ERK MAP kinase phosphorylates the F-actin cross-linking protein EPLIN
and regulates its interaction with F-actin

ERK MAP キナーゼによるアクチン結合タンパク質 EPLIN の
リン酸化およびアクチンとの相互作用の制御

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CONTENTS

Abstract .................................................. 2
Chapter 1: Introduction ................................. 3
Chapter 2: Results ....................................... 6
Chapter 3: Discussion .................................... 18
Chapter 4: Materials and methods ...................... 22
References .................................................. 27
Acknowledgements ........................................ 34
Figures .................................................... 35
Abstract

Extracellular signal-regulated kinase (ERK) is important for various cellular processes including cell migration. However, the detailed molecular mechanism by which ERK promotes cell motility remains elusive. Here I characterize epithelial protein lost in neoplasm (EPLIN), an F-actin cross-linking protein, as a novel substrate for ERK. ERK phosphorylates Ser360, Ser602 and Ser692 on EPLIN in vitro and in intact cells. Phosphorylation of the C-terminal region of EPLIN reduces its affinity for actin filaments. EPLIN colocalizes with actin stress fibers in quiescent cells, and stimulation with PDGF induces stress fiber disassembly and relocalization of EPLIN to peripheral and dorsal ruffles, wherein phosphorylation of Ser360 and Ser602 is observed. Phosphorylation of these two residues is also evident during wound healing at the leading edge of migrating cells. Moreover, expression of an ERK nonphosphorylatable mutant, but not wild type, EPLIN prevents PDGF-induced stress fiber disassembly and membrane ruffling, and also inhibits wound healing and PDGF-induced cell migration. I propose that ERK-mediated phosphorylation of EPLIN contributes to actin filament reorganization and enhanced cell motility.
Chapter 1: Introduction

Mitogen-activated protein kinases (MAP kinases) are activated by a wide variety of extracellular stimuli and regulate various cellular events by phosphorylating specific substrates (Sturgill and Wu, 1991; Nishida and Gotoh, 1993; Waskiewicz and Cooper, 1995; Zhou et al., 1995; Lewis et al., 1998; Chang and Karin, 2001; Pearson et al., 2001). MAP kinases contain four typical subfamilies: extracellular signal-regulated kinases (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38 kinases (p38α/β/γ/δ) and ERK5. ERK1/2 is principally activated by growth factor stimulation through the Ras-Raf-MEK pathway, and regulates many cellular processes such as proliferation, differentiation, migration and survival. Membrane receptors stimulated by various growth factors form multiprotein complexes, which activate the small GTPase Ras. Active GTP-bound Ras then interacts with Raf and recruits it to membrane (Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Jelinek et al., 1996). Although the mechanism by which Raf is subsequently activated has not been established, activated Raf phosphorylates MEK1/2 that directly phosphorylates and activates ERK1/2 (Seger and Krebs, 1995).

ERK MAP kinase phosphorylates sites based on the optimum consensus sequence Pro-Xaa-Ser/Thr-Pro but often phosphorylates sites conforming to the relaxed consensus Ser/Thr-Pro (Gonzalez et al., 1991). Several protein kinases, nuclear transcription factors and other proteins have been identified as ERK substrates: p90 ribosomal S6 kinase (RSK), myosin light chain kinase (MLCK), focal adhesion kinase (FAK), Elk-1, c-Myc, c-Fos, cytosolic phospholipase A2, caldesmon, paxillin, vinexin and calpain (Klemke et al., 1997; Hazzalin and Mahadevan, 2002; Liu et al., 2002; Hunger-Glaser et al., 2003; Glading et al., 2004; Mitsushima et al., 2004). Activated ERK1/2 translocates from the cytoplasm to the nucleus
and then phosphorylates nuclear substrates to regulate a wide variety of physiological processes. Besides its role in the nucleus, recent data show that ERK is involved as an essential component in the regulation of cell migration and invasion in cells of many different organisms (Klemke et al., 1997; Nguyen et al., 1999; Fincham et al., 2000; Stupack et al., 2000; Matsubayashi et al, 2004). Certain substrates such as MLCK, FAK, calpain and paxillin are known to function in ERK-mediated cell migration (Huang et al., 2004).

Cell migration requires dynamic reorganization of the actin cytoskeleton. Composite extracellular stimuli including growth factors and cell-matrix adhesions trigger signals for cell motility, which are then transduced by diverse intracellular components such as the MAP kinase family, protein kinase C, tyrosine kinases and Rho family small GTPases (Ridley, 2001; Etienne-Manneville and Hall, 2002). During dynamic remodelling of the actin system for cell migration, a number of actin cross-linking/bundling proteins are crucial (Pollard, 2002). In addition, actin bundles and cross-linked networks play key roles in the generation of tension and flexibility in the actin cytoskeleton (Stossel et al., 2001). Thus, ERK might mediate cell migration via phosphorylating some actin cross-linking/bundling proteins.

Epithelial protein lost in neoplasm (EPLIN) was initially identified as the product of a gene that is transcriptionally down-regulated in a number of human epithelial cancer cells including oral, prostate and breast cancer cell lines (Chang et al., 1998; Maul and Chang, 1999). EPLIN is expressed as two isoforms, \( \alpha \) and \( \beta \), the latter of which has an extra amino-terminal sequence of 160 amino acids. Both EPLIN\( \alpha \) and \( \beta \) contain a centrally located LIM domain that may mediate self-dimerization, and N- and C-terminal actin binding domains flanking the LIM domain (Maul et al., 2003). EPLIN cross-links and bundles actin filaments, thereby stabilizing actin stress fibers. Furthermore, EPLIN inhibits Arp2/3 complex-mediated
branching nucleation of actin filaments. Thus, EPLIN controls actin filament dynamics by stabilizing actin filament networks (Maul et al., 2003). It is therefore postulated that the loss of EPLIN expression in cancer cells may enhance the motility of these cells.

