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

Yao Huang,* ‡ Ji Li,* ‡ Yongjun Zhang,* ‡ and Chuanyue Wu‡

* Department of Cell Biology, and ‡ The Cell Adhesion and Matrix Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019

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 Enghave, 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; Olson and Klein, 1994; Andress and Walsh, 1996). During this process, the activity of MAP kinase is downregulated and expression of myogenic transcription factors such as myogenin is upregulated, followed by expression of other muscle-specific proteins such as myosin heavy chain (MHC) and subsequent formation of multinucleated myotubes (Lassar et al., 1994; Andress and Walsh, 1996; Bennett and Tonks, 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; Bunch 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; Bunch 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; Bunch 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; Bunch et al., 1998; Gullberg et al., 1998; Prokop et al., 1998).

The ILK-binding site has been mapped to the COOH-terminal ANK domain (Tu et al., 1999; Wu, 1999). Other studies 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; Dedhar et al., 1999) and PINCH, an adaptor protein comprising five LIM domains, through the NH2-terminal domain (Hannigan et al., 1996; Dedhar et al., 1999) and PINCH, an adaptor protein comprising five LIM domains, through the NH2-terminal domain (Hannigan et al., 1996; Dedhar 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 by 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 7.4, 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 (as described above) and phosphatase inhibitors (30 mM sodium fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin (Sastry et al., 1999)) unless otherwise specified. Duplicate membranes were analyzed for the total MAPK with an anti-p44/42 MAPK polyclonal antibody C-20 that specifically recognizes the active forms of MAPK (New England Biolabs). Duplicate membranes were analyzed for the total MAPK with an anti-p44/42 MAPK polyclonal antibody C-20 that specifically recognizes the active forms of MAPK (New England Biolabs). Duplicates were reprobed with an anti-FAK polyclonal antibody (New England Biolabs). Duplicates were reprobed with an anti-FAK polyclonal antibody (New England Biolabs). Duplicates were reprobed with an anti-FAK polyclonal antibody (New England Biolabs). Duplicates were reprobed with an anti-FAK polyclonal antibody (New England Biolabs).
Hoechst 33258 (Sigma Chemical Co.) in PBS for 5 min and observed in each experiment.

FLAG mAb M5, or anti-FAK polyclonal antibody C-20 as specified in anti-MyoD mAb 5.8A, 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 μg) were mixed with 10 μl of polyclonal anti-FAK antibody C-20 (2 μg) in a final volume of 500 μl. The samples were incubated at 4°C for 2 h with continuous agitation. 10 μl of Ultraproteolink 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 μl of SD-PAGE sample buffer for 5 min. Equal volumes of the samples were loaded onto SD-PAGE gels. 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/p42 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 μM 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-phospho (Thr202/Tyr204)-p44/p42 MAPK antibody and antimyogenin 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 μg/ml of Hoechst 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 bandunt 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-C-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 FLA G-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 FLA G-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 NH$_2$ 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 FLA G-tagged kinase-deficient (KD) ILK point mutant (Novak et al., 1998; Wu et al., 1998), in which the highly conserved Glu$^{399}$ within the ILK catalytic domain was substituted with lysine, in C2C12 cells. The expression of FLA G-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 wildtype ILK (Figs. 5, b and c, lane 4), did not inhibit the expression of myogenin (Fig. 5 b, lanes 6 and 8), M yoD (data not shown), or M HC (Fig. 5 c, lanes 6 and 8). Equal protein loading was confirmed by probing the same membranes with a polyclonal anti-F A K antibody (Fig. 5 d). Thus, ablation of the kinase activity relieves the inhibition on the expression of myogenin, M yoD, and M HC, 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 NH$_2$-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 FLA G-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 FLA G-ΔA NK1 (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). 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 FLA G-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 (Sastray 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 transfectants, 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 b, 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 b, 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.
Discussion

