Cell adhesion regulates Ser/Thr phosphorylation and proteasomal degradation of HEF1

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Summary
Human enhancer of filamentation 1 (HEF1), a multifunctional docking protein of the Cas family, participates in integrin and growth factor signaling pathways that regulate global cellular processes including growth, motility and apoptosis. HEF1 consists of two isoforms, p105 and p115, the larger molecular weight form resulting from Ser/Thr phosphorylation of p105HEF1. The molecular mechanisms that regulate the interconversion of the two HEF1 species as well as the function of HEF1 Ser/Thr phosphorylation are unknown. Our study reveals that cell adhesion and detachment regulate the interconversion of the two HEF1 isoforms. Experiments using various inhibitors of cytoskeletal organization indicated that disruption of actin microfilaments but not intermediate filaments or microtubules resulted in a complete conversion of p115HEF1 to p105HEF1. The conversion of p115HEF1 to p105HEF1 was prevented by inhibition of protein phosphatase 2A (PP2A), suggesting that cytoskeletal regulation of PP2A activity controlled the dephosphorylation of p115HEF1. Degradation of endogenous HEF1 was dependent on proteasomes with the p115 species of HEF1 being preferentially targeted for turnover. Dephosphorylation of HEF1 by suspending cells or disrupting actin filaments protected HEF1 from degradation. These results suggest that the adhesion-dependent actin organization regulates proteasomal turnover of HEF1 through the activity of PP2A.

Key words: Human enhancer of filamentation 1 (HEF1), Protein phosphatase 2A (PP2A), Actin cytoskeleton, Cell adhesion, Proteasome

Introduction
Cells adhere to matrix proteins through transmembrane receptors of the integrin family, which serve as anchors for both the extracellular matrix and the intracellular actin cytoskeleton to form a structural framework that defines cell morphology and function. Upon cell adhesion and integrin clustering, the cytoplasmic tail of the integrin receptor interacts with signaling molecules and docking proteins to form the focal adhesions that relay ‘outside in’ and ‘inside out’ signaling through the integrin receptors (Liddington and Ginsberg, 2002; Schwartz and Ginsberg, 2002). Multi-functional-domain docking proteins of the Cas family play a crucial role in adhesion signaling. Upon integrin ligation, Cas family proteins localize to focal adhesions and become tyrosine phosphorylated, resulting in the regulated recruitment of SH2-containing signaling proteins to focal adhesions (Cary and Guan, 1999; Nakamoto et al., 1997; O’Neill et al., 2000; Sakai et al., 1994). The members of the Cas family include p130Cas, Efs/Sin and human enhancer of filamentation 1 (HEF1, also known as Cas-L). HEF1 was initially identified as a human protein that promotes changes in actin organization of mammalian cells (Fashena et al., 2002; Law et al., 1996; O’Neill et al., 2000). The amino acid sequence of HEF1 is 64% similar to that of p130Cas. Both contain a number of protein-protein-interaction domains including an N-terminal SH3 domain, a substrate domain, a serine-rich domain and a helix-loop-helix motif in the C-terminus (O’Neill et al., 2000). In epithelial cells, HEF1 is post-translationally processed to p115, p105, p65, p55 and p28 protein forms. The p115 and p105 forms are generated through differential Ser/Thr phosphorylation whereas the last three low-molecular-weight forms are generated by a caspase-dependent cleavage (Law et al., 1998; Law et al., 2000). The p55 species of HEF1 is produced specifically at mitosis, and associates with the mitotic spindle where it interacts with Dim1p, a G2-M spindle-regulatory protein (Law et al., 1998). The p28 form is generated from the C-terminal of HEF1 during mitosis and regulates apoptosis by mediating the disassembly of the focal adhesion (O’Neill and Golemis, 2001). Cleavage of HEF1 to lower molecular-weight forms can be prevented by integrin clustering and focal-adhesion formation (O’Neill and Golemis, 2001). These findings suggest that, the generation of various forms of HEF1 is carefully regulated to allow for their participation in distinct cellular processes and cell adhesion is involved in this posttranslational modification process.

