The Association of CRKII with C3G Can be Regulated by Integrins and Defines a Novel Means to Regulate the Mitogen-activated Protein Kinases*

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In studies to define mechanisms of ERK activation in Chinese hamster ovary cells, we have observed an inverse correlation between CRKII-C3G complex formation and ERK activity. That is, we were able to coprecipitate the guanine nucleotide exchange factor C3G with the adaptor protein CRKII in lysates from suspended cells that had low ERK activity, but we could not do so or could do so less efficiently in lysates of adherent cells with increased ERK activity. Consistent with the presence of a functional CRKII-C3G complex, we detected more GTP-loaded RAP1 in suspension than adherent lysates. Overexpression of cDNAs encoding B-RAF, CRKII W109L, and PTP1B C215S activated ERK in suspension cells, the latter two constructs also disrupting CRKII-C3G complex formation. Finally, we have also observed that certain integrin α subunit cytoplasmic splice variants differentially regulate ERK1/2 but also in a manner that correlated with levels of a CRKII-C3G complex. Thus, these data suggest the involvement of integrins in an ERK suppression pathway mediated by CRKII-C3G complex formation and downstream signaling from activated RAP1.

Signal transduction pathways stimulated by growth factors and adhesion to matrix proteins influence cell behavior. Although the initial mechanics of these transduction pathways are distinct, several downstream effects and mediators are held in common. One of these common downstream mediators is the mitogen-activated protein kinase (the MAP3 kinases ERK1 and ERK2). Canonical pathways leading to ERK activation typically involve small GTPases. Principally, GTP loading or activation of RAS triggers a downstream kinase activation cascade from RAP1 to MEK and finally to ERK1 and ERK2. Integrin-mediated adhesion has been reported to impact this pathway at various and sometimes controversial points (1–4). A second GTPase that may affect ERK activity is RAP1. By competing with RAS for common downstream effectors, activated RAP1 is sometimes thought to be antagonistic to RAS-based signaling (5), although specific RAP1 effects may be species- or cell type-specific (6, 7).

Crucial in the activation of the small GTPases is the mobilization of adaptor protein-exchange factor complexes. Thus, the GRB2-SOS complex is recruited to the cell membrane in GRB2 SH2 domain-dependent interactions with tyrosine-phosphorylated growth factor receptors or phosphorylated intermediates of integrin signaling pathways, thereby enabling efficient guanine nucleotide exchange or activation of RAS. Likewise, RAP1 is activated by the relocation of the adaptor protein, CRKII, and its associated exchange factor C3G. A potential docking site for CRKII is p130CAS, a protein heavily phosphorylated upon integrin ligation (8). In addition to relocation of these complexes, recent studies suggest their association itself may be regulated, thereby affecting downstream signaling. In what can be thought of as a classical negative feedback loop, a kinase activity downstream of MEK, possibly MEK itself, can serine/threonine-phosphorylate SOS (9). Phosphorylated SOS can dissociate from GRB2 (10, 11), thereby limiting GTP loading of RAS and attenuating ERK activation. In a similar manner, as best exemplified with the insulin receptor, ligand occupancy can lead to tyrosine phosphorylation of the adaptor protein CRKII (12). Once tyrosine-phosphorylated, CRKII can form an intramolecular SH2 domain loop (13), altering its conformation and dissociating from C3G (12, 14).

Certain integrins are expressed with alternatively spliced cytoplasmic domains that often exist in a cell- or tissue-specific manner and can be induced upon differentiation (15). There are four β1 and β4 cytoplasmic variants, three of α7, and two each of β2a, α3, and α6. In some cases these isoforms confer phenotypic variation. For example, overexpression of the β1C and β2D variants slow cell cycle progression (16–18), whereas β2B appears to have dominant negative effects on cell adhesion and motility (19). Cells expressing the α6A as opposed to the α6B isoform are more migratory (20). Thus, the distribution and expression pattern of these splice variants may enable unique functional properties to constituent cells. Whereas phenotypic differences have been ascribed to certain splice variants, the intracellular signaling pathways utilized by them are less clear. Nevertheless, limited studies with the α6 variants have identified different patterns of adhesion-stimulated protein tyrosine phosphorylation (21) and MAP kinase activation (22).