How myoblastic cells control terminal myogenic differentiation is a fascinating and clinically important question. Although it has been well established that growth factors and extracellular matrix proteins, through interactions with their cell-surface receptors, provide crucial signals controlling terminal myogenic differentiation (McDonald et al., 1995; Sastry and Horwitz, 1996; Wewer and Engvall, 1996; Durbeej et al., 1998; Gullberg et al., 1998; Burkin and Kaufman, 1999), the intracellular events transducing the signals are not completely understood. In this study, we have identified ILK as an important regulator in the initial decision making process of myogenic differentiation. A correlation between downregulation of the ILK level in the Triton X-100-soluble fractions and terminal myogenic differentiation was observed. In further experimental studies, we found that overexpression of ILK effectively inhibits the expression of myogenic transcription factors and suppresses the subsequent myotube formation. The inhibition of myogenic differentiation requires both the PINCH-binding and the kinase activities of ILK, suggesting that the relative amount, subcellular localization, and the kinase activity of ILK are crucial elements in the cellular regulation of terminal myogenic differentiation.

The finding that ILK functions in the initial decision making process of terminal myogenic differentiation is consistent with recent studies by Sastry et al. (1999) who have demonstrated that overexpression of the β1 integrin cytoplasmic domain inhibits terminal myogenic differentiation. ILK was initially identified based on its interaction with the β1 integrin cytoplasmic domain (Hannigan et al., 1996). ILK is present in cell–matrix adhesion sites (Li et al., 1999a). Furthermore, the kinase activity of ILK can be activated by integrin-mediated cell adhesion to fibronectin (Delcommenne et al., 1998). In a recent study, we have found that MIBP, a muscle-specific β1 integrin binding protein, is critically involved in the regulation of myogenic differentiation (Li et al., 1999b). Because both ILK (Hannigan et al., 1996) and MIBP (Li et al., 1999b) interact with the β1 cytoplasmic domain, it is attractive to propose that ILK works in concert with MIBP and other integrin-proximal proteins such as FAK and paxillin (Sastry et al., 1999) in transducing signals from β1 integrins to downstream targets leading to the suppression of terminal myogenic differentiation.

A key downstream target of integrin-mediated regulation of terminal myogenic differentiation is MAP kinase (Sastry et al., 1999). Overexpression of the β1 integrin cytoplasmic domain enhances MAP kinase activation, which maintains the myoblasts in a proliferative, undifferentiated state (Sastry et al., 1999). Inhibition of MAP kinase activation, on the other hand, relieves the integrin-mediated suppression of myogenic differentiation (Sastry et al., 1999). Thus, inactivation of MAP kinases is an essential event in integrin-mediated regulation of myoblast cell cycle withdrawal and initiation of terminal myogenic differentiation (Sastry et al., 1999). In this study, we have demonstrated that overexpression of ILK, but not that of the PINCH-binding defective or the kinase-deficient ILK mutant, resulted in a sustained activation of MAP kinases (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; Roovers et al., 1999), a process that is corporately regulated by growth factors and integrins (Asoian, 1997; Schwartz, 1997; Howe et al., 1998; Giancotti 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 (+/+;−/−) 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 β-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.

We would like to thank Drs. Shoukath Daridy (Jack Bell Research Centre and University of British Columbia) for human ILK constructs, Richard H. Mayne for valuable discussion, and Benjamin Williams (University of Illinois at Urbana-Champaign) for sharing results before publication.

This work was supported by the National Institutes of Health grant D K 54639 (to C. Wu) and research project grant No. 98-220-01-CSM from the American Cancer Society (to C. Wu). C. Wu is a V Foundation Scholar.

Submitted: 17 February 2000
R evised: 15 June 2000
A ccepted: 7 july 2000

References

Alessi, D., A. Cuenda, P. Cohen, D. Udley, and A. Saltiel. 1995. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. J. Biol. Chem. 270:27489–27494.

Aird, V., and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. 132:657–666.

Asoian, R.K. 1997. A nchorage-dependent cell cycle progression. J. Cell Biol. 136:1–4.

Bennett, A.M., and N.K. Tonks. 1997. Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. Science. 278:1288–1291.

