The Roles of Integrin-linked Kinase in the Regulation of Myogenic Differentiation

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Abstract. Myogenic differentiation is a highly orchestrated, multistep process that is coordinately regulated by growth factors and cell adhesion. We show here that integrin-linked kinase (ILK), an intracellular integrin- and PINCH-binding serine/threonine protein kinase, is an important regulator of myogenic differentiation. ILK is abundantly expressed in C2C12 myoblasts, both before and after induction of terminal myogenic differentiation. However, a noticeable amount of ILK in the Triton X-100-soluble cellular fractions is significantly reduced during terminal myogenic differentiation, suggesting that ILK is involved in cellular control of myogenic differentiation. To further investigate this, we have overexpressed the wild-type and mutant forms of ILK in C2C12 myoblasts. Overexpression of ILK in the myoblasts inhibited the expression of myogenic proteins (myogenin, MyoD, and myosin heavy chain) and the subsequent formation of multinucleated myotubes. Furthermore, mutations that eliminate either the PINCH-binding or the kinase activity of ILK abolished its ability to inhibit myogenic protein expression and allowed myotube formation. Although overexpression of the ILK mutants is permissive for the initiation of terminal myogenic differentiation, the myotubes derived from myoblasts overexpressing the ILK mutants frequently exhibited an abnormal morphology (giant myotubes containing clustered nuclei), suggesting that ILK functions not only in the initial decision making process, but also in later stages (fusion or maintaining myotube integrity) of myogenic differentiation. Additionally, we show that overexpression of ILK, but not that of the PINCH-binding defective or the kinase-deficient ILK mutants, prevents inactivation of MAP kinase, which is obligatory for the initiation of myogenic differentiation. Finally, inhibition of MAP kinase activation reversed the ILK-induced suppression of myogenic protein expression. Thus, ILK likely influences the initial decision making process of myogenic differentiation by regulation of MAP kinase activation.

Key words: integrin • PINCH • MAP kinase • myogenin • myotubes

Introduction

Terminal myogenic differentiation is a highly orchestrated, multistep process that is controlled by environmental cues including growth factors and the extracellular matrix (McDonald et al., 1995; Sastry and Horwitz, 1996; Wewer and Enevall, 1996; Durbeej et al., 1998; Gullberg et al., 1998; Burkin and Kaufman, 1999). For example, it has been well described that differentiation of myoblasts plated on appropriate extracellular matrix proteins (e.g., collagens) into multinucleated myotubes can be induced by depriving the cells of growth factors (Weintraub, 1993; Lassar et al., 1994; A ndres and Walsh, 1996; B ennett and T onks, 1997).

Extensive studies over the last one and a half decades have demonstrated crucial roles of cell adhesion receptors, including integrins, in the regulation of terminal myogenic differentiation (McDonald et al., 1995; Sastry and Horwitz, 1996; Gullberg et al., 1998; Burkin and Kaufman, 1999). In genetic model systems such as Drosophila and...
Caenorhabditis elegans, it has been well documented that integrins are involved in sarcomere formation and stabilization or muscle cell attachment (Volk et al., 1990; Gettner et al., 1995; Martin-Bermudo and Brown, 1996; Bloor and Brown, 1998; Burch et al., 1998; Gullberg et al., 1998; Prokop et al., 1998). In vertebrates, a number of integrins are expressed in muscle cells, and the expression level, subtype, and activation state of the integrins are precisely regulated during myogenesis (Boettiger et al., 1995; Sastry and Horwitz, 1996; Gullberg et al., 1998; Burkin and Kaufman, 1999). A ntibody ligation of specific β1 integrins inhibits vertebrate myoblast differentiation (Menko and Boettiger, 1987; Rosen et al., 1992). Furthermore, gene transfer experiments have demonstrated that myoblast differentiation and proliferation are regulated by β1 integrins (Sastry et al., 1996) and this regulation is achieved through the β1 integrin cytoplasmic domain (Sastry et al., 1999). Studies using chimeric transgenic mice that were α5 integrin−/−; +/+ showed that the α5 −/− cells were able to contribute to skeletal muscle, but the myofibers were unstable, resulting in a form of muscular dystrophy (Taverna et al., 1998). Similar results showing a mild muscular dystrophy were obtained with a targeted deletion of the α7 integrin chain (Mayer et al., 1997), and mutations in the human integrin α7 gene lead to a congenital myopathy (Hayashi et al., 1998). Thus, integrins function in terminal myogenic differentiation by participating in both the initial decision making process and the later morphogenic processes such as cell fusion and maintaining the integrity of myotubes.

