Calpain Regulates Enterocyte Brush Border Actin Assembly and Pathogenic Escherichia coli-mediated Effacement*

Received for publication, May 2, 2003
Published, JBC Papers in Press, May 22, 2003, DOI 10.1074/jbc.M304616200

This study identifies calpain as being instrumental for brush border (BB) microvillus assembly during differentiation and effacement during bacterial pathogenesis. Calpain activity is decreased by 25–80% in Caco 2 lines stably overexpressing calpastatin, the physiological inhibitor of calpain, and the effect is proportional to the calpastatin/calpain ratio. These lines exhibit a 2.5-fold reduction in the rate of microvillus extension. Apical microvillus assembly is reduced by up to 50%, as measured by quantitative fluorometric microscopy (QFM) of ezrin, indicating that calpain recruits ezrin to BB microvilli. Calpain inhibitors ZLLYCHN2, MDL 28170, and PD 150606 block BB assembly and ezrin recruitment to the BB. The HIV protease inhibitor ritonavir, which inhibits calpain at clinically relevant concentrations, also blocks BB assembly, whereas cathepsin and proteasome inhibitors do not. Microvillus effacement is inhibited after exposure of calpastatin-overexpressing cells to enteropathogenic Escherichia coli. These results suggest that calpain regulates BB assembly as well as pathological effacement, and indicate that it is an important regulator involved in HIV protease inhibitor toxicity and host-microbial pathogen interactions.

Although it is accepted that actin-associated cross-linking and membrane linker proteins such as villin and ezrin, found in intestinal microvilli, are Ca\(^{2+}\)-sensitive, the role that Ca\(^{2+}\) plays in the assembly and stability of microvilli is undetermined. Interest in Ca\(^{2+}\) as a regulator of microvillus remodeling has been focused on its role in disrupting villin cross-links of the microvillus actin filaments (reviewed in Ref. 1) and the activation of the actin filament severing activity of villin. It has been suggested that the Ca\(^{2+}\)-dependent protease, calpain, cleaves the membrane linker protein ezrin, during cell motility-associated remodeling of cortical \(\beta\)-actin-containing structures (2–4). Because \(\beta\)-actin is the predominant actin isof orm of microvilli (5) and because ezrin, which is abundant in microvilli, associates with \(\beta\)-actin in an isoform-specific and calpain-sensitive fashion (2), the question has arisen whether calpain regulates \(\beta\)-actin-ezrin interactions in microvilli, and microvillus assembly. Additionally, the finding that calpain levels exceed calpastatin levels in intestinal epithelial cells (6) suggests that calpain could play a role in intestinal differentiation.

Calpain has been implicated in cytoskeletal remodeling, including disruption of cell-matrix interactions at the rear of the cell during crawling (7) and lamellipodial and protrusion formation during spreading (4). These examples illustrate the role of calpain in remodeling dynamic actin filament structures at the periphery of the cell. Calpain has not been implicated in assembly of filamentous actin structures in the BB apical domain of enteric epithelial cells or membrane recruitment of ezrin, where it may play an important role in assembly of actin filaments (2, 8).

A recently developed approach to study the role of calpain in cytoskeletal remodeling has been to create stable transfectants of established cell lines that overexpress calpastatin, calpain’s physiological inhibitor (4). Calpastatin inhibits the ubiquitous calpain isoforms, m- and \(\mu\)-calpain, so named for their respective millimolar and micromolar Ca\(^{2+}\) ion requirements for activity in vitro. Calpastatin is specific for calpain, regulates no other protease (9–11) and is the inhibitor of choice for implicating calpain in biological processes (12). Pharmacological inhibitors of calpain interact with the cysteine protease or the EF hand domains (13) of calpain and their respective millimolar and micromolar Ca\(^{2+}\) ion requirements for activity in vitro. Calpastatin inhibits the ubiquitous calpain isoforms, m- and \(\mu\)-calpain, so named for their respective millimolar and micromolar Ca\(^{2+}\) ion requirements for activity in vitro. Calpastatin is specific for calpain, regulates no other protease (9–11) and is the inhibitor of choice for implicating calpain in biological processes (12). Pharmacological inhibitors of calpain interact with the cysteine protease or the EF hand domains (13) of calpain and several are used to establish specific involvement of calpain proteases in cellular events. Fast-acting pharmacological inhibitors provide an advantage over antisense and siRNA approaches, which are limited by the 5-day half-life of calpain proteases (14). For the above reasons we chose to use calpastatin overexpression and combinations of pharmacologi-
Calpain inhibits to determine the role of calpain in BB assembly.

