Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 4374–4379, April 1998
Cell Biology

Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β-catenin signaling pathways

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Communicated by Harold E. Varmus, National Institutes of Health, Bethesda, MD, February 4, 1998 (received for review December 8, 1997)

ABSTRACT **The integrin-linked kinase (ILK) is an ankyrin repeat containing serine-threonine protein kinase that can interact directly with the cytoplasmic domains of the β1 and β3 integrin subunits and whose kinase activity is modulated by cell–extracellular matrix interactions. Overexpression of constitutively active ILK results in loss of cell–cell adhesion, anchorage-independent growth, and tumorigenicity in nude mice. We now show that modest overexpression of ILK in intestinal epithelial cells as well as in mammary epithelial cells results in an invasive phenotype concomitant with a down-regulation of E-cadherin expression, translocation of β-catenin to the nucleus, formation of a complex between β-catenin and the high mobility group transcription factor, LEF-1, and transcriptional activation by this LEF-1/β-catenin complex. We also find that LEF-1 protein expression is rapidly modulated by cell detachment from the extracellular matrix, and that LEF-1 protein levels are constitutively up-regulated at ILK overexpression. These effects are specific for ILK, because transformation by activated H-*ras* or v-*src* oncogenes do not result in the activation of LEF-1/β-catenin. The results demonstrate that the oncogenic properties of ILK involve activation of the LEF-1/β-catenin signaling pathway, and also suggest ILK-mediated cross-talk between cell–matrix interactions and cell–cell adhesion as well as components of the Wnt signaling pathway.**

The integrin-linked kinase (ILK) was identified from a yeast two-hybrid genetic screen by using as bait the cytoplasmic domain of the β_1 integrin subunit (1). ILK can interact with β_1 and β_3 integrins (1). ILK is a novel ankyrin-repeat containing serine-threonine kinase (1), which also contains sequence motifs found in pleckstrin homology domains capable of interacting with phosphoinositide lipids. The kinase activity of ILK can be modulated by interaction of cells with components of the extracellular matrix (1) or by integrin clustering. The activation or inhibition of ILK activity is cell-type dependent and can be modified by growth factors (M. Delcommenne and S. D., unpublished results). Overexpression of ILK in epithelial cells results in the stimulation of anchorage-independent cell growth (1) and cell cycle progression (2). The latter is caused by the constitutive up-regulation of expression of cyclin D_1 and cyclin A, resulting in the hyperphosphorylation of the retinoblastoma protein (2). Overexpression of ILK in epithelial cells also results in the induction of tumorigenicity in nude mice (3), indicating that ILK is a protooncogene. Surprisingly, transient, or stable expression of ILK in epithelial cells results in the rapid stimulation of fibronectin matrix assembly (3). This is a property unique to ILK as transfection of the same cells with other activated oncogenes such as H-*ras* or v-*src*, does not result in this stimulation (3). In addition to the stimulation of fibronectin matrix assembly, which is a property of mesenchymal cells, ILK overexpression in epithelial cells resulted in a disruption of cell–cell adhesion (1), likely caused by a profound inhibition in the level of expression of E-cadherin (3, 4).

During early stages of mouse embryonic development, the cell-fate determining protein, Wnt, which is a vertebrate homolog of *Drosophila* Wingless protein, activates a signaling pathway resulting in transient epithelial to mesenchymal transformation (5). This signaling pathway (6) involves the stabilization of the cytoplasmic pool of β-catenin, translocation of β-catenin to the nucleus, complex formation of β-catenin and the architectural transcription factor LEF-1 [T cell factor (TCF)] (7–9), and activation of this transcriptional complex leading to the stimulation of expression of mesenchymal genes (5, 6, 10). It also has been proposed that this transcription factor can simultaneously down-regulate the expression of E-cadherin, which contains LEF-1/β-catenin binding sites within its promoter (5).

To determine whether the ILK-induced disruption of cell–cell adhesion and stimulation of mesenchymal properties might involve components of the Wnt signaling pathway, we examined the fate of β-catenin in intestinal epithelial cells (IEC-18) (14) and mouse mammary epithelial cells (scp2) (11, 12) transfected with ILK cDNA expression vectors. We report here that, in these two independent epithelial cell systems, ILK overexpression results in the translocation of β-catenin to the nucleus in the absence of a a significant alteration in its expression levels of free pools. We also find that both loss of cell adhesion and overexpression of ILK up-regulate LEF-1 expression, resulting in its complex formation with β-catenin and activation of its transcriptional activity.

MATERIALS AND METHODS

Cells and Cell Culture. Rat IEC-18 (14) as well as scp2 (11) were stably transfected with a mammalian vector incorporating ILK to produce clones overexpressing wild-type (wt) ILK in the sense orientation (ILK-13) or antisense orientation (ILK-14) (1), or to produce a kinase-deficient form of ILK (IEC-18GH31RH). After selection under G418 (400 μg/ml), stable, independent clones were isolated by limited dilution cloning. IEC-18 cells also were stably transfected to overexpress H-*ras* (*Ras 33* and *Ras 37*) (14), and v-*src* (*Src2* and *Src4*) (14). Cells were grown in α-MEM containing 5% fetal calf serum, 2 mm L-glutamine, 3.6 mg/ml of glucose, and 10 μg/ml of insulin, and 40 μg/ml of G418 was added to transfected cells to maintain selection pressure.

Morphology and Invasion Assays. Confluent cells were trypsinized, and 7.5×10^4 cells in 1.5 ml of complete medium were seeded onto 1.5 ml of a three-dimensional collagen gel in

Abbreviations: ILK, integrin-linked kinase; TCF, T cell factor; IEC, intestinal epithelial cell; wt, wild type.

