Epidermal Growth Factor Receptor Activation of Calpain Is Required for Fibroblast Motility and Occurs via an ERK/MAP Kinase Signaling Pathway*

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Cell motility is a central process involved in many physiological events including tumor invasion, embryonic development, and wound healing. Cell motility has been found to be a very complex biophysical process involving multiple factors working together to accomplish concerted movement. To begin to understand the molecular bases of motility, fibroblast cell motility has been deconstructed into four separate events: extension of the lamellipod, formation of new focal adhesions at the leading edge, breaking of adhesions at the trailing edge, and translocation of the cell mass (1). Failure of any one of these steps is sufficient to prevent cell motility (2, 3). These motility events are regulated by both cell intrinsic and extrinsic properties (4, 5). Among the latter are regulatory inputs from growth factor receptors and the strength and nature of cell-substratum interactions. Previously, it had been demonstrated that motility of adherent cells occurs in a biphasic relationship to adhesiveness of the surface (6, 7). Recently, we have shown that this also holds for growth factor-induced motility, in that epidermal growth factor (EGF)* receptor-mediated fibroblast motility requires activation of M-calpain downstream of ERK/MAP kinase signaling. NR6 fibroblasts expressing full-length wild type epidermal growth factor receptor required both calpain and ERK activation, as demonstrated by pharmacological inhibitors (calpeptin and calpain inhibitor I and PD98059, respectively) for EGF-induced deadhesion and motility. EGF induced rapid activation of calpain that was prevented by molecular inhibition of the Ras-Raf-MEK but not phospholipase Cγ signaling pathway, and calpain was stimulated by transfection of constitutively active MEK. Enhanced calpain activity was not mirrored by increased calpain protein levels or decreased levels of its endogenous inhibitor calpastatin. The link between ERK/MAP kinase signaling and cell motility required the M-isoform of calpain (calpain II), as determined by specific antisense-mediated down-regulation. These data promote a previously undescribed signaling pathway of ERK/MAP kinases activating calpain to destabilize cell-substratum adhesions in response to EGF stimulation.

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2390
Calpain inhibition blocks EGF-induced motility. Effects of the pharmacological agents calpeptin (A) and calpain inhibitor-1 (CI-1) (B) on cell movement in the NR6 WT cell line are shown. Cells were treated with 1 nM EGF in the presence or absence of calpeptin (0.3, 1, 10, and 25 μM) or CI-1 (1, 3, and 5 μg/ml) for 24 h. The effects of calpeptin or CI-1 were calculated as a percentage of EGF-induced responses observed in the absence of calpeptin or CI-1. Values are mean ± S.E. (n = 4–5), each experiment performed in triplicate. *, p < 0.05; **, p < 0.01, as compared with EGF treatment alone. EGF-induced mitogenesis was inhibited only at higher concentrations of these inhibitors (data not shown).

Calpain inhibition blocks EGF-induced deadhesion. Effects of the pharmacological agents calpeptin (A) and calpain inhibitor-1 (B) on cell attachment of the NR6 WT cell line are shown. Cells were treated with or without EGF (10 nM) in the presence or absence of calpeptin (10 μM) or CI-1 (5 μg/ml) for 30 min. Cells were then subjected to centrifugation at 1643 × g. Cells were counted before and after centrifugation, and the percentage of cells remaining adherent was expressed as a percentage of adherent cells in the nontreated condition. Values are mean ± S.E. (n = 3 and n = 2, respectively), each experiment performed in triplicate or more. *, p < 0.05; **, p < 0.01, as compared with no treatment.
EGFR Activates Calpain via ERK/MAP Kinase

Fig. 3. MEK-dependent but not PLC-dependent signaling is required for EGF-induced deadhesion. Shown are the effect of the pharmacological agent PD98059 (A), which inhibits MEK1 activation of ERK/MAP kinase, and the NR6 cell line c’973 (B), which does not exhibit PLCγ activation, on cell attachment. Cells were treated with or without EGF (10 nM) in the presence or absence of PD98059 (2 μM) for 30 min. Cells were then subjected to an inverted centrifugal force of 1643 × g. Cells were counted before and after centrifugation, and the percentage of cells remaining adherent was calculated as a percentage of adherent cells in the non-treated condition. NR6 WT and c’973 cells were treated in the presence and absence of EGF (10 nM) for 30 min. Cells were then treated as above. Values are mean ± S.E. (n = 3 and n = 3, respectively). *, p < 0.05; **, p < 0.01, as compared with no treatment.