To determine the role of calpain in BB assembly, calpain activity levels, and corresponding BB microvillus elongation rates as well as ezrin and F-actin recruitment were measured for Caco 2 enterocyte cell lines stably overexpressing calpastatin. Down-regulation of calpain levels and activity were proportional to calpastatin overexpression, as was down-regulation of microvillus elongation. BB ezrin and F-actin recruitment were also blocked by calpastatin overexpression. The calpain inhibitors ZLLYCHN3, MDL28170, and PD 150606 also blocked BB assembly and ezrin recruitment. These results indicate that the calpain regulates assembly of the enterocyte BB, in part through regulation of ezrin recruitment. Calpastatin overexpression also conferred resistance to EPEC-initiated effacement. These findings indicate dynamic roles for calpain in epithelial morphogenesis and modulation of host-bacterial interactions during bacterial pathogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Caco 2a enterocytes (from Douglas Jefferson, Tufts University), were grown in Dulbecco’s Modified Eagle Medium (DMEM), with 10% heat-inactivated calf serum (Invitrogen), l-glutamine, penicillin, and streptomycin (complete medium, CM) at 5% CO2 (Revo Ultima Incubator). The Caco 2 enterocytes used in these studies are the Caco 2a subclone, which polarize and form tight junctions rapidly (15). Clones stably transfected with the calpastatin expression plasmid pRC/CMV-3CSN were grown in the above medium supplemented with 400 μg/ml G418. The cell lines, 0.5-11, 2-1, 2-3, 2-6, and 2-7, were horseradish peroxidase-coupled goat anti-mouse IgG (Bio-Rad) or were fixed with 400 μg/ml G418. G418-resistant clonal cell colonies were lifted with trypsin/EDTA and stored in liquid nitrogen. Passage of thawed cells was limited to 4–6 weeks.

Quantitation of Calpastatin, m-Calpain, and Cytoskeletal Calpain Substrates in Caco 2 Clonal Cell Lines—Cytosol and membrane/cytoskeletal extracts were made by Dounce homogenization of cells and differential centrifugation of the lysate of protein inhibitors, as described (4). The cytosolic extract contained ~95% of the extranuclear protein, and the membrane/cytoskeletal fraction, 5%. Immunoblot analysis of cytosolic extracts was performed after SDS-PAGE and transfer to 0.2 μm nitrocellulose filters. Blots were blocked (5% nonfat dry milk, 50 μM Tris-HCl, 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol) overnight and probed with primary antibodies, including monoclonal murine anti-human calpastatin PC-6 (17) (Reseach Diagnostics Inc., Flanders, NJ), rabbit anti-human m-calpain (D. E. Croall, University of Maine) (18), monoclonal murine anti-human ezrin-specific antibody 3C12 (Sigma), which reacted with a single 80-kDa band (D. E. Croall, University of Maine) (18), monoclonal murine anti-human calpastatin PC-6 (17) (Research Diagnostics Inc., Flanders, NJ), rabbit anti-human β-actin (5), and monoclonal murine anti-human villin (Chemicon International Inc., Temecula, CA). Secondary antisera were horseradish peroxidase-coupled goat anti-mouse IgG (Bio-Rad) or goat anti-rabbit IgG (Bio-Rad). Proteins were detected using the chemiluminescent peroxidase system (Amersham Biosciences) and exposed to film. The relative levels of antigens were quantified by densitometry and the Gelb-Plo-Pro program (UV Products, San Gabriel, CA).

Calpain Activity in Clonal Calpastatin-overexpressing Caco 2-derived Cell Lines—Calpain activity in cell suspensions was determined by measuring Ca2+-ionophore-specific hydrolysis of the peptidyl 7-amino bond of the calpain substrate succinyl-1-leucyl-1-leucyl-1-valyl-tirosyl-7-amino-4-methylcoumarin (succ-LLVY-AMC) (19) as previously described (4). Fluorimetry was performed with stirring at 37°C, with a PerkinElmer LS50B luminescence spectrometer. The initial rate of substrate cleavage in the absence of ionomycin was subtracted from the initial rate in the presence of ionomycin to determine the rate of Ca2+-dependent cleavage of the substrate.

Scanning Electron Microscopy—Caco 2 cells (5 × 104) were plated on silicon coverslips in 35-mm plate wells. After 16 h, the medium was changed to remove non-adherent cells. Confluent monolayers were fixed in 6% formaldehyde, 0.54% acetic acid, and 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1-hour washes. The Caco 2 lines stored were passed under identical conditions. The Caco 2 cell lines transfected with pRC/CMV were selected under identical conditions. The Caco 2 lines stored were passed under identical conditions. The C9 and C12 control cell lines transfected with pRC/CMV were selected under identical conditions. The Caco 2 lines stored were passed under identical conditions. The C9 and C12 control cell lines transfected with pRC/CMV were selected under identical conditions. The Caco 2 lines stored were passed under identical conditions.

Microvillus Morphometry and Measurement of Microvillus Elongation Rates—SEM images of Caco 2 cell monolayers photographed at 15,400× magnification were scanned from Polaroid photographs and stored as Adobe Photoshop TIFF files. Each image contained a size bar generated by the scanning electron microscope. At least two separate micrographs, each containing several hundred apical microvilli, were measured for each condition. Automated morphometry of the apical microvilli was done using Metamorph 5.0. Four filtering functions identified measurable apical microvilli. The first level of filtering involved thresholding to eliminate background surrounding the objects, which consisted primarily of pits and pockmarks in the apical membrane. Thresholding was done without significant loss of microvillus images, because of the high contrast of the microvilli. The second level of filtering involved gating objects to be measured on the basis of object width. Objects that were within ± 1 S.D. of the mean fiber width were retained for length measurement. The third level of filtering involved removal of objects touching the edges of the image. Filtering left 150–225 measurable microvilli per image and these were used to measure mean microvillus length. Calibration using the original size bar was used to convert all measurements to nanometers. The apical microvilli measured by automated morphometry were relatively comparable to curvilinear measurement of microvillus length of all measurable microvilli in the image, using NIH Image 1.61 (data not shown).

