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Integrin-Dependent Cell Growth and Survival Are Mediated by Different Signals in Thyroid Cells

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Cell adhesion to extracellular matrix regulates proliferation and survival of several cell types including epithelial thyroid cells. Activation of integrin receptors by binding to extracellular matrix generates a complex cell type-dependent signaling. Adhesion to extracellular matrix induces proliferation and survival in primary cultures of thyroid cells and induces survival in immortalized human thyrocytes. In this study we demonstrate that in immortalized human thyrocyte cells, adhesion to immobilized fibronectin (FN) stimulates DNA synthesis and proliferation through the p21Ras/MAPK pathway, whereas cell survival is mediated by phosphatidylinositol 3-kinase (PI3K) signal pathway. Integrin activation by immobilized FN induced phosphorylation of pp125 focal adhesion kinase and paxillin and induced the formation of focal adhesion kinase/Grb-2/Sos complex. Western blot and *in vitro* kinase assay demonstrated the activation of Ras and the p44/p42 MAPK/ERK1/2. Inhibition of p21Ras activity and inhibition of MAPK enzymatic activity completely arrested cell growth but did not induce cell death. Integrin activation by cell adhesion to FN also induced activation of PI3K. Inhibition of PI3K enzymatic activity induced apoptosis demonstrated by annexin V-binding assay and loss of cellular DNA content. These results demonstrate that in thyroid cells adhesion to FN regulates proliferation through the p21Ras/MAPK signal pathway, whereas integrin-mediated cell survival is mediated by PI3K. (*J Clin Endocrinol Metab* 88: 260–269, 2003)

TISSUE HOMEOSTASIS IS the result of contrasting stimuli that balance each other. Unbalanced cell proliferation and cell death accounts for increasing or decreasing tissue mass in both normal or tumor tissues. Thyroid cells are exposed to several mitogenic stimuli (TSH, epidermal growth factor, insulin and insulinlike growth factor), many of which also play a role in the control of apoptosis. We previously demonstrated that extracellular matrix (ECM) proteins control both thyroid cell proliferation and survival (1–3). Adhesion to fibronectin (FN) is mitogenic in thyroid cells in primary cultures and inhibits apoptosis induced by denied adhesion (anoikis) in thyroid cells in primary cultures as well as immortalized human thyrocytes (TAD-2). Control of cell proliferation and survival exerted by ECM is mediated by integrins, transmembrane receptors for ECM proteins that provide cell anchorage and regulate cellular architecture, motility, growth, and survival (4–10). Signals generated by ECM are important to neoplastic cells that must proliferate and survive in ectopic environments or even in denied adhesion. Integrin activation can directly generate signals that regulate cell behavior through the modulation of protein phosphorylation, ion concentration (Ca^{2+}, Na^{+}, H^{+}), or lipid metabolism. Alternatively, it can also generate signals that cooperate with other receptors, modulating other intracellular signals (11–18). The connection of integrins to the actin cytoskeleton occurs in subcellular structures known as focal adhesions, which contain several structural and signaling molecules.

In a number of different cell systems, the first signaling event following integrin clustering is the phosphorylation of the focal adhesion tyrosine kinase pp125FAK (19). A prototypical model of the pathway generated by cell adhesion leading to cell proliferation postulates that pp125FAK phosphorylation provides binding sites for the adapter molecules Shc and Grb2. They in turn recruit the guanine-nucleotide exchange factor Sos to the plasma membrane and activate p21 Ras (20, 21). Ras then activates the serine-threonine kinase Raf-1 that in turn activates the extracellular signal-related kinase (MEK) and MAPK. However, a number of studies argue against a role for pp125FAK in MAPK activation because inhibition of pp125FAK by a dominant negative truncated focal adhesion kinase (FAK) in fibroblast failed to block MAPK activation (22). The role of pp125FAK is complicated by the fact that its activation can play a critical role in integrin signaling controlling the activation of multiple MAPK family members such as the p44/p42 MAPK/ERK and the c-Jun NH2-terminal kinase through divergent signaling pathways. Besides the activation of p44/p42MAPK, pp125FAK phosphorylation induced activation of c-Jun NH2-terminal kinase through an alternative route involving paxillin and the small GTP-binding proteins of the ρ family (17) in the same cell system. Moreover, additional studies with a dominant negative Raf provided evidences in favor of the existence of a Ras-independent pathway of MAPK activation induced by integrins (23). The immediate phosphor-

Abbreviations: ECL, Enhanced chemiluminescence; ECM, extracellular matrix; FAK, focal adhesion kinase; FCS, fetal calf serum; FN, fibronectin; GFP, green lantern fluorescent protein; MAPKK, MAPK-activating kinase; MBP, myelin basic protein; PI3K, phosphatidylinositol 3-kinase; RBD, Ras-binding domain peptide; RGD, arginine-glycine-aspartic acid; TAD-2, immortalized human thyrocyte; TCA, trichloroacetic acid.

ylation of the focal adhesion components is not the only event elicited by integrin activation, as increase of intracellular calcium concentration, variations of intracellular pH, and phosphatidylinositol 3-kinase (PI3K) activation has been observed on cell attachment to ECM or binding of antiintegrin antibodies to platelets, macrophages, neutrophils, osteoclasts, and embryonic stem cells (24–26). Our study demonstrates that in thyroid cells, adhesion to FN regulates proliferation through the p21 Ras/MAPK pathway, whereas cell survival is mediated by PI3K.

Materials and Methods

Cell cultures and transfections

The TAD-2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells was generously donated by Dr. T. F. Davies (Mount Sinai Hospital, New York, NY) and cultured in a 5% CO_2 atmosphere at 37 C in DMEM (4.5 g glucose) and 10% fetal calf serum (FCS) (27). Medium was changed every 3–4 d. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free PBS with 0.05% trypsin. When needed, the cells were serum starved in 0.5% BSA and DMEM for 12–18 h before stimulation.

To obtain a FN or BSA coating, cell culture plates were filled with the appropriate FN (Collaborative Research, Bedford, MA) or BSA (Sigma, St. Louis, MO) dilution in PBS. After overnight incubation at 4 C, FN was removed, and the plates were washed with PBS and stored at 4 C. Coexpression of a dominant negative Ras (RasN17) gene and green lantern fluorescent protein (GFP) in TAD-2 cells was induced by cotransfecting pRSV-RasN17 and pEGFP (CLONTECH Laboratories, Inc., Palo Alto, CA) expression vectors with LipofectAMINE reagent (Life Technologies, Inc., Rockville, MD) according to manufacturer instructions. Twenty-four hours after transfection, the percentage of GFP-expressing cells was determined by flow cytometry using a FACScan (Becton Dickinson and Co., Mountain View, CA). The transfection efficiency ranged from 82–87%.

