed using cacodylate buffer to remove any remaining osmium. To aid in drying the cells were transferred through a regimen of graded ethanol solutions (35%, 50%, 70%, 85%, 95% and 100%) for 10 minutes each. The samples were desiccated in a critical point drier (Denton Vacuum) using liquid CO_2. After drying the cells were coated with a fine layer of carbon using the glass bell-jar sputter coater (Tousimis autosemelui) and examined in a Hitachi S5000 SEM.

Results

Expression of CFP-ezrin WT, T567A and T567D mutants in gastric parietal cells

To evaluate the efficiency of exogenous ezrin expression,

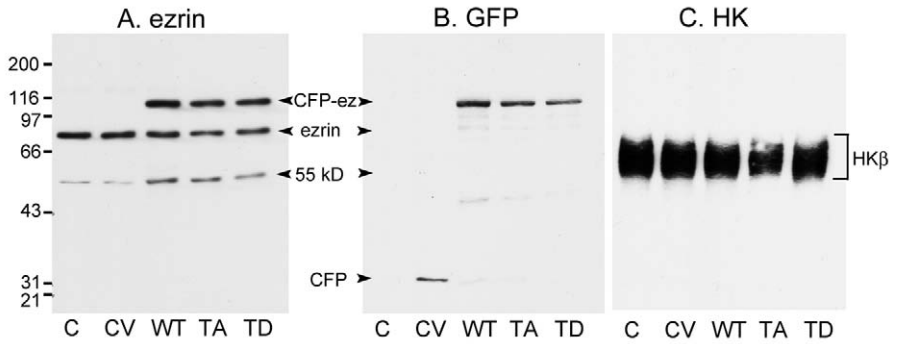

Fig. 1. Western blots of parietal cell cultures probed for ezrin, CFP-ezrin construct expression, and for H,K-ATPase. Conditions for cell culture included control cells (C, no treatment), control virus infected cells (CV), and cells infected with rAD including CFP-constructs as wild-type ezrin (WT), T567A mutant ezrin (TA), and T567D mutant ezrin (TD). (A) Blot probed with an antibody against ezrin that recognizes 80 kDa native ezrin, the CFP-constructs of ezrin (~106 kDa) and an N-terminal ~55 kDa breakdown product of ezrin. (B) Blot probed with antibody against GFP which also recognizes CFP. For blots A and B, protein bands are marked by arrowheads indicating lanes for ezrin (ezrin), the CFP-tagged ezrin constructs (CFP-ez), the approximately 55 kDa ezrin breakdown product (55 kD), and CFP alone (CFP) in the cells infected with the control rAd construct. (C) Blot probed with antibody against the β-subunit of H,K-ATPase (HKβ) to show the approximate equivalency of parietal cells in the assay. The antibody against the β-subunit of H,K-ATPase ordinarily migrates as a very broad band, 60-80 kDa, because of the high degree of glycosylation.

cultured parietal cells were infected with recombinant adenoviral constructs incorporating CFP-ezrin WT, and the T567A and T567D mutants. Western blot analysis of control and infected cell lysates were probed with an ezrin-specific antibody that recognized exogenously expressed as well as endogenous ezrin (Fig. 1A), with an anti-GFP antibody that also recognizes CFP (Fig. 1B), and with an antibody against the β-subunit of H,K-ATPase as a parietal cell normalizing factor (Fig. 1C). The ezrin blot in Fig. 1A shows that exogenously expressed ezrin WT, T567A and T567D can reach about the same expression level, and even appeared slightly more abundant than native ezrin. The increased level of the 55 kDa breakdown product of ezrin (typical calpain cleavage product) in the cells expressing the CFP-labeled ezrin proteins is consistent with an increased ezrin turnover in these cells.

Exogenously expressed CFP-ezrin WT and the T567A mutant localize heavily on apical membrane vacuoles of parietal cells

Live parietal cells expressing CFP-ezrin WT and CFP-T567A mutant are shown in Fig. 2A,B. Several laboratories commonly report that cultured parietal cells take on a different morphological form than in situ (Agnew et al., 1999; Chew, 1994; Mangeat et al., 1990). After a few hours in culture the apical membrane is sequestered in the form of easily identified vacuoles (2-5 μm in diameter), which we refer to as apical membrane vacuoles. Because the surrounding plasma membrane continues to serve as the site of receptor-mediated cell activation events, we refer to it as the basolateral membrane. Cultured parietal cells continue to maintain a secretagogue-dependent phenotype whereby H,K-ATPase-rich tubulovesicles are translocated to, and fuse with, apical membrane vacuoles and the resulting secretion of HCl produces gross vacuolar enlargement (Agnew et al., 1999; Mangeat et al., 1990; Zhou et al., 2003b). From the images of resting cells (i.e. nonsecreting) displayed in Fig. 2A it can be seen that the CFP signals are similar in cells infected with either CFP-ezrin WT or CFP-T567A mutant. CFP is heavily localized on the apical membrane vacuoles, with relatively small amounts of signal located on the basolateral membrane. The apical vacuoles of these infected cells remain relatively small as seen in typical resting parietal cells (Agnew et al., 1999). When the virus infected cells were stimulated with secretagogues (histamine and IBMX), the morphological response indicative of secretion was similar to that typically seen in normal, uninfected, parietals cells. The cells clearly show swollen apical membrane vacuoles, indicating that the cells were stimulated and secreting HCl and water. In both the WT and T567A expressing cells the CFP-ezrin remains on the apical membrane throughout the stimulation. These data indicate that both CFP-ezrin WT and CFP-T567A mutant ezrin are primarily targeted to the apical plasma membrane of parietal cells, and that they do not appear to interfere with the typical morphological response to stimulation of the cells.