Integrin-linked kinase (ILK) is a focal adhesion serine/threonine protein kinase that interacts with β1 integrins through the COOH-terminal domain (Hannigan et al., 1996; D’edhia et al., 1999) and PINCH, an adaptor protein comprising five LIM domains, through the NH2-terminal ankyrin (ANK1) repeat domain (Tu et al., 1999; Wu, 1999). The ILK-binding site has been mapped to the COOH-terminal zinc finger, which is located within the first LIM domain of PINCH (Li et al., 1999a; Wu, 1999). The ILK–PINCH interaction is required for proper subcellular localization of ILK (Li et al., 1999a; Wu, 1999). Furthermore, it may also connect ILK with components of the growth factor and small GTPase signaling pathways via other PINCH-binding proteins such as Nck-2 (Tu et al., 1998; Wu, 1999). Recent biochemical and functional studies have indicated that ILK serves as a mediator in integrin-mediated signal transduction (Hannigan et al., 1996; Radeva et al., 1997; Deconomene et al., 1998; Novak et al., 1998; Wu et al., 1998; Troussard et al., 1999; Tu et al., 1999; Wu, 1999). In this study, we have investigated the roles and potential mechanisms of ILK in cellular control of terminal myogenic differentiation.

Materials and Methods

Antibodies

Mouse monoclonal anti-ILK antibody 65.1 was generated as previously described (Li et al., 1999a). Mouse monoclonal anti-FLAG antibody M5 was from Eastman Kodak Co. Mouse monoclonal antimyogenin F5D and anti–MyoD 5.8A were from Pharmingen. Hybridoma for monoclonal anti–myosin heavy chain (MHC) antibody MF20 was obtained from the Developmental Studies Hybridoma Bank. Rabbit polyclonal anti-FAK antibody C-20 was purchased from Santa Cruz Biotechnology, Inc. Anti-tyrosine phosphorylation (2C6aHR PO) was purchased from Transduction Laboratories. Rabbit polyclonal anti-p44/42 MAPK and anti–phospho-p44/42 MAPK (Thr202/Tyr204) antibodies were purchased from New England Biolabs. HRP-conjugated goat anti–mouse IgG and goat anti–rabbit IgG were purchased from Jackson Immunoresearch Laboratories.

Cell Culture and Myogenic Differentiation

Mouse C2C12 myoblasts (American Type Culture Collection) were maintained at subconfluent densities in growth medium (GM) consisting of DMEM (Life Technologies) supplemented with 10% FBS (Sigma Chemical Co.). To induce terminal myogenic differentiation, cells were seeded into collagen I–coated plates (Becton Dickinson), and were shifted to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (Sigma Chemical Co.) when the cells reached 70–80% confluence.

Construction and Transfection of Wild-type and Mutant Forms of FLAG-tagged ILK

To create NH2-terminal FLAG epitope-tagged proteins, cDNA encoding the mouse wild-type ILK (residues 1–452) and the PINCH-binding defective ANK1 deletion mutant (residues 66–452, referred to as ANK1 N1), respectively, were cloned into a mammalian expression vector pFLAG-CMV-2 (Eastman Kodak Co.) as described previously (Li et al., 1999a). A cDNA encoding the human kinase-deficient ILK mutant (referred to as KD) containing a single mutation (Glu156→Lys) was PCR amplified from the recombinant plasmid GH31R (Novak et al., 1998; Wu et al., 1998) and cloned into pFLAG-CMV-2 using EcoRI-SalI sites.