Cell cultures from normal thyroids were prepared as previously described by collagenase digestion (1) and cultured in a 5% CO_2 atmosphere at 37 C, in F-12 medium supplemented with 10% FCS, with 1 mU/ml bovine thyreotropin (Sigma). The cells were used after 3 d.

[^{3}H]thymidine incorporation and proliferation assay

To determine the DNA synthesis, cells were plated in DMEM, 0.5% BSA, 0.5μCi [^{3}H]thymidine in 24-well plates coated with FN. After 24 h, the plates were gently washed with PBS avoiding cell loss and then with 10% trichloroacetic acid (TCA) and incubated 10 min with 20% TCA at 4 C. TCA was removed and cells were lysed with 0.2% SDS for 15 min at 4 C. Lysates were then resuspended in 5 ml scintillation fluid and counted in a β-counter (Beckton Dickinson). To determine the mitogenic effect of FN, cells were plated in DMEM and 0.5% BSA in 24-well plates coated with FN. After 48 h, the cells were detached with trypsin and counted with a hemocytometer.

Estimation of apoptosis

Cytofluorimetric estimation of cell death was performed as follows: Floating cells were collected, washed in cold PBS, added to adherent cells, and trypsinized. Cells were washed again in PBS and fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes, and cells were incubated in PBS, 50 μg/ml propidium iodide, 10 μg/ml ribonuclease A, and deoxyribonuclease-free overnight at 4 C. Cells were then analyzed by flow cytometry using a FACScan, and hypodiploid cells were counted. Annexin V assay for determination of early apoptosis was performed as follows: cells were washed twice with cold PBS, resuspended in 10 nM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$, and incubates for 15 min at room temperature with Annexin V-fluorescein conjugated (PharMingen, San Diego, CA) and 5 μg/ml propidium iodide. Cell were analyzed within 1 h by flow cytometry. The FN-integrin-binding inhibitor arginine-glycine-aspartic acid (RGD)-containing peptide (Gly-Arg-Gly-Asp-Ser-Pro) was purchased from Telios (San Diego, CA).

Western blot analysis and antibodies

Serum-starved cells (1×10^6) were plated on FN-coated Petri dishes in the presence and absence of serum. At variable times they were lysed in RIPA buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride]. A mouse monoclonal antibody was used to immunoprecipitate FAK or Paxillin (Transduction Laboratories, Inc., Lexington, KY) from 1 mg total lysate. The immunoprecipitates were then resolved by SDS-PAGE. Alternatively, the cells (3×10^5) were lysed in Laemmly buffer [125 mM Tris (pH 6.8), 5% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.006% bromophenol blue] and resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane (Immobilon P, Millipore Corp., Bedford, MA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine, and after three washes, the membranes were incubated for 1 h at 4 C with 0.5 μ/ml mouse monoclonal or rabbit polyclonal primary antibodies in PBS. After three washes filters were incubated for 1 h at 4 C with a horseradish peroxidase-conjugated antimouse secondary antibody. After a final wash, protein bands were detected by an enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech, Rainham, UK). For quantitation, relative intensities of hybridization signals were measured at 560 nm with a gel scan apparatus (PDI, Upper Saddle River, NJ). Mouse monoclonal antibodies to Grb-2, p42 MAPK, and phospho-p44/p42 MAPK were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

p21 Ras activity assay

Ras activity was assayed by affinity precipitation using a Ras activation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, 4×10^6 cells were plated on FN-coated plates and stimulated for 15 min as previously described. Cells were then lysed with MLB buffer [125 mM HEPES (pH 7.5), 750 mM NaCl, 5% Igepal CA630, 50 mM $MgCl_2$, 5 mM EDTA, and 10% glycerol] and incubated with 5 μl of a 50% slurry of Raf-1 RBD (Ras-binding domain peptide) for 30 min at 4 C. The beads were then boiled in reducing sample buffer, and adsorbed proteins were resolved by electrophoresis, transferred to nitrocellulose, and probed with a monoclonal anti-Ras (1 μg/ml) (MLB, Raf-1 RBD, and anti-Ras were included in a Ras activation assay kit, Upstate Biotechnology, Inc.). Proteins were visualized using a horseradish peroxidase-conjugated secondary antibody and ECL.

p44/p42MAPK activity

Cells were stimulated as described at 37 C for 15 min. Cells were then washed twice with ice-cold PBS, homogenized in RIPA buffer, and 0.5 mg of clarified cellular extract was immunoprecipitated in 1 ml RIPA buffer at 4 C for 1 h using an antibody to p44MAPK (Santa Cruz Biotechnology, Inc.) and protein A-agarose beads (Santa Cruz Biotechnology, Inc.): The beads were then washed once with 1 ml RIPA buffer and twice with 1 ml kinase buffer [20 mM HEPES (pH 7.0), 10 mM $MgCl_2$ 1 mM dithiotreithol]. Samples were then resuspended in 40 μl kinase buffer with 0.25 mg/ml myelin basic protein (MBP), 20 μM ATP, and 20 μCi/ml γ[^{32}P]ATP and incubated at 30 C for 15 min. The reaction was quenched with Laemmly buffer, and proteins were separated through a 4–20% polyacrylamide/Tris glycine gel. Radioactive-phosphorylated MBP on dried gels was quantified by PhosphorImager (Molecular Dynamics, Inc., Uppsala, Sweden).

PI3K activity

Cells were lysed for 40 min at 4 C in 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 10 mM $Na_4P_20_7$, 2 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. Total amount of proteins in each sample was determined. PI3K activity was determined in immunoprecipitated as described (28). Briefly, 0.5 mg cell lysates were incubated with polyclonal antibodies to p85 preadsorbed to protein G-Sepharose beads for 3 h at 4 C. Pellets were then washed in PBS containing 1%

(vol/vol) NP-40 and 0.2 mM, $NaVO_4$, then in 100 mM Tris-HCl (pH 7.4) supplemented with 500 mM LiCl and 0.2 mM $NaVO_4$, and finally in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.2 mM $NaVO_4$. Pellets were further resuspended in 40 mM HEPES (pH 7.4) and 20 mM MgCl2, and the kinase reaction was initiated by addition of phosphatidylinositol (0.2 mg/ml) and 75 μM $\gamma[^{32}P]$ATP (7000 Ci/mol) and performed for 20 min at room temperature. The reaction was stopped by addition of 4 M HCl, and the phosphoinositides were extracted with a methanol-chloroform (vol/vol) mix. Finally, phospholipids were separated by thin-layer chromatography and visualized and quantified by PhosphorImager (Molecular Dynamics, Inc.).

Statistical analysis

Results are presented as the mean ± SD. Standard deviations less than 10% are not reported in the diagrams. Statistical analysis was performed by using the *t* test. The level of significance was set at *P* less than 0.05.