HEF1 is a substrate for FAK and Src family tyrosine kinases and is thought to participate in signaling pathways regulated by integrin receptors (Kanda et al., 1999; Sattler et al., 1997; Tachibana et al., 1997). Phosphorylated HEF1 may function as a downstream effector of FAK to promote integrin-dependent cell motility (Law et al., 2000; van Seventer et al., 2001). Molecular genetic experiments have indicated that overexpression of HEF1 induces changes in cell morphology and motility (Fashena et al., 2002), suggesting a role for HEF1 in regulating cytoskeletal organization and integrin function. Earlier studies have proposed that HEF1 is expressed primarily in cells of lymphoid and epithelial origin (Law et al., 1996).
Recently, we have shown that both p105HEF1 and p115HEF1 are expressed at low levels in dermal fibroblasts, and that their expression in these cells is greatly upregulated by TGF-β1 (Zheng and McKeown-Longo, 2002). Functions of the two HEF1 isoforms are not well understood. Recent studies using overexpression systems have defined a possible role for p115HEF1 in modulating TGFβ1 signaling. In these studies, p115HEF1 was shown to undergo ubiquitination and proteasomal degradation following complex-formation with Smad3 and the ubiquitin ligase AIP4 (Feng et al., 2004; Liu et al., 2000). The molecular pathway that governs the interconversion between p105 and p115 HEF1, and the role of Ser/Thr phosphorylation in HEF1 function are unknown. In this study, we show that the interconversion between p105 and p115 HEF1 is determined by actin organization and protein phosphatase 2A (PP2A), and that the proteasomal degradation of HEF1 is regulated by HEF1 Ser/Thr phosphorylation.

Results
Proteasome inhibitors inhibit endogenous HEF1 degradation and result in p115HEF1 accumulation

We investigated the role of the proteasome on the degradation of endogenous p115 and p105HEF1 in two cell lines, human foreskin fibroblasts (A1-F) and human osteosarcoma (MG-63). As shown in Fig. 1A, treatment of both A1-F (left panel) and MG-63 cells (right panel) with either of two proteasome-specific inhibitors, lactacystine (LAC) or N-acetyl-leu-leu-norleucinal (LLnL), resulted in a large increase in the level of p115HEF1 protein. A control, N-acetyl-leu-leu-methional (ALLM) had no effect on HEF1 levels. The half-life of HEF1 protein was evaluated using cycloheximide-treated MG-63 cells (Fig. 1B). In the presence of cycloheximide, levels of both HEF1 isoforms rapidly decreased. By 8 hours after the addition of cycloheximide, p105 and p115HEF1 levels were reduced more than 90%. In the presence of the proteasome inhibitor LAC, p115HEF1 protein levels remained relatively stable, increasing slightly over the 8-hour period. By contrast, p105HEF1 protein levels decreased. The decrease in p105HEF1 was due to the conversion of p105HEF1 to p115HEF1 because total HEF1 protein was not significantly changed in the presence of proteasome inhibitor (data not shown). These data indicate that inhibition of proteasomal activity blocks HEF1 degradation, resulting in the accumulation of the p115 isoform and suggesting that Ser/Thr

Fig. 1. Proteasome inhibitors prevent HEF1 degradation and result in p115 HEF1 accumulation in A1-F and MG-63 cells. (A) MG-63 and A1-F cells were plated onto FN-coated tissue culture dishes in DMEM supplemented with 10% FBS and grown to confluence. Cell layers were treated with 10 μM lactacystin (LAC), 50 μM LLnL, 50 μM ALLM or carrier solvent DMSO (Cont) for 14 hours. (B) MG-63 cells on FN-coated dishes were serum-starved for 6 hours and then treated with TGF-β1 (2.5 ng/ml) for 12 hours to induce HEF1 expression (0 hours). TGF-β1-containing medium was removed and cell layers were washed twice with serum-free medium. Cells were then detached and suspended in serum-free medium for indicated times. Cell lysates were prepared and processed for immunoblotting with anti-HEF1 pAb. Nitrocellulose membranes were stripped and reprobed with anti-p130Cas mAb as a loading control.