To generate stable transfectants, C2C12 cells were cotransfected with pFLAG-CMV-2 vectors containing ILK, ANK1, or KD cDNAs, or pFLAG-CMV-2 vector lacking the ILK sequence as a control, and pcDNA3 (Invitrogen) carrying a neomycin-resistant marker at a ratio of 10:1, using the LipofectAmine PLUS reagent (Life Technologies). C2C12 cells expressing the FLAG-tagged wild-type and mutant forms of ILK were selected with 1 mg/ml of G418 (Life Technologies) and cloned as described previously (Li et al., 1999b). A total of five FLAG-ILK-expressing clones (C27, E13, F6, F31, and F41), three FLAG-ANK1-expressing clones (G2, G24, and G43), and three FLAG-KD-expressing clones (B38, E20, and H15) were isolated independently. The cells were maintained in culture medium containing 200 μg/ml of G418.

Immunoblotting

Cells were washed twice with PBS and lysed in ice-cold RIPA extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 0.25% sodium deoxycholate, 2 mM EDTA, and 2 mM EGTA) containing protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (0.2 mM), 10 μg/ml aprotinin, 1 μM pepstatin, and 5 μM leupeptin (Sastry et al., 1999) unless otherwise specified. The cell lysates were clarified by centrifugation at 10,000 g for 15 min. Protein concentration of the clarified lysates was determined using bicinchoninic acid (BCA) protein assay reagents (Pierce Chemical Co.). Proteins (5–15 μg) were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with TBS-T buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and incubated with primary antibodies (0.5–1 μg/ml) as specified in each experiment. A total of three to six washes with TBS-T, the membranes were incubated with secondary antibodies (1:10,000 dilution) and washed, and the bound antibodies were detected with a chemiluminescent substrate (Pierce Chemical Co.).

For immunoblotting analysis of phospha-p44/42 MAPK, cells cultured in GM or DM for 3 h were lysed in the RIPA buffer containing protease inhibitors (as described above) and phosphatase inhibitors (30 mM sodium pyrophosphate, 100 mM NaF, 2 mM sodium orthovanadate). A total amount (8 μg/lane) of the cell lysates was separated on 8% SDS-PAGE gels. A total of three to six washes with TBS-T, the membranes were blocked with 2% BSA in TBS-T. A total of three to six washes with TBS-T, the membranes were incubated with secondary antibodies (1:10,000 dilution) and washed, and the bound antibodies were detected with a chemiluminescent substrate (Pierce Chemical Co.).

For immunoblotting analysis of ILK, parental C2C12 cells were cul-
tered in GM or induced to differentiate in DM for 6 d and harvested. Half of the cells was lysed in PBS, pH 7.4, containing 1% Triton X-100 and protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (0.2 mM), 10 \( \mu \)g/ml aprotinin, 1 \( \mu \)g/ml leupeptin, and 5 \( \mu \)g/ml pepstatin. The other half was lysed in PBS, pH 7.4, containing 1% SDS and the protease inhibitors. An equal amount (10 \( \mu \)g/lane) of the cell lysates was separated on 10% SDS-PAGE gels, and ILK was detected by immunoblotting with a monoclonal anti-ILK antibody 65.1 (Li et al., 1999a).

For myogenin and MyoD immunoblotting, cells cultured in GM or induced to differentiate in DM for 4 d were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% SDS, 2 mM EDTA, 2 mM EGTA) containing protease inhibitors as described above. An equal amount (15 \( \mu \)g/lane) of the cell lysates was separated on 10% SDS-PAGE gels. The membranes were first immunoblotted with an antomyogenin mAb 5D5 or anti-MyoD mAb S8A, and were reprobed with anti-MHC mAb F20, anti-FLAG mAb M5, or anti-FAK polyclonal antibody C-20 as specified in each experiment.