Results

FN induces DNA synthesis and cell proliferation

[^{3}H]thymidine incorporation was used to measure the DNA synthesis in TAD-2 cells cultured in serum-free medium on immobilized FN for 24 h (Fig. 1A). To coat 24-well flat-bottom microtiter plates with FN, the plates were incubated overnight with PBS containing FN at a concentration ranged 0.1–100 μg/ml. In the control samples, in the absence of immobilized FN, [^{3}H]thymidine incorporation was very poor. The presence of FN coating induced a concentration-dependent increase in [^{3}H]thymidine incorporation reaching a 3.8-fold at 10 μg/ml FN. To test the ability of FN to induce proliferation, TAD-2 cells were cultured in DMEM without serum, with or without FN coating, or in 10% FCS (Fig. 1B). The number of adherent cells was determined by a hemocytometer after 3 h and then daily. Maximum cell proliferation was achieved in the presence of serum (approximately 69 h of doubling time), whereas in serum-free cultures without FN coating, the cell number remained unchanged. In serum-free cultures with FN coating, TAD-2 cells actively proliferated with a doubling time of 92 h.

FN induces FAK and paxillin phosphorylation and promotes FAK/Grb2-SoS association

After serum starvation in 0.5% BSA and DMEM for 12–18 h, TAD-2 cells were harvested by trypsin and left to deposit onto 10 μg/ml FN-coated plates. Then, the cells were recovered, lysed, and pp125FAK and paxillin immunoprecipitated by specific monoclonal antibodies. Proteins were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Total and phosphorylated FAK and paxillin were evidenced by specific antibodies and the antiphosphotyrosine antibody pY-20 (Fig. 2). Cell attachment to FN-induced FAK phosphorylation as early as 15 min. This phosphorylation was sustained for at least 90 min. In addition, paxillin phosphorylation was induced by adhesion to immobilized FN. Paxillin phosphorylation started later than FAK phosphorylation and displayed a peak activation at 30 min that was sustained for 90 min.

To assess whether FN induced the formation of the FAK/Grb2-SoS complex, thus driving Ras activation, cell extracts were immunoprecipitated with a specific anti-Grb2 antibody, and then, after protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated Grb2 and coprecipitated FAK were detected by anti-Grb2 and anti-FAK antibodies, respectively (Fig. 3). FN induced FAK association with Grb2 at 30 min, and this association was still detectable after 60 min of stimulation. This result implies the possibility of Ras activation by phosphorylated FAK following adhesion to FN.

FN induces p21 Ras and p44/p42 MAPK activation

We determined whether FN stimulation induces p21 Ras activation. Based on the ability of activated p21 Ras to bind Raf, extracts from cells stimulated by 10% FCS or FN for 15 min were incubated with Raf-1 RBD-conjugated agarose beads to allow the binding to activated Ras. Adsorbed Ras was then recovered from the beads and visualized by Western blot on a 12% polyacrylamide gel with anti-Ras antibody (Fig. 4A). The binding to FN induced a strong increase of activated p21 Ras, although lower than that induced by FCS stimulation. Because the activation of p44/p42MAPK (ERK1/2) is a pivotal event of the Ras signal pathway leading to cell proliferation, we determined whether this kinase was phosphorylated and activated by cell attachment to FN. Serum-starved cells were seeded onto FN-coated plates in serum-free medium or untreated plates in the presence of 10% FCS for the appropriate time and lysed. Cell extracts were analyzed by Western blot with anti-p44MAPK to visualize

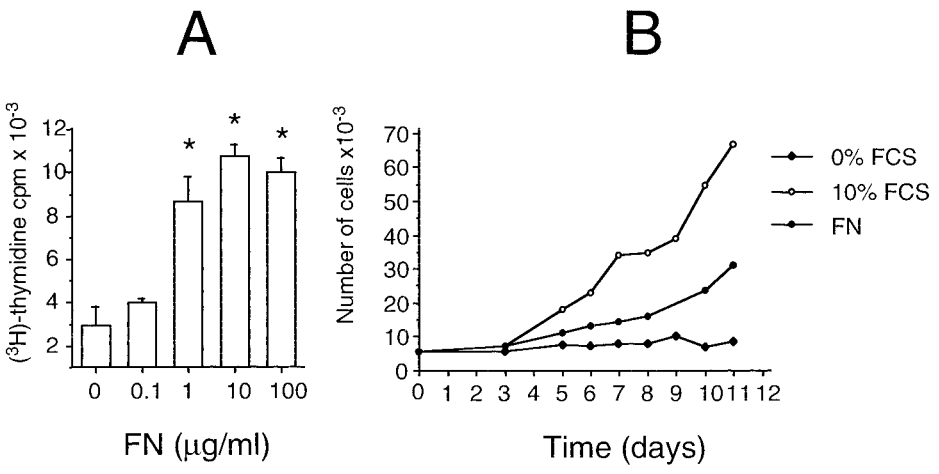

FIG. 1. Stimulation of DNA synthesis and cell proliferation by FN. A, TAD-2 cells were seeded in microtiter plates previously coated by overnight incubation with 0.1–100 μg/ml of FN in PBS. Cells were cultured for 24 h in serum-free medium, with 0.5 μCi [^{3}H]thymidine. Data are reported as mean ± SD of quadruplicate experiments. *, Significant *vs.* 0 time point. B, TAD-2 cells were seeded in plates previously coated with 10 μg/ml FN and cultured in serum-free medium *(full dots)* or uncoated plates in serum-free medium *(full rhombuses)* or uncoated plates in the presence of 10% FCS *(open dots)*. After 3 h and then after each day, the number of cells was determined by a hemocytometer. Data are reported as mean of quadruplicate plates. All time points of 10% FCS and FN *vs.* 0% FCS curves were statistically significant by the 6-d point.

