

## PEA-15 Binding to ERK1/2 MAPKs Is Required for Its Modulation of Integrin Activation\*

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Activation of Raf-1 suppresses integrin activation, potentially through the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). However, bulk ERK1/2 activation does not correlate with suppression. PEA-15 reverses suppression of integrin activation and binds ERK1/2. Here we report that PEA-15 reversal of integrin suppression depends on its capacity to bind ERK1/2, indicating that ERK1/2 function is indeed required for suppression. Mutations in either the death effector domain or C-terminal tail of PEA-15 that block ERK1/2 binding abrogated the reversal of integrin suppression. Furthermore, we used ERK/p38 chimeras and site-directed mutagenesis to identify ERK1/2 residues required for binding PEA-15. Mutations of residues that precede the  $\alpha$ G helix and within the mitogen-activated protein kinase insert blocked ERK2 binding to PEA-15, but not activation of ERK2. These ERK2 mutants blocked the ability of PEA-15 to reverse suppression of integrin activation. Thus, PEA-15 regulation of integrin activation depends on its binding to ERK1/2. To directly test the role of ERK1/2 localization in suppression, we enforced membrane association of ERK1 and 2 by joining a membrane-targeting CAAX box sequence to them. Both ERK1-CAAX and ERK2-CAAX were membrane-localized and suppressed integrin activation. In contrast to suppression by membrane-targeted Raf-CAAX, suppression by ERK1/2-CAAX was not reversed by PEA-15. Thus, ERK1/2 are

the Raf effectors for suppression of integrin activation, and PEA-15 reverses suppression by binding ERK1/2.

Changes in integrin affinity for ligand (activation) play a central role in integrin functions such as cell adhesion, migration and assembly of the extracellular matrix (1). Activation is transduced by a complex series of cellular signaling pathways that modify the interaction of cytoplasmic proteins, such as talin (2), with integrin cytoplasmic domains (3). Integrin activation is a tightly regulated event, suggesting the existence of pathways that can both promote and suppress integrin activation. One such pathway leads to a marked reduction in integrin affinity as a consequence of activation of the small GTP-binding protein H-Ras and its downstream effector kinase Raf-1 (4). Inhibition of extracellular signal-regulated kinases 1 and 2 (ERK1/2)<sup>1</sup> by the phosphatase MKP-1 (4) but not MKP-3 (6) reduces integrin suppression, indicating that ERK1/2 are likely downstream effectors of H-Ras/Raf-1-mediated suppression. R-Ras, a small GTP-binding protein with substantial homology to H-Ras, has the opposite effect of H-Ras on the integrins (*i.e.* promoting integrin activation) (5). Thus, the interplay between R-Ras and H-Ras could act as a rheostat for integrin function. Certain chimeras of H-Ras/R-Ras proteins that retain the ability to suppress integrin activation were weak activators of ERK1/2 (6). Moreover, inhibition of bulk ERK1/2 activation by use of a MEK inhibitor or transfection of MKP-3 does not abolish suppression (6). These observations suggest that Ras-mediated integrin suppression may be independent of ERK1/2 or that low level ERK1/2 activation in particular cellular areas is sufficient for suppression.

Interest in further characterizing the intracellular signaling pathways that regulate integrin activation led to the isolation of a small protein, previously designated PEA-15 (7, 8). Cellular expression of PEA-15, a 15-kDa protein expressed in a broad range of tissues and enriched in astrocytes (9), prevents activated H-Ras/Raf-1-mediated integrin suppression (8). PEA-15 binds ERK1/2 but does not inhibit ERK1/2 kinase activity or activation; instead, PEA-15 redirects the output of