### Immunoprecipitation

Cells cultured in GM or in DM for 4 d were lysed in the RIPA buffer containing protease inhibitors and phosphatase inhibitors as described above. The cell lysates (300 \( \mu \)g) were mixed with 10 \( \mu \)l of polyclonal anti-FAK antibody C-20 (2 \( \mu \)g) in a final volume of 500 \( \mu \)l. The samples were incubated at 4°C for 2 h with continuous agitation. 10 \( \mu \)l of U1ralink immobilized protein G (Pierce Chemical Co.) was added and incubated at 4°C for an additional 2 h. The beads were pelleted gently and washed four times with RIPA buffer. The precipitated proteins were released from the beads by boiling in 60 \( \mu \)l of SD-S-PAGE sample buffer for 5 min. Equal volumes of the samples were loaded onto SD-S-PAGE. Total FAK protein and the tyrosine-phosphorylated FAK were detected by immunoblotting with anti-FAK antibody C-20 and antiphosphotyrosine antibody RC20:HRPO (Transduction Laboratories), respectively.

### Inhibition of p44/42 MAPK Activity

The activation of MAPK in C2C12 cells was inhibited by treatment of the cells with specific MEK inhibitor PD98059 (New England BioLabs) based on a previously described method (Sastry et al., 1999). In brief, ILK-overexpressing and parental C2C12 cells were cultured in GM or DM in the presence or absence of 25 \( \mu \)M PD98059 for a period of time, as specified in each experiment, and lysed with the RIPA buffer. MAPK activation and myogenin expression were analyzed by immunoblotting with anti-phosphoThr202/Tyr204) p44/42 MAPK antibody and anti-myogenin antibody F5D, respectively, as described above.

### Nuclear Staining

Cells were cultured in GM or DM for 4 d in a 24-well tissue culture plate. The cells were rinsed with PBS, fixed with 4% paraformaldehyde solution in PBS for 20 min at room temperature, and incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min at 4°C. After rinsing with PBS, the cells were incubated with 20 \( \mu \)g/ml of Hoescht 33258 (Sigma Chemical Co.) in PBS for 5 min and observed under a fluorescence microscope.

### Results

#### ILK Regulates Terminal Myogenic Differentiation

To begin to investigate the roles of ILK in regulation of terminal myogenic differentiation, we analyzed the cellular levels of ILK in C2C12 myoblasts before and after induction of myogenic differentiation by immunoblotting with a monoclonal anti-ILK antibody. A bundant ILK was detected in Triton X-100 lysates of C2C12 myoblasts cultured in growth medium (Fig. 1, lane 1). A dititional slower migrating bands, which could represent either detergent-resistant ILK-containing complexes or other ILK-related proteins (Li et al., 1999a), were also detected in the C2C12 lysates (Fig. 1). A fter switching to differentiation medium for 6 d, the amount of ILK in the Triton X-100 lysates of C2C12 myoblasts was noticeably reduced (Fig. 1, compare lane 2 with lane 1). The reduction of the ILK level in the Triton X-100 lysates accompanying terminal myogenic differentiation could result from a decrease of overall cellular ILK or, alternatively, from a more selective reduction of ILK in the Triton X-100-soluble fractions including membrane and cytosolic fractions. To test this, we extracted the total cellular proteins from the C2C12 cells with SDS. Immunoblotting analyses of the SDS extracts indicated that the overall cellular level of ILK was not decreased after induction of terminal myogenic differentiation (Fig. 1, lanes 3 and 4). Taken together, these results indicate that the amount of ILK in the Triton X-100-soluble subcellular fractions, but not the overall cellular level of ILK, was decreased accompanying terminal myogenic differentiation.

