Drebrin E2 is differentially expressed and phosphorylated in parietal cells in the gastric mucosa

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ACTIN-BASED CYTOSKELETAL REARRANGEMENTS are fundamental events in the generation and remodeling of polarized cellular structures. The regulation of these processes is complex, involving the activity of multiple actin-binding proteins. Developmentally regulated brain proteins (drebrins) are members of an ancient ADF-H domain family of actin-binding proteins (31) first identified in chicken as the neuronal protein isoforms A (adult) and E1 and E2 (embryonic) (23, 42). Splice variants have since been identified in human (1a and 1b), mouse (A, A2, and E2), and rat (A and E). Specific functions of the various drebrin isoforms are unknown, but available evidence supports a regulatory role in actin filament formation and cellular morphogenesis (42). Overexpression of drebrin A induces the formation of neurite-like processes in fibroblasts and cultured neuroblastoma cells, increases the length of dendritic spines in cultured cortical neurons, and leads to striking changes in actin filament assembly (19, 21, 22, 43, 44). In vitro, drebrin binds to F-actin with relatively high affinity, competes with several actin-stabilizing proteins including troponyosin and α-actinin for actin binding, and inhibits fascin-dependent actin bundling and myosin-based ATPase activity (18, 23, 41, 43).

Initially, drebrins were thought to be brain specific. However, although they do not appear to be expressed in cardiac or skeletal muscles (37), there is increasing evidence that the drebrin E2 isoform is present in some epithelial cell lines (15, 37, 43) and in certain epithelial cell types including type A intercalated cells in kidney and in gastric parietal cells in mouse (26). Interestingly, both parietal cells and type A intercalated cells secrete protons and, in both cell types, the proton-pumping ATPases are localized to the apical plasma membrane and to intracellular vesicles and both are regulated through apical membrane recycling (6, 14). Compared with renal intercalated cells, considerably more is known about the mechanisms associated with activation of the parietal cell proton pump (H+–K+–ATPase). Parietal cell HCl secretion is potently stimulated in most species by cAMP-dependent agonists such as histamine, which elevates intracellular cAMP concentration ([cAMP]) by binding to G protein-coupled H2-type receptors, and forskolin, which directly activates adenyl cyclase, the G protein-coupled enzyme that catalyzes the production of cAMP from ATP. Increased HCl secretion is correlated with distinct well-characterized morphological changes in which the number and length of F-actin-rich microvilli in the apically directed intracellular canalicular membrane are significantly increased. The canalicular membrane is also the site of exocytotic insertion of the proton pump from an internal tubulovesicular compartment and the endocytic retrieval of the pump back into this compartment (see Refs. 34 and 48 for recent reviews). Currently, the best-characterized cellular models used to define intracellular events associated with activation of HCl secretion are derived from rabbit. In addition, in vivo acid secretory studies have confirmed that rabbit acid secretory responses to agonists are regulated by vagal-cholinergic ago-

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nists and histamine as in human and other mammalian species (40). The goal of this study was to define the pattern and type of drebrin isoform expression in different cell types within the acid-secreting (oxyntic) region of the rabbit gastric mucosa and to use cellular models derived from this region to provide further insights into the function(s) of this protein.

MATERIALS AND METHODS

Antibodies and fluorescently tagged reagents. Monoclonal anti-drebrin antibody (clone M2F6) was obtained from Medical and Biological Laboratories (Nagoya, Japan) or Stressgen (Victoria, BC, Canada). Monoclonal anti-hemagglutinin (HA) antibody (HA.11, clone 16B12) and anti-myc (clone 9E10) antibody were obtained from Babco/Covance (Berkeley, CA) and Developmental Studies Hybridoma Bank, Univ. of Iowa (Iowa City, IA), respectively. Anti-myc monoclonal antibody (clone 9B11) was purchased from Cell Signaling Technology (Beverly, MA). Affinity purified sheep polyclonal anti-Cdc42 was from Cytoskeleton (Denver, CO). Monoclonal anti-H-ATPase, α-subunit (clone HK12.18) was from Calbiochem, EMD Biosciences (La Jolla, CA), and monoclonal anti-actin (clone A40) was from Sigma (St. Louis, MO). Monoclonal antibodies directed against coronin [clone 2H9 (now named coronin 1B)] and lasp-1 (clone 3H8) were generated as previously described (12, 36). Secondary horseradish peroxidase-tagged donkey anti-rabbit Ig was from Amersham Biosciences (Piscataway, NJ). Fluorescently tagged probes used for subcellular immunolocalizations included Cy3-tagged donkey anti-sheep Ig, Cy5-tagged donkey anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 488-tagged chicken anti-mouse Ig, Oregon Green 488, and Alexa Fluor 647-tagged phalloidins (Molecular Probes, Eugene OR).

Cell isolation and culture. Gastric mucosal cells and glands were isolated from the stomachs of Nembutal-anesthetized male New Zealand White rabbits as previously described (7). Parietal cells were enriched to ~85% purity by Optiprep density gradient centrifugation for cell culture and ~95% purity for biochemical studies by combined density gradient centrifugation and centrifugal elutriation. Primary cultures of parietal cells used for plasmid transfection experiments were generated from enriched parietal cell isolates as previously described (10). Madin-Darby canine kidney (MDCK) cells were also used for transfection experiments as previously described (9). Controls for each experiment included exposure to transfection reagent alone and transfection with empty vector(s).

