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Spatial Localization of m-Calpain to the Plasma Membrane by Phosphoinositide Biphosphate Binding during Epidermal Growth Factor Receptor-Mediated Activation†

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Calpain activity is required for de-adhesion of the cell body and rear to enable productive locomotion of adherent cells during wound repair and tumor invasion. Growth factors activate m-calpain (calpain 2, CAPN2) via ERK/mitogen-activated protein kinases, but only when these kinases are localized to the plasma membrane. We thus hypothesized that m-calpain is activated by epidermal growth factor (EGF) only when it is juxtaposed to the plasma membrane secondary to specific docking. Osmotic disruption of NR6 fibroblasts expressing the EGF receptor demonstrated m-calpain being complexed with the substratum-adherent membrane with this increasing in an EGF-dependent manner. m-Calpain colocalized with phosphoinositide biphosphate (PIP_2) with exogenous phospholipase C removal of phosphoinositides, specifically, $PI(4,5)P_2$ but not $PI(4)P_1$ or PIP_3, releasing the bound m-calpain. Downregulation of phosphoinositide production by 1-butanol resulted in diminished PIP_2 in the plasma membrane and eliminated EGF-induced calpain activation. This PIP_2-binding capacity resided in domain III of calpain, which presents a putative C2-like domain. This active conformation of this domain appears to be partially masked in the holoenzyme as both activation of m-calpain by phosphorylation at serine 50 and expression of constitutively active phosphorylation mimic glutamic acid-increased m-calpain binding to the membrane, consistent with blockade of this cascade diminishing membrane association. Importantly, we found that m-calpain was enriched toward the rear of locomoting cells, which was more pronounced in the plasma membrane footprints; EGF further enhanced this enrichment, in line with earlier reports of loss of PIP_2 in lamellipodia of motile cells. These data support a model of m-calpain binding to PIP_2 concurrent with and likely to enable ERK activation and provides a mechanism by which cell de-adhesion is directed to the cell body and tail as phospholipase C-γ hydrolyzes PIP_2 in the protruding lamellipodia.

Cell motility is a complex process involving a sequence of events consisting of extension of a lamellipodium, formation of new adhesions at the leading edge, contraction of the cell body, and detachment of the rear of the cell (35, 47). These separate events must work in a coordinated effort to provide persistent cell movement in one direction. Rear detachment has been shown to be a rate-limiting step during both haptokinetic (26, 45) and growth factor-induced chemokinetic (22, 32, 56) motility. This subcellular asymmetry of processes occurs even in the absence of an externally imposed gradient of cues (35, 62), suggesting an intracellular segregation of biochemical controls. While progress has been made in deciphering the signaling gradients during ameboid movement in yeast (28, 37), the situation in mammalian fibroblasts and epithelial cells is less clear (47).

Extrinsic signals, including growth factors and the extracellular matrix, initiate intracellular signal cascades leading to biophysical changes in the cell (60, 62). At least two pathways have been identified as selective for growth factor-mediated motility. Phospholipase C-γ (PLC-γ) is activated downstream of several different receptor tyrosine kinases (5, 12, 33). Upon activation, PLC-γ cleaves phosphoinositide 4,5-biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (6). This hydrolysis, which is preferential just proximal to the extending lamellipodium (13), releases actin-binding proteins bound to PIP_2 that then reorganize the cytoskeleton and mediate protrusion of the lamellipodium at the front of the cell (11, 13, 43). This appears to be distinct from the PIP_2-dependent microdomain assembly seen in haptokinesis and during platelet-derived growth factor-driven motility in PC12 neuronal cells (25). The importance of the absence of PIP_2 in the front is seen in *Dictyostelium*, for lack of PIP_2 at the cell's front prevents lamellpodial shutdown by PTEN and thus continued motility (29). These two findings are not necessarily exclusive as epidermal growth factor receptor (EGFR) signaling causes a massive flux through PIP_2 with the activated PLC-γ leading to IP_3 generation but only a low steady-state level of available PIP_2 (13).

The second receptor-preferential pathway involves ERK/mitogen-activated protein kinase, which is activated via the divergent Ras-Raf-MEK pathway to mediate focal adhesion disassembly (44, 64). Previously, we have identified ERK activation of the cysteine protease m-calpain (calpain 2) as a crucial component of EGFR-mediated motility (22). m-Calpain cleaves a variety of proteins, with many of them being involved in focal adhesions, including ezrin, focal adhesion kinase (FAK), and principally talin (2, 15, 20, 23, 55). This activation

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of m-calpain is required for de-adhesion toward the rear of the cell; blocking of calpain during EGF exposure prevents the cell from pulling its trailing cell body forward (56, 57). Inhibition of either the PLC-γ or the ERK pathway leads to a loss of EGFR-mediated motility in fibroblasts (11, 64) and carcinoma cells (31, 32, 43). Thus, these two pathways are not only involved in cell motility but also must function in an asymmetric mode to maintain productive locomotion.

How EGF actuates m-calpain activity is still uncertain. However, both of the upstream effectors, ERK and EGFR, not only need to be present at the plasma membrane for m-calpain activation but also do not activate m-calpain when trafficked internal to the membrane (24). As ERK directly phosphorylates m-calpain (21), this suggests that m-calpain needs to be at the membrane as well in order to be activated, placing it near many, but not all, of its targets (2, 15, 20, 23, 55), where it is likely to be active in the regions of adhesions (2, 7, 8). This membrane localization may also solve the conundrum of the supraphysiological near-millimolar levels of calcium needed for m-calpain activation in vitro (17, 65). Localization to the membrane would either place m-calpain near the calcium channels (for highest local concentrations during puffs) or near other cofactors. Furthermore, EGF activates only m-calpain and not the more calcium-sensitive compound μ-calpain (CAPN1) (53), strongly suggesting that activation requires factors additional to calcium (22). It has been reported that binding to phospholipids lowers this Ca^{2+} requirement for m-calpain activation, although these in vitro investigations still necessitated supraphysiological levels of calcium (with half-maximal activation still occurring at Ca^{2+} levels as high as 0.1 mM) (1, 39, 46). Also in vitro, phosphoinositides have been shown to enhance binding of Ca^{2+} to domain III (D3) of m-calpain (61). These data suggest that m-calpain could be regulated at the membrane by phospholipids, although this has yet to be shown in cells under physiological activation.

What is attractive about phosphoinositides playing a role in m-calpain activity modulation is the possibility of explaining the quandary of the asymmetry of motility even in the absence of an external gradient and secondarily of placing activation at the presumed sites of action near adhesion-related targets (8, 23). Recently, in fibroblasts, we have identified a preference for PIP_2 hydrolysis at the leading edge and a decreasing gradient of PIP_2 from the cell body to the lamellipodium (13, 14). This rapid degradation of PIP_2 at the front of the cell essentially downregulates the steady-state levels of all phosphoinositides in the leading edge. A similar loss of steady-state levels of PIP_2 at the cell front is accomplished by PLC-γ and phosphoinositide 3 kinase in carcinoma cells (43) and by phosphoinositide 3 kinase in *Dictyostelium* (18, 29). Since m-calpain is involved in detachment at the rear during EGFR-mediated motility (22), we hypothesize that phosphoinositides play a role in restricting m-calpain activity to nonlamellipodial regions. Here we show that m-calpain localizes to phosphoinositide lipids in membranes in contact with the extracellular matrix. Inhibition of PIP_2 synthesis in membranes by 1-butanol blocks EGF-mediated m-calpain activity. Interestingly, we found that m-calpain accumulated toward the rear membrane of a moving cell in an EGF-dependent manner and that its activation was absent from forming lamellipodia.

MATERIALS AND METHODS

Isolation and immunofluorescent staining of cell footprints. Murine NR6 fibroblasts containing wild-type (WT) EGFR (63) were plated onto glass coverslips that had been covalently cross-linked with collagen I (Vitrogen; Cohesion, Palo Alto, CA) as described previously (38). These WT NR6 cells were passaged in minimum essential medium Eagle, alpha modification (MEMα), supplemented with 7.5% fetal bovine serum, 1× nonessential amino acids, and 400 μg/ml G418 (all culture reagents were from CellGro, Herndon, VA). Prior to treatment to expose substratum-bound membranes, cells were quiesced overnight in MEMα containing 0.1% dialyzed fetal bovine serum and then treated with 1 nM EGF for 18 h (or as stated in the legend to Fig. 2C). In some experiments, a denuded area was created with a rubber policeman to direct cell locomotion in one direction. Control cells were quiesced and wounded in parallel but not treated with EGF. The MEK inhibitor PD98059 (2 μM) and the calpain inhibitor *N*-acetyl-Leu-Leu-norleucinal (10 μg/ml) were added at the same time as the EGF (18 h ± EGF).

The apical (dorsal) aspect of cells was removed by a modification of a method described in detail previously (59). All procedures were performed while cells were on coverslips, in dishes, maintained on ice. All isolation solutions were maintained at 4°C. Cells were washed in morpholineethanesulfonic acid-buffered saline (MBS; 20 mM morpholineethanesulfonic acid [pH 5.5], 135 mM NaCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$) and then coated with a 1% solution of cationic colloidal silica (silica prepared as a 30% stock colloid as described previously [10]). (Cationic colloidal silica can be obtained by written request from Donna Beer Stolz, University of Pittsburgh, Pittsburgh, PA.) Cells were washed with MBS again and then coated with 1% polyacrylic acid in MBS (stock 25% aqueous polyacrylic acid solution, 100,000 average molecular weight; Aldrich, St. Louis, MO). Cells were finally washed with MBS and then swelled in hypotonic lysis buffer (2.5 mM imidazole [pH 7.0] supplemented with protease inhibitors [1:100 dilution, mammalian protease inhibitor cocktail; Sigma]) for 10 min. Cells were then unroofed by squirting the monolayers with lysis buffer through a 5-ml syringe fitted with a blunted, flattened 18-gauge needle. The degree of unroofing was monitored by observing cells with an inverted phase-contrast microscope.

Footprints were washed once with MBS and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS). In some experiments, the composition of the footprint membranes was altered prior to fixing. Footprints were treated with 25 μg/ml digitonin in PBS to extract cholesterol-rich areas for 10 min on ice or exposed to 100 μg/ml bacterial PLC in MBS for 10 min at 37°C to hydrolyze PIP_2. To determine if m-calpain interacts with the microtubular-cytoskeletal network associated with the membrane, membrane footprints were treated with 1 μM colchicine for 10 min at 37°C.