The correlation between the downregulation of ILK level in the Triton X-100-soluble fractions and the terminal myogenic differentiation suggests that ILK is likely involved in the regulation of myogenic differentiation. To investigate this, we overexpressed an epitope (FLAG)-tagged ILK in C2C12 myoblasts. C2C12 myoblasts were transfected with an expression vector containing the full-length ILK coding sequence under the CMV promoter (pFLAG-G-ILK). The FLAG-ILK transfectants were selected with G418 and cloned as described previously (Li et al., 1999b). A total of five FLAG-ILK-expressing C2C12 clones (C27, E1.3, F6.2, F31, and F41) were independently obtained. The expression of FLAG-ILK in the C2C12 cells before and after the induction of myogenic differentiation was confirmed by immunoblotting (Fig. 2 a, lanes 6–15). No FLAG-ILK was detected in the parental C2C12 cells (Fig. 2 a, lanes 1–5) or the vector-only control transfectants (Fig. 2 a, lanes 16–20).

One of the critical events at the initiation of terminal myogenic differentiation is induction of myogenin, which is a member of the MyoD family of skeletal muscle-specific, basic helix-loop-helix transcription factors (Lassar et al., 1994). As expected, myogenin was not detected in C2C12 cells grown in growth medium (Fig. 2 b, lanes 1, 6, 11, and 16). Myogenin expression was induced in the parental C2C12 cells and the vector-only control transfectants after they were shifted to differentiation medium (Fig. 2 b, lanes 1–5 and 16–20). By contrast, the induction of myogenin expression was almost completely inhibited in C2C12 cells overexpressing FLAG-ILK (Fig. 2 b, lanes
overexpress FLG-ILK failed to form multinucleated myotubes under identical experimental conditions (Fig. 4, f and h). Thus, consistent with an inhibitory role of ILK in myogenic protein expression, overexpression of FLG-ILK in C2C12 myoblasts suppresses the formation of multinucleated myotubes.

**The Kinase Activity of ILK Is Required for Suppression of Myogenic Differentiation**

ILK contains four ankyrin repeats at the NH₂ terminus and a protein kinase catalytic domain at the COOH terminus (Hannigan et al., 1996; Li et al., 1997; Dedhar et al., 1999; Wu, 1999). To test whether the ILK kinase catalytic activity is involved in suppression of myogenic differentiation, we expressed a FLG-ILK kinase-deficient (KD) ILK point mutant (Novak et al., 1998; Wu et al., 1998), in which the highly conserved Glu×39 within the ILK catalytic domain was substituted with lysine, in C2C12 cells. The expression of FLG-KD in the transfectants before and after induction of myogenic differentiation was confirmed by immunoblotting (Fig. 5, a, lanes 5–8). Overexpression of the kinase-deficient ILK mutant, unlike that of the wild-type ILK (Figs. 5, b and c, lane 4), did not inhibit the expression of myogenin (Fig. 5 b, lanes 6 and 8), MHC (data not shown), or MHC (Fig. 5 c, lanes 6 and 8). Equal protein loading was confirmed by probing the same membranes with a polyclonal anti-FAK antibody (Fig. 5 d). Thus, ablation of the kinase activity relieves the inhibition on the expression of myogenin, MHC, and MHC, indicating that ILK inhibits myogenic protein expression, at least in part, through its catalytic activity.

**Role of the PINCH-binding Activity of ILK in the Suppression of Myogenic Differentiation**

The NH₂-terminal ankyrin repeat domain of ILK mediates interaction with PINCH (Tu et al., 1999), an adaptor protein comprising five LIM domains (Rearden, 1994; Wu, 1999). To assess whether PINCH binding plays a role in suppression of myogenic differentiation, we expressed a FLG-tagged PINCH-binding defective ILK mutant (AA NK1; Li et al., 1999a), in which the first ankyrin repeat is deleted, in C2C12 cells. Expression of FLG-ΔNK1 (Fig. 6 a, lanes 9–12) in the transfectants, but not in the
parental C2C12 or the vector-only control (Fig. 6 a, lanes 1–4), before and after induction of differentiation was confirmed by immunoblotting. After induction of myogenic differentiation, the C2C12 cells that express the PINCH-binding defective ILK mutant (Fig. 6, lanes 10 and 12), like the parental C2C12 cells (Fig. 6, lane 2) or the vector-only transfectants (Fig. 6, lane 4), expressed myogenin (Fig. 6 b) and MHC (Fig. 6 c). In parallel control experiments, as expected, the induction of myogenin and myosin heavy chain was inhibited in cells expressing FLAG-ILK (Fig. 6, b and c, lane 8) but not in those expressing the kinase-deficient ILK mutant (Fig. 6, b and c, lane 6). A analysis of MyoD expression revealed that overexpression of the PINCH-binding defective ILK mutant, unlike that of the wild-type ILK, did not decrease MyoD expression (data not shown). Taken together, these results suggest that in addition to the kinase catalytic activity, the PINCH-binding activity is most likely also required for the suppression of myogenic protein expression.

