PALS1 Specifies the Localization of Ezrin to the Apical Membrane of Gastric Parietal Cells*

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The ERM (ezrin/radixin/moesin) proteins provide a regulated linkage between membrane proteins and the cortical cytoskeleton and also participate in signal transduction pathways. Ezrin is localized to the apical membrane of parietal cells and couples the protein kinase A activation cascade to regulated HCl secretion in gastric parietal cells. Here, we show that the integrity of ezrin is essential for parietal cell activation and provide the first evidence that ezrin interacts with PALS1, an evolutionarily conserved PDZ and SH3 domain-containing protein. Our biochemical study verifies that ezrin binds to PALS1 via its N terminus and is co-localized with PALS1 to the apical membrane of gastric parietal cells. Furthermore, our study shows that PALS1 is essential for the apical localization of ezrin, as either suppression of PALS1 protein accumulation or deletion of the PALS1-binding domain of ezrin eliminated the apical localization of ezrin. Finally, our study demonstrates the essential role of ezrin-PALS1 interaction in the apical membrane remodeling associated with parietal cell secretion. Taken together, these results define a novel molecular mechanism linking ezrin to the conserved apical polarity complexes and their roles in polarized epithelial secretion of gastric parietal cells.

The functions of an epithelium depend on the polarized organization of its individual epithelial cells. The acquisition of a fully polarized phenotype involves a cascade of complex events, including cell-cell adhesion, assembly of a lateral cortical complex, reorganization of the cytoskeleton, and polarized targeting of transport vesicles to the apical and basolateral membranes (1). Ezrin is an actin-binding protein of the ERM (ezrin/radixin/moesin) family of membrane-cytoskeleton linker proteins (2). 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. 3 and 4). Our previous studies showed that gastric ezrin is co-distributed with the β-actin isoform in vivo (5) and preferentially bound to the β-actin isoform in vitro (6). Because of its cytolocalization and observed stimulation-dependent phosphorylation, it was postulated that ezrin couples the activation of protein kinase A to the apical membrane remodeling associated with parietal cell secretion (4). Recently, we mapped the protein kinase A phosphorylation site on ezrin and illustrated the phosphoregulation of ezrin in gastric acid secretion (7). However, it is still not clear how ezrin links the apical membrane to the actin cytoskeleton during parietal cell activation.

ERM proteins all possess an ~300-residue N-terminal domain that shares sequence homology with the corresponding domain of erythrocyte band 4.1, followed by an ~170-residue region predicted to be largely α-helical and terminating in an ~100-residue domain in which an F-actin-binding site resides (6, 8, 9). Our previous study established an interrelationship between ezrin and calpain I (10). Further support for a membrane-cytoskeleton linking role came from a study in which calpain I-mediated proteolysis liberated the apical localization of ezrin and prevented activation of acid secretion in the apical membrane without altering the gross cytology (10).

To delineate the membrane-cytoskeleton linking role of ezrin, several groups have tried to identify ERM-binding proteins using pull-down assays. These include identification of hyaluronate receptor CD44 (11), ICAM (intercellular adhesion molecule-1) (12), and EBP50 (13). Because PDZ (PSD-95/Discs Large-ZO-1) domains are known to mediate associations with integral membrane proteins, it was hypothesized that membrane attachment of ezrin is likely to be mediated via EBP50, a PDZ domain-containing protein.

A recent study has demonstrated important roles for PDZ domain-containing proteins during cell polarization (14). One of the major groups of PDZ proteins is the membrane-associate guanylate kinase proteins. These proteins usually contain a non-catalytic guanylate kinase domain in combination with PDZ and SH3 1 domains. Many also contain a 4.1B or hook domain that allows these membrane-associated guanylate kinase proteins to bind members of the band 4.1 superfamily, such as protein 4.1, and ERM proteins (15, 16). PALS1 (protein associated with Lin seven-1) (17), a membrane-associated guanylate kinase protein, binds to a PDZ domain protein of PATJ (PALS1-associated tight junction protein), a human homolog of Drosophila Discs Lost (DGL) (18). DGL is crucial for epithelial

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1 The abbreviations used are: SH3, Src homology 3; GFP, green fluorescent protein; siRNA, small interfering RNA; GST, glutathione S-transferase; MBP, myelin basic protein; PBS, phosphate-buffered saline; MEM, minimal essential medium; SLO, streptolysin O.
cell polarity and exists in complex with the apical polarity determinant Crumbs in flies. It remains to be established how the PALS1-PATJ-Crumbs protein complex interacts with the actin-based cytoskeleton at the apical membrane during polarity establishment and/or maintenance.

To delineate the structure-function relationship of ezrin in the activation of gastric acid secretion, we took advantage of our recent development of streptolysin O-permeabilized gastric glands and assessed the requirement of ezrin in parietal cell activation by addition of recombinant ezrin and its deletion mutants. Our study demonstrates that the integrity of ezrin is critical for parietal cell activation. To identify the apical localization signal, we expressed full-length ezrin and its deletion mutants tagged with green fluorescent protein (GFP) in the cultured parietal cells. Our studies indicate that the N terminus of ezrin (amino acids 1–50) is sufficient for its localization to the apical membrane, whereas overexpression of this 50-amino acid fragment liberates endogenous ezrin from the apical membrane. Our pull-down assay revealed an association between ezrin and PALS1. Elimination of PALS1 by small interfering RNA (siRNA) diminished the apical localization of ezrin and effected a dominant-negative role in parietal cell activation. We propose that ezrin-PALS1 interaction provides a link between the apical plasma membrane and actin-based cytoskeleton in parietal cells.

