Characterization of Protein Kinase A-mediated Phosphorylation of Ezrin in Gastric Parietal Cell Activation*

Rihong Zhou‡§†, Xinwang Cao‡§, Charles Watson‡, Yong Miao‡, John G. Forte‡, and Xuebiao Yao‡¶

From the ‡Laboratory of Cell Dynamics, School of Life Science, University of Science and Technology of China, Hefei 230027, China, the ¶Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Received for publication, April 2, 2003, and in revised form, May 27, 2003
Published, JBC Papers in Press, July 2, 2003, DOI 10.1074/jbc.M303416200

Gastric ezrin was initially identified as a phosphoprotein associated with parietal cell activation. To explore the nature of ezrin phosphorylation, proteins from resting and secreting gastric glands were subjected to two-dimensional SDS-PAGE. Histamine triggers acid secretion and a series of acidic isoforms of ezrin on two-dimensional SDS-PAGE. Mass spectrometric analysis of these acidic ezrin spots induced by stimulation suggests that Ser66 is phosphorylated. To determine whether Ser66 is a substrate of protein kinase A (PKA), recombinant proteins of ezrin, both wild type and S66A mutant, were incubated with the catalytic subunit of PKA and [γ-32P]ATP. Incorporation of 32P into wild type but not the mutant ezrin verified that Ser66 is a substrate of PKA. In addition, expression of S66A mutant ezrin in cultured parietal cells attenuates the dilation of apical vacuolar membrane associated with stimulation by histamine, indicating that PKA-mediated phosphorylation of ezrin is necessary for acid secretion. In fact, expression of phosphorylation-like S66D mutant in parietal cells mimics histamine-stimulated apical vacuole remodeling. Further examination of H,K-ATPase distribution revealed a blockade of stimulation-induced proton pump mobilization in S66A but not S66D ezrin-expressing parietal cells. These data suggest that PKA-mediated phosphorylation of ezrin plays an important role in mediating the remodeling of the apical membrane cytoskeleton associated with acid secretion in parietal cells.

Ezrin is an actin-binding protein of the ezrin/radixin/moesin (ERM) family of cytoskeleton membrane linker proteins (1). Within the gastric epithelium, ezrin has been localized exclusively to parietal cells and primarily to the apical canalicular membrane of these cells (e.g. Refs. 2 and 3). Because of its cytolocalization and observed stimulation-dependent phosphorylation, an implied role for ezrin was suggested in the apical surface membrane remodeling associated with parietal cell activation via the protein kinase A pathway. Phosphorylation of ezrin has also been shown to be associated with surface membrane remodeling of A491 cells stimulated by epidermal growth factor, although activation in this case was via protein tyrosine kinase (4, 5). Our previous studies showed that gastric ezrin is co-distributed with the β-actin isoform in vivo (6) and preferentially bound to the β-actin isoform in vitro (7). However, it is still not clear how ezrin is involved in the membrane cytoskeletal dynamics triggered by histamine stimulation.

Using fluorescence resonance energy transfer monitored by fluorescence lifetime imaging microscopy and chemotaxis assays (8), it has been shown that protein kinase C-mediated phosphorylation of CD44 and ezrin modulates the interaction between these two proteins in vivo and that this phosphorylation was critical for CD44-directed cell motility, suggesting that phosphorylation of ezrin and its accessory proteins provides means to regulate the membrane cytoskeletal dynamics in response to stimulation. Whereas protein kinase C-mediated phosphorylation of CD44 was mapped to Ser291, the nature of ezrin phosphorylation is not characterized.

Phosphorylation has been proposed to regulate ERM activation, since phosphorylation of ERM proteins correlates with their cytoskeletal association, whereas dephosphorylation of ezrin is parallel to its liberation from actin-based cytoskeleton (e.g. Refs. 9 and 10). Ezrin is phosphorylated on tyrosine residues upon growth factor stimulation (11–13). In response to epidermal growth factor, ezrin phosphorylation on tyrosines 145 and 353 is concomitant with an increase in dimer formation, suggesting a causal relationship between phosphorylation and oligomerization (14, 15). However, mutations of these tyrosines into phenylalanines does not alter ezrin localization in microvilli, and production of this mutated ezrin does not affect cell morphology (13). Thus, it has been proposed that tyrosine phosphorylation of ezrin may serve in signal transduction rather than mediating its cytoskeletal association. This notion was supported by the experiments in which phosphorylation of tyrosine 353 was found to signal cell survival during epithelial differentiation (16).