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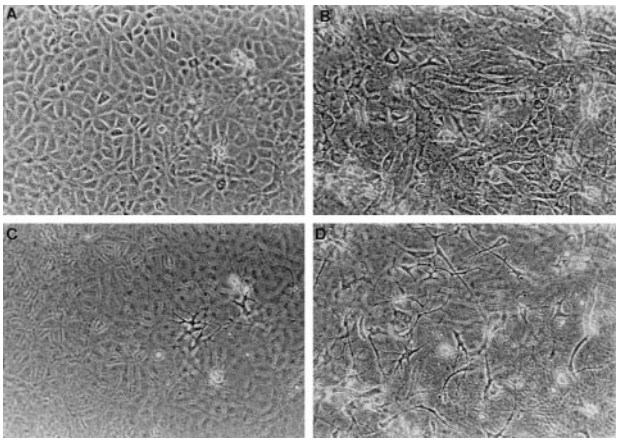

FIG. 1. Invasion of collagen gels. Seven days after seeding cells onto collagen, ILK-14A2c6 control transfectants (*A* and *C*) and ILK-13A4a, ILK-overexpressing cells (*B* and *D*) were photographed at the surface (*A* and *B*) and beneath the surface (*C* and *D*) to visualize cells that have penetrated the gel. (×150.)

a 35-mm tissue culture dish (13). After reaching confluence (3 days), the cultures were incubated for an additional 4 days, then fixed *in situ* with 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4), and photographed at different planes of focus. Invasion was quantitated by counting the number of cells that had migrated below the surface of the collagen gel.

Table 1. Invasion of collagen gels

Cell line	Invading cells/field
IEC-18	10 ± 0.87
ILK-14/A2c6	7.8 ± 1.32
ILK-13/A1a3	26.73 ± 2.61*
ILK-13/A4a	83.6 ± 4.68

Number of cells that invaded a collagen gel. After seeding 7.5×10^4 cells, the number of invading cells in five photographic fields from three separate experiments (total of 15 fields/cell line) were counted. Results are given as the mean number of invading cells ± SEM. *, $P \ll 0.01$ between ILK13A1a3 compared to IEC-18 and ILK-14 cells (Student's unpaired *t* test).

Five randomly selected fields measuring 1.0 mm × 1.4 mm were photographed at a single level beneath the surface monolayer by using a 10× phase contract objective. For scp2, morphology was examined in a reconstituted basement membrane gel. Liquid matrigel (200 μl/35-mm dish) was spread on ice and allowed to gel for 1 hr at 37°C before cell plating. Cells were plated at 2×10^5 per dish and maintained for 72 hr in serum-free medium (DMEM/F12 1:1) supplemented with 5 μg/ml of insulin, 1 μg/ml of hydrocortisone, and 3 μg/ml of prolactin for 72 hr. The cells then were photographed live, by using a Nikon inverted phase microscope.

Site-Directed Mutagenesis of ILK Kinase Domain. Mutations were introduced into wt ILK-cDNA (1) with the Promega Altered Sites II System (Promega). A mutant oligomer (with