Exogenously expressed CFP-ezrin T567D mutant is prominently localized to long finger-like projections on the basolateral surface of parietal cells

Parietal cells expressing CFP-ezrin T567D mutant ezrin showed a much different distribution of CFP than either CFP-ezrin WT or the T567A mutant. Fig. 2C shows that CFP-ezrin T567D is primarily expressed in the surrounding plasma membrane, which we usually refer to as the basolateral membrane. Sometimes the CFP signal appears as a simple and relatively smooth form, but more often the basolateral distribution appeared in the form of long and elaborate microvillar projections from the cell surface. Frequently the projections were localized to one surface of the cell, giving it kind of a polarized appearance. This T567D mutation is the only case in which we have ever seen a more prominent distribution of parietal cell ezrin to the basolateral, as compared with the apical, membrane. Unlike the case for cells expressing CFP-ezrin WT and CFP-T567A mutant, cells infected with CFP-T567D mutant did not show the typical morphological response to stimulation by histamine in the form of swelling of the apical membrane vacuoles (Fig. 2C). In fact, the apical membrane vacuoles tended to be diminished in number as well as depleted of CFP expression, whereas CFP expression was still prominent on basolateral membranes.

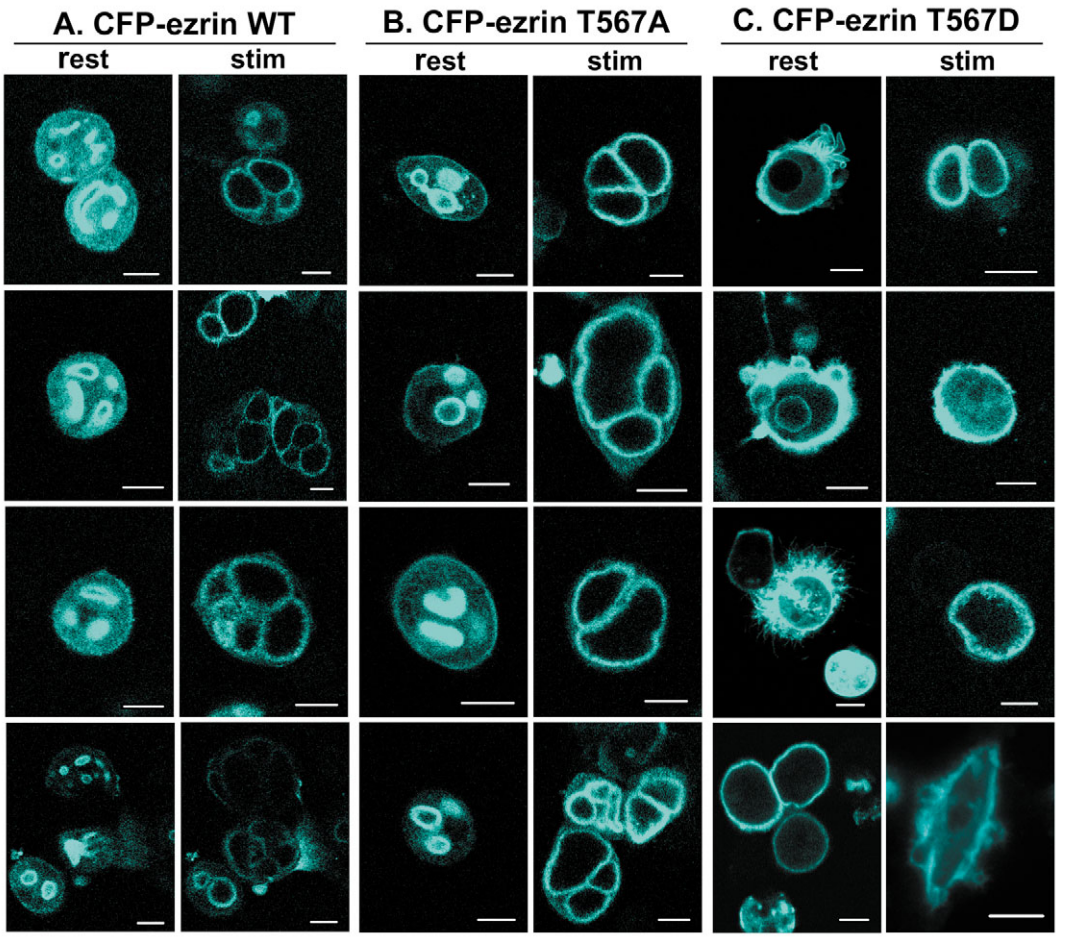

Fig. 2. Expression of CFP-ezrin constructs in live parietal. (A) Examples of cells infected with rAD-CFP-ezrin WT. (B) Cells infected with rAD-CFP-ezrin T567A. (C) Cells infected with rAD-CFP-ezrin T567D. For all cases images are shown for parietal cells maintained in the resting, nonsecreting, state (rest) and 20-30 minutes after cells had been stimulated to secrete acid (stim) by histamine plus IBMX. For WT and the T567A mutant, CFP-ezrin was mainly targeted to relatively large vacuoles that are derived from the apical plasma membrane when the cells are cultured, thus we refer to them as apical membrane vacuoles. There are lesser amounts of CFP-ezrin WT and T567A displayed on the basolateral membrane (surrounding plasma membrane) and in the cytoplasm. Cells expressing both CFP-ezrin WT and T567A respond well to stimulation with a large expansion of apical membrane vacuoles that fill with secreted acid. Note: the rest/stim images at the lower left are the same cells expressing CFP-ezrin WT at rest and after 20 minutes stimulation. The targeting of CFP-ezrin T567D mutant was totally different. The CFP signal was detected primarily at the basolateral membrane, often in the form of elaborate surface projections, and the cells did not display the normal secretory response to stimulation. Bars, 10 μm.