A phosphothreonine residue, originally identified in moesin (17), is localized in a conserved COOH-terminal region of ERM proteins (Thr567 in ezrin, Thr564 in radixin, and Thr558 in moesin). Using phosphospecific antibodies, this phosphorylated residue was detected in ezrin, radixin, and moesin from a variety of cells and tissues, and phosphorylated ERM proteins were shown to be present in actin-rich membrane structures (18–21). Two kinases, protein kinase C and Rho kinase, and
two phosphatases, myosin phosphatase and PP2C, were found in different systems to regulate the phosphorylation status of the conserved C-terminal threonine in ERM proteins (19, 21–23). The primary consequence of phosphorylating the COOH-terminal threonine is thought to regulate ezrin activity. Using an overlay assay, phosphorylation of Thr656 in the radixin COOH-terminal domain impaired its association with the NH2-terminal domain (19). Similarly, a T558D mutation of moesin, which mimics the phosphorylated state, was shown to alter the intra- and intermolecular interactions of ezrin (24). From the crystal structure, it appears that the phosphorylation of another Thr658 weakens the N/C-ERMAD interaction due to both electrostatic and steric effects (25). The phosphorylation of an isolated COOH-terminal fragment of ERM proteins does not affect its association with F-actin (19, 24). However, expression of Thr → Asp mutant forms of ezrin or moesin potentiates the formation of microvilli-like dorsal projections by growth factors (20, 26), whereas transfection of the nonphosphorylatable T558A moesin inhibits RhoA-induced formation of these structures (20, 27).

To explore the nature of ezrin phosphorylation associated with histamine-mediated stimulation of acid secretion, we carried out mass spectrometric analyses of ezrin phosphorylated in vivo and in vitro to identify a novel phosphorylation site on ezrin. Our studies show that Ser66 is a substrate of cAMP-dependent protein kinase and suggest that phosphorylation of Ser66 by PKA is critical for parietal cell activation. Given the fact that nonphosphorylatable ezrin prevents parietal cell activation, we proposed that PKA-mediated phosphorylation of ezrin provides a link between the activation of PKA to apical membrane cytoskeletal remodeling associated with acid secretion in parietal cells.

**EXPERIMENTAL PROCEDURES**

Reagents—[32P]GTP from PerkinElmer Life Sciences. Monoclonal antibody (JL-18) against GTP was purchased from Clontech (Palo Alto, CA), whereas ezrin antibody 4A5 was produced and described by Hanelz et al. (28). Antibody 2G11 against the β-subunit of HR-ATPase was described by Chow et al. (29). Monoclonal antibody against phosphoserine and all chemicals were ordered from Sigma. Rhodamine-coupled phosphoantim was purchased from Molecular Probes, Inc. (Eugene, OR). LipofectAMINE 2000 was obtained from Invivogen.

**DNA Construction**—The bacterial expression vectors containing human ezrin fused to glutathione S-transferase (GST) were generous gifts from Dr. Monique Arpin. GFP-ezrin was constructed by ligating an EcoRI-Sau1I PCR-amplified ezrin cDNA into pEGFP-N1 (Clontech, Palo Alto, CA). GEP-tagged ezrin mutants S66A and S66D were created by standard PCR methods and a site-directed mutagenesis kit (Takara Biotechnology, Dalian, China) according to the manufacturer’s manual using the following primers: S66D (forward), 5′-AAC AAG GTG GAT GCC CAG GAG TGC A-3′; S66D (reverse), 5′-ATC CAG CCT TCC CCA GGT AGG AAA T-3′; S66A (forward), 5′-AAC AAG GTG GTC GGC CAG GAG TGC A-3′; S66A (reverse), 5′-ATC CAG CCT TCC CCA GGT AGG AAA T-3′. The S66D mutant was created to mimic phosphorylated ezrin, whereas S66A was generated as nonphosphorylatable ezrin. All constructs were sequenced in full.

**Isolation of Gastric Glands and Aminopyrine Uptake Assay**—Gastric glands were isolated from New Zealand White rabbits as modified by Yao et al. (30). Briefly, the rabbit stomach was perfused under high pressure with PBS (2.25 mM K2HPO4, 6 mM Na2HPO4, 1.75 mM NaCl, and 136 mM NaCl) containing 1 mM CuCl2 and 1 mM MgSO4. The gastric mucosa was scraped from the smooth muscle layer, minced, and then washed twice with minimal essential medium (MEM) buffered with 20 mM HEPES, pH 7.4 (HEPES-MEM). The minced mucosa was then digested at 57°C for 30 min in a minimal amount (~20 μl) of HEPES-MEM containing 15 mg of collagenase (Sigma) and 20 μg each of bovine serum albumin (Sigma). Intact gastric glands were collected from the digestion mixture for 10–15 min and then washed three times in HEPES-MEM. In all subsequent gland experiments (aminopyrine uptake assay and two-dimensional SDS-PAGE analysis), glands were resuspended at 5% cyscot (v/v) in the appropriate buffer for final assay.