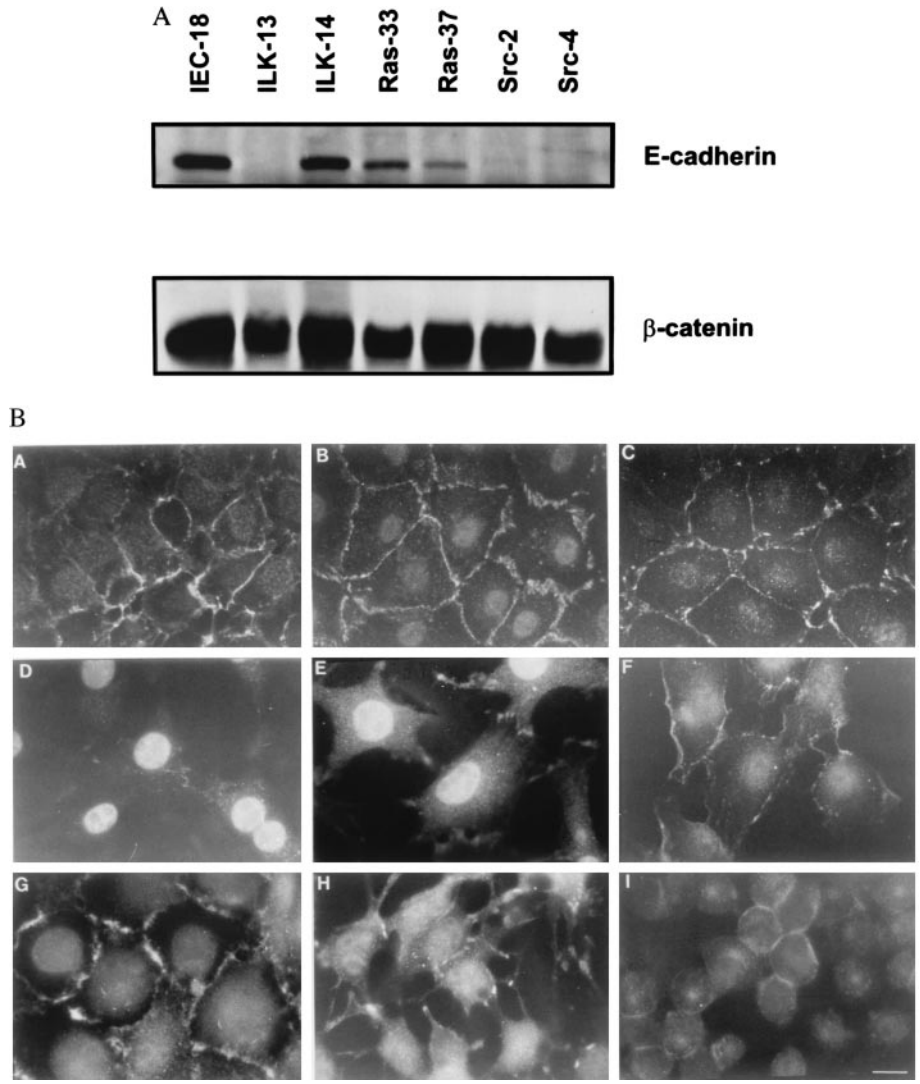

FIG. 2. (*A*) Immunoblot for E-cadherin and β-catenin. Cell lysates (10 μg) were analyzed for levels of E-cadherin and β-catenin expression by Western blotting as described in *Materials and Methods*. (*B*) Indirect immunofluorescence for β-catenin. Cells were plated out on coverslips and stained with antibody toward β-catenin and then with a fluorescent secondary antibody. *A*) Parental IEC-18; *B*) control transfected ILK-14 clone A2c3; *C*) control transfected ILK-14 clone A2c6; *D*) ILK-overexpressing ILK-13 clone A4a; *E*) ILK-overexpressing ILK-13 clone Ala3; *F*) IEC-18GH3IRH kinase deficient ILK; *G*) IEC-18 cells expressing activated H-*ras* oncogene (*Ras 33*) and (*Ras 37*), respectively; *H*) *Ras 37*; *I*) IEC-18 cells expressing v-*src* oncogene (*Src 2*). (Bar = 5 μm.) (×1,000.)

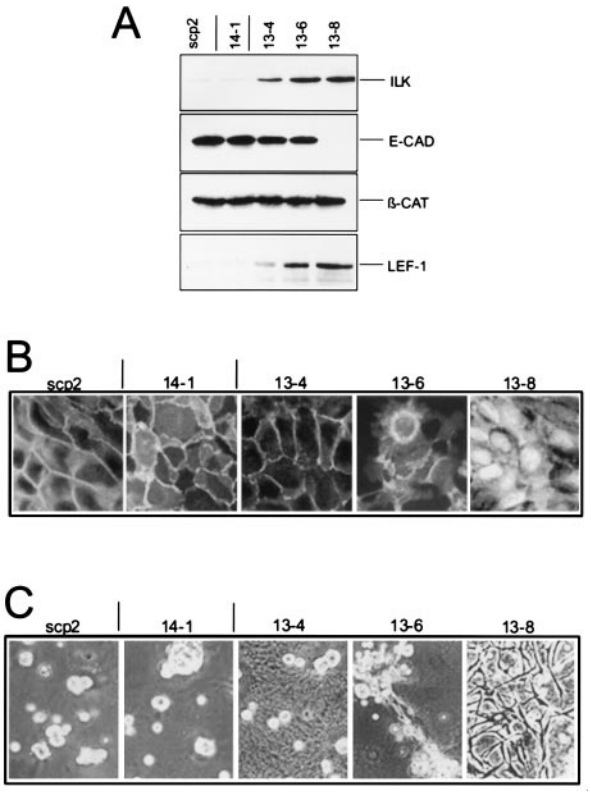

FIG. 3. (*A*) ILK, E-cadherin, β-catenin, and LEF-1 expression in scp2. Nonidet P-40 cell lysates (10 μg for ILK and LEF-1; 20 μg for E-cadherin and β-catenin) were analyzed by Western blotting as described in *Materials and Methods*. Clone 14–1: control transfectants, transfected with antisense ILK cDNA; Clones 13–4, 13–6, and 13–8: transfected with ILK-sense cDNA. (*B*) Indirect immunofluorescence for β-catenin. Cells were fixed in methanol and stained with a mouse mAb for β-catenin (Transduction Laboratories) that was visualized with a fluorescent secondary antibody. (×600.) (*C*) Morphology on a reconstituted basement membrane gel. Cells were plated on Matrigel, maintained for 72 hr in serum-free medium, and then visualized by phase contrast microscopy. (×150.)

the altered nucleotide underlined) was used to change glutamic acid at position 359 to lysine (E359 → K, 5′-CTGCAGAGCTTTGGGGGCTACCCAGGCAG GTG-3′). The mutant clone was confirmed by dideoxy sequencing and subcloned into pGEX4T-1 glutathione *S*-transferase (GST) fusion vector (Pharmacia) to express GST-ILK in *Escherichia coli* (BL21-DE3) and into pcDNA3 (Invitrogen) to stably transfect kinase-deficient ILK into the IEC-18 cell line (IEC-18GH31RH containing a E359 → K mutation). Kinase deficiency of the E359 → K mutant was confirmed by *in vitro* kinase assays of GST-fusion protein as described by us previously (1).

Western Blotting and Immunoprecipitation. Cells were lysed for 10 min on ice in Nonidet P-40 lysis buffer (1% Nonidet P-40/50 mM Hepes, pH 7.4/150 mM NaCl/2 mM EDTA/2 mM phenylmethylsulfonyl fluoride/1 mM Na-o-vanadate/1 mM NaF/10 μg/ml aprotinin/10 μg/ml leupeptin). Extracts were centrifuged with the resulting supernatants being the cell lysate used in assays. Lysates were electrophoresed through SDS/PAGE and transferred to Immobilon-P membranes (Millipore). Antibodies used to probe Western blots were: rabbit anti-ILK (1), monoclonal anti-E-cadherin and monoclonal anti-β-catenin (Transduction Laboratories, Lexington, KY), and rabbit anti-LEF-1 (21). Bands were visualized with ECL chemiluminescent substrate (Amersham). For immunoprecipitation, Nonidet P-40 lysates were rotated with primary antibody ON at 4°C, then rotated with Protein G-Sepharose (Pharmacia) for 2 hr at room temperature. Beads were pelleted, boiled in electrophoresis sample buffer (nonreducing), and centrifuged, and supernatants were electrophoresed. Protein concentrations were determined by the Bradford assay (Bio-Rad).

Indirect Immunofluorescence. Cells were grown on coverslips, washed with PBS, fixed in 4% paraformaldehyde in PBS for 12 min, washed with PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 min, blocked with 4% BSA in PBS for 30 min at room temperature, incubated with rabbit anti-β-catenin (29) diluted 1:400 in 0.1% Triton X-100 for 60 min at 37°C, washed with PBS, incubated with rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:50 in 0.1% Triton X-100 for 30 min at 37°C, washed with PBS, then mounted onto a slide with Slow-Fade Antifade (Molecular Probes). Cells were viewed at 100-fold magnification by using a Zeiss Axiovert 135 fluorescence microscope.