Expression of the PINCH-binding Defective or Kinase-deficient ILK Mutants Resulted in the Formation of Myotubes with Abnormal Morphology

Extracellular matrix and growth factors not only control the expression of myogenic transcription factors and other
myogenic proteins, but also influence cell fusion and organization of myotubes. Previous studies have suggested that cell adhesion receptors including integrins are involved in cell fusion and organization of myotubes (Menko and Boettiger, 1987; Volk et al., 1990; Rosen et al., 1992; Boettiger et al., 1995; Mayer et al., 1997; Durbeej et al., 1998; Gullberg et al., 1998; Taverna et al., 1998; Burkin and Kaufman, 1999; Montanaro et al., 1999; Tachibana and Hemler, 1999). To assess whether ILK plays a role in the myogenic morphogenesis, we analyzed myotube formation by C2C12 cells overexpressing the kinase-deficient and PINCH-binding defective ILK mutants. The results showed that C2C12 cells overexpressing the kinase-deficient mutant (Fig. 7, c and d) or the PINCH-binding defective mutant (Fig. 7, e and f), unlike those overexpressing FLAG-ILK (Fig. 7, g and h), were able to form multinucleated myotubes after induction of differentiation. However, the myotubes derived from the cells overexpressing the mutant forms of ILK frequently exhibited an abnormal morphology (Fig. 7, c–f). In contrast to myotubes derived from the parental C2C12 (Fig. 7 a) or the vector-only transfectants (Fig. 7 b), in which nuclei were well aligned along the myotubes, we have observed in cells overexpressing the ILK mutants many giant myotubes in which nuclei were clustered (Fig. 7, c–f). These results suggest that ILK, in addition to influencing the initial decision making process, may also play a role in the later stages (cell fusion or organization of myotubes) of terminal myogenic differentiation.

**ILK Influences Initiation of Terminal Myogenic Differentiation through Regulation of p44/42 MAP Kinase (Erk1 and Erk2) Activation**

Integrins control the terminal myogenic differentiation, at least in part, by regulation of MAP kinase activation (Sastry et al., 1999). Consistent with previous studies (Bennett and Tonks, 1997), the amounts of active forms of p44/42
MAP kinases (Erk1 and Erk2) in the parental C2C12 cells (Fig. 8 a, lanes 1 and 2) and the vector-only control cells (Fig. 8 a, lanes 7 and 8) were decreased upon induction of terminal myogenic differentiation. By contrast, the amount of active forms of p44/42 MAP kinases (Erk1 and Erk2) in C2C12 cells overexpressing the wild-type ILK remained high after shifting to differentiation medium (Fig. 8 a, lanes 3–6). Probing the same samples with an anti-p44/42 MAP kinase antibody showed that the total protein level of the p44/42 MAP kinases was not altered by overexpression of ILK or induction of differentiation (Fig. 8 b, lanes 1–8). Equal protein loading was confirmed by immunoblotting with an anti-FAK antibody (Fig. 8 c, lanes 1–8). Because the downregulation of MAP kinase activity is required for myoblasts to initiate terminal myogenic differentiation (Bennett and Tonks, 1997; Sastry et al., 1999), these results suggest that ILK suppresses myogenic differentiation, at least in part, by preventing inactivation of p44/42 MAP kinases.