To visualize calpain, footprints fixed in 2% paraformaldehyde in PBS for 1 h were rinsed three times in PBS, rinsed three times in PBG (PBS with 0.5% bovine serum albumin and 0.15% glycine), and then blocked in 5% nonimmune goat serum in PBG for 30 min at room temperature. Monoclonal antibody to m-calpain (Biomol, Plymouth Meeting, PA) at a 1:100 dilution in PBG was added to cells for 1 h at room temperature [alternatively, we used a monoclonal antibody to PIP_2, $PI(4)P_1$, or PIP_3 at 1:500 (PerSeptive Biosystems, Framingham, MA) to visualize these phospholipids]. Samples were washed five times in PBG, and then secondary antibodies (1:500 dilution of goat anti-mouse Alexa 488; Molecular Probes, Eugene, OR) in PBG were added for 1 h of incubation at room temperature. F-actin was visualized by incubation with a 1:250 dilution of rhodamine-phalloidin (Molecular Probes) for 40 min. Samples were washed three times in PBG and three times in PBS, and nuclei were stained with 0.01% Hoechst dye in PBS, coverslipped with Gelvatol (23 g polyvinyl alcohol 2000, 50 ml glycerol, 0.1% sodium azide, 100 ml PBS) and then viewed on an Olympus BX51 fluorescence microscope (Olympus, Malvern, PA) equipped with an Olympus color video camera.

Quantitative analysis of bound m-calpain in membrane footprints. To quantify the amount of bound m-calpain in the membrane footprints, the footprints were either stained with monoclonal m-calpain antibody or extracted with 1× sodium dodecyl sulfate (SDS) sample loading buffer. For immunostaining analysis, the fluorescence of 30 individual cells chosen randomly was separately measured with Photoshop Program. The average fluorescence was compared by the Student *t* test. For immunoblotting analysis, 50 μg total protein per treatment separated by 7.5% SDS-polyacrylamide gel electrophoresis was transferred to polyvinylidene difluoride membrane for further blot analysis with monoclonal m-calpain antibody. The amount of m-calpain binding to cellular bottom membrane attached to the dish also was estimated with an Olympus Fluoview 500 confocal laser scanning microscope (Biocompare Inc., South San Francisco, CA) following the immunostaining of m-calpain. Quantification of m-calpain was

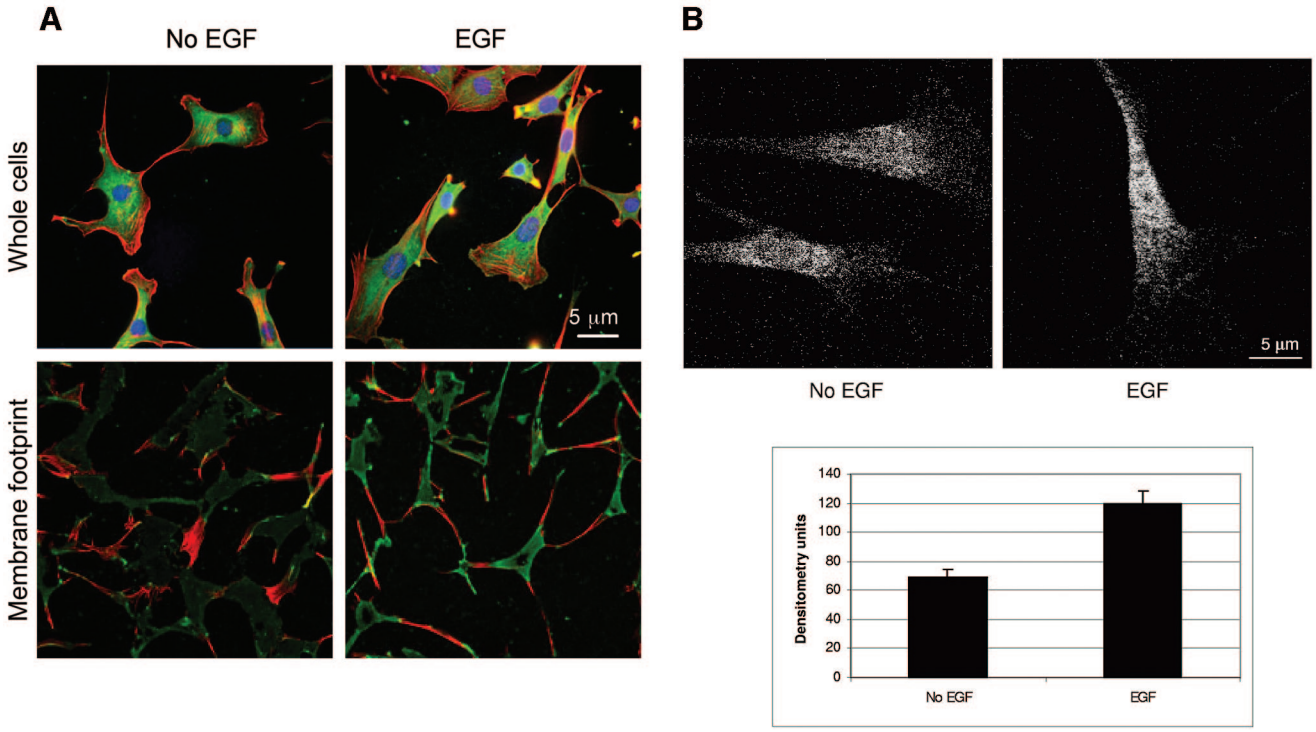


FIG. 1. m-Calpain localizes to cell membranes. Quiesced WT NR6 cells were exposed to diluent alone (−EGF) or 1 nM EGF (+EGF) overnight on coverslips. (A, top row) Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X, and stained for m-calpain (green) and F-actin (rhodamine-phalloidin, red; nuclei are Hoechst stained, blue). (A, bottom row) Cells were coated with silica gel and lysed, and the top membranes were removed. The remaining basolateral membranes were stained for m-calpain. Shown are representative cells from at least two independent experiments for the top row and at least three for the bottom row. (B) Cells were treated and fixed as above prior to staining for m-calpain. The fluorescence in the confocal slice containing the bottom membrane was observed by Olympus Fluoview 500 confocal laser scanning microscopy (Biocompare Inc., South San Francisco, CA). Quantification represents the average fluorescence (± the standard deviation; $P < 0.05$) of 30 individual cells chosen randomly.

performed with Metamorph analytical software (Universal Imaging Corp., Molecular Devices, Downingtown, PA).

Calpain activity assay. To detect calpain activity, the *tert*-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (BOC-LM-CMAC; Molecular Probes) assay (49) was used as previously described (22). Cells were plated on glass coverslips at 50% confluence and quiesced in 0.1% dialyzed fetal bovine serum for 24 h. They then were treated with 1.5% 1-butanol, 1.5% 2-butanol, 5 μg/ml brefeldin A (BFA), or diluent alone for 20 min. Subsequently, 50 μM BOC-LM-CMAC was added for 20 min of incubation. The cells were then stimulated with 10 nM EGF for 5 min and observed on an Olympus BX40 microscope with an M-NUA filter. Alternatively, some cells were treated for 45 min with 1.5% butanol. The butanol was then washed out with quiescing medium, and the cells were allowed to recover for 2 h. They then were treated with 50 μM BOC-LM-CMAC for 20 min and 10 nM EGF for 5 min and observed for fluorescence. In previous publications, we have demonstrated that under these conditions, BOC-LM-CMAC fluorescence in NR6 cells derives from m-calpain activation (22, 24, 56, 57) and thus serves as a specific indicator.

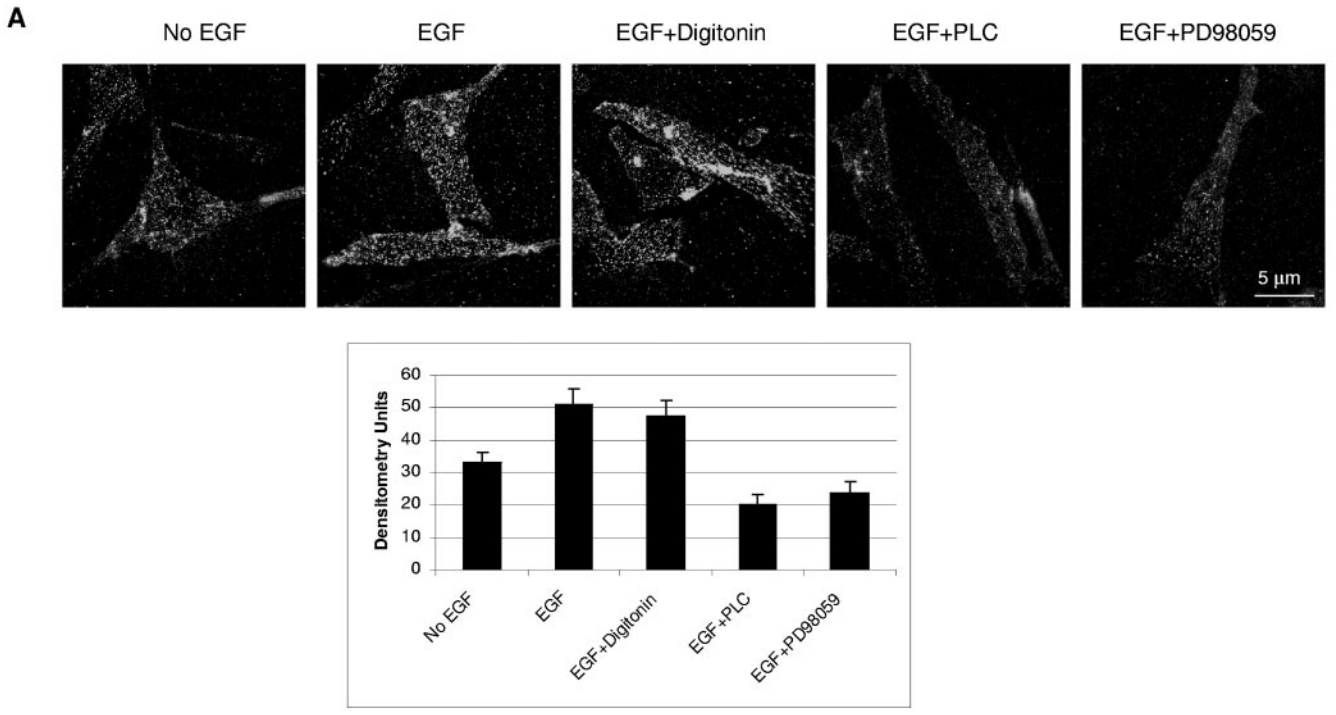
Assay of m-calpain binding to phospholipid-containing liposomes. Preparation of the liposomes and assessment of m-calpain-phospholipid binding were performed as previously reported (61). Briefly, liposomes were prepared from a crude bovine brain extract of phosphoinositides (Sigma, St Louis, MO) that contains 20 to 40% di- and triphosphoinositide (a minimum of 5 to 10% of each). Binding of native m-calpain or D3-eGFP (enhanced green fluorescent protein) to lipid vesicles was assayed by spin column size exclusion chromatography on MicroSpin S200 HR columns (Roche, Indianapolis, IN). The amount of protein in elute was detected by SDS-gel electrophoresis, followed by immunoblotting.

