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

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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 indicated 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 A431 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 Thr565 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...
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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 [14C]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.

EXPERIMENTAL PROCEDURES

Reagents—(14)[C]Aminopyrine and [32P]ATP were obtained from PerkinElmer Life Sciences. Monoclonal antibody (JL-18) against GFP was purchased from Clontech (Palo Alto, CA), whereas ezrin antibody 4A5 was produced and described by Hanzel et al. (28). Antibody 2G11 against the β-subunit of HK-ATPase was described by Chow et al. (29). Monoclonal antibody against phosphoserine and all chemicals were ordered from Sigma. Rhodamine-coupled phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR). LipofectAMINE 2000 was obtained from Invitrogen.

DNA Construction—The bacterial expression vectors containing hu-}

Preparation of Samples for Mass Spectrometry—Excised two-dimen-
sional protein spots were destained, chopped into small fragments with a razor blade, and subjected to digestion by modified porcine 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% acetonitrile/water. All supernatants were pooled and concentrated to 5 μl in a Speedvac concentrator and to 25 μl in 50% acetonitrile, 5% trifluoroacetic acid. The peptide mix was stored at 20 °C until analysis.

Matrix-assisted Laser Desorption Time-of-flight (MALDI-TOF) Mass Spectrometric Identification of Phosphopeptides of Ezrin—Aliquots of unseparated tryptic digesta were co-crystallized with cyano-4-hydroxy-
cinnamic acid and analyzed using a MALDI delayed extraction reflec-
tion TOF instrument (Bi-flex; Bruker-Daltonics, Framingham, MA) equipped with a nitrogen laser. Measurements were performed in a positive ionization mode. All MALDI spectra were externally calibrated using a standard peptide mixture (Sigma).

Data base interrogations based on experimentally determined pep-
tide masses 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 param-
eters included the putative molecular weight and a peptide mass
tolerance of 100–200 parts/million.

In Vitro Phosphorylation of Ezrin by PKA—Both the GST-null type
ezrin and GST-S66A ezrin proteins were expressed in Escherichia coli
BL21 (pLYS), and the purification of the GST fusion proteins was
HPLC 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-εzrin fusion protein, both wild type and S66A mutant, were
incubated with 10 units of the catalytic subunit of PKA (New England

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 Thr664 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 ankyrin
Thr668 weakens the N/C-ERMAD interaction due to both elec-
trostatic 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

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 KH2PO4, 6 mM NaH2PO4, 1.75 mM
Na2HPO4, and 136 mM NaCl) containing 1 mM CaCl2 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 37 °C for ~30 min in a minimal amount (~20 ml) of
HPLC column containing 15 mg of collagenase (Sigma) and 20 mg 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 resus-
pended at 5% cckytocrit (vv) in the appropriate buffer for final assay.

Assay and two-dimensional SDS-PAGE analysis), glands were resus-
peared with 10 units of the catalytic subunit of PKA (New England

Palo Alto, CA). GFP-tagged ezrin mutations S66A and S66D were
PCR-amplified ezrin cDNA into pEGFP-N1 (Clontech,

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Biolabs) in phosphorylation buffer containing 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, 10 mM ATP, and 5 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) in a total volume of 30 μl. Kinase reaction was carried out at room temperature for 15 min and terminated by the addition of 10 μl of 4 × SDS-PAGE sample buffer and separated by 6–16% gradient SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, dried, and quantified by a PhosphorImager (Amersham Biosciences).

Cell Culture and Transfection—Primary cultures of gastric parietal cells from rabbit stomach were produced and maintained as described (31). Separate cultures of parietal cells were transfected with plasmids encoding GFP-tagged wild type ezrin and two mutant forms of ezrin (S66A and S66D) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 1 μg of DNA was incubated with 600 μl of Opti-MEM (antibiotics-free) while 6 μl of LipofectAMINE 2000 was added and left at room temperature for 25 min. The cultured parietal cells (~3% cytocrit; 6-well plates) were washed once with Opti-MEM. The DNA-lipid mix was added to the plates and incubated for 4 h, followed by replacement of 1.5 ml of medium B. The transfected cells were then maintained in culture at 37 °C until use for protein expression, partition, immunoprecipitation, or immunofluorescence.