Reporter Gene Assay. Cells were transiently transfected with chloramphenicol acetyl transferase reporter gene constructs containing multimerized wt (5′CCCTTTGAAGCTCGCA) or mutated (5′-CCaaTTcAAGCTCGAA) LEF-1/TCF binding sites, fused to the *Drosophila* alcohol dehydrogenase promoter. As a control for transfection efficiency, a luciferase gene construct containing a Rous sarcoma virus promoter was included in each experiment. Extracts were prepared and assayed 48 hr after transfection.

Electrophoretic Mobility Shift Assay. Twenty micrograms of nuclear extract (30) were incubated with 1 fmol of ^{32}P-labeled duplex oligonucleotide probe specific for LEF-1, in 20 μl of binding buffer containing 200 ng of poly[d(I-C)] and 400 ng of salmon sperm DNA, and electrophoresed through a 5% native polyacrylamide gel (21). For DNA competition, an 800-fold molar excess of oligonucleotide containing a specific LEF-1 binding site (21, 22) or a nonspecific EBF-binding site (31) was included in the DNA-binding reaction. For antibody addition, 1 μl of polyclonal anti-LEF-1 antibody or 1 μl of monoclonal anti-β-catenin antibody (Transduction Laboratories) were used.

RESULTS

ILK Stimulates Collagen Gel Invasion of IECs and Inhibits E-Cadherin Expression. To determine whether the loss of cell–cell adhesion was accompanied by an increased invasive phenotype, we examined, in collagen gels (13) the invasiveness of IEC-18 parental cells and ILK-overexpressing (ILK-13) cells (1) that had been stably transfected with an ILK cDNA expression vector. As shown in Fig. 1 and Table 1, the ILK-13 cells, which have approximately 5-fold overexpression of ILK (1), are much more invasive than the parental and control transfected (ILK-14) cells that have been transfected with an ILK antisense cDNA expression construct and express the same level of ILK as the parental cells (1). Collagen-gel invasion by epithelial cells normally is associated with an epithelial to mesenchymal transformation characterized by the down-regulation of E-cadherin expression (4, 5). In agreement with this finding, the expression of E-cadherin protein is lost in ILK overexpressing cells (ILK-13), but is maintained in control transfected cells (Fig. 2*A*). E-cadherin expression also is reduced in IEC-18 cells transfected with activated H-*ras* cDNA (14) and in v-*src* transformed cells (Fig. 2*A*). In contrast, the steady-state levels of the expression of the intracellular E-cadherin binding protein, β-catenin, are approximately the same in all IEC transfectants examined (see Fig. 2*A* and 4*A*).

ILK Induces Nuclear Translocation of β-Catenin in IEC and Mammary Epithelial Cells. Because ILK overexpression resulted in a marked down-regulation of the expression of E-cadherin, we examined the subcellular localization of β-catenin in these cells. In sharp contrast to the localization of β-catenin at the plasma membrane and at cell–cell adhesion sites in the parental IEC-18 and control transfected ILK-14 cell clones (A2c3 and A2c6), (Fig. 2*B*, parts *A–C*), β-catenin is localized entirely in the nuclei of ILK overexpressing ILK-13 clones (A4a and A1a3) (Fig. 2*B*, parts *D* and *E*). This ILK-induced nuclear localization of β-catenin is