Ezin Immunofluorescent Antibody Staining of Assembling BB—Caco 2 cells were plated on collagen type I (10 μg/ml, 2 h, 37°C)-coated 8 well Lab-Tek II chamber slide system at 5 × 104 cells per well (0.7 cm2) and incubated in a tissue culture incubator under 5% CO2. Caco 2 cell plates were measured under these conditions, in fresh complete medium (Dulbecco’s modified Eagle’s medium with 10% fetal calf serum), form polarized monolayers within 2 h, exhibiting polygonal borders and apical microvilli. Slides were fixed at 2, 6, 12, and 24 h. SEM images were taken. SEM images indicated that BB assembly rates could be assayed by measurement of apical non-border microvilli. The fourth level of filtering involved removal of objects touching the edges of the image. Filtering left 150–225 measurable microvilli per image and these were used to measure mean microvillus length. Calibration using the original size bar was used to convert all measurements to nanometers. The apical microvilli measured by automated morphometry were relatively comparable to curvilinear measurement of microvillus length of all measurable microvilli in the image, using NIH Image 1.61 (data not shown).
Comparison of steady-state levels of calpain and calpastatin-associated proteins in the cytosolic fraction of calpastatin-overexpressing cell lines compared to an empty vector cell line

Measurements, normalized to the C9 control line, were determined by densitometry of Western blot analysis of protein levels. High calpastatin over-expressor cell line was 0.5-11. Lower over-expressor cell line was 2-3. Positive controls with BB extracts confirmed the reactivity of the ezrin antibody used to probe the cytosolic Western blots. At least three experiments were done to measure each protein in triplicate, except for β-actin, where the results presented are from triplicates in one experiment.

| Calpain-associated protein | Lower calpastatin over-expressor cell line | Higher calpastatin over-expressor cell line |
|---------------------------|------------------------------------------|------------------------------------------|
|                           | normalized mean ± S.D.                   | normalized mean ± S.D.                   |
| Calpain                   | 2.0 ± 0.22                               | 2.4 ± 0.04                               |
| m-Calpain                 | 0.59 ± 0.25                              | 0.58 ± 0.10                              |
| Calpastatin:m-Calpain ratio| 4.3                                       | 4.5 ± 0.27                               |
| Ezrin                     | 0.80 ± 0.49                              | 0.53 ± 0.27                               |
| Villin                    | 0.76 ± 0.07                              | 1.05 ± 0.07                               |
| β-Actin                   | 1.0 ± 0.014                              | 1.07 ± 0.013                              |

FIG. 1. Calpain activity is decreased in calpastatin-overexpressing Caco 2 Cells. Calpain activity was measured in intact cells by measuring hydrolysis of the cell permeant calpain substrate succinyl-L-lysyl-L-lysyl-L-lysine-AMC by fluorimetry. Calpain activities of the calpastatin-overexpressing 2-3 and 0.5-11 lines are normalized to mean calpain activities of the control Caco 2 line, C9. Error bars shown for 2-3 and 0.5-11 indicate the S.D. of the measurements. The results shown are duplicate measurements of a representative experiment.
TABLE II
Comparison of steady-state levels of calpain and calpastatin-associated proteins in the BB membrane/cytoskeleton fraction of calpastatin-overexpressing cell lines compared to an empty vector cell line

Measurements, normalized to the C9 control line, were determined by densitometry of Western blot analysis of protein levels. High calpastatin over-expressor cell line was 0.5-11. Lower over-expressor cell line was 2-3. At least three experiments were done to measure each protein in triplicate, except for β-actin, where the results presented are from triplicates in one experiment.

| Calpain-associated protein | Lower calpastatin over-expressor cell line | Higher calpastatin over-expressor cell line |
|----------------------------|------------------------------------------|------------------------------------------|
|                            | normalized mean ± S.D.                      | normalized mean ± S.D.                      |
| Calpastatin                | 0.92 ± 0.08                                | 1.6 ± 0.33                                |
| m-Calpain                  | 0.80 ± 0.24                                | 1.1 ± 0.45                                |
| Calpastatin:m-Calpain ratio| 1.2                                        | 1.4                                       |
| Ezrin                      | 0.86 ± 0.25                                | 0.54 ± 0.13                               |
| Villin                     | 0.44 ± 0.14                                | 0.77 ± 0.22                               |
| β-Actin                    | 0.93 ± 0.080                               | 0.91 ± 0.12                               |