**MATERIALS AND METHODS**

**Reagents**—[14C]Aminopyrine was obtained from PerkinElmer Life Sciences. Monoclonal antibody JL-18 against GFP was purchased from Clontech (Palo Alto, CA), and anti-ezrin antibody 4A5 was produced as described by Hanzel et al. (19). Anti-FLAG monoclonal antibody M2 and rabbit anti-Cdc42 antibody were purchased from Sigma and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Rhodamine-coupled phalloidin and Alexa Fluor 350-conjugated goat anti-rabbit IgG were purchased from Molecular Probes, Inc. (Eugene, OR). Lipofectamine 2000 was obtained from Invitrogen.

**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-Sall PCR-amplified ezrin cDNA into plGFP-N1 (Clontech) as described previously (7). The ezrin deletion mutants were constructed by a standard PCR method using the following primers: ezrin-N1, 5'-TCT CGG ACG GTA CCG CGG CCC GAT-3' (forward) and 5'-GAT GGT GTC ATT CCT CCG GCC CAT-3' (reverse); ezrin-C1, 5'-ATG GAG GTC GAG CAG ATG AAA CGG CCA-3' (forward) and 5'-CAG TAT AAG AAC GGT CTT GAG CTC GAC A-3' (reverse); ezrin-N2, 5'-TCT CGG ACG GTA CCG CGG CCC GAT-3' (forward) and 5'-CAG TAT AAG AAC GGT CTT GAG CTC GAC A-3' (reverse). All constructs were sequenced in full. GST-ezrin and its deletion mutant were constructed in pGEX-2T (Amersham Biosciences) as described (20).

Myelin basic protein (MBP)-tagged PALS1 was constructed in a modified pMAL-C2 vector as described by Fukata et al. (21). Briefly, PALS1 was PCR amplified by using primers 5'-CGG GAT CCA TGA CAA CAT CAT ATA TGA-3' and 5'-GGA ATT CTC ACC TTA GCC AGG TGG AT-3', digested with BamHI and EcoRI, and inserted into the pMAL-C2 vector digested with BamHI and EcoRI. Myc-tagged PALS1 (1–675), PALS1 (1–181), and PALS1 (182–675) constructs were generated as described previously (18).

**Isolation of Gastric Glands and Aminopyrine Uptake Assay**—Gastric glands were isolated from New Zealand White rabbits as described by Yao et al. (10). Briefly, rabbit stomach was perfused under high pressure with phosphate-buffered saline (PBS; 2.25 mM KH₂PO₄, 6 mM Na₂HPO₄, 1.75 mM NaCl, and 136 mM NaCl) containing 1 mM CaCl₂ and 1 mM MgSO₄. The gastric mucosa was scraped from the stomach and minced, and then washed twice with minimal essential medium (MEM) buffer containing 20 mM HEPES (pH 7.4) (HEPES/MEM). The minced mucosa was digested from the collection mixture for 20–25 min and then washed three times with HEPES/MEM. In all subsequent gland experiments (aminopyrine uptake assay), glands were resuspended at 5% (v/v) cytocrit in the appropriate buffer for final assay.

Stimulation of intact and streptolysin O (SLO)-permeabilized rabbit gastric glands was quantified using the aminopyrine uptake assay as described by Ammar et al. (22). Briefly, intact glands in HEPES/MEM were washed twice by setting at 4 °C in ice-cold K buffer containing 10 mM Tris base, 20 mM HEPES acid, 100 mM KCl, 20 mM NaCl, 1.2 mM MgSO₄, 1 mM NaH₂PO₄, and 40 mM mannitol (pH 7.4). SLO was added to a final concentration of 1 μg/ml, and the glands (at 5% cytocrit) were mixed by inversion and incubated on ice for 10 min. The glands were then washed twice with ice-cold K buffer to remove unbound SLO, and the permeabilization was initiated by incubating the gland suspension at 37 °C in K buffer containing 1 mM pyruvate and 10 mM succinate.

To evaluate the function of ezrin in parietal cell activation, we generated recombinant ezrin and its deletion mutants in bacteria. Briefly, GST-fused ezrin and deletion mutants were expressed in BL21(DE3) bacteria and purified using a glutathione affinity column as described (7). The fusion proteins were then eluted in PBS containing 10 mM glutathione and applied to a gel filtration column (Hi-Prep 16/10 DG, Bio-Rad) to remove glutathione and to exchange PBS for K buffer. The recombinant proteins were estimated to be 95% pure by SDS-PAGE; major contaminants were degraded fragments of ezrin. Protein concentrations were determined by the Bradford assay (23).

**Affinity Precipitation of PALS1 and Ezrin**—GST-ezrin-N2 was used as an affinity matrix to isolate proteins interacting with ezrin. A gastric gland preparation fraction from rabbit stomach was eluted from the GST-ezrin-N2 beads at room temperature for 2 h. The beads were then washed three times, followed by boiling in 1× sample buffer. The samples were resolved on 6–16% gradient SDS-polyacrylamide gel and analyzed by Western blotting.