For analyzing the exogenous protein expression, transfected parietal cells were harvested 30–36 h after transfection and lysed in 1 × SDS-PAGE sample buffer. To analyze the relative level of ezrin associated with cytoskeleton, transfected cells were raised with PHERIUM buffer (100 mM Tris, pH 6.8, 5 mM MgCl₂, 2 mM EGTA, and 4 mM glycerol) twice followed by incubation of PHERIUM buffer containing a protease inhibitor mixture (pepstatin-A, leupeptin, aprotinin, and chymostatin; final concentration 5 μg/ml for each inhibitor) plus 0.1% Triton X-100 for 1 min at room temperature to allow cytosolic proteins to be released into extracellular medium (33). The extracted cells were then harvested from a Petri dish and centrifuged at 1,500 × g for 5 min. The resulting pellets, designated as insoluble materials, were solubilized in 1 × SDS-PAGE sample buffer, whereas the supernatants, called the soluble fraction, were concentrated with 5% trichloroacetic acid and further precipitated with 15 μg of monomeric antibody (JL-18) at room temperature for 2 h followed by the addition of 10 μl of protein A/G beads for an additional 1 h (Pierce). The beads were collected and washed three times with Tris-buffered saline before boiling in SDS-PAGE sample buffer. Immunoprecipitates were then fractionated by SDS-PAGE, and proteins were transferred onto nitrocellulose membrane for Western blotting analyses. The blot was first labeled with ezrin antibody 4A5 to verify the efficiency of GFP immunoprecipitation. The blot was then stripped with SDS-PAGE sample buffer at 55 °C for 20 min followed by validation of serine phosphorylation on GFP-ezrin using a phosphoserine antibody.

Immunofluorescence Microscopy—For cytolocalization of exogenously expressed ezrin, cultured parietal cells were transfected with GFP, wild type GFP-ezrin, and GFP-ezrin mutants (S66A and S66D) and maintained in MEM for 30–36 h. Some cultures were treated with 100 μm cimetidine to maintain a resting state; others were treated with the same concentration plus 100 μM histamine plus 50 μM IBMX in the presence of SCH28098, a proton pump inhibitor (34). Treated cells were then fixed with 2% formaldehyde for 10 min and washed three times with PBS followed by permeabilization in 0.1% Triton X-100 for 5 min. Prior to application of primary antibody, the fixed and permeabilized cells were blocked with 0.5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.1% Tween 20, 0.1% gelatin, and 50 μM histamine plus 50 μM IBMX in the presence of SCH28098, a proton pump inhibitor (34). Treated cells were then incubated with primary antibodies, and the corresponding Alexa Fluor 488 or 594-labeled secondary antibody was applied. Coverslips were mounted on slides with a drop of 1:1 mix of Dako fluorescent mounting medium and were observed with a Zeiss LSM 510 NLO confocal microscope. Figures were constructed using Adobe Photoshop.

Confocal Microscopy—Immunostained parietal cells were examined under a laser-scanning confocal microscope LSM510 NLO (Carl Zeiss) scan head mounted transversely to an inverted microscope (Axiovert 200; Carl Zeiss) with a 40 × 1.0 numerical aperture PlanApo objective.

Measurement of the Diameter of the Apical Vacuoles—The diameter of phallolidin-stained apical vacuoles was measured using LSM 5 software (Carl Zeiss) and a Zeiss Axiovert 200 fluorescence microscope calibrated with a stage micrometer. When suitable vacuoles were identified, the image was enlarged 3-fold to facilitate accurate placement of a computer-generated cursor over the vacuoles outlined by phallolidin staining. Vacuole diameter was calculated as the mean of the major and minor axis of the F-actin-outlined vacuolar staining.

Western Blot—Samples were subjected to SDS-PAGE on 6–16% gradient gel and transferred onto nitrocellulose membrane. In some cases, samples were first separated by isoelectric focusing followed by SDS-PAGE on 6–16% gradient gel and subsequently transferred onto nitrocellulose membrane. Proteins were probed by appropriate primary antibodies and detected using ECL (Pierce). The band intensity was then quantified using a PhosphorImager (Amersham Biosciences).

