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Laminin-10/11 and Fibronectin Differentially Prevent Apoptosis Induced by Serum Removal via Phosphatidylinositol 3-Kinase/Akt- and MEK1/ERK-dependent Pathways*

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Cell adhesion to the extracellular matrix inhibits apoptosis, but the molecular mechanisms underlying the signals transduced by different matrix components are not well understood. Here, we examined integrin-mediated antiapoptotic signals from laminin-10/11 in comparison with those from fibronectin, the best characterized extracellular adhesive ligand. We found that the activation of protein kinase B/Akt in cells adhering to laminin-10/11 can rescue cell apoptosis induced by serum removal. Consistent with this, wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, or ectopic expression of a dominant-negative mutant of Akt selectively accelerated cell death upon serum removal. In contrast to laminin-10/11, fibronectin rescued cells from serum depletion-induced apoptosis mainly through the extracellular signal-regulated kinase pathway. Cell survival on fibronectin but not laminin was significantly reduced by treatment with PD98059, a specific inhibitor of mitogen- or extracellular signal-regulated kinase kinase-1 (MEK1) and by expression of a dominant-negative mutant of MEK1. Laminin-10/11 was more potent than fibronectin in preventing apoptosis induced by serum depletion. These results, taken together, demonstrate laminin-10/11 potency as a survival factor and demonstrate that different extracellular matrix components can transduce distinct survival signals through preferential activation of subsets of multiple integrin-mediated signaling pathways.

Cell adhesion to the ECM[1] generates intracellular signals that modulate cell proliferation, survival, and differentiation (1, 2). Normal epithelial cells deprived of matrix attachment undergo programmed cell death, a form of apoptosis termed anoikis (3). Malignant transformation by oncogenic Ras can prevent this process of apoptosis after denial of ECM attachment (3) or withdrawal of survival factors (4).

One approach to study the effects of ECM signals independently of signals from other extracellular sources has been to deprive cells of serum and then to analyze the effects of specific ECM ligands on cellular functions such as adhesion, migration, and survival. Using this approach, many cell biologic functions of ECM signals have been elucidated (2). Most studies of integrin-mediated signaling events have been performed on cells adhering to FN through the $\alpha_5\beta_1$ integrin, which seems to be involved in regulating apoptosis triggered by serum deprivation in many cell types (5, 6). This integrin has also been reported to protect neuronal cells against apoptosis triggered by β-amyloid peptide (7). In breast epithelial cells, the $\alpha_6\beta_1$ integrin receptor for laminin-1 has been shown to cooperate with insulin-signaling pathways to protect cells from apoptosis (8). In endothelial cells, functional inhibition of the $\alpha_v\beta_3$ integrin can lead to programmed cell death (9). Thus, several distinct integrins have been implicated in protection against apoptosis in different cell types. However, the signaling events transduced by the $\alpha_3\beta_1$ integrin, the major receptor for laminin-10/11 (LN-10/11) and LN-5, remain unclear. In addition, it is also not known whether $\alpha_3\beta_1$ integrin-mediated signals differ from those transduced by the $\alpha_5\beta_1$ integrin.

Several signal transduction components, including focal adhesion kinase (FAK) (10–12), phosphatidylinositol 3-kinase (PI 3-kinase) (8, 13, 14), extracellular signal-regulated kinase (ERK) (15–17), and c-Jun NH_2-terminal kinase (18), have been implicated in the mechanisms underlying anoikis (19). FAK has been proposed to couple integrins and cytoskeletal proteins to multiple signaling pathways. Several lines of evidence suggest that integrin activation of signaling pathways involving PI 3-kinase, ERK, and c-Jun NH_2-terminal kinase require FAK (12, 18, 20–23). However, some studies suggest that integrins are able to activate at least some of these pathways independently of FAK (24–26). Recently, increasing evidence has emerged showing that PI 3-kinase and its downstream effector Akt play key roles in the regulation of cell survival. For example, signals through the PI 3-kinase/Akt pathway protect Madin-Darby canine kidney cells against apoptosis mediated by denial of cell anchorage or by radiation (27). It was found that cell death in this system was inhibited by expression of a constitutively activated form of Akt (13, 27). On the other hand, the ERK pathway has also been found to enhance cell survival (28, 29).

Laminins are the major components of the basement membrane. Cells bind directly to laminins via a subset of integrins (30) and other nonintegrin receptors, such as α-dystroglycans (31). All laminins are composed of α, β, and γ chains. The

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[1] The abbreviations used are: ECM, extracellular matrix; Cas-ΔSD, $p130^{cas}$ lacking substrate domain; DMEM, Dulbecco's modified Eagle's medium; DN, dominant negative; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FN, fibronectin; HA, hemagglutinin; FRNK, FAK-related nonkinase; LN-10/11, laminin-10/11; MEK1, mitogen- or extracellular signal-regulated kinase kinase-1; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; PLL, poly-L-lysine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VSV, vesicular stomatitis virus glycoprotein; z-VAD, z-Val-Ala-Asp-flurometh ylketone; BSA, bovine serum albumin.

α_5-containing laminins, LN-10 ($\alpha_5\beta_1\gamma_1$) and LN-11 ($\alpha_5\beta_2\gamma_1$) are widely expressed in fetal and adult tissues (32, 33). Recently, we purified LN-10/11 from conditioned medium of A549 human lung carcinoma cells and found that the $\alpha_3\beta_1$ integrin is the preferred receptor for LN-10/11 (34, 35). LN-10/11 is more active than FN in promoting cell migration, and it preferentially activates Rac, but not Rho, via the p130^{cas}-CrkII-DOCK180 pathway. Cells adhering to FN develop stress fibers and focal contacts, whereas cells adhering to LN-10/11 do not, suggesting that LN-10/11 and FN have distinct effects on integrin-mediated cell spreading and migration (36).

In this study, the first goal was to determine whether $\alpha_3\beta_1$ integrin-mediated signals from LN-10/11 could rescue A549 cells from apoptosis induced by serum deprivation. We describe here that LN-10/11 has more survival potential than FN. The second goal was to identify pathway(s) that transduce the survival signals from LN-10/11. We report that survival signals from LN-10/11 are mainly through the PI 3-kinase/Akt pathway, whereas survival signals from FN are conveyed by MEK1/ERK through FAK.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—A549 human lung adenocarcinoma cells and HeLa S3 human cervix adenocarcinoma cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

Reagents and Antibodies—PD98059 (a specific MEK1 inhibitor) and wortmannin (a PI 3-kinase inhibitor) were purchased from Sigma. The caspase inhibitor I, z-VAD-FMK (z-Val-Ala-Asp-fluromethylketone, hereafter referred to as z-VAD), was obtained from Calbiochem. The DeadEnd Colorimetric Apoptosis Detection kit for the TUNEL assay was purchased from Promega (Madison, WI). Monoclonal anti-phospho-ERK1/2 as well as polyclonal anti-phospho-Akt and anti-Akt were purchased from New England BioLabs, Inc. (Beverly, MA). Monoclonal anti-FAK and anti-MEK1 were obtained from Transduction Laboratories (San Diego, CA). Polyclonal anti-ERK1/2 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Site-specific polyclonal antibodies against FAK specifically phosphorylated at Tyr-397, -407, -576, -577, or -925 were obtained from BIOSOURCE International (Camarillo, CA). Monoclonal anti-HA was purchased from Babco (Richmond, CA), and monoclonal antibodies against FLAG (M2) and VSV were from Sigma.