Fig. 2. The interconversion of p105 and p115 HEF1 is regulated by cell adhesion and detachment. (A) A1-F monolayers plated on FN-coated dishes were serum-starved overnight and treated with TGF-β1 (2.5 ng/ml) for 4 hours to induce HEF1 expression (0 hours). Cells were then detached and suspended in serum-free medium for indicated times. (B) After suspension for 2 hours (indicated as 0 hours of adhesion), A1-F cells were plated onto FN-coated dishes for indicated times. (C) After having been treated with TGF-β1 (2.5 ng/ml) for 4 hours, A1-F monolayers were detached and suspended in serum-free medium for 1 hour. Suspended cells were then plated onto tissue culture dishes that had been coated with polylysine (PL), fibronectin (FN), collagen (Col), laminin (LM) or vitronectin (VN) and incubated for 2 hours. Cell lysates were prepared and processed for immunoblotting with anti-HEF1 pAb. Nitrocellulose membranes were stripped and reprobed with anti-p130Cas mAb as loading control.
phosphorylation of HEF1 might regulate its turnover within the cell.

Actin organization regulates the conversion of p115HEF1 into p105HEF1

Experiments were designed to address the role of cytoskeletal organization on HEF1 Ser/Thr phosphorylation. As shown in Fig. 2, when human dermal fibroblasts were detached and maintained in suspension, the Ser/Thr-phosphorylated isoform p115HEF1 disappeared within 2 hours. The loss of p115HEF1 was accompanied by a corresponding increase in p105HEF1 (Fig. 2A). By contrast, p130Cas levels were unchanged by cell suspension and served as a loading control. Replating of suspended cells onto fibronectin-coated dishes resulted in a complete reappearance of p115HEF1 within 2 hours, accompanied by a concomitant decrease in p105HEF1 (Fig. 2B). These results suggest that suspension and readhesion regulate the interconversion of p105 and p115HEF1. To investigate whether the adhesion-dependent appearance of p115HEF1 correlated with ligation of a specific integrin receptor, cells were plated onto dishes that had been coated with fibronectin, collagen, laminin or vitronectin and incubated for 2 hours. As seen in Fig. 2C, all of these substrates supported the restoration of p115HEF1 protein levels – albeit not to exactly the same levels, suggesting some influence of extracellular matrix on the levels of each isoform. Adhesion of cells to polylysine was unable to support cell-spreading and did not restore p115HEF1 levels. These data suggest that the presence of p115HEF1 depends primarily on an appropriate cell morphology rather than ligation of a particular integrin receptor. To elucidate which cytoskeletal structures might be important for the maintenance of p115HEF1 levels, cells were incubated with reagents that selectively disrupt different cytoskeletal filaments. As shown in Fig. 3A, increasing doses of either nocodazole or colchicine, which disrupt microtubules at low concentration and microtubules as well as intermediate filaments at high concentration, did not change relative levels of p105 and p115HEF1; microtubules and intermediate filaments, however, were disrupted (Fig. 3B). Treatment with the actin-filament-disrupting drugs cytochalasin D or latrunculin A resulted in loss of p115HEF1 within two hours (Fig. 3A) without altering either microtubules or intermediate filaments (Fig. 3B). Similar to results obtained in cell suspension and replating experiments (Fig. 2), cytochalasin D or latrunculin A induced a loss of p115HEF1 that was accompanied by a corresponding increase in p105HEF1. These results suggest that the disruption of actin-filament organization activates a Ser/Thr phosphatase responsible for dephosphorylation of p115HEF1, thereby converting p115HEF1 into p105HEF1. To evaluate whether proteasomal degradation contributes to the cytochalasin-D-induced disappearance of p115HEF1, proteasome inhibitors were evaluated for their effect on the cytochalasin-D-induced decrease in p115HEF1. Cells were pre-treated with the proteasomal inhibitor LAC before being treated with cytochalasin D. Consistent with results shown in Fig. 1, LAC treatment resulted in an increase in p115HEF1 (Fig. 4, lane 2).
Adhesion regulates HEF1 degradation

However, LAC had no effect on the cytochalasin-D-induced loss of p115HEF1 (Fig. 4, lane 4), suggesting that the cytochalasin-D-induced disappearance of p115 HEF1 resulted from the dephosphorylation of p115 HEF1 rather than the selective proteasomal degradation of p115 HEF1. These data indicate that the level of p115HEF1 within the cell might be regulated by an actin-cytoskeleton-dependent phosphatase.

