Integrin-linked Kinase Controls Neurite Outgrowth in N1E-115 Neuroblastoma Cells*

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Mouse N1E-115 cells grown on a laminin matrix exhibit neurite outgrowth in response to serum deprivation. Treatment of cells with an antibody against β1 integrin inhibits neurite outgrowth. Thus, β1 integrin is involved in the neuritogenesis of N1E-115 cells on a laminin matrix. Integrin-linked kinase (ILK), a recently identified cytoplasmic serine/threonine protein kinase that binds to the cytoplasmic domain of β1 integrin, has an important role in transmembrane signal transduction via integrins. We report that ILK is expressed in N1E-115 cells, the expression levels of which are constant under both normal and differentiating conditions. A stable transfection of a kinase-deficient mutant of ILK (DN-ILK) results in inhibition of neurite outgrowth in serum-starved N1E-115 cells grown on laminin. On the other hand, a transient expression of wild type ILK stimulated neurite outgrowth. The ILK activity in the parental cells was transiently activated after seeding on the laminin matrix, whereas in the DN-ILK-transfected cells was not. These results suggest that transient activation of ILK is required for neurite outgrowth in serum-starved N1E-115 cells on laminin. Under the same conditions, p38 mitogen-activated protein (MAP) kinase, but neither MAP kinase/extracellular signal-regulated kinase kinase (MEK) nor extracellular signal-regulated kinases (ERK), was transiently activated after N1E-115 cell attachment to laminin, but not in the DN-ILK-expressed cells. The time course of p38 MAP kinase activation was very similar to that of ILK activation. Furthermore, a p38 MAP kinase inhibitor, SB203580, significantly blocked neurite outgrowth. Thus, activation of p38 MAP kinase is involved in ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth in N1E-115 cells.

Cell interactions with extracellular matrix (ECM)† proteins

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF256520.

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‡ The abbreviations used are: ECM, extracellular matrix; ILK, integrin-linked kinase; NGF, nerve growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; GFP, green fluorescent protein; PI, phosphatidylinositol; Pak, p21-activated kinase; DN, dominant negative; SH, Src homology; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium.
ILK-mediated signal transduction leading to integrin-dependent neurite outgrowth.

EXPERIMENTAL PROCEDURES

Reagents—LY294002 was obtained from Sigma. The rabbit polyclonal anti-ILK IgG (UB 06-550 and UB 06-592) and myelin basic protein were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-p38 MAP kinase, anti-p38 MAP kinase, anti-phospho-MEK, and anti-MEK antibodies were obtained from New England Biolabs (Beverly, MA). Anti-phospho-p44/42 MAP kinase (anti-phospho-ERK1/ERK2) and anti-p44/42 MAP kinase (anti-ERK1/ERK2) antibodies were obtained from Promega (Madison, WI). All other chemicals were of analytical grade and were obtained from Sigma or Wako Pure Chemical Co. (Osaka, Japan) unless otherwise specified.

Kinase Assays—Kinase assays were performed as described by Delcommenne et al. (17). Cells were lysed in 50 mM Hepes buffer (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μg/ml leupeptin, 2.5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, and 1 mM sodium orthovanadate. The lysates were incubated with anti-ILK antibody (UB 06-592) at 4 °C for 12 h. After incubation, the lysates were precleared and immune complexes were collected with Protein A-Sepharose. The immunoprecipitated ILK was incubated for 20 min at 30 °C in the presence or absence of 10 μg of the exogenous substrate myelin basic protein in a total volume of 50 μl of kinase reaction buffer (50 mM HEPES, pH 7.0, 10 mM MnCl₂, 10 mM MgCl₂, 2 mM NaF, 1 mM Na₃VO₄) containing 10 μCi of [γ-³²P]ATP (6000 Ci/mmol, NEN Life Sciences). The reaction was stopped by the addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The kinase reaction products were analyzed using SDS-PAGE (5–20% polyacrylamide) and autoradiography. For detection of the immunoprecipitated ILK and DN-ILK proteins, the precipitated proteins were released from the immunobeads by boiling in 80 μl of SDS-PAGE sample buffer for 5 min. Equal volumes of the samples were loaded onto SDS-PAGE. Total ILK and DN-ILK proteins were detected by immunoblotting with an anti-ILK antibody (UB 06-550) that recognizes both ILK and DN-ILK proteins.