In exploring the role of adaptor protein-exchange factor complexes in the regulation of the MAP kinases, we have identified a correlation between CRKII-C3G complex formation, levels of GTP-loaded RAP, and ERK activity in CHO cells. That is, in lysates of suspension cells, we observed low ERK activity but an immunoprecipitable CRKII-C3G complex and high levels of activated RAP. In contrast lysates generated from CHO cells adherent to fibronectin (fn) demonstrated increased ERK ac-
Integrins and MAP Kinases

Reagents—The characterization of the anti-α5β2 antibodies D57, anti-LIBS6, and PAC1 have been described previously (23–25). The antibody D57 was biotinylated with biotin-N-hydroxysuccinimide (Sigma) according to the manufacturer’s directions. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM and FITC-conjugated goat anti-mouse IgG were from Tago (Burlingame, CA), and phycoerythrin-streptavidin was from Molecular Probes Inc. (Junction City, OR). The peptide mimic compound Ro43-5054, which specifically inhibits binding to α5β2 was a generous gift from Beat Steiner (Hoffmann-La Roche). Antibodies to ERK1, ERK2, CRKII, C3G, B-Raf, and the HA epitope were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-PTPIB antibody was from Transduction Laboratories (Lexington, KY). Phospho-ERK-specific antibodies were from Promega (Madison, WI) and Santa Cruz Biotechnology. Fibrinogen (fg) was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and G418 (Geneticin) was obtained from Life Technologies, Inc.

DNA Constructs—The construction of the chimeric integrins β6a, α5δ6A, and α5α6b has been described previously (23). pCDM8 constructs expressing the chimeric integrins α5δ6A, and α5α6b were created by a similar strategy. Briefly, cytoplasmic sequences of the integrin variants α5 and α6b were generated from full-length cDNA clones by polymerase chain reaction with appropriately designed oligonucleotide primers, digested with HindIII and XbaI, and subcloned into pCDM8. The resulting construct was digested with HindIII and fused to a HindIII fragment of CD2b (26) containing the extracellular and transmembrane domains of α5. A cDNA construct encoding an α5α6b variant with an SH3 domain mutation has been described previously (27, 28), whereas an expression construct for wild-type CRKII was obtained from Jeffrey Pessin (University of Iowa). A catalytically inactive mutant of PTPIB (C215S) was generated from the wild-type clone (gift of Nicholas Tonks; Cold Spring Harbor Laboratory) by Quick Change Mutagenesis (Stratagene) with appropriately designed oligonucleotides. Cell lines expressing integrin chimera, plasmids encoding these sequences, and the appropriate negative controls were transfected into CHO cells. Briefly, the cells were plated at a density of 1 × 10⁶ cells/100-mm dish, and 24 h later, 2 ml of media containing 0.5% fetal bovine serum. At this time, the cells were harvested by incubation with trypsin-EDTA (Irvine Scientific) and collected. The media were changed after 24 h, and the cells were allowed to incubate for 10 min at 37 °C before washing in PBS (suspension cells). The remaining cells were allowed to adhere for 10 min to tissue culture dishes that had been coated with substrate proteins (overnight incubation with 15 μg/ml fg or fn) and washed once with PBS (adherent cells).

MAP Kinase Assays—Suspended and adherent cells prepared as described above were lysed on ice in M2 buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5 mM Nonidet P-40, 3 mM EGTA, 5 mM EDTA, 20 mM NaF, 3 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1× complete phosphate inhibitor (Roche Molecular Biochemicals)), the lysates clarified by centrifugation at 15,000 × g for 10 min at 4 °C, and the supernatants were determined for the BCA assay (Pierce). For phospho-ERK immunoblotting, 60 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 2 h at room temperature, incubated with an anti-phospho-ERK antibody (1:2000 dilution in 5% milk) for 1 h at room temperature, and then washed with TBS containing 0.5% Tween 20 (TBS-T). The membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000 dilution) for 1 h, washed with TBS-T, and immunoreactive bands visualized using ECL reagent (Amersham Pharmacia Biotech). To confirm equal loading of ERK1 and ERK2, the membrane was stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 μg/mi β-mercaptoethanol for 30 min at 65 °C, blocked with 5% non-fat milk, and re-probed with antibodies to ERK1 and ERK2.