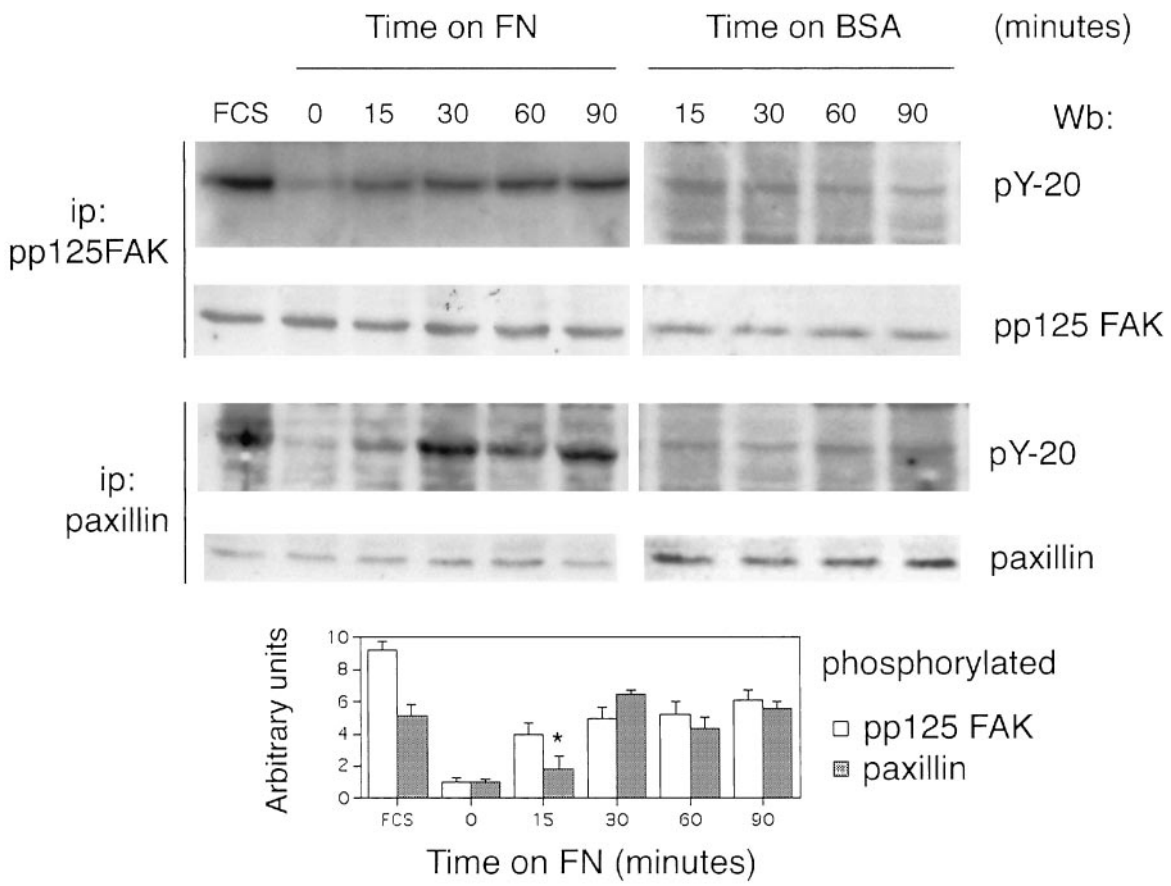

FIG. 2. FAK and paxillin phosphorylation are induced by cell attachment to FN. TAD-2 cells were starved from FCS for 12–18 h and harvested by trypsin. The cells were seeded onto FN-coated plates without serum (0–90) or uncoated plates with 10% FCS. After the indicated time, the cells were collected, lysed, and 1 mg cell extract subjected to immunoprecipitation by anti-FAK or anti-paxillin monoclonal antibodies. The immunoprecipitated proteins were divided into equal aliquots, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Total FAK or paxillin was visualized by specific antibodies, and phosphotyrosines were evidenced by the antiphosphotyrosine antibody pY-20. Antibody binding was evidenced by ECL. Averages and SDs of relative expressions of phosphorylated FAK and paxillin were also determined by scanning densitometry of three immunoblots. In each diagram, a value of one arbitrary unit was assigned to 0 time point. *, *vs.* 0 time point.

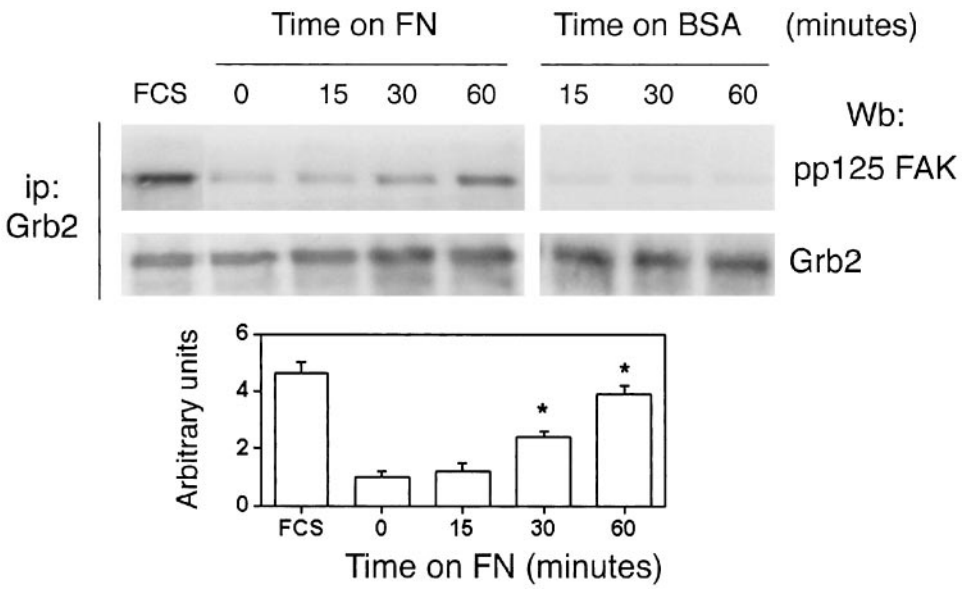

FIG. 3. FN promotes FAK/Grb2-SoS association. TAD-2 cells were starved from FCS for 12–18 h and then harvested by trypsin. The cells were plated onto FN coated without serum or in uncoated plates with 10% FCS. After the indicated time, 1 mg cell extracts were immunoprecipitated with a specific anti-Grb2 antibody, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with an anti-FAK or with anti-Grb2 antibody. Antibody binding was evidenced by ECL. Averages and SD of relative expressions of FAK were also determined by scanning densitometry of three immunoblots. In each diagram, a value of one arbitrary unit was assigned to 0 time point. *, The only data not significant *vs.* 0 time point.