Stimulation of rabbit gastric glands was quantified using the aminopyrine (AP) uptake assay as modified by Yao et al. (30). Briefly, glands were loaded with [3H]Aminopyrine followed by treatment with 100 μM cimetidine. Stimulation of glands was achieved with 50 μM IBMX and 100 μM histamine. Gland preparations were incubated for different time intervals at 37°C with shaking (~160 oscillation/min) followed by a brief spin to separate the glands from supernatant. The gland pellets were dried and weighed, and aliquots of both the supernatant and pellet were counted in a Beckman liquid scintillation counter. These data were used to calculate the AP accumulation ratio (ratio of intracellular to extracellular AP concentration) as described (30). To calculate the stimulation index of AP uptake, the data are expressed as a fold level of the resting control from each individual time point.

**Preparation of Samples for Mass Spectrometry**—Excised two-dimensional protein spots were destained, chopped into small fragments with a razor blade, and subjected to digestion by modified pericorne trypsin (50–100 ng/digestion; Promega, Madison, WI) according to Zhou et al. (31). Peptides were recovered by three extractions of the digestion mixture with 50% acetonitrile plus 5% trifluoroacetic acid and desalted and concentrated using C18 ZipTips (Millipore Corp., Bedford, MA), eluting peptides in 50% (v/v) acetonitrile/water. All supernatants were pooled and concentrated to 5 μM using Speedvac and stored at 5°C until analysis.

**Data Base Interrogations Based on Experimentally Determined Peptide Masses**—The sequence data were carried out using mass spectrometry (MS–Fit) and PSD data interrogation was performed using MS-Tag; both software programs were developed in the University of California San Francisco MS Facility and are available on the World Wide Web at prospector.ucsf.edu. Both the National Center for Biotechnology Information protein data base and Swiss-Prot data base were searched. Search parameters, including the putative molecular weight and a peptide mass tolerance of 100–200 parts/million.

**In Vitro Phosphorylation of Ezrin by PKA**—Both the GST-wild type ezrin and GST-S66A ezrin proteins were expressed in Escherichia coli BL21 (pLYS), and the purification of the GST fusion proteins was done by affinity chromatography using glutathione-Sepharose beads (Sigma) as described (32). The fusion proteins bound to glutathione-Sepharose beads were suspended in phosphorylation buffer prior to use.

To verify whether Ser66 is a substrate for PKA, 5 μg of purified GST-ezrin fusion protein, both wild type and S66A mutant, were incubated with 10 units of the catalytic subunit of PKA (New England
PKA Phosphorylates Ezrin

RESULTS

Identification of Phosphoerzin Associated with Parietal Cell Secretion—Separation of ezrin by two-dimensional gel electrophoresis is shown in Fig. 1A for resting and stimulated gastric glands. The Coomassie Brilliant Blue-stained gel shows that stimulation with histamine plus IBMX resulted in a shift of ezrin spots to more acidic pH, consistent with the stimulation-dependent phosphorylation of ezrin reported previously (e.g., Ref. 10). Stimulation through the histamine/cAMP pathway induced a reduction in the relative intensity of the major alkaline spot (0; estimated pI of 6.7) and a shift toward a series of more acidic isoforms 1, 2, and 3, an estimated pI varying from 6.6 to 6.4, respectively. Treatment with H89, a selectively sensitive inhibitor of PKA, prevented such shifts in ezrin isoforms on twodimensional SDS-PAGE (not shown), verifying that the phosphorylation of ezrin is downstream from the activation of protein kinase A.

Histamine stimulation induced three acidic ezrin spots separating with a distance equivalent to −0.1 pH unit, which reflects multiple phosphorylation sites involved. The labeling of 1, 2, and 3 represents the number of phosphomodification sites on ezrin proteins based on the shift of pI. Over the years, monoclonal antibodies against phosphoamino acids (e.g., serine, threonine, and tyrosine) have been generated as useful probes to detect phosphoprotein and the nature of the phosphorylated amino acid. To test if any of these acidic spots are related to phosphoserine induced by histamine stimulation, we carried out Western blotting using two-dimensional SDS-PAGE of separated ezrin spots. As shown in Fig. 1B, phosphoerzin antibody selectively reacts strongly with acidic spot 3 of ezrin from the stimulated preparation, although there was also very minor reactivity with spot 2. Since an isoelectric shift is characteristic of protein phosphorylation, the acidic phosphoserine-positive spots suggest that phosphorylation occurred on a serine residue of ezrin.