For in vitro kinase reactions, ERK1 and ERK2 were immunoprecipitated from 150 μg of lysate by incubation with 0.4 μg each of anti-ERK1 and anti-ERK2 antibodies and 30 μl of a 50% slurry of protein-G Sepharose for 3 h at 4 °C. Immunoprecipitates were collected by centrifugation, washed with kinase buffer (20 mM HEPES, pH 7.6, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphatase, 10 mM DTT, 5 mM Na3VO4), and then resuspended in KB. Kinase reactions were initiated by addition of 2 μg of myelin basic protein (MBP), 20 μM cold ATP, and 5 μCi of [γ-32P]ATP and allowed to incubate for 20 min at 30 °C. Reactions were stopped by addition of SDS sample buffer, and labeled products were resolved by SDS-PAGE and autoradiography. Equal immunoprecipitation of ERK1 and ERK2 was confirmed by Western blotting with antibodies to these proteins.

Western Blotting—Suspension and adherent lysates prepared as described above were clarified by centrifugation, and the resulting supernatants were collected. For immunoprecipitations, 300 μg of cell extract was incubated with 0.4 ml in lysis buffer and incubated overnight at 4 °C with the appropriate antibodies. Proteins were recovered after a 2-h incubation with protein G-Sepharose, resolved by SDS-PAGE, and electro-photographically transferred to nitrocellulose membranes. Protein bands were identified after incubating the membranes with specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and finally visualized with the ECL reagent (Amersham Pharmacia Biotech).

GTP-Protein Loading Assays—To determine the GTP-loading of Ras and Rap GTPases, we performed pull-down assays. GST fusion proteins with the minimal RAP-biding domain of RaIGDS and the Ras-binding domain of Raf-1 (RBD) were generated in Escherichia coli strain BL21 following induction with 1 mM isopropyl-1-thio-β-D-galactoside and isolated by immobilizing bacterial lysates at 4 °C with glutathione-Sepharose beads. The beads were then washed three times with RBD buffer (20 mM HEPES, pH 7.5, 120 mM NaCl, 10 mM glycerol, 0.5% Nonidet P-40, 20 mM EDTA, 10 μg/ml aprotinin), and then resuspended in RBD buffer to make a 50% slurry.

CHO cells were transfected with expression vectors for HA-tagged wild-type Rap1 or wild-type Ha-Ras and pHOK as described above. After 48 h, transfectants were isolated by magnetic sorting and sus-
RESULTS

Low ERK Activity in CHO Cells Is Correlated with CRKII-C3G Association and Activation of Rap—As noted above, the association of SH2 and SH3-containing adaptor proteins with guanine nucleotide exchange factors can be regulated by signals arising from occupancy of growth factor receptors. To determine if integrin occupancy also regulates these associations, we have examined the properties of these molecules in suspension and adherent lysates. As expected, when CHO cells were serum-starved overnight and then kept in suspension for 2 h, we observed low levels of ERK1/2 activity that were significantly increased upon adhesion to fn (Fig. 1A). When we immunoprecipitated CRKII from CHO lysates, we consistently observed C3G in Western blots from suspension lysates but could not do so or could do so less efficiently from adherent lysates (Fig. 1B; also Figs. 4C and 5C). Similar amounts of CRKII were immunoprecipitated from both lysates. In contrast we saw no change in levels of the GRB2-SOS complex from suspended or adherent lysates (data not shown). Thus low ERK1/2 activity correlated with the presence of an immunoprecipitable CRKII-C3G complex.