Kinase Cloning—A cDNA library was constructed in Uni-ZAP XR using cDNA synthesis kits (nos. 200400–200402, Stratagene, La Jolla, CA). Poly(A)⁺ RNA prepared from the whole brain of an adult guinea pig was converted to cDNA by oligo(dT)-primed reverse transcription. The obtained cDNAs were ligated between EcoRI and XhoI sites of Uni-ZAP XR as described under "Experimental Procedures." The expression level of ILK was examined after culturing cells in either normal or differentiating conditions. Cells were seeded on laminin-coated plates, cultured in serum-free conditions for the indicated time period, and then lysed. ILK expression levels were determined using Western blotting methods with an affinity-purified polyclonal anti-ILK antibody as described under "Experimental Procedures.”

cDNA Cloning—A cDNA library was constructed in Uni-ZAP XR using cDNA synthesis kits (nos. 200400–200402, Stratagene, La Jolla, CA). Poly(A)⁺ RNA prepared from the whole brain of an adult guinea pig was converted to cDNA by oligo(dT)-primed reverse transcription. The obtained cDNAs were ligated between EcoRI and XhoI sites of Uni-ZAP XR as described by the manufacturer. This cDNA library was screened with ³²P-labeled antisense oligonucleotides, 3’-ATACGTGGACGGACCACAT-5’ and 3’-GGACTTCTGTGTTTGTCT-5’, both of which were designed to bind to the encoding sequences for the amino acids sequences, YAPA(W/V) and PEPTDNR respectively,
within the catalytic domain of human ILK (13). One positive clone containing a 1.8-kilobase pair insert, which binds both oligonucleotide probes, was isolated from 2 × 10⁶ plaques. After excision of the pBluescript phagemid vector from the Uni-ZAP XR, the CDNA insert was cloned into the pBluescript SK plasmid as described by the manufacturer and sequenced. The nucleotide sequence of guinea pig ILK has been submitted to the GenBank database with accession number AF256520. The encoded protein has a 98.9% amino acid homology with the human ILK.

Construction of cDNA—The kinase-deficient ILK (DN-ILK) was generated by site-directed mutagenesis (Glu to Lys) at amino acid residue 359 within the kinase domain using the polymerase chain reaction (PCR) as follows. A 1.159-kilobase pair cDNA containing the 5′-untranslated sequence and the coding region for Met⁷-Glu⁸⁸⁹ of guinea pig ILK, in which Glu⁷³⁹ was mutated to Lys, was synthesized using PCR with two oligonucleotide primers, 5′-TGGCCGCGGCTCTAGAACTAGTG-3′ (pBluecript vector primer, the NotI site is underlined) and 5′-TTCAGTTTCTCTGCAAGCTTATGGCGCTA-3′ (a unique HindIII site of ILK cDNA is underlined, and a single mutation site C to T is shown in parentheses), and ILK cDNA in pBluescript vector as a template. The PCR fragment was digested with NotI/HindIII restriction enzymes, and was then placed back into a full-length ILK cDNA in a pBluecript vector. The nucleotide sequence of the PCR fragment was determined using a standard DNA sequencing technique (18). Wild type ILK and DN ILK cDNAs were ligated into the poly linkers in a mammalian expression vector, pTracerTM-CMV2 (V885-01, Invitrogen Corp., Carlsbad, CA), and were introduced into N1E-115 cells.

Stable Expression and Cloning of Cell Lines—DN-ILK cDNA was transfected into N1E-115 cells (5 × 10⁴ cells/dish) using the calcium/phosphate precipitation method as described by Graham and van der Eb (19), and 48 individual zeocin-resistant cell lines were selected on the basis of detection of the GFP fluorescence and protein blotting for detection of these phosphorylated MAP kinases. The final protein-IgG complexes were visualized following the reaction with horseradish peroxidase. The final protein-IgG complexes were visualized following the reaction with 3,3′-diaminobenzidine tetrahydrochloride. For detection of the phosphorylation status of p38 MAP kinase and MEK, polyclonal antibodies reactive with the phosphorylated form of p38 MAP kinase and MEK were purchased from New England Biolabs. For detection of the phosphorylation status of p44/42 MAP kinase (ERK1/ERK2), polyclonal antibodies reactive with the phosphorylated form of p44/42 MAP kinase (ERK1/ERK2) were purchased from Promega. Preparation of cell lysate and protein blotting for detection of these phosphorylated MAP kinases were conducted according to the manufacturer’s instructions.