HEF1 turnover under adhesion, suspension or cytochalasin-D-treatment conditions was evaluated to determine whether the conversion of p115HEF1 to p105HEF1 prolonged HEF1 half-life. Adherent cells in the presence or absence of cytochalasin D and suspended cells were incubated with cycloheximide to inhibit protein synthesis. HEF1 levels were detected by western blot and analyzed with densitometry (Fig. 5). After 8 hours of cycloheximide treatment, there was little detectable HEF1 in the control culture of adherent cells. By contrast, there was only a slight change in the levels of p130Cas protein, which served as a loading reference. Cells placed in suspension or treated with cytochalasin D showed a complete loss of p115HEF1 within 2 hours. Loss of p115HEF1 was associated with an increase in p105HEF1 levels, which, however, remained relatively constant during 8 hours of cycloheximide treatment (Fig. 5). In control experiments, these treatments did not affect degradation of Smad3, which has been shown to be degraded via the proteasomal pathway (Fukuchi et al., 2001), suggesting that suspension and cytochalasin D had no inhibitory effect on proteasome activity. Taken together, these data indicate that the conversion of p115 HEF1 to p105HEF1 prolongs the half life of HEF1 by removing HEF1 from proteosomal degradation pathways.

Protein PP2A-dependent dephosphorylation of p115HEF1

Preliminary experiments using 32P-labeled myelin basic protein (MyBP) as a substrate indicated that PP1 and PP2A accounted for 95% of the total Ser/Thr-protein-phosphatase activity in human dermal fibroblasts (data not shown). Okadaic acid, calyculin A and tautomycin are potent inhibitors of PP1 and PP2A (Favre et al., 1997). To determine whether PP1 and/or PP2A are involved in the conversion of p115HEF1 to p105HEF1, selective inhibitors of each of these phosphatases were compared for their effects on the cytochalasin-D-induced loss of p115HEF1. As shown in Fig. 6, pretreatment of cells with either 10 nM calyculin A (panel A) or 200 nM okadaic acid (panel B) – both selective inhibitors of PP2A (Cohen, 1991) – was able to block the cytochalasin-D-induced dephosphorylation of p115HEF1. The dephosphorylation of p115HEF1 induced by cytochalasin D was unaffected by pretreatment of cells with 7.5 μM tautomycin (Fig. 6C) – a selective inhibitor of PP1 (MacKintosh and Klumpp, 1990). These data suggest that PP2A regulates the cytochalasin-D-induced dephosphorylation of p115HEF1. To confirm the inhibitory effects of the reagents on intracellular PP2A and PP1, the activities of PP2A and PP1 were measured in the extracts of cells that had been previously treated with various conditions.

Fig. 4. Cytochalasin-D-induced conversion of p115HEF1 to p105HEF1 is not due to selective degradation of p115 HEF1. A1-F monolayers on FN-coated dishes were serum-starved overnight and pre-treated with TGF-β1 (2.5 ng/ml) for 4 hours in the presence of 10 μM lactacystin (LAC) or 50 μM ALLM as indicated. 2 μM cytochalasin D (CD) or equal volumes of carrier solvent DMSO (control) were then directly added into pre-treatment medium, and cells were further incubated for 6 hours. Cell lysates were generated and processed for immunoblotting with anti-HEF1 pAb. Nitrocellulose membranes were stripped and reprobed with anti-p130Cas mAb as loading control.