Subcellular localization of fluorescently tagged proteins. For in vivo studies, rabbits were fasted overnight, tranquilized with a cocktail of ketamine/xylazine, and then injected with either the H2-receptor blocker ranitidine (2 mg/kg; 1 h before death under Nembutal anesthesia) or with histamine (0.2 ml, 100 mM, pH 7; two successive doses, 15 min apart; 30 min before death) after the administration of the H1-receptor blocker (chlorpheniramine maleate, 2 mg/kg) as described by Crothers et al. (13). Oxyntic mucosal tissue sections were fixed by perfusion in situ with 4% paraformaldehyde and then processed and stained as previously described (11, 12).

Cultured cells and isolated gastric glands were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 5 min. For drebrin immunolocalization, non-specific binding was blocked with 3% BSA in PBS (1 h, room temperature) and antibodies were diluted in the same solution (45). Controls for primary and secondary antibodies were included in each experiment as previously described (11, 12). Cells and tissue sections were incubated overnight with M2F6 (1 μg/ml) followed by Cy5-labeled donkey anti-mouse secondary antibody (1:100 dilution) plus Oregon Green phalloidin (1:400 dilution) to label F-actin. In transfected cells, nonspecific binding was blocked with 5% nonfat milk (Bio-Rad, Hercules, CA) in PBS, and antibodies were diluted in 1% milk, PBS (11, 12). In dual-labeling experiments, HA-tagged drebrin was localized with an anti-HA monoclonal antibody (1:2,000 dilution) in conjunction with Alexa Fluor 488 chicken anti-mouse secondary antibody (1:100 dilution) and F-actin was localized with Alexa Fluor 647 phalloidin (1:40 dilution). Myc-tagged Cdc42 constructs were similarly localized with anti-myc antibody (1:500) and Alexa Fluor 647 phalloidin. In triple labeling experiments, Cy3 tagged donkey anti-mouse and Cy5 donkey anti-sheep antibodies (1:100) were used in conjunction with mouse anti-HA and sheep polyclonal anti-Cdc42 antibodies (1:100) along with Oregon Green phalloidin (to localize F-actin). (In pilot experiments, the anti-Cdc42 antibody was found not to be sufficiently sensitive to detect native Cdc42 in nontransfected cells but to be sufficiently sensitive to detect overexpressed Cdc42 in transfected cells). Parietal cell H-ATPase was localized in tissue sections with anti-H-ATPase, α-subunit (1:1,000) as previously described (11). Fluorescently tagged endogenous and transiently expressed drebrin were analyzed along with fluorescently tagged F-actin with a Zeiss LSM 510 META confocal microscope.

Relative levels of expressed proteins in transfected cells were analyzed by confocal microscopy using a Zeiss LSM 510 confocal microscope and Meta (version 3.2) software. Laser powers and pinhole openings were kept constant, and scans were performed in the multitrack mode. Low expression was arbitrarily defined as the weakest signal above background that could be visually identified. Increasing levels of expression were defined from this set point based on increases in detector gain settings with a constant amplifier gain setting of one. Gain settings used for the lowest to highest levels of expression ranged from 425 to 750, respectively. In direct comparisons, the gain settings for actin were similar in transfected vs. nontransfected parietal cells, ranging from 480 to 540.

Differential centrifugation, Western blot analysis, and immunoprecipitation analyses. For differential centrifugation experiments, cells were extracted in 1% Nonidet P-40 (NP-40), 150 mM NaCl, 2 mM EDTA, 0.5 mM DTT, 20 mM HEPES, pH 7.4 with protease inhibitors (10 μg/ml pepstatin, 1 Roche mini-EGTA-free tablet/7 ml) and then centrifuged at 14,000 g, 30 min, 4°C. The resulting supernatants were centrifuged at 100,000 g, 2 h, 4°C.

One (1D)- and two-dimensional (2D) Western blot analyses with enhanced chemiluminescence (ECL) detection (Amersham Biosciences) were performed as previously described (9, 11) using the following monoclonal antibodies: anti-drebrin (1:1,000), anti-HA tag (1:1,000), anti-coronin 1B (1:1,000), anti-lasp-1 (1:1,000), anti-H-ATPase (1:20,000), and anti-actin (1:1,000).

Immunoprecipitation of native parietal cell drebrin was attempted using the M2F6 antibody (1:50–1:1,000 dilutions) under both denaturing and non-denaturing conditions. For denaturing immunoprecipitations, 0.05–0.1 ml packed cells (5–10 × 106 cells) were lysed by heating 5 min, 100°C with 1% SDS, 1 ml freshly added DTT, and 15 mM HEPES, pH 7.4. Lysates were diluted 1:10 with a buffer containing 1% sodium deoxycholate, 0.5% Triton X-100, 0.15 M NaCl, 10 mM EDTA, and 15 mM HEPES, pH 7.4, and clarified by centrifugation (14,000 g, 20 min, room temperature). Protein A + G Sepharose beads (Calbiochem) were added after incubating clarified lysates with the M2F6 antibody for 1 h. Incubation was continued for 16 h at 4°C. Beads were then washed four times with the same buffer used to dilute lysates, and bound proteins were eluted by heating in SDS/PAGE stop buffer for 1D SDS-PAGE or 1 × SDS/BME for 2D Western blot analyses (9). Non-denaturing immunoprecipitations were performed in a similar fashion with the following exceptions: 1) cells were lysed for 5 min at 4°C in 1 ml of a buffer composed of 1% NP-40, 1 mM EDTA, 50 mM KCl, 20 mM HEPES-KOH, pH 7.8; 2) lysates were centrifuged at 4°C, and 3) beads were washed with PBS. HA-tagged drebrin was immunoprecipitated from transfected MDCK cells (see below) under the same denaturing immunoprecipitation conditions used for parietal cells.