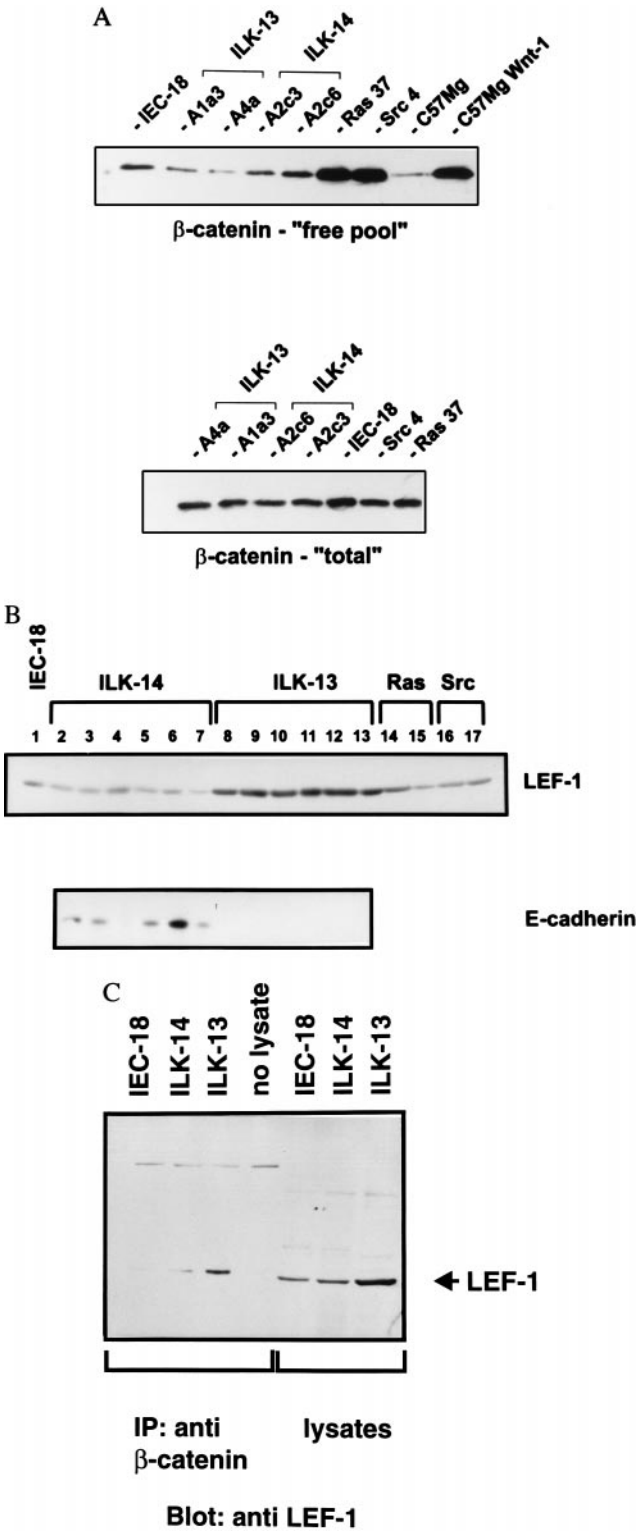

FIG. 4. (*A*) Immunoblot of uncomplexed and total β-catenin. Cell lysates were precipitated with Sepharose coupled to a glutathione *S*-transferase fusion protein containing the cytoplasmic domain of E-cadherin to bind uncomplexed pools of β-catenin (19). The pelleted beads were solubilized and electrophoresed through SDS/PAGE then Western-blotted with antibody toward β-catenin. (*Lower*) Comparison of the total concentrations of β-catenin. C57Mg is a mouse mammary epithelial cell line and C57Mg Wnt-1 is its counterpart stably transfected to constitutively express Wnt-1 (C57805) (32). (*B*) Immunoblot for LEF-1 and E-cadherin. Supernatants of cells lysed in Nonidet P-40 lysis buffer (40 μg) were electrophoresed through 8% SDS/PAGE and Western-blotted with antibody toward LEF-1 and E-cadherin. Lane 1, IEC-18; lanes 2–7, ILK-14 clones A2a3, A2c3, A2c6, A2 g3, A2 g8, and A3a1; lanes 8–13, ILK-13 clones, A1a3, A1d11, A4a, A4a3, A4c, and A4i; lanes 14 and 15, Ras clones 33, 37; and lanes 16 and 17) Src clones 2,4. (*C*) Coimmunoprecipitation of LEF-1 with β-catenin. Cell extracts (500 μg in Nonidet P-40 lysis buffer) were immunoprecipitated with 4 μg of β-catenin mAb and electrophoresed though 8% SDS/PAGE along with 20 μg of cell lysates alone. The gel was Western-blotted with antibody toward LEF-1.

dependent on an active kinase, because overexpression of a kinase-deficient ILK (E359 → K) did not induce nuclear translocation of β-catenin, which remains localized largely to the plasma membrane (Fig. 2*B*, part *F*). Likewise, overexpression of kinase-deficient ILK also did not result in a loss of E-cadherin expression (data not shown). The translocation of β-catenin to the nucleus is a specific property of ILK, because in IEC-18 cells transfected with activated H-*ras* (Fig. 2*B*, parts *G* and *H*) or v-*src* (Fig. 2*B*, part *I*) oncogenes, β-catenin is not translocated to the nucleus, but either is localized to the plasma membrane or is expressed diffusely in the cytoplasm. Although these oncogenes also disrupt the epithelial morphology of IEC-18 cells and result in the down-regulation of E-cadherin expression, the translocation of β-catenin to the nucleus is observed specifically in ILK-expressing cells, suggesting that loss of E-cadherin expression and β-catenin nuclear translocation may be regulated differentially.

To determine whether overexpression of ILK in other epithelial cells also would induce similar changes in phenotypic properties, as well as alterations in E-cadherin expression and β-catenin localization, we transfected and overexpressed ILK in scp2 (11, 12), whose differentiative response is mediated by laminin in an integrin-dependent manner (11, 12). Three stably ILK overexpressing cell clones (ILK-13) were identified, and as shown in Fig. 3*A*, subclone ILK 13–8 expressed the highest level of ILK, whereas ILK 13–4 and ILK 13–6 expressed intermediate levels. As was observed in the IEC-18 cells, transfection with an antisense ILK cDNA (ILK-14) did not result in alterations in ILK expression levels compared with the parental levels. Interestingly, as was the case in ILK-overexpressing IEC-18 cells, a reduction in E-cadherin expression is observed in clone 13–8, although there does not appear to be a strict correlation between ILK expression levels and E-cadherin expression (Fig. 3*A*). Likewise, the level of expression of β-catenin protein is unaltered by ILK, but its cellular localization is dramatically altered and is almost entirely associated with the nucleus in the highly ILK-overexpressing cells (ILK 13–8). Interestingly, β-catenin is associated with the plasma membrane in the moderate ILK-overexpressing (ILK 13–4) cells and is cytoplasmic or perinuclear in the intermediate ILK-overexpressing (ILK 13–6) cells, suggesting a role for a threshold level of ILK in promoting the nuclear localization of β-catenin. In addition, scp2 overexpressing ILK (13–8) are also highly invasive, as shown in Fig. 3*C*, which shows the morphology of the ILK cell clones in matrigel. These data demonstrate similar effects of increasing ILK expression in epithelial cell lines derived from different tissue origins, and suggest that, although ILK overexpression does result in decreases in E-cadherin expression, the levels of ILK expression correlate better with β-catenin nuclear localization and LEF-1 expression (see below) rather than with E-cadherin expression.

ILK Stimulates LEF-1 Expression But Does Not Alter β-Catenin "Free Pools." Translocation of β-catenin to the nucleus can be induced by the activation of the Wnt signaling pathway (6, 10), which initially results in an elevation of free cytosolic β-catenin caused by decreased degradation normally regulated by ubiquitination of β-catenin. Alternatively, loss of expression or mutations in the tumor suppressor protein APC (15) and certain mutations in the β-catenin gene itself can lead to cytosolic β-catenin stabilization and nuclear translocation (16, 17). The nuclear translocation of β-catenin is associated with complex formation between β-catenin and members of the high mobility group transcription factors, LEF-1/TCF, which then activate (or si-