RESULTS

Stimulation of Neurite Outgrowth in Serum-starved N1E-115 Cells Grown on a Laminin Matrix Requires Cell Adhesion via β₁ Integrin—Mouse N1E-115 neuroblastoma cells exhibit neurite outgrowth in response to serum deprivation (8, 9), which is strongly affected by the substrates to which the cells adhere in the extracellular matrix (10, 20). We observed neurite outgrowth of cells grown on laminin-coated plates under serum-free conditions. In this condition, cells became flattened and then gradually extended neurites within 8 h, and ~88% of the cells possessed neurites after 16 h (Fig. 1B; see also Fig. 3). The number of neurite-bearing cells grown on noncoated plates, however, was quite low (8.6 ± 3.0% at 8 h and 18.3 ± 5.2% at 16 h). Sarner et al. (10) found that pre-treatment of N1E-115 cells with β₁ inte-
Grin antibody blocks adhesion and neurite outgrowth of the cells plated on laminin-coated glass slide in serum-free condition, suggesting that β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on a laminin matrix. We also examined the effect of β1 integrin antibody on neurite outgrowth in serum-starved N1E-115 cells grown on the laminin-coated plastic plate. Cells were briefly pretreated for 7 min with varying concentrations of an antibody directed against β1 integrin and then seeded on laminin-coated plates under serum-free conditions. As shown in Fig. 1, higher concentrations of antibody significantly blocked adhesion. On the other hand, lower concentrations of antibody (0.3 and 1.0 μg/ml) did not affect cell adhesion but significantly inhibited neurite outgrowth 8 and 16 h after antibody-treatment. Our observations are similar to those of Sarner et al. (10). Thus, β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on a laminin matrix under serum-free conditions.

**ILK Is Highly Expressed in N1E-115 Cells and Its Expression Level Remains Constant during Neuronal Differentiation**—ILK is a serine/threonine protein kinase that interacts directly with the cytoplasmic domain of the β1 integrin subunit (13) and whose kinase activity is modulated by cell-extracellular matrix interactions and insulin in a PI 3-kinase-dependent manner (17). ILK has important roles in integrin- and growth factor-mediated signal transduction in several different cells, leading to regulation of cell adhesion, growth, migration, survival, proliferation, and differentiation (13, 16, 21–23). Little is known, however, regarding the role of ILK in neuronal cells. Because β1 integrin is involved in both adhesion and neuritogenesis of N1E-115 cells grown on the laminin matrix (Fig. 1), we further examined whether ILK is expressed in the cells. We found that ILK is highly expressed in N1E-115 cells and also that the expression level of ILK did not change during neuronal differentiation (Fig. 2).
Transient Activation of ILK Activity after Seeding on a Laminin Matrix Is Required for Neurite Outgrowth in Serum-starved N1E-115 Cells—We first examined whether endogenous ILK is involved in integrin-dependent neurite outgrowth. To inactivate the endogenous ILK, cells were stably transfected with a kinase-deficient mutant of ILK (DN-ILK) that behaves as a dominant negative (17). Based on the results obtained from immunoblotting (Fig. 4, inset), the expression level of DN-ILK protein in DN-ILK-transfected cells could be estimated to be at least twice more than that of endogenous ILK protein. As shown in Figs. 3 and 4, neurite outgrowth of the DN-ILK-transfected cells was significantly inhibited compared with that of untransfected parental cells 8 and 16 h after seeding on the laminin matrix under serum-free conditions. This result suggests that the kinase activity of endogenous ILK is critical for neurite outgrowth in serum-starved N1E-115 cells grown on a laminin matrix. We also examined the effect of transient expression of the wild type ILK on neurite outgrowth of the cells. Overexpression of the wild type ILK stimulated neurite outgrowth in the cells even grown on noncoated plates (Fig. 5). We next examined whether ILK is activated after seeding on a laminin matrix under serum-free conditions. As shown in Fig. 6, the ILK activity in the parental cells was transiently activated after seeding on the laminin matrix, whereas that in the DN-ILK-transfected cells did not change after cell attachment to the laminin. Maximal stimulation of ILK activity in the parental cells occurred 60 min after plating, and then rapidly disappeared. Moreover, ILK activation was prevented by treatment of the cells with LY294002, a specific inhibitor of PI 3-kinase (24). Thus, ILK activation following cell attachment to the laminin under serum-free conditions is PI 3-kinase-dependent. These results suggest that transient activation of ILK activity after cell attachment to a laminin matrix is required for neurite outgrowth in serum-starved N1E-115 cells.