cDNA cloning, plasmid construction, and cellular transfections. Drebrin cDNA (GenBank accession no. AY 170852) was cloned from
rabbit parietal cell mRNA using a RT-PCR-based approach (11). Primers were designed with Oligo 6.8 and were initially based on conserved regions in human and mouse drebrin DNAs identified by BLAST alignments of human drebrin 1, transcript variant 1 (NM_004395.2) and mouse drebrins (NM_098131.1 and AF187148.1). Cellular mRNA was isolated from parietal cells with the Promega PolyATtract mRNA isolation system IV and used as a template to generate cDNA using oligo(dT) and a gene-specific antisense primer with an EcoRI site (5′-GGG GAA TTC GCT GTA AAA GTC AGG CCC TGT-3′; position 2246–2266 in NM_004395.2). RNA was converted to single-stranded DNA with a Superscript first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA), which was then used as a template for PCR (Advantage-GC cDNA PCR kit, BD Biosciences). Primers for the complete open reading frame were based on the rabbit drebrin sequence. The sense primer was engineered to contain a BamHI site (5′-GGG CCT CGA GCT AAT CGC CGC CCT CGA A-3) and cellular transfections

RESULTS

Localization of drebrin in gastric mucosal cell extracts and tissue sections. In vivo, epithelial cells within the oxyntic (acid-secreting) gastric mucosa are organized into glandular “units” composed of pit, isthmus, neck, and base regions. The mucosal surface is lined with columnar, mucus-secreting surface epithelial cells, which also line the pits. The parietal cell lineage is thought to originate from precursor cells in the isthmus from where parietal cells migrate upward into the pit and downward to the base of gland units (24). In the pit and isthmus, parietal cells are interspersed with mucus-secreting cells. In the neck region, parietal cells are the predominant cell type with decreasing numbers in the basal regions where the majority of pepsinogen-secreting chief cells reside. There is accumulating evidence to suggest that the function of parietal cells varies depending on their location within gland units (25, 33). Because the pattern of drebrin expression along the gland axis had not been previously defined, experiments were designed to address this question. Initially, Western blot analyses were performed to confirm that drebrin E2 could be detected in rabbit with the M2F6 antibody. As shown in Fig. 1, the drebrin antibody cross-reacted with a prominent band with an apparent molecular weight (M r) of ~120 kDa. Although the predicted Mr of drebrin in human and mouse is ~70,000 kDa, drebrin also migrates at ~120 kDa in these species (26, 37), presumably because of the high proline content and acidic nature of the molecule. The 120-kDa band was prominent in parietal cell extracts enriched to >95% purity, but was not detectable in gel tryptic digested, analyzed by MALDI-TOF mass spectrometry to check digestion efficiency then fractionated on nanopurification columns followed by precursor ion scanning and MS/MS analyses using a QSTAR XL equipped with Protana NanoES Model II nanospray and oMALDI sources.

Statistical analyses. For confocal microscopic analyses, duplicate slides for each treatment group (derived from 3–10 independent experiments) were prepared and scanned at random. Images were acquired from 25–50 cells/slide and analyzed as dichotomous observations as previously described (11). In biochemical experiments, values were expressed as means ± SE with n = the number of cellular isolates from different animals. Paired comparisons were analyzed with Student’s t-test. Multiple comparisons were analyzed with ANOVA and Duncan’s multiple range tests.
extracts of mucosal cell fractions depleted of parietal cells (<1%). To further validate differential drebrin expression in parietal cell fractions and to compare protein transfer efficiencies, the same blots were reprobed for the parietal cell-specific H⁺-K⁺-ATPase and for coronin 1B, a protein ubiquitously expressed in the gastric oxyntic (acid-secreting) mucosa (36). As expected, there was pronounced H⁺-K⁺-ATPase cross-reactivity in the parietal cell-enriched fraction but no detectable signal in the parietal cell-depleted fraction. In contrast, similar amounts of coronin 1B were detected in both fractions (Fig. 1).

To define the cellular and subcellular distributions of drebrin in cells within the oxyntic gastric mucosa, tissue sections were dual labeled for drebrin and F-actin. Because both the drebrin and H⁺-K⁺-ATPase antibodies were mouse monoclonals, adjacent tissue sections were dual labeled for F-actin and H⁺-K⁺-ATPase to provide additional confirmation that F-actin staining appropriately localized parietal cells in the glands (not shown). As shown in Fig. 2, A–E, drebrin staining was prominent in parietal cells in the neck and base of glands, but was undetectable in mucous cells in the surface and pit regions, in