To characterize the interaction between PALS1 and ezrin, GST-tagged ezrin and deletion mutants C1 and N1 were used as an affinity matrix to isolate MBP-tagged recombinant PALS1 from bacteria cells lysates using the protocol described by Lou et al. (24). The beads were washed with PBS three times, followed by boiling in 1× sample buffer. The samples were resolved on 6–16% gradient SDS-polyacrylamide gel and analyzed by Western blotting.

**Cell Culture and Transfection**—Primary cultures of gastric parietal cells from rabbit stomach were produced and maintained as described (7). Separate cultures of parietal cells were transfected with plasmids encoding GFP-tagged wild-type ezrin and/or deletion mutants using Lipofectamine 2000 according to the manufacturer’s instructions. Briefly, 1 μg of DNA was incubated in 600 μl of Opti-MEM (antibiotic-free), and 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 mixture was added to the plates and incubated for 4 h, followed by replacement of the media. The transfected cells were harvested 24 h after transfection using 0.1% saponin solution in saponin-buffered medium (Dulbecco’s modified Eagle’s medium/F-12 [Invitrogen] supplemented with 20 mM HEPES, 0.2% bovine serum albumin, 10 mM glucose; 8 mM epidermal growth factor, 1× selenite, insulin, and transferrin (SITE) medium (Sigma), 1 mM glutamine, 100 units/ml penicillin-streptomycin, 400 μg/ml gentamicin sulfate, and 15 μg/liter genetin or 20 μg/liter novobiocin, pH 7.4). The transfected cells were then maintained in culture at 37 °C until used for protein expression, partition, immunoprecipitation, or immunofluorescence.

To confirm the interaction between PALS1 and ezrin, GFP-ezrin- and FLAG-PALS1-cotransfected parietal cells were harvested and lysed in 1.5 ml of Tris-buffered saline (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EGTA, and 0.1% Triton X-100) containing protease inhibitor mixture. The cell lysates were clarified using an Eppendorf centrifuge at 13,000 rpm for 10 min. The resulting supernatant was incubated with 30 μl of GST-ezrin-N2 beads at room temperature for 2 h. The beads were then washed three times, followed by boiling in 1× sample buffer. The samples were resolved on 6–16% gradient SDS-polyacrylamide gel and analyzed by Western blotting.

**siRNA Treatment and Assay for Knockdown Efficiency**—siRNA sequences used for silencing PALS1 correspond to coding regions 504–524 (siRNA1-1) and 488–508 (siRNA2-1). As a control, either a duplex...
targeting cyclophilin or a scrambled sequence was used (24). The 21-mer oligonucleotide RNA duplexes were synthesized by Dharamco Research, Inc. (Boulder, CO). In trial experiments, different concentrations of siRNA oligonucleotides were used for different treatment times as detailed previously, and transfection efficiency was judged based on the uptake of fluorescein isothiocyanate-conjugated oligonucleotides (24). In brief, cultured parietal cells were transfected with siRNA-1 and siRNA-2 oligonucleotides or control scrambled oligonucleotides, and the efficiency of this siRNA-mediated protein suppression was judged by Western blot analysis.

Immunofluorescence Microscopy—For cytolocalization of exogenously expressed ezrin, cultured parietal cells were transfected with GFP, GFP-tagged wild-type ezrin, and GFP-tagged ezrin deletion mutants (N1, N2, C1, and C2) 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 secretory stimulants 100 μm histamine and 50 μm isobutylmethylxanthine in the presence of SCH28080, a proton pump inhibitor (7). 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 the primary antibody, the fixed and permeabilized cells were blocked with 0.5% bovine serum albumin in PBS, followed by incubation with the primary antibody against ezrin (4A5) or GFP. The endogenous and exogenous ezrin proteins were labeled with fluorescein isothiocyanate-conjugated goat anti-mouse antibody and counterstained with rhodamine-coupled phalloidin to visualize filamentous actin. Coverslips were supported on slides by grease pencil markings and mounted in Vectashield (Vector Laboratories). Images were taken with a Zeiss Axiovert 200 fluorescence microscope using a 63 × 1.3 numerical aperture PlanApo objective. Figures were constructed using Adobe Photoshop.

Western Blotting—Samples were subjected to SDS-PAGE on 6–16% gradient gel and transferred to nitrocellulose membrane. Proteins were probed with the appropriate primary antibodies and detected using an ECL kit (Pierce). The band intensity was then quantified using a PhosphorImager (Amersham Biosciences).

