Membrane Proximal ERK Signaling Is Required for M-calpain Activation Downstream of Epidermal Growth Factor Receptor Signaling*

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Localization of signaling is critical in directing cellular outcomes, especially in pleiotropic signaling pathways. The extracellular signal-regulated kinase (ERK)/microtubule-associated protein kinase, which promotes cell migration, proliferation, and differentiation, is found in the nucleus and throughout the cytoplasm. Recently, it has been shown that nuclear translocation of ERK is required for transcriptional changes and cell proliferation. However, the cellular consequences of cytoplasmic signaling have not been defined. We explored whether cytoplasmic, specifically membrane-proximal, ERK signaling is involved in growth factor-induced cell motility. We previously have demonstrated that increased M-calpain activity downstream of epidermal growth factor receptor (EGFR)-mediated ERK activation is necessary for epidermal growth factor (EGF)-induced motility. Calpain isoforms also have been found in nuclear, cytosolic, and plasma membrane-associated compartments in a variety of cell types. We now employ cell engineering approaches to control localization of the upstream EGFR and ERK activities to examine the spatial effect of upstream signal localize on downstream calpain activity. With differential ligand-induced internalization and trafficking-restricted receptor variants, we find that calpain activity is triggered only by plasma membrane-restricted activated EGFR, not by internalized (although still active) EGFR. Cells transfected with membrane-targeted ERK1 and ERK2, which sequester endogenous ERKs, exhibited normal EGF-induced calpain activity. Transfection of an inactive ERK phosphatase (MKP-3/Pyst1) that sequesters ERK in the cytoplasm prevented calpain activation as well as de-adhesion. These data strongly suggest that EGF-induced calpain activity can be enhanced near sites of membrane-proximal EGF-mediated ERK signaling, providing insights about how calpain activity might be regulated and targeted to enhance its effects on adhesion-related substrates.

The individual biophysical processes of extension, adhesion, de-adhesion, and contraction must be finely regulated in a temporal and spatial manner to enable productive fibroblast motility (3). A change in cell motility is just one of the many pleiotropic effects of signaling mediated by the epidermal growth factor receptor (EGFR), and it has been suggested that such specific cellular responses are determined by the spatial targeting of downstream signaling events. This simple concept is complicated by the fact that EGFR-mediated cell motility requires signaling through the ubiquitous intracellular effector, ERK (4), which is present in both the cytoplasmic and nuclear compartments. Furthermore, our previous studies have demonstrated that ERK activates the ubiquitously distributed intracellular neutral protease calpain to affect de-adhesion during epidermal growth factor receptor (EGFR)-mediated cell motility (5, 6). This alteration in adhesiveness is coincident with EGFR-mediated focal adhesion disassembly (4), suggesting that the target of calpain is a component of the adhesion plaque. In support of this, calpain has been shown to cleave many adhesion plaque proteins, such as talin, ezrin, pp125FAK, and the cytoplasmic tail of β1 and β2 integrins (7–13). However, calpain does cleave other cytosolic and nuclear targets (14) and is involved in other responses such as proliferation and apoptosis (15–19).

Localization of calpain has been described in many cell types. This is best documented in erythrocytes, in which calpain translocates to the membrane from the cytosol and complexes with a number of cytoskeletal proteins when activated by ionophore or thrombin (20–23). Unfortunately, these studies investigated a nonmotile cell type and focused on the predominant isoform in erythrocytes, μ-calpain (calpain-I). In nucleated, adherent cells, this isoform has been reported to translocate to the membrane upon injury, whereas the isoform critical for EGF-induced motility (5), M-calpain (calpain-II), remains cytoplasmic (24). Direct action on adhesion sites is supported by findings in BS-C-1 epithelial cells that M-calpain co-localized with talin in the periplasma membrane space as visualized by indirect immunofluorescence (25). However, these findings have been disputed in recent publications, which have not

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The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP, microtubule-associated protein; MEK, MAP kinase/ERK kinase; TGF, transforming growth factor; DTAF, dichlorotriazinylamino-fluorescein; Boc-LM-CMAC, N-Boc-L-leucyl-L-methionine-7-amino-4-chloromethyl coumarin amid; MEM, minimal essential medium; PIPES, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; WT, wild type; notx, mock-treated.