Fig. 2. Cellular localization of drebrin and F-actin in rabbit gastric mucosal tissue sections. Gastric mucosal tissue sections were prepared from control and histamine-stimulated rabbits, dual-labeled for drebrin (red; A, C, F, and I) and F-actin (green; B, D, G, and J), and analyzed by confocal microscopy as described in MATERIALS AND METHODS. Images are representative of data obtained from 3 animals. A and B: composite image showing the pattern of drebrin and F-actin staining from the serosa (right) to the lumen (left). Arrowheads indicate positions of several parietal cells in the pit region that stained for F-actin but not drebrin. Arrows point to blood vessels and smooth muscle present in the base of the glands that stain strongly for F-actin but not drebrin. The solid circle plus arrow points to a region near the base that contains chief cells where F-actin staining is prominent on apical membranes facing the gland lumen but no drebrin staining is present. The solid square plus arrow points to mucous cells in the pit that stain for F-actin on their cortical membranes but do not stain for drebrin. C–E: higher magnification image of parietal cells in the pit region showing differential staining for drebrin (C) and F-actin (D). E: merged images. Bar, 20 μm. F–H: image of a parietal cell in a tissue section from a fasted rabbit showing similar localization of drebrin (F) and F-actin (G) in the intracellular canalicular region and the cortical plasma membrane. H, merged images. Bar, 5 μm. I–J: image of a parietal cell in a tissue section from a histamine-stimulated animal. Note that drebrin (I) and F-actin (J) distributions are similar to the control, but the intracellular canalculus is highly convoluted as expected with histamine stimulation. K, merged images. Bar, 5 μm.
chief cells, and in a subpopulation of parietal cells within the pit region. Interestingly, parietal cells in the pit region that stained strongly for drebrin were frequently located immediately adjacent to parietal cells that did not stain for drebrin (Fig. 2, A–E).

Drebrin localization in parietal cells is not grossly altered by elevation of [cAMP]. Histamine elevates parietal cell [cAMP], and both histamine- and cAMP-dependent activation of HCl secretion are correlated with the formation of a large number of elongated, F-actin-rich microvilli on parietal cell intracellular canaliculi. These actin-based morphological changes are so dramatic that they can be visualized with a light microscope and have been shown to be correlated with the translocation of the H\(^+\)-K\(^+\)-ATPase from an internal tubulovesicular or tubular, Golgi-like stacks to F-actin-rich, apically directed membrane projections on the intracellular canaliculi (see Refs. 34, 35, 48 for recent reviews). In liver, there is some evidence to suggest that drebrin is present, along with F-actin, on Golgi transport vesicles (15). This suggested the possibility that drebrin might also be involved in vesicle trafficking in the parietal cell. To determine whether drebrin is recruited to the canalicular membrane in a stimulus-dependent manner, the subcellular distribution of this protein was analyzed in parietal cells within oxyntic mucosal tissue sections from histamine-stimulated and control animals (see MATERIALS AND METHODS). Similar experiments were performed with isolated gastric glands that contain parietal cells and other mucosal cell types in a normal polarized morphology (5, 11).

As expected, in vivo histamine stimulation induced the formation of highly convoluted intracellular canaliculi, which stained more strongly for F-actin compared with unstimulated controls (Fig. 2, F–K). Under these conditions, the pattern of drebrin immunostaining was similar to that for F-actin with prominent distribution on the F-actin-rich parietal cell canalicular membrane and, to a lesser degree, on cortical cell membranes along with F-actin (Fig. 2, F–K). Drebrin and F-actin staining patterns were faithfully reproduced in parietal cells within isolated gastric glands. Differences in the level of drebrin expression in parietal cells were also apparent in segments from appropriate mucosal regions (Fig. 3). Treatment with either histamine (10 \(\mu\)M, 30 min) or forskolin (10 \(\mu\)M, 30 min; not shown) induced the expected morphological changes in canicular F-actin, but did not grossly alter the subcellular distribution of drebrin (Fig. 3, A–D). Exposure to cytochalasin D (10 \(\mu\)M, 30 min) partially disrupted cortical F-actin to the extent that glands from the neck region had a “stretched” appearance, and aggregates of F-actin formed on basal membranes of mucous cells in the pit region (Fig. 3, E and G). With cytochalasin D treatment, there was also variable clumping of F-actin in intracellular canaliculi. Under these conditions, drebrin remained colocalized with F-actin within the canicular membrane region (Fig. 3, E and F). Thus subcellular localization data from tissue sections and isolated glands support the conclusion that drebrin is prominently localized within the intracellular canaliculi and, to a lesser extent, on the cortical plasma membrane of parietal cells. Studies with cytochalasin D further support the conclusion that drebrin is tightly associated with F-actin in the intracellular canalicular compartment.

To provide further confirmation that elevation of intracellular cAMP does not grossly alter the subcellular distribution of drebrin, control and forskolin-stimulated parietal cells were lysed in the presence of 1% NP-40 and then subjected to differential centrifugation as described in MATERIALS AND METHODS. Forskolin stimulation was confirmed by analyzing changes in the electrophoretic mobility pattern of the phosphoprotein, lasp-1 (Fig. 4, inset). As shown previously (9), cAMP-dependent phosphorylation of lasp-1 induces a M, “band shift” on SDS-PAGE gels. In these experiments (\(n = 5\)), there was no significant difference in the subcellular distribution of drebrin in control vs. forskolin-stimulated cells with 95.7 ± 1.2% (in controls) and 95.6 ± 0.8% (with forskolin) of the drebrin signal being localized within the detergent-insoluble low-speed cytoskeletal fraction. This fraction also contained 79 ± 2.0% of the total actin and 46.2 ± 1.8% of the actin-binding protein coronin 1B (36). Less than 5% of total drebrin was present in the cytosolic (100,000 g supernatant) fraction (1.7 ± 0.4% controls; 1.7 ± 0.5%, forskolin) along with 15.7 ± 1.9%, actin and 50.9 ± 2.1%, coronin 1B (Fig. 4). In fractions in which drebrin was highly enriched (Fig. 4), two immunoreactive
bands, one strong and one weak, were detected. The band producing the strong signal comigrated with \( \sim 120 \) kDa bands in other parietal cell fractions. The weaker band migrated at a predicted frame coding for a protein of 668 amino acids. The cDNA from positive clones identified a 2007-base open reading frame. Sequence analysis of the isolated rabbit drebrin cDNA clones. Sequence analysis of the cause drebrin isoforms had not been previously characterized.