finding that calpain is downstream of ERK and that activation of calpain is necessary for EGFR-mediated cell deadhesion from substratum, we also could ascertain that this deadhesion was critical for EGFR-mediated cell motility. Last, we demonstrate that the calpain isomorph required for EGFR-mediated motility is the M-isoform (22, 23).

EXPERIMENTAL PROCEDURES

Materials—Calpeptin, calpain inhibitor-1, E-64-d, all calpain (M-clone 107–82, Mu-clone 9A4H83d) and calpastatin (clone 1F7E3D10) antibodies, U73122, and ERK antisense and control oligonucleotides (Oligos International, Santa Barbara, CA) were obtained from Stratagene (La Jolla, CA). Human recombinant EGF was obtained from Collaborative Biomedical Products, Bedford, MA. All other buffer reagents, ionophores, and bovine serum albumin were obtained from Sigma.

Cell Culture—NR6 mouse fibroblasts transfected with either wild-type human EGFR (WT NR6) or a signaling-restricted construct lacking all autophosphorylation motifs c’973 (c’973 EGFβ) (24, 25) were cultured using minimum essential medium (MEM)-α plus 26 mM sodium bicarbonate with 7.5% fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM, nonessential amino acids, and the antibiotics penicillin, streptomycin, and G418 (350 μg/ml) as the growth medium. Cells were quiesced at subconfluence using restricted serum conditions without G418 (MEM-α plus 26 mM sodium bicarbonate with 1% dialyzed fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, and the antibiotics penicillin/streptomycin) for 18–24 h prior to experiments.

Motility Assay—EGF-induced migration was assessed by the ability of the cells to move into an acellular area (24). WT NR6 cells were plated on a six-well plastic dish and grown to confluence in MEM-α with 7.5% fetal bovine serum. After 48 h quiescent in the media with 1% dialyzed fetal bovine serum, an area was denuded by a rubber policeman at the center of the plate. The cells were then treated with or without EGF (1 nM) and incubated at 37 °C; inhibitors or diluent alone were added at the same time as EGF. Photographs were taken at 0 and 24 h, and the relative distance traveled by the cells at the acellular front was determined.

Adhesion Assay—Cell-substratum adhesiveness was quantitated using an inverted centrifugation detachment assay. 24-well plates were coated with the human extracellular matrix Amgel (0.5 mg/ml) (26) for 1 h at room temperature, after which they were blocked with 1% bovine serum albumin for 60 min at room temperature. The plates were washed twice with phosphate-buffered saline and utilized for the following experiment. Cells were plated at a concentration of 106 cells/ml with quiescent medium into Amgel coated plates and incubated for 12 h at 37 °C. Plates were filled with MEM-α with 1% bovine serum albumin and 25 mM HEPES. Then plates were sealed with enzyme-linked immunosorbent assay sealing tape (Corning, Cambridge, MA) and centrifuged inverted for 5 min at 3000 rpm at 37 °C using a Beckman CS6R plate centrifuge; 3000 rpm (1643 × g) was chosen empirically as the force required to detach approximately half of the EGF-treated cells. Before and after the centrifugation, the number of cells on the plates was counted by phase-contrast microscopy.

Calpain Activity Assays—Three different assays were used to assess calpain activity in stimulated cells. The first assay, referred to in this paper as microtubule-associated protein 2 (MAP2)-DTAF, measures calpain activity in cell lysates; the second, succinyl-Leu-Leu-Val-Tyr-aminoethylcoumarin (AMC), determines calpain activity in whole cells after treatment; the last assay, Boc, detects calpain activity in individual living cells. Thus, these three assays measure calpain activity in distinct but complementary manners, using three different calpain-preferential substrates.