To further analyze the mechanism by which ILK regulates myogenic differentiation, we examined the effects of overexpression of the kinase-deficient or the PINCH-binding defective ILK mutants on activation of p44/42 MAP kinases. In contrast to C2C12 cells overexpressing the wild-type ILK (Fig. 8 a, lanes 3–6), the amounts of the active forms of p44/42 MAP kinases in C2C12 cells overexpressing the ILK mutants (Fig. 8 a, lanes 9–16) were downregulated after shifting to differentiation medium.
The total protein level of the p44/42 MAP kinases was not altered by overexpression of the ILK mutants (Fig. 8 b, lanes 9–16). Equal protein loading was further confirmed by immunoblotting with an anti-FAK antibody (Fig. 8 c, lanes 9–16). These results indicate that ablation of the kinase activity or the PINCH-binding activity of ILK eliminated its ability to regulate MAP kinase activation. Because neither the kinase-deficient mutant nor the PINCH defective mutant inhibits terminal myogenic differentiation, these results provide additional evidence suggesting that p44/42 MAP kinases serve as downstream effectors of ILK in the regulation of terminal myogenic differentiation. In contrast to the major difference in MAP kinase activation, FAK activation, as indicated by the tyrosine phosphorylation level of FAK, in the ILK-overexpressing C2C12 cells, parental C2C12 cells and the vector-only transfecteds, did not differ under either growth (Fig. 9 a) or differentiation (Fig. 9 b) condition, suggesting that ILK likely regulates MAP kinase activation via a pathway independent of FAK activation. This result is consistent with previous findings showing that the FAK tyrosine phosphorylation level is not altered during the α5β1 integrin-mediated suppression of myogenic differentiation (Sastry et al., 1999).

We reasoned that if ILK indeed suppresses myogenic differentiation through sustaining MAP kinase activation, inactivation of MAP kinase should reverse the ILK-induced suppression of terminal myogenic differentiation. To test this, we treated the cells with PD98059, a MEK inhibitor that specifically inhibits MAP kinase activation (Alessi et al., 1995). As expected, p44/42 MAP kinases were inactivated in parental C2C12 cells after mitogen deprivation, either in the absence or presence of the MEK inhibitor (Fig. 10 a, lanes 1–3). In ILK-overexpressing C2C12 cells, whereas p44/42 MAP kinases remained active after mitogen deprivation in the absence of the MEK inhibitor (Fig. 10 a, lanes 5, 8, and 11), the amounts of active forms of p44/42 MAP kinases were significantly reduced in the presence of the MEK inhibitor (Fig. 10 a, lanes 6, 9, and 12). Thus, activation of p44/42 MAP kinases that were induced by ILK overexpression was effectively inhibited by the specific MEK inhibitor PD98059, indicating that ILK activates p44/42 MAP kinases through MEK. In further supporting a key role of MAP kinase in the ILK-induced suppression of myogenic differentiation, inhibition of MAP kinase reversed the ILK-induced suppression of myogenin (Fig. 10 a, lanes 6, 9, and 12) and myosin heavy chain (data not shown) expression. In control experiments, myogenin was readily detected in parental C2C12 cells after mitogen deprivation, either in the absence or presence of the MEK inhibitor (Fig. 10 a, lanes 2 and 3). We conclude from these results that ILK influences the initial decision making process of myogenic differentiation by regulation of MAP kinase activation.
In a recent study, we have identified ILK as an important regulator in the initial decision making process of myogenic differentiation. These results provide strong evidence for the notion that ILK is an important component of the integrin signaling pathway that regulates MAP kinase activation and, ultimately, the decision of proliferation versus differentiation. Because MAP kinase activation is critically involved in cell cycle progression through the G1 phase (Bottazzi et al., 1999; Rovers et al., 1999), a process that is corporately regulated by growth factors and integrins (Assoian, 1997; Schwartz, 1997; Howe et al., 1998; Gancotti and Ruoslahti, 1999), the finding that ILK enhances MAP kinase activation is also consistent with recent observations that overexpression of ILK in epithelial cells promotes anchorage-independent cell cycle progression (Radeva et al., 1997) and tumor formation (Wu et al., 1998).