Fig. 5. Cell suspension or cytochalasin D treatment inhibit HEF1 degradation. MG-63 cells on FN-coated dishes were serum-starved for 6 hours and then treated with TGF-β1 (2.5 ng/ml) for 12 hours to induce HEF1 expression. TGF-β1-containing medium was removed and cell layers were washed twice with serum-free medium. (A) Cell layers were then treated with 20 μg/ml cycloheximide in the absence (adhesion) or presence (adhesion + CD) of 2 μM cytochalasin D for indicated times, or detached and plated onto agar-coated dishes in the presence 20 μg/ml cycloheximide (suspension). Times indicate hours after addition of cytochalasin D or placement in suspension. Aliquots of cell lysates containing equal amounts of protein were processed for immunoblotting with anti-HEF1 antibody. Membranes were stripped and reprobed for p130 Cas, which served as a loading control. (B) The levels of total HEF1 as shown in A were analyzed by densitometry and the time at 0 hours was set as 100%.
concentrations of inhibitor. As shown in Fig. 7A, the concentration of okadaic acid required to inhibit PP2A activity, exactly correlated with the concentration required to prevent the cytochalasin-D-induced p115HEF1 dephosphorylation. Treatment of A1-F cells with 200 nM okadaic acid, which abolished the effect of cytochalasin D on p115HEF1 dephosphorylation, resulted in almost complete inhibition of PP2A activity with little effect on PP1. The protein levels of PP1 and PP2A did not change under the cell-treatment condition (Fig. 7B). Treatment of A1-F cells with the PP1 inhibitor tautomycin induced a dose-dependent inhibition of PP1 activity but had less effect on PP2A (Fig. 7C). Although treatment of A1-F cells with 3 μM tautomycin completely inactivated PP1, the dephosphorylation of p115HEF1 induced by cytochalasin D was not affected, even at 7.5 μM tautomycin (Fig. 6C). The protein levels of PP1 and PP2A did not change when cells were treated with various concentrations of tautomycin (Fig. 7D). These results indicate that PP2A but not PP1 is involved in the cytochalasin-D-induced dephosphorylation of p115HEF1. Taken together, these data indicate that p115HEF1 levels are regulated through a cell-adhesion-dependent actin-cytoskeleton organization, which regulates the activity of PP2A and that the PP2A-mediated conversion of p115HEF1 to p105HEF1 regulates the half-life of HEF1 in the cell.

Discussion

HEF1 is a Cas-family-member docking protein, which serves as a convergence point for signaling pathways that originate from integrins, growth factors and cytokines (Manie et al., 1997; Zhang et al., 2000; Zhang et al., 1999; Zheng and McKeown-Longo, 2002). HEF1 undergoes complex post-translational modifications, which direct its subcellular localization and promote its participation in diverse biological activities including apoptosis and motility (Fashena et al., 2002; Law et al., 1998; Law et al., 2000). HEF1 localizes to focal adhesions and has been identified as a downstream component of integrin signaling. Integrin ligation results in the tyrosine phosphorylation of HEF1, which occurs through the activity of integrin-dependent tyrosine kinases such as FAK, Src or PTK2B, also known as RAFTK (Astier et al., 1997; Manie et al., 1997; Tachibana et al., 1997; Zheng and McKeown-Longo, 2002). These phosphorylation events are important for the docking function of HEF-1 because the phosphorylated form of HEF1 provides binding sites for the SH-2 domains of crk and zyxin family members (Sattler et al., 1997; Yi et al., 2002), which probably contribute to HEF1’s role in lymphocyte migration (Ohashi et al., 1999). Ser/Thr phosphorylation of p105HEF1 results in the p115 isoform of HEF1 (Law et al., 1998; Zheng and McKeown-Longo, 2002). A distinct function for this isoform of HEF1 has been suggested based on studies, which show that p115HEF1 but not p105HEF1 forms complexes with Smad3. These complexes are cleared from the cell by proteosomal degradation, implicating HEF1 in modulating TGF-β signaling pathways (Liu et al., 2001). Half-life experiments indicate that there is a rapid turnover of a few hours of both the p105 and p115 species of endogenous HEF1. Treatment of either osteosarcoma or fibroblast cells with proteosome inhibitors results in a several-fold increase in the level of p115HEF1, consistent with this species of HEF1 being preferentially degraded by proteosomes (Liu et al., 2001).