Identification of the phosphoinositide-binding domain of m-calpain. A plasmid containing the full-length human m-calpain cDNA was kindly provided by Richard Bodnar in our laboratory. To construct eGFP-fused m-calpain or its fragments in a transient-expression vector, standard PCR amplification was performed with sequence-specific oligonucleotides. PCR products digested with restriction enzymes were cloned upstream of eGFP in the mammalian expression vector pEGFP-N1 (Clontech, Palo Alto, CA). All recombinant plasmids were verified by DNA sequencing. In order to determine their membrane-binding capacity, mutant forms of m-calpain isolated with a Max Plasmids isolation kit (QIAGEN, Valencia, CA) were transfected into WT NR6 cells with Superfect transfection reagent (QIAGEN) as recommended by the manufacturer. Twelve hours after transfection, membrane footprints were prepared by the deroofing method described above and the fluorescence retained on the footprint was observed under an Olympus BX51 fluorescence microscope equipped with an Olympus color video camera. To observe the localization of the endoplasmic reticulum (ER), we stained whole WT NR6 cells transfected with WT m-calpain–eGFP or D3-eGFP and the membrane footprints of WT NR6 cells prepared as described above with ER-Tracker red dye (Molecular Probes); fluorescence images were obtained with an Olympus fluorescence microscope.

Statistical considerations. All pairwise comparisons were performed with Student's *t* test with minimum significance deemed to be $P < 0.05$.

RESULTS

m-Calpain localizes to cell membranes. The localization of an enzyme impacts both how it is activated and its subsequent function. In carcinoma cells, m-calpain is widely distributed throughout the cytoplasm and nucleus (34); this was also observed for WT NR6 fibroblasts (Fig. 1, top row) (24). Earlier, we had found that EGF-induced calpain activity was limited to membrane-localized ERK, with cytosolic active ERK incapable of triggering m-calpain (24), and that ERK activates m-calpain by direct phosphorylation (21). These data suggest that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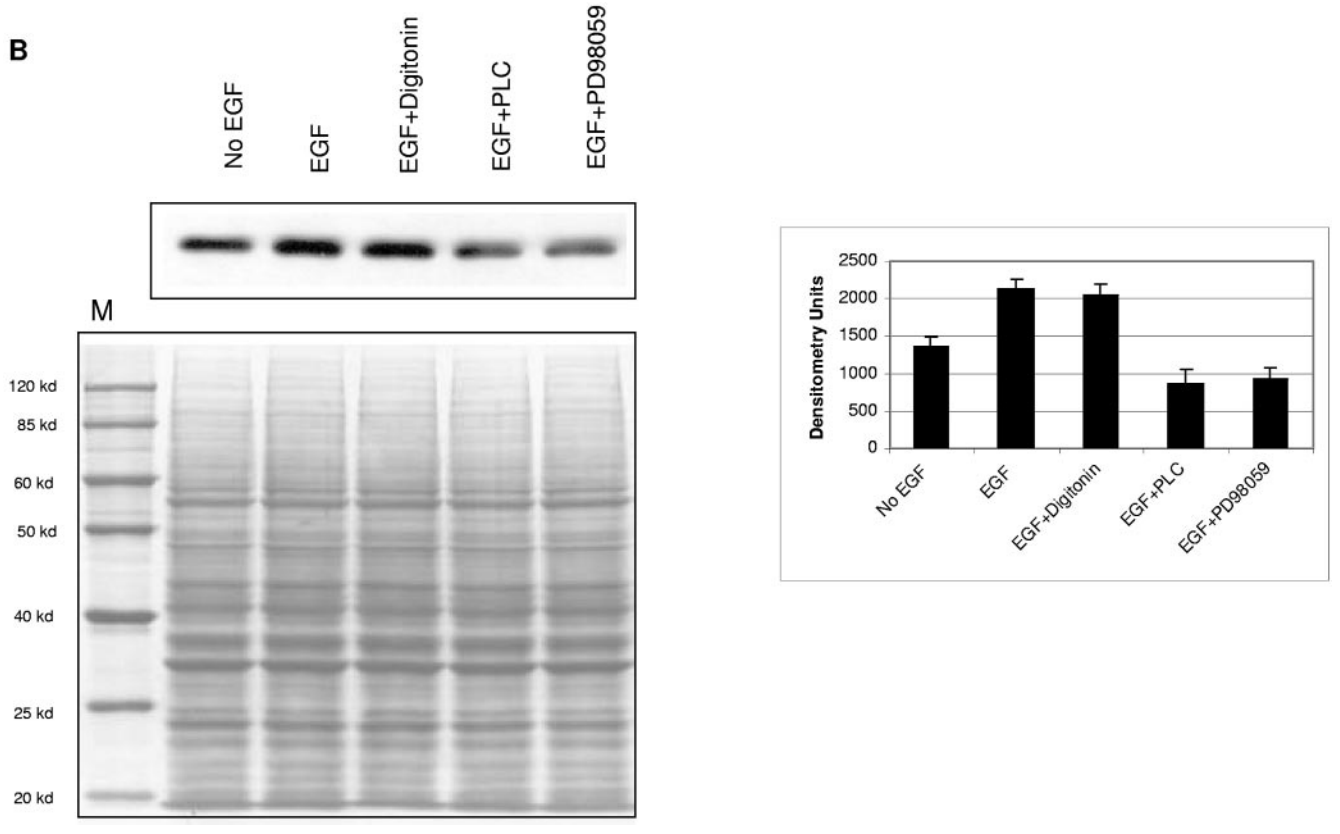
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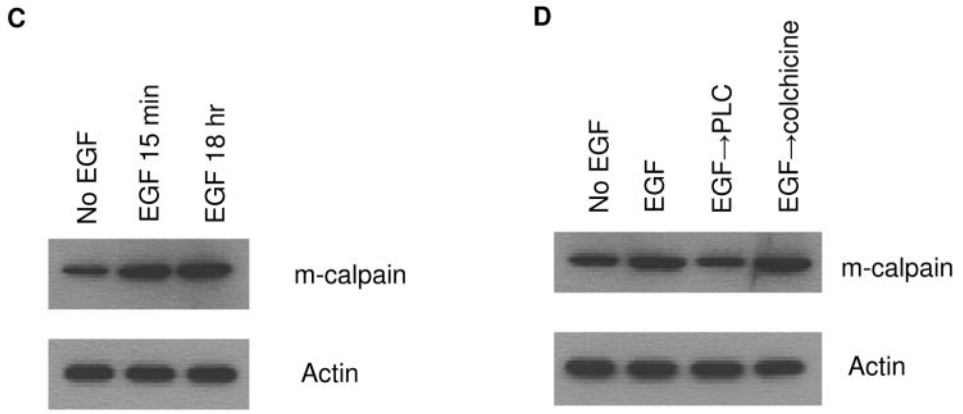

FIG. 2. EGF increases the amount of m-calpain on membrane footprints. The remaining basolateral membranes on coverslips were further treated with digitonin or bacterial PLC (see Materials and Methods) prior to fixation and quantitative analysis by immunostaining (A) or immunoblotting (B); alternatively, the cells were exposed to EGF in the presence of the MEK inhibitor PD98059. This translocation to the membrane occurs rapidly (C) and is not dependent on an intact microtubule network (D). The quantification in panel A represents the average fluorescence of 30 individual cells chosen randomly. Panel B represents the immunoblotting of m-calpain extracted with SDS sample buffer from membrane footprints treated with digitonin or bacterial PLC (or diluent). The bar graph represents the average value (± the standard deviation) of two independent experiments for each treatment. In both situations, the differences between diluent and EGF treatments and between EGF treatment and PLC treatment or PD98059 coexposure were statistically significant ($P < 0.05$). In panel C, WT NR6 cells were treated with 10 nM EGF for 15 min or with 1 nM EGF for 18 h, with the EGF dosages adjusted to activate the majority of EGFR without long-term receptor downregulation. The immunoblots in panels C and D are representative of two similar ones with the actin controls demonstrating that the PLC treatment does not release footprint-associated actin. M, molecular mass; kd, kilodaltons.

during motility, the m-calpain utilized in response to EGF exposure is in the periplasma membrane locale. We disrupted cells to determine whether calpain was associated with the plasma membrane during EGF-induced motility (Fig. 1). WT NR6 cells exposed overnight to 1 nM EGF, at which point some 90% of the cells were locomoting, were unroofed as described in Materials and Methods. The basolateral membranes retained on the substratum were immunostained for m-calpain. EGF-stimulated cells had more m-calpain bound to the basolateral membranes than did nonstimulated cells, although nonstimulated cells presented detectable staining (Fig. 1A, bottom row). EGF increased the amount of m-calpain retained in the membrane footprint by approximately 60% in immunostaining compared to the non-EGF treatment ($P < 0.05$ for both situations) (Fig. 2). This increase in m-calpain retained on the membrane fraction was rapid, occurring within 15 min after EGF exposure, and sustained throughout the continued presence of EGF (Fig. 2C).

It is possible that the unroofing also removes some of the less adherent cell membranes, such as extended lamellipodia, thereby skewing the results. We analyzed confocal sections (~2 μm thick) to localize the m-calpain translocation to a membrane-proximal region. The shift in m-calpain seen with the footprints was also noted in intact cells as increased m-calpain levels in the confocal slice taken just above the substratum (Fig. 1B, $P < 0.05$).

We sought to determine if this increased association was driven by calpain activation. Interestingly, the EGF-induced increase in m-calpain binding to membrane was significantly inhibited by the MEK inhibitor PD98059 (Fig. 2), which prevents EGF-induced calpain activation (22). PD98059 inhibited EGF-mediated m-calpain binding to less than 45% of the EGF-stimulated level. Similar results were found by semiquantitative imaging and immunoblotting of populations of footprints. This level is below that in unstimulated cells, suggesting that there is some substratum-driven ERK-mediated localization of m-calpain. These qualitative and repeatable changes suggest that activation or postactivation processing may strengthen m-calpain binding to the membrane, although more extensive investigation is needed to fully decipher this observation.