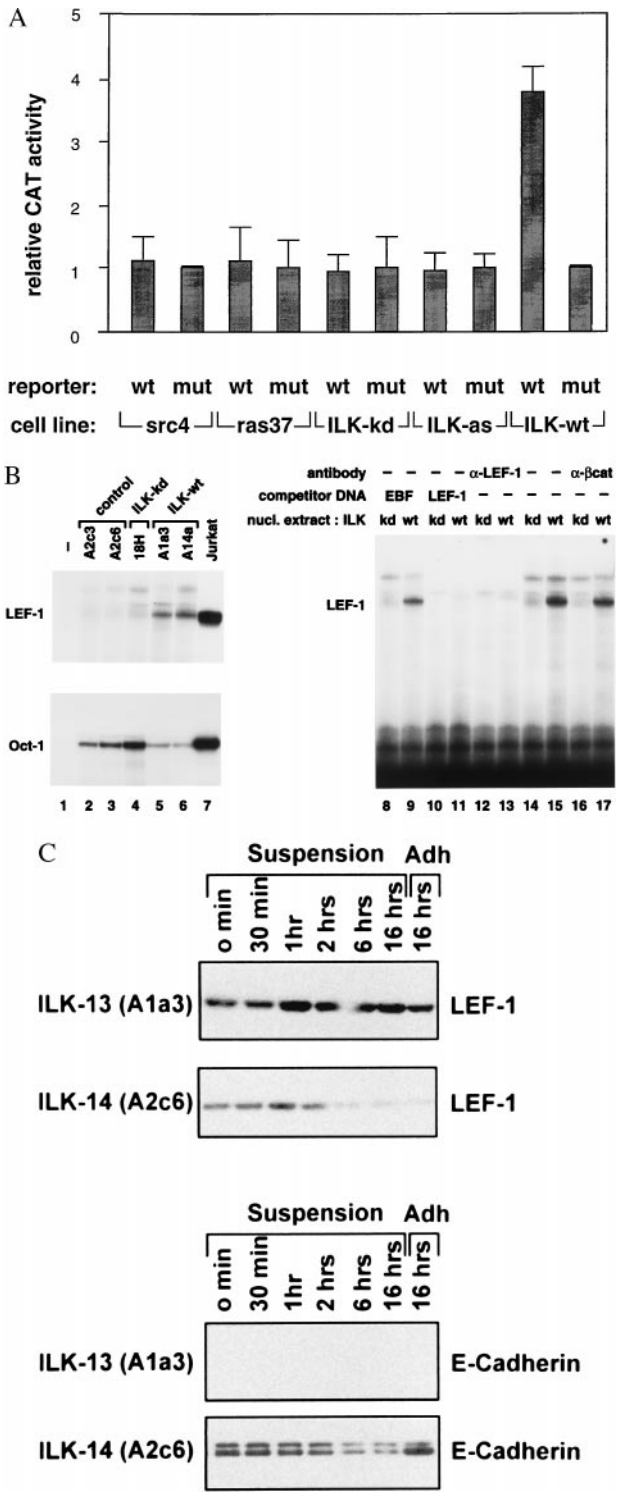

FIG. 5. (*A*) Transcriptional activation of a LEF-1 responsive reporter construct. IEC-18 cell transfectants (described in *Materials and Methods*) were transiently cotransfected with a chloramphenicol acetyl transferase (CAT) reporter construct containing either wt or mutated (mut) LEF-1/TCF binding sites, and a control luciferase reporter construct. CAT activities were standardized relative to their corresponding luciferase activities. (*B*) Gel mobility shift assays for LEF-1. Nuclear extracts of ILK14 control transfectants (control), the kinase-deficient transfected cell line (kd), ILK-overexpressing ILK13 cell lines (wt), and Jurkat T cells, were assayed for LEF-1 (*Upper*, lanes 1–7) and Oct-1 (*Lower*, lanes 1–7) DNA binding activity, the latter to serve as a control for nuclear extract preparations. Nonspecific EBF competitor DNA (lanes 8 and 9), specific LEF-1 competitor DNA (lanes 10 and 11), anti-LEF-1 antibody (lanes 12 and 13), and anti-β-catenin antibody (lanes 16 and 17) were added to determine the specificity of the LEF-1 bandshift. A complex migrating more slowly than LEF-1 is present in both the wt ILK13 and the kinase-deficient transfected cells, and this may represent binding by another member of the LEF-1/TCF family. (*C*) Immunoblot showing the effect of cell detachment on the levels of LEF-1 and E-cadherin. Cells were detached from tissue culture plates with 5 mm EDTA/PBS, placed on ice ($t = 0$) or incubated in suspension in complete medium containing fetal calf serum at 37°C with 5% CO_2 for up to 16 hr, then pelleted and extracted with Nonidet P-40 lysis buffer. Lysates (50 μg) were electrophoresed through 8% SDS/PAGE and Western-blotted with antibody toward LEF-1 and E-cadherin.

lence) transcription of target genes (7–9). However β-catenin contains "importin"-like sequences (18) that may mediate nuclear localization independently of LEF-1/TCF proteins. Because the steady-state levels of β-catenin were not changed by ILK overexpression in IEC-18 cells, we measured "uncomplexed" or "free" β-catenin levels, as determined by binding to a cytoplasmic domain peptide of E-cadherin (19). Surprisingly, "uncomplexed" pools of β-catenin in ILK-overexpressing (ILK-13) clones were found to be lower or unaltered compared with IEC-18 cells or control ILK 14 clones (Fig. 4*A*), and were never higher in any of the clones examined. This finding indicates that most of the β-catenin in the ILK-overexpressing cells may be complexed with nuclear components, such as transcription factors, because these cells do not express E-cadherin. In contrast, free β-catenin pools in *Ras*- and *Src*-transformed cells were high, although this did not result in nuclear translocation of β-catenin (Fig. 2*B*), suggesting that ILK may activate alternative pathways for the nuclear translocation of β-catenin, other than stabilization of cytosolic β-catenin. We therefore examined the expression levels of LEF-1, a member of the family of high mobility group transcription factors (7–9, 20) that bind β-catenin. We found that the expression of LEF-1 is dramatically higher in six independent IEC-18 ILK-overexpressing (ILK-13) cell clones as compared with six independent control transfected ILK-14 clones, as well as two activated H-*ras* transfected and v-*src* transfected IEC-18 clones (Fig. 4*B*). Furthermore, LEF-1 expression also is elevated in scp2 ILK-overexpressing cells, with the highest level of LEF-1 expressed in ILK 13–8 cells, which also express the highest level of ILK (Fig. 3*A*).