RESULTS

Integrity of Ezrin Is Required for Parietal Cell Activation—The gastric parietal cell offers an excellent system to study ezrin function, when other ERM proteins are absent. We have recently established a permeable gland model that permits entry of relatively large molecular components (e.g. actin and syntaxin) (22). Our previous study suggested that the integrity of ezrin is required for parietal cell activation, as calpain-mediated proteolysis of ezrin is correlated with inhibition of acid secretion (10). To directly assess the role of ezrin in parietal cell activation, we introduced recombinant full-length ezrin and its deletion mutants into SLO-permeabilized glands. Recombinant proteins expressed in bacteria were purified to homogeneity using a glutathione column (Fig. 1A). Recombinant proteins were added to the SLO-permeabilized glands in the presence and absence of cAMP/ATP. Addition of full-length ezrin caused relatively small changes in aminopyrine uptake (at most, an ~7% decrease), and there was no dose-dependent inhibitory effect. In contrast, N-terminal ezrin mutants caused a dose-dependent inhibition of acid secretion in SLO-permeabilized glands as measured by aminopyrine uptake. No significant inhibition was noted at 2.5 μg/ml protein, but 5 μg/ml protein caused a 23.9% reduction in acid secretion, and maximal inhibition (89–91%) occurred at 10 and 20 μg/ml (Fig. 1C). Interestingly, addition of C-terminal ezrin caused relatively small changes in aminopyrine uptake (at most, an ~9.7% decrease), and there was no dose-dependent inhibitory effect. These experiments support the notion that the integrity of ezrin is required for parietal cell activation.

The N Terminus of Ezrin Specifies Its Apical Localization in Parietal Cells—We reasoned that the inhibition seen in the SLO-permeabilized glands is due to a dominant effect exerted by addition of ezrin deletion mutants. Because ezrin is postulated to be a membrane-actin skeleton linker, we next searched for the apical targeting domain of ezrin. Our previous study showed that exogenously expressed ezrin bears the same biochemical characteristics as those of endogenous protein (7). To determine whether there were any major changes in the behavior of exogenously expressed GFP-ezrin mutants, we measured the partitioning of exogenously expressed full-length ezrin and its deletion mutants into the digitonin-soluble fraction compared with the insoluble “cytoskeletal” fraction based on the Western blot analyses using anti-GFP antibody. As summarized in Fig. 2A, 63.3 ± 4.5% of full-length ezrin resided in the digitonin-insoluble fraction, consistent with previous reports (e.g. Refs. 7 and 25). Partitioning of GFP-ezrin-N1 and GFP-ezrin-N2 was similar to that of full-length ezrin; 62.1 ± 4.7% of ezrin-N1 and 60.7 ± 5.1% of ezrin-N2 were associated with the digitonin-insoluble fraction. Interestingly, the majority of GFP-ezrin-C1 and GFP-ezrin-C2 was partitioned into soluble fractions: 87.5 ± 7.3% of ezrin-C1 and 89.7 ± 8.7% of ezrin-C2 were distributed to the digitonin-soluble fraction. The distinct distribution profiles between the N- and C-terminal deletion mutants may reflect their difference in modulating acid secretion in SLO-permeabilized glands.

To determine the subcellular localization of ezrin deletion mutants in parietal cells, the transfected cells were double-stained for GFP using anti-GFP monoclonal antibody (green) and for F-actin using phalloidin (red). Fig. 2B shows optical sections from GFP-ezrin-transfected cells, all maintained in the non-secreting state. Similar to what has been noted in a recent study (7), full-length exogenous GFP-ezrin was localized to the plasma membranes, most prominently to the apical membrane vacuoles sequestered to the cell interior and somewhat more sparsely to the basolateral membrane surrounding the cells. The GFP-ezrin signal was relatively co-localized with F-actin (Fig. 2B, panels a–c). The distribution of the signal for the GFP-tagged N-terminal ezrin mutant constructs (N1 and N2) was similar to that for full-length ezrin, i.e. primarily associated with apical membrane vacuoles and to a lesser extent with the basolateral membrane (Fig. 2B, panels d and g). The distribution of F-actin also was not altered by the transfections (Fig. 2B, panels b, e, and h). However, the distribution of the signal for the GFP-tagged C-terminal ezrin mutants (C1 and C2) was distinctly different from that for full-length ezrin, i.e. primarily diffused throughout the cytoplasm, with no apparent localization to the apical membrane (Fig. 2B, panels j and m). Whereas the distribution of F-actin was also not altered by the transfections (Fig. 2B, panels k and n), the distribution of ezrin-C1 and ezrin-C2 no longer co-localized with actin (Fig. 2B, panels l and o). These data demonstrate that transfected GFP-tagged ezrin and N-terminal ezrin fragments are sufficient to localize to the apical plasma membrane of parietal cells.

Ezrin Interacts with PALS1 via Its N Terminus—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. Our recent study demonstrated that ezrin couples activation of protein kinase A to the apical membrane-actin skeleton remodeling associated with parietal cell activation (7). To identify the protein responsible for ezrin localization to the apical membrane, we employed GST-tagged recombinant ezrin as an af-
finity matrix to isolate proteins from the gastric epithelial cell lysates as described (13). To this end, GST-ezrin-N2 was immobilized on agarose beads, mixed with detergent-soluble tissue lysates, and washed extensively, and any binding proteins were eluted and fractionated on SDS-polyacrylamide gel. As shown in Fig. 3A (lane 3), there were two polypeptide bands with apparent molecular masses of 80–83 kDa that specifically bound to GST-ezrin-N2. Mass spectrometric analyses suggested that the 80-kDa band is ezrin, whereas the 83-kDa band is PALS1-like protein, a putative band 4.1-binding protein in the apical polarity determinant protein complex of flies (17). To confirm the results from the mass spectrometric analyses, we carried out Western blot analyses using ezrin- and PALS1-specific antibodies. As shown in Fig. 3B, immunoblot analyses confirmed that ezrin was pulled down by FLAG immunoprecipitation of PALS1. Conversely, Western blotting using anti-FLAG antibody revealed that PALS1 was pulled down by GFP-ezrin (Fig. 3C, lane 4). No FLAG-tagged PALS1 was precipitated with control IgG (Fig. 3C, lane 3), and no actin was detected in any of the immunoprecipitates. Thus, we concluded that the interaction between PALS1 and ezrin is specific.

