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Calcium/Calmodulin-dependent Protein Kinase II Binds to Raf-1 and Modulates Integrin-stimulated ERK Activation*

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Integrin activation generates different signalings in a cell type-dependent manner and stimulates cell proliferation through the Ras/Raf-1/Mek/Erk pathway. In this study, we demonstrate that integrin stimulation by fibronectin (FN), besides activating the Ras/Erk pathway, generates an auxiliary calcium signal that activates calmodulin and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This signal regulates Raf-1 activation by Ras and modulates the FN-stimulated extracellular signal-regulated kinase (Erk-1/2). The binding of soluble FN to integrins induced increase of intracellular calcium concentration associated with phosphorylation and activation of CaMKII. In two different cell lines, inhibition of CaMKII activity by specific inhibitors inhibited Erk-1/2 phosphorylation. Whereas CaMK inhibition affected neither integrin-stimulated Akt phosphorylation nor p21Ras or Mek-1 activity, it was necessary for Raf-1 activity. FN-induced Raf-1 activity was abrogated by the CaMKII specific inhibitory peptide autocamtide. Integrin activation by FN induced the formation of a Raf-1/CaMKII complex, abrogated by inhibition of CaMKII. Active CaMKII phosphorylated Raf-1 *in vitro*. This is the first demonstration that CaMKII interplays with Raf-1 and regulates Erk activation induced by Ras-stimulated Raf-1. These findings also provide evidence supporting the possible existence of cross-talk between other intracellular pathways involving CaMKII and Raf-1.

Integrins are widely expressed transmembrane receptors for extracellular matrix (ECM)[1] proteins that provide a physical linkage between ECM and cytoskeletal structures (1–3). Modulation of integrin binding affinity to ECM by integrin itself and intracellular directed signals are of fundamental importance not only to cell adhesion but also to cellular architecture, motility, growth, and survival (4–7). We demonstrated previously that the ECM protein fibronectin (FN) controls both cell proliferation and survival in thyroid cells in primary cultures and in the immortalized human thyroid cell line TAD-2 (8, 9). In TAD-2 cells, integrin activation by FN promotes the activation of p21Ras. Ras activates the serine-threonine kinase Raf-1, which in turn activates the extracellular signal-related kinase kinase (Mek) and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk). This pathway represents a prototypical model of the pathway initiated by cell adhesion that leads to cell proliferation (10, 11). Integrin activation also induces phosphatidylinositol 3-kinase (PI3-K) activation and accumulation of PI3-K products (phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate) (12, 13). Activation of PI3-K by integrins regulates phosphorylation of Raf-1 Ser 338 through the serine/threonine kinase Pak-1, providing a co-stimulatory signal that enhances Raf-1 activation by Ras and increases stimulation of cell growth by integrins (14). After integrin activation, increase of intracellular calcium concentration $[Ca^{2+}]_i$ has been observed upon cell attachment to ECM or binding of anti-integrin antibodies to platelets, macrophages, neutrophils, osteoclasts, and embryonic stem cells (15–17). Elevation of $[Ca^{2+}]_i$ upon activation of $\alpha7\beta1$ integrin in skeletal muscle cells results from both inositol triphosphate-evoked Ca^{2+} release from sarcoplasmic/endoplasmic reticulum and extracellular Ca^{2+} influx through voltage-gated, L-type plasma membrane Ca^{2+} channels (18). $[Ca^{2+}]_i$ mediates important components of the integrin signaling pathway by controlling cell functions, such as migration in vascular smooth muscle cells, and phagocytosis and migration in macrophages (19, 20). These Ca^{2+} signals are mediated by Ca^{2+}/calmodulin protein kinase II (CaMKII), a ubiquitous serine/threonine protein kinase that is activated by Ca^{2+} and calmodulin (CaM) to phosphorylate diverse substrates involved in metabolism, neurotransmitter release and cell cycle control.

Emerging data on potential connections between Ca^{2+} signaling and the MAPK pathway in multiple cell systems lead us to explore possible cross-talk between these two signal pathways upon integrin activation. Indeed, it has recently been reported that pharmacological inhibition of CaM decreased epidermal growth factor-induced MAPK activity by interfering with Raf-1 (21). In PC-12 cells, calcium and CaM were both necessary for the activation of Erk after epidermal growth factor receptor stimulation and TrkA stimulation by nerve growth factor. The same report suggested that CaM might regulate the full activation of Raf-1 after Ras activation (22). In

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[1] The abbreviations used are: ECM, extracellular matrix; FN, fibronectin; Mek, extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PI3-K, phosphatidylinositol 3-kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaM, Ca^{2+}/calmodulin; FCS, fetal calf serum; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; TFP, trifluoperazine W7, *N*-(6-aminohexyl)-5-chloro-1-nafthalene-sulfonamide; MBP, myelin basic protein.

hippocampal neurons, activation of *N*-methyl-D-aspartate-type glutamate receptors induces CaMKII phosphorylation that inhibits the Ras GTPase activating protein (p135 Syn-GAP) (23). Therefore, Syn-GAP represents another potential intersection point between the Ca^{2+} signaling and the MAPK pathway. Our data demonstrate that integrin stimulation by FN, besides activating the Ras/Raf/Mek/Erk pathway, also generates a Ca^{2+}/CaMK signal that modulates the Ras/Erk pathway through the regulation of Raf-1 activity.

MATERIALS AND METHODS

Cell Culture—Thyroid TAD-2 cells and the hepatoma cell line Hep3B were cultured in a 5% CO_2 atmosphere at 37 °C, in Dulbecco's modified Eagle's medium (4.5 g of glucose) and 10% fetal calf serum (FCS). When needed, the cells were serum-starved in 0.5% BSA-Dulbecco's modified Eagle's medium for 12–18 h before stimulation. To obtain FN or BSA coating, cell culture plates were filled with the appropriate FN (Collaborative Research, Bedford, MA) or BSA (Sigma) dilution in PBS, incubated overnight at 4 °C and washed with PBS.