Significant amounts of m-calpain were retained in a punctate pattern in the membrane suggesting lipid rafts, a pattern accentuated upon EGF exposure (Fig. 1). Although it is difficult to determine the direction of cell movement in these unroofed cells, this may implicate m-calpain localization toward the rear as lipid rafts have been shown to polarize to the rear of motile neutrophils (54). In order to identify the membrane components interacting with m-calpain, we treated the membrane footprints with digitonin, which has been shown to remove cholesterol from plasma membrane. We found that the capacity of m-calpain to bind to the plasma membrane footprint was not affected by digitonin extraction (Fig. 2) or methyl-β-cyclodextrin (data not shown), suggesting that m-calpain does not associate with cholesterol on the plasma membrane. Lastly, m-calpain appears not to bind to microtubules or the actin cytoskeleton as disruption of the tubule network with colchicine had no effect on retained m-calpain, and PLC treatment, which reduced m-calpain binding (see below), did not similarly remove footprint-associated actin (Fig. 2D). These findings suggest that m-calpain binds directly to moieties in the membrane.

m-Calpain binds to phosphoinositides. The above data show that m-calpain is complexed to the plasma membrane but do not suggest a particular binding target. We hypothesized a direct interaction between m-calpain and membrane lipids, rather than membrane-associated proteins, on the basis of reports in the literature. First, phospholipids have been shown

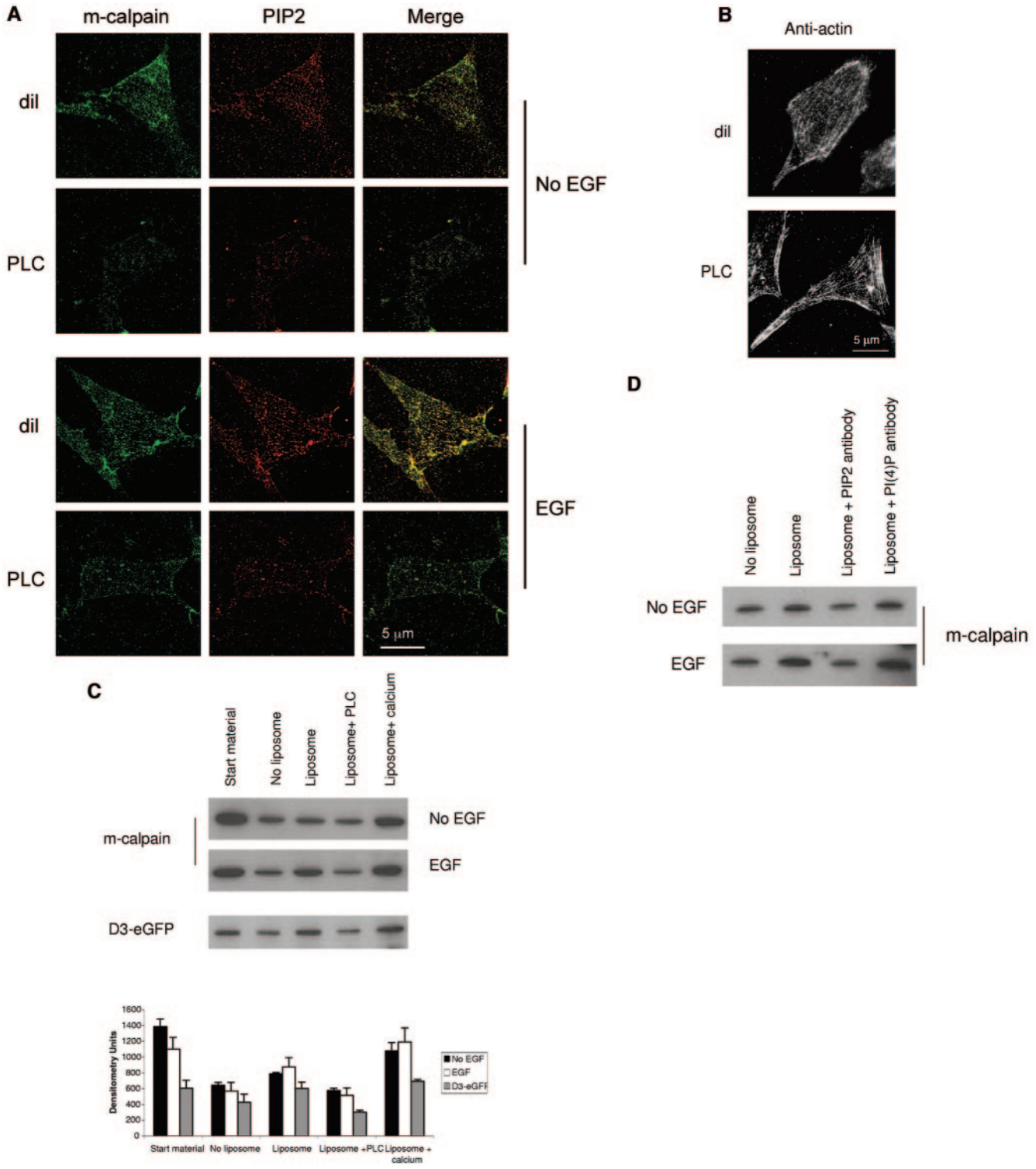

FIG. 3. m-Calpain binding to membranes is eliminated by PLC. (A) Quiesced WT NR6 cells were exposed to 1 nM EGF overnight on coverslips, and substratum-associated membranes were prepared. The membrane footprint further treated with or without (dil for diluent) bacterial PLC (100 μg/ml for 10 min at 37°C) was dually stained by m-calpain (green) and PIP_2 (red) antibodies. (B) The footprints were stained simply for F-actin (by antibody, green) to demonstrate that the PLC treatment did not remove the actin cytoskeleton (see also Fig. 2 for total membrane protein). Shown are representative cells from at least three independent experiments for each treatment. (C and D) The specificity of m-calpain binding to PIP_2 was detected by assay of protein-liposome binding. The upper two rows of panels C and D show the immunoblotting of native m-calpain extracted from WT NR6 cells, without and with treatment with EGF, and the bottom row of panel C represents immunoblotting of the D3-eGFP construct. The graphic representation of densitometry scanning in panel C is the average ± the standard deviation of two experiments. Shown in each immunoblot is one of two similar experiments.

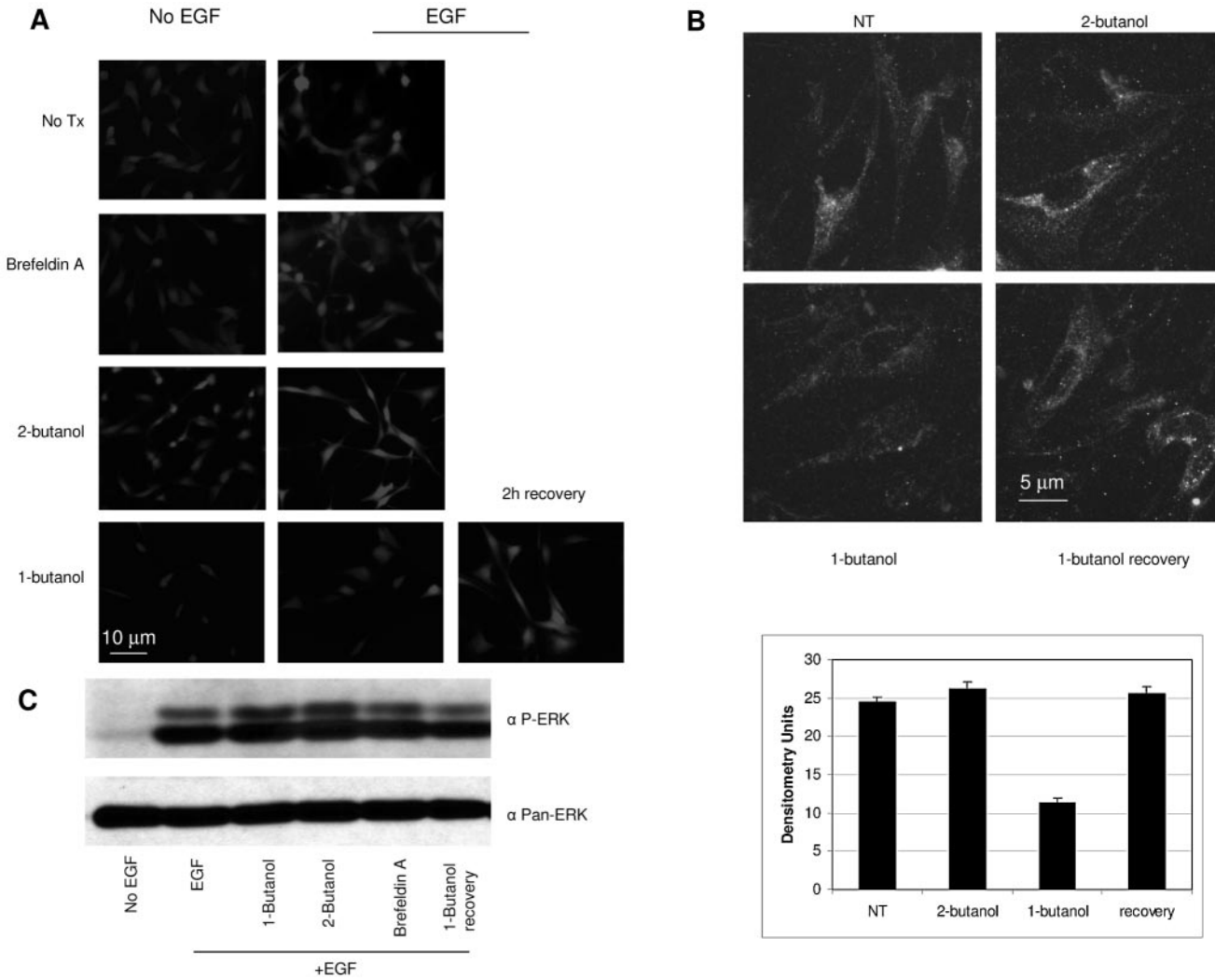

FIG. 4. Inhibiting PIP synthesis eliminates EGF-induced calpain activity. (A) Quiesced WT NR6 cells were exposed to 5 mg/ml BFA or 1.5% 1-butanol or 2-butanol for 20 min and then 50 μM BOC-LM-CMAC for an additional 20 min. They were then treated with 10 nM EGF and observed for fluorescence after 5 min. In addition, WT NR6 cells treated with 1-butanol were placed into 1-butanol-free medium for 2 h, stimulated with 10 nM EGF for 5 min, and then observed for fluorescence. (B) Footprint of cells left unexposed, exposed to 1-butanol or 2-butanol, or exposed to 1-butanol and allowed to recover for 2 h prepared and then stained by PIP$_2$ antibody. The quantification represents the average fluorescence (± the standard deviation) of 30 individual cells chosen randomly. Shown are representative cells from three independent experiments in panels A and B. (C) In parallel, cells left unexposed (No Tx); exposed to 1-butanol, 2-butanol, or BFA; or exposed to 1-butanol and allowed to recover for 2 h were tested for EGF-induced ERK activation by immunoblotting for phospho-ERK. Equal amounts of protein from cell lysates from each treatment were loaded. Shown is one of two identical blots. NT, no treatment.