ILK Stimulates LEF-1/β-Catenin Complex Formation and LEF-1 Transcriptional Activity. The increased levels of LEF-1 and the nuclear translocation of β-catenin are associated with enhanced complex formation between LEF-1 and β-catenin in the IEC-18 ILK-overexpressing cells (Fig. 4*C*), as determined by coimmunoprecipitation of LEF-1 and β-catenin from extracts of ILK-13 cells.

LEF-1 is a transcription factor that is by itself unable to stimulate transcription from multimerized sites, although in association with β-catenin LEF1/TCF proteins can augment promoter activity from multimerized binding sites (21, 22). We examined transcriptional activation from a synthetic LEF/β-catenin responsive promoter construct containing multimerized LEF/TCF binding sites in IEC-18 ILK-overexpressing cells and in control kinase-deficient ILK-expressing cells (Fig. 5*A*). As a control, we also transfected the promoter construct into IEC-18 cells together with activated *Ras* and *Src* oncogenes. High promoter activity was observed only in ILK-overexpressing cells, and the extent of transcriptional activation was substantially reduced with promoter constructs containing mutations in the multimerized LEF-1/TCF binding sites (Fig. 5*A*). Moreover, we analyzed nuclear extracts from IEC-18 ILK-overexpressing cell clones and from cell clones transfected with an antisense or kinase-deficient ILK cDNA to identify proteins that bind the LEF/TCF binding site in an electrophoretic mobility shift assay (Fig. 5*B*). In ILK-overexpressing cells, a nuclear factor was detected that displays the same electrophoretic mobility as LEF-1 in Jurkat T cells. To control for variability in the preparation of nuclear extract, we examined the abundance of the unrelated Oct-1 protein (Fig. 5*B*, *Lower*). The identity of LEF-1 was confirmed by addition of anti-LEF-1-antibody (Fig. 5*B*, lanes 12 and

13) or excess of specific oligonucleotide (Fig. 5*B*, lanes 10 and 11), or nonspecific oligonucleotide (Fig. 5*B*, lanes 8 and 9). We also examined whether the slower-migrating complex contains β-catenin by adding anti-β-catenin antibody (Fig. 5*B*, lanes 16 and 17). No significant change in the LEF-1/DNA complex and the slower-migrating complex was detected, suggesting that the slower-migrating complex may contain another member of the LEF-1/TCF protein family.

Cell Adhesion Regulates LEF-1 Expression. ILK binds to the cytoplasmic domain of β_1 and β_3 integrin subunits (1), and its kinase activity is down-regulated at epithelial cell attachment to fibronectin (1). Overexpression of constitutively activated ILK overcomes this regulation of ILK activity by integrin occupation and results in decreased cell adhesion to extracellular matrix (ECM) (1). Fig. 5*C* shows that cell adhesion to ECM suppresses LEF-1 expression, which is rapidly, but transiently, elevated at cell detachment in IEC-18, ILK-14, and ILK-13 cells. However, in ILK-overexpressing ILK-13 cells the elevation in LEF-1 levels are more robust and are maintained at high levels for as long as 16 hr in suspension (Fig. 5*C*). Furthermore, LEF-1 levels are also higher in adherent ILK-13 cells compared with ILK-14 cells. These data suggest that ILK overexpression overcomes the regulation of LEF-1 expression by adhesion-deadhesion, and that the maintenance of constitutively high levels of LEF-1 result in enhanced complex formation between LEF-1 and β-catenin, translocation of β-catenin to the nucleus, and transcriptional activation of responsive genes.

DISCUSSION

The transformation of epithelial cells to mesenchymal cells is of critical importance during embryonic development. Furthermore, such changes in adult tissue are important to the oncogenic and invasive properties of tumor cells derived from epithelial origins. Carcinomas frequently exhibit loss of expression of E-cadherins, which predisposes them to be more invasive (4). The molecular basis for this increased invasiveness is not understood but may include the activation of mesenchymal gene expression and transformation to a more mesenchymal phenotype. Thus the expression of E-cadherin in *Xenopus* fibroblasts results in a down-regulation of expression of extracellular matrix proteins such as fibronectin and its cognate integrin receptors (33). In the ILK overexpressing cells, the loss of E-cadherin expression correlated with increased expression of fibronectin and increased fibronectin matrix assembly (3).

Another means of regulating epithelial and mesodermal cell fate is via certain Wnt proteins, which activate a signaling pathway that results in the stabilization of the cytosolic pool of β-catenin. The resulting complex formation with LEF-1/TCFs, and the activation of the β-catenin/LEF-1 (TCF) transcription factor seems to up-regulate mesenchymal genes but also may down-regulate E-cadherin expression (5). For example, X-TCF-3/β-catenin has been shown to induce transcription of genes encoding homeobox proteins that regulate mesenchymal genes, e.g. Siamois in *Xenopus* (23–25), consistent with the possibility that this pathway may mediate the observed epithelial to mesenchymal transformation, as well as the oncogenic properties of ILK in epithelial cells. In addition, constitutive activation of TCF/β-catenin is oncogenic in human colon carcinomas and melanomas (15–17). The data presented here suggest a connection between the expression levels of ILK and LEF-1 and the signaling properties of β-catenin in mesenchymal induction because the expression and the nuclear translocation of β-catenin are tightly coupled in ILK-transformed cells. On the other hand, although ILK overexpression does result in a loss of E-cadherin expression, a tight correlation does not exist between ILK and E-cadherin expression, and β-catenin nuclear localization, as exemplified by the results shown in Fig. 3*A*. Furthermore, the loss of E-cadherin expression does not correlate with nuclear β-catenin translocation, e.g. in the v-*src*- and H-*ras*-transformed cells, suggesting a specific property of ILK in regulating this function, which may include the ability of ILK to stimulate the expression of LEF-1. However, the work of others has shown that E-cadherin can antagonize Wnt-induced β-catenin signaling (26, 27). Preliminary evidence suggests that this regulation is most likely at the posttranslational level because LEF-1 mRNA levels are unchanged in the ILK-overexpressing cells (A.N. and S.D., unpublished observations).