Molecular characterization of parietal cell drebrin E2. Because drebrin isoforms had not been previously characterized in rabbit gastric mucosa, a PCR-based approach was used to isolate rabbit drebrin cDNA clones. Sequence analysis of the cDNA from positive clones identified a 2007-base open reading frame coding for a protein of 668 amino acids. The predicted Mr and pl of this protein were 72,986 and 4.39, respectively. These data provided further confirmation that the drebrin E2 isoform is present in the rabbit parietal cell and demonstrated that the deduced amino acid sequence of rabbit drebrin E2 is highly homologous with human, mouse, and rat sequences (Fig. 5A). ADF/cofilin-like (ADF-H) domains are present (residues 8–134) and 100% conserved in all four species. Thus it is likely that this domain plays an essential role in drebrin function(s), which could include phosphorylation.

Phosphobase analyses identified a number of kinase-susceptible residues that were conserved across human, rabbit, mouse, and rat including 14 serine residues and four threonines with \( P > 0.9 \) phosphorylation potential. The majority of these sites are clustered within a region that falls within the COOH-terminal end of the coil-coil region and immediately upstream of the PRM1 motif (Fig. 5, A and B). There are also four candidate serine residues in the ADF-H domain, two in the coiled-coil region, and two in the far COOH terminus. Two of the threonine candidates are located in this latter region (Fig. 5A). Of the nine conserved tyrosine residues, two (Y\(^{80}\) and Y\(^{615}\)) had phosphorylation potentials with \( P \geq 0.6 \).

Drebrin phosphorylation analyses. To determine whether the drebrin E2 isoform is phosphorylated in vivo, parietal cell extracts were fractionated on phosphoprotein affinity columns, and column eluates were analyzed by Western blotting. The pattern of drebrin migration in cell lysates was also analyzed on 2D Western blots (Fig. 6). Drebrin was significantly enriched in phosphoprotein column eluates (Fig. 6A) in which there was a small but significant decrease in the recovery of drebrin from extracts of forskolin-stimulated cells (10 \( \mu M \), 15 min) compared with paired controls (DMSO vehicle) (Fig. 6B). In these same eluates, there was a significant enrichment of the cAMP-dependent phosphoprotein, lasp-1 (9) in extracts from forskolin-stimulated cells (Fig. 6). However, although 2D Western blot analyses of parietal cell lysates demonstrated that drebrin migrates as a series of spots with an average pl of \( \sim 4.3 \), drebrin was inefficiently solubilized in the absence of SDS (first dimension isoelectric focusing buffer), and migration patterns were not sufficiently consistent to demonstrate reproducible agonist-dependent changes in phosphorylation in whole cell extracts.

In related experiments, the M2F6 antibody was not found to immunoprecipitate drebrin specifically under either denaturing or non-denaturing conditions. With high protein concentrations (6–8 mg), a small amount of drebrin coprecipitated with protein A + G Sepharose beads along with actin and other proteins, two of which had molecular weights similar to gelatin and myosin identified in a previous report (18); however, these proteins also coprecipitated along with the Sepharose beads when the M2F6 antibody was omitted (data not shown). To circumvent this problem, a monoclonal anti-HA antibody was used to immunoprecipitate HA-tagged drebrin E2 from MDCK cells 24–48 h after transfection with the pcDNA3 construct. These experiments were not attempted with parietal cells, because the transfection efficiency in these cells is too low to produce sufficient amounts of expressed protein (36). After confirming efficient immunoprecipitation of HA-tagged drebrin by Western blot analysis (Fig. 8), the HA antibody was
Fig. 5. A: sequence alignment of human (H. Sap), rabbit (Rab), mouse (Mus), and rat drebrin E2 and surface accessibility plots and diagrams comparing major domains in drebrin E2 and the Ena/VASP protein family member Mena. Conserved phosphorylation sites with significant phosphorylation potential are identified with asterisks. Dotted line, ADF-H domain; black lines, class III PDZ domain binding motifs; red lines, Class I SH3 domain recognition motif; green line, Class IV WW domains interaction motif region; cyan lines, EVH1 domain binding sites; red box, proline-rich profilin binding region. B: diagrammatic representation of drebrin showing major domains and locations of 3-phosphoinoside-dependent protein kinase-1 (PDK1) consensus binding site, profilin binding site, potential lipid binding site, and clustered phosphorylation sites upstream of the proline-rich region that bridges the coiled-coil and extensin 2-like domain (green) regions. C: surface accessibility plot. Diagrammatic representations for Mena show similarities between drebrin and Mena in extensin 2 (green) and proline-rich domains as well as in surface accessibilities in coiled-coil regions. Extensins are hydroxyproline-rich proteins present in plant extracellular matrix. Detection of this region in drebrin and Mena presumably reflects the concentration of proline residues within this region.
used to immunoprecipitate expressed drebrin from ~10^6 MDCK cells for mass spectrometry analyses (see MATERIALS AND METHODS). In two independent immunoprecipitates, phosphate ion scans detected several major peaks that contained the following peptide residues (based on the native rabbit drebrin sequence) under the following elution conditions: 1) 20% MeOH eluate, 140–147, 337–351; 2) 50% MeOH eluate, 140–149, 211–226, 224–236, 225–237, 238–252, 262–291, 253–270, 238–354, 328–351; and 3) 5% NH_4OH/50% MeOH, 140–149, 224–236, 225–237, 238–252, 262–291. These results are summarized in Table 1, which also lists predicted protein kinase consensus sites present in these peptides. All residues except combined residues 328–354, which contained one (337–361) to four (328–354) predicted phosphate additions, had one predicted phosphate addition. Further analysis by oMALDI confirmed the presence of a phosphorylated species in one peptide (residues 140–147) with a tentative assignment of phosphorylation to serine 142 in this peptide. As indicated in Table 1, serine 142 is a proline-directed protein kinase consensus site. The predicted phosphorylation potential for this site is high (P = 0.87). Serine 141 is a PKA consensus site with a low phosphorylation potential (P = 0.329). Interestingly, all of the peptides with potential phosphorylation sites fall within the region found to have a similar surface accessibility and to share significant sequence homology with the Ena/VASP family member Mena (Fig. 5B). It should be noted that phosphopeptides frequently do not ionize efficiently in the presence of nonphosphorylated peptides. Thus the oMALDI (MS/MS) analysis may not have resolved all of the phosphopeptide residues present in drebrin E2.