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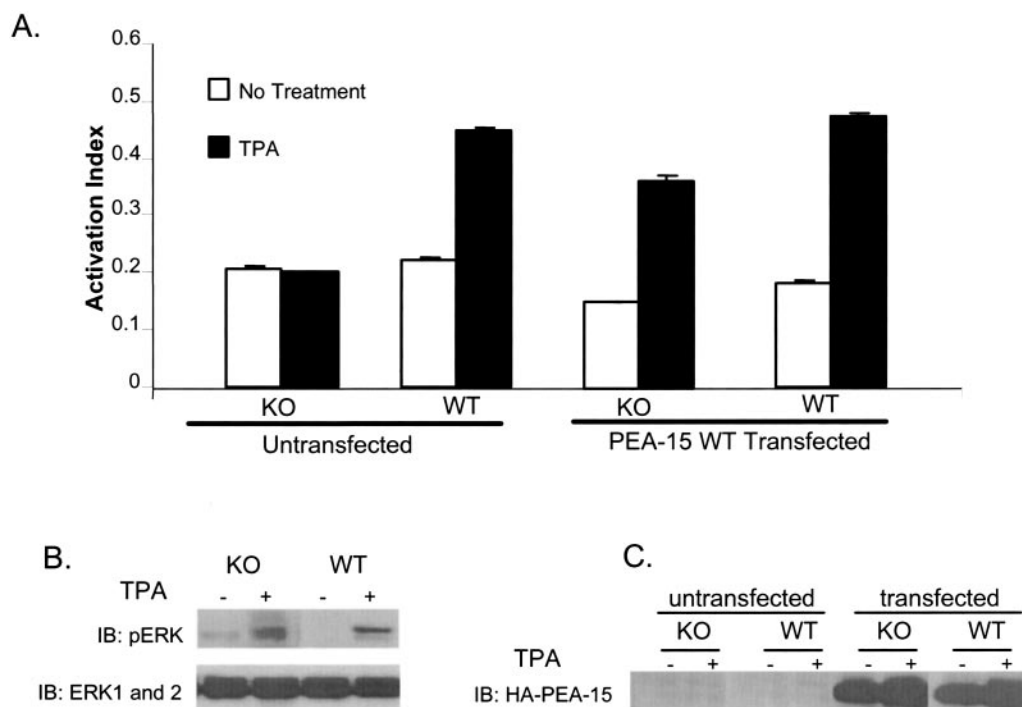
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<sup>1</sup> The abbreviations used are: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; DED, death effector domain; CHO, Chinese hamster ovary; GST, glutathione S-transferase; GFP, green fluorescent protein; AI, activation index; GMF, geometric mean fluorescence; FACS, fluorescence-activated cell sorting; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MAPK, mitogen-activated protein kinase; HA, hemagglutinin.



**FIG. 1. PEA-15 regulates integrin activation.** *A*, PEA-15 null (*KO*) or wild type (*WT*) mouse fibroblasts were incubated at room temperature for 30 min in the presence or absence of TPA (100 nM). The cells were suspended, and the binding of 3Fn-(9–11) was assayed by two-color flow cytometry as described under “Materials and Methods.” In the *left panel*, TPA elicited an increase in 3Fn-(9–11) binding in wild type cells but not in the null cells. However, when the cells were transfected with PEA-15, both null and wild type exhibited similarly enhanced 3Fn-(9–11) binding in response to TPA (*right panel*). *B*, cell lysates from the untransfected cells in *A* were fractionated by SDS-PAGE and immunoblotted (*IB*) with antibodies against phospho-ERK (*pERK*) or total ERK. Note the similar degrees of TPA-induced phosphorylation of ERK in the wild type and null cells. *C*, the cell lysates from *A* were immunoblotted for the expression of the HA epitope to verify similar quantities of recombinant PEA15 expression in all of the transfected cell preparations.

ERK1/2 signaling by altering the subcellular localization of ERK1/2 (10). Thus, it is plausible that PEA-15 may be one of a class of proteins capable of modulating ERK1/2 function and thereby integrin suppression. PEA-15 is composed of an N-terminal death effector domain (DED), a protein interaction module more commonly found in proteins that regulate apoptosis, and a C-terminal tail of irregular structure. NMR “footprinting” and mutagenesis studies identified residues in both the DED and tail that are required for ERK1/2 binding (11). In this study, we explored the relationship between PEA-15 interaction with ERK1/2 and the role of ERK1/2 in integrin suppression. Single point mutations in either the DED or tail of PEA-15, which inhibit interaction with ERK1/2, resulted in a concomitant loss of the ability of PEA-15 to reverse suppression of integrin activation. Furthermore, we used a series of ERK/p38 chimeras (12, 13) in combination with site-directed mutagenesis to identify ERK1/2 residues required for PEA-15 binding. Point mutation of residues preceding the  $\alpha$ G helix or within the MAPK insert blocked the capacity of ERK2 to bind PEA-15 but did not adversely effect ERK2 phosphorylation. Cellular expression of each of these mutant ERKs blocked the ability of PEA-15 to reverse suppression of integrin activation. Thus, the capacity of PEA-15 to regulate integrin activation depends on its binding to ERK1/2. To directly test the role of ERK1/2 in suppression, we enforced membrane association of ERK1 and 2 by joining a membrane-targeting CAAX box sequence to them. Both ERK1-CAAX and ERK2-CAAX were membrane-localized and suppressed integrin activation. In contrast to suppression by membrane-targeted Raf-CAAX, suppression by ERK1/2-CAAX was not reversed by PEA-15. Thus, ERK1/2 are the Raf effectors that lead to suppression of integrin activation, and PEA-15 reverses suppression by binding ERK1/2.