The results reported here demonstrate an additional pathway to that by activated Wnt-1 leading to nuclear localization of β-catenin and increased transcriptional activation by LEF-1. These data also corroborate recent reports showing that overexpression of LEF-1 by itself can result in LEF-1/β-catenin-mediated transcription independently of Wnt signaling (20, 28). Here it is shown that in contrast to the effects of Wnt-1, activated ILK can dramatically induce nuclear translocation of β-catenin and formation of LEF-1/β-catenin complex without a corresponding increase in the free pool of β-catenin. This ILK-regulated pathway may be modulated via cell adhesion to extracellular matrix, but can be constitutively activated by ILK overexpression. Because ILK overexpression induces anchorage-independent cyclin D1 and cyclin A expression, it will be interesting to determine whether the expression of the cell cycle genes can be regulated by ILK via activation of LEF-1/β-catenin, and whether ILK is overexpressed or activated in certain carcinomas.

We thank Dr. Walter Birchmeier for his kind gift of anti-β-catenin antibody, and Mina Viscardi and Marc Coppolino for help in preparing the manuscript. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council (to S.D.), as well as by a grant from the Swiss National Science Foundation (to R.M.). R.G. is an Investigator of the Howard Hughes Medical Institute. S.D. is a Terry Fox Scientist of the National Cancer Institute of Canada.

1. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C. & Dedhar, S. (1996) *Nature (London)* **379,** 91–96.
2. Radeva, G., Petrocelli, T., Behrend, E., Leung-Hagesteijn, C., Filmus, J., Slingerland, J. & Dedhar, S. (1997) *J. Biol. Chem.* **272,** 13937–13944.
3. Wu, C., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Coppolino, M., Goicoechea, S., McDonald, I. A. & Dedhar, S. (1998) *J. Biol. Chem.*, **273,** 528–536.
4. Brache, M. E., Van Roy, F. M. & Mareel, M. M. (1996) *Curr. Top. Microbiol. Immunol.* **213,** 123–161.
5. Huber, O., Bierkamp, C. & Kemler, R. (1996) *Curr. Opin. Cell Biol.* **8,** 685–691.
6. Miller, J. R. & Moon, R. T. (1996) *Genes Dev.* **10,** 2527–2539.
7. Behrens, J., von Kries, J. P., Kuhl, M., Bruher, L, Weldich, D., Grosschedl, R. & Birchmeier, W. (1996) *Nature (London)* **382,** 638–642.
8. Huber, O., Korn, R., McLaughlin, J., Oshugi, M., Hermann, B. G. & Kemler, R. (1996) *Mech. Dev.* **59,** 3–10.
9. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Madaire, J., Godsave, S., Korinek, V., Roose, J., Destree, O. & Clevers, H. (1996) *Cell* **86,** 391–399.
10. Barth, A. I. M., Nathke, I. S. & Nelson, W. J. (1997) *Curr. Opin. Cell Biol.* **9,** 683–690.
11. Desprez, P., Roskelley, C., Campisi, J. & Bissell, M. (1993) *Mol. Cell Diff.* **1,** 99–110.
12. Streuli, C. H., Bailey, N. & Bissell, M. J. (1991) *J. Cell Biol.* **115,** 1383–1395.
13. Montesano, R. & Orci, L. (1985) *Cell* **42,** 469–477.
14. Filmus, J., Church, J. G. & Buick, R. N. (1988) *Mol. Cell. Biol.* **8,** 4243–4249.
15. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* **275,** 1784–1787.
16. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K. W. (1997) *Science* **275,** 1787–1790.
17. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. & Polakis, P. (1997) *Science* **275,** 1790–1792.
18. Kussel, P. & Frasch, M. (1996) *J. Cell Biol.* **129,** 1491–1507.
19. Papkoff, J., Rubinfeld, B., Schryver, B. & Polakis, P. (1996) *Mol. Cell. Biol.* **16,** 2128–2134.
20. Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R. & Biong, M. (1997) *Cell* **88,** 777–787.
21. Travis, A., Amsterdam, A. & Belanger, C. (1991) *Genes Dev.* **5,** 880–894.
22. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., VanEs, J., Loureiro, J., Ypma, A., Hursch, D., Jones, T., Bejsovec, A. & Clevers, H. (1997) *Cell* **88,** 789–799.
23. Carnac, G., Kodjabochian, L., Gurdon, J. B. & Lemaire, P. (1996) *Development (Cambridge, U.K.)* **122,** 3055–3065.
24. Moon, R. T., Brown, J. D. & Torres, M. (1997) *Trends Genet.* **13,** 157–162.
25. Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T. & Kimelman, D. (1997) *Genes Dev.* **11,** 2359–2370.
26. Fagotto, F., Funayama, N., Gluck, V. & Gumbiner, B. M. (1996) *J. Cell Biol.* **132,** 1105–1114.
27. Heasman, J., Crawford, A., Gladstone, K., Garner, H. P., Gumbiner, B., McRea, P., Kintner, C., Novo, C. Y. & Wylie, C. (1994) *Cell* **79,** 791–803.
28. Nusse, R. (1997) *Cell* **89,** 321–323.
29. Hulsken, J., Birchmeier, W. & Behrens, J. (1994) *J. Cell Biol.* **127,** 2061–2069.
30. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
31. Hagman, J., Travis, A. & Grosschedl, R. (1991) *EMBO J.* **10,** 3409–3417.
32. Blasband, A., Schryver, B. & Papkoff, J. (1992) *Oncogene* **7,** 153–161.
33. Finnemann, S., Kuhl, M., Otto, G. & Weldich, D. (1995) *Mol. Cell Biol.* **15,** 5082–5091.