Recently, I have identified EPLIN as a candidate substrate for ERK by a proteomic approach using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with phosphoprotein enrichment (Kosako et al., manuscript in preparation). In this study, I have shown that ERK phosphorylates Ser 360, 602 and 692 on EPLIN in vitro and in intact cells. The C-terminal region of EPLIN reduces its affinity to F-actin upon ERK-mediated phosphorylation. Immunostaining of NIH3T3 fibroblasts reveals that EPLIN colocalizes with actin stress fibers in quiescent cells. Stimulation with PDGF induces stress fiber disassembly and relocation of EPLIN to the peripheral and dorsal ruffles, wherein phosphorylation of Ser 360 and 602 is observed. The phosphorylation of these two serine residues is also evident during wound healing at the leading edge of migrating cells. Moreover, expression of EPLINα(S360/602/692A) but not wild-type EPLIN protects PDGF-induced stress fiber disassembly and membrane ruffling, and also reduces the motility of NIH3T3 cells during PDGF-induced cell migration and wound healing. These results suggest that phosphorylation of EPLIN by ERK leads to reorganization of actin filaments and stimulation of cell motility.
Chapter 2: Results

Proteomic identification of EPLIN as a candidate substrate for ERK

To globally identify factors involved in the ERK MAP kinase cascade, I developed a system consisting of phosphoprotein purification, fluorescent two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometric identification of proteins. Phosphoprotein profiles of ERK-activated and ERK-suppressed cells were compared, and candidate ERK substrates were identified as phosphoprotein spots that increased in ERK-activated samples (Fig. 1B).

I used ΔB-Raf:ER cells to prepare lysates. This cell line is a derivative of NIH3T3 cells in which the protein kinase domain of mouse B-Raf (that most strongly activate MEK-ERK pathway compared with c-Raf1 and A-Raf) is expressed as a fusion protein with the hormone-binding domain of the human estrogen receptor (hbER) (Pritchard et al., 1995). ERK MAP kinase was activated by 4-hydroxy-tamoxifen (4-HT), an antagonist of estrogen. To suppress the ERK MAP kinase pathway, the MEK inhibitor U0126 (Davies et al., 2000) was used (Fig. 1A). Phosphoproteins were enriched from whole cell lysates by immobilized metal ion affinity chromatography (IMAC) using a commercially available purification kit. This affinity separation technique uses solid chromatographic supports with covalently bound chelating compounds that complex metal ions and serve as affinity ligands for nucleophilic groups in peptides/proteins. The oxygen atoms in the phosphorylated side chains of serine, threonine and tyrosine show high affinity for hard metal ions such as Fe$^{3+}$, Ca$^{2+}$ and Al$^{3+}$, and this has been exploited to isolate and enrich phosphopeptides under acidic conditions (Fig. 1C) (Chaga, 2001; Guerrera et al., 2005). Purification of phosphoproteins enabled sensitive
detection of minor phosphorylated signaling components that would otherwise be obscured by abundant cellular proteins.

Phosphoprotein-enriched fractions from ERK-suppressed and ERK-activated cells were labeled with cyanine dyes, Cy3 and Cy5, respectively, combined, and run on the same two-dimensional gel. The Cy3 (ERK-suppressed cells) and Cy5 (ERK-activated cells) signals were individually scanned with nonoverlapping excitation/emission wavelengths, which are exhibited as green and red pseudocolores on a merged image (Fig. 2A). There were a large number of red spots with a higher intensity in ERK-activated cells than in ERK-suppressed cells. These spots were considered candidates for proteins phosphorylated in the ERK signaling pathway. Most of the remaining spots were yellow as a merge of red and green, demonstrating their equal quantities between two samples. These spots may be phosphoproteins unrelated to the ERK pathway or proteins nonspecifically trapped by the IMAC column.

Quantitative analyses detected 72 reproducibly changed protein spots, some of which were recognized by the anti-MAPK substrate monoclonal antibodies (data not shown). Proteins in these spots were subjected to in-gel tryptic digestion followed by peptide mass fingerprinting using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. The identified proteins included MEK1, MEK2, ERK1 and ERK2, as well as previously known ERK substrates such as p90 ribosomal S6 kinase 2 (RSK2), cytosolic phospholipase A2 (cPLA2), heterogeneous nuclear ribonucleoprotein K (hnRNP K), caldesmon, cortactin and vinexin, demonstrating the feasibility of our approach. I also identified 24 candidates for novel ERK pathway components. The information obtained through Scansite and PhosphoSite, I purified 11 proteins fused to GST. Ten proteins were
phosphorylated by active ERK \textit{in vitro}. EPLIN was one of the proteins identified as above (Fig. 2B) and most strongly phosphorylated by ERK \textit{in vitro}.

As shown in Figs. 2 and 3, EPLIN did not focus as a discrete spot, but rather showed a broad, band-like pattern. Volumes of the EPLIN spot in ERK-activated (Fig. 3, bottom right) and ERK-suppressed (bottom left) samples are schematically shown together with 2-D gel profiles surrounding the EPLIN spot (upper). EPLIN pre-mRNA is alternatively spliced to produce two mRNAs coding for EPLIN\(\alpha\) and EPLIN\(\beta\) (EPLIN\(\beta\) has an amino-terminal extension). The identified spot contained EPLIN\(\alpha\).

**ERK-mediated phosphorylation of EPLIN in living cells**

To determine whether EPLIN is phosphorylated in living cells, Myc-tagged EPLIN was expressed in \(\Delta B\)-Raf:ER cells, then the cells were treated with 4-HT or U0126. The lysates of these cells were subjected to immunoblotting with the anti-Myc (A-14) antibody (Fig. 4A, left). Both Myc-EPLIN\(\alpha\) and Myc-EPLIN\(\beta\) showed mobility shifts on SDS-PAGE upon treatment with 4-HT compared to U0126. To confirm phosphorylation as the cause of these mobility shifts, I examined the effect of phosphatase treatment. Myc-tagged EPLIN\(\beta\) was immunoprecipitated from transfected \(\Delta B\)-Raf:ER cells treated with 4-HT, and the immunoprecipitates were treated with calf intestinal alkaline phosphatase (CIAP). Myc-EPLIN\(\beta\) again showed a shift in mobility upon 4-HT treatment and this shift disappeared on the treatment with CIAP (Fig. 4A, right). This suggests that EPLIN is phosphorylated upon activation of the ERK signalling pathway.

I generated polyclonal antibodies by immunizing a rabbit with the bacterially expressed full-length EPLIN\(\alpha\) fused to GST. Affinity-purified anti-EPLIN antibodies specifically
recognized EPLINα, and also EPLINβ as a faint band in immunoblot analysis of mouse NIH3T3 cell lysates (Fig. 4B). These signals completely disappeared when NIH3T3 cells were transfected with siRNA for EPLIN, demonstrating the specificity of the generated antibodies (Fig. 4B). When ΔB-Raf:ER cells were treated with 4-HT, or NIH3T3 cells were stimulated with platelet-derived growth factor (PDGF) for 30 min, endogenous EPLINα and β were phosphorylated, which was prevented upon pretreatment with U0126 (Fig. 4C).