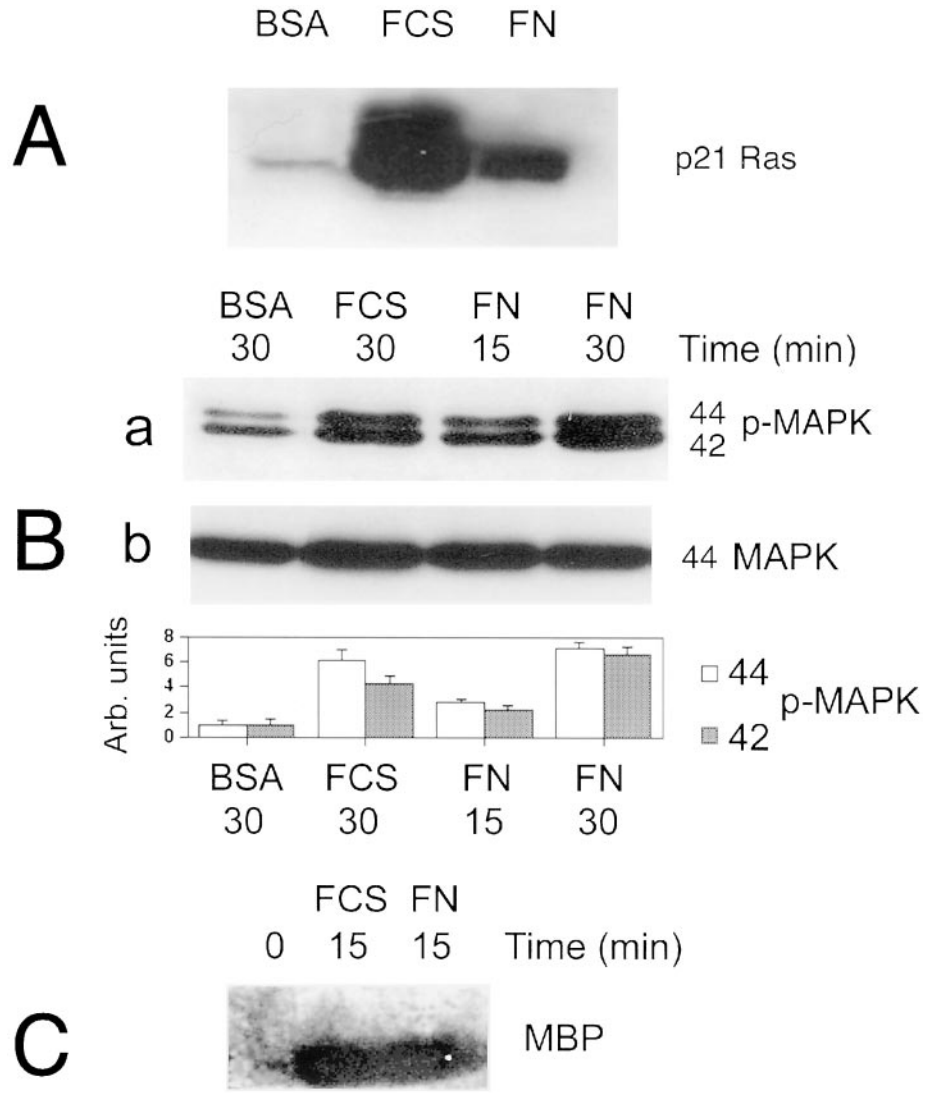

FIG. 4. Activation of p21 Ras and p44/p42 MAPK by cell attachment to FN. Serum-starved cells were seeded in serum-free medium onto BSA-coated plates or FN-coated plates or untreated plates in the presence of 10% FCS for the indicated time and lysed. A, After 15 min cell extracts were incubated with Raf-1 RBD-conjugated agarose beads, and the activated p21 Ras adsorbed onto the beads was recovered and visualized by Western blot with anti-p21 Ras antibody. B, Cell extracts were analyzed by Western blot with antibodies anti-p44MAPK (44MAPK) (b) and anti-phosphotyrosine-p44/p42MAPK (p-MAPK) (a). Averages and SD of relative expressions of phosphorylated p44 and p42 MAPK isoforms were also determined by scanning densitometry of three immunoblots. In each diagram, a value of one arbitrary unit was assigned to BSA 30 point. All experimental points *vs.* BSA 30 $P < 0.05$. C, MAPK was immunoprecipitated by specific antibody from cell extracts and allowed to react for 15 min with $\gamma[^{32}P]$ATP and MBP. The reaction products were subjected to SDS-PAGE, and radioactivity incorporated into MBP was detected by autoradiography.

the protein and with antiphosphothyrosine-p44/p42MAPK to visualize the activated kinase (Fig. 4B). Both FCS and immobilized FN induced p44/p42MAPK phosphorylation. MAPK activation by FN was confirmed by *in vitro* incorporation of radioactive ATP into MBP. MAPK was immunoprecipitated by a specific antibody from cell extracts and incubated with $\gamma[^{32}P]$ATP and MBP. The reaction mixture was resolved on SDS-PAGE, and radioactive MBP was detected by autoradiography (Fig. 4C). Strong MAPK activation was induced by FCS and attachment to FN.

FN induces PI3K activation

The effect of integrin activation by FN on PI3K enzymatic activity was evaluated at different time points (Fig. 5). TAD-2 cells were starved from FCS for 12 h, trypsinized, and retained in suspension for 1 h in plates coated with a thin layer of 2% agarose. Then, the cells were seeded onto plates coated by overnight incubation with 10 μg/ml FN or 1 mg/ml BSA solution. Serum starvation and 1-h suspension were necessary to decrease the background activity of PI3K. The enzyme was immunoprecipitated by antibodies anti-p85 reg-

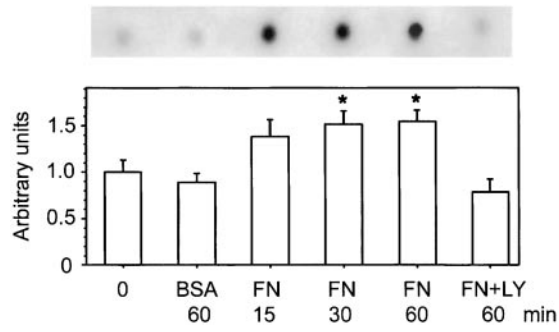

FIG. 5. Activation of PI3K. Cells were starved from FCS, trypsinized, and retained in suspension for 1 h in agar-coated plates (0) and then plated for the indicated time onto plates coated with BSA or FN with or without 10 μM LY294002. PI3K was immunoprecipitated by antibodies against the p85 regulative subunit of PI3K and incubated for 20 min with phosphatidylinositol and $\gamma[^{32}P]$ATP. Radioactive phospholipids were separated by thin-layer chromatography, visualized, and quantified by PhosphorImager. A value of one arbitrary unit was assigned to 0 point. Averages and SD of three experiments are reported in the diagram. *, Significant *vs.* 0 time point.

ulative subunit and incubated with phosphatidylinositol and $\gamma[^{32}P]$ATP, and finally generation of radioactive phospholipids were analyzed by thin-layer chromatography. PI3K activity remained unchanged after 60 min in BSA-coated plates, whereas increase of PI3K enzymatic activity was observed on FN stimulation. PI3K activation was completely inhibited in the presence of 10 μM LY294002 in the culture medium.