Exogenously expressed CFP-ezrin WT and T567A promote the translocation of H,K-ATPase to the apical membrane while apical membrane vacuoles remain small as in resting cells

The subcellular location of exogenously expressed CFP-ezrin WT and T567A was compared with that of endogenous H,K-ATPase by fluorescence microscopy (Fig. 3). Control uninfected parietal cells were double stained for endogenous ezrin using an ezrin monoclonal antibody MAb 4A5 and for H,K-ATPase using a monoclonal antibody MAb 2G11 coupled with the Zenon kit. Cells infected with rAD-CFP-ezrin, including either WT or mutant constructs of ezrin, were fixed and subjected to staining for CFP-ezrin using a GFP polyclonal antibody and for H,K-ATPase using MAb 2G11. Fig. 3 shows that in control uninfected parietal cells the endogenous ezrin is mainly localized to apical vacuoles with low levels of staining in the cytosol, whereas the HK-ATPase appears randomly spread through the cytosol. This pattern of H,K-ATPase distribution in resting cells is similar to what we and others have reported many times, and is consistent with H,K-ATPase localization in the numerous tubulovesicles throughout the cytoplasm (Forte et al., 1977). However, in cells heavily expressing either CFP-ezrin WT or CFP-ezrin T567A, there was a different pattern of H,K-ATPase distribution in the resting cells. When CFP expression level was high, H,K-ATPase appeared to be depleted from the cytoplasm and redistributed to the apical membrane vacuoles. Moreover, redistribution of H,K-ATPase occurred without significant vacuole swelling, thus retaining the appearance of resting cells. For both WT and T567A, the exogenously expressed CFP-ezrin was heavily localized to the apical membrane vacuoles. When cells were not expressing CFP-ezrin, or expression was at very low levels, then H,K-ATPase presented the typical distribution seen in control resting cells: generally spread throughout the cytoplasm.

To verify that these virus infected cells were functionally competent, even when H,K-ATPase was already localized to the apical membrane, they were stimulated with histamine and IBMX. The stimulated cells were submitted to the same fluorescence staining as above. Images in Fig. 3 show that both rAD-CFP-ezrin WT and rAD-CFP-ezrin T567A infected cells can be stimulated to the same extent as control cells. The exogenously expressed CFP-ezrin and endogenous H,K-ATPase have the same localization pattern as control cells. This experiment indicates that exogenous overexpressed ezrin leads to accumulation of cargo H,K-ATPase at the apical membrane, but the H,K-ATPase remains in the inactive status until the stimulus is applied.

When cells infected with rAD-CFP-ezrin T567D were fixed, subsequent immunostaining presented a pattern for CFP-ezrin staining similar to the distribution seen in live cells. The CFP-T567D ezrin was expressed principally at the basolateral membrane, often in the form of dense long projections from the membrane surface (Fig. 3). Interestingly, immunostaining indicated a similar basolateral distribution of H,K-ATPase to these basolateral membrane projections. Moreover, parietal cells expressing the T567D mutant did not respond morphologically to stimulation by the normal secretagogue pathway.

Exogenously expressed CFP-ezrin T567D significantly inhibits the acid secretory response

We proceeded to test whether cells infected with rAD-CFP-ezrin T567D were capable of a secretory response. Fig. 4 shows images from a time course of live cell stimulation with histamine and IBMX. Typically we found that for cells heavily expressing ezrin T567D, as noted by the strong CFP signal revealed during microscopy, there was little or no change even after 60 minutes of treatment. It was of interest that when we looked very carefully in the field, we sometimes found neighboring cells expressing little or no CFP that were clearly visualized by DIC optics. For these nonexpressing cells the response to stimulation was normal, i.e. progressive swelling of vacuoles was nearly complete within 20 minutes of stimulation. To demonstrate this phenomenon we took one area of the image in Fig. 4 (inset) and digitally greatly enhanced the contrast so that nearby cells expressing little or no CFP could be visualized. Although the image quality of the inset is very poor, it is clear that the noninfected cells are undergoing a morphological response to stimulation.