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found either calpain isoform in focal adhesions, even in the face of μ-calpain overexpression (26, 27). These diverse findings may result from the fact that calpain can perform diverse functions and that most studies of calpain localization are directed solely to identify the distribution of the protein and not the distribution of its activity. However, for an enzyme distributed at high levels throughout the cellular space, one mode of determining selective cellular outcome might be to activate only preresident calpain in a spatially restricted locale. Recent advances in cell and molecular engineering now enable us to localize the upstream activators of M-calpain and thus begin to define the active subcellular compartments. Thus, localizing the subcellular locale of calpain activation by ERK would provide insight into how a cell selects from among multiple possible responses to a pleiotropic signal and suggest physiological targets to probe.

Binding of ligand to the EGFR leads to activation of M-calpain subsequent to ERK/MAP kinase signaling (5). This allows us to exploit the ability to produce differential EGFR signaling between internalized and cell surface EGFR. Upon ligand binding, EGFR is internalized into an early endosomal compartment before being either degraded or recycled to the cell surface. Two EGFR ligands differentially segregate in the acidic pH of the endosome; EGF remains bound to the receptor, whereas transforming growth factor-α (TGF-α) dissociates. Consequently, EGFR retains activity in the early endosome when bound by EGF but not by TGF-α (28). This has profound effects on downstream signaling, since from the endosome, EGFR is able to activate Ras (29), but not phospholipase C-γ (28). Both signaling pathways are triggered by cell surface-localized EGFR. However, since M-calpain is activated by ERK signaling, which is three steps downstream from Ras, it is quite possible for actual localization to be different between the EGFR signal, ERK activation, and subsequent calpain functioning. Furthermore, while Ras is membrane-localized due to farnesylation and thus one might favor a membrane location of the Ras/Raf/MEK/ERK signaling complex, Ras can be fully

**FIG. 1.** A, schematic of EGFR signaling compartmentation protocol. EGFR ligands TGF-α or EGF are added to NR6 fibroblasts expressing wild type EGFR. During a 20-min incubation at 37 °C, the receptors are internalized to the early endosomal compartment. In this compartment, with a pH of ~6.4, TGF-α dissociates from the receptor. However, EGF remains bound and continues to signal Ras activation. After this initial incubation, the cells are washed and then stripped of accessible (surface) ligand with a mild acid buffer for 2 min. TGF-α is then added back to the cells to produce four different conditions in which EGFR signaling is differentially localized. B, time course of EGF-induced calpain activity. Cells were treated with 10 nM EGF for the times shown and then lysed, and fluorescein-tagged MAP2-calpain substrate was added. An increase in fluorescence of the substrate was measured using a spectrofluorometer. n = 4.

**FIG. 2.** Internalization-deficient EGFR signals EGF-induced calpain activity. NR6 cells expressing WT or c'973 EGFR were plated on glass coverslips at ~50% confluence. The cells were then loaded with 10 μM of the calpain substrate Boc-LM-CMAC. The cells were treated with or without 10 nM EGF for 5 min, mounted on glass slides, and observed using a fluorescent microscope (BX40, Olympus). Images were false colored to grayscale; therefore, brightness correlates with increased fluorescence intensity and calpain activity.