To define the binding domain of ezrin responsible for its association with PALS1, we expressed and purified GST-tagged ezrin and ezrin deletion mutants C1 and N1 from bacteria using glutathione-agarose beads. Purified GST-ezrin proteins were used as an affinity matrix for absorbing MBP-tagged PALS1 protein from bacterial lysates. As shown in Fig. 3D, the association of recombinant PALS1 with ezrin

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**Fig. 1.** Integrity of ezrin is required for parietal cell acid secretion. **A,** shown is a schematic drawing of ezrin and its deletion mutants used for secretory activity assay in SLO-permeabilized gastric glands. **B,** GST-tagged recombinant ezrin protein samples were purified using glutathione affinity beads, separated by SDS-PAGE, and stained with Coomassie Blue to assess the purity of the preparations. The protein samples were desalted for the secretory activity assay. **C,** shown is the suppression of secretory activity by N-terminal ezrin in SLO-permeabilized glands. Recombinant ezrin and its deletion mutants (C1, C2, N1, and N2) were purified as GST fusion proteins from bacteria and added to permeabilized gland suspensions at the indicated amounts (micrograms of protein). Glands were then stimulated with cAMP and ATP, and aminopyrine (AP) uptakes were measured. Aminopyrine uptakes are shown for resting and stimulated controls and for stimulated glands treated with either full-length ezrin or deletion mutants N1 and C1. Aminopyrine data are plotted as a percentage of the stimulated control for each experiment. Error bars represent S.E. (n = 4 for all experiments). *, p < 0.01 compared with stimulated controls. max stim, maximal stimulation.
Ezrin-PALS1 Interaction

PALS1 Is Co-localized with Ezrin to the Apical Membrane in Parietal Cells—Because PALS1 is part of the apical polarity determinant protein complex conserved from flies to humans, the biochemical interaction between ezrin and PALS1 propelled us to test whether PALS1 is an apically distributed protein. Fig. 3F (panels a–c) shows optical sections from non-secreting (resting) parietal cells. Similar to what has been noted in earlier studies, ezrin was localized to the plasma membranes, most prominently to the apical membrane vacuoles sequestered to the cell interior as rings (Fig. 3F, panel b, red). PALS1 staining appeared as six clearly resolved rings of apical membrane vacuoles (Fig. 3F, panel a, green). The PALS1 signal was co-localized with ezrin as seen in the merged image from two channels (Fig. 3F, panel c, yellow). The co-distribution of PALS1 with ezrin to the apical membrane confirms the biochemical interaction between these two proteins.

The stimulation of parietal cell acid secretion involves insertion of H,K-ATPase-containing tubulovesicular membrane into apical canalicular membrane, resulting in dilation of apical membrane vacuoles as active HCl and water transport occur (7). Because of this swelling, stimulated parietal cells are considerably larger in diameter than their resting counterparts (7). Because PALS1 was co-distributed with ezrin to the apical membrane, we tested whether PALS1 and ezrin remain co-localized as the apical cytoskeleton remodeling occurs. Fig. 3F (panels d–f) shows optical sections taken from parietal cells treated with the secretagogues histamine and isobutylmethylxanthine and probed for PALS1 and ezrin. As seen in resting parietal cells (Fig. 3F, panels a–c), ezrin and PALS1 were primarily co-localized to the same regions in secreting cells. However, in the case of secreting cells, the dilated apical canalicular vacuoles occupied most of the cytoplasmic space (Fig. 3F, panels d and e), as noted in our recent study (7). Thus, PALS1 remains associated with ezrin at the apical plasma membrane of secreting parietal cells.