To evaluate the stimulus-induced ezrin phosphorylation in relation to activation of acid secretion, we used gastric glands to measure the time course of ezrin phosphorylation judged by a phosphoserine antibody, and in a parallel set of glands we measured acid secretion by the AP uptake assay (Fig. 1C). A typical two-dimensional Western blot used to quantify the extent of ezrin phosphorylation is shown as Fig. 1B, demonstrating that the phosphoserine antibody rather selectively reacts with acidic ezrin spots (2 and 3). For each time point after stimulation with histamine, the summed intensity of the acidic,
phosphoserine-positive, ezrin spots was compared with the resting control, normalized by ezrin spot intensity, and plotted in Fig. 1C (solid circles). The acid stimulation index, defined as $\frac{\text{AP}_{\text{stimulated}}}{\text{AP}_{\text{resting}}}$, is also plotted on the same scale (Fig. 1C, open circles). The relative acid secretory response reached a maximal level after about 15 min of stimulation and was sustained for the 35 min of measurement. On the other hand, the index of protein phosphorylation peaked earlier, at about 7.5 min, and slowly returned to near resting level by 35 min. The temporal profiles of AP uptake and ezrin protein phosphorylation are consistent with reports in the literature (e.g. Ref. 10) and suggest that the phosphorylation event may be required for parietal cell activation.

To identify the phosphoamino acids associated with histamine stimulation, the acidic ezrin isotype spots (e.g. 1–3 from the histamine-stimulated samples) were removed from Coomassie Brilliant Blue-stained two-dimensional gel, combined, and subjected to in-gel digestion with trypsin. The corresponding regions of two-dimensional gel from resting samples were used as control. The resulting phosphopeptides were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. These multiple spots represent the same ezrin protein with different modifications. The tryptic peptides recovered from mass spectrometric analysis indicate that Thr$^{36}$, Ser$^{66}$, and Tyr$^{193}$ are possible substrates accounting for ezrin phosphorylation in parietal cell activation stimulated by histamine.

Ser$^{66}$ Is a Substrate of Protein Kinase A—Early analysis of the amino acid sequence of ezrin suggested three potential PKA phosphorylation sites, such as Ser$^{66}$ (11). Our mass spectrometric analysis also pointed to the possibility of Ser$^{66}$ phosphorylation in response to histamine stimulation. To test whether Ser$^{66}$ is a substrate of PKA, we performed in vitro phosphorylation on recombinant GST-ezrin fusion proteins, including both wild type ezrin and a mutant ezrin in which serine 66 was replaced by alanine (S66A). Both GST fusion proteins, wild type and S66A mutant ezrin, migrate at about the predicted 105 kDa as shown in Fig. 2A. Incubation of the fusion proteins with [32P]ATP and the catalytic subunit of PKA resulted in the incorporation of 32P into wild type but not S66A mutant ezrin (Fig. 2B). This PKA-mediated phosphorylation is specific, since incubation of ezrin with [32P]ATP in the absence of the kinase resulted in no detectable incorporation of radioactivity into the wild type protein. Thus, Ser$^{66}$ of ezrin is probably a substrate for PKA.

To verify whether Ser$^{66}$ of ezrin is phosphorylated in response to histamine stimulation, we transfected GFP-ezrin, both wild type and nonphosphorylatable S66A mutant, into cultured parietal cells followed by stimulation and immunoprecipitation of GFP-ezrin fusion proteins from the stimulated cells. GFP antibody absorbed a small portion of exogenously expressed GFP-ezrin proteins, but not endogenous ezrin, from the parietal cell lysates as labeled by ezrin antibody 4A5 (Fig. 2C, ezrin blot). However, anti-phosphoserine antibody only marks wild type GFP-ezrin and not mutant S66A ezrin from stimulated parietal cells, indicating that Ser$^{66}$ is responsible for histamine-stimulated phosphorylation on serine. Thus, we conclude that Ser$^{66}$ of ezrin is involved in histamine stimulation of parietal cells.

Exogenously Expressed GFP-Ezrin Is Primarily Associated with Cytoskeleton—To evaluate the efficacy of exogenous ezrin expression, cultured parietal cells were transfected with a GFP-tagged wild type ezrin plasmid. Western blotting analysis carried out using transfected cells showed that exogenously expressed ezrin protein was about twice the level of endogenous ezrin in cultured parietal cells (Fig. 3A). Assuming a transfection efficiency of about 45–50%, the actual expression level of GFP-ezrin in positively transfected cells is about 4-fold higher than that of endogenous protein.
To determine whether there were any major changes in the behavior of exogenously expressed GFP-ezrin, we measured the partitioning of endogenous ezrin and exogenously expressed GFP-ezrin into the Triton X-100-soluble fraction compared with the insoluble "cytoskeletal" fraction based on the Western blotting analyses. In the case of transfected cells, only GFP-ezrin content was measured. As summarized in Fig. 3, 71.7 ± 3.3% of endogenous ezrin resides in the Triton X-100-insoluble fraction, consistent with previous reports (e.g. Refs. 31 and 35).