Morphological effects of drebrin overexpression in a MDCK cell line and in parietal cells in primary culture. Several studies have examined the effects of drebrin overexpression in fibroblasts and cell lines (19, 21, 22, 26, 43, 44), but none, to our knowledge, have analyzed the effects of drebrin overexpression on normal, terminally differentiated epithelial cells or compared effects of drebrin overexpression under confluent vs. nonconfluent conditions in cell lines. Thus the same pcDNA3 plasmids containing the HA-tagged rabbit drebrin E2 cDNA insert were transfected into both MDCK and parietal cells for direct comparisons. In confluent MDCK cells, HA-tagged drebrin expression induced the formation of relatively simple microspikes/filopodia (Fig. 7, A–C), whereas nonconfluent MDCK cells underwent more striking and variable changes in their morphologies. In contrast, nontransfected MDCK cells and MDCK cells transfected with empty vector had morphologies typical of this cell line (Fig. 7, D–O). Within 24 h, essentially all nonconfluent cells expressing HA-drebrin displayed actin-associated abnormalities, including the formation of highly branched neurite-like processes, both thick and thin actin-rich filopodial protrusions, F-actin bends and waves, and intracellular F-actin rings. Some of these morphologies are depicted in Fig. 7, D–L. Thus rabbit drebrin E2 isoform overexpression in nonconfluent MDCK cells produced morphological transformations similar to those in fibroblasts overexpressing the drebrin A isoform (3, 17, 22). High levels of expression were correlated with HA-drebrin accumulation at cell peripheries and the formation of massive, amorphous aggregates of F-actin within cell interiors (Fig. 7, J–L). None of the drebrin-transfected cells formed lamellipodial projections, suggesting a regulatory role for drebrin in the formation of parallel arrays of actin rather than in the formation of orthogonal actin networks characteristic of lamellipodia.

In parietal cells expressing intermediate levels of HA-drebrin, there was also prominent localization within cell cortices (Fig. 8). However, although the length of microspike projec-

Table 1. Identification of potential phosphorylation sites in drebrin E2

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Sequence*</th>
<th>Kinase Consensus Site †</th>
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<tbody>
<tr>
<td>140–149</td>
<td>LSSPVLR(LR)</td>
<td>PKA/Proline-directed kinase</td>
</tr>
<tr>
<td>211–237</td>
<td>(MEQERQEQEER(RYR)EREQQIEEHR)(R)‡</td>
<td>?</td>
</tr>
<tr>
<td>238–252</td>
<td>KQQTLEAEKRRLK</td>
<td>PKCa, CAMKII</td>
</tr>
<tr>
<td>253–291</td>
<td>(EQSGDQRDDDEETQMK)(KSEVEEAAAIAQRDPNDPR</td>
<td>?CKII/PKCγ/CKII</td>
</tr>
<tr>
<td>328–354</td>
<td>MAPTIPARSPDASTATPVTIER</td>
<td>Cdc2&amp;5,ERK1/GSK3,Cdc2&amp;5,ERK1/CKI,ERK1/GSK3, Akt,ERK1/GSK3</td>
</tr>
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*Phosphorylation consensus sites are underlined. Sites with highly significant phosphorylation potentials are in bold. Parentheses indicate positions where peptide data were combined to simplify the data presentation. †Protein kinase consensus sites are listed in the order in which they fall within the peptides. ‡A tyrosine residue (Y235) is the only potential phosphorylation site in this peptide. This residue has a low predicted phosphorylation potential (P = 0.239). Drebrin, developmentally regulated brain protein.
pression of drebrin E2 prevents cAMP-dependent, F-actin-associated morphological changes that occur upon activation of HCl secretion.