To further evaluate the role of these ezrin T567 mutants on the physiological secretory response we used the aminopyrine (AP) uptake assay as a measure of acid secretion in response to secretagogues and pharmacological inhibitors. Fig. 5 shows the AP uptake data for cultures of cells infected with adenovirus alone, rAD-CFP-ezrin WT, rAD-CFP-ezrin T567A and rAD-CFP-ezrin T567D. The data for infection with adenovirus alone was used as a control, and the AP uptake data were normalized by setting the maximum secretory response within each individual experiment at 100% and then averaging over the number of different experiments. The cultures were

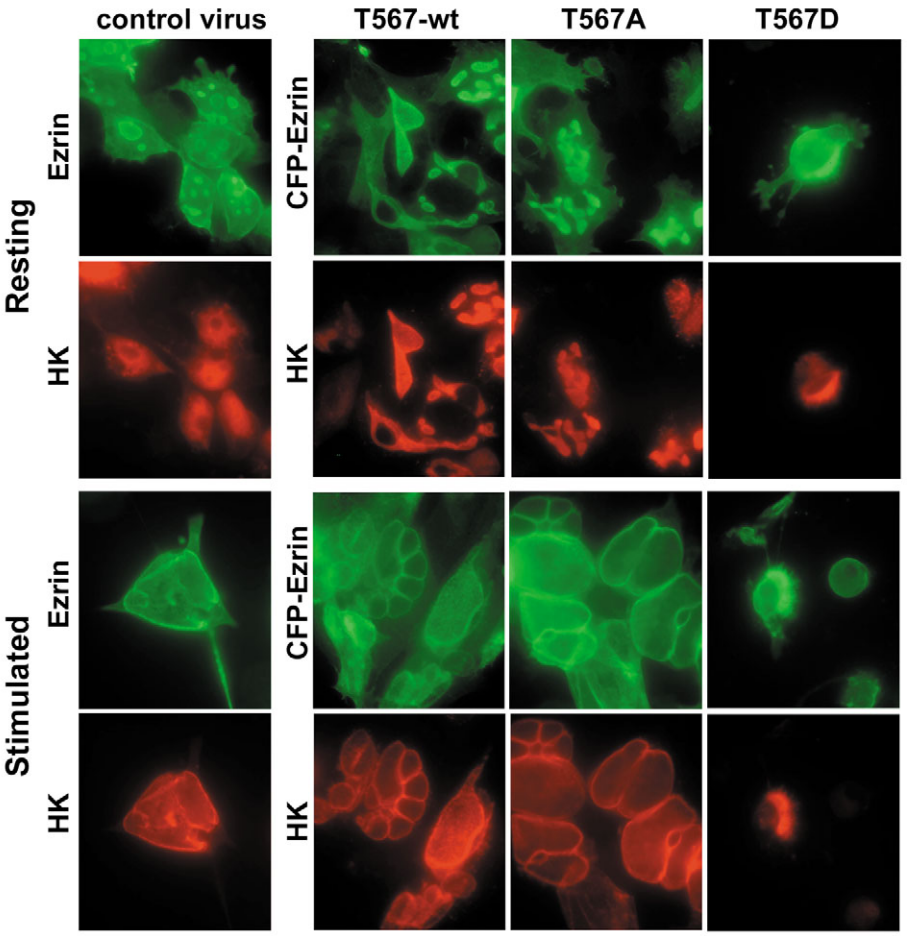

Fig. 3. Parietal cell cultures double stained for expression of H,K-ATPase (HK) and ezrin (or CFP-ezrin). Resting cells infected with control virus displayed native ezrin primarily on apical membrane vacuoles and basolateral membrane to some extent, and HK staining was distributed throughout the cytoplasm, presumably on tubulovesicles. After stimulation ezrin and HK were localized to some extent on expanded apical secretory vacuoles. Cells infected with rAD-CFP-ezrin WT, CFP-ezrin T567A mutant and CFP-ezrin T567D mutant were stained for HK and CFP (using a GFP antibody). In resting cells expressing CFP-ezrin WT and T567A mutant where it colocalized with a subset of CFP staining. This was highly unusual because the resting cells displayed none of the morphological characteristics of the secretory phenotype (which promptly occurred after the cells were stimulated with histamine plus IBMX). The targeting of CFP-ezrin T567D mutant was similar to that described in Fig. 2, where the CFP signal was detected primarily at the basolateral membrane. Not only was the normal secretory response absent in the T567D-expressing cells, the HK was completely 'repolaraized' to the basolateral membrane, there colocalizing with CFP-ezrin T567D.