Preparation of LN-10/11 and FN—LN-10/11 was purified to homogeneity from conditioned media of A549 cells as previously described (34). Monoclonal antibody 5D6 recognizing the human laminin α_5 chain was used for immunoaffinity chromatography as described (37). FN was purified from human plasma by gelatin-Sepharose affinity chromatography.

Expression Plasmids—VSV-tagged FAK and VSV-tagged FRNK were generated from the green fluorescent protein vector pGZ21δxZ (38) by excision of the green fluorescent protein marker and the adjacent Kozak sequence using *Cla*I and *Bam*HI and replacement by a PCR-generated insert flanked by *Cla*I and *Bam*HI sites containing a Kozak consensus sequence, two adjacent repeats of the VSV epitope, and either full-length mouse FAK (to generate VSV-FAK) or FAK nucleotides 2185–3268 (VSV-FRNK); the fidelity of each construct was confirmed by DNA sequencing. FLAG-Cas and FLAG-ΔSD-Cas were constructed as described (38). The expression plasmid for the dominant negative HA-tagged MEK1 (HA-MEK1) was kindly provided by Dr. Natalie G. Ahn (Department of Chemistry and Biochemistry, University of Colorado); transfection of A549 cells with this construct decreased the level of ERK activation by 80% when assayed 10 min after plating on fibronectin by immunoblotting with anti-phospho-ERK1/2. Dominant-negative Akt (Akt-K179A) in the pCIS2 expression vector was provided by Dr. Michael J. Quon (Hypertension-Endocrine Branch, NIDDK, National Institutes of Health). The puromycin resistance plasmid pHA262pur was provided by Dr. Hein te Riele (The Netherlands Cancer Institute, Amsterdam).

Transfection and Cell Selection—Thirty μg of each expression plasmid was co-transfected with 5 μg of pHA262Puro into 3×10^6 A549 cells by electroporation at 170 V and 960 microfarads with a Bio-Rad Gene Pulser (Hercules, CA). To increase expression of transfected genes, 5 mM sodium butyrate was included in the culture medium. Cells were subcultured at a 1:3 dilution 12 h after transfection and maintained for 48 h in 1 μg/ml puromycin-containing medium. Before use, cells were cultured overnight in the absence of puromycin.

TUNEL Assay and Cell Viability—The TUNEL assay for labeling breaks in DNA strands was performed using the DeadEnd Colorimetric Apoptosis Detection kit (Promega). Samples were prepared according to the manufacturer's protocol. In brief, glass coverslips were coated with LN-10/11 (10 nM), FN (40 nM), or poly-L-lysine (PLL; 100 nM) in PBS overnight at 4 °C, and then nonspecific binding sites were blocked with 1% BSA. The coating concentration of FN was 4-fold higher than that of LN-10/11 to attain comparable levels of cell-adhesive activity for A549 cells (36). A549 cells were serum-starved overnight and then replated on the coverslips and incubated for specified times in DMEM containing 1% BSA. Cells were then fixed with 4% paraformaldehyde in PBS for 25 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After washing with PBS, the coverslips were incubated with biotinylated nucleotide mixture with terminal deoxynucleotidyl transferase enzyme; incorporated nucleotides were detected using diaminobenzidine and hydrogen peroxide and developed until there was a light brown background. Apoptotic nuclei were stained brown.

To assess loss of cell viability, the proportion of nonviable cells was determined by a trypan blue exclusion assay. Briefly, A549 cells were serum-starved overnight, detached with trypsin-EDTA, and kept in suspension in DMEM containing 1% BSA for 90 min. Cells were plated on dishes coated with either LN-10/11 or FN in serum-free DMEM with or without chemical inhibitors as indicated. At the times indicated, cells were harvested with trypsin-EDTA and then stained by trypan blue or photographed by phase-contrast microscopy. Comparisons with results of the TUNEL assay confirmed that rounded cells with a bright circumference indicative of high refractility by phase-contrast microscopy were all apoptotic cells, which were also nonviable cells that stained with trypan blue.

Western Blotting—After 24 h of serum starvation, A549 cells were detached from culture dishes with 0.05% trypsin-EDTA, washed with serum-free DMEM containing 1% BSA, and resuspended in the same medium. Cells were kept in suspension for 90 min at 37 °C on a rotator. 5×10^5 cells were allowed to spread on 35-mm tissue culture dishes coated with 5 nM LN-10/11 or 20 nM FN for the indicated times. After washing in ice-cold PBS, cells were solubilized in 250 μl of 1% Triton lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenymethylsulfonyl fluoride). The cell lysates were clarified by centrifugation at 20,000 × *g* for 15 min at 4 °C. Immunoblots for phospho-ERK, phospho-Akt, ERK, Akt or site-specific tyrosine-phosphorylated FAK epitopes were visualized by ECL (Amersham Biosciences).

RESULTS

Integrin-mediated Survival Signals from LN-10/11 Prevent Apoptosis Induced by Serum Depletion—Many epithelial cells require appropriate cell-ECM interactions for survival, and they undergo apoptosis in the absence of anchorage to the basement membrane (39). To determine whether LN-10/11 was able to serve as a survival ligand, cells were plated on substrates coated with LN-10/11, FN, or PLL in serum-free medium and then subjected to the TUNEL assay for apoptosis after specific periods of time. Cells cultured on PLL started to die even 7 h after replating, with ~30% of the cells becoming rounded and positively stained by this apoptosis assay, while more than 90% of the cells plated on FN or LN-10/11 remained spread and were negative for the assay (Fig. 1). After 20 h of incubation in the absence of serum, cells on PLL underwent apoptosis near the maximal level, but a majority of cells on LN-10/11 and FN remained spread and viable. However, after 27 h of incubation, not only cells on PLL but also a number of those on FN displayed a rounded morphology and underwent apoptosis. In contrast, most of the cells on LN-10/11 remained spread and viable, demonstrating that survival signal(s) from LN-10/11 are more potent than from FN in A549 cells. The prolonged cell survival on LN-10/11 but not on PLL was also observed with HeLa S3 cells (data not shown). We also found that the percentage of apoptotic cells as determined by TUNEL assay was nearly identical to the percentage of rounded, brightly phase-refractile cells, making it practical to use the