**FIG. 3.** ERK is activated by both internal and surface EGFR. The immunoblot shows anti-phospho-ERK. Lane 1, notx; lane 2, TGF-α for 5 min; lane 3, EGF for 20 min (internal only); lane 4, EGF for 20 min, strip, TGF-α for 5 min (internal and external); lane 5, TGF-α for 20 min (no signal); lane 6, TGF-α for 20 min, strip, TGF-α for 5 min (external only). Results shown are representative of at least three separate experiments.
activated both from plasma membrane EGFR and endosomal EGFR (29). Obviously, the accessibility of focal adhesion components would differ greatly from these two sites. Two recent molecular constructs can localize ERK signaling to permit a cell engineering approach to control localization. ERK chimeras that express the Ras farnesylation sequence at their C terminus (ERK1-CAAX and ERK2-CAAX), and are membrane-bound sequester endogenous ERK at the plasma membrane (1) and provide for plasma membrane-localized signaling. A catalytically inactive cytoplasmic MAP kinase phosphatase, MKP-3/Pyst1, which binds and sequesters ERK in the cytoplasm, and does not affect its activity (2, 30), provides for cytosolic ERK signaling. Using a combination of these cell and molecular engineering approaches, we have found the site of ERK-mediated calpain activation to be localized at the plasma membrane, and this localization is required for EGF-induced de-adhesion. This constraint supports a model that emphasizes calpain activation targeting focal adhesion components during cell motility and provides insight into how ERK signaling might differentially induce specific cellular responses.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant TGF-α, dichlorotriazinylaminofluorescein (DTAF), digitonin, and all other buffer reagents were obtained from Sigma. Human recombinant EGF was obtained from Collaborative Biomedical Products (Bedford, MA). All cell culture reagents were obtained from Life Technologies, Inc. PD098059, anti-phospho-ERK, and anti-phospho-p90RSK antibodies were obtained from New England Bio- technologies, Inc. PD098059, anti-phospho-ERK, and anti-phospho-p90RSK antibodies were obtained from New England Biosciences, Inc. (Buckingham, UK). PD098059 was used during the first 20 min incubation to prevent a loss of calpain activity during the internalization process. PD098059 prevents calpain activation by ERK but does not have any direct effect on calpain. NR6 fibroblasts containing high levels of wild type EGFR were treated with 20 nM EGF or TGF-α in the presence of 2 μM PD098059 for 20 min. Cells were then washed once with ice-cold WHIPS buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2, and 1 mg/ml polyvinylpyrrolidone, pH 7.4) and then incubated for 2 min in an ice-cold acid wash (50 mM glycine-HCl, 100 mM NaCl, 1 mg/ml polyvinylpyrrolidone, pH 3.0). This stripped any remaining ligand from the EGFR on the cell surface (34). The cells were then washed again with ice cold WHIPS buffer and then reincubated with or without 20 nM TGF-α in binding buffer for 5 min. The cells were then either lysed for Western blotting of active ERK or loaded with 10 μM Boc-LM-CMAC and used for the following calpain activity assay.

Calpain Activity Assay—NR6 WT cells plated on glass coverslips were loaded for 20 min at 37 °C with 10 μM Boc-LM-CMAC, a synthetic calpain substrate (5, 35). After loading, the cells were treated with growth factor for 5 min and then mounted on glass slides, and images of the Boc-LM-CMAC fluorescence were obtained. The substrate is designed so that fluorescent quenching is removed upon calpain cleavage, resulting in an increase in fluorescence. Images were then false colored to grayscale; increased lightness correlates with increased calpain activity.

Expression of Engineered Constructs—Cells were electroporated to express the various ERK and MAPK-3/Pyst1 constructs in the presence of green fluorescent protein (GFP) to mark expressing cells following standard protocols. Cells were trypsinized, pelleted, and resuspended in Opti-MEM medium (Life Technologies, Inc.) in electroporation cuvettes, and the appropriate plasmid DNA was added to a total of 20–40 μg. pEFGFP plasmid was co-transfected at a 0.1 molar ratio. The cells were electroporated at 0.220 V and 960 microfarads for 5 s. The cells were then replated and allowed to grow for 48 h before use in experiments, with one change of medium.

To ensure that the constructs functioned as previously demonstrated (1, 30), indirect immunofluorescence was utilized to identify the distribution of active ERK (phosphorylated ERK) in transfected cells. Cells were transfected as above and plated on glass coverslips. The cells were treated 48 h after transfection in the presence and absence of 10 nM EGF for 10 min. The cells were fixed with 3% paraformaldehyde for 30 min. at room temperature and then washed with PBS four times. To

![FIG. 4. Internalized EGFR does not signal EGF-induced calpain activity. A, calpain activity (lightness correlates with activity). Note that the MEK inhibitor PD98059 was present in these experiments during the first 20 min incubation. Lane 1, notx; lane 2, TGF-α for 5 min; lane 3, EGF for 20 min (internal only); lane 4, EGF for 20 min, strip, TGF-α for 5 min (internal and external); lane 5, TGF-α for 20 min (no signal); lane 6, TGF-α for 20 min, strip, TGF-α for 5 min (external only). B, calpain activity in the presence of PD98059 for the total treatment time. Images are representative of at least three separate experiments, five fields per condition, average of 12 cells per field.](Image 128x596 to 477x729)
remove cell cytosolic contents but retain membrane and cytoskeleton
associated molecules, some of the coverslips were exposed to 8 μM
digitonin plus 100 μM MgCl₂ and 200 μM ATP for 10 min at room
temperature and washed twice with PBS. Control coverslips were per-
meabilized with 0.05% Triton X-100, which enables antibody access to
intracellular targets without removal of cytosolic macromolecules. To
visualize the activated ERK, primary antibody (anti-phospho-ERK,
clonE10) diluted in 1% bovine serum albumin (1:400) was applied to
the coverslips, which were then incubated in a humidified chamber for
30 min at 37 °C. The cells were washed twice with PBS, and secondary
antibody (Alexa 594 goat anti-mouse) diluted in 1% BSA (1:1000) was
applied for 30 min at 37 °C. The cells were observed for Alexa 594
fluorescence using an Olympus BX40 microscope (× 40 magnification),
and images were captured using a SPOT CCD camera. Transfected
cells were identified by GFP expression.