As C3G functions as an exchange factor for the RAS family member RAP1, we were interested if the state of integrin occupancy, and variable levels of the CRKII-C3G complex, affected the activation or GTP loading of this G protein. To determine this, we used a pull-down assay with a GST-RalGDS fusion protein that selectively binds the GTP as opposed to the GDP-bound form of RAP. When analyzed in this way, we found that levels of activated RAP were approximately 10-fold greater in suspension lysates than in adherent lysates (Fig. 2). In analogous experiments using a GST-RBD fusion protein to isolate GTP-bound RAS, we found activated RAS was mostly present in adherent but not suspended cell lysates (Fig. 2). In both experiments we used an activated variant (G12V) of these GTPases as a positive control (data not shown). Thus, the state of integrin occupancy determines the nucleotide loading of the RAS and RAP GTPases, and GTP-loaded RAP is also found in lysates with low ERK activity.

The downstream signaling consequences of activated RAP are dependent upon the predominant isoform of RAP present. As noted above, in certain cell types, GTP-loaded RAP is thought to be antagonistic to RAS-mediated ERK activation by competing for common effectors such as RAP1 (5). However, in many neuronal cell types that express B-RAF, activated RAP stimulates rather than suppresses ERK activity (29, 30). As RAP is GTP-loaded in suspended CHO cells, we would predict that overexpression of B-RAF would activate ERK in this condition. To examine this possibility we transiently transfected CHO cells with B-RAF cDNA or a vector control and determined ERK activity levels upon suspension or adhesion to fn. While CHO cells transfected with the empty vector construct demonstrated background levels of ERK activity, those transfected with B-RAF had significantly greater levels of active ERK in suspension lysates (Fig. 3A). As expected, ERK activity increased upon adhesion of both cell types. Although the absolute level of ERK activity was greater in the B-RAF transfectants, when analyzed quantitatively, adhesion stimulated a 50% increase in the vector transfectants but only a 5% increase in the B-RAF transfectants. Thus ERK phosphorylation, and presumably activity, was nearly maximal in suspension lysates of the B-RAF transfectants. These results are consistent with the idea that RAP is GTP-loaded when CHOs are in suspension and that the state of RAP loading is an important determinant of ERK activity.
Disruption of the CRKII-C3G Complex and ERK Activity—
The above data suggested that activation of RAP and inhibition of ERK activity in CHO cells was dependent upon the regulated formation of the CRKII-C3G complex. Blocking this association would therefore be predicted to activate ERK1/2 in suspension cells. To look at this possibility we have made use of a CRKII variant (W109L) with an SH3 domain mutation that abolishes interaction with C3G. When we expressed this construct in CHO cells, we observed that levels of ERK activity in suspension lysates were increased relative to cells transfected with wild-type CRKII (Fig. 4, A and B), despite comparable expression of both CRKII constructs (Fig. 4D). Consistent with this observation and the described functional properties of this amino acid substitution, we detected little of the CRKII-C3G complex in suspension lysates of the W109L transfectants (Fig. 4C). In contrast, cells transfected with wild-type CRKII, like untransfected CHO’s, did demonstrate an immunoprecipitable CRKII-C3G complex in suspension lysates (Fig. 4C). Thus blocking the interaction of CRKII with C3G activates ERK1/2.