RESULTS

Identification of Phosphoezrin 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/AMP 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 isofoms 1, 2, and 3, an estimated pI varying from 6.6 to 6.4, respectively. Treatment with H89, a selectively inhibitory 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 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, phosphoserine 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 phosphoserinepositive 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,
from mass spectrometric analysis indicate that Thr36, Ser66, and but with different modifications. The tryptic peptides recovered by peptide mass fingerprinting using MALDI-TOF mass spectrometry. These multiple spots represent the same ezrin protein region of two-dimensional gel from resting samples were removed from Coo- massie 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 but with different modifications. The tryptic peptides recovered from mass spectrometric analysis indicate that Thr36, Ser66, and Tyr191 are possible substrates accounting for ezrin phosphorylation in parietal cell activation stimulated by histamine.

Ser66 Is a Substrate of Protein Kinase A—Early analysis of the amino acid sequence of ezrin suggested three potential PKA phosphorylation sites, such as Ser66 (11). Our mass spectrometric analysis also pointed to the possibility of Ser66 phosphorylation in response to histamine stimulation. To test whether Ser66 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, Ser66 of ezrin is probably a substrate for PKA.

To verify whether Ser66 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 Ser66 is responsible for histamine-stimulated phosphorylation on serine. Thus, we conclude that Ser66 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 endogeneous 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 endogeneous 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...
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**Localization of GFP-Ezrin to the Apical Membrane Independent of Ser<sup>66</sup>**—The subcellular localization of the exogenously expressed GFP-ezrin constructs was compared with that of endogenous ezrin by fluorescence microscopy (Fig. 4A). 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 Ser<sup>66</sup> 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 Ser<sup>66</sup> 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 Ser<sup>66</sup> is not required for apical localization of ezrin protein but may be involved in apical membrane cytoskeletal remodeling.

**Phosphorylation of Ser<sup>66</sup> 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 Ser<sup>66</sup> 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-
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**Table I**

| Treatment (No. of cells counted) | Vacuole diameter* | No. of vacuoles |
|----------------------------------|-------------------|----------------|
| Resting (Cimetidine)             |                   |                |
| Wild type (83 cells)             | 3.5 ± 0.3         | 229           |
| S66A (85 cells)                  | 3.4 ± 0.3         | 215           |
| S66D (87 cells)                  | 7.2 ± 0.5          | 197           |
| Stimulated (histamine + IBMX)    |                   |                |
| Wild type (89 cells)             | 15.7 ± 1.1        | 137           |
| S66A (86 cells)                  | 6.3 ± 0.5          | 173           |
| S66D (84 cells)                  | 16.3 ± 1.0         | 151           |

* Diameters of apical vacuoles were measured as an index for apical membrane extension associated with acid secretion. Data were obtained from resting and stimulated parietal cells in which apical vacuoles were in the same focal plane. These measurements were made from three different preparations in which more than 80 cells from each category were examined. In resting cells, measurements were carried out on 2–5 vacuoles/cell, whereas 1–3 vacuoles/cell were selected for stimulation. Data are expressed as mean ± S.E.

p < 0.05; the diameter of S66D was compared with that of wild type ezrin-expressing resting cells.

p < 0.05; the diameter of S66A was compared with that of wild type ezrin-expressing secreting cells.

**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 vitro and in vivo. 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.
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, b' and b''). 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', and 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, a membrane vacuoles, where ezrin is primarily localized (b, b' and b'').

showed that ezrin phosphorylation alters intramolecular interactions. T567D ezrin is a strongly morphogenetic 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\textsuperscript{567} is phosphorylated and involved in parietal cell activation. It is possible that an upstream kinase responsible for Thr\textsuperscript{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\textsuperscript{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 ERB 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.\textsuperscript{2} 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\textsuperscript{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\textsuperscript{66}, results in phenotypes of partially stimulated parietal cells, suggesting that phosphorylation of Ser\textsuperscript{66} alone is not sufficient for maximal activation. It is likely that a parallel pathway distant from the Ser\textsuperscript{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\textsuperscript{666} and Ser\textsuperscript{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\textsuperscript{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.

\textsuperscript{2} X. Cao, E. Chen, and X. Yao, unpublished results.
PKA Phosphorylates Ezrin

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