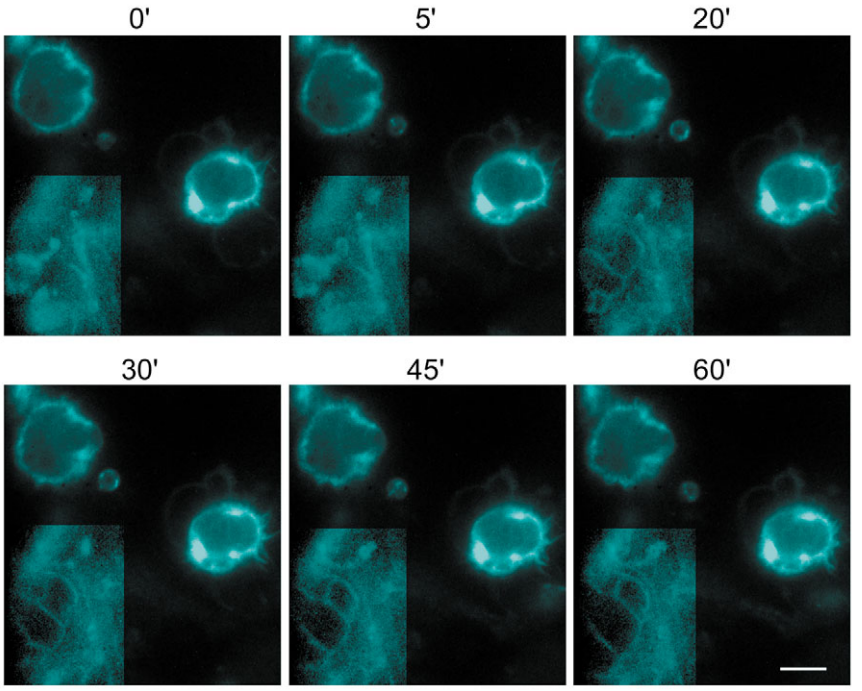

Fig. 4. Time course of stimulatory response for cells infected with rAD-CFP-ezrin T5687D mutant. Cells were placed in an incubating chamber (37°C) and viewed by spinning disc confocal microcopy using the CFP channel. Histamine plus IBMX was added at zero time and the cells were periodically examined with images being selected at the times (in minutes) shown. For the two parietal cells expressing CFP-ezrin T567D in the main panel the CFP signal was at the basolateral surface in the form of long surface projections; there was no significant morphological response to stimulation. The rectangular inset at the lower left has digitally amplified an extremely faint image (virtually autofluorescence) of that very region and the signal to show that 'hidden' parietal cells on the same slide, but not expressing T567D, respond normally to stimulation. Bar, 20 μm for all images.

subjected to three conditions of treatment: (1) cells maintained in a resting state (by the addition of a histamine H2-receptor blocker, cimetidine), (2) cells treated with histamine and IBMX to effect maximum secretory response, and (3) cells treated with histamine and IBMX as well as a specific inhibitor of the H,K-ATPase, SCH28080. Cells infected with control adenovirus showed a robust (nearly tenfold over resting) secretory response to histamine and IBMX that was totally abolished by the H,K-ATPase inhibitor. Cells expressing rAD-CFP-ezrin WT were somewhat less responsive than the control cells, perhaps because of ezrin overexpression; however, the secretory response of these cells was still quite robust, and was used to evaluate the relative responses of the mutant ezrin forms. Cells infected with the T567A mutant reported a reduced AP uptake that was 70% of that measured for WT (P=0.05). Cells infected with the T567D mutant showed a much greater and highly significant (P<0.004) reduction of the measured secretory response (44% of WT). Recognizing that not all parietal cells were infected, the inhibitory effect on cells expressing specifically CFP-ezrin T567D was probably even more severe.

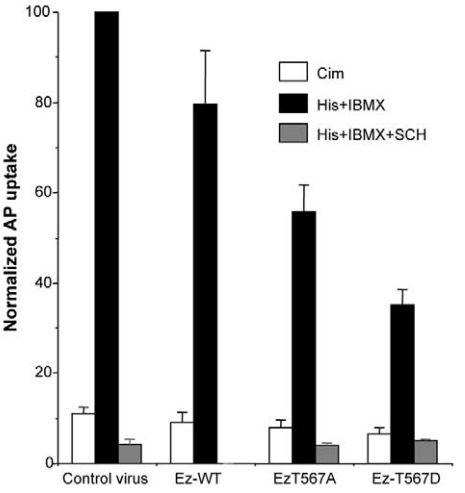

Fig. 5. Acid secretory response of parietal cells infected with control rAd and with CFP-ezrin constructs expressing WT and the T567A and T567D mutants. The aminopyrine (AP) uptake was measured for the various cultures of parietal cells in the presence of the presence of the H2-receptor blocker cimetidine (Cim), maximally stimulated by histamine plus IBMX (His+IBMX), and stimulated in the presence of the H,K-ATPase blocking drug SCH-28080 (His+IBMX+SCH). The data were collected from assays on six separate culture preparations, and normalized within each experimental run by setting the AP uptake of His+IBMX for control virus at 100% and shown as mean±s.e.m. All preparations showed highly significant AP uptakes in response to stimulation by His+IBMX. Statistical analysis (t-test) of the maximally stimulated AP uptake revealed that P>0.05 between control virus and ezrin-WT; P=0.05 between ezrin-WT and T567A; and P=0.004 between ezrin-WT and T567D.

Exogenously expressed CFP-ezrin T567D mutant directs the H,K-ATPase to the finger-like surface projections

The mis-sorting of the ezrin T567D mutant and the marked secretory inhibition was consistent with a defect in the normal stimulation-associated recruitment of H,K-ATPase. We therefore further studied rAD-CFP-ezrin T567D-infected cells double stained for CFP-ezrin and either H,K-ATPase or F-actin. As evidenced by confocal images in Fig. 6, cells heavily expressing CFP-ezrin T567D displayed H,K-ATPase colocalized with the mutant ezrin in the long filamentous spikes projecting from the plasma membrane; there was almost no H,K-ATPase staining remaining in the cytoplasm or on apical membrane vacuoles. As previously noted, cells with the