Studies from another group suggested that overexpression of the protein tyrosine phosphatase, PTP1B, impaired integrin-mediated up-regulation of ERK activity in 3Y1 fibroblasts, whereas a CAS binding-deficient variant of this phosphatase reversed these effects (31). In another study, expression of a catalytically inactive form of this phosphatase resulted in altered morphology, reduced spreading, and decreased focal contacts in mouse L fibroblasts (32). Therefore, to determine if this enzyme affected ERK activity levels or CRKII-C3G complex formation in CHO’s, we transiently transfected these cells with wild-type PTP1B or its catalytically inactive variant (C215S). Wild-type PTP1B transfectants, as well as vector (pCDNA3.1)-transfected cells, had basal levels of ERK activity in suspension that increased upon adhesion (Fig. 5, A and B). On the other hand, cells overexpressing the C215S variant had increased levels of active ERK in both suspension and adherent lysates (Fig. 5, A and B). Both PTP1B constructs expressed equally well (Fig. 5D). Next we examined levels of the CRKII-C3G complex in the PTP1B transfectants. Whereas wild-type transfectants had high levels of the CRKII-C3G complex in suspension lysates, the C215S transfectants had reduced levels of this complex under the same conditions (Fig. 5C). Equivalent amounts of CRKII were immunoprecipitated in all cases. High ERK activity and low levels of the CRKII-C3G complex were also observed in suspension lysates of cells transfected with a substrate-trapping mutant (D181A) of PTP1B (data not shown). Thus PTP1B regulates CRKII-C3G complex formation with subsequent effects on ERK activity.

Integrin α Subunit Cytoplasmic Splice Variants Differentially Regulate ERK1/2—In an attempt to identify potential “outside in” signaling differences mediated by integrin α subunit cytoplasmic splice variants, we have constructed chimeric integrins containing these sequences. These α subunit chimeras consisted of the extracellular and transmembrane domains of the platelet integrin αIIbβ3 and the cytoplasmic sequences of the splice variants α6A, α6B, α6C, α6A, and α6B (Fig. 6A). These constructs were then cotransfected into CHO cells with a β subunit chimera consisting of the extracellular and transmembrane domains of β3 joined to the cytoplasmic domain of βA. After G418 selection, lines of similar expression levels were identified by flow cytometry (Fig. 6B) and carried on for further study. Similar to cells expressing either αc variant (23), cells expressing the α3 variants conferred a high affinity ligand-binding state (Fig. 7) as determined by the binding of the activation-specific monoclonal antibody, PAC1 (25).

We next examined the ERK activation properties of these cells after they were kept in suspension or allowed to adhere to fibrinogen (fg). We observed that cells expressing the α3 isoform had low or undetectable levels of ERK1/2 activity in suspension but increased levels after 10 min of adhesion (Fig. 8). A gradual diminution of activity was observed at longer time points, with a return to basal levels by 20 min. Surprisingly,
formed immunoprecipitation experiments. C3G was coprecipitated from lysates with CRKII from cells expressing the α5A isoform when they were put into suspension but not when these cells were allowed to adhere to fn (Fig. 8C). In contrast for cells expressing the α5B, α6A, and α6B isoforms, C3G was mostly coprecipitated with CRKII from adherent but not suspended cell lysates (Fig. 8C). Similar amounts of CRKII were precipitated in all cases. Thus, as with untransfected CHO cells, these splice variant lines demonstrated a direct correlation between low ERK activity and the presence of an immunoprecipitable CRKII-C3G complex.

**DISCUSSION**

The results presented here suggest the existence of an ERK1/2 suppression pathway that involves the regulated association of CRKII with C3G and downstream events mediated by GTP-loaded RAP. These conclusions are drawn from the following results.

First, we have observed a direct correlation between low ERK activity and an immunoprecipitable CRKII-C3G complex in untransfected CHO cells. Second, we have also detected high levels of GTP-loaded RAP in lysates with low ERK activity, consistent with the idea that C3G is an exchange factor for RAP and that RAP can be antagonistic to RAS-based signaling. Third, overexpression of B-RAF activates ERK in suspension CHO cell lysates. Fourth, dissociation of the CRKII-C3G complex by overexpressing CRKII W109L or PTP1B C215S up-regulated ERK activity. Finally, we have found that certain integrin α subunit splice variants differentially regulate ERK1/2 but also in a manner correlating with CRKII-C3G complex formation. Thus, these studies define a novel means for integrin regulation of downstream signaling by affecting the association of adaptor proteins with guanine nucleotide exchange factors. Integrin-mediated adhesion can therefore stimulate ERK not only by activating RAS or one of the components of the canonical RAS-ERK pathway but also by relieving the suppressive effects of a RAP-based pathway.