PALS1 Is Essential for the Localization of Ezrin to the Apical Membrane—Previous studies established the importance of the actin-based cytoskeleton (26) and the integrity of ezrin (10) in parietal cell activation. The function of ezrin in parietal cell activation has been directly demonstrated in our recent study (7). Given the observed interaction between PALS1 and ezrin in vitro and their co-distribution in vivo, it was of great interest to investigate the possible influence of PALS1 on the localization of ezrin to the apical membrane. To this end, we expressed Myc-tagged full-length and N-terminal PALS1 in gastric parietal cells and assessed their subcellular distribution profiles. Typically, we achieved a 3–4-fold expression in positively transfected parietal cells (7). As shown in Fig. 3G (panel a), Myc-tagged PALS1 was localized mainly to the parietal cell apical plasma membrane, which is superimposed with ezrin distribution (panel b). Despite an apical localization of N-terminal PALS1 (amino acids 1–181) (Fig. 3G, panel d), ezrin distribution became diffused in the PALS-(1–181)-transfected cells (panel e, green), suggesting that full-length PALS1 is required for ezrin localization to the apical membrane.
Fig. 3. Identification of PALS1 as a major ezrin-binding protein in gastric epithelial cells. A, ezrin pulls down two major high molecular mass polypeptides from gastric lysates. GST-ezrin-N2 coupled to glutathione-agarose beads was loaded with lysates of gastric epithelial cells. Bound proteins were fractionated by SDS-PAGE and stained with Coomassie Blue. Note that there are two polypeptides with approximate molecular mass of 81 and 85 kDa, respectively. B, confirmation of the results from mass spectrometric analyses of ezrin and PALS1. A duplicate of the SDS-polyacrylamide gel in A was used for transblotting. The blot was used for probing ezrin, PALS1, and actin using three different mono-specific antibodies, respectively. Western blot analyses confirmed that ezrin and PALS1, but not actin, were pulled down by the GST-ezrin fragment. C, co-immunoprecipitation of ezrin and PALS1 from transfected parietal cells. Gastric parietal cells cotransfected with FLAG-PALS1 and GFP-ezrin were extracted and subjected to immunoprecipitations using anti-GFP and anti-FLAG monoclonal antibodies, respectively. Control immunoprecipitations were performed using nonspecific mouse IgG. Starting fractions, non-binding fractions, and immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotting using anti-FLAG and anti-GFP antibodies. In the experiment shown in A, Western blotting verified co-immunoprecipitation of ezrin and PALS1. No actin was detected in the GST-ezrin and FLAG-ezrin IP. D, N-terminal ezrin binds to PALS1. GST-tagged ezrin and deletion mutants C1 and N1 were expressed in bacteria and purified using glutathione-agarose beads. Purified GST-ezrin proteins were used as an affinity matrix for absorbing MBP-tagged recombinant PALS1 protein from bacterial lysates. An aliquot of bacterial lysates (starting) was used as positive control for probing MBP-tagged recombinant PALS1 protein. The retention of recombinant PALS1 on three sets of ezrin affinity beads was analyzed by SDS-PAGE and immunoblotting using anti-PALS1 antibody, and the content of GST-ezrin proteins on the affinity matrix was confirmed using anti-GST antibody. Western blotting of PALS1 verified an association between ezrin and PALS1. No PALS1 protein was detected in the GST-ezrin-N2 pull-down in the starting fractions, non-binding fractions, and immunoprecipitates (IP) using anti-Myc monoclonal antibody. Starting fractions, non-binding fractions, and immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using anti-ezrin and anti-actin antibodies. Western blotting verified co-immunoprecipitation of ezrin with full-length and C-terminal PALS1, but not the N-terminal fragment (amino acids 1–181). No actin was detected in either the GFP or FLAG immunoprecipitates. E, C-terminal PALS1 binds to ezrin in transfected parietal cells. The gastric parietal cells cotransfected with Myc-tagged full-length PALS1, N-terminal PALS1 (amino acids 1–181), and C-terminal PALS1 (amino acids 182–675) were extracted and subjected to immunoprecipitations (Immunoprep.) using anti-Myc monoclonal antibody. Starting fractions, non-binding fractions, and immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using anti-ezrin and anti-actin antibodies. Western blotting verified co-immunoprecipitation of ezrin with full-length and C-terminal PALS1, but not the N-terminal fragment (amino acids 1–181). No actin was detected in Myc immunoprecipitates. F, co-distribution of ezrin with PALS1 to the apical membrane of gastric parietal cells. This triple montage represents confocal images collected from resting and secreting gastric parietal cells doubly stained for PALS1 (green) and ezrin (red), including their merged images. Ezrin was localized mainly to the apical plasma membrane of parietal cells, seen as rings, in a pattern suggestive of the apical plasma membrane invaginations that form the intracellular canaliculi (panel b). PALS1 labeling was localized mainly to the parietal cell apical membrane (panel a), which is superimposed with ezrin distribution (panel b, red). This becomes readily evident when the two images are merged (panel c, yellow).
To test this hypothesis, we introduced siRNA oligonucleotides (siRNA-1) of PALS1 by transfection into parietal cells. To determine the efficient timing for knocking down PALS1 protein levels suppressed by the RNA interference, we transfected parietal cells with 100 nM siRNA-1 and collected cells at different post-transfection times. As shown in Fig. 4A (left panels), Western blotting with anti-PALS1 antibody revealed that siRNA-1 caused remarkable suppression of PALS1 protein levels at 48 h, whereas control cells treated with an irrelevant oligonucleotide (e.g., cyclophilin) expressed normal PALS1 levels. Quantitative analysis revealed that the siRNA-1 treatment caused a 5.9-fold suppression of PALS1 protein without altering the levels of other proteins such as actin. Further extension of the siRNA treatment interval to 72 h did not significantly improve the efficiency of PALS1 protein suppression. As PALS1 synthesis in ~40% of the untransfected cells with little or no oligonucleotide was unlikely to be markedly diminished, the observed 5.9-fold inhibition at 100 nM must represent almost complete inhibition of PALS1 in 61 ± 3% of the successfully transfected cells. Another siRNA oligonucleotide (siRNA-2) targeted to a different sequence of PALS1 gave a similar suppression profile for PALS1 protein accumulation (Fig. 4A, right panels). This suppression was relatively specific, as it did not alter the levels of other proteins such as ezrin and actin.