Inhibition of Ras/MAPK pathway arrests cell growth

FN-induced DNA synthesis and cell proliferation were evaluated in the presence of inhibitors of Ras and MAPK-activating kinase (MAPKK). The cells were transiently transfected with a control plasmid (GFP) alone or with a dominant-negative Ras expression vector (RasN17) and serum starved for 24 h. The cells were then seeded in serum-free medium onto 10 μg/ml FN-coated plates. GFP-transfected cells were alternatively treated with or without 40 μM PD98059, a molecular inhibitor of MAPKK. Then, [^{3}H]thymidine incorporation was evaluated after 24 h (Fig. 6A), and the cell number was determined after 48 h (Fig. 6B) of culture. Both DNA synthesis and cell proliferation were inhibited almost completely by RasN17 and PD98059, thus demonstrating that Ras/MAPK pathway is needed for FN-induced growth in TAD-2 cells. The effect of PD98059 on cell cycle was evaluated also in primary cultures of thyroid cells by FACScan analysis. Three-day-old primary cultures from normal thyroids were plated onto FN-coated plates and cultured for 48 h in serum-free medium with PD98059 or left untreated. Then, the cells were collected, stained with propidium iodide, and analyzed by FACScan (Fig. 6C). Primary cultures of thyroid cells are characterized by slow and limited growth. The number of cells in S-phase decreased fom 13–14% in untreated cells to 1–3% after PD98059 treatment, demonstrating that MAPKK is necessary to FN-stimulated cell growth also in primary cultures.

Inhibition of PI3K but not of Ras/MAPK pathway induces apoptosis

We previously showed that denied adhesion to ECM rapidly induced apoptosis in thyroid cells (2). To evaluate the role of integrin signaling in cell survival and growth, we investigated the effect of the inhibition of PI3-K and Ras/MAPK pathway to TAD-2 cells. Cells transfected with GFP alone or in combination with RasN17 were seeded in serum-free medium onto 10 μg/ml FN-coated plates. Alternatively, cells were plated on FN and treated with 40 μM PD98059, 10 μM LY294002, and 50 nM wortmannin (not shown) or 100 μg/ml of the FN integrin-binding inhibitor RGD-containing peptide. After 48 h, GFP- and RasN17-transfected cells and cells treated with PD98059 were adherent and displayed a flattened shape, whereas cells treated with RGD-containing

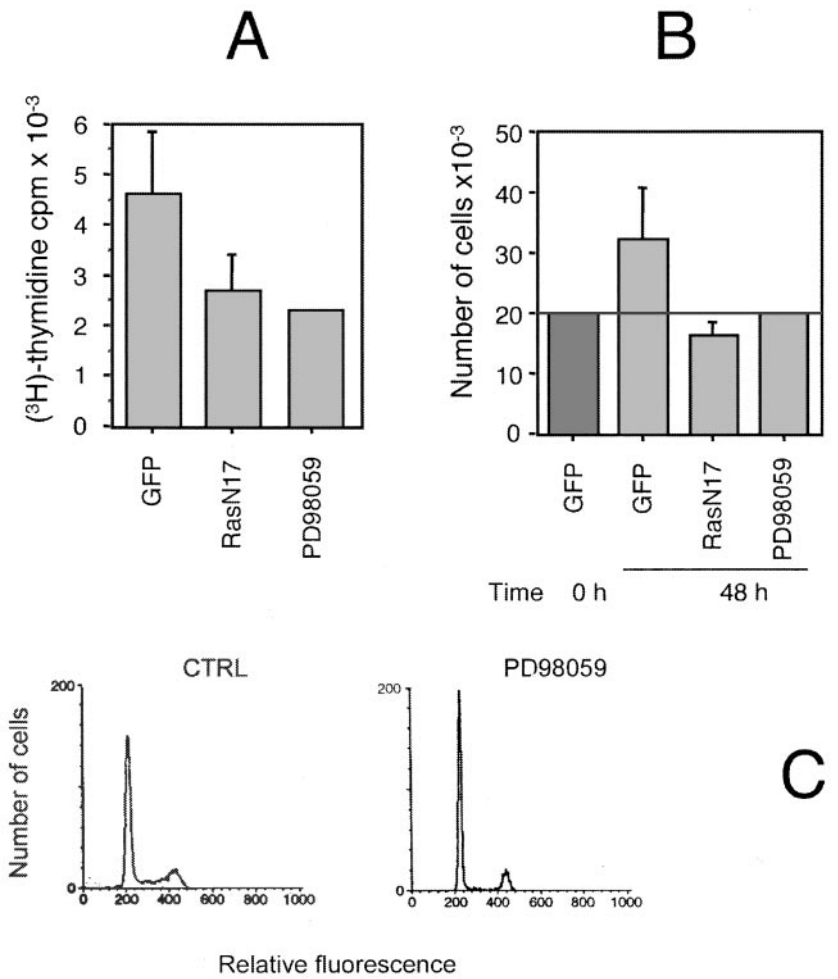

FIG. 6. Role of Ras/MAPK pathway in the FN-induced thyroid cell proliferation. Cells were transiently transfected with a control plasmid (GFP) alone or with a dominant-negative Ras expression vector (RasN17). The cells were then plated onto 10 μg/ml FN-coated plates. An MAPK inhibitor (PD98059) was added to GFP-transfected cells. [^{3}H]thymidine incorporation was evaluated after 24 h of culture (A) and cell number was determined after 48 h (B). Data are reported as mean ± SD of three independent experiments of quadruplicate plates. In all experimental points, $P < 0.05$ *vs.* GFP. C, Primary cultures from normal human thyroids were plated onto FN-coated plates and cultured in serum-free medium with PD98059 or left untreated for 48 h. Then, the cells were collected, stained with propidium iodide, and analyzed by FACScan.

peptides or the PI3K inhibitor were nonadherent and displayed a spherical shape. Both adherent and floating cells were collected and apoptosis was estimated by flow cytometric analysis. Hypodiploid cells were observed only in the presence of LY294002 and RGD-containing peptides, whereas they were absent in the presence of PD98059 and in RasN17-transfected cells (Fig. 7A). The percentages of hypodiploid cells induced by 48 h of treatment with increasing concentrations of PD98059 and LY294002 were calculated by flow cytometry (not shown). Increasing concentrations of PD98059 up to 50 μM did not induce cell death, whereas LY294002 demonstrated a dose-dependent effect, inducing cell death in 68% of the cells at 10-μM concentration. Because loss of fragmented DNA is a late apoptotic and necrotic phenomenon, plasma membrane phosphatidylserine exposure, an early and specific apoptotic phenomenon, was an-