The first assay was adapted from literature reports (27). Briefly, microtubule-associated protein 2 (MAP2) (Cytoskeleton, Denver, CO) was labeled with DTAF by incubation of MAP2 and DTAF in pH 8.5 PIPES buffer for 30 min at 4 °C. Labeled MAP2 was then isolated by size exclusion column chromatography and dialyzed against pH 7.5 HEPES buffer overnight. Cells were grown to confluence in 10-cm tissue culture plates and quiesced for 24 h. After a 1-min treatment with EGF (10 nM), cells were washed twice with ice-cold phosphate-buffered saline and lysed with cell lysis buffer (20 nM HEPES (pH 7.4), 10% glycerol, 0.1% Triton X-100, 500 mM sodium chloride, 1 mM sodium vanadate). After removing the cell debris by centrifugation, 0.9 μg of DTAF-labeled MAP2 was added to the samples with either 0 or 0.1 mM free Ca2+ concentration. Fluorescence was immediately measured by an Amino-Bowman Series II spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY) at excitation and emission wavelength of 490 and 520 nm, respectively, for 3 min at room temperature.

The AMC assay was modified from literature reports (18). In short, cells were grown to confluence in 10-cm tissue culture plates and quiesced overnight. EGF (10 nM) was then added for 5 min at room temperature. The cells were washed with cold phosphate-buffered saline and then treated with 3 mL of 0.05% trypsin with 0.53 mM EDTA for 3 min. Three mL of complete medium was added to neutralize the trypsin, and the cells were collected. Cells were pelleted in microcentrifuge tubes (7.5 × 107 cells/tube) and washed with Hanks’ buffered salt solution twice. Cells were resuspended in Hanks’ buffered salt solution at 2.5 × 106 cells/mL. Cells were then held on ice for up to 1 h. The Amino-Bowman spectrofluorimeter was warmed to 37 °C using an attached water bath. Of each sample, 96 μl was added to a 0.2-mL quartz cuvette and allowed to warm in the fluorometer to 37 °C. At time −1 min, ionomycin (2.5 μM) in ME6 SO was added to the cell suspension (ME6 SO alone was the control). At time 0, 50 μM AMC (Enzyme Systems Products) was added to bring the total volume to 100 μL, and fluores-
cience was measured immediately at excitation of 360 nm and emission of 460 nm for 3 min.

To determine calpain activity in single cells, enabling transient transfections of dominant-negative and constitutively active constructs, as well as antisense oligonucleotides, we utilized the Boc assay (28). Briefly, cells were plated at 50% confluence on glass coverslips. The cells were then either treated with drug inhibitors and incubated at 37 °C for 30 min to 1 h or transfected using the manufacturer’s protocol for Lipofectin (Life Technologies) or Fugene (Amersham Pharmacia Biotech) lipid transfection reagents and incubated for 18–24 h. The cells (drug-treated or transfected) were then incubated for 20 min in the presence of 50 μM t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (Boc-LM-CMAC) (Molecular Probes) and treated with 10 nM EGF for 10 min; control cells were not treated with EGF. Boc-LM-CMAC is retained within the cells by conjugating with intracellular thiol groups. Cleavage of the substrate results in retention of the chloromethylaminocoumarin portion of the molecule in the cell and results in increased fluorescence. The coverslips were then wet mounted on glass slides and observed for chloromethylaminocoumarin fluorescence using an Olympus fluorescence microscope (model BX40) with an Olympus M-NUA filter. Representative images of each slide were captured using a SPOT CCD camera. The image exposure settings were identical within each experiment (i.e. for treatment with and without EGF) but did vary slightly between experiments; thus, one can directly compare fluorescence intensity within an experiment but not between experiments. Transfected cells were identified by observing expression of green fluorescent protein, which was co-transfected with the plasmid or oligonucleotide of interest (Olympus M-NIBA filter).

Levels of calpain I, calpain II, and calpastatin were assessed by immunoblotting with antibodies (Biomol) at specific times after EGF (10 nM) exposure but prevent de novo calpain synthesis. Subsequently, calpain activation by EGF was assessed after a brief (10-min) pulse with EGF. Isoform-specific calpain degradation was assessed by immunoblotting with antibodies (Biomol) at specific times after EGF (10 nM) exposure in the presence of puromycin (40 μM).