Indirect immunofluorescence of phospho-p90RSK was performed on
mock (GFP only)- and MKP-3/Pyst1-transfected cells. Cells were fixed
and treated as above, using Triton X-100 to permeabilize the cells for

FIG. 5. Activated ERK can be local-
ized to plasma membrane or cytoso-
lic compartments. A, indirect immuno-
fluorescence staining of phospho-ERK.
Cells were transfected with either both
ERK1 and ERK2 CAAX constructs,
ERK1 and ERK2 SAAAX control con-
structs, or MKP-3/Pyst1. Transfected
cells were identified by co-transfection
with GFP (data not shown). Cells were
fixed after treatment with or without
EGF and permeabilized with 0.05% Tri-
ton X-100 for standard immunofluores-
cence or digitonin to allow extraction of
the cytosolic components. The arrows in-
dicate transfected cells. Images are repre-
sentative of two separate experiments. B, indirect immunofluorescence of phospho-
p90RSK. p90RSK is a cytosolic target of
ERK. Cells transfected with MKP-3/Pyst1
were stained for phospho-p90RSK in the
presence and absence of EGF treatment.
The arrows indicate transfected cells. Im-
ages are representative of two separate
experiments.

Membrane-localized ERK Signaling
staining. Primary antibody (anti-phospho-p90RSK, Thr250/Thr252/Tyr253) diluted in 1% bovine serum albumin (1:1000) was applied for 30 min at 37 °C. The cells were washed twice, and secondary antibody (Alexa 594 goat anti-rabbit, 1:1000) was applied for 30 min at 37 °C. The cells were observed for fluorescence as above. Transfected cells were identified by GFP expression.

**Adhesion Assay**—Cell-substratum adhesiveness was quantitated using an inverted centrifugation detachment assay. Transfected cells were plated on 16-well glass slides (Nunc, Rochester, NY). At 48 h post-transfection, cells were treated with and without 10 nM EGF for 5 min, and images were taken. The arrows indicate transfected cells as observed by co-transfection of GFP. Farnesylation-negative ERK-SAAX chimeras were also transfected into cells and also exhibited EGF-induced calpain activity (data not shown). Shown is a representative of three experiments.

**RESULTS**

**Calpain Is Activated by an Internalization-deficient EGFR.** c′973—NR6 fibroblasts expressing both WT and internalization-deficient (c′973) EGFR were used in the Boc-LM-CMAC calpain activity assay. c′973 NR6 cells exhibited calpain activity equivalent to that seen in WT cells (Fig. 2). This indicated that internalization of the EGFR was not required for EGFR-mediated calpain activity.

**EGFR Activates ERK from both the Plasma Membrane and Internal Sites**—Previous studies had shown that both internalized and cell surface EGFR signaled Ras activation (29). However, the activation of downstream molecules, such as ERK, was not shown. That elements downstream from Ras would be activated was not assumed, since EGFR phosphorylates phospholipase C-γ from the endosome but this fails to hydrolyze phosphatidylinositol 4,5-bisphosphate (28). To address this, we determined whether ERK was activated under conditions that produced differential external and internal signaling (Fig. 1A). ERK was phosphorylated by both internal and external active EGFR (Fig. 3). Thus, there are a number of ways in which ERK activation can be linked to the activation of calpain. First, ERK may activate calpain globally within the cell. Second, ERK may translocate to a specific location within the cell to target calpain activation. Third, upstream components of the MAP kinase pathways such as Raf or MEK may translocate to the site of ERK/calpain activation. Finally, only one site of ERK activation may be relevant for activation of M-calpain.