Fig. 2. Apical microvillus density and length are decreased in calpastatin-overexpressing Caco 2 lines. A, SEM images of the apical surface and microvilli of low and high calpastatin over-expressor Caco 2 lines (lines 2-3 and 0.5-11) are compared with the control line (C9). The cells were plated at 4-fold over confluence, fixed, and coated for SEM at 54 h of culture. The size bar shown is applicable for all images (1 µm). B, microvillus length (nm) of low and high calpastatin over-expressor Caco 2 lines (lines 2-3 and 0.5-11) is compared with the control line (C9), as a function of time following plating. Microvillus length was measured by morphometric analysis. Between 150 and 225 microvilli were measured for each time point, except for the 0.5-11 line at 6 h, where 25 microvilli were measured because of the low density of microvilli. The error bars indicate the S.D. of the mean.
and 0.5-11 lines were plated at 1000, 500, and 100 cells per well in 96-well plates and cell number was determined by MTT assay. Cell recovery, measured at 24 h was highest for the C9 line, intermediate for the 2-3 line, and lowest for the 0.5-11 line, at all densities tested. At a plating density of 100 cells per well, no 0.5-11 cells could be detected at 24 h and the 2-3 cells were reduced in recovery compared with the C9 line. These results indicate that calpastatin overexpression, as low as 2.4-fold, blocks recovery of clonal calpastatin over-expressing Caco 2 cell lines, and this may explain the near absence of Caco 2 lines expressing high levels of calpastatin.

Calpastatin overexpression stably down-regulates the predominant calpain isoform, m-calpain (Table I), in proportion to the calpastatin/calpain ratio. The lower and higher over-expressor lines demonstrate calpastatin levels 2-fold or 2.5-fold background, respectively (Table I). Cytosolic m-calpain levels are reduced by 40% in the lower and higher calpastatin over-expressors (Table I). The lower and higher over-expressor lines, 2-3 and 0.5-11, respectively, demonstrate calpastatin/calpain ratios of 3:1 and 4:1 (Table I) and have calpain activities 73 and 22% of control, as determined by fluorometric measurement of cleavage of suc-LLVY-AMC (Fig. 1). These results indicate that a small change of the calpastatin/calpain ratio markedly affects calpain activity. This is consistent with the notion that the calpastatin/calpain ratio is tightly regulated in mammalian cells and directly modulates calpain activity (4, 23).

Membrane/Cytoskeletal Ezrin and Villin Are Decreased by Calpastatin Overexpression—Membrane/cytoskeleton levels of calpastatin and the microvillus proteins villin and ezrin were measured by Western blot analysis of the calpastatin-overexpressing lines. This fraction represents about 5% of the total extranuclear protein. Membrane/cytoskeleton calpastatin was increased 1.6-fold in the highest over-expressor line, 0.5-11, and the calpastatin/calpain ratio was increased 1.4-fold, while the low over-expressor line, 2-3, exhibited a 1.2-fold increase of the calpastatin/calpain ratio (Table II). Membrane/cytoskeletal ezrin and villin were decreased up to 50% in calpastatin-overexpressing lines (Table II). These results indicate that calpastatin overexpression changes the calpastatin/calpain ratio in the cytosolic rather than in the membrane/cytoskeletal fraction and that trafficking of ezrin and villin to the membrane/cytoskeletal fraction is impaired by inhibition of calpain. The

---

A. Srirangam and D. Potter, unpublished results.
decrease of ezrin in the membrane/cytoskeletal fraction is consistent with impairment of BB assembly in calpastatin-overexpressing Caco 2 cell lines (Fig. 2, A and B).

**Calpastatin Overexpression Decreases the Rate of Apical Microvillus Elongation During BB Assembly**—Because calpain promotes assembly of actin and ezrin-rich cortical structures during cell motility (4, 24), we tested the hypothesis that calpain also regulates cortical actin remodeling involved in differentiation. Specifically, we tested whether BB assembly during enterocyte polarization is calpain regulated by measuring apical microvillus elongation in calpastatin-overexpressing cells. Calpastatin-overexpressing Caco 2 cells were plated under conditions that resulted in monolayer formation and BB assembly within 2 h (Fig. 2A). Qualitative assessment of SEM images indicated that the apical microvilli of the control cell line elongated continuously during the first 24 h of BB assembly (Fig. 2A). The high calpastatin-overexpressing Caco 2 line, 0.5-11 displayed shorter microvilli at each time, while the microvilli of the low calpastatin over-expressor line 2-3 were of intermediate length. To determine apical microvillus length at each time by automated image analysis, a microvillus morphometry method was developed, using the program Metamorph 5.0r. Images of the Caco 2 apical domain captured at ×18,400 magnification were analyzed as described (see “Experimental Procedures”). 

Calpain-overexpressing cells demonstrated a 30–80% reduction in the length of apical microvilli during the first 24 h after plating (Fig. 2B) and remained decreased by 18–29% even at 54 h (data not shown). The initial rate of microvillus elongation was 31 nm/h for the C9 control line, 12 nm/h for the 2-3 line and 38 nm/h for the 0.5-11 line, indicating microvillus shortening for the highest calpastatin-overexpressing line. The rates of microvillus elongation were similar for all three lines at 24 h, but there was a lag in length of 33% for the 2-3 line and 40% for the 0.5-11 line, relative to the C9 control line. All 5 clonal lines overexpressing calpastatin exhibited similar defects in microvillus assembly (data not shown). These results indicate that calpain plays a regulatory role in the initiation of apical microvillus elongation.