**Fig. 5.** Effect of transient expression of wild type ILK on neurite outgrowth in N1E-115 cells grown on the noncoated plate. N1E-115 cells were seeded at a density of 5 × 10⁴/dish onto 35-mm noncoated culture dishes and grown in DMEM containing 10% FBS. At 20 h following plating, cells were transfected with the wild type ILK cDNA (Wild-type ILK) using LipofectAMINE transfection reagent, as described under “Experimental Procedures.” Control cells were transfected with the empty plasmid (Vector). At 18 and 36 h following the transfection, the morphological changes produced by the transfection of the plasmid were examined using fluorescence microscopy. At least 100 randomly selected GFP-positive cells were assessed, and the cells that have a process longer than 2-fold the length of a cell body were categorized as being neurite bearing cells. The neurite bearing cell was assessed as the percentage of the total number of GFP-expressing cells. Each experiment was repeated at least three times. Values are the means ± S.D.
The involvement of PI 3-kinase in the stimulation of ILK activity, cells were treated with 40 μM LY294002, a specific inhibitor of PI 3-kinase, for 1 h by direct addition to the culture medium. Total ILK and DN-ILK proteins in the immunoprecipitates were detected by immunoblotting with an anti-ILK antibody that recognizes both ILK and DN-ILK proteins, as described under "Experimental Procedures." To examine the involvement of PI 3-kinase in the stimulation of ILK activity, cells were treated with 40 μM LY294002, a specific inhibitor of PI 3-kinase, for 1 h by direct addition to the culture medium. Total ILK and DN-ILK proteins in the immunoprecipitates were detected by immunoblotting with an anti-ILK antibody that recognizes both ILK and DN-ILK proteins, as described under "Experimental Procedures."

### Immunoprecipitated protein

| Endogenous ILK protein | Parental cell | DN-ILK |
|------------------------|--------------|--------|
| 0                      | 30           | 60     | 120    |
| Endogenous ILK protein | Parental cell | DN-ILK |
| 0                      | 30           | 60     | 120    |

**Fig. 6. Stimulation of ILK activity after cell adhesion on laminin in serum-free condition.** Cells were seeded on laminin-coated plates in serum-free medium and cultured for the indicated time periods. The cells were lysed, and ILK was immunoprecipitated from cell extracts. ILK activity was determined using myelin basic protein as an exogenous substrate, as described under "Experimental Procedures." (Top) To examine the involvement of PI 3-kinase in the stimulation of ILK activity, cells were treated with 40 μM LY294002, a specific inhibitor of PI 3-kinase, for 1 h by direct addition to the culture medium. Total ILK and DN-ILK proteins in the immunoprecipitates were detected by immunoblotting with an anti-ILK antibody that recognizes both ILK and DN-ILK proteins, as described under "Experimental Procedures." (Bottom).

**p38 MAP Kinase, but Neither MEK Nor ERK, Is Activated after Seeding on a Laminin Matrix under Serum-Free Conditions, and Endogenous ILK Is Involved in This Activation—**