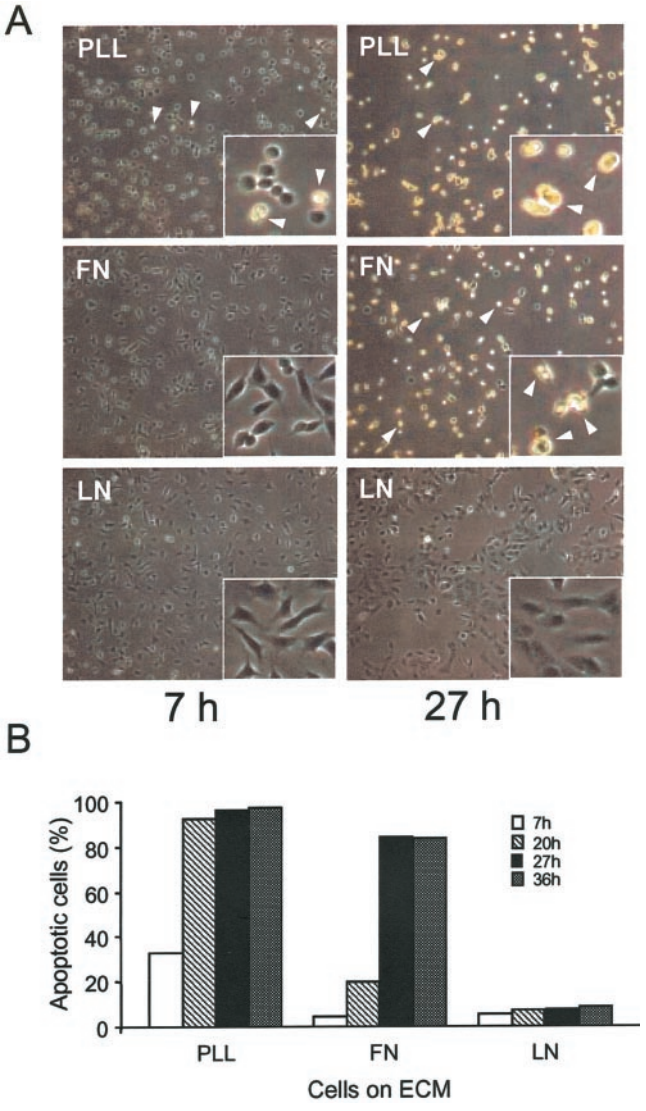

FIG. 1. **LN-10/11 protects A549 cells against apoptosis induced by serum depletion.** *A*, serum-starved A549 cells were allowed to spread on coverslips coated with PLL (100 nM), FN (40 nM), or LN-10/11 (10 nM) for 7 h (*left panel*) or 27 h (*right panel*) in DMEM containing 1% BSA. After fixation and permeabilization, cells were incubated with biotinylated nucleotide mixture, followed by incubation with streptavidin horseradish peroxidase and then with diaminobenzidine and hydrogen peroxide as described under "Experimental Procedures." The *arrowheads* indicate apoptotic cells stained brown. *B*, the data show the percentage of apoptotic cells quantified and expressed as the mean from two independent experiments.

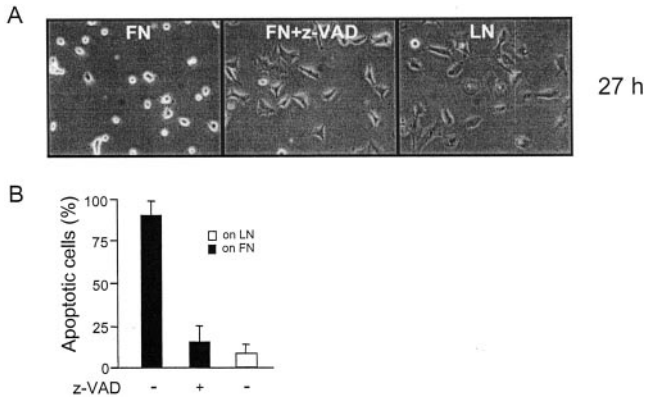

FIG. 2. **Serum deprivation-induced apoptosis is caspase-dependent.** *A*, serum-starved A549 cells were detached and then replated on 24-well culture dishes coated with FN (20 nM, *left* and *middle panel*) or LN-10/11 (5 nM, *right panel*) in 1% BSA medium with (*middle panel*) or without (*left* and *right panels*) 20 μM z-VAD. The coating concentrations of FN and LN-10/11 were reduced by half to obtain comparable cell-adhesive activity as obtained on glass coverslips. After incubation for 27 h, cells were photographed using a phase-contrast microscope. The rounded, phase-refractile cells shown in the *left panel* were considered apoptotic and nonviable as described under "Experimental Procedures." *B*, the percentages of apoptotic cells were quantified and expressed as the mean ± S.D. from three independent experiments.

percentage of rounded, phase-bright cells as an index of apoptotic cell death.

Serum Depletion-induced Apoptosis Is Caspase-dependent—There is extensive evidence for the involvement of caspases in apoptosis (15, 16, 40). To confirm whether the apoptosis induced by serum depletion was caspase-dependent, A549 cells were cultured on FN in the presence of z-VAD, a broad spectrum caspase inhibitor. As expected, cells cultured on FN in the presence of z-VAD remained spread and viable, as was the case for the cells on LN-10/11 in the absence of z-VAD (Fig. 2*A*). The percentage of apoptotic cells was significantly decreased from 87 to 15% when cells were plated on FN and incubated in the serum-free medium containing z-VAD (Fig. 2*B*), indicating that the apoptosis induced by serum removal is caspase-dependent.

LN-10/11 and FN Selectively Activate Akt and ERK Pathways—Several lines of evidence indicate that activation of PI 3-kinase/Akt or ERK pathways can block various apoptotic stimuli (8, 13–17, 27, 41). To explore the role of these signaling pathways in anchorage-dependent survival of cells, we examined the levels of activated Akt and ERK in cells adhering to LN-10/11 or FN in the absence of serum. Adhesion to LN-10/11 induced a rapid and strong stimulation of Akt; a high level of activated Akt persisted up to 1 h, followed by decline over 27 h (Fig. 3*A*). A low but significant level of activated Akt was still detectable after 27 h of incubation in the absence of serum. In contrast, Akt was only moderately activated on FN, with a peak at 10 min after replating. The levels of activated Akt on FN declined rapidly to undetectable levels within 3 h after replating (Fig. 3*A*). The stimulation of Akt on both substrates was completely blocked by wortmannin, a specific inhibitor of PI 3-kinase (Fig. 3*A*), indicating that the activation of Akt on LN-10/11 and FN was PI 3-kinase-dependent.