to reduce the calcium requirement of m-calpain (1, 39, 46), suggesting an interaction. Second, D3 contains C2-like domains that, in other molecules, bind phospholipids (48, 61). Lastly, D3 has been shown to bind to liposomes containing di- and triphosphoinositides (61). To determine, as a simple proof of principle, whether m-calpain protein would interact directly with phospholipids, two approaches were taken. First, bacterially expressed m-calpain was incubated with a nitrocellulose strip spotted with various phosphoinositides and then immunoblotted (data not shown). Under these nonphysiologic conditions, m-calpain bound to phosphoinositides but not other phospholipids. However, we found that m-calpain expressed in cells bound to phosphoinositide-containing liposomes, and this binding was increased after EGF exposure (Fig. 3C). To ascertain if there is a specificity to this interaction, we found that liposome-binding capacity was diminished by monoclonal antibodies to PIP$_2$ but not by monoclonal antibodies to PI(4)P$_1$ (Fig. 3D). These data support the earlier study with liposomes (61) and serve simply as proof of the concept, which needs to be validated within cells, that m-calpain is capable of directly interacting with phosphoinositides in the absence of other molecules.

The postulate that m-calpain binds to phosphoinositides was tested by removing the major cellular phosphoinositide species, PIP$_2$, from the membrane footprints. High levels of exogenously added PLC hydrolyzed the phosphoinositol head groups from the footprint membranes. Staining for PIP$_2$ demonstrated that these procedures removed most of the detect-

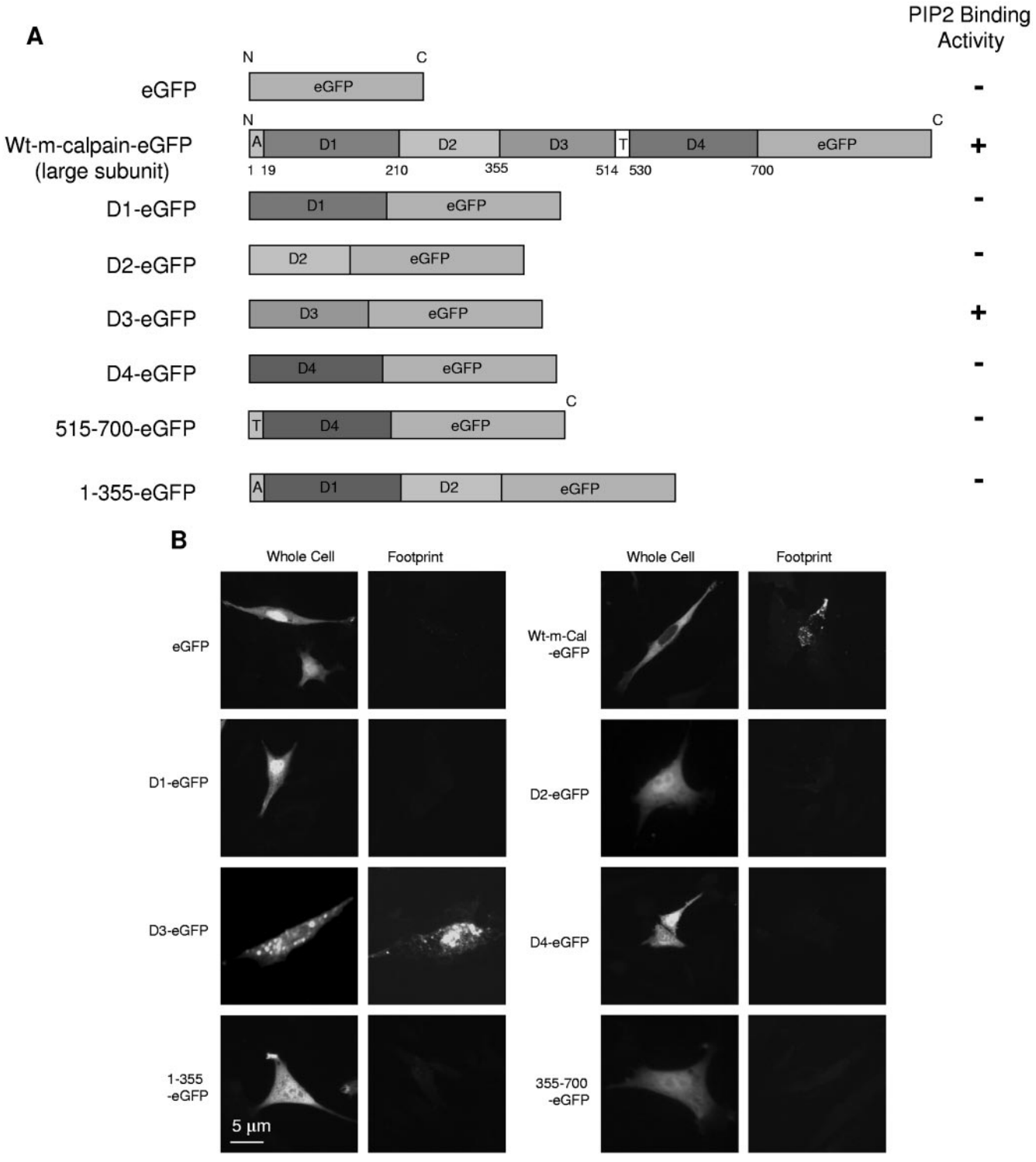

FIG. 5. The membrane-binding domain of m-calpain maps to D3. (A) Schematic representation of full-length human m-calpain and different mutant forms as eGFP-fusion proteins and their phosphoinositide-binding activities. The positions of anchor (A), protease D-I (D1), protease D-II (D2), D3, transducer (T), and D4 are shown. (B) WT NR6 cells were transiently transfected with eGFP, WT m-calpain–eGFP, and m-calpain domains fused with eGFP, respectively. The membrane footprint of transfected cells was prepared, and the fluorescence retained on the footprint was viewed under a fluorescence microscope (see Materials and Methods). (C) Membrane footprints of WT NR6 cells transfected with WT m-calpain–eGFP and D3-eGFP were prepared and then treated with either digitonin (DIG) or bacterial PLC prior to observation under a fluorescence microscope. (D) WT NR6 cells transfected with WT m-calpain–eGFP or D3-eGFP (green) were stained with ER-Tracker red dye and visualized under fluorescence microscopy. (E) Membrane footprint of WT NR6 cells prepared and stained by either F-actin antibody (green) or ER-Tracker red dye (red). The negativity of staining in the right half is because no ER is detectable in the footprint. Shown are representative cells from at least three independent experiments for each treatment. FT, footprint.