In addition to demonstrating a prominent role in the initial decision making process of terminal myogenic differentiation, our results suggest that ILK may also play a role in the later stages of myogenic differentiation, namely modulation of cell fusion or maintaining the integrity of myotubes. Overexpression of the PINCH-binding defective or the kinase-deficient ILK mutants, resulted in a sustained activation of MAP kinas (Erk1 and Erk2). Furthermore, inhibition of MAP kinase activation reverses the ILK-induced suppression of myogenic differentiation. These results provide strong evidence for the notion that ILK is an important component of the integrin signaling pathway that regulates MAP kinase activation and, ultimately, the decision of proliferation versus differentiation. Because MAP kinase activation is critically involved in cell cycle progression through the G1 phase (Bottazzi et al., 1999; Rovers et al., 1999), a process that is corporately regulated by growth factors and integrins (Assoian, 1997; Schwartz, 1997; Howe et al., 1998; Gancotti and Ruoslahti, 1999), the finding that ILK enhances MAP kinase activation is also consistent with recent observations that overexpression of ILK in epithelial cells promotes anchorage-independent cell cycle progression (Radeva et al., 1997) and tumor formation (Wu et al., 1998).

In addition to demonstrating a prominent role in the initial decision making process of terminal myogenic differentiation, our results suggest that ILK may also play a role in the later stages of myogenic differentiation, namely modulation of cell fusion or maintaining the integrity of myotubes. Overexpression of the PINCH-binding defective or the kinase-deficient ILK mutants, which is permissive for the initiation of myogenic differentiation, resulted in the formation of myotubes with altered morphology (giant myotubes containing clustered nuclei). ILK is a multi-domain protein with several distinct biochemical activities including integrin-binding, PINCH-binding, and catalysis of serine/threonine phosphorylation (Dedhar et al., 1999; Wu, 1999). Thus, ILK mutants, in which one of the activities (e.g., PINCH-binding or kinase activity) is ablated, could function as dominant negative inhibitors of endogenous ILK. Indeed, a dominant negative inhibitory effect of the kinase-deficient ILK mutant in ILK signaling has been observed in previous studies (Delcommenne et al., 1998; Troussard et al., 1999). A role of ILK in the modulation of
myogenic morphogenesis is further supported by previous studies showing that alterations in the expression or functions of β1 integrins, to which ILK binds (Hannigan et al., 1996), resulted in abnormal muscle structure. For example, dystrophic muscles with giant muscle fibers or increased numbers of nuclei per fiber with altered position and size have been observed in α5 integrin (α5; +; −) chimeric mice (Taverna et al., 1998) and mice lacking α7 integrin (Mayer et al., 1997). Treatment of myoblasts with an antibody that alters α5β1 integrin function also results in the formation of myotubes with an altered morphology (e.g., myotubes with clustered nuclei; Boettiger et al., 1995). The similar effects of ILK and the β1 integrins on myogenic morphogenesis strongly suggest that ILK functions in this process through, at least in part, modulation of integrin signaling. Recent studies in C. elegans have provided strong genetic evidence for a critical role of ILK and its binding partner PINCH in integrin functions during muscle development. Deficiency in β1-integrin/pat-3 results in a specific developmental arrest phenotype termed Pat (paralyzed and arrested elongation at the twofold stage), which is caused by a dysfunction of body wall muscles (Gettner et al., 1995). The loss of expression of either ILK/pat-4 (Mackinnon, A., C., and B. Williams, personal communication) or PINCH/unc97 (Hobert et al., 1999) causes a similar body wall muscle-defective Pat phenotype. The dual functions of ILK in myogenesis suggest that ILK may play a crucial role in the regulation of normal muscle regeneration as well as pathological conditions, such as muscular dystrophies or other myopathies.

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