**Calpastatin Overexpression Blocks BB Assembly and Ezrin Recruitment to the BB**—To determine whether calpain regulates BB assembly by promoting ezrin recruitment to apical microvilli, as suggested by the membrane/cytoskeletal fraction (Table II), ezrin IF staining was used to develop a QFM assay for BB assembly. Ezrin IF staining has been used to visualize...
apical microvilli in epithelial cells (20, 21, 25). Ezrin IF staining of epithelial monolayers reveals a “belt-like” border structure at cell-cell junctions and a convex non-border apical domain, the highest region of which overlays the cell nucleus (20, 21, 25). IF staining of ezrin in the non-border apical domain correlates with BB (25–27) and was chosen to assay BB assembly. Caco 2 cell lines were plated at high density on a collagen IV for 6 h, fixed and stained for ezrin. Three images representative of at least 30 cells for each condition are shown for the C9 control line and the 2-3 and 0.5-11 calpastatin over-expressing lines (Fig. 3A). There were fewer apical microvilli in the apical domains of the calpastatin-overexpressing lines (Fig. 3A), confirmed by QFM. Both calpastatin-overexpressing lines exhibited a 50% reduction of the apical ezrin normalized mean pixel intensity per cell (NMPI) (line 2-3, p < 0.0023; line 0.5-11, p < 0.00010) suggesting that calpain regulates BB assembly and the recruitment of ezrin to the BB. These results suggest that reduced ezrin recruitment to apical microvillous structures leads to reduced ezrin in the cytoskeletal/membrane fraction.

**Calpain Inhibitors Block BB Assembly and Ezrin Recruitment to the BB**—To confirm that calpain regulates BB assembly and ezrin recruitment to apical microvilli, calpain inhibitors that specifically target the protease and EF-hand domains of calpain were tested for inhibition of BB assembly, by assaying incorporation of ezrin into apical microvilli. The selective calpain inhibitor, ZLLYCHN₂, which binds irreversibly to the active site, does not inhibit the proteasome at concentrations less than 100 μM (28) and has been used to demonstrate the role of calpain in lamellipodial protrusion formation (4). At concentrations selective for calpain inhibition, ZLLYCHN₂ blocks BB assembly and apical ezrin recruitment (Fig. 4, B and C). The QFM assay demonstrates a 20% reduction of the NMPI (Fig. 4J, p < 0.0034). Another selective active site inhibitor of calpain, MDL 28,170 (29), also blocks BB assembly and apical ezrin recruitment (Fig. 4, E and F), resulting in a 25% reduction of the NMPI (Fig. 4J, p < 0.0020). The HIV protease inhibitor, ritonavir, which competitively inhibits m-calpain (Kᵢ = 9 μM) (30), blocks BB assembly (Fig. 4f), resulting in a 15% reduction of the NMPI (Fig. 4J, p < 0.042). Calpain activity was blocked by ritonavir under these conditions, by fluorometric assay of succ-LLVY-AMC cleavage in intact cells (data not shown). PD150606, which binds to the calcium-binding EF hand motif of calpain and inhibits its proteolytic activity (31) also blocks BB assembly (Fig. 4H), resulting in a 25% reduction of the NMPI (Fig. 4J, p < 0.00010). Inhibition of cathepsins by the lysosomotropic agent, NH₄Cl (1 mM) had no effect on BB assembly (Fig. 4, D and J). The proteasome inhibitor, lactacystin, which binds specifically to the Xβ6 subunit of the proteasome, does not inhibit calpain (28), and has no inhibitory effect on BB assembly (Fig. 4, G and J). In summary, pharmacological calpain inhibitors that work through different mechanisms confirm that calpain regulates ezrin recruitment to the BB.

**Apical Microvillus Actin Filament Assembly Is Inhibited by Calpastatin Overexpression**—Because microvillus assembly is blocked by calpastatin, the β-actin content of the BB cytoskeleton was measured by Western blotting of membrane/cytoskeletal extracts of cells plated for 54 h (Table II). Although BB assembly is impaired in both calpastatin over-expressor lines, the BB β-actin content is reduced only 10%. The length of microvilli is decreased by 18–29% in confluent monolayers plated for 54 h, suggesting that cytoskeletal/membrane β-actin of the BB may be in structures other than microvilli. To test this hypothesis, confocal microscopy of fluorescein-phalloidin-stained monolayers was conducted. F-actin was localized in calpastatin over-expressing Caco 2 lines, to assess morphologic perturbation of the apical actin cytoskeleton induced by calpastatin overexpression. These images, assembled as a reconstruction looking down the z-axis, as well as a cross-sectional x-z plane, (Fig. 5) confirm that calpastatin over-expression decreases the density of apical microvilli and the apical microvillus F-actin content. These images also reveal abnormal deposits of actin filament deposits at the apical surface, localization of which is confirmed by an x-z plane cross-section, which provides a side view of the monolayer (Fig. 5). These findings suggest that calpain plays a role in localization and organization of β-actin in the microvilli of enterocytes, and that loss of calpain activity results in mislocalization of actin in abnormal filamentous structures at the enterocyte apical domain.