Activation of the MEK/ERK pathway is required for NGF-induced neuronal differentiation in PC12 cells (25, 26). Unlike the NGF-induced neuronal differentiation in PC12 cells, however, activation of MEK and/or ERK was not detected in the integrin-dependent neuronal differentiation of N1E-115 cells (Fig. 7, A and B). We next examined the possible involvement of another member of the MAP kinase family, p38 MAP kinase, in the signaling pathway of integrin-dependent neuronal differentiation of N1E-115 cells. As shown in Fig. 7 (A and C), the p38 MAP kinase in the parental control cells was transiently activated after seeding on the laminin matrix under serum-free conditions. The activation of p38 MAP kinase in the parental cells reached a maximum level within 60 min and then declined rapidly. Weak activation was still detected 90 min after plating. Thus, the time course of the activation of p38 MAP kinase was very similar to that of the ILK. In contrast, activation of p38 MAP kinase in the DN-ILK-transfected cells was not detected (Fig. 7, A and C). Thus, endogenous ILK is somehow involved in the activation of p38 MAPK. These results suggest that the ILK activation after cell attachment to laminin is necessary for the activation of p38 MAP kinase in serum-starved N1E-115 cells.

**Activation of p38 MAP Kinase Is Critical for Integrin-dependent Neurite Outgrowth in Serum-starved N1E-115 Cells—**

To evaluate the specific role of the p38 MAP kinase signaling pathway in integrin-dependent neurite outgrowth of N1E-115 cells, the cells were treated for 3 h with varying concentrations of SB203580, a specific inhibitor of p38 MAP kinase (27). SB203580 was applied after seeding the cells on laminin-coated plates under serum-free conditions. Treatment was terminated by changing the medium. As shown in Fig. 8, treatment of the cells with SB203580 markedly, but not completely, inhibited integrin-dependent neurite outgrowth in a dose-dependent manner with a maximal inhibition obtained at 10 μM. PD98059, a specific inhibitor of MEK (28), did not affect neurite outgrowth (Fig. 8). On the other hand, the same treatment of DN-ILK-transfected cells with SB203580 did not affect neurite outgrowth (Fig. 9), suggesting that p38 MAP kinase is not involved in ILK-independent neurite outgrowth. Moreover, SB203580 (10 μM) maximally blocked only 75% of the ILK-dependent neurite outgrowth (Figs. 8 and 9), suggesting that signaling pathways other than p38 MAP kinase, which can be activated via ILK activation, might also be involved in integrin-dependent neurite outgrowth. These results suggest that p38 MAPK activated via the PI 3-kinase-dependent activation of ILK, but neither MEK nor ERK, is required for integrin-dependent neurite outgrowth in serum-starved N1E-115 cells.

**PI 3-Kinase Is Involved in Both ILK-dependent and -independent Signaling Pathways in Neurite Outgrowth of Serum-starved N1E-115 Cells on Laminin—**

Although neurite outgrowth in the DN-ILK-transfected cells was markedly inhibited in comparison with that in the parental cells, ~30% of the DN-ILK-transfected cells, in which the kinase activity of ILK is negligible, extended neurites 16 h after plating (Fig. 4). Indeed, ILK and p38 MAP kinase activity in the DN-ILK-transfected cells was not stimulated at all after plating the cells on laminin-coated plates under serum-free conditions (Figs. 6 and 7). In this condition, neurite outgrowth in the DN-ILK-transfected cells was not affected by SB203580, but was significantly inhibited by LY294002 (Fig. 9). These results suggest that PI 3-kinase, but not p38 MAP kinase, is also involved in ILK-independent neurite outgrowth. Thus, it seems that PI-3 kinase activates not only ILK-dependent but also ILK-independent signaling pathways in neurite outgrowth of serum-starved N1E-115 cells on laminin.

**DISCUSSION**

The results of the present study demonstrate that ILK participates in integrin-dependent neuritogenesis in serum-
starved N1E-115 cells grown on a laminin matrix, and also that activation of p38 MAP kinase is critical to the effect of ILK. These results are based on the following observations: 1) both ILK and p38 MAP kinase were activated in a similar time course after seeding on a laminin matrix under serum-free conditions, 2) neurite outgrowth and p38 MAP kinase activation were inhibited in N1E-115 cells expressing the dominant negative ILK, and 3) treatment of cells with SB203580, a specific inhibitor of p38 MAP kinase, significantly blocked neurite outgrowth.