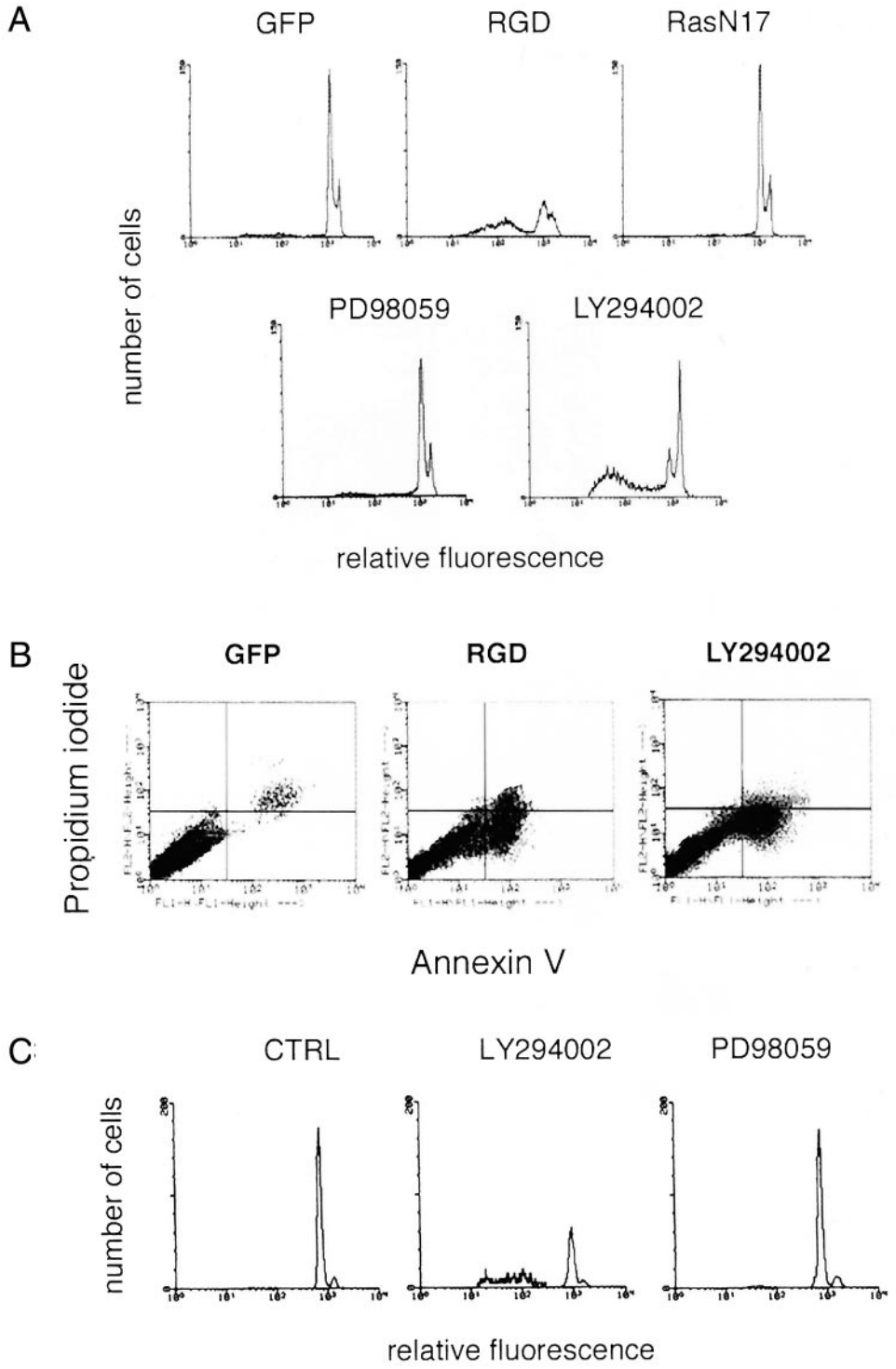

FIG. 7. Role of Ras/MAPK pathway and PI3K in cell survival. TAD-2 cells were transfected with GFP alone (GFP) or in combination with RasN17 and plated in serum-free medium onto FN-coated plates. Alternatively, the cells were treated with PD98059, LY294002, or the FN-integrin binding inhibitor RGD-containing peptide. After 48 h, both adherent and floating cells were collected and apoptosis was estimated by flow cytometric analysis. A, Cells were stained by propidium iodide and hypodiploid cells were searched. B, Cells were stained with annexin V and propidium iodide for determination of early apoptosis. Intact cells are located in the lower left quadrant, necrotic cells permeable to propidium iodide are in the upper right and left quadrants, and the early apoptotic cells stained by annexin V and unstained by propidium iodide are in the lower right quadrant. C, Three-day-old primary cultures of normal thyroid cells were plated in serum-free medium onto FN-coated plates and treated for 72 h with PD98059 or LY294002 or left untreated. Adherent and floating cells were collected, and apoptosis was estimated by flow cytometric analysis.

alyzed by annexin V-binding assay (Fig. 7B). Annexin V-binding assay demonstrated apoptosis in LY294002- and RGD-treated cells but not in cells treated with PD98059 (not shown). The effect of PI3K and MAPKK inhibition on survival was studied also in thyroid primary cultures. Three-day-old primary cultures from normal thyroids were plated onto FN and left untreated or were cultured in serum-free medium with 40 μM PD98059 or 10 μM LY294002 for 72 h. Then, the cells were collected, stained with propidium iodide, and analyzed by FACScan (Fig. 7C). While MAPKK inhibiton by PD98052 did not produce hypodiploid cells, PI3K inhibition induced cellular loss of DNA. The analysis by annexin V/propidium iodide demonstrated that cell death was due to apoptosis (not shown). These data confirm in primary cultures the results obtained in TAD-2 cells and extends the role of PI3K to normal thyroid cells.

Discussion

It is known from studies in other cell types that integrins not only mediate cell anchorage but also affect several cellular functions including cell growth and apoptosis through a complex signaling. We analyzed the integrin signaling in an immortalized human thyroid cell line and primary cultures of normal thyrocytes. Most of the data reported in this study has been performed in immortalized cells; however, similar results have been obtained in primary cultures (Illario, M., A. Cavallo, G. Fenzi, G. Rossi, and M. Vitale, manuscript in preparation). Our results demonstrated that activation of the integrin receptors by FN binding in thyroid cells induces different signals with different biological effects (Fig. 8). The complexity of cell functions regulated by integrins is made possible by the complexity and the tissue specificity of signalings generated in response to integrin engagement. Some but not all integrins activate the Ras/MAPK signal pathway through the adapter molecules Shc and Grb2. In fibroblasts, endothelial cells, and transformed cells, the laminin receptor $\alpha_6\beta_4$, laminin/collagen receptor $\alpha_1\beta_1$, and FN/vitronectin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ activate p44/p42MAPK through the p21 Ras signaling and the assembly of Shc/Grb-2/Sos (21, 29, 30). In our study, FN binding to integrins induced FAK and paxillin phosphorylation, inducing the formation of the FAK/Grb-2/Sos complex, thus confirming the involvement of these upstream components of the integrin pathway also in the thyroid cell. This pathway initiates with FAK activation and proceeds to SH2-domain adapter proteins, guanine nucleotide exchange factors, Ras, Raf-1, MAPKK, and finally to MAPK. However, the direct involvement of FAK, the first element of this kinase cascade, is not certain, and alternative pathways leading to MAPK activation without FAK involvement have been proposed (29). In our cell system, FAK is phosphorylated following adhesion to FN. Nevertheless, its role in the activation of the signal pathway leading to cell proliferation has not been documented, and further direct experiments are needed to address this question. The proposed FAK-independent routes from integrins to MAPK include direct involvement of Src kinase or the membrane protein caveolin, which both interact with Shc (31).