**Calpastatin Overexpression Blocks EPEC-mediated Brush Border Effacement**—To test whether calpain regulates BB effacement, which occurs during the first 30 min of EPEC infection and before intimate association between EPEC and Caco 2 cells (32), confluent monolayers of calpastatin over-expressing cells were infected with EPEC strain 2348/69 (EAF+/EAE+), in log-phase growth. Infection proceeded for 30 min and the monolayers were fixed, stained and evaluated by TEM. During this period of infection, pedestal formation does not occur (32). The control lines are sensitive to EPEC-mediated effacement (Fig. 6A), with few microvilli left 30 min after infection. The calpastatin over-expressing lines (Fig. 6, B and C) exhibit nearly intact microvilli, suggesting a role for calpain in EPEC-mediated effacement.

**DISCUSSION**

These studies demonstrate the Ca²⁺ and calpain dependence of intestinal epithelial cell BB assembly and BB disassembly initiated by a common enteric pathogen, EPEC. Calpastatin overexpression blocks calpain activity up to 80%, in proportion to increase of the calpastatin/m-calpain ratio. Calpastatin overexpression inhibits the rate of apical microvillus elongation 2.5-fold, blocks ezrin recruitment to the BB and decreases apical microvillus density. The pharmacological calpain inhibitors ZLLYCHN₂, MDL 28,170 and ritonavir, which selectively block the sulfhydryl protease domain, and the PD150606 inhibitor, which blocks the Ca²⁺-binding EF hand domain, simi-
ilarly inhibit BB assembly and ezrin recruitment. The BB proteins ezrin and villin are decreased in the membrane/cyto-skeletal fraction, while BB content of β-actin, the microvillus isoactin, is unchanged. The F-actin content of the apical domain is disorganized in calpastatin over-expressing cells, shifted from microvillus structures to irregular clumps. These results indicate a regulatory role for calpain in microvillus actin assembly and in the nucleation and extension of microvilli. Thus calpain is involved not only in motility-associated actin remodeling, but also actin remodeling associated with cell differentiation. We find also that calpain regulates adherens junction formation, suggesting a general role for calpain in intestinal epithelial cell differentiation. The finding that EPEC-mediated BB effacement is regulated by calpain provides an alternative explanation for the Ca²⁺-sensitivity of microvilli (33), previously attributed to Ca²⁺ activation of the actin severing activity of villin. These findings are consistent with the notion that the BB is not static, in agreement with studies of BB protein turnover (34).

There are at least three mechanisms by which calpain could facilitate actin filament, and thus microvillus, extension: (a) uncapping of actin filaments to foster microvillus extension, (b) disruption of ezrin and myosin I linkages between bundled microvillus actin filaments and the membrane needed for movement of the microvillus core relative to the membrane, thus creating space for addition of actin monomers at the barbed ends of actin filaments, or (c) remodeling of the terminal web or cortical membrane to facilitate microvillus actin filament nucleation. Supporting the first mechanism, ezrin is an important component of microvilli and associates specifically with the microvillus actin isotype, β-actin, through an indirect interaction at the actin filament barbed end (2, 35). This complex of ezrin and β-actin is Ca²⁺- and calpain-sensitive, and involves the ubiquitous β-actin capping protein cap73 (3).

Because the binding of ezrin and cap73 to the barbed end of actin filaments is calpain sensitive, it has been proposed that activation of calpain mediates Ca²⁺-regulated exposure of barbed ends (3).

Prior studies have argued against capping of actin filaments in microvilli (36), despite fact that microvilli tend to be uniform in length, arguing in favor of some form of capping complex. Isolated, permeabilized, microvilli have free barbed ends that can serve as sites of elongation of actin filaments (37). Nonetheless, ezrin is easily extractable under the membrane permeabilization conditions used in these studies, and capping proteins may be extractable as well (3). When isolated BB, not treated with detergent, is soaked in solutions of actin monomer, addition of monomer at the barbed ends indicates uncapped actin structure (37). Nonetheless, the added actin monomers have an altered filament structure, not found in control tips, suggesting that the tip structure of the microvillus is altered as a result of BB preparation. Our finding of calpain in the membrane/cytoskeletal fraction of Caco 2 enterocytes provides an alternative explanation. Calpain activation during BB isolation could disrupt ezrin-containing capping structures and promote addition of actin monomer to barbed ends.

A second mechanism by which calpain could facilitate mi-

**FIG. 6.** Calpastatin overexpression blocks EPEC-mediated effacement of the BB. Calpastatin-overexpressing Caco 2 cell lines plated at confluence and cultured for 2 weeks on Permanox microscope slides were infected with log phase EPEC in complete medium (CM) for 30 min, without antibiotics. Infected monolayers were fixed and stained for TEM. A, control Caco 2 cell line C9 stably transfected with pRC/CMV vector is shown. The upper panel shows cells infected with 1.5 × 10⁸ EPEC/ml. The lower panel shows mock-infected cells. B, calpastatin-overexpressing Caco 2 line 0.5-11 is shown. The upper panel shows cells infected with 1.5 × 10⁸ EPEC/ml. The lower panel shows mock-infected cells. C, calpastatin-overexpressing Caco 2 line 2-1 is shown. The upper panel shows cells infected with 1.5 × 10⁸ EPEC/ml. The lower panel shows mock-infected cells. The size bar is 0.8 μm.