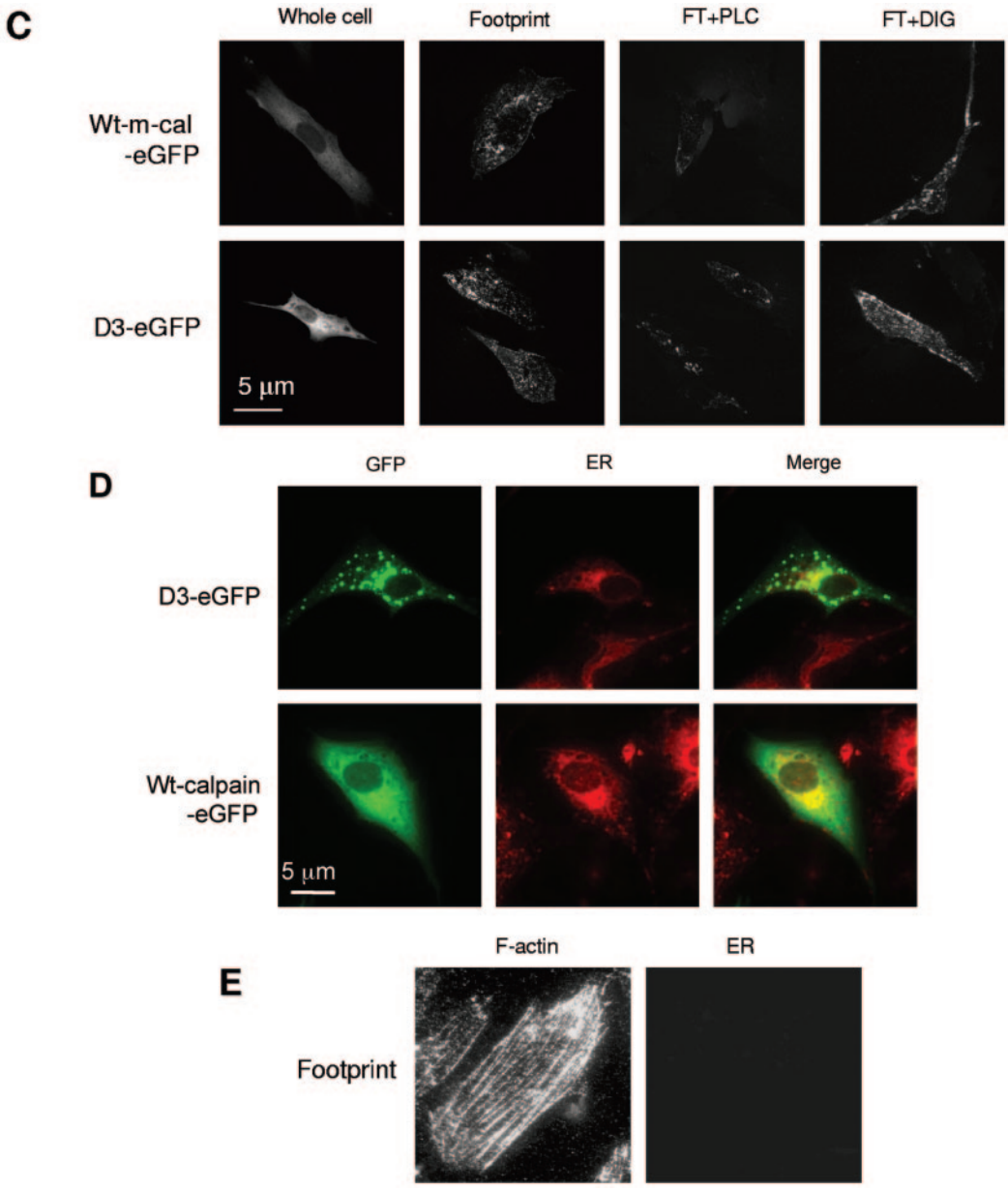

FIG. 5—*Continued.*

able PIP$_2$ (Fig. 3A); this was verified by detection of PIP$_2$ with the PLC-δ pleckstrin homology domain (see Fig. S1 in the supplemental material). Similar treatment did not decrease the levels of PIP$_3$ or PI(4)P$_1$ (see Fig. S2 in the supplemental material). In parallel, antibodies to m-calpain detected similarly decreased amounts of m-calpain protein in the footprint membranes treated with PLC to remove PIP$_2$ (Fig. 3A); not only did exogenous PLC remove m-calpain from the membrane footprint to less than 40% of the EGF-stimulated level, but it also decreased the basal level by more than a third ($P < 0.05$ in both situations) (Fig. 2). This PLC removal of bound m-calpain was noted in the in vitro liposome-binding assay also (Fig. 3C). In order to see if a membrane footprint treated with PLC lost membrane protein, we stained the footprint with F-actin antibody (Fig. 3B) and examined the total membrane-associated proteins (Fig. 2B) or actin (Fig. 2C and 2D) and found that there was no decrease compared to no treatment. Interestingly, m-calpain was colocalized with PIP$_2$ (Fig. 3A), as would be expected if these molecules were binding partners. While it might be expected that m-calpain binding to the inositol head groups would block antibody detection of these species, fixation of the membrane prior to staining may unmask PIP$_2$ or m-calpain interactions with a subpopulation of molecules within a region of high PIP$_2$ content. These data strongly support the contention that m-calpain associates with the membrane through direct or indirect binding to phosphoinositides in general and PIP$_2$ in particular.

PIP$_2$ is required for EGF-induced calpain activity. If m-calpain interaction with phosphoinositides is important for in

vivo function, elimination of these sites should abrogate EGF-induced calpain activation. We downregulated cellular PIP_2 levels in membranes with 1-butanol, which inhibits phosphatidic acid synthesis, required for production of PIP_2, thus preventing replacement of hydrolyzed PIP_2 (58). Since 1-butanol also fragments the Golgi apparatus, BFA, which disrupts the Golgi by a different mechanism of dispersing Golgi enzymes into the ER, was used as a control. Another control was 2-butanol, which does not affect PIP_2 synthesis. The efficacy and relative specificity of lowering membrane PIP_2 levels were validated by staining of membrane footprints (Fig. 4). BOC-LM-CMAC is a substrate for calpain that fluoresces when cleaved, providing an in vivo indicator of calpain activation (22, 49). WT NR6 cells were treated with 5 μg/ml BFA or 1.5% 1-butanol or 2-butanol for 20 min, and then 50 μM BOC-LM-CMAC was added for an additional 20 min. Cells were then treated with 10 nM EGF for 5 min and observed for fluorescence (Fig. 4A). EGF-induced calpain activity was inhibited only in 1-butanol-treated cells; BFA and 2-butanol did not affect calpain activity. To determine whether the downregulation of m-calpain activity in WT NR6 cells treated with 1-butanol correlates with the amount of PIP_2 localizing in the plasma membrane, we prepared the membrane footprint and performed immunostaining with PIP_2 antibody. As shown in Fig. 4B, the amount of PIP_2 retained in the membrane footprint was significantly decreased by 1-butanol, but not 2-butanol, suggesting that 1-butanol blocked the m-calpain activation secondary to the diminution of PIP_2 in membranes. To show that 1-butanol-mediated loss of calpain activity was not due to general detrimental effects on cells, medium containing 1-butanol was replaced with normal growth medium for an additional 2 h and EGF-induced calpain activity returned to normal levels. Additionally, 1-butanol did not disrupt other signaling from EGFR (Fig. 4C); EGF-induced ERK activation, as assessed by phospho-ERK staining, was similar in 1-butanol-treated and recovered cells. Thus, while we cannot eliminate the possibility of a bridging molecule between the phosphoinositides and m-calpain, we have defined the need for cellular phosphoinositides, particularly PIP_2, for normal activation in living cells of m-calpain by EGF.

Identification of m-calpain phosphoinositide-binding domain. Our findings thus far show that m-calpain has intrinsic phosphoinositide-binding activity. In order to determine the phosphoinositide-binding domain of m-calpain, we constructed a series of m-calpain mutant fusions to eGFP for visualization (Fig. 5A). After transfection of these constructs into WT NR6 cells, we prepared plasma membrane footprints. We found that D3-eGFP was retained on the plasma membrane footprint to even a higher degree than full-length m-calpain–eGFP (Fig. 5B). However, the other domain-specific constructs, D1-eGFP, D2-eGFP, D4-eGFP, 515-700–eGFP, 1-355–eGFP, and eGFP itself, were not retained on the footprints. Immunofluorescence demonstrated that all of the mutants were expressed, and immunoblotting showed that the eGFP fusion proteins had the predicted molecular weights (data not shown). That D3 binds via phosphoinositides was expected since m-calpain was removed from membrane footprints by PLC treatment but not by digitonin treatment (Fig. 5C). Together, these findings suggest that D3 is the membrane-associating domain.

The staining of D3-eGFP appeared, if anything, more punctate than that of WT m-calpain–eGFP (Fig. 5C and D). Two possibilities that would score falsely include vesicular sequestration and ER retention. Staining of membrane footprints with lipid-intercalating dyes does not present evidence of vesicles being attached to this membrane (data not shown). The ER was visualized in intact cells with ER-Tracker and shown not to colocalize with m-calpain (Fig. 5D), nor was ER found associated with the deroofed basolateral membranes (Fig. 5E). The nature of the membrane subdomains on which m-calpain appears to cluster awaits further investigation.

Activation of m-calpain enables increased D3 association with the membrane. EGF exposure increases m-calpain association with the membrane. To determine if this occurs via alteration of D3, we treated D3-eGFP-transfected WT NR6 cells with 1 nM EGF overnight and then prepared membrane footprints to evaluate D3 binding. Figure 6A shows that EGF does not increase D3-eGFP binding to membrane. In contrast, the membrane-binding activity of WT m-calpain–eGFP was significantly enhanced by EGF. Interestingly, these data were confirmed by the in vitro liposome-binding assay in that full-length m-calpain required EGF for liposome binding but the D3 fragment bound to liposomes even without prior EGF treatment of the cells (Fig. 3C); increased binding because of calcium exposure demonstrates the functionality of the constructs as previously reported (61). This direct evidence was very consistent with the immunostaining result of native m-calpain (Fig. 1 and 2) and was further confirmed by immunoblotting against the GFP retained on the membrane footprint (Fig. 6B). Greater EGF-independent binding of D3 to membranes suggests that in the holoenzyme, the amino acid residues that interact with the membrane are partially constrained or masked and that the m-calpain phosphorylation by ERK (21) enables these residues to interact more stably with phosphoinositides.

To test this postulate, we evaluated the membrane footprint binding of ERK-independent m-calpain mutant forms S50A and S50E; these either cannot be phosphorylated or mimic EGF-induced ERK phosphorylation, respectively (21). These mutant forms were fused to eGFP. ERK-resistant S50A m-calpain bound to membrane footprints at low levels, and this binding was not increased by EGF (Fig. 6B). On the other hand, the phosphorylation mimic S50E m-calpain bound at increased levels even in the absence of EGF and, as predicted, EGF exposure did not alter this high level of membrane interaction. These decreased or increased binding abilities were not due to the different levels of S50A-eGFP and S50E-eGFP expression (Fig. 6C). These data are consistent with our model of ERK phosphorylation of m-calpain enabling D3 to interact with membrane phosphoinositides either more stably or more avidly.

m-Calpain enriches toward the rear during cell motility. m-Calpain controls de-adhesion of the cell's trailing body and tail during EGF-induced motility (56). Thus, if PIP_2 binding is critical for activation, then m-calpain should be found as membrane associated toward the rear part of migrating cells and relatively lacking in the lamellipodia. We examined the localization of m-calpain in cells exposed to EGF for 18 h to stimulate locomotion (Fig. 7). An in vitro wound-healing scrape assay was used to align the direction of the locomoting cells and footprints as instantaneous vector information is often difficult to obtain during chemokinesis because of the low