**Calpain Is Activated Downstream of EGFR Located on the Plasma Membrane**—We applied cellular engineering first to sort through the possibilities. If we spatially isolated EGFR activation, we could determine the likelihood of various translocations of ERK or its upstream mediators to sites of calpain action. Therefore, we asked whether internal EGFR could activate calpain. NR6 WT cells were treated with either TGF-α or EGF (20 nM) for 5 min to drive internalization of the receptor. Under normal experimental conditions, EGFR-mediated calpain activity peaks at 5 min of stimulation with EGF and is greatly reduced at 20 min (Fig. 1B) probably due to autophagy. In order to observe calpain activity after internalization of the EGFR, the MEK inhibitor PD98059 was added during the first 20 min of incubation. After internalization, any remaining ligand was removed by a mild acid strip (34), and the cells were then treated with or without TGF-α for an additional 5 min. Control experiments demonstrated that the washing procedure removed the PD98059 and allowed full EGFR activation of ERK as determined by phospho-ERK detection (data not shown). Under these conditions, calpain activity using the Boc-LM-CMAC substrate was assessed (Fig. 4). The presence of external signaling only (TGF-α, strip, TGF-α) induced calpain activity as robustly as internal and external signaling. Internal signaling only did not result in calpain activity, although ERK was phosphorylated equivalently to external only (Fig. 3) and Ras was strongly activated under these conditions (29). Thus, the phosphorylation of ERK following internal signaling does not lead to activation of calpain. This strongly suggests that the subcellular localization of ERK may be a critical determinant in regulating the activity of calpain.

**Sequestering ERK1 and ERK2 at the Membrane Does Not Affect EGFR-mediated Calpain Activity**—Having rapidly determined that ERK localized to the plasma membrane is probably responsible for calpain activation, molecularly engineered ERK chimeras expressing the Ha-Ras farnesylation sequence were transfected into NR6 WT cells. Control nonlocalizing chimeras that have a nonfunctional farnesylation sequence (SAAX) were also transfected. Both ERK1-CAAX and ERK2-CAAX chimeras sequester endogenous ERK at the plasma membrane (1). Control experiments demonstrate that transfer of both ERK chimeras induces localization of active ERK at the plasma membrane, which is retained even in the face of digitonin treatment, which causes loss of cytoplasmic contents. Nonlocalizing chimeras did not have the ability to localize active ERK to the membrane or retain it upon digitonin treatment (Fig. 5A). Cells expressing the CAAX chimeras exhibited EGF-in-
duced calpain activity at the same level as mock-transfected cells (Fig. 6). In addition, expression of both ERK1 and ERK2 chimeras together to localize all ERK signaling to the plasma membrane did not diminish EGF-induced calpain activity. These findings strongly supported the contention that membrane-localized ERK can activate calpain.

**Sequestering ERK in the Cytoplasm Reduces EGFR-mediated Calpain Activity**—The counterpoint to the foregoing is whether non-plasma membrane-associated ERK also can activate calpain, providing for a model of global calpain activation. An inactive form of MAPK phosphatase, MKP-3/Pyst1, binds endogenous ERK and retains it in its cytosolic location (30). Control experiments show that, in cells expressing MKP-3/Pyst1, phosphorylated ERK is localized in the cytosol and that phosphorylated ERK is lost with the cytosolic contents upon digitonin treatment (Fig. 5A). Importantly, MKP-3/Pyst1 does not interfere with the ability of ERK to phosphorylate its cytoplasmic targets. To verify that MKP-3/Pyst1-bound ERK was still functional in our system, we used immunofluorescence to stain for phosphorylated p90RSK, a cytoplasmic target of ERK phosphorylated in response to growth factor stimulation (2). Phospho-p90RSK was observed in mock-transfected cells as well as MKP-3/Pyst1-transfected cells, treated with EGF (Fig. 5B). Expression of MKP-3/Pyst1 in NR6 WT cells inhibited EGF-induced calpain activity compared with mock-transfected cells, using the Boc-LM-CMAC assay (Fig. 7). A conundrum is presented by the clear inhibition of calpain activation by the inactive MKP-3/Pyst1 construct. The construct has been reported to be capable of phosphorylating a membrane-anchored target (2), and thus one might expect membrane-associated M-calpain to be accessible. While lacking a demonstrated explanation, numerous possibilities render this deficit less than disconcerting; threshold considerations, steric accessibility, and inability to attain a protein-protein interaction in addition to merely phosphorylating all have precedents. Despite these limitations, these results, combined with our observation that ERK activation mediated by internal signaling does not lead to calpain activation strongly suggest that a plasma membrane-associated signaling complex that includes activated ERK is required.