Unlike the activation of Akt, levels of activated ERK were slightly higher in cells adhering to FN than those to LN-10/11 over the period of 27-h incubation (Fig. 3*B*). Phosphorylated ERK was detectable even after 20–27 h of incubation on FN but not on LN-10/11. Although the levels of activated Akt and ERK in the cells stably adhering to LN-10/11 or FN were low and often only faintly detectable, it should be emphasized that both preferential activation of Akt on LN-10/11 and that of ERK on FN were reproducibly observed in repeated experiments.

Inhibition of PI 3-Kinase/Akt and MEK1/ERK Pathways Differentially Facilitates Apoptosis on FN and LN-10/11—Although both LN-10/11 and FN serve as cell survival factors, they may suppress apoptosis by triggering separate signaling pathways. To further explore the roles of the PI 3-kinase/Akt and MEK1/ERK pathways in cell survival on LN-10/11 and FN, we examined the effects of wortmannin and PD98059, specific inhibitors of PI 3-kinase and MEK1, respectively. Blockade of Akt activation by wortmannin was found to facilitate cell death on LN-10/11, as evidenced by the emergence of rounded cells with phase-bright circumferences, but it did not significantly affect cell death on FN (Fig. 4). The percentage of apoptotic cells increased from 4 to ~53% on LN-10/11 upon treatment with wortmannin. In contrast, treatment with PD98059 significantly increased the percentage of rounded, apoptotic cells on FN but exerted only a marginal effect on the viability of cells

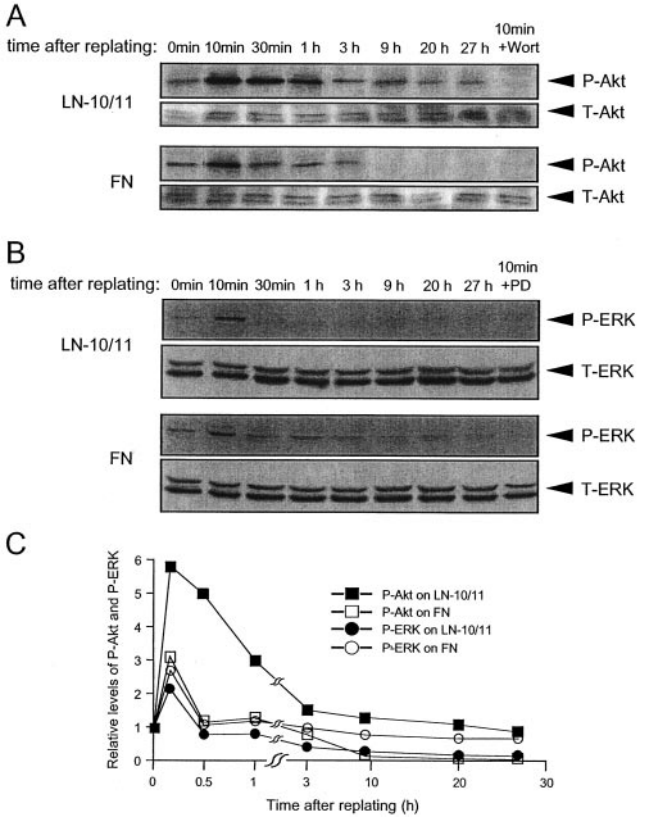

FIG. 3. **Activation of Akt and ERK in cells adhering to LN-10/11 and FN.** Serum-starved A549 cells were detached and maintained in suspension for 90 min and then replated on dishes coated with LN-10/11 (5 nM) or FN (20 nM) for the indicated times. Cell lysates were subjected to 10% SDS-PAGE. After electroblotting, blots were analyzed for phosphorylated Akt (*P-Akt*; *A*) and ERK (*P-ERK*; *B*) as well as total Akt (*T-Akt*; *A*) and ERK (*T-ERK*; *B*). *PD*, PD98059; *Wort*, wortmannin. *C*, densitometric quantification of the levels of phosphorylated Akt and ERK. The data are shown as -fold increases relative to the levels of the unstimulated control (*i.e.* cells before plating) after normalization against the levels of T-Akt and T-ERK. The data shown are the means of three separate experiments.

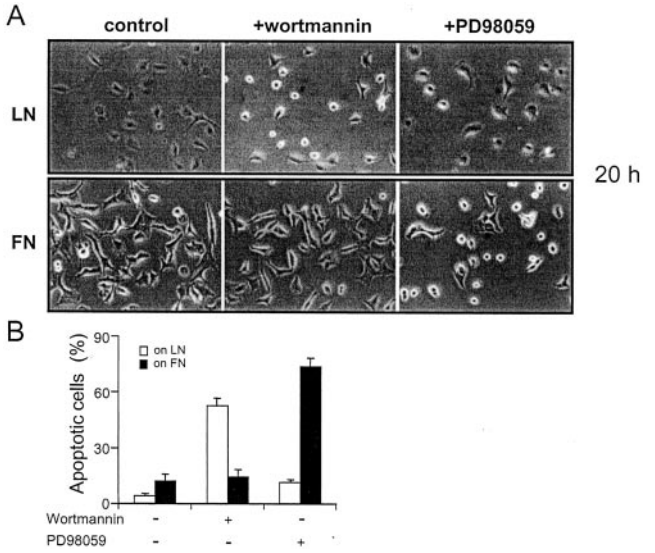

FIG. 4. **Wortmannin and PD98059 selectively block survival signals from LN-10/11 and FN.** *A*, serum-starved A549 cells were detached and replated on dishes coated with LN-10/11 (5 nM, *upper panel*) or FN (20 nM, *lower panel*) in 1% BSA medium without (*left panel*) or with 50 nM wortmannin (*middle panel*) or 20 nM PD98059 (*right panel*) for 20 h and then were photographed by phase-contrast microscopy. The percentage of rounded cells was taken as an index of apoptosis. *B*, data shown are the mean ± S.D. from three independent experiments.