Integrin signaling is required for neuritogenis in serum-starved N1E-115 cells (10, 20). We demonstrated that neurite
outgrowth in serum-starved N1E-115 cells on laminin depends on an integrin-dependent signal pathway, because the anti-\(\beta_1\) integrin antibody inhibited neurite outgrowth (Fig. 1). How signals are transduced into the cell via integrin and what intracellular events occur after the cell-extracellular matrix interaction, however, are not fully understood in N1E-115 cells. Although higher concentrations of the antibody blocked cell adhesion, lower concentrations of the antibody inhibited neurite outgrowth without affecting cell adhesion (Fig. 1). These results imply that mechanical stresses brought by the direct interaction of integrin with the antibody have influences on the formation of focal adhesion complexes or the integrin/ECM-mediated intracellular signaling without affecting cell adhesion via integrin/ECM ligand interaction. Indeed, previous study using beads coated with anti-\(\beta_1\) antibodies demonstrated that the binding of anti-\(\beta_1\) antibody to integrin induces a rapid recruitment of mRNA and ribosomes to focal adhesion (28). Thus, such an integrin-mediated unknown signaling might be involved in the inhibition of neurite outgrowth by lower concentrations of antibody.

In the present study, we determined that ILK transduces signals derived from the cell-laminin matrix interaction into the cell and functions as an important regulator in the initial process of neuronal differentiation in N1E-115 cells. ILK is located in the cell-matrix adhesion site (29). The kinase activity of ILK can be activated by integrin-mediated cell adhesion to the extracellular matrix and insulin in a PI 3-kinase-dependent manner (17). ILK is expressed in N1E-115 cells; additionally, adhesion of the cells to laminin stimulates ILK activity under serum-free conditions (Figs. 2 and 6). The stimulation was maximal 60 min after plating and then declined rapidly (Fig. 6). The activation of ILK was prevented by treatment with the PI 3-kinase inhibitor, LY294002 (Fig. 6). These results suggest that activation of ILK after cell adhesion to laminin is mediated through a PI 3-kinase-dependent mechanism, and not likely through direct interaction between ILK and integrin, even though ILK interacts directly with the \(\beta_1\) integrin cytoplasmic domain (13). To investigate the role of endogenous ILK in the neuronal differentiation of N1E-115 cells, we cloned cell lines that highly express a kinase-deficient mutant of ILK (DN-ILK), which behaves as a dominant negative. Indeed, activation of ILK was completely blocked in DN-ILK expressed cells (Fig. 6). The overexpression of DN-ILK results in a significant inhibition of neurite outgrowth (Figs. 3 and 4), suggesting that endogenous ILK is an important mediator for integrin-dependent neuritogenesis in N1E-115 cells.

The pheochromocytoma cell line PC12 has been well studied for neuronal differentiation induced by neurotrophic factors such as NGF. These studies suggest that growth factor-induced activation of MAP kinase pathways is critical for neuronal differentiation of the cells (26, 30–33). On the other hand, integrin-dependent adhesion induces MAP kinase activation (34–36) and also cooperates with growth factors to efficiently activate MAP kinase pathways (3). A recent study of myogenic differentiation demonstrated that overexpression of ILK induces sustained activation of MAP kinase (21). Cross-talk between ILK and MAP kinase has also been reported in studies on anchoring-independent cell cycle progression (37). Because
MAP kinase serves as a downstream effector of β1 integrin (38), integrin clustered in focal adhesions on EMC is considered to recruit ILK via β1 integrin and activate its enzyme activity, which in turn regulates MAP kinase activity. In the present study, both β1 integrin and ILK were necessary for integrin-dependent neurite outgrowth in serum-starved N1E-115 cells. Therefore, we further examined the possible involvement of MAP kinase in the neuronal differentiation of N1E-115 cells. Two different MAP kinase inhibitors were used, a specific inhibitor of p38 MAP kinase, SB203580, and a specific inhibitor of MEK, PD98059, to examine whether p38 MAP kinase or the ERK pathway is involved in integrin-dependent neuronal differentiation of N1E-115 cells. SB203580 (10 μM) significantly inhibited neurite outgrowth of N1E-115 cells with a maximal inhibition of 50.3%, which compares to 75% inhibition in ILK-dependent neurite outgrowth. PD98059 had no effect on neurite outgrowth (Fig. 8). These results suggest that p38 MAP kinase, but not the ERK pathway, is necessary for integrin-dependent neurite outgrowth. Indeed, we demonstrated that p38 MAP kinase, but not MEK or ERK, was activated after cell attachment to laminin under serum-free conditions (Fig. 7). The results also suggest, however, that activation of p38 MAP kinase alone is not sufficient for neurite outgrowth in serum-starved N1E-115 cells on laminin because SB203580 did not completely inhibit neurite outgrowth. On the other hand, LY294002 completely blocked ILK-dependent neurite outgrowth and also significantly inhibited ILK-independent neurite outgrowth (Fig. 9). Thus, PI 3-kinase is critical for the activation of not only the ILK-p38 MAP kinase pathway but also other pathways involved in the neuronal differentiation of N1E-115 cells. Recently, Sarner et al. (10) suggested that PI 3-kinase-dependent activation of Cdc42 and Rac1 via Ras signals, but not ERK or JNK, is critically involved in neurite outgrowth in N1E-115 cells. This report supports the present results in which activation of ERK is not required for neurite outgrowth in N1E-115 cells. Thus, it seems that both p38 MAP kinase and Ras signal pathways might be necessary for full induction of integrin-dependent neurite outgrowth in serum-starved N1E-115 cells, although the interaction and/or the cross-talk between ILK and Ras signaling pathways remains unknown.