Immunoprecipitation and Western Blot Analysis and Antibodies—For immunoprecipitation, the cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride). Rabbit polyclonal antibody reactive to all CaMKII isoforms (Santa Cruz Biotechnology, Santa Cruz, CA), Raf-1 (Santa Cruz Biotechnology), and protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate CaMKII or Raf-1 from 1 mg of total lysate. Non-immune rabbit IgG were also used as a control. For Western blot analysis, the cells were lysed in Laemmli buffer (125 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.006% bromphenol blue), and proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon P; Millipore Corporation, Bedford, MA). Membranes were blocked by 5% nonfat dry milk, 1% ovalbumin, 5% FCS, and 7.5% glycine; washed and incubated for 1 h at 4 °C with primary antibodies; and washed and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. Then, protein bands were detected by an enhanced chemiluminescence system (Amersham Biosciences). Computer-acquired images were quantified using ImageQuant software (Amersham Biosciences). Mouse monoclonal antibodies to p42 MAPK, phospho-p44/p42 MAPK, Akt, and phospho-threonine-308-Akt were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286) was from (Promega, Madison, WI).

p21Ras Activity Assay—Ras activity was assayed by affinity precipitation using a Ras activation assay kit (Upstate Biotechnology). Briefly, 4×10^6 cells were lysed with Mg^{2+} lysis 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 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) (Mg^{2+} lysis buffer, Raf-1 Ras binding domain peptide, and anti-Ras were included in a Ras activation assay kit; Upstate Biotechnology). Proteins were visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

CaMKII Activity Assay and Inhibitors—The cells were lysed in 200 μl of RSB buffer (24) with 10 mM CHAPS and 20 μl of the extracts were assayed in 50 μl of reaction mixture consisting of 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0,5 mM dithiothreitol, 2 μM CaM, 100 nM microcystin, 50 μM ATP (1500 cpm/pmol [γ-^{32}P]ATP), and 0.1 mM substrate peptide Autocamtide II. Total CaMK activity was determined by including 1 mM $CaCl_2$ in the mixture, whereas autonomous activity was measured in the presence of 2.5 mM EGTA. Ionomycin (Sigma) at a concentration of 500 ng/ml was used as a positive control for CaMKII activation. The reaction was carried out for 2 min at 30 °C, and 20-μl aliquots of the reaction mixture were spotted onto p81 phosphocellulose filters (Upstate Biothechnology) as described previously (25). Purified CaM and Autocamtide II were a kind gift from Dr. A. R. Means (Durham, NC). The CaMK inhibitor KN93 and the CaM inhibitors trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7) were purchased from Sigma. The CaMKII specific inhibitor ant-CaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN (26) and was made cell-permeable by N-terminal addition of an Antennapedia-derived sequence (ant-CaNtide: RQIKIWFQNRRMKWKKRPPKLGQIGRSKRVVIEDDRIDDVLK). The reversed ant-CaNtide peptide was also used as a control.

Calcium Measurements—A total of 3×10^5 cells harvested by trypsin were loaded with cell-permeant Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR), by incubating the cells with Dulbecco's modified Eagle's medium, 10 μM Fura-2, 0.5% BSA, and 10 mM HEPES for 30 min at 37 °C. The cells were then washed twice for 10 min with 1 mM $CaCl_2$ in Hanks' balanced salt solution (118 mM NaCl, 4.6 mM KCl, 10 mM glucose, and 20 mM HEPES, pH 7.2). When indicated, cells were incubated with integrin-FNH-binding RGD-containing peptides (Gly-Arg-Gly-Asp-Ser-Pro) or control peptides (RGE-containing, Gly-Arg-Gly-Glu-Ser-Pro) (Calbiochem). Fluorescence was measured with a fluorimeter (PerkinElmer Life Sciences). Excitation was at 345 and 380 nm, emission was at 510 nm. R_{min} and R_{max} were obtained by adding 10 mM EDTA and 2% Triton X-100 or 10 mM EDTA, 2% Triton X-100, and 10 mM $CaCl_2$, respectively. The nanomolar concentration of Ca^{2+} was obtained by the Grynkiewiez formula considering a 225 K_d for Fura-2 (27).

Raf-1, Pak, and Mek-1 Activity Assay—Raf-1 activity was evaluated by a Raf-1 immunoprecipitation-kinase cascade assay kit (Upstate Biotechnology). Briefly, Raf-1 was immunoprecipitated from 1 mg of cell extracts. The immunocomplexes were washed and incubated in the presence of magnesium/ATP, inactivated Mek-1, and Erk-2 for 30 min at 30 °C. An aliquot of the mixture was then incubated with 20 μg of myelin basic protein (MBP) in the presence of [γ-^{32}P]ATP. The reaction was quenched with Laemmli buffer, and proteins were separated through a 10% polyacrylamide/tris glycine gel. Radioactive-phosphorylated MBP on dried gels was quantified with the use of a PhosphorImager (Amersham Biosciences).

Pak activity was determined as follows. Pak was immunoprecipitated by specific antibodies (Santa Cruz Biotechnology). Immunocomplexes were washed and incubated for 20 min at 30 °C in 20 mM HEPES, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 μg of MBP in the presence of [γ-^{32}P]ATP. The proteins were separated through a 10% polyacrylamide/tris glycine gel, and radioactive-phosphorylated MBP was quantified by PhosphorImager.

To measure Mek-1 activity, Mek-1 was immunoprecipitated by specific antibodies and incubated with purified Erk-2. Erk-2 phosphorylation was evaluated by Western blot with mouse monoclonal antibodies to phospho-p44/p42 MAPK. All antibodies were from Santa Cruz Biotechnology.

Statistical Analysis—Student *t* test was performed as appropriate. analysis of variance with post hoc correction according to Bonferroni was performed on multiple comparison.

RESULTS

Integrin Activation by Soluble FN Induced $[Ca^{2+}]_i$ Increase and CaMKII Activation—Activation of CaMK requires binding of Ca^{2+}/CaM and thus a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To determine whether integrin activation by FN can produce such a Ca^{2+} signal, $[Ca^{2+}]_i$ was measured by fluorimetric analysis in TAD-2 cells in suspension in response to soluble FN binding (Fig. 1*A*). Cells were incubated with soluble FN in the presence of peptides inhibiting the integrin-FN binding (RGD) or in the presence of control peptides (RGE). $[Ca^{2+}]_i$ remained unchanged for 30 min and then slightly increased in untreated cells. After 30 min of suspension, FN + binding inhibitor or control peptides were added to the cells. In the cells treated with FN + control peptide, $[Ca^{2+}]_i$ began to increase after a few minutes and continued to rise for the duration of the assay, reaching a 7.5-fold increase by 60 min. The FN receptor antagonist peptide added at 500 μg/ml completely inhibited the $[Ca^{2+}]_i$ increase. Antagonist peptide alone did not produce any effect on $[Ca^{2+}]_i$ (data not shown).