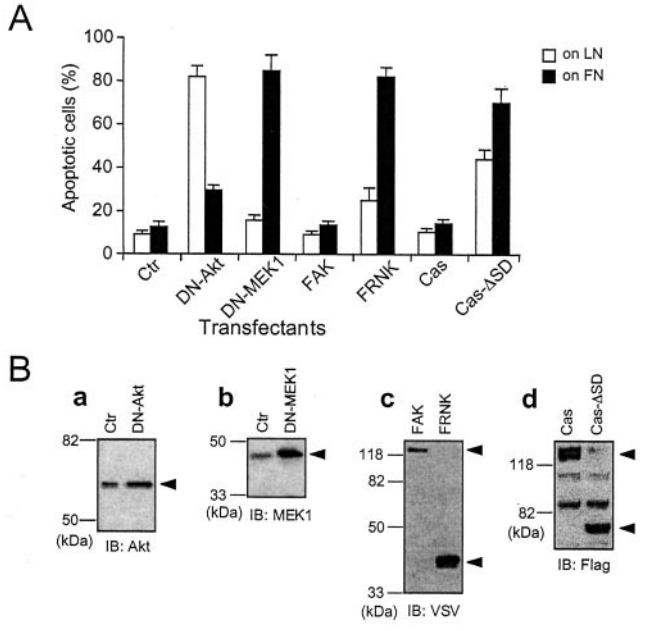

FIG. 5. **Effects of dominant-negative mutants on cell viability.** A549 cells were cotransfected with pHA262pur with or without DN-Akt, DN-MEK1, VSV-FAK, VSV-FRNK, FLAG-Cas, or FLAG-ΔCas-SD. After selection with puromycin, cells were serum-starved for 24 h. Cells were detached and replated on dishes coated with LN-10/11 (5 nM) or FN (20 nM) in 1% BSA medium for 20 h, and then viable cells were quantified by phase-contrast microscopy. *A*, data shown are the mean ± S.D. from three independent experiments. *B*, the expression levels of DN-Akt (*a*), DN-MEK1 (*b*), VSV-FAK and VSV-FRNK (*c*), or FLAG-Cas and FLAG-Cas-ΔSD (*d*) were detected by immunoblotting (*IB*) with anti-Akt, anti-MEK1, anti-VSV, and anti-FLAG antibodies, respectively. *Ctr*, cells transfected with pHA262pur alone.

adhering to LN-10/11 (Fig. 4). These results suggest that LN-10/11 and FN differentially rescue serum deprivation-induced apoptosis by preferentially activating PI 3-kinase/Akt and MEK1/ERK pathways, respectively.

LN-10/11-mediated Survival Is Linked to the PI 3-kinase/Akt Pathway, whereas FN-mediated Survival Is Linked to the MEK1/ERK Pathway—To assess further the role of PI 3-kinase/Akt and MEK1/ERK pathways in the antiapoptotic actions of LN-10/11 and FN, we overexpressed dominant negative mutants of Akt (DN-Akt) and MEK1 (DN-MEK1) to test whether these DN mutants could mimic the effects of wortmannin and PD98059, respectively. We cotransfected a puromycin resistance plasmid with DN-Akt or DN-MEK1 and then selected transfectants for 2 days in medium containing puromycin. This puromycin selection procedure routinely yields ~90% positive populations of transfectants as previously described (38). The surviving cells were cultured in medium containing 10% fetal bovine serum without puromycin overnight and starved in medium without serum for an additional 24 h. These selected cells were then replated on LN-10/11 or FN-coated substrates for cell viability assays. Consistent with the results shown in Fig. 4, overexpression of DN-Akt substantially increased the percentage of nonviable cells to ~84% on LN-10/11, but only ~30% of the cells became apoptotic on FN (Fig. 5*A*). Conversely, transfection with DN-MEK1 significantly potentiated cell apoptosis on FN but not on LN-10/11. Immunoblot quantification of DN-Akt and DN-MEK1 relative to their endogenous counterparts showed that the levels of DN-Akt and DN-MEK1 were 3- and 4-fold greater than those of endogenous Akt and MEK1, respectively (Fig. 5*B*). Taken together, these results strongly indicate that LN-10/11 and FN differentially

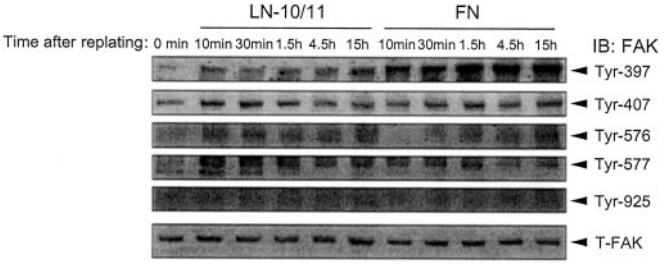

FIG. 6. **Differential phosphorylation of FAK on LN-10/11 and FN.** Serum-starved A549 cells were detached and held in suspension for 90 min and then replated on dishes coated with LN-10/11 (5 nM) or FN (20 nM) and incubated for the indicated times. Cell lysates were subjected to 8% SDS-PAGE, followed by immunoblotting (*IB*) with polyclonal antibodies against FAK phosphorylated at Tyr-397, -407, -576, -577, or –925. Total quantities of FAK (*T-FAK*) were also determined using anti-FAK antibody (*bottom panel*) to demonstrate equal amounts of loaded protein. *0 min*, cells kept in suspension.

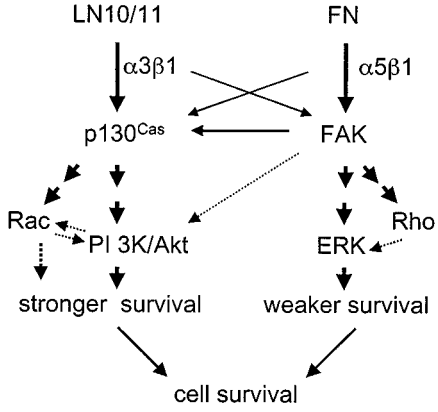

FIG. 7. **Proposed scheme of distinct survival signaling pathways on LN-10/11 and FN.** Cell adhesion to ECM triggers integrin-mediated tyrosine phosphorylation of FAK and p130^{cas}, leading to activation of downstream cascades involving PI 3-kinase/Akt and MEK1/ERK that prevent apoptosis. LN-10/11 preferentially induces phosphorylation of p130^{cas}, whereas FN induces phosphorylation of FAK (Ref. 36; also this study). The survival signals from LN-10/11 are mainly through the PI 3-kinase/Akt pathway, whereas FN survival signals are conveyed by FAK through the MEK1/ERK pathway. Cross-talk between PI 3-kinase and Rac has been described (43, 44) (*dashed lines*). Recently, it has been shown that Rho activation may contribute to sustained ERK activation (48) (*dashed line*). Cross-talk has also been reported between FAK and PI 3-kinase (12, 21) (*dashed line*), although this process was not observed in the present study.

protect cells from apoptosis induced by serum removal through distinct signaling pathways.

FAK Is Not Critically Involved in Survival Signals from LN-10/11—FAK plays a central role in linking integrin receptors to intracellular signaling pathways (23). To determine whether FAK is required for the antiapoptotic action of FN or LN-10/11, we cotransfected FAK or FRNK with the puromycin resistance plasmid. The expression level of recombinant FRNK was found to be ~4-fold greater than the level of recombinant FAK (Fig. 5*B*); levels of the latter were comparable with those of endogenous FAK (data not shown). Transfection of FAK alone did not alter cell viability on either FN- or LN-10/11-coated substrates. Expression of FRNK, however, significantly potentiated serum depletion-induced apoptosis of cells adhering to FN, whereas only a small increase in cell death was observed with cells adhering to LN-10/11 (Fig. 5*A*). These results indicated that FAK is involved in FN-mediated, but not LN-10/11-mediated, survival signaling.