Accordingly, we examined the distribution of ezrin in cultured parietal cells treated with PALS1 siRNA-1. Fig. 4B shows optical sections taken from resting parietal cells simultaneously probed with fluorescein-conjugated anti-ezrin antibody 4A5 (panel a'), rhodamine-conjugated phalloidin (panel b'), and Alexa Fluor 350-labeled anti-Cdc42 antibody (panel d'). The F-actin distribution was similar to that shown in control oligonucleotide-treated cells (Fig. 4B, panel a), outlining the apical membrane vacuoles and the basolateral membrane surface as noted above (Fig. 2B). In PALS1 siRNA-1-treated resting cells, ezrin staining was distributed throughout the cytoplasm, as noted above in N-terminal deletion mutants of ezrin (Fig. 2B, panels j and m), although we also noted some accumulation of ezrin signal near the apical membrane vacuoles (Fig. 4B, panel c'). The Cdc42 distribution in PALS1-suppressed cells was similar to that shown in control oligonucleotide-treated cells (Fig. 4B, panel d'), outlining the apical membrane vacuoles as noted in a previous study (26). Thus, suppression of PALS1 results in no apparent alteration of the actin and Cdc42 distribution profiles, but liberates ezrin from the apical membrane of parietal cells.

Treatment of parietal cells with siRNA-2 suppressed PALS1 protein accumulation and effected a dislocation of ezrin localization to the apical membrane (Fig. 4C, panel a') without alteration of F-actin and Cdc42 distribution (panels b' and d'). A control scrambled oligonucleotide did not alter the distribution of ezrin.

**FIG. 4. PALS1 is essential for ezrin localization to the apical membrane of parietal cells.** A, shown is a time course of RNA interference of PALS1. Cultured parietal cells were transfected with the PALS1 siRNA oligonucleotides (siRNA-1 and siRNA-2) for different intervals and subjected to SDS-PAGE and immunoblotting. Upper panel, immunoblot for PALS1; middle panel, immunoblot for ezrin; lower panel, immunoblot for actin. Scrambled oligonucleotides were used as controls (0 hr.). B, this set of confocal images was collected from resting and secreting gastric parietal cells triply stained for PALS1 (green), ezrin (red), and Cdc42 (blue). Ezrin was localized mainly to the apical vacuole membrane of parietal cells, seen as rings in control siRNA-treated cells (panel b). However, elimination of PALS1 by siRNA-1 abolished the apical localization of ezrin (panel a'), but not actin (panel b') and Cdc42 (panel d'). It becomes apparent that suppression of PALS1 liberated ezrin from the apical membrane as ezrin and F-actin images are merged (panel c'). In addition, elimination of PALS1 attenuated the dilation of apical vacuoles (panel f versus panel f'), as ezrin was essential for linking the apical membrane to the actin-based cytoskeleton. Scale bars = 15 μm. C, this set of optical images was collected from resting gastric parietal cells treated with another siRNA oligonucleotide (siRNA-2) and triply stained for PALS1 (green), ezrin (red), and Cdc42 (blue). Ezrin was localized mainly to the apical vacuole membrane of parietal cells, seen as rings in control (scrambled) oligonucleotide-treated cells (panel b). However, elimination of PALS1 by siRNA-2 abolished the apical localization of ezrin (panel a'), but not actin (panel b') and Cdc42 (panel d'). It becomes apparent that suppression of PALS1 liberated ezrin from the apical membrane as ezrin and F-actin images are merged (panel c'), indicating the functional role of PALS1 in the apical localization of ezrin. Scale bars = 15 μm.
**TABLE I**

**Integrity of ezrin is required for apical membrane extension**

The 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 taken from three different preparations in which 83 cells from each category were examined. In resting cells, measurements were carried out on two to five vacuoles/cell, whereas one to three vacuoles/cell were scaled in stimulated preparations. Data are expressed as means ± S.E. IBMX, isobutylmethylxanthine.

| Treatment                  | Vacuole diameter | No. of vacuoles |
|----------------------------|------------------|-----------------|
| Resting cells              |                  |                 |
| Full-length ezrin          | 5.3 ± 1.3        | 263             |
| Ezrin-N1                   | 5.5 ± 1.1        | 247             |
| Ezrin-C1                   | 5.3 ± 1.7        | 251             |
| Control siRNA              | 5.7 ± 1.7        | 183             |
| PALS1 siRNA-1              | 4.7 ± 0.9        | 255             |
| Secreting (histamine plus IBMX) cells |                  |                 |
| Full-length ezrin          | 15.3 ± 1.3       | 223             |
| Ezrin-N1                   | 5.5 ± 1.1        | 197             |
| Ezrin-C1                   | 13.9 ± 1.9       | 291             |
| Control siRNA              | 15.7 ± 1.9       | 193             |
| PALS1 siRNA-1              | 4.7 ± 0.5        | 205             |

* a p < 0.05. The vacuole diameter of ezrin-N1-expressing cells was compared with that of full-length ezrin-expressing stimulated cells.

b p < 0.05. The vacuole diameter of PALS1 siRNA-treated cells was compared with that of scrambled oligonucleotide-treated 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, adhesion, motility, and survival (2). Here, we provide the first evidence that ezrin interacts with PALS1, a PDZ and SH3 domain-containing protein. Ezrin binds to PALS1 via its N-terminal 50 amino acids and is co-localized with PALS1 to the apical membrane of gastric parietal cells. Furthermore, we have shown that PALS1 is essential for the apical localization of ezrin, as either suppression of PALS1 or deletion of the PALS1-binding domain eliminated the apical targeting of ezrin. Finally, our study has demonstrated the essential role of ezrin-PALS1 interaction in the apical membrane remodeling associated with parietal cell secretion.