To determine whether the FN-induced $[Ca^{2+}]_i$ increase can activate CaMK enzymatic activity, we evaluated the T286/287-phosphorylation level of CaMKII, a CaMK highly expressed in brain but present in every mammalian cell type examined (Fig. 1*B*). Phospho-T286/287-CaMKII is generated by auto-phosphorylation and thus can be used as a reporter for CaMKII's history of activation within cells. Serum-starved cells were plated onto FN or BSA, and CaMKII phosphorylation was evaluated by Western blot. Although at 15 min only a slight

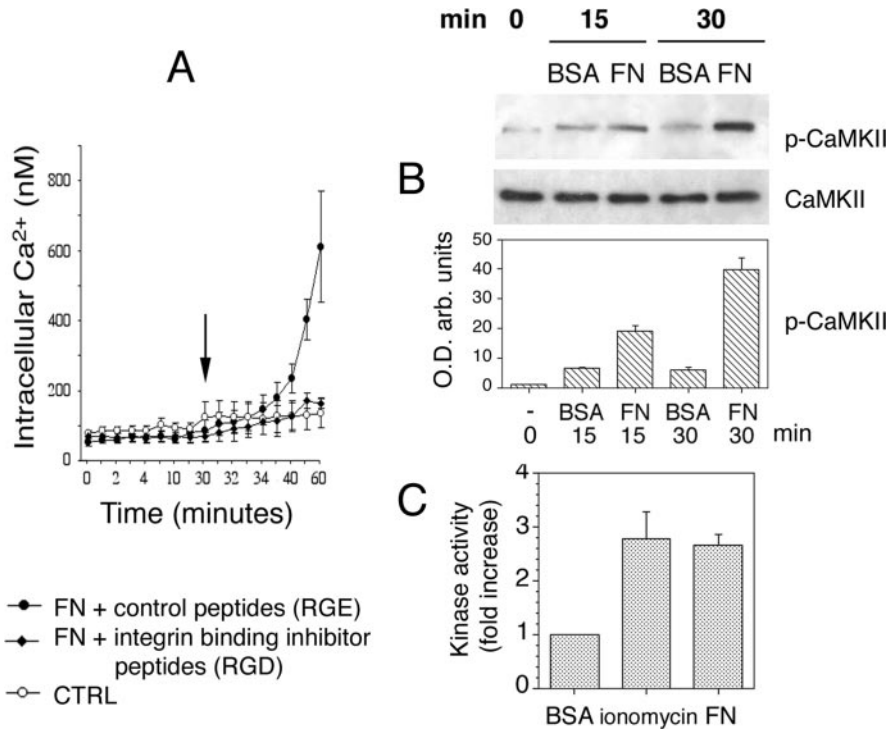

FIG. 1. **Integrin activation by FN induces a Ca^{2+} signal and CaMKII activation in TAD-2 cells.** *A*, soluble FN induced $[Ca^{2+}]_i$ increases in thyroid cells in suspension. The cells were serum-starved for 24 h, harvested by trypsin, washed with culture medium, and loaded with Fura-2. At the time indicated by the *arrow*, soluble FN with integrin binding inhibitor (*RGD*) or with control peptide (*RGE*) was added to the cells, and $[Ca^{2+}]_i$ was measured by fluorimetric analysis. *CTRL*, untreated cells. Results are presented as mean ± S.D. nanomolar concentration of Ca^{2+} from quadruplicates. *B*, generation of the T286-phosphorylated form of CaMKII in response to FN stimulation in TAD-2 cells. Serum-starved TAD-2 cells were seeded onto FN- or BSA-coated plates in the absence of serum and analyzed by Western blot. Total CaMKII was visualized by specific antibody and phosphorylated CaMKII (pT286-CaMKII) by an anti-phosphothreonine CaMKII antibody. Averages and S.D. of relative expressions of phosphorylated CaMKII were also determined by scanning densitometry of three immunoblots. In each diagram, a value of 1 OD arbitrary unit was assigned to 0 point. FN *versus* BSA at 15 and 30 min, $p < 0.001$. *C*, CaMK activity after 30 min of FN treatment was measured by a phosphorylation assay of the CaMKII peptide substrate autocamtide II. Results are presented as -fold increase compared with cells plated onto BSA. A maximal Ca^{2+} signal was induced by ionomycin treatment. The kinase activities induced by ionomycin and FN were comparable.

difference between BSA and FN was made visible with the use of an anti-phosphothreonine CaMKII antibody, by 30 min, increased phosphorylation was clearly evident in the FN-stimulated cell lysates.

Activation of CaMKs by cell attachment to FN was confirmed by *in vitro* kinase assay using Autocamtide II as substrate (Fig. 1*C*). The cells were stimulated with immobilized FN or treated with ionomycin, a powerful ionophore that induces a rapid $[Ca^{2+}]_i$ increase. FN induced a 2.8-fold increase of CaMK activity, a magnitude comparable with that induced by ionomycin. These results clearly demonstrate that FN can induce an intracellular Ca^{2+} signal sufficient for activation of CaMK.

Inhibition of CaMKII Activity Inhibits FN-induced Erk Phosphorylation—Our initial data indicated that Ras/Erk and Ca^{2+}/CaMK signalings are both generated by FN-activated integrins. To investigate where the two pathways converge, we first tested the effects of CaMK inhibitors on the phosphorylation of Erk, the immediate downstream target of Mek. In this analysis, we used one CaMK inhibitor (KN93) and two CaM inhibitors (W7 and TFP); CaM is needed as the Ca^{2+} sensor for CaMK activation. TAD-2 cells were plated onto immobilized FN with different concentrations of KN93, W7, or TFP, and the level of Erk-1/2 phosphorylation was evaluated by Western blot (Fig. 2). Both 10% FCS and FN induced a comparable increase of Erk-1/2 phosphorylation. KN93 was inhibitory at 0.5 μM and completely abolished FN-induced Erk-1/2 phosphorylation at 5 μM. In addition, W7 and TFP displayed dose-dependent inhibitory effects. To determine whether within CaMK the isoform II is required for FN-stimulated Erk phosphorylation, the experiments were reproduced with the specific inhibitor ant-CaNtide. This short peptide is derived from the endogenous CaMKII inhibitor-protein CaMKIIN and was made cell permeable by the Antennapedia N-terminal sequence. Ant-CaNtide inhibited FN-induced Erk-1/2 phosphorylation at a 5 μM concentration. The ant-CaNtide reversed peptide displayed no inhibitory effect. Together, these data demonstrate that Erk phosphorylation is dependent on CaMKII activation. Thus, CaMK- and MAPK-pathways are simultaneously required for FN-stimulated cell proliferation and seem to converge upstream of Mek.