ERK phosphorylates Ser 360, 602 and 692 on EPLIN in vitro and in vivo

As described in the previous section, EPLIN is phosphorylated upon ERK activation. To test whether EPLIN is a direct substrate of ERK, I prepared GST-fusion proteins of full-length EPLINα, the amino- and carboxy-terminal portion of EPLINα (EPLIN-N and EPLIN-C), which are illustrated schematically in Fig. 5. Then in vitro kinase assay was performed using recombinant active ERK, γ-32P-ATP and recombinant GST-EPLINα, GST-EPLIN-N and GST-EPLIN-C as substrates (Fig. 6A). Both GST-EPLINα and GST-EPLIN-C were strongly phosphorylated by ERK, whereas GST-EPLIN-N phosphorylation was rather weak (Fig. 6A; lanes 1, 3 and 6). These observations indicate that EPLIN is a novel ERK substrate in vitro.

It has been established that ERK preferentially phosphorylates Ser or Thr just before Pro. EPLIN has seven Ser-Pro sequences that are conserved between mouse and human EPLIN. To identify the phosphorylation sites on EPLIN, I substituted each Ser residue by Ala (EPLIN-N-S360A or -S372A, and EPLIN-C-S485A, -S488A, -S602A, -S607A or -S692A). Recombinant GST-EPLINα, GST-EPLIN-N, GST-EPLIN-C and their Ala substitutes were incubated with recombinant ERK in the presence of γ-32P-ATP in vitro. As shown in Fig. 6A, the S360A substitution completely abolished ERK phosphorylation of EPLIN-N (Fig. 6A, lanes 3-5). For
EPLIN-C, substitution of either Ser 602 or 692 by Ala partially abolished phosphorylation, and substitution of both residues by Ala (EPLIN-C-S602/692A) markedly reduced phosphorylation (Fig. 6A, lanes 6-12). When full-length EPLINα was employed as a substrate, substitution of Ser 360, 602 and 692 by Ala (EPLINα-S360/602/692A) greatly impaired phosphorylation by ERK (Fig. 6A, lanes 1 and 2). The result indicates that Ser 360, 602 and 692 are the primary sites on which ERK phosphorylates EPLIN in vitro.

To determine the phosphorylation site on EPLIN that induced the electrophoretic mobility shift, HA-tagged EPLINβ or its Ala mutants were expressed in ΔB-Raf:ER cells, then the cells were treated with 4-HT or U0126. HA-EPLINβ-S360A did not show the mobility shift (Fig. 6B), suggesting that the shift is due to the phosphorylation of Ser 360.

To further confirm the phosphorylation sites, EPLIN phosphorylated by ERK in vitro was digested with trypsin or V8 protease, and peptides were subjected to LC-MS/MS analysis. As shown in Fig. 7, retention of charge on the N-terminal portion of the peptide ion yields a b-type ion; retention on the C-terminal portion of the peptide ion yields a y-type ion. For example, in Fig. 7A, the b ions, b13 and b15 but neither b8 nor b10 showed the characteristic loss of neutral phosphoric acid (-98); the y ions, y4 was observed, as well as a series of y ions resulting from the loss of phosphoric acid (y7, y9 and y17). Because the loss of phosphoric acid is often seen in phosphoserine- and phosphothreonine-containing peptides and is thus used as a marker for phosphopeptides, therefore, the site of phosphorylation was identified as Ser 360. Phosphorylation sites determined by Ser to Ala replacement were confirmed by this analysis (Fig. 7A, C and D). Phosphorylation of Ser 488 and 607 was also observed, which may be minor phosphorylation sites (Fig. 7B and C).

Next, I produced phospho-specific antibodies using phosphopeptides that harbour
phosphorylated Ser 360, 602 or 692. The anti-pS360 antibodies recognized wild-type and S372A mutant of GST-EPLIN-N upon ERK-mediated phosphorylation, but did not recognize the S360A mutant of GST-EPLIN-N (Fig. 8A). Similarly, anti-pS602 and anti-pS692 antibodies recognized wild-type and S692A, and wild-type and S602A of GST-EPLIN-C, respectively. However, anti-pS602 and anti-pS692 antibodies did not recognize S602A and S692A mutants of GST-EPLIN-C, respectively. These findings indicate that the anti-pS360, anti-pS602 or anti-pS692 antibodies specifically recognize EPLIN phosphorylated at Ser360, 602 or 692, respectively.

I next asked whether the anti-pS360, anti-pS602 and anti-pS692 antibodies could detect endogenous EPLIN phosphorylated in response to external stimuli that physiologically activate ERK. As shown in Fig. 8B, on addition of PDGF to NIH3T3 cells, all of the anti-pS360, anti-pS602 and anti-pS692 antibodies detected two bands that corresponded to EPLINα and EPLINβ. Since the phosphorylation of Ser 360 caused the mobility shift, anti-p602 antibodies detected EPLINα and β both as doublets (the upper band being phosphorylated on Ser 360). The time course of Ser 602 and 692 phosphorylation was similar to that of ERK activation, but Ser 360 phosphorylation proceeded slowly and increased up to 240 min. The phosphorylation of EPLIN was strongly inhibited when cells were pretreated with U0126. I therefore concluded that EPLIN is phosphorylated by ERK in living cells.

Phosphorylation of the C-terminal region of EPLIN by ERK reduces its affinity to F-actin in vitro and in vivo

Because EPLIN has two actin-binding domains and cross-links actin filaments into bundles (Maul et al., 2003), I next examined whether phosphorylation of EPLIN regulates its
association with F-actin. First, F-actin binding properties of nonphosphorylated and ERK-phosphorylated C-terminal region of EPLIN (GST-EPLIN-C) were compared in a co-sedimentation assay with F-actin \textit{in vitro} (Fig. 9). In this assay, GST-EPLIN-C was mixed with F-actin, the sample was ultracentrifuged, and the distribution of GST-EPLIN-C in the supernatant and pellet was examined. A fraction of GST-EPLIN-C was recovered in the pellet only in the presence of F-actin, demonstrating the F-actin binding activity of GST-EPLIN-C. The recovery of phosphorylated GST-EPLIN-C in the pellet with F-actin was less than that of the nonphosphorylated form, whereas the recovery of the nonphosphorylatable mutant of GST-EPLIN-C (GST-EPLIN-C(S602/692A)) did not show such effect by ERK phosphorylation (Fig. 9A). To determine their stoichiometries to F-actin at saturation level and dissociation constants for F-actin, varying concentrations of these proteins were incubated with a fixed amount of F-actin, and the amount of bound proteins was determined. As shown in Fig. 9 B, binding of GST-EPLIN-C to F-actin increased as the concentration of GST-EPLIN-C increased. The dissociation constants (Kds) of nonphosphorylated and phosphorylated forms GST-EPLIN-C for F-actin were calculated to be 0.66 µM and 0.96 µM, respectively. In contrast, the nonphosphorylatable form of GST-EPLIN-C(S602/692A) with or without phosphorylation by ERK showed similar binding properties to F-actin with Kds of 0.65 µM. This result suggests that phosphorylation of the C-terminal half of EPLIN by ERK reduces its affinity to F-actin. In contrast, phosphorylation of full-length and N-terminal EPLIN by ERK did not reduce their affinity to F-actin in the similar co-sedimentation assay (data not shown).