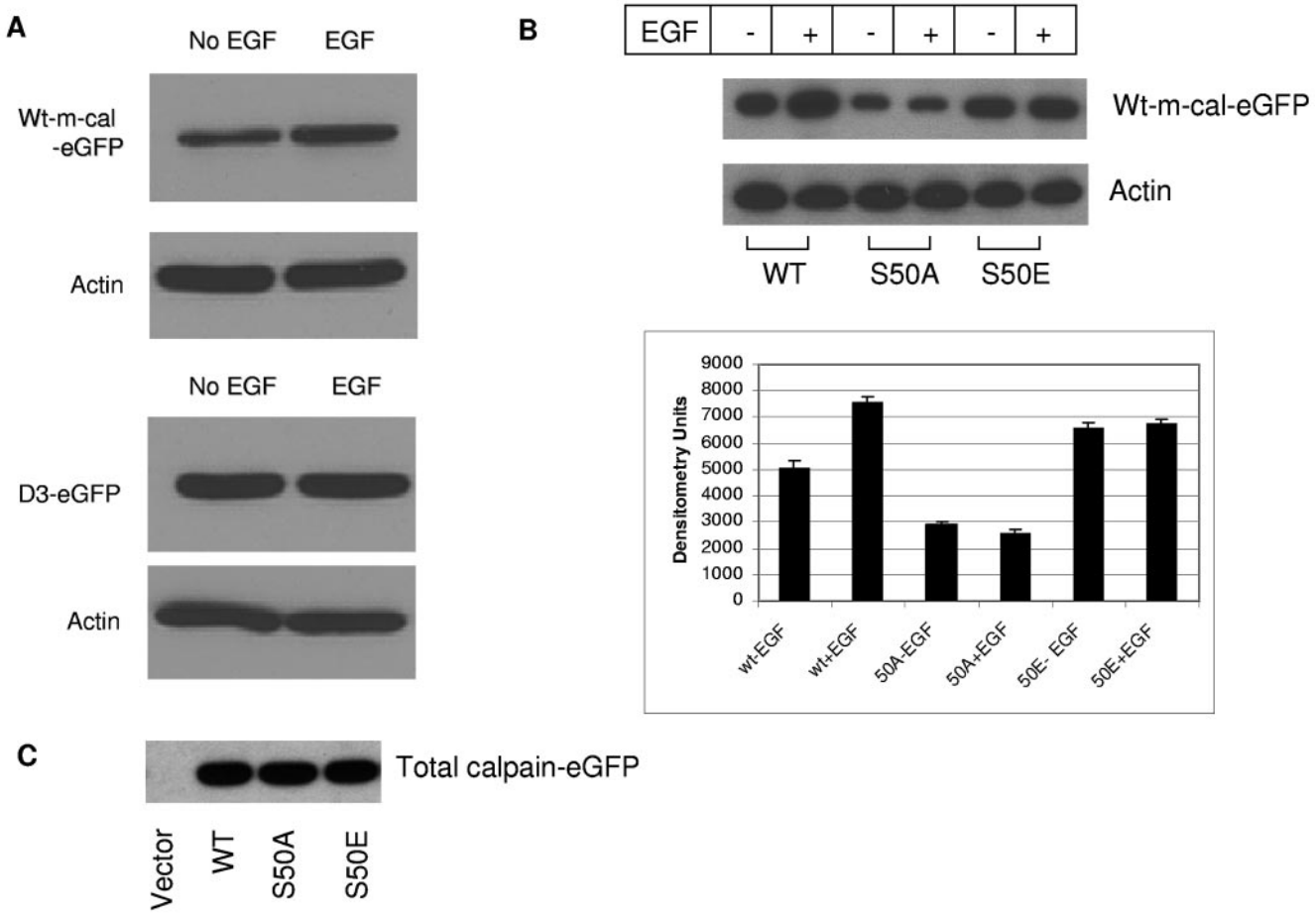

FIG. 6. Activation of m-calpain enables increased D3 association with the membrane. WT NR6 cells transfected separately with WT m-calpain–eGFP, D3-eGFP, S50A-eGFP, or S50E-eGFP were quiesced overnight and then stimulated with 1 nM EGF for 18 h. Cells were coated with silica gel and lysed, and the top membranes were removed. (A and B) The amounts of bound WT m-calpain–eGFP and D3-eGFP (A) or WT m-calpain–eGFP, S50A-eGFP, and S50E-eGFP (B) on the membrane footprint were detected by immunoblotting against GFP. Immunoblotting against F-actin was used as a loading control. The quantification represents the average value (± the standard deviation) of two independent experiments. (C) Total WT m-calpain–eGFP, S50A-eGFP, and S50E-eGFP expressed in WT NR6 cells as detected by immunoblotting against GFP. Shown is one of two identical blots.

persistence of cells under the influence of EGF. m-Calpain accumulates to the body and rear of actively locomoting whole cells (Fig. 7A), e.g., in regions in which adhesions are noted as broken during locomotion. The localization of m-calpain was not simply in the tail, as the entire body and tail must de-adhere to move forward. m-Calpain did partly co-localize with the fraction of membrane-associated phospho-ERK, with pERK also being found in the cell front separated from m-calpain (see Fig. S3 in the supplemental material). This enrichment of m-calpain in the cell and toward the rear was not due to generalized sequestration of adhesion-related molecules in these cell regions as FAK and vinculin were found evenly distributed across the periplasma membrane region regardless of EGF exposure; if anything, FAK was found more toward the front of the locomoting cells (Fig. 7B; see Fig. S4 in the supplemental material). That FAK and m-calpain did not closely colocalize is not unexpected as activated m-calpain drives adhesion complex disassembly, possibly in part through FAK proteolysis (7, 8), with subsequent loss of FAK from that site. This does not argue against FAK acting as a scaffold for m-calpain but rather that, once activated, the adhesion disassembly and m-calpain dissociation may be too labile to be maintained during the generation of the footprints. PLC treatment of the membrane footprints did not remove FAK or vinculin, further suggesting that PIP$_2$ is requisite for membrane translocation, even if proteins such as FAK and pERK are involved in the membrane complex or recruitment.

Interestingly, m-calpain enrichment in the cell body and rear was more pronounced on membrane footprints (Fig. 7C), again colocalizing with PIP$_2$ (Fig. 7D). This distribution of m-calpain was reflected by a similar localization of PIP$_2$ toward the rear of the cell as detected by both antibody to PIP$_2$ (Fig. 7D) and binding of the PLC-δ pleckstrin homology domain (see Fig. S1 in the supplemental material). Detection of m-calpain was much more limited in the lamellipodia of locomoting cells even in the whole-cell mounts. These data are consistent with the exclusion of calpain activity from the nascent ruffles (data not shown), often considered acutely activated transient structures that correspond to the later stable lamellipodia.

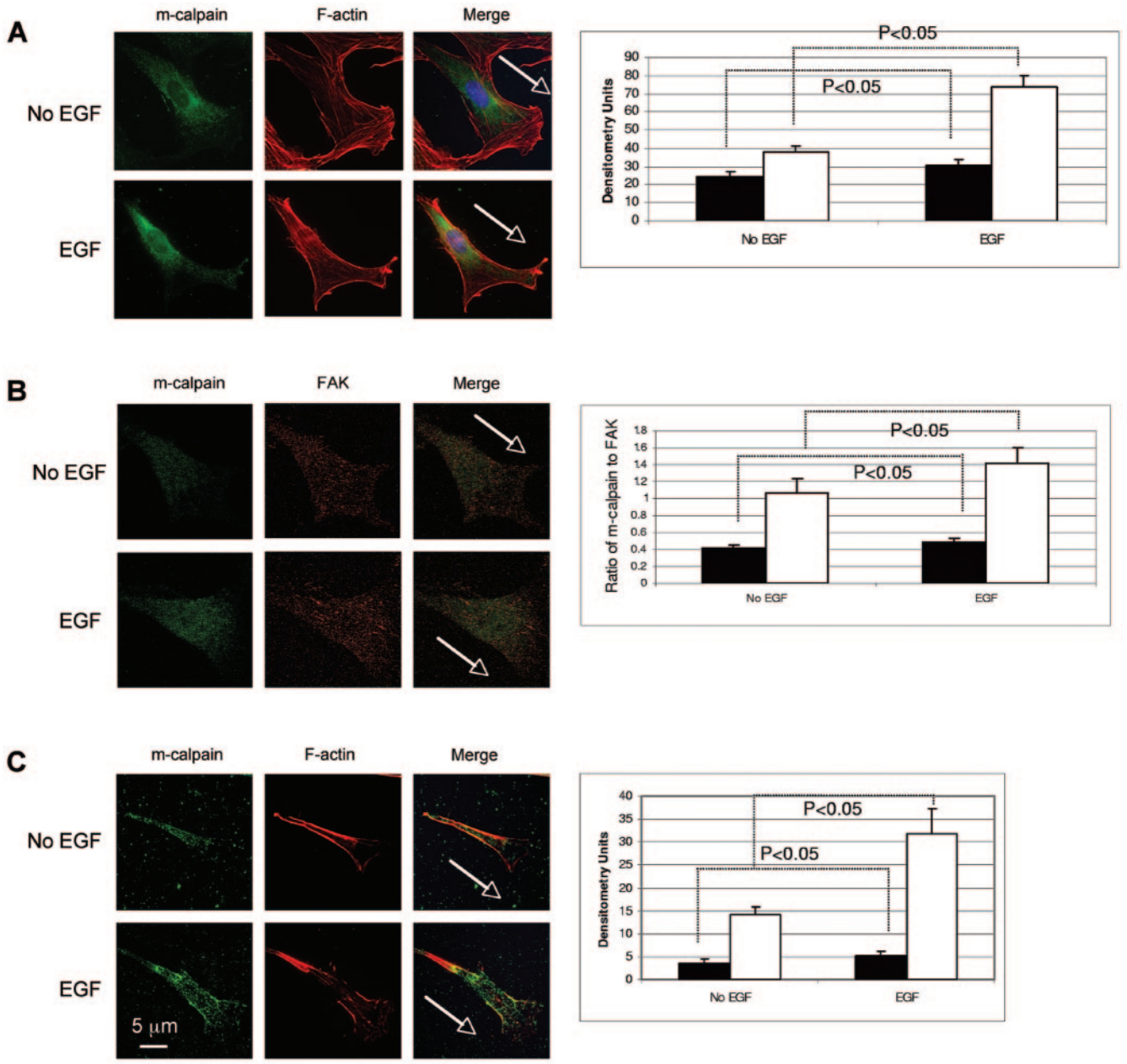

FIG. 7. m-Calpain is enriched toward the rear of locomoting cells. A cell-denuded area was created by physical scraping across a monolayer of quiesced WT NR6 cells; motility was augmented by stimulation with 1 nM EGF for 18 h. (A and B) Whole cells fixed with 2% paraformaldehyde were permeabilized with 0.1% Triton X and stained for m-calpain (green) and F-actin (rhodamine-phalloidin, red; nuclei are DAPI stained, blue) (A) or stained for m-calpain (green) and FAK (red) (B). In panel B, the fluorescence in the bottom membrane was observed with an Olympus Fluoview 500 confocal laser scanning microscope tuned to the substratum-adjacent region. (C and D) Cells were coated with silica gel and lysed, and the top membranes were removed. The remaining basolateral membranes were stained for m-calpain (green) and F-actin (rhodamine-phalloidin, red in panel C) or PIP_2 (red in panel D; nuclei are DAPI stained, but no nuclei are noted in the regions of membrane footprints). The quantification represents the average fluorescence of membrane subdomains for m-calpain (total in front of the nucleus [black bars] versus total behind [open bars]; in panel B, it is the ratio of m-calpain to FAK in the front half versus the rear half of the cell) taken from 30 individual cells chosen randomly. Shown are representative cells from three independent experiments. Arrows denote the direction of cell migration.