We also examined the role of p130^{cas} in cell survival signaling pathways on FN or LN-10/11 by cotransfecting cells with plasmids for p130^{cas} or its mutant form lacking the substrate domain (Cas-ΔSD) with the selectable puromycin resistance plasmid. The expression levels of wild-type p130^{cas} and its mutant Cas-ΔSD were comparable (Fig. 5*B*) and ~2-fold greater than that of endogenous p130^{cas}, as determined by immunoblotting with anti-p130^{cas} antibody (data not shown). Expression of Cas-ΔSD reduced the viability of cells adhering to both FN- and LN-10/11-coated substrates (Fig. 5*A*), suggesting that phosphorylation of p130^{cas} is an important mediator of survival signals on both LN-10/11 and FN.

To investigate further whether FAK is needed for survival signals on LN-10/11, we examined FAK phosphorylation patterns using a panel of polyclonal antibodies recognizing tyrosine phosphorylation of FAK at Tyr-397, -407, -576, -577, and -925. There were no apparent differences in the phosphorylation levels of any of the tyrosine residues between cells on LN-10/11 and FN except for Tyr-397; the level of the Tyr-397 phosphorylation was much lower in cells adhering to LN-10/11 than in cells adhering to FN (Fig. 6). These results, together with the data shown in Fig. 5, strongly suggest that phosphorylation of FAK is not critically involved in transmitting survival signals on LN-10/11, in contrast to its role in FN-dependent protection from apoptosis.

DISCUSSION

The $\alpha_3\beta_1$ integrin-mediated signaling events triggered by cell adhesion to LN-10/11 are quite different from those triggered by adhesion to FN. LN-10/11 preferentially activates Rac, but not Rho, through an $\alpha_3\beta_1$ integrin-dependent pathway involving a p130^{cas}-CrkII-DOCK180 complex, thereby strongly promoting cell migration through enhanced formation of lamellipodia. FN, however, preferentially activates Rho rather than Rac, leading to enhanced stress fiber and focal contact formation (36). In this study, we analyzed intracellular signaling pathways regulating cell survival of A549 human lung adenocarcinoma cells by focusing on two distinct signaling pathways involving PI 3-kinase/Akt and MEK1/ERK; although separate, these pathways might potentially engage in cross-talk. We found that LN-10/11 is more potent than FN in suppressing apoptosis induced by serum deprivation. The antiapoptotic effects of LN-10/11 could be inhibited by the PI 3-kinase inhibitor wortmannin, whereas the antiapoptotic effects of FN were inhibited by the MEK1 inhibitor PD98059; these contrasting findings indicate that different ECMs selectively modulate different intracellular signaling pathways to sustain cell survival. These findings were further confirmed by expression of dominant negative Akt and MEK1, which compromised the ability of LN-10/11 and FN to transduce survival signals, respectively. Our results provide clear evidence that different signaling pathways leading to cell survival are activated on different ECM ligands (*i.e.* on FN and LN-10/11) (Fig. 7). Since laminins are the major components of the basement membrane of epithelium, our work supports the notion that a function of the basement membrane is to provide distinctive cell survival signals for establishment and maintenance of epithelial tissue.

LN-10/11 Is More Potent Than FN in Protecting Cells against Apoptosis-induced by Serum Depletion—Interactions of cells with the ECM through integrins are known to suppress apoptosis in many cell types. Mammary epithelial cells cultured on collagen I show extensive apoptosis over periods of several days, whereas the same cells do not when in contact with LN-1 or Matrigel, a basement membrane-like gel containing laminin-1, collagen IV, nidogen, and perlecan (39). However, LN-1 may not be a survival ligand for other cells, since endothelial cells undergo apoptosis on an LN-1 substrate while being protected from apoptosis on FN or vitronectin substrates (24). Thus, different cell types may have their own favored ECM for protection from apoptosis, depending on the repertoire of inte-

grins expressed on their cell surface, which in turn may define the types of ECM ligands most potent for protection from apoptosis. Our present studies are based on comparisons of the signaling events and abilities of LN-10/11 and FN to rescue A549 cells from serum depletion-induced apoptosis. Since $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins serve as the dominant adhesion receptors for LN-10/11 and FN, respectively, the distinct apoptotic responses of cells on LN-10/11 and FN mirror the distinct signaling pathways downstream of the $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins (Fig. 7). Our data show that LN-10/11 is more potent than FN in preventing apoptosis induced by serum depletion, suggesting that the $\alpha_3\beta_1$ integrin transduces potent survival signals when bound to LN-10/11. This is consistent with the closely overlapped distribution of $\alpha_3\beta_1$ integrin and its major ligand LN-10/11. In fact, the $\alpha_3\beta_1$ integrin is predominantly expressed on many kinds of epithelial cells that deposit laminin-10 and laminin-11 as the major components of their basement membrane. It should be noted, however, that signaling pathways on distinct ECM ligands are usually context-dependent and may not be the same in different cell types. Our conclusions based on analyses of A549 lung carcinoma cells remain to be generalized to other cell types.

PI 3-Kinase/Akt Pathway Is Essential for LN-10/11 Survival Signals—Cell adhesion to ECM triggers integrin-mediated downstream phosphorylation cascades involving the ERK type of mitogen-activated protein kinase and PI 3-kinase, providing possible mechanisms for ECM-dependent cell survival (1, 2). It remains to be determined, however, whether the survival signals from different ECM ligands are transduced by distinct signaling pathways. Activation of the PI 3-kinase/Akt pathway provided a potent antiapoptotic signal in cells adhering to LN-10/11, whereas activation of the MEK1/ERK pathway was necessary for survival of cells adhering to FN. To our knowledge, this is the first report to provide a clear distinction between the signaling pathways that rescue a cell from apoptosis on different ECM ligands.

Accumulating evidence indicates that the PI 3-kinase/Akt pathway is critical for preventing apoptosis (42). Anoikis resulting from denial of integrin-mediated adhesion involves reduced signaling through the PI 3-kinase/Akt pathway (42). Our results show that the $\alpha_3\beta_1$ integrin, when compared with $\alpha_5\beta_1$, selectively activates the PI 3-kinase/Akt pathway, thereby exerting its potent antiapoptotic effects. The evidence for a specific connection between $\alpha_3\beta_1$ integrin and the PI 3-kinase/Akt pathway includes the facts that the antiapoptotic effects of LN-10/11 but not FN are reversed by wortmannin or expression of a dominant-negative Akt mutant. The stronger activation of Rac in cells adhering to LN-10/11 than in those adhering to FN (36) may also contribute to enhance cell survival on LN-10/11; Rac activation has been shown to protect epithelial cells against anoikis through activation of the PI 3-kinase/Akt pathway (43, 44), although the precise mechanisms of the cross-talk between PI 3-kinase and Rac in cell survival remain to be clarified. Protection of Madin-Darby canine kidney cells from anoikis by overexpression of a membrane-anchored, constitutively activated form of FAK has been described (10); the mechanism might involve cross-talk activation between the hyperactivated FAK and PI 3-kinase pathways (45) or some alternative mechanism.