FN-independent CaMKII Activity Is Necessary for Erk phosphorylation Induced by FN in Hep3B Cells—To determine whether the role played by CaMK in the regulation of the MAPK-pathway and in the control of FN/integrin-dependent cell growth is restricted to the thyroid TAD-2 cells or is a more general phenomenon, we analyzed the effect of FN on Erk- and CaMKII-activity in an additional cell line. Erk-1/2 phosphorylation was evaluated by Western blot in Hep3B cells plated onto FN or BSA in absence of serum or in uncoated plates with 10% FCS (Fig. 3*A*). As in TAD-2 cells, FN and FCS alone stimulated Erk-1/2 phosphorylation, inducing a 3-fold increase. Then, the effect of integrin-stimulation on CaMKII activation was determined in cells stimulated by FN and FCS. CaMKII phosphorylation at T286/287 was determined by Western blot (Fig. 3*B*). Activated CaMKII was clearly detected in both FN- and FCS-stimulated cells. However, essentially the same level of activated kinase was seen without such stimulation under the basal conditions used in our experiments.

To test whether CaMKII activity is necessary for Erk phosphorylation in Hep3B, the cells were plated onto FN with or

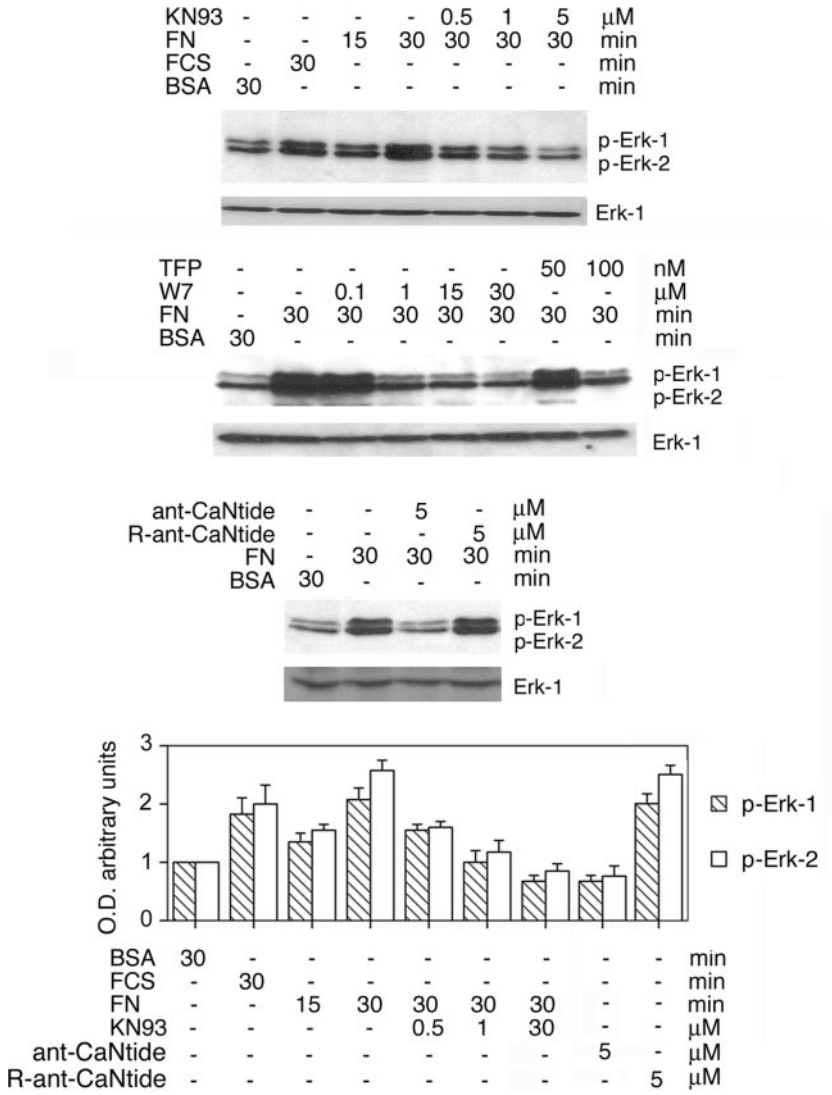

FIG. 2. **Inhibition of CaMKII blocks FN-dependent Erk phosphorylation.** TAD-2 cells were plated onto BSA-coated plates for 30 min in serum-free medium, in uncoated plates in the presence of 10% FCS, or in FN-coated plates for 15 or 30 min in serum-free medium with or without KN93, W7, TFP, ant-CaNtide, or reverse ant-CaNtide (*R-ant-CaNtide*). Cell extracts were analyzed by Western blot with anti-phosphotyrosine-Erk-1/2 (*p-Erk*) or anti-total-Erk-1 (*Erk-1*) antibodies. Averages and S.D. of relative expressions of phosphorylated Erk1/2 were also determined. A value of 1 OD arbitrary unit was assigned to 0 point. FN *versus* FN+KN93 or FN+ant-CaNtide, $p < 0.001$.

without KN93, W7, TFP, ant-CaNtide, or R-ant-CaNtide, and the level of Erk-1/2 phosphorylation was evaluated by Western blot (Fig. 4). FN-stimulated Erk phosphorylation was inhibited by the CaMK inhibitor KN93, the CaMKII inhibitor ant-CaNtide, or the CaM inhibitors TFP and W7 in Hep3B, as it was in TAD-2 cells. These results demonstrate that in Hep3B cells, as in TAD-2 cells, CaMKII modulates the signaling pathway generated by integrins; here, however, integrin activation is not required for CaMKII activation. They also demonstrate that CaMKII activity is required but not sufficient for Erk activation in both Hep2B and in TAD-2 cells.

CaMK Does Affect Akt Phosphorylation—Besides Ras/Erk pathway activation, Akt/protein kinase B is phosphorylated by PI3-K after integrin activation. To determine whether inhibition of CaMK modulates this pathway, TAD-2 cells were stimulated with FN in the presence of KN. Akt phosphorylation in threonine-308 was not affected by the presence of the CaMK inhibitor (Fig. 5*A*).

Modulation of the FN-dependent Erk Activation by CaMK Occurs Downstream of p21Ras—Our results showed that CaMK activity is required for the FN-induced Erk-pathway in two different cell types. Thus, we next examined whether CaMK was required for p21Ras activation by integrins. The activity of p21Ras was determined in extracts from TAD-2 cells stimulated by FN with or without KN93 (5 μM), W7 (30 μM), and TFP (0.1 μM). Cell extracts were incubated with Raf-1 Ras binding domain peptide-conjugated agarose beads to allow the binding to activated Ras, and adsorbed active-p21Ras was recovered and visualized by Western blot (Fig. 6*B*). Both FCS and integrin engagement activated p21Ras. The CaMK inhibitor KN93 and the CaM inhibitors W7 and TFP were ineffective on the induction of Ras activation by FN, demonstrating that the CaMK involvement in the integrin-Ras-Erk pathway occurs downstream of Ras.