To examine the effect of ERK-mediated phosphorylation of EPLIN on its affinity to F-actin in living cells, either Myc-tagged full-length, N-terminal and C-terminal region of EPLIN was
co-expressed in 293T cells with constitutively active (SDSE) or dominant-negative (SASA) HA-tagged MEK. Myc-tagged EPLIN was immunoprecipitated with the anti-Myc (9E10) antibody, and immunoprecipitates were subjected to immunoblotting with anti-actin (Fig. 10). The co-expression of MEK-SDSE markedly decreased the binding of EPLIN-C to actin, as compared to MEK-SASA, while the binding to actin of EPLIN-C(S602/692A) did not change upon ERK activation (Fig. 10, right). Full-length Myc-EPLINα and Myc-EPLIN-N did not show such reduction in their actin binding activity upon ERK activation (Fig. 10, left and middle). EPLIN homo-dimerizes through a LIM domain, and cross-links actin filaments through two actin-binding domains (Maul et al., 2003). Therefore, it is reasonable that the reduction in the actin binding activity of C-terminal region does not lead to a decrease in the actin binding of full-length EPLIN.

**Stimulation with PDGF induces relocalization of EPLIN to peripheral and dorsal ruffles**

ERK phosphorylates EPLIN *in vitro* and in living cells, and reduces the affinity of C-terminal region of EPLIN to actin filaments. To examined whether EPLIN phosphorylation by ERK regulates EPLIN function in living cells, I first investigated the cellular localization of EPLIN during ERK activation. Serum-starved NIH3T3 cells were stimulated with PDGF, then immunostained with anti-EPLIN (Fig. 11). EPLIN co-localized with the actin stress fibers in quiescent cells. Upon PDGF stimulation, the cells started to polarize, then extended lamellipodia. In cells treated with PDGF for 15 min (Fig. 11), EPLIN localized on the peripheral and dorsal ruffles. When cells were treated with PDGF in the presence of U0126, stress fiber disassembly was partially inhibited, and EPLIN localized on the remaining actin stress fibers.
Stimulation with PDGF induces phosphorylation of Ser 360 and 602 at peripheral and dorsal ruffles

To gain insight into the roles of EPLIN phosphorylation by ERK, I examined the location of phosphorylated EPLIN in PDGF-treated NIH3T3 cells by staining EPLIN with anti-pS360 and anti-pS602 antibodies (Figs. 12 and 13). In quiescent cells, phosphorylation of EPLIN at Ser 360 was hardly detected. However, when cells were stimulated with PDGF, immunostaining showed that phosphorylated EPLIN appeared in the peripheral and dorsal ruffles, and lasted for at least 120 min, consistent with the result of immunoblot analysis (Fig. 8B). When cells were treated with PDGF in the presence of U0126, signals of phosphorylated EPLIN were completely abolished.

Immunostaining with the anti-pS602 antibodies revealed that phosphorylation of EPLIN at Ser 602 proceeded earlier than that at Ser 360 (Fig. 13). At 5 min treatment with PDGF, signals appeared at the peripheral and dorsal ruffles. The time course of signal intensity of immunostaining with the anti-pS602 antibodies also correlated well with the result of immunoblot analysis. Activated phospho-ERK (p-ERK, Fig. 13) was observed throughout the cell body at 5 min, then translocated into the nucleus at 30 min, and returned to the cytoplasm at 120 min. Most of the phospho-specific antibodies show staining in nucleus and centrosomes, but this is nonspecific. Similarly, nuclear staining with anti-pS602 antibodies was shown, but that was observed in quiescent cells and in cells treated with U0126, suggesting that this signal may be nonspecific.
pS360- and pS602-EPLIN localize to the leading edge of migrating cells

The localization of phosphorylated EPLIN at membrane ruffles prompted us to test whether the phosphorylation of EPLIN is involved in cell motility. Six hours after wounding a confluent monolayer of NIH3T3 cells, cells were immunostained with the anti-pS360 or anti-pS602 antibodies (Fig. 14). Both pS360 and pS602 signals were clearly visible in cells at the leading edge. As expected, pretreatment of cells with U0126 completely abolished these signals, further supporting the specificity of the immunostaining. During wound healing, ERK1/2 had a rapid and transient activation at 3 min in NIH3T3 fibroblasts, which was inhibited by U0126 (data not shown). These results suggest that EPLIN is phosphorylated by ERK at the leading edge of migrating fibroblasts.

Phosphorylation of EPLIN by ERK is required for PDGF-induced stress fiber disassembly and membrane ruffling

To determine whether the phosphorylation of EPLIN by ERK is essential for cell motility, I generated enhanced green fluorescent protein (EGFP)-fused wild-type (EPLINα-EGFP) and a mutant (EPLINα(S360/602/692A)-EGFP) EPLIN in which three major phosphorylation sites were replaced with Ala. Overexpression of both types of EPLIN increased the number and size of actin stress fibers in quiescent cells (Fig. 15), as reported previously using MCF-7 cells (Maul et al., 2003). When cells were stimulated with PDGF for 5 min, the EPLINα-EGFP-transfected cells formed markedly more lamellipodia/membrane ruffles than surrounding cells, but EPLINα(S360/602/692A)-EGFP-transfected cells still retained stress fibers and formed few membrane ruffles (Fig. 15).

I categorized cells into four classes according to the degree of ruffling: type I showed no
ruffles, type II had little ruffles, type III showed both marked ruffles and stress fibers, and type IV had only marked membrane ruffles (Fig. 16). Before PDGF treatment, most of both wild-type- and the Ala mutant-transfected cells were classified into type I. When cells were stimulated with PDGF for 5 min, EPLINα-EGFP transfected cells were mostly in type III and IV with marked lamellipodia/membrane ruffles, but in EPLINα(S360/602/692A)-EGFP-transfected cells ruffle formation was significantly impaired. These data suggest that phosphorylation of EPLIN by ERK is required for PDGF-induced lamellipodia/membrane ruffle formation.