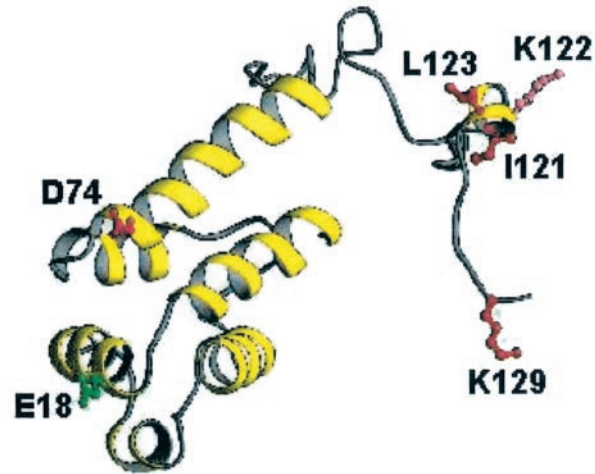
#### MATERIALS AND METHODS

**Cell Culture**—Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 1% nonessential amino acids (Sigma), 1% glutamine (Sigma), and 1% penicillin and streptomycin (Sigma).

**Antibodies, Reagents, and cDNA Constructs**—The activating  $\beta_1$  antibody 9EG7 was purchased from Pharmingen. The anti-ERK1 and anti-ERK2, anti-Ras, anti-RhoGDI, and anti-phospho-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology, Inc. The mouse monoclonal anti-HA antibody (12CA5) and anti-integrin  $\beta_1$  (7E2) were as described (4). Anti-lamin A/C was a generous gift from Dr. Larry Gerace (The Scripps Research Institute). Anti-PEA-15 (3099) was previously described (14). All PEA-15 mutants were generated by using the QuikChange kit (Stratagene) using HA-PEA-15-pcDNA3 as template. ERK2 mutants were generated by the same strategy using HA-ERK2-pcDNA3 as template. All constructs were confirmed by DNA sequencing. The p38/ERK1 chimeric constructs have been described (12). The p38/ERK2 constructs were gifts from Dr. Melanie Cobb (13). The HA-ERK1-CAAX-pEF and HA-ERK1-SAAX-pEF constructs were as described (15). Kinase-dead HA-ERK1(K71R)-CAAX-pEF was generated by using the QuikChange™ kit (Stratagene) using HA-ERK1-CAAX-pEF as template. GST-PEA-15 expression vector was constructed by subcloning the full-length PEA-15 from HA-PEA-15-pcDNA3 into the pGEX-2T vector (Amersham Biosciences). Purification of recombinant 3Fn-(9–11) from the pGEX expression vector encoding Type III repeats 9–11 of fibronectin was previously described (16).