---

3 D. Potter, D. Acheson, and I. Herman, unpublished data.
Calpain Regulates Brush Border Assembly and Effacement

...but short... The mechanism of nucleation of microvilli is unknown, and microvillus growth is by facilitating microvillus actin nucleation. Tethering of these oligomers to the plasma membrane could then result in increased ezrin binding capacity, consistent with our observation that calpain activates ezrin at the plasma membrane could function as isoactin nucleation activity... Elongation of the filaments could then result in increased ezrin binding capacity, consistent with our observation that calpain activation of villin (50, 51). Baldwin hypothesized that the effacement lesion may be due to Ca2+-sensitivity of microvilli... Thus, calpain may play regulatory roles in both the physiological and pathological dissolution of the BB.

Acknowledgments—We thank...}

REFERENCES

1. Bretscher, A. (1983) Ciba Found. Symp. 95, 164–174.
2. Shuster, C. B., and Herman, I. M. (1995) J. Cell Biol. 128, 837–848.
3. Shuster, C. B., Lin, A. Y., Nayak, R., and Herman, I. M. (1996) Cell Motil. Cytoskeleton 35, 175–182.
4. Potter, D. A., Tiranauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco, K. A., Mier, J. W., Maki, M., and Herman, I. M. (1998) J. Cell Biol. 141, 647–662.
5. Hooke, T. C., Newcomb, P. M., and Herman, I. M. (1991) J. Cell Biol. 112, 653–664.
6. Ibrahim, M., Upreti, R. K., and Kidwai, A. M. (1994) Mol. Cell Biochem. 131, 49–59.
7. Hutton-locher, A., Palecek, S. P., Lu, Q., Zhang, W., Mellgren, R. L., Lauffenburger, D. A., Ginsberg, M. H., and Horwitz, A. F. (1997) J. Biol. Chem. 272, 32719–32727.
8. Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marrietti, G., Pannistl, J., Faulstich, H., and Griffiths, G. (2000) EMBO J. 19, 1961–1972.
9. Maki, M., Hatanaka, M., Takano, E., and Murachi, T. (1990) in Intracellular Calcium-Dependent Proteolysis (Mellgren, R. L., and Murachi, T., eds) pp. 37–54, CRC Press, Boca Raton, FL.
10. Croall, D. E., and DeMartino, G. N. (1991) Physiol. Rev. 71, 831–847.
11. Croall, D. E., and McGregor, K. S. (1994) Biochemistry 33, 13223–13230.
12. Carafoli, E., and Molinari, M. (1998) Biochem. Biophys. Res. Commun. 247, 193–203.
13. Wang, K. K., Posner, A., Raser, K. J., Burode-Kilgore, M., Rath, N., Hajimohammadmehra, I., Probert, A. W., Marczuk, F. W., Lunn, E. A., Haya, S. J., and Yuen, P. W. (1996) Adv. Exp. Med. Biol. 389, 95–101.
14. Zhang, W., Lane, R. D., and Mellgren, R. L. (1996) J. Biol. Chem. 271, 18825–18830.
15. Griffiths, J. K, Moore, B., Doubly, S., Keusch, G. T., and Tapson, S. (1994) Infect. Immun. 62, 4506–4514.
16. Assada, K., Ishino, Y., Shimada, M., Shimoto, T., Endo, M., Kimazuka, K., Kato, M., Maki, M., Hatanaka, M., and Murachi, T. (1989) J. Enzyme Inhib. 3, 49–56.
17. Lane, R. D., Allan, D. M., and Mellgren, R. L. (1992) J. Biol. Chem. 267, 351–356.
18. Croall, D. E., Slaughter, C. A., Wirtham, H. S., Skelly, C. M., DeOggy, L., and Moosaw, C. R. (1992) Biochim. Biophys. Acta 1121, 47–53.
19. Bronk, S. F., and Gores, G. J. (1993) Ann. J. Physiol. 264, 5744–5751.
20. Kondo, T., Takeuchi, K., Doi, Y., Yonemura, S., Nagata, S., and Tsukita, S. (1993) J. Cell Biol. 120, 749–760.
21. Tsukita, S., and Yonemura, S. (1999) J. Biol. Chem. 274, 34507–34510.
22. Herman, I. M., and Pollard, T. D. (1981) J. Cell Biol. 88, 346–351.
23. Barney, S., Glaser, T., and Koozer, N. S. (1988) Biochim. Biophys. Acta 940, 52–60.
24. Dourdin, N., Bhut, A. K., Dutt, P., Greer, P. A., Arthur, J. S., Elec, J. S., and Bretscher, A. (2001) J. Biol. Chem. 276, 13475–13482.
25. Berryman, M., Franck, Z., and Bretscher, A. (1995) J. Cell Sci. 105, 1025–1043.
26. Berryman, M., Gary, R., and Bretscher, A. (1995) J. Cell Biol. 131, 1211–1214.