**Phosphorylation of EPLIN by ERK is required for cell migration**

Dynamic phosphorylation and dephosphorylation of cytoskeletal proteins are essential for effective cell motility. To evaluate the potential role of EPLIN phosphorylation in cell migration, wound healing assays were performed using EPLINα-EGFP- and EPLINα(S360/602/692A)-EGFP-transfected NIH3T3 cells. The proportion of EGFP-positive cells at the wound edge was assessed over an 8-h time period. The ratio of EPLINα-EGFP-expression at the wound edge (approximately 20%) did not change during this period, indicating that EPLINα-EGFP-expressing cells and surrounding untransfected cells migrated at similar velocity (Fig. 17). In contrast, the EPLINα(S360/602/692A)-EGFP-expressing cells showed a marked decrease in motility and gradually fell behind the wound edge during recovery (Fig. 17). These results indicate that EPLIN phosphorylation by ERK is required for cell migration during wound healing.

The roles of EPLIN phosphorylation by ERK in cell motility were evaluated in another experiment, a modified Boyden chamber assay (Fig. 18). Overexpression of
EPLINα(S360/602/692A)-EGFP significantly inhibited the ability of NIH3T3 cells to migrate in response to PDGF added to the lower chamber, compared with the GFP or EPLINα-EGFP expressing cells. These results suggest that EPLIN phosphorylation by ERK is required for PDGF-induced cell migration.
Chapter 3: Discussion

In the present study, I have characterized an F-actin cross-linking protein, EPLIN, as a novel ERK MAPK substrate. First, ERK phosphorylates EPLIN on Ser360, Ser602 and Ser692 in vitro and in living cells. Second, ERK phosphorylation of EPLIN decreases the affinity of its C-terminal region for actin filaments. Third, EPLIN localizes to actin stress fibers in quiescent fibroblasts, and stimulation with PDGF induces relocalization of EPLIN to lamellipodia/membrane ruffles. Fourth, phosphorylated EPLIN localizes to membrane ruffles both upon PDGF stimulation and during wound healing. Fifth, an ERK nonphosphorylatable mutant of EPLIN inhibits PDGF-dependent actin stress fiber disassembly, membrane ruffling and cell migration. ERK thus controls actin organization and cell motility by phosphorylating EPLIN.

ERK phosphorylation sites within EPLIN were identified by site-directed mutagenesis. I determined that Ser 360, 602 and 692 residues of EPLIN are the major phosphorylation sites for ERK. These phosphorylation sites were confirmed by MS/MS analysis of in vitro phosphorylated EPLIN (Fig. 7). I raised phospho-specific antibodies against these phosphorylation sites (anti-pS360, anti-pS602 and anti-pS692) and confirmed that these sites are indeed phosphorylated by ERK in intact cells (Fig. 8). However, ERK still phosphorylated EPLIN-C(S602/692A) and EPLINα(S360/602/692A) to some extent (Fig. 6A, lanes 2 and 12), suggesting that EPLIN contains other phosphorylation sites. In agreement with this result, minor phosphorylation sites such as Ser 488 and 607 residues were observed on MS/MS analyses (Fig. 7).

EPLIN contains two actin-binding domains in the N- and C-terminal half, and a LIM domain between these two actin-binding regions may allow EPLIN to homo-dimerize. EPLIN
therefore cross-links and bundles actin filaments, but the two actin-binding domains may have
different functions in the cell (Maul et al., 2003). In co-sedimentation assays with F-actin, I
found that the C-terminal half of EPLIN but neither the N-terminal half nor full-length of
EPLIN reduces its association with F-actin upon ERK-mediated phosphorylation. Since
EPLIN has two actin-binding regions, it is reasonable that the amount of full-length EPLIN
co-precipitated with F-actin did not vary upon phosphorylation. This observation was
confirmed by in vivo experiment that the amount of actin co-immunoprecipitated with the C-
terminal half but neither full-length nor the N-terminal half of EPLIN reduced upon activation
of ERK.

The phosphorylation-dependent reduction of the affinity of the C-terminal region for F-
actin may affect the actin bundling activity of EPLIN to facilitate dynamic remodeling of
actin filament networks. Thus, I investigated the effects of EPLIN phosphorylation on its
localization, actin dynamics and cell motility. It has been reported that endogenous EPLIN is
distributed predominantly along actin stress fibers in U2OS cells (Song et al., 2002). Consistent
with this finding, immunostaining showed that EPLIN co-localized with F-actin
stress fibers in quiescent NIH3T3 cells (Fig. 11). Stimulation with PDGF induced stress fiber
disassembly and localization of EPLIN to lamellipodia within 15 min. When cells were
treated with PDGF in the presence of U0126, stress fiber disassembly was partly inhibited by
blocking the ERK signalling pathway (Orr et al., 2002), and EPLIN remained localized on the
resultant actin stress fibers.

I further demonstrated that both Ser 360 and 602 are phosphorylated in intact cells by
PDGF stimulation or during cell migration, suggesting the significance of phosphorylation in
physiological cellular events (Figs. 8, 12-14). Both signals were not detectable when the cells

19
were pretreated with U0126. PDGF treatment induced the phosphorylation of EPLIN at ruffling membranes. In migrating NIH3T3 fibroblasts, phosphorylated EPLIN preferentially localized to the leading edge, which is consistent with previous observations that activated ERK is also localized at the leading edge during migration of fibroblastic 3Y1 cells (Matsubayashi et al., 2004). These findings suggest the involvement of ERK-mediated phosphorylation of EPLIN in cell migration.

To clarify the effects of EPLIN phosphorylation on actin organization and cell motility, I used wild type and an ERK nonphosphorylatable mutant EPLINα fused to EGFP. The nonphosphorylatable mutant inhibited both membrane ruffling and cell motility. These data along with our unpublished results suggest the importance of ERK-mediated phosphorylation of EPLIN during cell migration. Since nonphosphorylated EPLIN dimer can form thick actin bundles through the N- and C-terminal actin-binding sites, EPLIN in quiescent cells may stabilize stress fibers and inhibit cell migration (Fig. 19, upper). Phosphorylated EPLIN dimer can cross-link actin filaments through only the N-terminal actin-binding sites, and thereby EPLIN in migrating cells may form a dynamic actin meshwork in membrane ruffles (Fig. 19, lower). Taken together, PDGF stimulation activates ERK, which phosphorylates EPLIN to reduce the affinity of its C-terminal region for actin filaments, and then phosphorylated EPLIN causes destabilization of stress fibers and reorganization of actin cytoskeleton to form membrane ruffles and to enhance cell migration.