CaMKII Regulates FN-dependent Raf-1 Activation—If the CaMK and MAPK pathways converge upstream of Erk and downstream of Ras, this leaves Raf-1 and Mek as possible targets for CaMK regulation. We next examined whether CaMKII was required for FN-induced activation of Raf-1 in both TAD-2 and Hep3B cells (Fig. 6). Raf-1 activity was measured by a Raf-1 inositol phosphate kinase cascade assay in lysates from cells plated on BSA or FN alone or in the presence of KN93 or ant-CaNtide. FN stimulation induced a 4-fold increase Raf-1 activity, an effect completely inhibited by the treatment with KN93 or ant-CaNtide, demonstrating that FN/integrin-dependent Raf-1 activation requires CaMKII activity. To exclude Mek phosphorylation or a direct MBP phosphorylation by co-immunoprecipitated CaMKII, Mek was omitted in the assay mix, together with MBP and [γ^{32}P]ATP in parallel reaction tubes. In the absence of Mek, MBP was not phosphorylated, and the CaMKII inhibitor did not reduce MBP phosphorylation by FN-stimulated cell extracts (data not shown).

CaMK Inhibitors Have No Direct Effect on Raf-1, Pak, and Mek—Control experiments were performed to exclude a direct

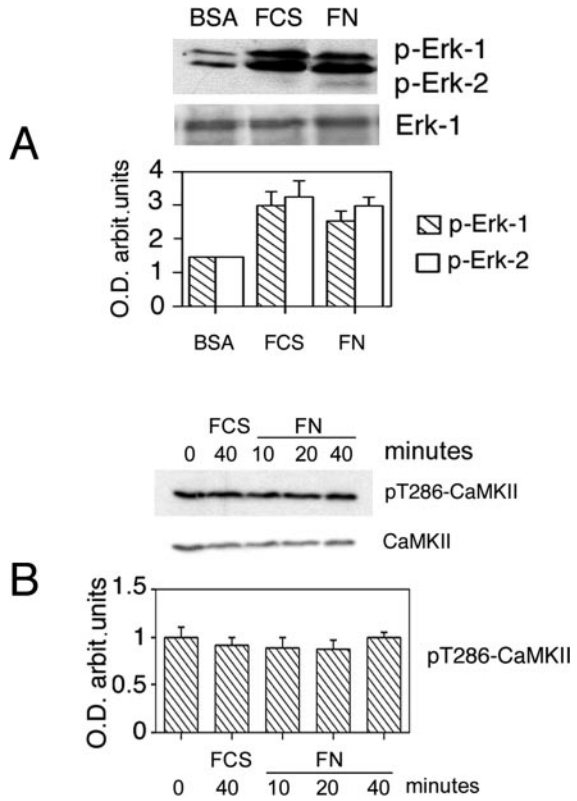

FIG. 3. **Binding to FN activates Erk-1/2 but does not increase CaMKII phosphorylation in Hep3B cells.** *A*, binding to FN activates Erk-1/2. Serum-starved Hep3B cells were seeded in serum-free medium onto BSA-coated plates (*BSA*) or FN coated plates (*FN*) or in untreated plates in the presence of 10% FCS (*FCS*) for 30 min and lysed. Cell extracts were analyzed by Western blot with anti-total-Erk-1 (*Erk-1*) or with anti-phosphotyrosine-Erk-1/2 antibodies (*p-Erk*). Relative expressions of phosphorylated Erk-1 and Erk-2 isoforms were determined by scanning densitometry. A value of 1 arbitrary unit was assigned to BSA point. All experimental points *versus* BSA, $p < 0.001$. *B*, binding to FN does not increases CaMKII phosphorylation. Hep3B cells were starved from the serum and harvested (0). Then the cells were seeded onto uncoated plates for 40 min in the presence of 10% FCS or in FN-coated plates in serum-free medium. Total CaMKII (CaMK) was visualized by specific antibody and phosphorylated CaMKII (*pT286-CaMKII*) by an anti-phosphothreonine CaMKII antibody in Western blot assay. Scanning densitometry demonstrated that differences between FN and 0 time point were not significant.

effect of CaMK inhibitors on Raf-1, Pak, and Mek. Raf-1, Pak, and Mek-1 were immunoprecipitated by specific antibodies from FN-stimulated cells. Inhibitors [γ-^{32}P]ATP and MBP were included in the assay mix, together with immunoprecipitated Pak (Fig. 7*A*) or with Raf-1 in the Raf-1 inositol phosphate kinase cascade assay (Fig. 7*B*). The ability of immunoprecipitated Mek-1 to phosphorylate Erk-2 in the presence of CaMK inhibitors was evaluated by Western blot (Fig. 7*C*). Results show that CaMK inhibitors have no direct effect on Raf-1, Pak, and Mek-1.

FN Promotes Raf-1/CaMKII Association—Kinase-to-target interaction generates protein complexes that, when sufficiently stable, can be co-precipitated. To assess whether CaMKII might be associated with Raf-1 in a protein complex, CaMKII was immunoprecipitated in extracts from cells plated on BSA or FN. Immunoprecipitated CaMKII and co-precipitated Raf-1 were detected by Western blot by specific antibodies (Fig. 8). In the absence of FN stimulation, co-immunoprecipitation of CaMKII and Raf-1 was not visible in TAD-2 cells, whereas it was clearly evident in Hep3B cells. In both cell lines, Raf-1 and CaMKII were co-immunoprecipitated after adhesion to FN. The CaMKII inhibitor KN93 inhibited both the constitutive and the FN-induced Raf-1/CaMKII association. These results suggest that CaMKII, either activated constitutively or activated by integrins, participates in the formation of a protein complex with Raf-1, directly or indirectly.

Raf-1 Is Phosphorylated by CaMKII in Vitro—To determine whether CaMKII can phosphorylate Raf-1, the latter was immunoprecipitated from non-integrin-activated TAD-2 cells and incubated *in vitro* with active purified CaMKII and [γ-^{32}P]ATP. KN93 or ant-CaNtide were added in the mix, and phosphorylated Raf-1 was resolved by SDS-PAGE and visualized by autoradiography (Fig. 9). Raf-1 was phosphorylated by active CaMKII and ant-CaNtide inhibited Raf-1 phosphorylation, whereas KN93 was ineffective. Ant-CaNtide binds the substrate-binding site of CaMKII blocking the active enzyme. KN93 binds the CaMKII auto-phosphorylation binding site required for CaMKII activation; hence, it is ineffective on already active CaMKII. These data demonstrate that CaMKII can phosphorylate Raf-1 *in vitro*.