27. Melendez-Vazquez, C. V., Rios, J. C., Zunzanni, G., Lambert, S., Bretscher, A., and Salzer, J. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1245–1249.
28. Mellgren, R. L. (1997) J. Biol. Chem. 272, 29899–29903.
29. Mehdi, S., Angelastro, M. R., Wiseman, J. S., and Bey, P. (1988) Biochem.
30. Wan, W., and DePetrillo, P. B. (2002) Biochem. Pharmacol. 63, 1481–1484
31. Wang, K. K., Nath, R., Pasner, A., Raser, K. J., Buroker-Kilgore, M., Hajimohammadreza, I., Preburt, A. W., Jr., Marcoux, F. W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J. L., Fergus, A. Lee, K. S., Lunney, E. A., Hays, S. J., and Yuen, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6687–6692
32. Francis, C. L., Jerse, A. E., Kaper, J. B., and Falkow, S. (1991) J. Infect. Dis. 164, 693–703
33. Glenney, J. R., Jr., Bretscher, A., and Weber, K. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6458–6462
34. Stidwill, R. P., Wysolmerski, T., and Burgess, D. R. (1984) J. Cell Biol. 98, 641–645
35. Yao, X., Chapponnier, C., Gabbiani, G., and Forte, J. G. (1995) Mol. Biol. Cell 6, 541–557
36. Schafer, D. A., Mooseker, M. S., and Cooper, J. A. (1992) J. Cell Biol. 118, 335–346
37. Mooseker, M. S., Pollard, T. D., and Wharton, K. A. (1982) J. Cell Biol. 95, 223–233
38. Howe, C. L., and Mooseker, M. S. (1983) J. Cell Biol. 97, 974–985
39. Hanzel, D., Reggio, H., Bretscher, A., Forte, J. G., and Mungrait, P. (1991) EMBO J. 10, 2363–2373
40. Mooseker, M. S., Graves, T. A., Wharton, K. A., Falco, N., and Howe, C. L. (1988) J. Cell Biol. 97, 869–882
41. Espindola, F. S., Espreafico, E. M., Coelho, M. V., Martins, A. R., Costa, F. R., Mooseker, M. S., and Larson, R. E. (1992) J. Cell Biol. 118, 359–368
42. Nascimento, A. A., Cheney, R. E., Tashata, S. B., Larson, R. E., and Mooseker, M. S. (1996) J. Biol. Chem. 271, 17561–17569
43. Burgess, D. R., and Grey, R. D. (1974) J. Cell Biol. 62, 566–574
44. Pearl, M., Fishkind, D., Mooseker, M., Keene, D., and Keller, T., III (1984) J. Cell Biol. 98, 66–78
45. Markowitz, M., Saag, M., Powderly, W. G., Hurley, A. M., Hsu, A., Valdes, J. M., Henry, D., Sattler, F., La Marca, A., Leonard, J. M., and Ho, D. D. (1985) N. Engl. J. Med. 313, 1534–1539
46. Mueller, B. U., Nelson, R. P., Jr., Steasman, J., Zuckerman, J., Heath-Chiozzi, M., Steinberg, S. M., Balis, F. M., Brouwers, P., Hsu, A., Saulis, R., Sei, S., Wood, L. V., Zeichner, S., Katz, T. T., Higham, C., Aker, D., Edgerly, M., Jarosinski, P., Serckhuiz, L., Whiteop, S. M., Pazruti, D., and Pizzo, P. A. (1998) Pediatrics 101, 335–343
47. Bode, H., Schmidt, W., Schulzke, J. D., Fromm, M., Riecken, E. O., and Ulrich, R. (2000) Ann. N. Y. Acad. Sci. 915, 117–122
48. Kenny, B., Abe, A., Stein, M., and Finlay, B. B. (1997) Infect. Immun. 65, 2606–2612
49. Burgess, D. R., and Prum, B. E. (1982) J. Cell Biol. 94, 97–107
50. Baldwin, T. J., Ward, W., Aitken, A., Knutton, S., and Williams, P. H. (1991) Infect. Immun. 59, 1599–1604
51. Knutton, S., Baldwin, T., Williams, P. Manjares-Hernandez, A., and Aitken, A. (1993) Zentralbl Bakteriol. 278, 209–217
52. Pinson, K. I., Dunbar, L., Samuelson, L., and Gumucio, D. L. (1998) Dev. Dyn. 211, 109–121
53. Matsudaira, P. T. (1983) Ciba Found. Symp. 95, 233–252
Calpain Regulates Enterocyte Brush Border Actin Assembly and Pathogenic 
Escherichia coli-mediated Effacement
David A. Potter, Anjaiah Srirangam, Kerry A. Fiacco, Daniel Brocks, John Hawes, Carter Herndon, Masatoshi Maki, David Acheson and Ira M. Herman

J. Biol. Chem. 2003, 278:30403-30412.
doi: 10.1074/jbc.M304616200 originally published online May 22, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304616200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 30 of which can be accessed free at 
http://www.jbc.org/content/278/32/30403.full.html#ref-list-1