Partitioning of wild type GFP-ezrin is similar to endogenous ezrin; 68.5 ± 3.7% of wild type GFP-ezrin is associated with the Triton X-100-insoluble fraction.

To probe for the potential role of Ser 66 phosphorylation in promoting the association of ezrin with the cytoskeleton, we generated two mutant ezrin plasmids that encode mutant proteins mimicking nonphosphorylatable Ser66 (S66A) and permanently phosphorylated ezrin (S66D), respectively. As shown in Fig. 3B, both mutant ezrin proteins have a distribution pattern consistent with previous reports (e.g. Refs. 31 and 35).

PKA Phosphorylates Ezrin

FIG. 2. Serine 66 of ezrin is a substrate of PKA. For A and B, bacterially expressed GST-ezrin fusion proteins, both wild type and mutant S66A, were purified on glutathione-Sepharose beads and phosphorylated in vitro using [γ-32P]ATP and purified catalytic subunit of PKA as described under "Experimental Procedures." Samples were separated by 6–16% gradient SDS-PAGE gel. A, Coomassie Brilliant Blue-stained gel of samples of wild type GST-ezrin without PKA added (Ezrin only), wild GST-ezrin plus PKA (Ezrin + PKA), and mutant S66A GST-ezrin plus PKA (S66A + PKA). Note that roughly equivalent amounts of GST-ezrin protein was present in the three reactions. B, the same gel was dried gel and subsequently incubated with x-ray film. Note that in the presence of PKA there was dramatic incorporation of 32P into wild type, but not mutant, ezrin protein. C, the cultured parietal cells transfected with GFP-ezrin (wild type and S66A) were collected 36 h post-transfection for immunoprecipitation as outlined under "Experimental Procedures." Equivalent amounts of proteins from the lysates and nonbinding fraction were applied to SDS-PAGE. Following separation by 6–16% gradient SDS-PAGE and subsequent transblotting onto nitrocellulose membrane, the blot was first probed with an anti-ezrin antibody 4A5 and developed with an ECL kit (Pierce). Because GFP adds about 25 kDa in mass to the fusion proteins, GFP-tagged ezrin migrates as upper bands (arrow), whereas endogenous ezrin protein migrates as lower bands (arrowhead). After ezrin probing, the blot was stripped and reprobed with a monoclonal antibody reacting with phosphoserine and visualized by ECL. The phosphoserine antibody recognizes wild type GFP-ezrin but not nonphosphorylatable GFP-ezrin. Note that endogenous ezrin from stimulated parietal cells was also labeled by the phosphoserine antibody.

FIG. 3. Exogenously expressed GFP-ezrin protein behaves like endogenous ezrin. A, samples from both mock-transfected and GFP-ezrin-transfected parietal cells were prepared and separated on SDS-PAGE, blotted to nitrocellulose, and probed by an ezrin antibody 4A5. Note that the ezrin antibody recognizes both endogenous (80-kDa) and exogenously expressed GFP-ezrin (105-kDa) proteins. B, cultures of parietal cells were either mock-transfected (endogenous) or transfected with one of several GFP-ezrin constructs, including wild type ezrin, S66A substitution mutant, and S66D substitution mutant. 30–36 h post-transfection, the cells were extracted with 0.1% Triton X-100 solution and separated into soluble (s) and insoluble (i) fractions as described under "Experimental Procedures." Equivalent amounts of proteins from the soluble and insoluble fractions were applied to SDS-PAGE. Following separation on 6–16% gradient SDS-PAGE and transblotted onto nitrocellulose membrane, the blots were probed with an anti-ezrin antibody 4A5 and developed with an ECL kit (Pierce). GFP-tagged ezrin migrates around 105 kDa, whereas endogenous protein situates around 80 kDa on SDS-PAGE gel, it was then possible to distinguish endogenous protein and exogenously expressed ezrin as exemplified in A. The signals were quantified using a PhosphorImager with values expressed as a percentage of total (soluble + insoluble). The error bars represent S.E.; n = 3 preparations.
similar to the wild type. These results indicate that exogenously expressed GFP-ezrin bears biochemical characteristics similar to endogenous protein.