DISCUSSION

Our results demonstrate that activation of the integrin receptors by FN binding stimulates the Ras/Erk pathway and that this pathway is controlled by CaMKII-mediated Ca^{2+} signaling through the regulation of Raf-1 activity (Fig. 10). We demonstrated previously that FN binding to integrins induces FAK and Paxillin phosphorylation with the formation of the FAK/Grb-2/Sos complex in the thyroid cell line TAD-2. This pathway mediates the integrin-dependent cell growth as inhibition of Ras or Mek blocks FN-induced proliferation (13).

Several studies suggested the existence of a connection between Ca^{2+} signaling and MAPK pathway at multiple steps and with possible divergent final effects. Both these signals can be generated simultaneously by integrin activation in some cell types. Therefore, we decided to investigate whether Ca^{2+} signaling and the Ras/Erk pathway interact and together regulate ECM-stimulated cell growth. To determine whether a Ca^{2+} signaling is generated by integrin activation in this cell line, we measured $[Ca^{2+}]_i$ upon soluble FN binding. Although $[Ca^{2+}]_i$ increase is fast and transient upon most hormone-receptor binding, in our experiments, $[Ca^{2+}]_i$ increase was delayed, slow, and long-lasting. These results are in agreement with the data obtained in umbilical vein cells (16). Integrins do not have intrinsic kinase activity, and ECM binding leads to their clustering into the focal adhesions. The progressive recruitment of integrins into the focal adhesions by FN leads to the progressive activation of kinases and generates unsynchronized signals that produce a delayed slow $[Ca^{2+}]_i$ wave. The biological effects of the integrin-induced $[Ca^{2+}]_i$ changes depends upon the cell type and include cell spreading in endothelial cells, modulation of response to inflammatory mediators in neutrophils, spreading and morphological changes in platelets, but no effects on cell growth have been reported so far (16, 28, 29).

Changes in $[Ca^{2+}]_i$ are sensed by CaM, a ubiquitous Ca^{2+} binding protein that binds to and regulates target enzymes such as CaMK. In our study, CaMKII was activated upon FN-integrin binding in TAD-2 cells, whereas in Hep3B cells, CaMKII seemed to be phosphorylated in basal conditions, and FN failed to increase its phosphorylation. The events that determined the basal CaMKII phosphorylation in Hep3B cells were not investigated in this study. However, we observed in primary cultures of normal and tumor thyroid cells that activation of CaMKII requires integrin engagement only in normal cells.[2] Activating mutations of CaMKII have not been described thus far, and the presence of constitutive up-stream activating signals is a possible explanation for the high basal CaMKII

[2] M. Illario, A. L. Cavallo, and M. Vitale, unpublished observations.

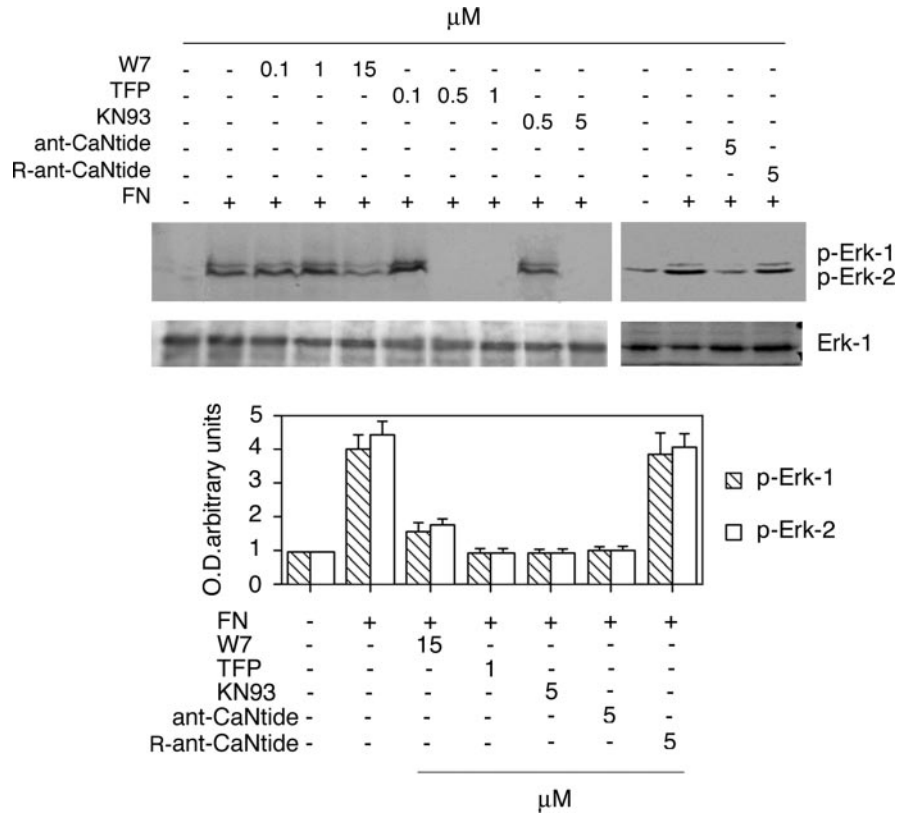

FIG. 4. **Inhibition of CaMKII blocks Erk activity in Hep3B cells.** Serum-starved Hep3B cells were plated for 30 min in serum-free medium onto BSA- or FN-coated plates with or without KN93, W7, TFP, ant-CaNtide, or reverse ant-CaNtide (*R-ant-CaNtide*). The cells were then lysed and the extracts were analyzed by Western blot with anti-phosphotyrosine-Erk (*p-Erk*) or anti-total-Erk-1 (*Erk*) antibodies. Relative expressions of phosphorylated Erk-1 and Erk-2 isoforms were determined by scanning densitometry. A value of 1 OD arbitrary unit was assigned to BSA point. FN *versus* FN+KN93/W7/TFP/ant-CaNtide, $p < 0.001$.