RESULTS
Calpain Is Required for EGF-mediated Cell Motility—A central postulate of our model of EGF-induced motility is that calpain activity is required for fibroblast morphology changes and locomotion. Therefore, we first determined whether inhibition of calpain would affect that motility. Using an in vitro wound healing assay, the calpain inhibitor, calpeptin, limited EGF-induced cell migration in a dose-dependent manner (Fig. 1). Since calpeptin can also block activation of Rho-kinase (30),...
EGF Activates Calpain via ERK/MAP Kinase

(a) A second inhibitor was also utilized, calpain inhibitor I. This agent also prevented EGF-induced cell migration (Fig. 1). Inhibition of calpain limited EGF-induced mitogenesis only at higher concentrations of agents (data not shown). These data show that calpain activity is needed for EGFR-mediated motility but do not yet define active involvement in EGFR signaling.

Calpain Is Needed for EGF-induced Deadhesion—The next question was which biophysical event was dependent on calpain activity. Our model, based on the reports of calpain in other systems including fibroblasts and cancer cells (27), implicates calpain during deadhesion (18). This method allows the measurement of calpain to occur under physiological conditions, since it identifies cells in which calpain is activated but does not allow easy quantification (28).

By all three methods we can detect a rapid increase in calpain activity within 1–10 min after exposure to EGF (10 nM) in WT NR6 fibroblasts (Fig. 4). The increase in activity was 2.5-fold over basal activity as shown in the MAP2 and AMC assays. This increase in fluorescence was blocked by preincubation with the calpain inhibitors calpeptin (10 μM) and the less specific thiol protease inhibitor E-64-d (75 μM) (Fig. 4). This activation of calpain was at the level of activity, since we did not detect an increase in calpain levels or a decrease in calpastatin levels, which was not unexpected given the acute time course of EGF exposure during which increased activity was observed (data not shown). This demonstrates that EGF can induce calpain activity in fibroblasts, and calpain may be an important downstream effector of EGF-mediated cell motility.

EGF-induced Calpain Activity Lies Downstream of ERK Activation—We have previously described two pathways from the EGFR that were required for motility, one involving ERK/MAP kinase pathway (13) and the other utilizing PLCγ leading to cytoskeletal reorganization (24, 29). We first investigated whether calpain was downstream of PLCγ activation, since calpain is calcium-dependent, and PLCγ cleavage ofPIP2 leads to stimulation by inositol 1,4,5-trisphosphate of calcium release. Somewhat surprisingly, PLCγ activation was not required for EGF-induced calpain activity (Fig. 5). This was...
demonstrated by three approaches. First, calpain activity was measured in c’973 NR6 cells, which are signaling-restricted and do not activate PLCγ (24, 32); EGF exposure led to robust calpain activity in these cells. Inhibition of PLCγ in WT NR6 cells by transfection of a dominant negative PLCγ, PLCz, also had no effect on EGF-induced calpain activity, as shown by the Boc-LM-CMAC assay. Last, PLCγ signaling was inhibited by U73122, which specifically inhibits PLCγ (33), with no effect on EGF-induced calpain activity (data not shown).

ERK/MAP kinase signaling was then investigated as the link between EGFR and calpain activation (Fig. 6). Disruption of the Ras-ERK signaling pathway by the MEK inhibitor PD98059 prevented calpain activation in response to EGF in all three assays. Using the Boc-LM-CMAC assay, we found the converse, that transfection of a constitutively active MEK was able to activate calpain in the absence of EGF (Fig. 6), and this activity was no longer inhibitable by PD98059 (data not shown). Transfection of ERK antisense oligonucleotides also blocked EGF-induced calpain activity to basal levels, whereas the control scrambled oligonucleotide had no effect. Taken together, these data demonstrate that the ERK/MAP kinase pathway links EGFR signaling to calpain activation.