Localization of GFP-Ezrin to the Apical Membrane Independent of Ser66—The subcellular localization of the exogenously expressed GFP-ezrin constructs was compared with that of endogenous ezrin by fluorescence microscopy. Control cultured parietal cells were double stained for endogenous ezrin using an ezrin antibody (green) and for F-actin using phalloidin (red). The transfected cells were double-stained for GFP-ezrin using a monoclonal GFP antibody (green) and double-stained for F-actin using phalloidin (red). Fig. 4A shows optical sections from control and transfected cells, all maintained in the nonsecreting state. Similar to what has been noted in earlier studies, endogenous ezrin in control cells is localized to the plasma membranes, most prominently to the apical membrane vacuoles that have been sequestered into the cell interior and somewhat more sparsely to the basolateral membrane that surrounds the cells. The ezrin signal is relatively co-localized with F-actin (Fig. 4, a, a’, and a”). The distribution of the signal for all three GFP-ezrin constructs (wild type, S66A, and S66D) was similar to that of endogenous ezrin (i.e. primarily associated with apical membrane vacuoles and to a lesser extent with basolateral membrane) (Fig. 4A, b, c, and d). The distribution of F-actin was also not altered by the transfections (Fig. 4A, b’, c’, and d’). These data demonstrate that transfected GFP-ezrin is targeted to the same loci as endogenous ezrin and that phosphorylation of Ser66 is not responsible for targeting of ezrin to actin-based cytoskeleton at the apical plasma membrane.

There was, however, at least one striking difference in the morphology of nonsecreting parietal cells transfected with the Ser66 mutants. Cells expressing mutant S66D, a mutant protein mimicking phosphorylated ezrin, have dilated apical vacuole membranes, characteristic of secreting parietal cells, although no stimulant was added. As shown in Table I, the average diameter of apical vacuoles in S66D-expressed cells (7.2 ± 0.5 μm) is about twice as big as those of cells expressing wild type GFP-ezrin (3.5 ± 0.3 μm) or S66A mutant ezrin (3.4 ± 0.3 μm). These data suggest that phosphorylation of Ser66 is not required for apical localization of ezrin protein but may be involved in apical membrane cytoskeletal remodeling.

Phosphorylation of Ser66 Is Required for Apical Membrane Dynamic—Stimulation of parietal cells by histamine results in dramatic expansion of the apical canalicular plasma membrane due to insertion of H,K-ATPase-containing vesicular membranes and subsequent proton pumping into the canalicular space. In cultured parietal cells, the same membrane transformations occur, but, because the apical canalicular membrane has been incorporated into the vacuolar forms, stimulation results in dilation of apical membrane vacuoles as active HCl and water transport occur (34). Because of this swelling, stimulated parietal cells are considerably larger in diameter than their resting counterparts (34). Since the expression of S66D mutant ezrin affected relatively dilated apical membrane vacuoles, we tested whether phosphorylation of Ser66 is critical for apical cytoskeletal remodeling. To this end, we assessed the effects of stimulation on parietal cells transfected with wild type, S66A, and S66D ezrin tagged with GFP. Fig. 4B shows optical sections taken from parietal cells treated with the secretagogues histamine plus IBMX and probed for F actin and for ezrin (using either anti-ezrin or anti-GFP antibodies, similar to the protocol used in Fig. 4A). As for resting parietal cells, GFP-ezrin and F-actin are primarily co-localized to the same regions in secreting cells. For all conditions, F-actin labeling (Fig. 4B, a’–d’) outlines the dilated apical membrane in addi-
PKA Phosphorylates Ezrin

Phosphorylation of Ser66 is required for the apical membrane extension

<table>
<thead>
<tr>
<th>Treatment (No. of cells counted)</th>
<th>Vacuole diameter* μm</th>
<th>No. of vacuoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting (Cimetidine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (83 cells)</td>
<td>3.5 ± 0.3</td>
<td>229</td>
</tr>
<tr>
<td>S66A (85 cells)</td>
<td>3.4 ± 0.3</td>
<td>215</td>
</tr>
<tr>
<td>S66D (87 cells)</td>
<td>7.2 ± 0.5*</td>
<td>197</td>
</tr>
<tr>
<td>Stimulated (histamine + IBMX)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (89 cells)</td>
<td>15.7 ± 1.1</td>
<td>137</td>
</tr>
<tr>
<td>S66A (86 cells)</td>
<td>6.3 ± 0.5*</td>
<td>173</td>
</tr>
<tr>
<td>S66D (84 cells)</td>
<td>16.3 ± 1.0</td>
<td>151</td>
</tr>
</tbody>
</table>

* Diameters of apical vacuoles were measured as an index for apical membrane extension associated with acid secretion. Values are presented as mean ± S.E. A Student’s t test indicated that the diameter of S66D was compared with that of wild type ezrin-expressing resting cells, whereas 1–3 vacuoles/cell were scaled stimulation.