Our results do not imply that the $\alpha_3\beta_1$ integrin is the only integrin capable of activating the PI 3-kinase/Akt pathway, since cell type-specific differences are known. Mammary epithelial cells utilize the $\alpha_6\beta_1$ integrin to transduce cell survival signals that are dependent on the PI 3-kinase/Akt pathway (8). In contrast to our results, $\alpha_5\beta_1$ integrin regulation of cell survival in rat intestinal epithelial cells has been shown to modulate the PI 3-kinase/Akt pathway (14), whereas our data showed that FN protected A549 cells against apoptosis mainly through the MEK1/ERK pathway. The central role of the MEK1/ERK pathway in survival of A549 cells on FN was supported by the proapoptotic effects of PD98059 or the overexpression of dominant negative MEK1 mutant on cells adhering to FN. Consistent with our observations, the Ras/mitogen-activated protein kinase cascade has been shown to function as a survival signaling pathway; thus, sustained activation of this pathway efficiently rescues fibroblasts and epithelial cells from anoikis (15). Besides PI 3-kinase/Akt and ERK pathways, activation of c-Jun NH_2-terminal kinase has also been shown to be involved in apoptosis (46, 47). Almeida *et al.* (18) reported that activation of the c-Jun NH_2-terminal kinase pathway, but not the PI 3-kinase/Akt or ERK signaling pathways, is essential for protecting primary rabbit synovial fibroblasts against apoptosis induced by serum depletion on FN-coated substrates, indicating that distinct signaling pathways play critical roles in integrin-mediated survival signals.

Prolonged Activation of Akt or ERK Is Substrate-dependent—Although this study showed that the initial activation of Akt or ERK (*e.g.* 10 min after replating; see Fig. 3) was observed in cells adhering to either LN-10/11 or FN, prolonged basal activation of Akt or ERK was observed only in cells adhering to LN-10/11 or to FN, respectively. The basal levels of activated Akt and/or ERK seem to be important for protecting cells against apoptosis induced by serum removal, as demonstrated by the experiments using wortmannin and PD98059 and expression of their dominant negative mutants. The precise mechanisms of specific activation of Akt and ERK by different ECM ligands remain to be elucidated. Several lines of evidence indicate that integrins with different α subunits activate mitogen-activated protein kinases via different signaling pathways (1). For example, a subset of integrins including the $\alpha_5\beta_1$ integrin can recruit the transmembrane protein caveolin-1 and the adaptor protein Shc, thereby activating the ERK pathway. Recently, it has been reported that Rho has an essential role in integrin- and growth factor receptor-mediated signaling pathways that lead to sustained ERK activation and subsequent cyclin D1 regulation (48). Thus, the prolonged basal activation of ERK on FN could be explained by the observations that in certain cells, FN preferentially activates Rho but not Rac, whereas LN-10/11 preferentially activates Rac but not Rho (36). The α subunits of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ associate with a group of TM4SF proteins (49). The TM4SF proteins may associate with protein kinase C and phosphatidylinositol 4-kinase, linking these integrins to phosphoinositide signaling pathways (50). It remains to be examined, however, whether TM4SF proteins are involved in the prolonged basal activation of Akt on LN-10/11 via association with $\alpha_3\beta_1$.

FN Survival Signals Are FAK-dependent, whereas LN-10/11 Survival Signals Are FAK-independent—A rapid increase in the tyrosine phosphorylation of FAK at multiple sites has been identified as a prominent early event in integrin-mediated cell adhesion that regulates cell proliferation, migration, and apoptosis (23, 51). Autophosphorylation of FAK at Tyr-397 has emerged as a crucial event in FAK-mediated signal transduction, since the phosphorylation of FAK at Tyr-397 triggers the formation of molecular complexes with other signaling proteins including Src family kinases (52, 53), the p85 regulatory subunit of PI 3-kinase (54), Shc (55), and tumor suppressor PTEN (38, 56). Our data showed that FAK phosphorylation at Tyr-397 was more prominently induced in cells adhering to FN than to LN-10/11, supporting the previous observation that the level of overall tyrosine phosphorylation of FAK was lower in cells adhering to LN-10/11 than in those adhering to FN (36).

Together with the observation that expression of FRNK substantially impaired survival of cells on FN with minimal effects when cells were on LN-10/11, our results suggest that FAK is an essential component in survival signaling on FN but not on LN-10/11.

The role of FAK in integrin-mediated ERK activation is complex. Schlaepfer *et al.* (55, 57) found that FAK was involved in integrin-triggered ERK signaling, but differing findings have been reported in other systems (24–26). Since the time course of phosphorylation of FAK did not correlate with the time course of ERK activation (Figs. 3 and 6), FAK may act collaboratively by other mechanisms with other signaling molecules to ensure prolonged basal activation of ERK on FN. In fact, B-Raf has been shown to be required for the sustained activation of ERK in a FAK-dependent manner (58). On the other hand, p130^{cas} appeared to be involved in both LN-10/11 and FN survival signaling pathways, since the expression of p130^{cas} lacking the substrate domain significantly reduced cell viability on both LN-10/11 and FN. Consistent with this observation, the role of p130^{cas} in integrin $\alpha_3\beta_1$-dependent Rac activation on LN-10/11 has been demonstrated (36), while the involvement of p130^{cas} in integrin $\alpha_5\beta_1$-dependent ERK activation has also been demonstrated in different cell types (58).

In conclusion, our results strongly suggest that different ligands differentially activate integrin-mediated signaling pathways to protect against apoptosis in A549 cells, although it remains to be examined whether the distinct signaling pathways transduced by those different ECMs exist in other cell types. LN-10/11 was more potent than FN for protection against apoptosis induced by serum depletion by selectively activating the PI 3-kinase/Akt pathway rather than the MEK1/ERK pathway. The importance of anchorage to the basement membrane is well established for the maintenance of epithelial architecture and survival of epithelial cells; this study provides insight into the molecular basis of basement membrane-triggered signaling events regulating epithelial cell function.