M-calpain Is Required for EGF-induced Calpain Activity and Motility—One last point in deciphering the signaling pathway is whether both ubiquitous isoforms of calpain are required for EGFR-mediated motility (24, 29). Using isoform-specific antisense oligonucleotides, we were able to down-regulate M-calpain independently of Mu-calpain; this molecular approach is
necessary, since the calpain inhibitors work on both isoforms. Antisense oligonucleotides to M-calpain, but not Mu-calpain, limited EGFR-mediated motility similarly to the calpain inhibitors (Fig. 7A); Mu-calpain-directed antisense oligonucleotides had a slight effect on basal, haptokinetic motility, as one might predict from published reports (10). Again, M- but not Mu-specific oligonucleotides blocked EGF-induced calpain activity as determined by the Boc-LM-CMAC assay (Fig. 7B). These...
data, for the first time to our knowledge, demonstrate a qualitative separation of, rather than solely a quantitative difference in effect on, biological functions for the different isoforms of calpain.

One might be surprised that antisense oligonucleotides prevented EGFR-mediated motility, since the half-life for calpain is reported to be greater than 5 days (34). To ascertain levels of calpain and calpain activity during the period of sustained motility (greater than 4–6 h post-EGF (9)), we first treated cells in the presence of antisense oligonucleotides for 6 h with EGF. EGF, but not antisense oligonucleotide, was then withdrawn overnight for the cells to reequilibrate before challenging them acutely with EGF. Immunoblotting for the M isoform verified that the calpain protein level was reduced in the M- but not Mu-calpain antisense-treated cells (Fig. 7C). We reconciled this finding to data previously obtained, showing no change in the protein level of either calpain isoform after 6 h of EGF exposure, by postulating that EGFR signaling causes a turnover in calpain due to autoproteolysis, which, matched by de novo synthesis, is thus seen as no net change in calpain level (data not shown). That EGF could stimulate the degradation of calpain is supported by previous reports that calpain is degraded shortly after activation (35). To determine that EGF does cause increased calpain turnover, cells were treated with EGF in the presence of the protein synthesis inhibitor puromycin (40 μM). Immunoblotting for M and Mu-calpain levels demonstrated that EGF leads to decreased M but not Mu-calpain (Fig. 7D). This further supports the preferential involvement of the M-isoform in EGFR-mediated, fibroblast motility-related calpain activity.

**DISCUSSION**

Our studies demonstrate that EGFR signaling actively induces M-calpain activity downstream of ERK/MAP kinase and that this activation is required for both EGFR-mediated fibroblast deadhesion and motility. Thus, we have described a physiological role for calpain in modulating motility and adhesion in intact cells. There have been few previous reports on the physiological activation and role of calpain in response to extracellular signaling factors. The most relevant is an ancillary study of ours (31), in which we report that calpain is the point of integration of promotility signals from EGFR and counterregulatory signals from the CXCR3 receptor for ELR-CXC chemokines (IP-10, PF4, and MIG). Earlier investigations (36–38), using neuronal and glial cell types, demonstrated long-term (1–3 days) down-regulation of calpain activity, potentially due to modulation of calpain and calpastatin levels by neuronal growth factor and fibroblast growth factor. These earlier reports did not determine the mechanism of modulation of calpain and whether it was direct or indirect and are difficult to extrapolate from one cell type and extended time course to another. Therefore this is the first time, to our knowledge, that an intracellular kinase cascade was shown to be important to the regulation of calpain, that calpain was shown to have a distinct role in downstream signaling from a growth factor receptor, and that calpain was described in a role mediating chemokinetic motility.

It was surprising that calpain was activated secondary to the ERK/MAP kinase pathway and not the PLCγ signaling pathway, since the latter pathway leading to calcium mobilization from intracellular stores, since calpain has been reported to require high levels of calcium for activation (22, 39, 40). The two isoforms of calpain, Mu and M, differ most notably by their requirement for calcium, with the M isoform needing nearly millimolar concentrations of calcium. A conundrum arises, since such high levels of calcium are not noted in cells, or they exist only transiently in very localized areas. Our finding leaves open the question of the presence of increased intracellular calcium, for there is no apparent source of increased calcium levels in our experimental system. Further investigation will be required to determine whether calcium fluxes are required in this system and, if so, where these fluxes originate.