Phosphorylation of Ser66 Is Important for Parietal Cell Activation — A large part of parietal cell activation involves translocation and insertion of H,K-ATPase-containing vesicular membranes into the apical membrane for subsequent proton pumping into canalicular space (e.g. Refs. 3 and 36). Several earlier studies established the importance of the actin-based cytoskeleton (e.g. Ref. 37) and the integrity of ezrin (30) in parietal cell activation. Given the observed morphological responses to the mutant forms, it was of interest to ascertain the function of the phosphorylation of ezrin Ser66 on the translocation and/or insertion of H,K-ATPase from cytoplasmic vesicles to the apical membrane. Accordingly, we examined the distribution of H,K-ATPase in cultured parietal cells expressing wild type, S66A, and S66D mutant ezrin proteins. Fig. 5 shows optical sections taken from secreting parietal cells simultaneoulsy probed with fluorescein-coupled H,K-ATPase antibody (2G11; Fig. 5, a, b, e, and c) and rhodamine-conjugated phalloidin (a’, b’, e’). The F-actin distribution is similar to that shown in Fig. 4, outlining the apical membrane vacuoles and the basolateral membrane surface. In resting cells, H,K-ATPase staining is distributed throughout the cytoplasm as previously documented, although we also noted some accumulation on the apical membrane vacuoles (e.g. Ref. 34; Fig. 5d). Stimulation of cells transfected with wild type ezrin revealed a diminution of H,K-ATPase in the cytoplasmic compartment concomitant with an enrichment in the apical membrane of vacuoles (Fig. 5e), indicating that stimulation-induced translocation of H,K-ATPase occurs in cells expressing exogenous ezrin. Overlapping images demonstrate a superimposition of the F-actin and H,K-ATPase probes in the apical membrane of parietal cells, verifying the apical trafficking of H,K-ATPase (yellow, Fig. 5e’). However, for cells expressing S66A mutant ezrin, stimulation failed to mobilize H,K-ATPase to the apical membrane, since the staining remains in cytoplasm (Fig. 5b), suggesting that phosphorylation of Ser66 might be involved in the recruitment of H,K-ATPase. Indeed, examination of parietal cells expressing S66D ezrin show that the mutant does facilitate the stimulation-induced translocation of H,K-ATPase. We therefore conclude that PKA-mediated phosphorylation of ezrin Ser66 is important for proton pump translocation essential for parietal cell activation.

DISCUSSION

Ezrin, a founding member of the membrane-cytoskeleton linker family of ERM proteins, has been implicated in a variety of dynamic cellular functions such as determination of cell shape, cell adhesion, motility, and survival (e.g. Ref. 1). Gastric ezrin was identified by virtue of its phosphorylation correlated with histamine-stimulated gastric acid secretion in parietal cells (10). Despite numerous investigations on the structure-functional interrelationships, the PKA-mediated phosphorylation on ezrin has not been well characterized. Here we provide the first evidence that ezrin is substrate of PKA in vivo and in vitro. Moreover, we have mapped an important PKA-mediated phosphorylation site on ezrin to Ser66. Furthermore, our studies show that heterologously expressed ezrin linked to GFP is targeted to the apical membrane of cultured parietal cells, similar to endogenous ezrin. However, mutations of Ser66 on ezrin alter apical membrane dynamics associated with histamine stimulation, suggesting that phosphorylation of Ser66 on ezrin is required for proton pump mobilization and polarized secretion in gastric parietal cells. A major role for the actin cytoskeleton in the secretory processes of parietal cells has been inferred from studies using actin disruptors that disorganize actin filaments and act to inhibit acid secretion (37). Highly organized microfilaments are typical features of microvilli at the apical membrane within the parietal cell canalculus. In going from rest to the secreting state, there are major changes at the apical canalicular surface, including elongation of microvilli. Interestingly, as the parietal cell returns to the resting state after withdrawal of stimulants, microfilament ultrastructural changes become apparent as a disorganization of actin filaments along with collapse of the apical canalicular surface (36, 38). These morphological studies indicate that reversible actin-based cytoskeletal dynamics are tightly linked to the secretory cycle in parietal cells.