Our previous studies indicate that cell adhesion and cytoskeletal organization regulate the interconversion of p105 and p115 HEF1 (Zheng and McKeown-Longo, 2002). Reversible phosphorylation of proteins is an important mechanism for the regulation of cellular signal transduction (Hunter, 1995). Although the role of protein kinases in the regulation of adhesion-related signaling has been extensively studied (Howe et al., 1998; Schwartz and Assoian, 2001), the role of protein phosphatases, especially Ser/Thr phosphatases, in the transduction of cell-adhesion dependent signals is not well understood. We show here that the conversion of p115HEF1 to p105HEF1 is regulated by actin-microfilament organization because agents that disrupt intermediate filaments or microtubules did not affect the levels of either HEF isoform. The loss of p115HEF1 in response to cytochalasin D is not due to preferential degradation of p115HEF1 because proteosomal...

Fig. 6. The effects of Ser/Thr phosphatase inhibitors on the cytochalasin-D-induced loss of p115HEF1. (A–C) A1-F cells were plated onto FN-coated dishes, serum-starved overnight and then incubated for 4 hours with DMEM containing 0.1% heat-inactivated BSA and 2.5 ng/ml TGF-β1 (Contr). After incubation of cells with indicated concentration of calyculin A for 0.5 hour (A), or okadaic acid for 2 hours (B), or tautomycin for 14 hours (C), the cells were treated with 2 μM cytochalasin D for 2 hours in the presence of the inhibitors. Aliquots of cell extracts containing equal amount of protein were analyzed by western blotting with anti-HEF1 pAb. Nitrocellulose membranes were stripped and reprobed with anti-actin pAb to ensure equal loading.
inhibitors had no effect on the conversion of p115 to p105HEF1. Treatment of cells with inhibitors of the Ser/Thr protein phosphatase PP2A, completely inhibited the conversion of p115 to p105HEF1 in response to either cell-detachment or treatment with cytochalasin D. PP2A is an abundant Ser/Thr protein phosphatase, which participates in pathways regulating cell-cycle progression and apoptosis (Millward et al., 1999; Schonthal, 1998; Sontag, 2001). In this study, we demonstrate that PP2A-dependent dephosphorylation of p115HEF1 is induced by cell detachment or actin-disrupting drugs. Because p115HEF1 forms a complex with Smad3 to dampen TGF-β signal transduction, PP2A might be a positive regulator of TGF-β signaling pathways. A similar pathway has been described in HEK299 cells where cell detachment upregulates PP2A activity and attenuated IFN-γ signaling (Ivaska et al., 2003). PP2A activity has been linked to specific integrin signaling pathways in T lymphocytes, where α2β1-dependent activation of PP2A inhibits Fas-induced apoptosis (Gendron et al., 2003). The mechanism by which PP2A triggers dephosphorylation of p115HEF1 is not known. Placing cells in suspension did not result in an increase in the activity of PP2A (M.Z. and P.J.M.-L., unpublished observations). This suggests that PP2A exerts its effect on p115HEF1 following a change in the subcellular localization of either PP2A or p115HEF1. Correlations between the subcellular distribution of PP2A and changes in actin organization have been recently reported (Nakajima et al., 2004). The association of PP2A with β1 integrins is modulated during the differentiation of myoblasts to myotubes (Kim et al., 2004) and during cell mitosis. It is possible that the changes in actin organization that occur during mitosis and during muscle differentiation contribute to the disruption of β1/PP2A complexes.

Adhesion of either fibroblasts or osteosarcoma cells to a range of extracellular matrices results in the conversion of p105 to p115 HIF. These results suggest that HIF1 is a downstream target of integrin dependent Ser/Thr kinases. Integrins are known to regulate the activity of several Ser/Thr kinases including MAP kinases, protein kinases B and C and integrin-related kinases (Aplin et al., 2001; Miranti et al., 1999; Tian et al., 2002; Wu, 1999). Conversion of p105HEF1 to p115HEF1 following cell adhesion to various matrix molecules occurred over a period of 2 hours, consistent with the Ser/Thr phosphorylation of HIF1 being linked to the process of cell spreading. This therefore, suggests that the Ser/Thr kinase lies downstream of actin organization. Agents that disrupt microtubules and intermediate filaments did not affect the levels of either HIF1 isoform, indicating that actin organization is the primary effector of p105HEF1 Ser/Thr phosphorylation. The kinase responsible for the conversion of p105HEF1 to p115HEF1 has not yet been identified but kinases, such as AKT/PKB, that have been shown to be regulated by actin organization are possible candidates (Enomoto et al., 2005). Further studies are needed to elucidate the role of actin-dependent-kinases and -phosphatases in the regulation of HIF1 protein function.