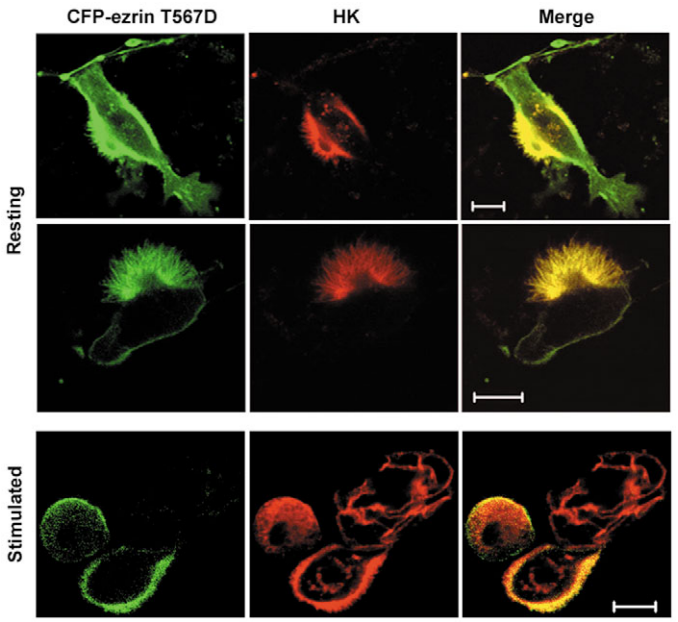

Fig. 6. Confocal microscopy of parietal cell cultures double stained for expression of H,K-ATPase (HK, red) and CFP-ezrin T567D mutant (green). Resting cells displayed T567D ezrin primarily on the surface basolateral membrane often appearing as extensive surface projections. HK staining was distributed (relocated) to the basolateral membrane colocalized as a subset of T567D staining. After treating the cultures with histamine plus IBMX, parietal cells prominently expressing T567D ezrin did not respond to the stimulation, but neighboring cells expressing little or no T567D ezrin assumed the typical secretory phenotype. Bars, 10 μm.

mis-targeted T567D ezrin and H,K-ATPase did not respond to stimulants. In the few cells where there was little or no visible CFP-ezrin expression (i.e. no infection), the H,K-ATPase was distributed as in native, control cells throughout the cytoplasm with some expression on apical membrane vacuoles, and these cells showed the typical morphological response to stimulation (Fig. 6). Staining of cells with phalloidin along with CFP-ezrin T567D showed that F-actin was also distributed to the filamentous basolateral projections containing the mutant ezrin, in sharp contrast to the normal pattern (data not shown).

SEM reveals a system of long and extensive microvillar structures projecting from the plasma membrane of cells infected with rAD-CFP-ezrin T567D

Control cells and those infected with various rAD ezrin constructs were examined by scanning electron microscopy. All cultured parietal cells displayed an abundance of membrane folds and surface projections at the basolateral membrane surface (Fig. 7). However, these tended to be relatively short except for cells infected with rAD-CFP-ezrin T567D. For the latter cells the basolateral extensions were abundant and frequently very long and filamentous. This morphological surface display pattern was consistent with the immunostaining data.

Discussion

This study has examined the role of phosphorylation of ezrin at T567 on the targeting and functional participation of ezrin in the secretory activity of the parietal cell. The approach used rAd-CFP-ezrin constructs efficiently introduced into primary parietal cell cultures, including WT ezrin and site-directed phosphorylation mutants of ezrin in which T567 was modified to simulate a nonphosphorylatable T567A mutation, and a T567D mutation to mimic a permanently phosphorylated ezrin. Similar to endogenous ezrin, CFP-WT ezrin and CFP-T567A ezrin were found to localize heavily to apical membrane vacuoles with considerably lower levels associated with the surrounding basolateral membrane. Cells expressing WT or T567A ezrin were also morphologically and functionally responsive to stimulation by appropriate secretagogues. Histamine plus IBMX (via cAMP pathway) produced secretion

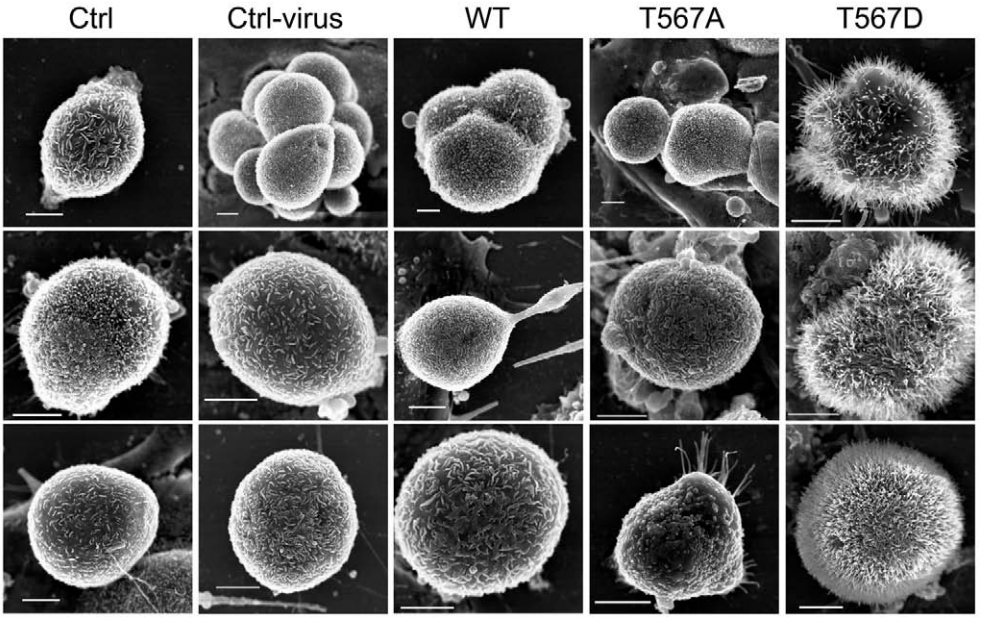

Fig. 7. Scanning electron microscopy of parietal cell cultures in control conditions and those infected with rAd-CFP-ezrin constructs expressing WT and the T567A and T567D mutants. The cells were cultured on silicon wafers, exposed to virus (or not) for 48 hours, then fixed, dried, coated with carbon and examined in a Hitachi scanning microscope as described in Materials and Methods. For most of the cells/treatments the plasma membrane (basolateral membrane) is studded with short projections and folds, with occasional lamellipodia and longer microvillar projections. In the case of T567D the basolateral membrane was often found to be replete with extensive long microvillar projections. Bars, 5 μm.