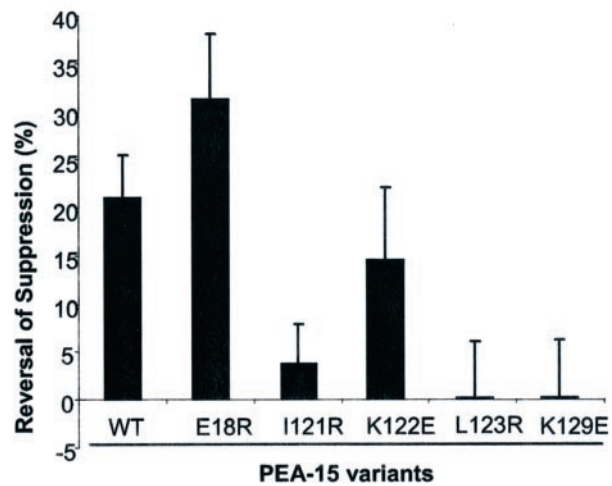
**In Vitro PEA-15 Pull-down Assay**—CHO cells were transfected with p38/ERK1/2 chimeras using LipofectAMINE reagents as per the manufacturer’s protocol (Invitrogen). 40–48 h post-transfection, cells were washed with 1× phosphate-buffered saline (pH 7.4) and lysed in lysis buffer (20 mM HEPES, pH 7.2, 2 mM  $MgCl_2$ , 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Total lysates were homogenized with binding buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 2 mM EGTA, 2 mM  $MgCl_2$ ) and centrifuged at 12,000 rpm for 10 min. The cleared lysates (supernatant) were mixed with 10  $\mu$ l of GSH-Sepharose-bound GST-PEA-15 for 2 h at 4 °C. After washing four times with binding buffer, bound proteins were eluted with SDS sample buffer and resolved by SDS-PAGE under reducing conditions.

A.

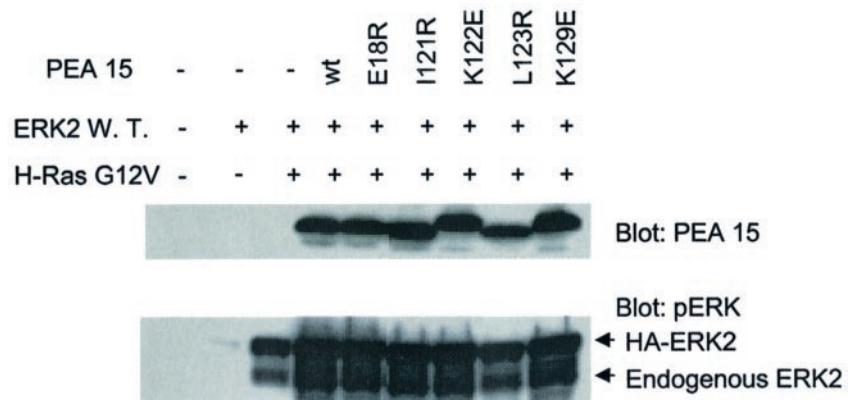


**FIG. 2. PEA-15 reversal of H-Ras(G12V) suppression requires residues implicated in ERK1/2 binding.** *A*, ribbon representation of the three-dimensional structure of PEA-15 (10) (Protein Data Bank accession code 1N3K) with helices colored in yellow and the loops and C-terminal tail in gray. The side chains of selected residues are drawn as ball-and-stick models with Asp<sup>74</sup>, Ile<sup>121</sup>, Leu<sup>123</sup>, and Lys<sup>122</sup> shown in red, Lys<sup>122</sup> in pink, and Glu<sup>18</sup> in green. *B*, summary of FACS analysis of the effect of point mutations on the ability of PEA-15 to rescue H-Ras-induced suppression. To measure reversal of suppression, various PEA-15 mutants were transfected with wild-type ERK2 and H-Ras(G12V). Percentage reversal is calculated as described under "Materials and Methods." Mutations of PEA-15 that block ERK1/2 binding (I121R, L123R, and K129E) abolish reversal. In contrast, mutants that partially inhibit ERK1/2 binding either partially reduce (K122E) or fail to reduce (E18R) reversal. *C*, each of the transfected cells from *B* were lysed in SDS sample buffer and fractionated by SDS-PAGE, and the expression of wild-type or mutant PEA-15 was assessed by immunoblotting. Blotting with anti-phospho-ERK1/2 is shown in the lower panel. Note that neither wild type nor mutant PEA-15 reduces the extent of ERK1/2 phosphorylation induced by H-Ras(G12V).

B.



C.