To more directly examine the role of ILK in integrin-dependent neuronal differentiation, we attempted to express the wild type ILK in either transient or stable overexpression systems. We clearly showed that transient overexpression of the wild type ILK results in significant stimulation of neurite outgrowth in the cells even grown on noncoated plate (Fig. 5). Transient ILK-expression in the cells grown on laminin-coated plate, however, strongly inhibited cell adhesion on a laminin matrix, and all ILK-expressed cells, but not vector-expressed control cells, were detached from the plate 24 h after transfection but still survived in suspension for some time (data not shown). Furthermore, we failed to generate stable cell lines that highly expressed the wild type ILK, whereas a kinase-deficient mutant readily yielded such cell lines. Therefore, we could not exactly determine the effect of exogenous overexpression of wild type ILK on the laminin/integrin-dependent neuronal differentiation of N1E-115 cells. Previous studies demonstrated...
that overexpression of ILK in epithelial cells suppresses suspension-induced apoptosis and stimulates anchorage-independent cell cycle progression (16). Thus, ILK in N1E-115 cells might have an important roles in bidirectional transmembrane signaling pathways via integrin to regulate a variety of functions, such as cell adhesion, growth, and survival besides neuronal differentiation.

Overexpression of DN-ILK completely prohibited the activation of p38 MAP kinase (Fig. 7, A and C). Furthermore, the time course of p38 MAP kinase activation was almost identical to that of ILK activation after cell attachment to laminin (Figs. 6 and 7). These results strongly suggest that ILK activation is necessary for p38 MAP kinase activation in N1E-115 cells. The mechanism by which ILK might trigger p38 MAP kinase activation after adhesion to laminin under serum-free conditions, however, remains unknown. ILK interacts with β1 integrin through the carboxyl-terminal domain (13) and also with PINCH, an adaptor protein comprising five LIM domains, through the amino-terminal ankyrin repeat domain (39). PINCH can interact with the SH2/SH3 domain-containing adaptor protein Nck-2, which associates with ligand-activated growth factor receptor kinases or intracellular components of growth factor signaling pathways such as insulin receptor substrate (40, 41). Thus, ILK-PINCH interaction is critical not only for proper subcellular localization of ILK but also formation of a signaling complex coupling integrins and growth factor receptors. On the other hand, p21-activated kinase (Pak), a family of Ser/Thr kinases, activates p38 MAP kinase (42). Nck-2 can recognize and activate Pak, the activation process of this phenomenon.