and pronounced expansion of apical membrane vacuoles, although more quantitative assessment using the [^{14}C]AP assay indicated that cells expressing T567A had about 30% less (P=0.05) acid secretion than cells expressing WT ezrin. This could imply that impairment of T567 phosphorylation plays a role in the acid secretory response, but it is clear that the principal secretagogue-mediated responses, as measured by AP uptake and morphological changes, remain largely intact for rAd-CFP-ezrin T567A mutant.

Cells expressing the T567D mutant ezrin gave quite a different set of responses. CFP-T567D ezrin was primarily targeted to what we ordinarily refer to as the basolateral membrane, as if the new targeting were totally reorienting the polarity of the parietal cells. Moreover, the 'basolateral' ezrin was usually associated with long filamentous extensions from that surface, and the cells were incapable of a normal secretagogue-mediated acid secretory response. Thus, it is apparent that the T567 phosphorylation-mimic leads to mis-targeting of ezrin and changes the secretory phenotype of the parietal cell.

Before interpreting these data there is additional information to be considered. For all three of the exogenously expressed CFP-tagged ezrin proteins, WT, T567A and T567D, there was a totally surprising relocation of the principal membrane cargo of the parietal cell, by which the H,K-ATPase tended to follow the targeting of the expressed CFP-ezrin construct. For WT and T567A mutant, H,K-ATPase was relocated from its typical distribution in cytoplasmic tubulovesicles to the apical membrane vacuoles, giving the impression of a cell that was activated by apical recruitment of H,K-ATPase, but without the swollen vacuoles or increased AP uptake, which are the normal indices of acid secretion. We know that acid secretion requires both recruitment of H,K-ATPase and activation of apical K^+ and Cl^- channels (Reenstra and Forte, 1990; Wolosin and Forte, 1984), so we might assume that the latter does not occur until appropriate secretagogues are added. In the case of cells expressing the T567D mutant ezrin, H,K-ATPase tended to colocalize with T567D at the basolateral membrane indicating recruitment of the cargo to a totally different surface than has ever been seen in vivo. Predictably, cells expressing T567D were incapable of secreting acid in the normal way. From all these observations, two main issues rise to the surface. First, there is the role of T567 phosphorylation in the activation and targeting of ezrin. Second, the issue of why and how ezrin can influence the recruitment of H,K-ATPase, and ultimately the secretory phenotype of the parietal cell.

Data presented here clearly show that T567 phosphorylation is not involved in the apical targeting of ezrin and has very little role in the activation of ezrin associated with the secretory activity of the parietal cell. This is consistent with the finding that 'activated' ezrin from parietal cells had ^{32}P incorporated into serine residues but no ^{32}P appeared in threonine residues (Urushidani et al., 1989). Given that phosphorylation of T567 on ezrin is known to involve the Rho activation pathway (Matsui et al., 1998; Shaw et al., 1998), the data offer additional interpretation on experiments testing the more general role of Rho in parietal cell function. In experiments performed on parietal cells by two laboratories (Pausawasdi et al., 2000; Tashiro et al., 2003), it was observed that treatments to inhibit Rho activity augmented acid secretion, and treatments that activate Rho inhibited acid secretion. For example, inhibition of Rho using C3 botulinum toxin, which ADP-ribosylates Rho, substantially increased carbachol- and histamine-stimulated acid secretion in isolated parietal cells (Pausawasdi et al., 2000) and in gastric gland preparations (Tashiro et al., 2003). The obverse experiment, in which the constitutively active mutant of Rho was expressed in parietal cells, resulted in significant inhibition of acid secretion (Tashiro et al., 2003). It is important to note that in this latter study the actin cytoskeleton at the apical membrane vacuoles was seriously disrupted with no diminution of basolateral actin filaments. These data support an hypothesis that the Rho-activation pathway has a negative effect on parietal cell secretory activity, and one of the downstream effects of Rho is phosphorylation of T567 on ezrin (Matsui et al., 1998; Shaw et al., 1998) and is consistent with the present results for which we have used point mutation on T567. Mis-targeting of ezrin and the loss of the secretory phenotype is thus a consistent result of introducing the T567D ezrin mutant. The Rho activation path has generally been associated with the formation of actin stress fibers and focal adhesions (Etienne-Manneville and Hall, 2002; Ridley, 2001), and in the parietal cell this may be manifest by motile cytoskeletal activity within the 'volatile' actin pool at the basolateral surface (Ammar and Forte, 2002; Ammar et al., 2001). It may well be that T567 phosphorylation of ezrin, and/or other ERM proteins, represents a major effector event in the redirection, or even repolarization, of the actin cytoskeletal activity.