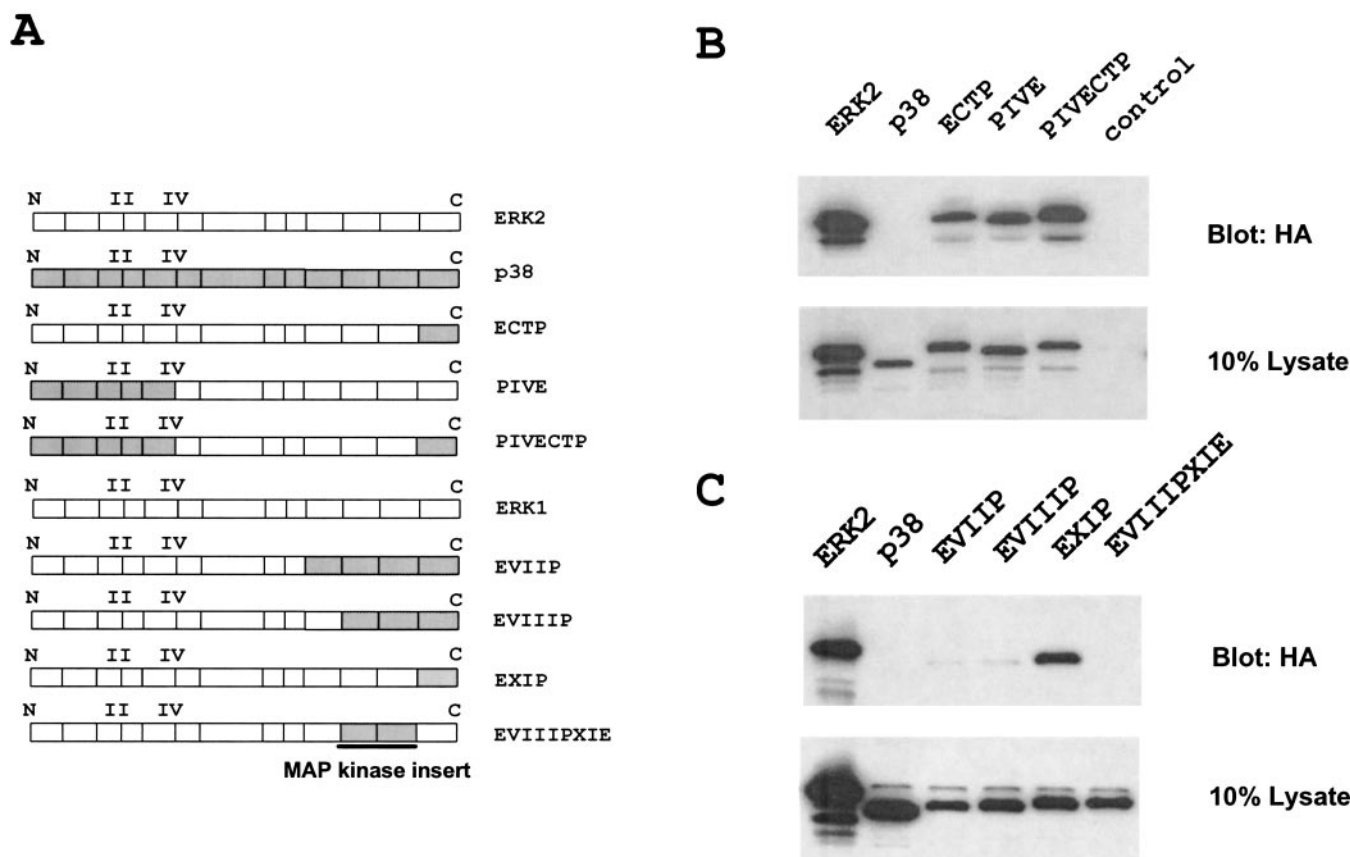


Bound proteins were visualized by Western blotting with anti-HA to detect transfected chimeric proteins. The same protocol was used for GST-PEA-15 pull-down of HA-ERK1-CAAX, HA-ERK1-SAAX, and HA-ERK1(K71R)-CAAX.

Wild-type and mutant ERK2 contained in a NpT7-5 vector (17) were expressed *in vitro* using the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of [<sup>35</sup>S]methionine. GST-PEA-15 bound to glutathione-agarose beads (Sigma) was incubated with <sup>35</sup>S-labeled wild-type and mutant ERK2 proteins in 150  $\mu$ l of 50 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 10% glycerol. Samples were incubated for 2 h at 4  $^{\circ}$ C and then washed three times

with 0.5 ml of the same buffer. The bound proteins were separated on 16% Tris-Tricine gels and visualized by phosphorimaging.