Phosphorylation of C-terminal ezrin (e.g. Thr567) has been shown to regulate its association with the actin cytoskeleton (39). This observation was supported by the finding that phosphorylation of the homologous threonine 558 in moesin is required for F-actin binding in vitro (21, 40). Using mutant ezrin T567D, mimicking the phosphorylated protein, Matsui et al.
showed that ezrin phosphorylation alters intracellular interactions. T567D ezrin is a strongly morphogenic variant that triggers the formation of wide lamellipodia, extensive membrane ruffles, and microvilli-rich projections when overexpressed, indicating that T567D ezrin promotes actin cytoskeletal dynamics. Our studies revealed no evidence that Thr^{567} is phosphorylated and involved in parietal cell activation. It is possible that an upstream kinase responsible for Thr^{567} phosphorylation is not related to the parietal cell activation cascade. Our present studies, however, do show that phosphorylation of ezrin is dynamic and correlated with histamine-stimulated parietal cell secretion. Alteration of Ser^{66} phosphorylation did not change ezrin association with the cytoskeleton but did modulate the activity of apical membrane dilation in response to stimulation, consistent with the notion that the C-terminal domain of ezrin is responsible for actin binding, whereas its N-terminal region is responsible for the association of ezrin with other proteins proximal to the plasma membrane. To search for ERM binding partners potentially involved in membrane association, Reczek et al. (41) used GST-ezrin as an affinity matrix to isolate a 50-kDa phosphoprotein named EBP50 from human placental cell lysates. These authors further showed that the N-terminal ezrin binds to the C-terminal PDZ domain of EBP50 (42). However, an initial search for EBP50 in gastric parietal cells was negative. Thus, it is likely that ezrin binds to a functional homologue of EBP50, which mediates the association of ezrin with the apical plasma membrane of parietal cells. Since parietal cell activation involves translocation of H,K-ATPase from cytoplasm to the apical plasma membrane, it would be of great interest to illustrate how mutant ezrin S66A blocks the apical membrane dynamics and H,K-ATPase translocation process. Since the translocation of H,K-ATPase onto the apical membrane involves multiple steps, including the possible trafficking over actin filaments, docking to secretory sites, insertion of the pump into the apical membrane, and perhaps maintenance of the pump in apical membrane during active secretion, it will be important to distinguish precisely where the phosphorylation of Ser^{66} participates.

Our studies show that histamine-stimulated incorporation of phosphate onto ezrin peaks in about 8 min and then declines. Interestingly, acid secretion reaches its maximum about 15 min after the stimulation, suggesting that phosphorylation of serine is an early event of the activation process. In fact, expression of ezrin mutant S66D, which mimics phosphorylated Ser^{66}, results in phenotypes of partially stimulated parietal cells, suggesting that phosphorylation of Ser^{66} alone is not sufficient for maximal activation. It is likely that a parallel pathway distant from the Ser^{66} phosphorylation cascade is required for complete activation. Alternatively, phosphorylation of ezrin in other residues may be synergistic for the activation process. In fact, we have noticed other suggestive phosphorylation sites including Ser^{66D} and Ser^{412} in some of our preparations. In any event, further characterization of ezrin phosphorylation associated with histamine stimulation will provide detailed structure-function relationships of the role of ezrin in parietal cell secretion.

Taken together, the present work reveals that ezrin is phosphorylated by PKA on Ser^{66} and that this PKA-induced phosphorylation is essential for parietal cell activation. Finally, we show that nonphosphorylatable ezrin blocks translocation of H,K-ATPase to the apical membrane. We propose that phosphorylation of ezrin links proton pump trafficking to apical membrane-cytoskeletal dynamics required for polarized secretion in epithelial cells.

\[\text{PKA Phosphorylates Ezrin}\]

**Fig. 5.** *Exogenous expression of nonphosphorylatable GFP-ezrin diminishes H,K-ATPase trafficking.* A, this pair of triple montage represents confocal images collected from resting and stimulated gastric parietal cells doubly stained for H,K-ATPase (green), ezrin (red), and their merged images. As described in the legend to Fig. 4B, stimulation induces remodeling of the apical membrane due to the fusion of H,K-ATPase-containing vesicles, which was readily apparent by a co-localization of H,K-ATPase with ezrin at the periphery of the expanded apical membrane vacuoles (b, c, and d). In contrast, H,K-ATPase was seen throughout the cytoplasm of resting parietal cells with some localization to the apical membrane vacuoles, where ezrin is primarily localized (a, a', a''). B, this set of triple montage represents confocal images collected from histamine-stimulated parietal cells, expressing exogenous ezrin (wild type, S66A, and S66D), doubly stained for H,K-ATPase (green), F-actin (red), and their merged results. Stimulation of parietal cells expressing exogenous ezrin triggers mobilization of H,K-ATPase (c) to the apical membrane vacuoles, which is evident by superimposition of H,K-ATPase staining onto that of apical F-actin staining (c' and c''). However, mutant ezrin (S66A but not S66D) attenuates the dilation of apical vacuoles and prevents the translocation of H,K-ATPase from cytoplasm to the apical membrane vacuoles (d, d', and d''), suggesting that PKA-mediated phosphorylation of Ser^{66} is necessary for mobilization of the proton pump. Note that histamine stimulation induces remodeling of apical membrane and H,K-ATPase translocation in S66D-expressing cells (e, e', and e''). Bar, 20 μm.

\[\text{References:}\]

2 X. Cao, E. Chen, and X. Yao, unpublished results.
Acknowledgments—We thank Dr. Monique Arpin for ezrin cDNA constructs. We also thank members of our group for insightful discussion during the course of this study. The mass spectral data were obtained in part at the University of California San Francisco Mass Spectrometry Facility (A. L. Burlingame, Director).

REFERENCES