Kinase activity has not been fully investigated as a means of regulation of calpain. Calpain as purified from a number of sources is phosphorylated, but the amount and source of phosphorylation has not been reported to affect the activity of the purified calpain. Therefore, it has previously been thought unlikely that calpain is directly regulated by intracellular kinases (41, 42). However, we have found that not only does EGFR signaling lead to calpain activation, but it does so via the ERK/MAP kinase cascade. This was shown by pharmacological inhibition using the specific MEK1 inhibitor PD98059 and by molecular techniques involving transient transfection of ERK antisense oligonucleotides, which inhibited ERK activation, and an active MEK construct, which increased calpain activity even in the absence of EGF stimulation. This showed that calpain activity was regulated downstream of ERK/MAP kinase. However, the exact mechanism by which ERK/MAP kinase leads to calpain activation has not been determined. Regulation may occur by many mechanisms, direct phosphorylation by ERK/MAP kinases being one of them. Alternatively, there may be other downstream effector molecules present that mediate the ERK/MAP kinase-calpain interaction. Even molecular association without phosphorylation by ERK/MAP kinase could lead to increased calpain activity through conformational changes. A second possible scenario would involve not calpain but negative regulation of its endogenous inhibitor, calpastatin, either by direct phosphorylation or other means. In addition, ERK/MAP kinase may affect gene transcription of calpain or calpastatin. However, while this may have a significant impact on calpain activity over the long run, it is unlikely to play a role in our assays, which show an acute increase in calpain activity. This linkage between ERK and calpain is an obvious target of intense investigation.

Previous work describing calpain in motility dealt with haptokinetic motility mediated through integrin signaling. Thus, it was of interest to determine whether chemokinetic motility also utilizes calpain, since these two modes of motility signal both share biochemical pathways and have distinct intermediates (5). Furthermore, much of the data implicating calpain in modulating adhesion and motility describe it as a molecule required for adhesion formation and alteration (18, 19, 21). Our present results do not conflict with the published findings. Calpain's involvement in adhesion and motility is probably delicately orchestrated in regulating adhesion turnover. Calpain may play distinct roles in haptokinetic motility, as signaled by integrin adhesion, and in chemokinetic motility, as signaled by growth factors and other receptors. Indeed, the regulation of calpain in motility may also integrate spatial and temporal constraints in order to allow overlapping functions of the molecule during one round of detachment, translocation, and attachment of the cell during motility. Of interest is that we find that M-calpain but not Mu-calpain is required for EGFR signaled chemokinetic motility, whereas Mu-calpain was reported to be necessary for integrin-mediated haptokinetic motility (10). This is consistent with our antisense experiments wherein anti-M oligonucleotides decreased basal motility but anti-M oligonucleotides blunted EGF-induced motility (Fig. 7A). These findings provide the first evidence for physiologically distinct roles for these two closely related and equivalently ubiquitous enzyme isoforms.

Our studies implicate calpain in the disassembly of focal adhesions in premotile cells. Calpain activation downstream of
ERK/MAP kinase and a subsequent decrease in adhesion place the role of calpain somewhere between ERK and the focal adhesion. This is consistent with what is currently known about the targets of calpain in vivo. Calpain cleaves many focal adhesion proteins, including talin, ezrin, and pp125FAK (18, 20, 21, 43, 44). Cleavage of these proteins is limited and often results in active molecules with perhaps altered binding affinities. Cleavage of talin, for example, results in a 190-kDa fragment that retains its protein binding sites but loses its membrane association domain (45). Although the biophysical consequences of this cleavage have not been directly examined, it is possible that this cleavage acts to facilitate the dissociation of talin from the focal adhesion, along with its companion molecules. This would provide a mechanism whereby calpain could regulate focal adhesion disassembly. In preliminary experiments in this direction, we have found that calpain cleavage of talin occurs in response to EGF stimulation. Despite these provocative initial reports, the delineation of the role of calpain somewhere between ERK and the focal adhesion and a subsequent decrease in adhesion place the two isoforms of calpain are differentially activated by both a positive and negative manner. One may speculate that the cell motility cycle, modulating adhesion and motility in a positive and negative manner. One may speculate that feedback.

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