Boor, J.W., and N.H. Brown. 1998. Genetic analysis of the Drosophila alphaPS2 integrin subunit reveals discrete adhesive, morphogenetic and sarcomeric functions. Genetics. 148:1127–1142.

Boettiger, D., M. Emonts-Iwamoto, H.Y. Yoon, U. Hofer, A.S. Menko, and R. Chiquet-Ehrismann. 1995. Regulation of integrin alphaBeta1 affinity during myogenic differentiation. Dev. Biol. 169:261–272.

Bottazzi, M., E. X. Zhu, R.M. Bohmer, and R.K. A. Sosian. 1999. Regulation of p21cip1 expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. J. Cell Biol. 146:1255–1264.

Bunch, T.A., M. Sweiger, L.I. Fessler, K.D. Schneider, A. Kirsch, L.P. Choy, B.W. Burgess, and D.L. Brown. 1998. The PS2 integrin ligand tiggin is required for proper muscle function in Drosophila. Development. 125:1679–1689.

Burkin, D.J., and S.J. Kaufman. 1999. The alpha7beta1 integrin in muscle development and disease. Cell Tissue Res. 286:183–190.

Dedhar, S., D. Williams, P.L. Howell, and G. Hannigan. 1999. Integrin linked kinase (ILK): a regulator of integrin and growth-factor signaling. Trends Cell Biol. 9:319–323.

Delcommenne, M., C. Tan, V. Gray, L. Rue, J. Woodgett, and S. Dedhar. 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycerol synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. USA. 95:12111–12116.

Durbeej, M., M.D. Henry, and K.P. Campbell. 1999. Dystroglycan in development and disease. Curr. Opin. Cell Biol. 11:594–602.

Giancotti, F.G., and E. Ruoslahti. 1999. Integrin function and cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature. 397:91–96.

Hobert, O., G.D. Merman, K.A. Clark, M.C. Beckerle, and G.R. Ruvkun. 1999. A conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in Caenorhabditis elegans. J. Cell Biol. 144:45–57.

Howe, A., A.E. Aplin, S.K. Alahari, and R.L. Juliano. 1998. Integrin signaling and cell growth control. Curr. Opin. Cell Biol. 10:220–231.

Lassar, A.B., S.X. Sapek, and B. Novitch. 1994. Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. Curr. Opin. Cell Biol. 6:788–792.

Li, F., J. Liu, R. Mayne, and C. Wu. 1997. Identification and characterization of a mouse protein kinase that is highly homologous to human integrin-linked kinase. Biochim. Biophys. Acta. 1258:215–220.

Li, F.-Y. Zhang, and C. Wu. 1999a. Integrin-linked kinase is localized to cellular adhesion sites and the focal adhesion localization of integrin-linked kinase is regulated by the PINCH-binding domain. J. Cell. Sci. 112:4589–4599.

Li, J.-R. Mayne, and C. Wu. 1999b. A novel muscle-specific beta1 integrin binding protein (MIBP) that modulates myogenic differentiation. J. Cell Biol. 147:1391–1397.

Martin-Bermudo, M.D., and N.H. Brown. 1996. Intracellular signals direct integrin localization to sites of function in embryonic muscles. J. Cell Biol. 134:217–226.

Mayer, U., G. Saher, R. Fassler, A. Bremann, F. Echerlmeyer, H. von der Mark, N. Miosge, E. Poschl, and K. von der Mark. 1997. Absence of integrin alpha7 causes a novel form of muscular dystrophy. Nat. Genet. 17:318–323.

McDonald, K.A., A.F. Horswill, and K.A. Nudson. 1995. A dshon molecules and skeletal myogenesis. Semin. Dev. Biol. 6:105–116.

Menko, A.S., and D. Boettiger. 1997. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell. 51:51–57.

Montanaro, F., M. Indenbaum, and S. Carbonetto. 1999. α-Dystroglycan is a laminin receptor involved in extracellular matrix assembly on myoblasts and muscle viability. J. Cell Biol. 145:1325–1340.