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REFERENCES
1. Hynes, R. O. (1992) Cell 69, 11–25
2. Pledger, W. G., McNamace, H. P., Dike, L. E., Bojanowski, K., and Inger, D. E. (1995) Mol. Biol. Cell 6, 1349–1365
3. Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996) J. Cell Biol. 135, 1635–1642
4. Hotchin, N. A., and Hall, A. (1995) J. Cell Biol. 131, 1857–1865
5. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) J. Cell Sci. 113, 3563–3571
6. Turner, D. C., and Flier, L. A. (1989) Dev. Neurosci. 11, 300–312
7. Turner, D. C., Flier, L. A., and Carbonetto, S. (1989) J. Neurosci. 9, 3227–3296
8. Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S., and Carbonetto, S. (1994) J. Cell Biol. 126, 801–810
9. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) Mol. Cell. Biol. 17, 1201–1211
10. Sarner, S., Kozma, R., Ahmed, S., and Lim, L. (2000) Mol. Cell. Biol. 20, 158–172
11. Rosato, P., Gavazzi, I., Timpl, R., Aumailley, M., Abbadini, M., Giancotti, F., Silengo, L., Marchisio, P. C., and Taronne, G. (1990) Exp. Cell Res. 189, 100–108
12. Zhu, Y., Han, Y., and Bradley, A. (1993) J. Biol. Chem. 268, 5557–5565
13. Hannigan, G. H., Leung-Hagesteijn, C., Fitz-Gibbon, L., Copolov, M. G., Radeva, G., Filus, J., Bell, J. C., and Dedhar, S. (1996) Nature 379, 91–96
14. Dedhar, S., Williams, B., and Hannigan, G. (1999) Trends Cell Biol. 9, 319–323
15. Huang, Y., and Wu, C. (1999) Int. J. Mol. Med. 3, 563–572
16. Radeva, G., Petrocchi, T., Behrend, E., Leung-Hagesteijn, C., Filus, J., Slingerland, J., and Dedhar, S. (1997) J. Biol. Chem. 272, 17287–17294
17. Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12111–12116
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
19. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456–467
20. van der Eb, A. J., and Graham, F. L. (1977) J. Virol. 23, 11642–11646
21. Tu, Y., Li, F., and Wu, C. (1998) J. Cell Biol. 140, 268–282
22. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3207–3212
23. Wu, C., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Copolov, M., Giococlea, S., McDonald, J. A., and Dedhar, S. (1998) J. Biol. Chem. 273, 5287–5296
24. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 2541–2548
25. Cowley, S., Paterson, M., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
26. Palecek, P. S., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Copolov, M., Giococlea, S., McDonald, J. A., and Dedhar, S. (1998) J. Biol. Chem. 273, 13583–13588
27. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) FEBS Lett. 364, 229–233
28. Chiche, M. E., Singer, R. H., Meyer, C. J., and Ingber, D. E. (1989) Nature 392, 730–733
29. Li, F., Zhang, Y., and Wu, C. (1999) J. Cell Sci. 112, 4589–4599
30. Lange-Carter, C. A., and Johnson, G. L. (1994) Science 265, 1458–1464
31. Leppa, S., Saffrich, R., Ansorge, W., and Bohmann, D. (1998) EMBO J. 17, 4404–4413
32. Morooka, T., and Nishida, E. (1998) J. Biol. Chem. 273, 24285–24288
33. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McLeskey, E. W., and Stork, P. S. J. (1998) Nature 392, 622–626
34. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) J. Biol. Chem. 269, 26602–26605
35. Clark, E. A., and Brugge, J. S. (1995) Science 268, 233–239
36. Zhu, X., and Assoian, R. K. (1995) Mol. Biol. Cell 6, 273–282
37. Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14821–14826
38. Sastry, S. K., Lekanovishok, M., Wu, S., Tu, Y., Li, F., Gavazzi, I., Timpl, R., Aumailley, M., Abbadini, M., Giancotti, F., Silengo, L., Marchisio, P. C., and Taronne, G. (1990) Exp. Cell Res. 189, 100–108
39. Tu, Y., Li, F., Gavazzi, I., and Wu, C. (1999) Mol. Biol. Cell 10, 2425–2434
40. Tu, Y., Li, F., and Wu, C. (1998) Mol. Biol. Cell 9, 3387–3392
41. Wu, C. (1999) J. Cell Sci. 112, 4485–4489
42. Manser, E., Leung, T., Salibuddin, H., Zhao, L., and Lim, L. (1994) Nature 367, 40–46
43. Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) Curr. Biol. 7, 85–94
44. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. A., Ulevitch, R. J., and Bokoch, G. M. (1995) J. Biol. Chem. 270, 23934–23936