In addition to these molecules, growth factor receptors also can cooperate with integrins to generate intracellular signals. Integrins can induce tyrosine phosphorylation of epidermal growth factor receptor in the absence of this factor, leading to activation of MAPK (32). However, no matter which are the upstream phosphorylation events, the proposed pathways include Ras as a critical link between integrins and MAPK. Inhibition of p21 Ras by dominant negative N17-mutated Ras in thyroid cells blocked MAPK phosphorylation and cell proliferation, thus supporting a pivotal role for Ras (13, 31). The existence of a Ras-independent pathway that links integrins to growth in certain cell types has been proposed and is supported by the evidence that inhibition of Raf signaling by dominant negative truncated Raf-1 did not inhibit MAPKK activation in NIH-3T3 cells (23). The apparent disagreement of these data is explained by the tissue specificity of integrin signalings. Expression of a constitutively activated MAPKK1 in primary human thyroid cells demonstrated that both activated Ras and MAPK are necessary to generate a mitogenic signal, whereas activation of MAPK alone is not sufficient (32). Our results in TAD-2 cells with RasN17 and PD98059 demonstrate that the mitogenic effect of integrin activation requires both Ras and MAPK activation and demonstrate that a Ras-independent pathway that links integrins to growth does not exist in thyroid ep-

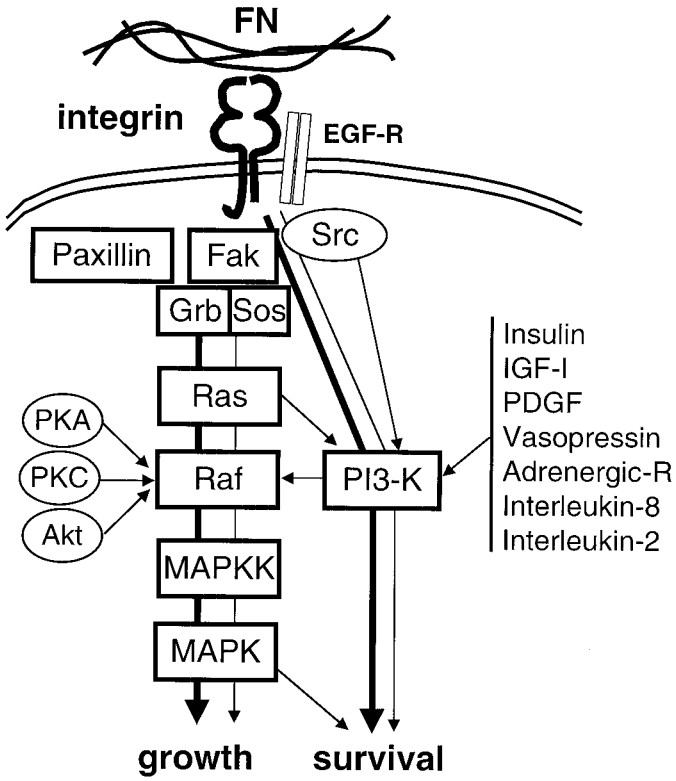

FIG. 8. Integrin-mediated signaling pathways leading to cell growth and survival in thyroid cells *(thick lines)* and nonthyroid cells *(thin lines).* Cell proliferation is promoted by integrins via Ras/ERK. This pathway is involved in survival of nonthyroid cells, whereas it is not necessary for thyroid cell survival. In both cell types, integrins promote survival through the PI3K pathway. See the text for more details. EGF-R, Epidermal growth factor receptor; Fak, focal adhesion kinase; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; Akt, protein kinase B/Akt.

ithelial cells. However, this signal pathway alone could not be sufficient for the mitogenic action of integrins.

In several other receptor-generated signal pathways, Ras and other kinases provide costimulatory signals that together lead to MAPK activation. Besides Ras, PI3K activity is necessary to phosphorylate Raf-1 for activation of MAPK by insulin, IGF-I, IL-2 and -8, PDGF, vasopressin, T-cell receptor, and adrenergic receptor, and several other kinases can phosphorylate Raf-1 (Src, protein kinases A and C, Akt) (33–38). The role of PI3K as a costimulatory factor also was demonstrated in the integrin signal pathway, as attenuation of integrin-dependent MAPK activation by PI3K inhibition was demonstrated in COS 7 cells (39). Following integrin-FN binding in these cells, PI3K regulates the phosphorylation of Raf-1 Ser338 through the serine/threonine kinase Pak (40). By this costimulatory mechanism, integrins can simultaneously generate and modulate a growth stimulus. The possibility that other hormones and growth factors operate a similar control mechanism in the thyroid cell is worthy of thorough investigation.

Although inhibition of integrin activation by RGD-containing peptides induced cell death (anoikis), inhibition of the Ras/MAPK pathway did not affect thyroid cell survival, but inhibition of PI3K induced apoptosis. PD98059 and LY294002 or wortmannin induce apoptosis in the liver cancer cells HepG2 and vascular smooth muscle cells, indicating that both the MAPKK/MAPK and the PI3K pathways are required for survival in these cells (41, 42). However, inhibition of Ras or MAKK in PC12 cells does not promote apoptosis, suggesting that the role of the Ras/MAPK pathway in survival is cell type and context specific. In TAD-2 cells, RasN17 and PD98059 induced growth arrest but did not induce apoptosis, indicating that the Ras/MAPK pathway is essential for proliferation but is not required for survival. Although the Ras/MAPK pathway is not necessary for TAD-2 cell survival, it can have synergistic effects with other signaling, protecting the cell from anoikins. Ras oncogene in transformed epithelial cells can mimic the protective effect of matrix through a direct interaction with the catalytic p110 subunit of PI3K or downstream cytochrome *c* release from the mitochondria at a point distinct from PI3K/Akt (43–45). Evidences of coexisting antiapoptotic pathways are provided by the demonstration that growth factors inhibit BAD-mediated apoptosis through BAD phosphorylation in tree serine residues, Ser155, Ser136, and Ser112, by protein kinase A or PI3K (46). Thus, although the Ras/MAPK pathway may only marginally participate in FN-dependent survival, PI3K plays a pivotal role in integrin-dependent cell survival in thyroid cell.

Altered integrin expression or abnormal modulation of integrin signalings could lead to unregulated proliferation sustained by ECM or integrins in pathological situations such as neoplastic transformation (47). Indeed, an altered pattern of integrin expression has been demonstrated in thyroid neoplastic cells, and its role in tumor growth must be investigated (48).

Acknowledgments

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2003 Certifying Examination in Pediatric Endocrinology

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