ERK is known to regulate actin organization and cell motility by phosphorylating a number of proteins including MLCK, FAK, paxillin, actopaxin and vinexin. I demonstrate in this study that EPLIN is also a mediator of ERK-regulated cytoskeletal dynamics. Because expression of phosphomimetic mutants of EPLIN had weak effects on these processes (data
not shown), many actin-binding proteins phosphorylated by ERK are likely to act in concert
to regulate actin dynamics. Furthermore, various extracellular stimuli induce actin
reorganization and cell migration through other ERK-independent pathways. For example, it
has recently been reported that Akt regulates these processes via phosphorylation of Girdin,
an F-actin cross-linking protein (Enomoto et al., 2005). Other actin cross-linking proteins,
such as fascin (Yamakita et al., 1996; Vignjevic et al., 2006) and L-plastin (Janji et al., 2006),
are also shown to be regulated by phosphorylation to control actin cytoskeletal assembly and
cell motility.

EPLIN was originally identified as a protein whose expression is either down-regulated or
lost in a number of oral, prostate and breast cancer cell lines (Maul and Chang, 1999). Since
EPLIN regulates cell motility, it may be possible that the loss of EPLIN expression is
involved in the enhanced motility of transformed cells. Further experiments such as siRNA-
mediated EPLIN depletion may be needed to clarify the role of EPLIN in the regulation of
actin dynamics.

Previously, it has been reported that ectopic expression of EPLIN can suppress anchorage-
independent growth of NIH3T3 cells transformed by Cdc42-V12 or EWS/Fli-1 but not by
Ras-V12 (Song et al., 2002). This can now be explained by the actin reorganization and
enhanced cell motility through the Ras-Raf-MEK-ERK-EPLIN pathway. Ras-mediated
phosphorylation of EPLIN may be involved in invasion of tumor cells with Ras mutations.
EPLIN is highly conserved from zebrafish to human, containing multiple Ser/Thr-Pro motifs
that can be potentially phosphorylated by ERK. The ERK-EPLIN pathway may play
important roles in diverse physiological processes in vertebrates.
Chapter 4: Materials and methods

Cell culture and transfection. ΔB-Raf:ER cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. NIH3T3 cells were cultured in DMEM containing 10% calf serum. 293T and HeLa cells were cultured in DMEM containing 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) for 293T and ΔB-Raf:ER cells or Lipofectamine LTX (Invitrogen) for NIH3T3 cells according to the manufacturer’s instructions.

Antibodies and reagents. Anti-mouse EPLINα rabbit polyclonal antibodies were generated against GST-fused full-length EPLINα expressed in E. coli and affinity purified. The antiphospho EPLIN antibodies were raised by immunizing rabbits with the KLH-conjugated phosphopeptides corresponding to 11 amino acids of EPLINα and purified from antiserum as a bound fraction of the phosphopeptide-conjugated column and an unbound fraction of the nonphosphopeptide-conjugated column. The following antibodies were also used: 9E10 anti-Myc mouse mAb (monoclonal antibody), A-14 anti-Myc rabbit pAb (polyclonal antibody) and K-23 anti-ERK1 rabbit pAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin mouse mAb (Chemicon, Temecula, CA) and 3F10 anti-HA rat mAb (Roche, Basel, Switzerland). PDGF and 4-hydroxy-tamoxifen (4-HT) were obtained from Sigma-Aldrich (St. Louis, MO). U0126 was purchased from Promega (Madison, WI).
2D-DIGE. U0126- or 4-HT-treated ΔB-Raf:ER cells (1x10⁷) were subjected to PhosphoProtein Purification Kit (Qiagen, Hilden, Germany). Fifty micrograms of phosphoprotein-enriched fractions were labeled with Cy3 or Cy5 (CyDye DIGE Fluors; GE Healthcare, Buckinghamshire, UK). The paired samples were mixed, and first-dimension isoelectric focusing was performed on immobilized pH gradient strips (24 cm; pH 4-7) using an Ettan IPGphor II system (GE Healthcare). Second-dimension separation was performed on 10% SDS-PAGE gels (20x24 cm). Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare) at 100-μm resolution, and image analysis was carried out with DeCyder software (version 5.01; GE Healthcare).

Plasmids and protein expression. The DNA fragment encoding mouse EPLINα, EPLINβ, EPLIN-N or EPLIN-C was amplified with PCR, and cloned into pCMV-Tag3-Myc vector (Stratagene, La Jolla, CA) or pGEX-4T-3 vector (GE Healthcare). For EPLINα-EGFP, the DNA fragment encoding EGFP was amplified with PCR, and cloned into the C-terminus of pCMV-Tag3-Myc-EPLINα. Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Phosphatase treatment. Myc-EPLINβ was transfected into ΔB-Raf:ER cells. Cells were then treated with 4-HT for 2 h, and cell lysates were immunoprecipitated with the anti-Myc (9E10) antibody. Immunoprecipitates were resuspended with a reaction buffer containing 4 units of calf intestine alkaline phosphatase (TaKaRa, Shiga, Japan) and incubated at 37°C for 60 min. The reaction was stopped by adding Laemmli’s sample buffer and boiling. Proteins were separated by SDS-PAGE and subjected to immunoblotting with the anti-Myc (A-14) antibody.
**In vitro kinase assays.** Phosphorylation of recombinant GST-EPLINα, GST-EPLIN-N, GST-EPLIN-C or their Ala substitutes by ERK was performed by incubation of 50 ng of recombinant active ERK2 (New England Biolabs, Beverly, MA) with 3.0 μg each of GST-EPLINα, N, C or its mutants and 2.5 μCi of γ-32P-ATP (6000 Ci/mmol, GE Healthcare) in 30 μl of a kinase buffer (50 mM Tris-HCl, pH 7.5, 15 mM MgCl₂, 1 mM EGTA and 2 mM DTT) for 20 min at 30°C. The reaction was stopped by adding Laemmli’s sample buffer and boiling. Half the sample was subjected to 10% SDS-PAGE, and phosphorylation reaction was visualized by autoradiography.

**LC-MS/MS analysis.** GST-EPLINα phosphorylated by ERK in vitro was separated by SDS-PAGE. In-gel digestion was performed using sequencing grade trypsin (Promega) or endoproteinase Glu-C (Roche). The peptides were subjected to liquid chromatography for purification and analyzed by a MALDI-TOF/TOF tandem mass spectrometer (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA). Detected masses and peptide sequences were subjected to database searches with the Mascot search engine (Matrix Science, London, UK).