Materials and Methods

Reagents

TGF-β1 was from R&D Systems. Cytochalasin D and phosphorylase b were from Sigma (St Louis, MO). Latrunculin A, nocodazole, colchicine, okadaic acid, calyculin A, tautamycin and proteasome inhibitors were from Biomol (Plymouth Meeting, PA). Anti-smad3 polyclonal antibody (pAb) was from Zymed Laboratories (South San Francisco, CA). Anti-protein phosphatase 2A monoclonal antibody (mAb) (Clone 24) was from Upstate Biotechnology (Lake Placid, NY). Anti-p130Cas mAb and anti-protein phosphatase 1 mAb (Clone 24) were from BD Transduction Laboratories (Lexington, KY). Anti-vimentin pAb and anti-β-tubulin...
mAb (Clone D10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein phosphatase inhibitor-2 was from New England Biolabs (Beverly, MA). Dye-conjugated secondary antibodies were from Molecular Probes. Phosphorylase kinase was from ICN (Aurora, OH). Laminin was from Chemicon International Inc (Temecula, CA). Type I collagen was from Cohesion Technologies (Palo Alto, CA). Human laminin fibronectin (FN) was purified from a fibronectin- and fibrogenogen-rich byproduct of factor VIII production by ion-exchange chromatography as previously described (McKeown-Longo and Etzler, 1987) and further purified by affinity chromatography with gelatin-agarose and subsequently with heparin-agarose (Engvall and Ruoslahti, 1977). Anti-HEF1 antisera was generated in rabbits with peptide (C1)-PAPTESKVRNPQER linked to KLH as antigen (Zheng and McKeown-Longo, 2002). The specific IgG fraction was purified from antisera by affinity chromatography with peptide-coupled thiopropyl-Sepharose 6B. Vitronecetin was purified from human serum by heparin-Sepharose (Amersham Biosciences) affinity chromatography (Yatohgo et al., 1988).

Cell culture
Human foreskin fibroblasts (A1-F) were obtained from Lynn Allen-Hoffman (University of Wisconsin, Madison, WI) and used between passage 6 to 12. Human osteosarcoma, MG-63 cells were from ATCC. Both cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in an atmosphere of 7.5% CO2. DMEM containing 0.1% heat-inactivated BSA was used as serum starvation medium.

Cell lysis and immunoblot analysis
Cell layers were washed with cold washing buffer (PBS containing 1 mM PMSF, 0.1 mM Na3VO4, and 10 mM NaF) before solubilization in cell-lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% NP-40, 0.1 M NaCl, 40 mM NaF, 30 mM Na3PO4, 2 mM EDTA, 1 mM Na3VO4, 0.5 mM PMSF and one tablet of complete-protease-inhibitor per 10 mL). After incubation on ice for 30 minutes, cell lysates were centrifuged at 20,800 g for 15 minutes at 4°C and the insoluble pellets were discarded. The protein concentration of the lysate was determined using a BCA protein assay reagent (Pierce, Rockford, IL). Aliquots of cell lysates containing equal amount of protein were subjected to SDS-PAGE on a 6.5-8% gradient polyacrylamide gel under reducing conditions, followed by transfer to nitrocellulose membrane. Proteins were detected by western blot with a chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Densitometric analysis was performed by using the Personal Densitometer SI and ImageQuant program (Molecular Dynamics, Sunnyvale, CA). The density-volume was converted to the amount of protein by comparison with a standard protein-densitry curve established (Molecular Dynamics, Sunnyvale, CA) and processed with Photoshop-7 (Intelligent Imaging Innovation, Denver, CO) and processed with Photoshop-7 program (Adobe).

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