DISCUSSION

One of the great conundrums of cell motility is how the intracellular machinery dictates the asymmetry of cellular processes that result in productive movement (23, 35, 43, 62). This is especially vexing for chemokinesis, in which the external signals are not directionally partitioned. Recent advances have shown that calpain-mediated de-adhesion is required for both haptokinesis (27) and chemokinesis (22) by enabling retraction of the rear. During haptokinesis, one can imagine stretch-activated calcium channels (30) opening only near the tail (36)

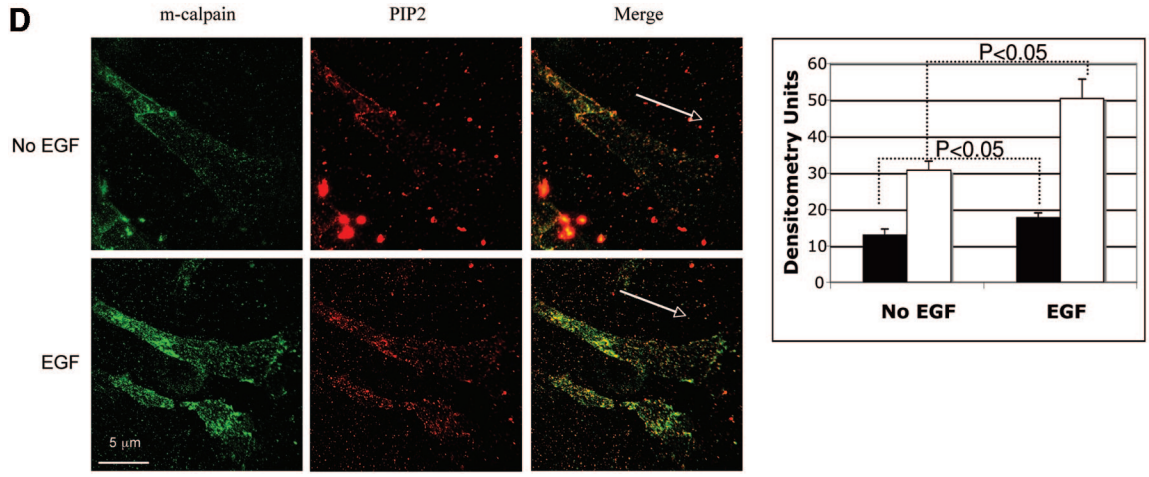

FIG. 7—*Continued.*

and thus activating μ-calpain only in the tail (53). This localization of active calpain would prevent retraction of the newly extended and surface-tethered lamellipodium (23). However, during growth factor-induced motility, ERK is required for activation of m-calpain, which appears incapable of being activated solely by these calcium sparks (53). Herein, we provide a model by which m-calpain-mediated de-adhesion might be preferentially localized during growth factor-mediated motility. Our data suggest that m-calpain localizes to the membranes underlying the cell body and rear. In cells, the production and presence of PIP_2 in the membranes are necessary for EGF-induced m-calpain activity. We can visualize EGF-induced m-calpain activation as occurring initially at or near the membrane in cell regions other than the lamellipodia (data not shown). Lastly, we find that m-calpain is enhanced in the body and toward the rear of locomoting cells, where PIP_2 is located in motile cells (13). While no single experimental intervention demonstrates the specificity of this binding, the totality of the various investigations points to PIP_2 being required for m-calpain membrane association in response to EGF. These data support our model of differential subcellular activation of rate-limiting processes being critical in cell motility.

Our findings herein provide avenues as to how and where m-calpain is activated in cells. Calpains are a family of (in vitro) Ca^{2+}-dependent cysteine proteases, with the two ubiquitous forms being the highly homologous m-calpain and μ-calpain proteins. These are named according to the levels of Ca^{2+} needed for activation in vitro. Near-millimolar levels (200 to 1,000 μM) of Ca^{2+} are required to activate m-calpain, while near-micromolar levels (i.e., high hundreds nanomolar) are needed for μ-calpain activation (17, 65); the latter might be reachable in calcium sparks and puffs (4), whereas the former is not attainable in viable cells. In keratinocytes, peptide factor-induced calcium fluxes can activate μ-calpain but not m-calpain to accomplish cell de-adhesion and motility (53). μ-Calpain appears critical in integrin- and chemokine-mediated cell motility and adhesion (3, 27, 53), while m-calpain is the isoform required for growth factor-mediated cell motility (22, 53), although recent evidence suggests that haptokinetic signaling may also activate m-calpain (19). In vitro, calcium activates calpains by shifting the molecule to close the misalignment of the active cleft (41). How m-calpain is activated in cells is uncertain, but a number of cofactors have been proposed to lower the levels of calcium to ambient cellular levels. Some of these cofactors for activation include phospholipids (61) and direct phosphorylation (21). Ca^{2+} and phosphoinositides mutually promote each other's binding to D3 (61). However, in all of these studies, the extent of reduction of the calcium requirement was only about half—still well above physiological levels. One might argue that final adjudication of these mechanisms requires direct mutagenesis of D3, and this is a goal of numerous groups. However, this is premature at present for a number of reasons. As D3 has been implicated in multiple roles (binding calcium [61], interacting with phosphoinositides [61], and regulating D1 and D2 juxtapositioning [56]), such manipulations are technically challenging, especially as the model posits that all of these roles are required for activation in cells. Demonstrating that enzymatic inactivation is not nonspecific will be a tour de force. Secondly, as D1 and D2 can form an active, calcium-dependent calpain fragment in vitro (41), the interpretation of D3 deletions would be highly confounded by overexpression artifacts. Thus, the progression to this worthy goal of site-directed mutagenesis studies must await the time when structural data are in hand that pinpoint phospholipid interaction sites on D3.

We provide data that strongly suggest that at least part of the functional mechanism of EGF-induced m-calpain phosphorylation is to position m-calpain at the membrane. Replacement of serine 50 with alanine decreased, but did not eliminate, the membrane-binding ability of m-calpain, while replacement of the this amino acid with a phosphorylation mimic glutamic acid residue leads to increased and EGF-independent binding to the membrane. We cannot tell whether ERK phosphorylation increases translocation to the membrane or stabilizes the interaction when at the membrane, although we favor the latter as earlier studies showed that only membrane-associated ERK activated m-calpain, suggesting that phosphorylation occurs in the perimembrane region (24). Still, in either case, ERK phosphorylation of S50 allows D3 to attain a more favorable membrane-interacting conformation or exposure. D3 alone binds to

the membrane footprint in an EGF-independent manner, suggesting that the phosphorylation status at S50 functions through indirect mechanisms to increase the level of PIP_2 binding. It is interesting that blocking ERK signaling reduces the level of membrane-bound m-calpain below that noted in cells not exposed to EGF, down to the same level as removal of PIP_2 (Fig. 2); this EGF-independent binding might represent low-level activation of ERK and m-calpain by engaged integrins (19) or the background of nonspecific binding. However, m-calpain interaction with the membrane does not in itself lead to m-calpain activation since there is basal membrane interaction in the absence of calpain activity, and phosphorylated m-calpain is active in vitro in the absence of phosphoinositides or membranes (21).

Our data suggest that cytosolic and membrane localization of m-calpain is dynamic. There is precedent for intracellular transport of calpains upon activation. Calpains are predominantly found in the cytosol, presumably in an inactive state (16). When μ-calpain is exposed to Ca^{2+}, it translocates to membranes (42, 52), where it then undergoes autolysis (40). The 80-kDa subunit of μ-calpain has been shown to associate with membranes in the presence of Ca^{2+}, followed by autolysis to an intermediate, active 78-kDa form found at the membrane, which then autolyzes to the 75-kDa form found only in the cytosol with subsequent proteolytic degradation (40). This mechanism of translocation is unlikely to be fully mimicked by m-calpain as we find this isoform associated with the membrane even in the absence of EGF exposure or in the presence of MEK inhibitors, with membrane interaction dependent on the presence of phosphoinositides. Furthermore, such phospholipids have been shown to retard autolysis of the 78-kDa species, suggesting a role for the membrane in the stabilization of this alternative, active form of μ-calpain (39). Calpain also has been shown in vitro to bind to several different phospholipids (1, 9, 46). In one study, phosphatidylinositol (PI) and dioleoylglycerol, but not phosphatidylserine (PS), phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid (PA), PI(4)P, or PIP_2, lowered by half the Ca^{2+} requirement for m-calpain autolysis. PI, PS, and dioleoylglycerol stimulated m-calpain activity, and PI also stimulated μ-calpain in another study (9). Other data added PA, phosphatidylglycerol, and sphingomyelin as potential activators of m-calpain (1). In another study, PI, phosphatidylcholine, phosphatidylethanolamine, PS, and PA all reduced the Ca^{2+} requirement for μ-calpain autolysis (46). μ-Calpain autolysis (50) and activity (51) were stimulated by, in order of increasing activating strength, PI, PI(4)P, and PIP_2. However, all of these the studies examined calpains in vitro and did not account for their subcellular dynamics in living cells.

Despite the fact that phospholipids have been implicated in the regulation of both ubiquitous calpain isoforms, we focused on m-calpain as this is the isoform activated by EGF and other growth factors (22, 53, 57). We have identified m-calpain binding to phosphoinositides in vitro. Because of its prevalence in cells in relation to the other phosphoinositides, PIP_2 is mostly likely the species that is mediating m-calpain localization toward the rear. We find that removal of PIP_2 from liposomes and membranes by PLC results in the loss of most of the calpain staining (and the PIP_2 but not the PIP_3 staining), further supporting the key role of this particular phosphoinositide. That we have not eliminated the possibility that other molecules serve to bridge m-calpain to phospholipids at the membrane is an evident caveat. It is likely that such third-party interactions play some role in this localization and need to be identified in studies that lie beyond the scope of the present report. Still, even if this were the case, the operative conclusion is that membrane phosphoinositides are essential for EGF-induced functioning of active m-calpain.

Since Ca^{2+} never reaches levels adequate to activate m-calpain in cells, even in the presence of these phospholipid coactivators, m-calpain needs to be activated through other means such as phosphorylation by ERK/mitogen-activated protein kinase (21, 22). In our system of EGF-induced m-calpain activation, it is plasma membrane-associated EGFR and ERK that trigger calpain (24). We have attempted unsuccessfully to analyze the activation status of membrane-associated m-calpain directly; this failure is likely due to rapid autoproteolysis upon EGF activation (24). Still, in preliminary experiments with a calpain-sensitive fluorescence resonance energy transfer reporter, we note that acutely upon EGF addition, calpain is activated first at the membrane in an ERK- and calpain-dependent fashion. We note m-calpain activation in the absence of increased calcium fluxes (21, 53) and also in calcium-free medium with Ca^{2+} chelators (22), suggesting a separate mechanism that lowers calpain's calcium requirement to or below ambient levels. Perhaps in ERK-mediated m-calpain activation, PIP_2 acts in the same way as with Ca^{2+}-activated calpain and synergistically promotes m-calpain activation. This would explain the absence of calpain in ruffles and lamellipodia, where there is a deficiency of steady-state levels of PIP_2 because of rapid PLC-γ hydrolysis, and more activity in the cell body, where there is more PIP_2 (13). Therefore, we propose a model of m-calpain membrane association with plasma membrane phosphoinositides only in nonlamellipodial regions which results in a polarized pattern of focal contact disassembly in the body and toward the rear of the cell. This would present the cells with enhanced attachment directed toward the leading lamellipodium with the result that transcellular contractility would lead to tail detachment and retraction.

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