**RNA interference.** EPLIN siRNA and siCONTROL non-targeting siRNA were obtained from Dhharmacon (Lafayette, CO). The sequences of siRNA duplexes that target mouse EPLIN are as follows: sense, 5’-GGACGAAUCUACUGUAAGCUU-3’; antisense, 5’-GCUUACAGUAGAUUCCGUCCU-3’. These siRNA duplexes were transfected into NIH3T3 cells using DharmaFECT 1 reagent (Dhharmacon), and cells were cultured for 72 h.
**Immunofluorescence microscopy.** Cells were grown on coverslips coated with poly-L-lysine and fixed with 3.7% formaldehyde for 10 min at room temperature. Fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. After washing with PBS, the cells were incubated with primary antibodies in PBS containing 2% goat serum for 2 h, followed by incubation with Alexa Fluor-conjugated secondary antibodies (1:1,000 dilution; Invitrogen) for 1 h at 37°C. F-actin was detected by staining with rhodamine-phalloidin or Alexa Fluor 647-conjugated phalloidin (Invitrogen).

**Boyden Chamber Assay.** Cell migration was assayed in Boyden chambers (8.0 μm pore size polyethylene terephthalate membrane, FALCON cell culture insert; Becton Dickinson, Mountain View, CA). NIH3T3 cells transfected with GFP, EPLINα-EGFP or EPLINα(S360/602/692A)-EGFP were serum starved with DMEM containing 0.2% CS, and then trypsinized and counted. Cells (5-10×10⁴) in DMEM containing 0.2% CS (1.2 ml) were added to the upper chamber, and 2 ml of appropriate medium with or without 30 ng/ml PDGF was added to the lower chamber. Transwells were incubated for 6 h at 37°C. Cells transfected with GFP, EPLINα-EGFP or EPLINα(S360/602/692A)-EGFP on the transwell inserts were counted, and then cells on the inside of the insert were removed with a cotton swab, and GFP-positive cells on the underside of the insert were counted. The number of cells in five randomly chosen fields per filter was counted by microscopic examination.

**Actin co-sedimentation assays.** G-actin (Cytoskeleton, Denver, CO) and GST-EPLIN-C or GST-EPLIN-C(S602/692A) were first precleared by centrifugation at 100,000 g for 30 min at
4°C. Actin was polymerized in 5 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 0.2 mM ATP and 0.5 mM DTT at room temperature for 30 min and then incubated with ERK-phosphorylated or nonphosphorylated GST-EPLIN-C or GST-EPLIN-C(S602/692A) for 30 min at 4°C. After centrifuging at 100,000 g for 30 min at 4°C, the supernatant and pellet were separated and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

**Immunoprecipitation.** Cells were lysed with IP buffer (20 mM Tris-HCl at pH7.5, 150 mM NaCl, 10 mM NaF, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM PMSF, 20 μg/ml aprotinin and 2 mM DTT) for 15 min on ice. Lysates were clarified by centrifugation and incubated with agarose beads conjugated with the 9E10 anti-Myc antibody (Santa Cruz) for 1 hr at 4°C. The beads were then washed three times with IP buffer and finally resuspended with Laemmli’s sample buffer. Bound proteins were subjected to a phosphatase treatment or resolved by SDS-PAGE for immunoblot analysis.
References