Characterization of effects of coexpression of the Rho-GTPase, Cdc42, and dominant negative Cdc42 with drebrin. The dramatic effects of drebrin overexpression on actin filament formation in MDCK cells suggested a potential involvement of the Rho GTPase family member Cdc42 which is known to promote the formation of actin-rich cell membrane extensions (16). To further explore this possibility, a series of cotransfections were performed using rabbit HA-tagged drebrin in conjunction with either myc-tagged Cdc42 or dominant negative myc-tagged Cdc42 (T17N) constructs. As shown in Fig. 9, A–C, wild-type Cdc42 overexpression induced the formation of thin, cortical microspikes of relatively uniform length (~2 μm) and distribution. HA-drebrin E2 overexpression also produced morphological changes similar to those depicted in Fig. 7 D–L. Coexpression of both proteins induced the formation of large numbers of long (5–10 μm), partially branched actin-rich processes that resembled a synthesis of drebrin and Cdc42 effects (Fig. 9, D–G). In contrast, filopodial elongation in cells expressing dominant negative Cdc42 was limited, and this myc-tagged protein accumulated at cell peripheries and within internal F-actin-rich “clumps” (Fig. 9, H–J). Coexpression of drebrin E2 with the dominant negative Cdc42 construct was correlated with the formation of long, branched filaments on cortical cell membranes (Fig. 9, K–N). These results suggest that drebrin is capable of regulating actin microfilament formation and elongation independent of the

Fig. 7. Effects of HA-tagged drebrin expression on morphologies of confluent and subconfluent Madin-Darby canine kidney (MDCK) cells. Twenty-four to 48 h after transfection with the HA-drebrin construct, cells were fixed, dual labeled with anti-HA primary antibody/Alexa Fluor 488 chicken anti-mouse secondary antibody and Alexa Fluor 647 phalloidin to label F-actin, and then examined by confocal microscopy. A, D, G, J, M: HA-drebrin (green); B, E, H, K, N: F-actin (red); C, F, I, L, O: merged images. Asterisks in figure mark some of the surrounding nontransfected cells. Bars, 10 μm. A–C: confluent cells transfected for 24 h. D–F: subconfluent cells transfected for 24 h. G–I: subconfluent cells transfected for 48 h. J–L: subconfluent cells transfected for 48 h. M–O: example of subconfluent cell morphologies following transfection with empty vector for 24 h.

Fig. 8. Effects of HA-tagged drebrin expression on the morphology of parietal cells in primary culture. Cells were transfected with the HA-drebrin construct and analyzed after 24 h as described in Fig. 7. [A, D, G, J: F-actin (red); B, E, H, K: HA-drebrin (green); C, F, I, L: merged images]. Bars, 5 μm. A–C: unstimulated parietal cell, empty vector. D–F: forskolin-stimulated (10 μM, 30 min) parietal cell, empty vector. G–I: transfected unstimulated parietal cell, 24 h. J–L: transfected unstimulated parietal cell, 48 h.
Cdc42 signaling pathway; however, the findings do not preclude the possibility that drebrin-induced microfilament formation can be modulated by this pathway.

DISCUSSION

In this study, we provide the first evidence that a specific population of parietal cells in the gastric oxyntic mucosa express high levels of a drebrin isoform homologous with drebrin 1 (isoform a), also known as drebrin E/E2. The cloning, sequencing, and analysis of rabbit drebrin 1a/E2 indicates this protein is highly conserved in mammals and also possesses numerous conserved protein-protein interaction domains and phosphorylation consensus sites. Thus drebrin 1a/E2 is primed to play an important role in regulating the functions of cells in which it is highly expressed.

The finding that drebrin E2 expression is most prominent in parietal cells in the neck and base of intact gland units and is undetectable in a subpopulation of parietal cells within the pit region supports the concept that parietal cell populations are functionally heterogeneous (26, 33). In this context, the subcellular localization of drebrin E2 to parietal cell intracellular canaliculi and the ability of drebrin E2 to induce the formation of F-actin-rich projections independent of Cdc42 indicate that this actin-binding protein has the capacity to play a central role in the regulation of microfilament formation within the intracellular canaliculus. The ability to induce actin microfilament formation independent of Cdc42 raises intriguing questions about the function of drebrin E2 with respect to the activation of HCl secretion. Cdc42 was recently proposed to be a mediator of parietal cell secretion (49). It will be important to determine whether drebrin plays an independent role in the secretagogue-dependent formation of elongated actin filaments in intracellular canaliculi, or whether drebrin interacts with Cdc42-dependent signaling pathways. It will also be important for future studies to determine whether drebrin functions are directly regulated by secretagogue-dependent alterations in phosphorylation and/or indirectly by alterations in protein-protein interactions. Although the present study does not provide unequivocal evidence that drebrin 1a/E2 is acutely regulated by agonist-dependent alterations in phosphorylation, the phosphoprotein affinity column as well as the mass spectrometry data presented herein strongly support this likelihood. Furthermore, the identification of numerous phosphorylation consensus sites and conserved protein-protein interaction domains support the proposal that drebrin 1a/E2 is regulated by multiple intracellular signaling pathways. The inability to dem-