**EGF Induces Cell De-adhesion in the Presence of Membrane-anchored but Not Cytosolic ERK**—Previously, we demonstrated that calpain is required for EGF-induced de-adhesion during active motility (5, 6). Therefore, we queried EGF-induced de-adhesion in cells expressing either both ERK CAAX chimeras, both ERK SAAX control chimeras, or MKP-3/Pyst1. Cells were co-transfected with GFP, which allowed the adhesion of transfected cells alone to be studied. Cells were treated with or without EGF for 30 min and then placed inverted onto a plate centrifuge rotor and spun at 3000 rpm for 5 min. The number of transfected cells adherent was counted before and after centrifugation in three representative, marked fields. The number of cells remaining adherent was expressed as a percentage of mock-transfected, untreated cells remaining. Data are mean ± S.E. from two separate experiments, with four wells per condition, three fields per well. 

$$p < 0.01$$ comparing the EGF-treated transfected cells with the EGF-treated control transfectants.
ated calpain activation through ERK, in that ERK and EGF activities are required at the plasma membrane for calpain activity and for EGF-induced de-adhesion. This strongly suggests that calpain’s role in EGF-induced motility is to facilitate adhesion turnover, and its action occurs at the plasma membrane.

**DISCUSSION**

Herein we provide evidence that EGF-induced calpain activity begins at the plasma membrane. Both EGFR and ERK must be present and active at the plasma membrane in order for EGF-induced calpain activity to be stimulated. Plasma membrane-localized EGFR drove calpain activity, whereas internalized EGFR, although able to activate ERK, did not activate calpain, suggesting that ERK needed to be at the plasma membrane. Furthermore, active ERK confined to the cytoplasmat was not able to activate calpain, but membrane-tethered ERK was. Finally, EGF-induced de-adhesion, which is required for induced cell migration (36), was inhibited by restricting ERK to the cytoplasm but not by restricting ERK to the plasma membrane. This suggests a model whereby EGF signaling at the plasma membrane drives the activation of M-calpain, which in turn cleaves focal adhesion proteins and mediates de-adhesion. One caveat in this model is that we were unable to directly visualize M-calpain activation. Using immunofluorescence and GFP-tagged constructs, we found that M-calpain has a pancellular distribution as previously reported (37–40), which does not change appreciably with EGF exposure (data not shown). We did see some punctate aggregates of GFP-tagged calpain in the overlay of a pancellular distribution that may reflect the reported localization of calpain with focal adhesions (25). Visualization of calpain activity using the Boc-LM-CMAC fluorescent substrate has also proved challenging even with rapid analyses that allow for detection of calcium transients, since the substrate appears to not be restricted spatially before or after cleavage. Despite these limitations, the data provide a model in which ERK activation of calpain is segregated by subcytoplasmic locale to dictate a specific outcome of cell signaling.

The ability to spatially localize specific signals broadens the range of cell responses from any one pleiotropic signaling element. ERK has two isoforms that are present throughout the range of cell responses from any one pleiotropic signaling element. ERK has two isoforms that are present throughout the range of cell responses from any one pleiotropic signaling element. ERK can be activated at sites of focal adhesion. The concerted action of these activities is required at the plasma membrane for calpain activity and for EGF-induced de-adhesive processes during motility. µ-Calpain (calpain-I) has been suggested to be active in integrin-mediated motility and also in cell spreading and lamellipodial formation (10, 42). Our current findings do not exclude this role for µ-calpain but suggest that M- and µ-calpain may play complementary roles in the motile cell. Differential activation and localization of not only protein, but activity, would therefore serve to regulate the adhesive and de-adhesive properties of the cell. µ-Calpain, which has a much lower calcium requirement in vitro, could be activated by integrin receptors forming adhesions at the leading edge or by stretch-activated calcium receptors (43), which can provide adequate local increases in calcium concentration. M-calpain, on the other hand, which has a millimolar requirement for calcium in vitro, would then be activated by ERK at sites of focal adhesion. The concerted action of these two proteases would present a mechanism by which the highly regulated motility machinery could be fine tuned during hapto kinase and chemokinesis.

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