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REFERENCES

1. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
2. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599
3. Frisch, S. M., and Francis, H. (1994) *J. Cell Biol.* **124,** 619–626
4. Downward, J. (1998) *Curr. Opin. Genet. Dev.* **8,** 49–54
5. Zhang, Z., Vuori, K., Wang, H., Reed, J. C., and Ruoslahti, E. (1996) *Cell* **85,** 61–69
6. O'Brien, V., Frisch, S. M., and Juliano, R. L. (1996) *Exp. Cell Res.* **224,** 208–213
7. Matter, M. L., Zhang, Z., Nordstedt, C., and Ruoslahti, E. (1998) *J. Cell Biol.* **141,** 1019–1030
8. Farrelly, N., Lee, Y. J., Oliver, J., Dive, C., and Streuli, C. H. (1999) *J. Cell Biol.* **144,** 1337–1348
9. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) *Cell* **79,** 1157–1164
10. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. (1996) *J. Cell Biol.* **134,** 793–799
11. Xiong, W., and Parsons, J. T. (1997) *J. Cell Biol.* **139,** 529–539
12. Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S., and Yamada, K. M. (1999) *J. Biol. Chem.* **274,** 20693–20703
13. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) *EMBO J.* **16,** 2783–2793
14. Lee, J. W., and Juliano, R. L. (2000) *Mol. Biol. Cell* **11,** 1973–1987
15. Le Gall, M., Chambard, J. C., Breittmayer, J. P., Grall, D., Pouyssegur, J., and Van Obberghen-Schilling, E. (2000) *Mol. Biol. Cell* **11,** 1103–1112
16. Jost, M., Huggett, T. M., Kari, C., and Rodeck, U. (2001) *Mol. Biol. Cell* **12,** 1519–1527
17. Tran, S. E., Holmstrom, T. H., Ahonen, M., Kahari, V. M., and Eriksson, J. E. (2001) *J. Biol. Chem.* **276,** 16484–16490
18. Almeida, E. A., Ilic, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D., and Damsky, C. H. (2000) *J. Cell Biol.* **149,** 741–754
19. Frisch, S. M., and Screaton, R. A. (2001) *Curr. Opin. Cell Biol.* **13,** 555–562
20. King, W. G., Mattaliano, M. D., Chan, T. O., Tsichlis, P. N., and Brugge, J. S. (1997) *Mol. Cell. Biol.* **17,** 4406–4418
21. Zhao, J. H., Reiske, H., and Guan, J. L. (1998) *J. Cell Biol.* **143,** 1997–2008
22. Dolfi, F., Garcia-Guzman, M., Ojaniemi, M., Nakamura, H., Matsuda, M., and Vuori, K. (1998) *Proc. Natl. Acad. Sci.* **95,** 15394–15399
23. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) *Prog. Biophys. Mol. Biol.* **71**, 435–478
24. Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) *Cell* **87,** 733–743
25. Lin, T. H., Aplin, A. E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R. L. (1997) *J. Cell Biol.* **136,** 1385–1395
26. Wary, K. K., Mariotti, A., Zurzolo, C., and Giancotti, F. G. (1998) *Cell* **94,** 625–634
27. Khwaja, A., and Downward, J. (1997) *J. Cell Biol.* **139,** 1017–1023
28. Erhardt, P., Schremser, E. J., and Cooper, G. M. (1999) *Mol. Cell. Biol.* **19,** 5308–5315
29. Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998) *Science* **282,** 1911–1914
30. Colognato, H., and Yurchenco, P. D. (2000) *Dev. Dyn.* **218,** 213–234
31. Montanaro, F., Lindenbaum, M., and Carbonetto, S. (1999) *J. Cell Biol.* **145,** 1325–1340
32. Miner, J. H., Lewis, R. M., and Sanes, J. R. (1995) *J. Biol. Chem.* **270,** 28523–28526
33. Sorokin, L. M., Pausch, F., Frieser, M., Kroger, S., Ohage, E., and Deutzmann, R. (1997) *Dev. Biol.* **189,** 285–300
34. Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (1998) *J. Biol. Chem.* **273,** 15854–15859
35. Kikkawa, Y., Sanzen, N., Fujiwara, H., Sonnenberg, A., and Sekiguchi, K. (2000) *J. Cell Sci.* **113,** 869–876
36. Gu, J., Sumida, Y., Sanzen, N., and Sekiguchi, K. (2001) *J. Biol. Chem.* **276,** 27090–27097
37. Fujiwara, H., Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (2001) *J. Biol. Chem.* **276,** 17550–17558
38. Gu, J., Tamura, M., Pankov, R., Danen, E. H., Takino, T., Matsumoto, K., and Yamada, K. M. (1999) *J. Cell Biol.* **146,** 389–403
39. Pullan, S., Wilson, J., Metcalfe, A., Edwards, G. M., Goberdhan, N., Tilly, J., Hickman, J. A., Dive, C., and Streuli, C. H. (1996) *J. Cell Sci.* **109,** 631–642
40. Bachelder, R. E., Ribick, M. J., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. R., and Mercurio, A. M. (1999) *J. Cell Biol.* **147,** 1063–1072
41. Walker, F., Kato, A., Gonez, L. J., Hibbs, M. L., Pouliot, N., Levitzki, A., and Burgess, A. W. (1998) *Mol. Cell. Biol.* **18,** 7192–7204
42. Downward, J. (1998) *Curr. Opin. Cell Biol.* **10,** 262–267
43. Coniglio, S. J., Jou, T. S., and Symons, M. (2001) *J. Biol. Chem.* **276,** 28113–28120
44. Zugasti, O., Rul, W., Roux, P., Peyssonnaux, C., Eychene, A., Franke, T. F., Fort, P., Hibner, U., Coniglio, S. J., Jou, T. S., and Symons, M. (2001) *Mol. Cell. Biol.* **21,** 6706–6717
45. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., and Chen, H. C. (1999) *J. Biol. Chem.* **274,** 12361–12366
46. Frisch, S. M., Vuori, K., Kelaita, D., and Sicks, S. (1996) *J. Cell Biol.* **135,** 1377–1382
47. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
48. Welsh, C. F., and Assoian, R. K. (2000) *Biochim. Biophys. Acta* **1471,** M21–M29
49. Hemler, M. E. (1998) *Curr. Opin. Cell Biol.* **10,** 578–585
50. Yauch, R. L., Berditchevski, F., Harler, M. B., Reichner, J., and Hemler, M. E. (1998) *Mol. Biol. Cell* **9,** 2751–2765
51. Hanks, S. K., and Polte, T. R. (1997) *Bioessays* **19,** 137–145
52. Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) *Mol. Cell. Biol.* **14,** 147–155
53. Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D., and Guan, J. L. (1994) *Mol. Biol. Cell* **5,** 413–421
54. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J. Biol. Chem.* **271,** 26329–26334
55. Schlaepfer, D. D., Jones, K. C., and Hunter, T. (1998) *Mol. Cell. Biol.* **18,** 2571–2585
56. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. (1998) *Science* **280,** 1614–1617
57. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) *Nature* **372,** 786–791
58. Barberis, L., Wary, K. K., Fiucci, G., Liu, F., Hirsch, E., Brancaccio, M., Altruda, F., Tarone, G., and Giancotti, F. G. (2000) *J. Biol. Chem.* **275,** 36532–36540

Laminin-10/11 and Fibronectin Differentially Prevent Apoptosis Induced by Serum Removal via Phosphatidylinositol 3-Kinase/Akt- and MEK1/ERK-dependent Pathways

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