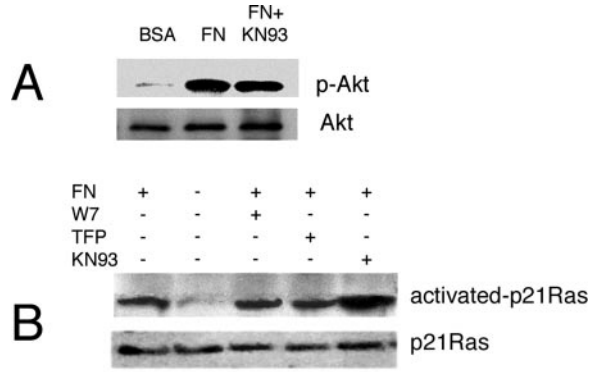

FIG. 5. **Integrin-dependent Akt and Ras activation are not modulated by CaMK inhibition.** TAD-2 cells were starved from serum and plated for 30 min in serum-free medium onto BSA- or FN-coated plates with or without KN93, W7 or TFP. *A*, phosphorylated-Akt (*p-Akt*) and total Akt (*Akt*) were visualized by Western blot with specific antibodies. *B*, activated p21Ras was immunoprecipitated with Raf-1 Ras binding domain peptide-conjugated agarose beads and visualized by Western blot (*activated-p21Ras*). Total Ras in cell extracts was also visualized by Western blot (*p21Ras*).

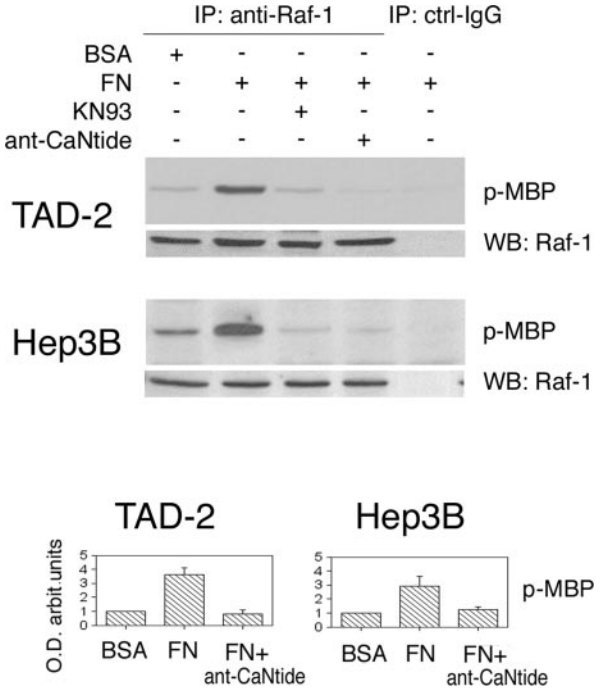

FIG. 6. **CaMKII regulates FN-dependent Raf-1 activity.** The cells were plated for 30 min in serum -free medium on BSA or FN alone or on FN in the presence of the general CaMK inhibitor KN93 or the selective CaMKII inhibitor ant-CaNtide. Then, Raf-1 in cell lysates was immunoprecipitated by specific antibodies. IgG from non-immune rabbit serum were used in place of anti-Raf as a control (*ctrl-IgG*). The immunoprecipitates (*IP*) were incubated with inactive Mek and Erk in the presence of cold ATP, and an aliquot of the mixture was then incubated with MBP in the presence of [γ-^{32}P]ATP. Phosphorylated MBP (*p-MBP*) was resolved by SDS-PAGE and visualized by autoradiography. Total Raf-1 (*Raf-1*) in each immunoprecipitate was visualized by Western blot (*WB*). Averages of relative MBP phosphorylation were also determined by scanning densitometry. In each diagram, a value of 1 OD arbitrary unit was assigned to 0 point. FN *versus* FN+ant-CaNtide, $p < 0.001$.

phosphorylation in tumor cells. However, active CaMKII alone is not sufficient to induce Erk-1/2 activation, as indicated by the observation that Erk-1/2 results in phosphorylation only upon integrin activation in Hep3B cells. In both TAD-2 and Hep3B cells, inhibition of CaMKII activity by the broad CaMK enzymatic inhibitor KN93, the CaM inhibitors W7 and TFP, and the cell-permeant, CaMKII-specific inhibitory peptide ant-CaNtide demonstrated that CaMKII enzymatic activity is required for Erk-1/2 phosphorylation induced by FN-integrin binding. A role for a Ca^{2+}/CaM-dependent pathway in the activation of Erk-1/2 has been described in a number of studies. Calcium and CaM are both necessary for Erk-1/2 stimulation by thapsigargin in foreskin fibroblasts, for the short-term activation of Erk after nerve growth factor stimulation in PC-12 cells; recently, it has been reported that CaM regulates at

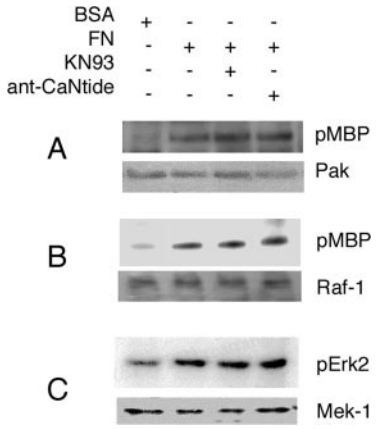

FIG. 7. **CaMK inhibitors have no direct effect on Raf, Pak, or Mek.** TAD-2 cells were plated for 30 min in serum-free medium on BSA or FN. Raf-1 and Mek-1 were immunoprecipitated by specific antibodies. *A*, CaMK inhibitors were included in the assay mix, together with MBP, [γ-^{32}P]ATP, and immunoprecipitated Pak (*A*) or Raf-1 in the Raf-1 inositol phosphate-kinase cascade assay (*B*). Phosphorylated MBP (*pMBP*) was resolved by SDS-PAGE and visualized by autoradiography. Total Pak (*Pak*) and Raf-1 (*Raf-1*) in each immunoprecipitate was visualized by Western blot. *C*, CaMK inhibitors, immunoprecipitated Mek-1, and purified Erk-2 were incubated together, and phosphorylated Erk-2 (*pErk-2*) was visualized by Western blot with anti-phospho-Erk-1/2 antibodies. Total Mek-1 (*Mek-1*) in each immunoprecipitate was visualized by Western blot. Results show that both CaMK inhibitors have no direct effect on Raf-1 or on Mek-1.

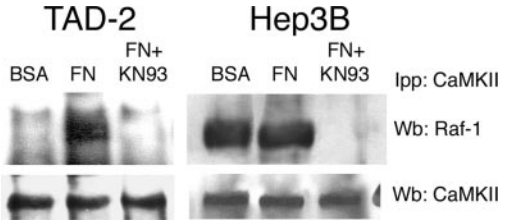

FIG. 8. **Phosphorylated CaMKII binds to Raf-1 in a regulated manner and phosphorylate it *in vitro*.** TAD-2 and Hep3B cells were plated for 30 min onto BSA- or FN-coated plates in serum-free medium without or with KN93. Cell extracts were immunoprecipitated (*Ipp*) with a specific anti-CaMKII antibody. After protein separation by SDS-PAGE and transfer to a nitrocellulose membrane, immunoprecipitated CaMKII and co-precipitated Raf-1 were detected by anti-CaMKII and anti-Raf-1 antibodies respectively. *Wb*, Western blots.