**Flow Cytometry**—Analytical two-color flow cytometry was carried out as previously described (4). Briefly, CHO cells were transfected with GFP (0.1  $\mu$ g) as transfection marker and a combination of HA-ERK2-pcDNA3, ERK2 mutants in the pcDNA3 vector, H-Ras(G12V)-pcDNA3, and HA-PEA-15-pcDNA3. A total of 5  $\mu$ g of DNA was used for each transfection. Transient transfections were carried out using LipofectAMINE Plus reagent as per the manufacturer's protocol (Invitrogen). 24 h post-transfection, cells were harvested and analyzed for transfection efficiency (GFP) and integrin binding to 3Fn(9-11). In



**FIG. 3. PEA-15 binding to ERK/p38 chimeras.** A, schematic representation of ERK/p38 chimeras. MAPKs are composed of 12 conserved subdomains; the N-terminal domain comprises kinase subdomains I–IV together with the C-terminal loop, and the C-terminal domain includes kinase subdomains V–XI (also see Fig. 4A). Sequence derived from ERK1/2 and p38 are represented by *white* and *gray* boxes, respectively. The chimeras are named according to the subdomains that were exchanged (12, 13). Chimera PIVE contains p38 sequence constituting the N terminus up to and including subdomain IV with the remaining sequence from ERK2. Chimera PIVECTP is similar to PIVE except that it also contains the C-terminal loop of p38. Chimeras EVIIP, EVIIIP, and EXIP comprise N-terminal ERK1 sequence up to and including subdomain VII, VIII, and XI, respectively, with p38 sequence constituting the remaining C terminus. Chimera EVIIIPXIE is similar to EVIIIP except that it also includes the C-terminal loop of ERK1. B and C, lysates from CHO cells transfected with each chimeric ERK/p38 construct were incubated with GST-PEA-15 fusion protein-coated beads. Bound proteins were detected by Western blotting with anti-HA. ERK1 and ERK2 bind to GST-PEA-15, whereas p38 does not. Only those chimeric ERK/p38s containing subdomains X and XI of ERK1/2, which include the MAPK insert (*underlined*) bind to GST-PEA-15.