Although we have not yet identified the means whereby adhesion affects CRKII-C3G complex formation, previous reports suggested adaptor protein-exchange factor interactions might be regulated by phosphorylation. As already noted above, the serine/threonine phosphorylation of SOS can result in its dissociation from GRB2, (9), and phosphorylation of tyr221 in CRKII alters its conformation and association with C3G (12). Thus, it will be entertaining to see if adhesion by wild-type CHO or by our splice variant lines does alter CRKII phosphorylation levels and if this accounts for variable association with C3G. Additional phosphorylation events may also play a role in CRKII-C3G complex formation. For example, it is not clear if C3G, like SOS, can be serine/threonine-phosphorylated and whether this might affect its ability to interact with CRKII. Furthermore, it has been demonstrated that C3G can be tyrosine-phosphorylated with resulting effects on its exchange activity (33). The efficient and proper phosphorylation of p130CAS may also be important, as this is required to create an SH2-docking site for CRKII. CAS-CRKII interactions may be a prerequisite for CRKII-C3G association in our cell lines. Finally, the phosphorylation of additional, unknown, molecule(s) may be involved in CRKII-C3G complex formation.

Our finding that a catalytically inactive form of PTP1B-activated ERK in suspended cell lysates is consistent with other observations that this enzyme can dephosphorylate CRKII in vitro (12). Thus one physiological role for this enzyme might be to regulate CRKII-C3G complex formation and hence the downstream effects of activated RAP. In support of this idea, PTP1B has been reported to bind to p130CAS in an SH3-dependent manner (34), placing it physically near potential substrates, such as CRKII. This mode of action would also explain the findings of Liu et al. (31) where overexpression of
wild-type PTP1B abolished adhesion stimulation of ERK, whereas a variant deficient in CAS binding did not. Interestingly, in our system, we have also observed somewhat reduced ERK responses in wild-type PTP1B as opposed to vector transfectants (Fig. 5B). Of further interest, PTP1B has been reported to be proteolytically cleaved and relocated in activated and aggregated platelets, both modifications believed to influence its activity and function (35). It is therefore conceivable that differential recruitment or activation of this phosphatase by the splice variants may initiate a signaling cascade resulting in variable ERK activity. However, it should be noted that additional kinases or phosphatases might also be involved in regulating MAP kinase activity through CRKII-C3G complex formation. For instance, the CRK proteins are substrates for the ABL kinase (36), and the oncogenic effects of BCR-ABL may be explained by constitutive phosphorylation of CRKII, its dissociation from C3G, and reversal of the suppressive effects of RAP on ERK activity.

Those experiments with the splice variant lines comprise the second report of integrin occupancy mediating down-regulation of ERK1/2. In an earlier study, activated, aggregated platelets treated with RGD-containing peptides demonstrated an increase in the activity of these kinases compared with untreated controls (37). By using a synthetic and specific inhibitor of αIbβ3 binding function (Ro435054), we have reproduced those platelet findings (data not shown). Although other studies do not specifically demonstrate down-regulation, there is evidence that certain integrins are unresponsive to adhesion in regulating MAP kinases. For instance, Wei and co-workers (22) have shown that laminin adhesion of macrophages transfecteed with

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**Fig. 6. Sequence and expression of chimeric α subunit splice variants.** A, partial sequences of the chimeric α subunits. These constructs consist of the cytoplasmic domains of αA, αB, αA, and αB fused to the extracellular and transmembrane domains of αIb. Shown are the partial transmembrane sequences of αA, and the cytoplasmic sequences of the splice variants. B, CHO cells stably transfected with the indicated α chimera and a β, construct containing βI, cytoplasmic sequences were analyzed for surface expression by flow cytometry with the anti-αIbβ3 antibody D57 (solid line). Clonal lines demonstrating comparable levels of expression are depicted and were carried on for further study. Non-transfected CHO cells (dotted lines) were used as a negative controls in the flow cytometric analysis.
The ligand binding properties of the clonal lines illustrated in Fig. 6 were analyzed by their ability to bind the activation-specific, anti-α6β1, monoclonal PAC1. PAC1 binding was determined in the presence (dotted line) and absence (solid line) of the ligand mimetic Ro43-5054 to demonstrate specificity. The shift in fluorescence intensity to the right indicates that the transfected integrins are in an active state.