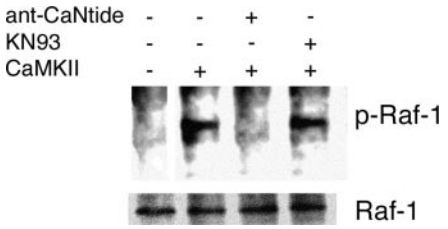

FIG. 9. **Raf-1 was immunoprecipitated from unstimulated TAD-2 cells.** An equal amount of Raf-1 was incubated with active CaMKII and [γ-^{32}P]ATP alone or in the presence of KN93 or ant-CaNtide. Phosphorylated Raf-1 (*p-Raf-1*) was resolved by SDS-PAGE and visualized by autoradiography. Total Raf-1 (*Raf-1*) in each immunoprecipitate was visualized by Western blot.

multiple steps the activation of Erk by stimulation of the epidermal growth factor receptor, whereas studies that demonstrate CaMKII requirement for Erk activity are lacking (21, 22, 30). In a different cell system, inhibition of CaM synergizes with different stimuli to induce protein kinase C-dependent Erk activation (31, 32). In Swiss 3T3 cells, CaM binds the GTP-bound K-RasB isoform and down-modulates Erk phosphorylation induced by epidermal growth factor, bombesin, platelet-derived growth factor, and serum. Together, these data

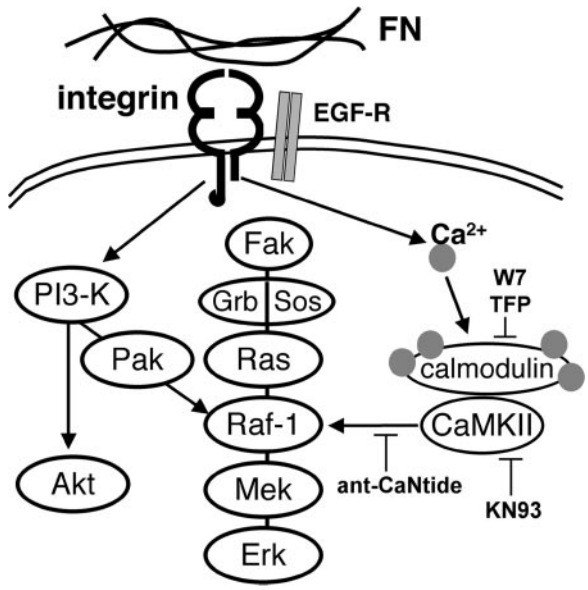

FIG. 10. **Integrin-mediated signaling pathways.** Integrin activation promotes three signaling pathways: Ras/Raf-1/Mek/Erk, PI3-K/Akt, and Ca^{2+}/CaMKII. The last is necessary to Raf-1 to activate Mek, thus inducing Erk activation. Ca^{2+}/CaMKII signal is necessary but not sufficient for Raf-1 activation. *EGF-R*, epidermal growth factor-receptor; *Fak*, focal adhesion kinase; *Akt*, protein kinase B/Akt; *PAK*, p21-GTPase activated kinase; modified from Ref. 13.

demonstrate that modulation of Ras/Erk signaling by Ca^{2+}/CaM signal depends on the balance between divergent effects in a defined cell context.

In our study, the effect of the CaMKII-specific inhibitor ant-CaNtide demonstrated the pivotal role of CaMKII in the regulation of integrin-dependent Erk-1/2 activation. A link between integrins and CaMKII in the regulation of cell motility and spreading has been documented in some cell types (20, 33). However, evidence that cell proliferation induced by ECM-activated integrins requires the activation of the Ca^{2+}/CaMK pathway has not yet been produced.

In our study, integrin activation induced CaMKII phosphorylation and its association with Raf-1 in thyroid cells. In Hep3B cells, where CaMKII phosphorylation did not require FN stimulation, CaMKII and Raf-1 were already associated in unstimulated conditions. In both cell types, CaMK inhibitors prevented CaMKII/Raf-1 association and inhibited Raf-1 activity. Although a direct Raf-1 phosphorylation by CaMKII has not yet been described, the existence of seven canonical R/KXXS/T consensus sequence for CaMKII makes Raf-1 phosphorylation by CaMKII a possibility worthy of consideration (34). Indeed, our *in vitro* experiments demonstrate that Raf-1 can be phosphorylated by CaMKII. Although this does not demonstrate that Raf-1 is a CaMKII substrate *in vivo*, it supports this possibility. Raf-1 is a substrate of several kinases (Src, protein kinase C, protein kinase A, and Akt) that, together with Ras, provide co-stimulatory signals that lead to Erk activation in response to several receptor signals (35). Activation of PKC contributes to Raf/Erk-1/2 activation in response to cyclic pressure-induced strain in endothelial cells (36). Ras-mediated activation of Erk-1/2, either by insulin, insulin like-growth factor I, interleukin-8 and -2, platelet-derived growth factor, vasopressin, T cell receptor, and adrenergic receptors is increased by Raf-1 co-activation by PI3-K (37–43). Although a direct effect of CaM on Raf-1 activity has been proposed in response to epidermal growth factor receptor activation, a direct CaMKII/Raf-1 interaction has not yet been described (21, 22, 44).

Our results demonstrate for the first time that Raf-1 is a

target of CaMKII in the pathway that regulates activation of Erk-1/2 downstream of integrin receptors. Based on available evidence, we suggest that integrins activate the Ras/Erk pathway, but this is modulated by the interplay of a Ca^{2+}/CaMKII signaling pathway. The evidence of co-stimulatory signals generated by integrins has already been provided. Severe attenuation of integrin-dependent Erk-1/2 activation by PI3-K inhibition was demonstrated in COS-7 cells (12). After integrin-FN binding, PI3-K is activated and regulates the phosphorylation of Raf-1 Ser338 through the serine/threonine kinase Pak, participating in the ensuing Erk activation (14).

In conclusion, our data demonstrate that: 1) CaMKII is activated after integrin engagement; 2) CaMKII regulates Ras-stimulated Raf-1 activity leading to Erk-1/2 activation in the integrin pathway; and 3) CaMKII is necessary for ECM-stimulated Erk activation. The control of Raf-1 activity by CaMKII might not be restricted to integrin signaling but could play a more general and pivotal role in the cross-talk between intracellular signaling pathways. It will be of considerable interest to investigate the role of Ca^{2+} and CaMKII in other signaling pathways that comprise Raf-1 as a pivotal factor.

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