Novak, A., S.C. Hsu, C. Leung-Hagesteijn, G. Radeva, J. Papkoff, R. Montesano, C. Roskelley, R. Grossehled, and S. Dedhar. 1998. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. Proc. Natl. Acad. Sci. USA. 95:4374–4379.

Olson, E.N., and W.H. Klein. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. 8:1–8.

Prokop, A., M.D. Martin-Bermudo, M. Bate, and N.H. Brown. 1998. A balance of PS integrins or laminin A affects extracellular adhesion, but not intracellular assembly, of hemiadhesers and neuromuscular junctions in Drosophila embryos. Dev. Biol. 196:289–296.

Radeva, G., T. Petrocilli, E. Behrend, C. Leung-Hagesteijn, J. Filmus, J. Slingler, and S. Dedhar. 1997. Overexpression of the integrin-linked kinase promotes anchorage-independent cell cycle progression. J. Biol. Chem. 272:13937–13944.

Reardon, A. 1994. A new LIM protein containing an autoepitope homologous to “sequestrin cell antigen.” Biochem. Biophys. Res. Comm. 201:1124–1131.

Roversi, K., G. Davey, X. Zhu, M.E. Battazzi, and R.K. A. Sosian. 1999. alphaBeta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. Mol. Biol. Cell. 10:3197–3204.

Rosen, G.D., J.R. Sannes, L. LaChance, J.M. Cunningham, J. Roman, and D.C. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor in muscle development and disease. Curr. Opin. Cell Biol. 4:269–274.

Sastry, S.K., and A.F. Horwitz. 1996. A dhesion-growth factor interactions during differentiation: an integrated biological response. Dev. Biol. 180:455–
Sastry, S.K., M. Lakonishok, S. Wu, T.Q. Truong, A. Huttenlocher, C.E. Turner, and A.F. Horwitz. 1999. Quantitative changes in integrin and focal adhesion signaling regulate myoblast cell cycle withdrawal. J. Cell Biol. 144:1295–1309.

Schwartz, M.A. 1997. Integrins, oncogenes, and anchorage independence. J. Cell Biol. 139:575–578.

Tachibana, I., and M.E. Hemler. 1999. Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. J. Cell Biol. 146:893–904.

Taverna, D., M.H. Disatnik, H. Rayburn, R.T. Bronson, J. Yang, T.A. Rando, and R.O. Hynes. 1998. Dystrophic muscle in mice chimeric for expression of α5 integrin. J. Cell Biol. 143:849–859.

Troussard, A.A., C. Tan, T.N. Yoganathan, and S. Dedhar. 1999. Cell extracellular matrix interactions stimulate the AP-1 transcription factor in an integrin linked kinase (ILK) and glycogen synthase kinase-3 dependent manner. Mol. Cell. Biol. 19:7420–7427.

Tu, Y., F. Li, S. Goicoechea, and C. Wu. 1999. The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol. Cell. Biol. 19:2425–2434.

Tu, Y., F. Li, and C. Wu. 1998. Nick-2, a novel Src homology2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase signaling pathways. Mol. Biol. Cell. 9:3367–3382.

Volk, T., L.I. Fessler, and J.H. Fessler. 1990. A role for integrin in the formation of sarcomeric cytoarchitecture. Cells. 63:525–536.

Weintraub, H. 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell. 75:1241–1244.

Wewer, U.M., and E. Engvall. 1996. Merosin/laminin-2 and muscular dystrophy. Neuromuscul. Disord. 6:409–418.

Wu, C. 1999. Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction. J. Cell Sci. 112:4485–4489.

Wu, C., S.Y. Kightley, C. Leung-Hagesteijn, G. Radeva, M. Coppolino, S. Goicoechea, J.A. McDonald, and S. Dedhar. 1998. Integrin-linked protein kinase regulates fibronecin matrix assembly, E-cadherin expression, and tumorigenicity. J. Biol. Chem. 273:528–536.