A major role for the actin cytoskeleton in the secretory processes of parietal cells has been inferred from a study using actin disrupters that disorganize actin filaments and act to inhibit acid secretion (26). Highly organized microfilaments are typical features of microvilli at the apical membrane within the parietal cell canalculus. In going from the resting to the secreting state, there are major changes at the apical microvillar 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 microvillar surface (28, 29). These morphological studies indicate that reversible actin-based cytoskeletal dynamics are tightly linked to the secretory cycle in parietal cells. Our recent study demonstrated that ezrin couples protein kinase A-mediated phosphorylation to the remodeling of the apical membrane-cytoskeleton associated with acid secretion in parietal cells (7). However, our preliminary data showed that protein kinase A-mediated ezrin protein phosphorylation of Ser66 did not modulate ezrin-PALS1 interaction, consistent with our previous study in which alteration of Ser66 phosphorylation did not change ezrin distribution to the apical membrane (7). To search for ERM binding partners potentially involved in membrane association, Recek et al. (13) use GST-epsilon ezrin as an affinity matrix to isolate a 50-kDa PDZ domain-containing phosphoprotein named EB50 from human placental cell lysates. However, our search for EB50 in isolated rabbit gastric parietal cells was unsuccessful (7). Thus, the interaction between ezrin and PALS1 established here represents a functional link between the ezrin molecule and the apical plasma membrane of gastric parietal cells. Our preliminary assessment of ezrin-PALS1 binding interface(s) revealed that the C-terminal region of PALS1 is essential for ezrin association. Precise mapping of the respective binding interfaces between these two proteins will help to delineate the molecular mechanisms underlying polarity establishment and/or maintenance in gastric parietal cells.

Parietal cell activation involves translocation of H,K-ATPase from the cytoplasm to the apical plasma membrane via 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 the apical membrane during active secretion. It will be essential to distinguish precisely where the ezrin-PALS1 interaction operates. Recent work has shown that the membrane fusion machinery component alpha-SNAP (g-soluble N-ethylmaleimide-sensitive factor attachment protein) is localized to the apical membrane of neuroepithelial cells (30). The alpha-SNAP knockout experiment revealed a liberation of atypical protein kinase C, PALS1, and VAMP7 (vesicle-associated membrane protein-7) from the apical membrane, which suggests an interactive nature of the membrane fusion machinery and the apical membrane polarity determinants in neuroepithelial cells. Our previous study established the respective roles of the actin-based cytoskeleton and membrane fusion machinery in mediating H,K-ATPase translocation to the apical membrane (22); it

Furthermore, we have shown that PALS1 is essential for the apical localization of ezrin, as either suppression of PALS1 or deletion of the PALS1-binding domain eliminated the apical targeting of ezrin. Finally, our study has demonstrated the essential role of ezrin-PALS1 interaction in the apical membrane remodeling associated with parietal cell secretion.
remains to be established how the ezrin-PALS1 interaction operates the apical membrane-cytoskeleton dynamics to facilitate the docking and insertion of H,K-ATPase into the apical membrane of parietal cells for proton pumping.

In *Drosophila*, the Crumbs-Stardust-Discs Lost complex is required during the establishment of polarized epithelia. Embryos that lack a component of this complex or that overexpress Crumbs exhibit defects in epithelial morphogenesis (31–33). Two evolutionarily conserved multiprotein complexes, PALS1-PATJ-Crumb and Cdc42-Par6-Par3-atypical protein kinase C, have been implicated in the polarization of *Drosophila* melanogaster epithelia. In fact, it has been shown that these two complexes are linked by Par6-PALS1 interaction (33). Our demonstration that suppression of PALS1 did not alter the apical distribution of Cdc42 and parietal cell polarity supports the notion that an alternative mechanism exists for linking Cdc42 to the apical plasma membrane (27). Interestingly, expression of C-terminal ezrin mutants in cultured parietal cells or addition of C-terminal ezrin mutants to SLO-permeabilized glands did not effect a dominant action, suggesting that the recombinant proteins are unable to compete with endogenous protein for actin binding. In any event, further characterization of the ezrin-PALS1 apical membrane-cytoskeleton complex will provide detailed information regarding the molecular composition and structure-function relationships of the ezrin-containing apical signaling complex in cAMP-dependent polarized secretion in gastric parietal cells.

Taken together, the results presented in this work reveal that ezrin interacts with the evolutionarily conserved apical protein PALS1 and that this interaction specifies the apical localization of ezrin. Finally, we have shown that disruption of ezrin-PALS1 interaction blocks the remodeling of the apical membrane-cytoskeleton associated with the translocation and insertion of H,K-ATPase into the apical membrane. We propose that ezrin-PALS1 interaction links proton pump H,K-ATPase trafficking to the apical membrane-cytoskeleton remodeling required for polarized secretion in gastric parietal cells.

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