Another major event within the present data is the redistribution of H,K-ATPase from a resting pool of cytoplasmic tubulovesicles to the ezrin targeted membrane. To a large extent this may be a problem of overexpression of the various ezrin forms. In the normal resting cell native ezrin is heavily represented in association with actin-rich microvilli at the apical membrane, with relatively minor presence at the basolateral membrane. Cell activation, possibly including ezrin activation via phosphorylation, leads to the well characterized apical recruitment of H,K-ATPase. In ezrin knockdown mice, where expression of ezrin was reduced to about 5% of the wild-type level, parietal cells were replete with H,K-ATPase-rich tubulovesicles; however, the apical canalicular surface and resident microvilli were very much reduced (Tamura et al., 2005). Moreover, the parietal cells did not respond to secretagogues with the translocation of H,K-ATPase and acid secretory response, suggesting that ezrin plays an important role in the character of the apical membrane and the membrane recruitment process. We propose the possibility that ezrin overexpression may promote a response opposite to ezrin knockdown. Excess ezrin would be targeted to the plasma membrane where it would promote the production of the actin-rich microvillar structures. The polarity for targeting ezrin is a function of T567 phosphorylation. In the parietal cell under conditions of relatively low Rho activity, ezrin is targeted to the apical surface; when T567 is phosphorylated, microvillar expansion would occur at the basolateral membrane. Now the question becomes, where does the newly expanded membrane come from? In the short term it cannot come from new membrane synthesis, so the requisite expansion would have to come from existing cellular pools, and clearly, the most abundant pool is the H,K-ATPase-rich tubulovesicles. When tubulovesicles are recruited to the apical membrane vacuoles, as in the case of WT and T567A mutant ezrin, the unresolved

issue is that the cells appear to maintain small apical membrane vacuoles rather than the enlarged ones associated with the proton secreting state. As mentioned above this may be due to the absence of appropriate K^+ and Cl^- channel activation. However, the situation becomes distinctly abnormal in the case of the T567D mutant ezrin, which is directed to the surrounding (basolateral) plasma membrane, and according to the hypothesis, H,K-ATPase is recruited to the basolateral membrane.

Functional activation of ezrin and other ERM proteins is highly dependent on phosphorylation (Bretscher, 1989; Bretscher, 1999; Gautreau et al., 2002; Urushidani et al., 1989). Ultimately, functional activation has to be the state in which the ERM protein can form a linkage between the actin cytoskeleton via a C-terminal actin binding site and the plasma membrane via an N-terminal binding site, albeit there may be one or more adaptor proteins participating in the latter binding. One of the possible steps in the activation probably involves the dissociation of intra- and inter-molecular interactions within and between the proteins themselves in what has been termed ERM association domains (ERMAD) via the N- (N-ERMAD) and C- (C-ERMAD) terminal binding. Several studies have suggested that phosphorylation may be essential for dissociating N- and C-ERMADs, thus promoting 'open' and active ezrin, radixin and moesin monomers (Gautreau et al., 2000; Matsui et al., 1998). The present results show that T567 phosphorylation is not essential for normal targeting and functional activity of ezrin in the parietal cell. This is consistent with the recent conclusion of Chambers and Bretscher (Chambers and Bretscher, 2005), albeit their conclusions were based entirely on binding and structural studies of ezrin fusion protein constructs.

Studies on ezrin (radixin and moesin) with site directed mutations at T567 (T564 and T558) have produced striking surface morphological anomalies in a variety of cell systems expressing these mutant forms. COS7 cells and LLC-PK1 cells display extensive formation of microvillar structures when expressing T558 moesin or T567 ezrin mutants, respectively (Gautreau et al., 2000; Oshiro et al., 1998). In early mouse embryo development, phosphorylation of T567 on ezrin was shown to play an important and time-dependent role in the compaction and polarization events (Dard et al., 2004). T567D ezrin accumulated at the basolateral surface of blastomeres at the 8- and 16-cell stages and appeared to induce the formation of long microvilli, thus impairing polarization and morphogenesis of the blastocyst. This recurrent theme of elaboration of long microvilli promoted by the mimicking N-terminal Thr phosphorylation is analogous to what we have seen for the parietal cell, although there appear to be some ambiguities regarding sidedness of the microvillar expansion.

Na,K-ATPase is the ubiquitous Na/K exchange transporter that is highly homologous to H,K-ATPase in much of its primary (65% identity) and secondary structure. In addition to the obvious difference in the transported ions, the two proteins are distinctly different in their cytological targeting. Na,K-ATPase is almost always targeted to the membrane facing the blood or body fluid (plasma membrane in nonpolarized cells; basolateral membrane in epithelial cells). Thus, in renal epithelial cells Na,K-ATPase is normally found on the basolateral membrane, but there are genetic diseases, such as renal polycystic disease, in which there is a high proportion of Na,K-ATPase mis-targeted to the apical membrane (Maeda et al., 2002; Orellana et al., 2003; Wilson et al., 2000; Wilson et al., 1991). When cultured NRK-52E renal epithelial cells were microinjected with a constitutively active Rho mutant, Na,K-ATPase was found to be translocated to the apical plasma membrane where it was colocalized with ERM proteins phosphorylated at the N-terminal Thr site, e.g. T567 ezrin (Maeda et al., 2002). At this point there is no evidence to link the etiologic basis for polycystic disease and the Rho-mediated effect in renal cell lines, but they both represent aberrant mis-targeting of an important transport protein. It will be of interest to determine the extent to which phosporylation of ERM proteins may be related to mis-polarization of Na,K-ATPase.

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