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Fig. 1. (A) The pathway to activation of ERK. ERK MAP kinase activated by 4-HT through the Raf-MEK pathway phosphorylates several substrates such as RSK, Elk-1 and MNK in ΔB-Raf:ER cells expressed the protein kinase domain of mouse B-Raf. The pathway is suppressed by U0126.
(B) Schematic showing the protocol used. Total lysates from ΔB-Raf:ER cells treated with U0126 or 4-HT for 30 min were subjected to IMAC. The purified phosphoproteins were then labeled with the cyanine dyes (Cy3 or Cy5) and analyzed by 2D electrophoresis.
(C) Schematic representation of the protocol used for IMAC. The phosphorylated side chains of serine, threonine and tyrosine that have the oxygen atoms show high affinity for hard metal ions such as Fe(III) and this has been exploited to isolate phosphoproteins.
Fig. 2. (A) Combination of IMAC and 2D-DIGE detects multiple Raf/MEK/ERK-mediated phosphorylation events. The 2D gel was scanned with different wavelengths to visualize protein patterns corresponding to proteins labeled with Cy3 (green) and Cy5 (red). The experiments were repeated three times including one dye-swap experiment, and reproducible results were obtained. The ovals denote the position of several identified proteins.
(B) The 2D-DIGE representations (left) and immunoblotting images (right) of the EPLIN spot.
Fig. 3. The 2D gel images (upper) and 3D representations (lower) of the EPLIN spot.
Fig. 4. ERK-mediated phosphorylation of EPLIN in living cells. (A) ΔB-Raf:ER cells transfected with Myc-EPLINα or β were treated with 20 μM U0126 or 1 μM 4-HT for 2 h, and the lysates were immunoblotted with the A-14 anti-Myc rabbit antibody (left). Myc-EPLINβ-transfected cell lysates obtained in the left were immunoprecipitated with the 9E10 anti-Myc mouse antibody, and immunoprecipitates were incubated with or without calf intestine alkaline phosphatase (right).
(B, C) Affinity-purified anti-mouse EPLINα antibodies specifically recognized mouse EPLIN but not human EPLIN. NIH3T3 cells were transfected with EPLIN siRNA, control siRNA or buffer as indicated. The expression of EPLINα/β, ERK1/2 and actin were analyzed by immunoblotting with the indicated antibodies (B). ΔB-Raf:ER cells were treated with 20 μM U0126 or 1 μM 4-HT for 30 min, and serum-starved NIH3T3 cells were stimulated with 50 ng/ml PDGF for 30 min in the presence or absence of a 30-min pretreatment with 20 μM U0126. The lysates were immunoblotted with anti-EPLINα and anti-ERK1/2 antibodies (C).
Fig. 5. The domain structure of EPLIN and its truncation mutants. Potential ERK phosphorylation sites are indicated. For bacterial expression, EPLIN\(\alpha\), EPLIN-N and EPLIN-C were tagged with GST at the N-terminus.
Fig. 6. ERK phosphorylates Ser 360, Ser 602 and Ser 692 of EPLIN. (A) *In vitro* kinase assay was performed using GST-EPLINα, GST-EPLIN-N, GST-EPLIN-C or their Ala substitutes as substrates and recombinant active ERK as a kinase in the presence of γ-32P-ATP. After electrophoresis, the gel was stained with Coomassie Brilliant Blue and subjected to autoradiography (upper). The relative intensities of the phosphorylation bands were quantified by a FUJIX Bio-imaging analyzer BAS2000 (lower).
(B) Phosphorylation of Ser 360 is required for the electrophoretic mobility shift. ΔB-Raf:ER cells transfected with wild-type or Ala substitutes of HA-EPLINβ were treated with U0126 or 4-HT for 2 h. Cell lysates were immunoblotted with anti-HA and anti-ERK1/2 antibodies.
Fig. 7. Tandem mass (MS/MS) spectra of tryptic (upper and lower left) and V8 (lower right) phosphopeptides derived from GST-EPLINα phosphorylated by ERK in vitro.
Fig. 8. (A) GST-EPLIN-N, GST-EPLIN-C and their Ala substitutes were incubated with or without ERK in vitro and analyzed by immunoblotting with anti-pS360, anti-pS602 or anti-pS692 antibodies as indicated.
(B) Serum-starved NIH3T3 cells treated with 10 ng/ml PDGF for the indicated times were analyzed by immunoblotting with anti-pS360, anti-pS602, anti-pS692, anti-EPLIN, anti-p-ERK1/2 and anti-ERK1/2 antibodies.
Figure 9. Phosphorylation of the C-terminal region of EPLIN by ERK reduces its affinity to F-actin in vitro. (A) Phosphorylated (+ERK) or non-phosphorylated (-ERK) GST-EPLIN-C or GST-EPLIN-C(S602/692A) was mixed with (+) or without (-) 2.5 μM polymerized actin derived from rabbit skeletal muscle and ultracentrifuged. Supernatants (S) and pellets (P) were analysed by SDS-PAGE followed by Coomassie blue staining. (B) Quantitative analysis for binding of the C-terminal region of EPLIN to F-actin. The co-sedimentation assay was performed by mixing of 2.5 μM polymerized actin with various amounts of phosphorylated (red) or non-phosphorylated (black) GST-EPLIN-C (left) or GST-EPLIN-C(S602/692A) (right). Amounts of the free and bound GST-EPLIN-C in the supernatant and pellet fractions were determined from a digitized Coomassie-stained gel.
Figure 10. Phosphorylation of the C-terminal region of EPLIN by ERK reduces its affinity to actin in vivo. 293T cells were co-transfected with Myc-EPLINα, Myc-EPLIN-N, Myc-EPLIN-C or their Ala substitutes, and dominant negative (SASA) or constitutive active (SDSE) HA-MEK as indicated. The lysates were immunoprecipitated with the anti-Myc (9E10) antibody, followed by immunoblot analysis with anti-actin and anti-Myc (A-14) antibodies (upper). The total lysates were immunoblotted with anti-Myc (9E10) and anti-ERK1/2 antibodies (lower).
Figure 11. Stimulation with PDGF induces localization of EPLIN to peripheral and dorsal ruffles. Serum-starved NIH3T3 cells were stimulated with 50 ng/ml PDGF for 0 or 15 min in the presence or absence of a 30-min pretreatment with 20 μM U0126. Cells were fixed and stained with anti-EPLINα antibodies (green), rhodamine-phalloidin (red) and DAPI (blue). The arrows and arrowhead indicate peripheral and dorsal ruffles, respectively. Bar, 30 μm.
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**Figure 12.** Stimulation with PDGF induces phosphorylation of Ser 360 of EPLIN at peripheral and dorsal ruffles. Serum-starved NIH3T3 cells were stimulated with 50 ng/ml PDGF for the indicated times in the presence or absence of a 30-min pretreatment with 20 μM U0126. Cells were fixed and stained with anti-pS360 antibodies (green), rhodamine-phalloidin (red) and DAPI (blue). The arrow and arrowhead indicate peripheral and dorsal ruffles, respectively. Bar, 30 μm.
Figure 13. Stimulation with PDGF induces phosphorylation of Ser 602 of EPLIN at peripheral and dorsal ruffles. Serum-starved NIH3T3 cells were stimulated with 50 ng/ml PDGF for the indicated times in the presence or absence of a 30-min pretreatment with 20 μM U0126. Cells were fixed and stained with anti-pS602 antibodies (green), Alexa Fluor 647-phalloidin (red) and anti-p-ERK antibodies (grey). The arrowheads indicate dorsal ruffles. Bar, 30 μm.
Figure 14. EPLIN is phosphorylated at the leading edge of migrating fibroblasts during recovery from wounding. After scratching a monolayer of NIH3T3 cells for 0 or 6 h in the presence or absence of a 30-min pretreatment with 20 μM U0126, cells were fixed and doubly stained with the anti-pS360 (A) or anti-pS602 (B) antibody and rhodamine-phalloidin (red). The wound site is at the left of each panel. Bars, 50 μm.
Figure 15. Expression of EPLINα(S360/602/692A)-EGFP reduces PDGF-induced membrane ruffling. NIH3T3 cells transfected with EPLINα-EGFP or EPLINα(S360/602/692A)-EGFP were stimulated with 50 ng/ml PDGF for 0 or 5 min. Cells were fixed and stained with rhodamine-phalloidin and DAPI to detect F-actin (red) and DNA (blue), respectively. Bar, 30 μm.
Figure 16. NIH3T3 cells transfected with EGFP, EPLINα-EGFP or EPLINα(S360/602/692A)-EGFP were stimulated with 50 ng/ml PDGF for 0 or 5 min. The degree of ruffling was categorized into four classes exemplified by the bottom panel. At least 100 cells were counted per sample in three independent experiments. Error bars represent s. d.
**Figure 17.** At 6 h after wounding a confluent monolayer of NIH3T3 cells transfected with EPLInα-EGFP or EPLInα(S360/602/692A)-EGFP, cells were fixed and stained with rhodamine-phalloidin and DAPI to detect F-actin (red) and DNA (blue), respectively (upper). The wound site is at the left of each panel. Bar, 50 μm. At the indicated times after wounding, the proportion of EGFP-positive cells at the wound margin was assessed (lower).
Figure 18. NIH3T3 cells were transfected with GFP, EPLINα-EGFP or EPLINα(S360/602/692A)-EGFP. After 24 h, a modified Boyden chamber assay was performed in the presence or absence of 30 ng/ml PDGF in the lower chamber. At least 100 cells were counted per sample in three independent experiments. Error bars represent s. d.
Figure 19. A schematic representation of the proposed mechanism by which ERK-mediated phosphorylation of the C-terminal region of EPLIN leads to reorganization of actin filaments.