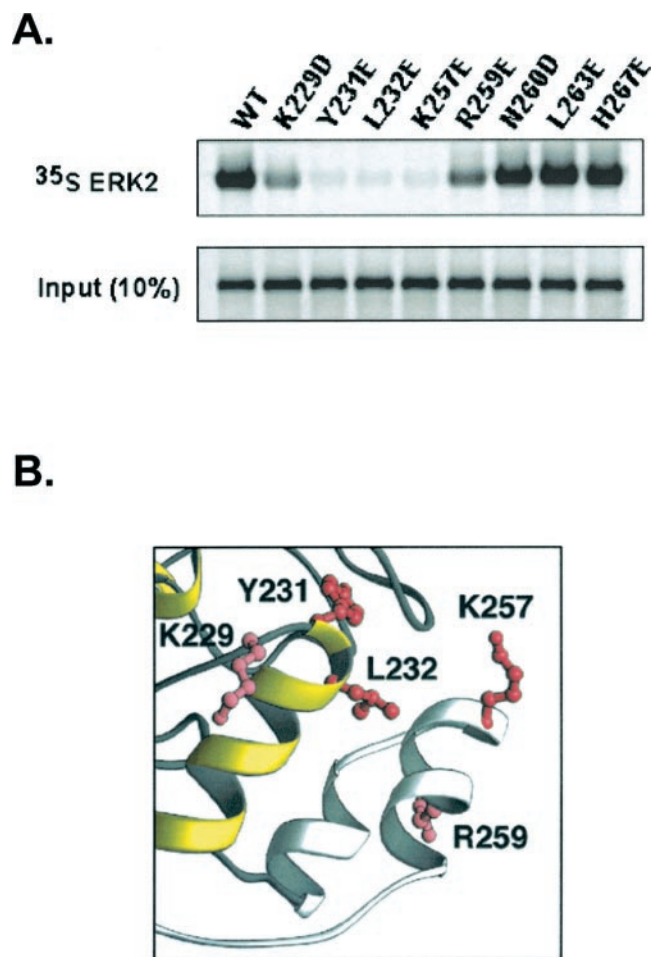
brief, for each transfection, harvested cells were divided into three tubes. The three preparations were used to assay for binding to 3Fn-(9–11) alone, binding in the presence of EDTA (10 mM), or in the presence of 9EG7 (10 ng/ $\mu$ l). All incubation and washes were carried out using 1 $\times$  Tyrode buffer (10 mM HEPES, 10 g of NaCl, 1.015 g of NaHCO<sub>3</sub>, 0.195 g of KCl, 1 mg/ml dextrose, 1 mg/ml bovine serum albumin, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4). Cells were first incubated with EDTA or 9EG7 for 10 min at room temperature, and 3Fn-(9–11) was then added to all samples and allowed to incubate at room temperature for 20 min. After washing twice with 1 $\times$  Tyrode buffer, cells were incubated with streptavidin-R-phycoerythrin (20 ng/ $\mu$ l) on ice for 20 min. Propidium iodide (1 ng/ $\mu$ l) was added to each sample for the last 5 min. Samples were washed twice before analysis. Integrin activation was quantified as an activation index (AI) as previously defined (4).  $AI = 100 * (F - F_o) / (F_m - F_o)$ , where  $F$  represents the geometric mean fluorescence (GMF) of 3Fn-(9–11) binding alone,  $F_o$  is the GMF of 3Fn-(9–11) binding in the presence of EDTA (10 mM), and  $F_m$  is the GMF of 3Fn-(9–11) binding in the presence of 9EG7. The percentage of suppression was calculated as  $100 * (AI_M - AI_T) / AI_M$ , where  $AI_M$  is the activation index of the mock-transfected cells, and  $AI_T$  is the activation index in the presence of a transfected cDNA. The percentage of reversal of suppression was calculated as  $100 * (AI - AI_R) / AI$ , in which AI is the activation index of the control cells, and  $AI_R$  is the activation index in the presence of a transfected rescuing cDNA. In addition to FACS analysis, the cell lysate of each transfection was analyzed for expression of the transfected constructs and phosphorylation of ERK1/2 by immunoblotting. The same protocol was used to assay integrin suppression by Raf-CAAX, HA-ERK1-CAAX, HA-ERK1-SAAX, and HA-ERK1(K71R)-CAAX.

**PEA-15 Knockout Fibroblasts**—PEA-15 knockout fibroblasts derived

from PEA-15 null mice (18) or wild-type fibroblasts were transiently transfected with cDNA encoding a reporter, GFP (0.3  $\mu$ g), and HA PEA-15-pcDNA3 (3  $\mu$ g). As a control, knockout and wild-type fibroblasts were transiently transfected with GFP and empty pcDNA3 vector. Transient transfections were carried out using LipofectAMINE and Plus reagents per the manufacturer's protocol (Invitrogen). 24 h post-transfection, cells were incubated in 0.5% serum Dulbecco's modified Eagle's medium overnight. After stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (100 nM) for 1 h, cells were harvested and analyzed for transfection efficiency (GFP) and integrin binding to 3Fn-(9–11) by two-color flow cytometry as described above. Additionally, cell lysates were analyzed for expression of transfected HA-PEA-15 and phosphorylation of ERK1/2 induced by TPA treatment.

**Subcellular Fractionation**—CHO cells were transfected as described above with HA-ERK1-CAAX, HA-ERK1-SAAX, or HA-ERK1(K71R)-CAAX. 24 h post-transfection, cells were harvested in 0.5 ml of lysis buffer (20 mM HEPES-KOH, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 5 mM KCl, 0.2 mM NaVO<sub>4</sub>, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). After a 10-min incubation on ice to allow for swelling, cells were disrupted using 40 strokes in a Dounce homogenizer. A fraction of the total cell lysate was saved prior to centrifugation at 2000 rpm for 10 min to pellet the nuclei. The supernatant was subjected to additional centrifugation at 13,000 rpm for 30 min to pellet the membrane fraction. The resultant supernatant is the cytosolic fraction. The membrane pellet was extracted at 4  $^{\circ}$ C with lysis buffer containing 1% Nonidet P-40. After centrifuging at 14,000 rpm for 10 min, the membrane-soluble supernatant was removed, and the insoluble pellet was resuspended in 100  $\mu$ l of 1 $\times$  SDS-PAGE buffer. Total lysate, membrane-soluble, and cytosolic fractions were analyzed for protein expression by immunoblot.