α6β1 stimulated ERK1/2, whereas the same cells expressing α6β3 did not stimulate these kinases. In another study, although it is not clear which isoform was examined, cross-linking of α6β1 on NIH3T3 cells did not stimulate the activity of transfected HA-ERK2, whereas cross-linking of α6β1 and α6β3 did (38). Wary and co-workers (4, 38) suggested the failure of α6β1 to stimulate ERK1/2 was related to its inability to phosphorylate the adaptor protein SHC and recruit the GRB2-SOS complex to initiate the classical RAS-MAP kinase pathway. Our data suggest that an alternative mechanism to actively maintain basal levels of or down-regulate MAP kinases is through the regulated association of CRKII with C3G and the downstream effects of activated RAP. In a similar manner, it has been proposed that dissociation of a CRKII-C3G complex is partly responsible for activation of ERK1/2 by insulin receptor occupancy (12, 14). Although it is not clear if platelets regulate exchange factor function, the activation of RAP may also account for ERK down-regulation in these cells that express high levels of this G protein. It should be noted, however, that although our data are consistent with this model, there is ample evidence that the consequences of C3G and RAP activation might be determined locally or in a cell type-dependent manner. For instance, membrane localization of Drosophila C3G and activation of RAP stimulate MAP kinases in that organism (6), whereas growth factor-induced activation of RAP1 in Rat-1 fibroblasts appeared to have no effect on RAS-dependent ERK activation (7). Furthermore, as mentioned above, in those cell types that express B-RAP, GDP-loaded RAP is also thought to activate rather than inhibit ERK1/2 (29).

The differential capacity of the α6 subunit splice variants to regulate ERK1/2 may have important consequences on cell behavior. MAP kinases play a role in stimulating cell migration, through their phosphorylation of myosin light chain kinase and, in turn, the phosphorylation of myosin light chains by this kinase (39). Interestingly, cells expressing the α6A isoform are more motile than those expressing α6B (20). The differential ability of these isoforms to stimulate ERK1/2 upon laminin adhesion has been proposed as a mechanism for these migration differences (22). MAP kinases can also function to suppress the ligand binding affinity of activated integrins (40). This mechanism may constitute an important negative feedback loop for integrin function and may serve as a means for integrin cross-talk. It has recently been shown that α6β1-mediated adhesion is increased in α6β1-deficient cells, and it has been proposed that adhesion through this latter integrin negatively influences α6β1 in normal cells (41). Similar to the findings of Hughes and co-workers (40), it is conceivable that variable ERK activity stimulated by α6β1 binding might globally or locally influence α6β1 binding function. Finally, MAP kinases are important in establishing the growth properties of developing cells. It has been observed that engagement of a
subset of integrins and activation of ERK activity through SHC promotes growth factor-dependent transit from the G1 cell cycle phase (38). Downstream substrates of the MAP kinases are believed to be involved in these effects on cell cycle progression and proliferation. On the other hand, those integrins that fail to stimulate SHC and MAP kinases promote exit from the cell cycle. Related studies from another group suggested the relative abundance of αβ1 or αβ2 is an important determinant of myoblast proliferation or differentiation, respectively (42, 43). As the α subunit splice variants are often expressed in a tissue- or differentiation state-specific manner, their capacity to variably regulate ERK activity may be likewise be important in establishing the fate of these developing cells.

In conclusion we have shown that low ERK1/2 activity correlated with CRKII-C3G complex formation and activation of RAP in CHO cells. These data suggest that these cells may use active mechanisms to maintain basal levels of or suppress these kinases and that integrin-mediated adhesion may variably affect this suppression pathway. Mechanisms of this sort may be involved in determining the migratory, adhesive, and proliferative capacity of developing cells.

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