Fig. 9. Comparison of effects of coexpression of HA-drebrin with myc-tagged wild-type and dominant negative Cdc42. MDCK cells were transiently transfected for 24 h; fixed; dual- or triple-labeled for the myc-tag or HA-tag, F-actin, and/or Cdc42; and analyzed by confocal microscopy as described in MATERIALS AND METHODS. In controls transfected with empty vector(s), morphologies were typical of images shown in Fig. 7, M–O. Bars, 10 μm. A–C, cells expressing wild-type Cdc42 stained with the myc antibody/Alexa Fluor 488 chicken anti-mouse secondary antibody (A, green) and Alexa Fluor 647 phalloidin (B, red). C, merged images (colors apply to the merged image). D–G, cells expressing HA-tagged drebrin and myc-tagged wild-type Cdc42 stained with the HA antibody/Alexa Fluor 488 chicken anti-mouse secondary antibody (D, purple); Oregon Green phalloidin (E, green); and Cdc42 polyclonal antibody/Cy3 donkey anti-sheep secondary antibody (F, red). G, merged images. H–J, cells expressing myc-tagged dominant negative Cdc42 stained with the myc antibody/Alexa Fluor 488 chicken anti-mouse secondary antibody (H, green) and Alexa Fluor 647 phalloidin (I, red). J, merged images. K–N, cells expressing HA-tagged drebrin and myc-tagged dominant negative Cdc42 stained with the HA antibody/Cy3 donkey anti-mouse secondary antibody (K, purple); Oregon Green phalloidin (L, green); and Cdc42 polyclonal antibody/Cy5 donkey anti-sheep secondary antibody (M, red). N, merged images.
onstrate consistent agonist-dependent alterations in drebrin E2 phosphorylation with current two-dimensional Western blot technology may be related to the poor solubility of drebrin in SDS-free isoelectric focusing buffer. Drebrin is ineffectively solubilized in nonionic detergents (Fig. 4), and the solubility of this molecule could be further compromised, for example, by oligomer formation similar to that which occurs in kidney and testes (38). Along these lines, it has been proposed that high local concentrations of drebrin directly regulate local actin dynamics and the formation of cellular protrusions (38). If this is indeed the case, there could be an important interplay between oligomeric and less complex forms of drebrin. Although we cannot yet propose a specific pathway with the capacity to regulate alterations in drebrin phosphorylation directly, it should be noted that the actin severing protein coflin with which drebrin shares a high degree of homology in the ADF-H domain region is activated by dephosphorylation of a site phosphorylated by LIM kinase and testicular protein kinase (2, 46, 47). Therefore, it is not unreasonable to suggest that a similar mechanism may be involved in drebrin regulation.

With respect to drebrin functions, it is important to note that although drebrins bind F-actin with high affinity in vitro, these proteins have thus far not been found to possess F-actin severing, bundling, capping, or nucleating activities (23, 27). Thus drebrins may regulate F-actin stability by interacting with other proteins that are either directly or indirectly involved in regulating one or more actin-associated activities. The identification of multiple conserved protein-protein interaction motifs in drebrin 1a/E2, including those for proteins possessing EVH1, SH3, PDZ, and WW binding domains, provides strong support for this proposal. With respect to possible functions, EVH1 domains are present in the Wiscott-Aldrich syndrome proteins WASP and N-WASP and in the Ena/VASP family members VASP, Mena, and Ev1 (20, 39). Ena/VASP proteins link cell membrane proteins, signal transduction pathways, and multiple actin-dependent processes (28). WASP and N-WASP play an important role in Cdc42-induced filopodia formation. Given the striking similarities between surface accessibilities of drebrin and Mena, it is particularly interesting to note that Mena plays an essential role in modulating Cdc42-dependent organization and assembly of actin filaments (30). Drebrin E2 also contains PRM1 and PKD1 consensus motifs. PRM1 motifs are present in proteins that associate with the multifunctional regulator of actin assembly, profilin (20), and there is in vitro evidence that these specific drebrin residues bind profilin (32). The presence of a potential binding site for a PKD1-binding site in the ADF-H domain of drebrin E2 provides further opportunity for regulation as PKD1 phosphorylates and activates AGC kinase members regulated by phosphatidylinositol 3-kinase (cf., Ref. 1). In addition to regulation by protein-protein interactions, drebrin E2 may be regulated by differential phosphorylation/dephosphorylation. Such a mechanism could provide for a balance between actin-polymerizing (EVH1 domain/PRM1 motif) vs. F-actin severing (ADF/cofilin domain) functions. Further studies are necessary to resolve these important questions.

In summary, the finding that drebrin E2 is phosphorylated in vivo in conjunction with the identification of highly conserved phosphorylation consensus sites and protein-protein interaction motifs predicts that multiple intracellular signaling pathways have the capacity to regulate the functions of this actin-binding protein. The differential expression of drebrin E2 in subpopulations of parietal cells further suggests that drebrin is involved in the regulation of parietal cell maturation and function. Finally, complementary studies with parietal cells in primary culture and in an MDCK cell line support a role for drebrin E2 in the modulation of physiologically important actin-based cytoskeletal rearrangements including those that accompany the activation of parietal cell HCl secretion and, by inference, similarly regulated ion transport activities in other drebrin-rich polarized epithelial cell types.

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The nucleotide sequence reported in this paper has been submitted to GenBank with accession no. AY 170852.

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REFERENCES

DREBRIN E2 IS DIFFERENTIALLY EXPRESSED IN PARIETAL CELLS