**FIG. 5. Identification of ERK2 point mutations that disrupt PEA-15 binding.** *A*, effects of point mutations of ERK2 on interaction with PEA-15, determined by the binding activity of [<sup>35</sup>S]methionine-labeled wild-type (WT) or mutant ERK2 to GST-PEA-15. The *Input* lane represents 10% of the total protein added to the GST-PEA-15-coated beads. *B*, structure of a PEA-15 binding region of ERK2. The side chains of residues important for PEA-15 binding are drawn as *ball-and-stick* models with Tyr<sup>231</sup>, Leu<sup>232</sup>, and Lys<sup>257</sup> shown in *red* and Lys<sup>229</sup> and Arg<sup>259</sup> in *pink*.

been shown to block the effects of PEA-15 on integrin activation (8). Similarly, each of the three new mutants that block ERK1/2 binding also abrogated the effects of PEA-15 on integrin activation (Fig. 2*B*). Mutations within PEA-15 that were only partially defective for ERK1/2 binding (11) either partially (K122E) or fully (E18R) reversed integrin suppression, which correlates with the relative defect in PEA-15/ERK2 interaction (11) (Fig. 2*B*). These observations were not a consequence of differential expression levels of the mutants (Fig. 2*C*). Furthermore, ERK1/2 was phosphorylated when each of the mutants was co-transfected with H-Ras(G12V) (Fig. 2*C*). Thus, the capacity of PEA-15 to bind to ERK1/2 closely correlates with its ability to reverse H-Ras suppression of integrin activation.

**Identification of ERK1/2 Mutants That Fail to Bind PEA-15**—The foregoing experiments showed that PEA-15 mutations that strongly inhibit ERK1/2 binding also abrogated the capacity of PEA-15 to block suppression of integrin activation. However, these mutations may perturb other functions of PEA-15 in addition to ERK1/2 binding. To exclude this possibility, we sought to identify mutations in ERK1/2 that disrupt PEA-15 binding and to learn whether they block the effect of PEA-15 on integrin activation. PEA-15 binds specifically to ERK1/2 and not to the related MAPKs p38 or c-Jun N-terminal kinase (10). Furthermore, there is a high degree of sequence identity be-

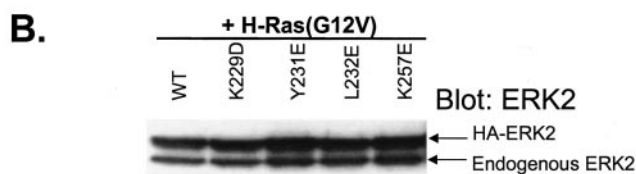
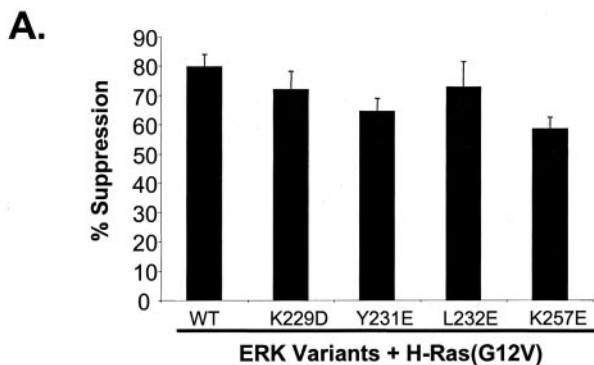
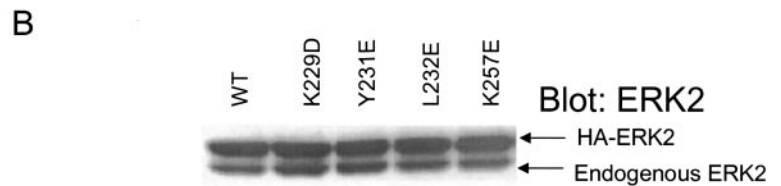
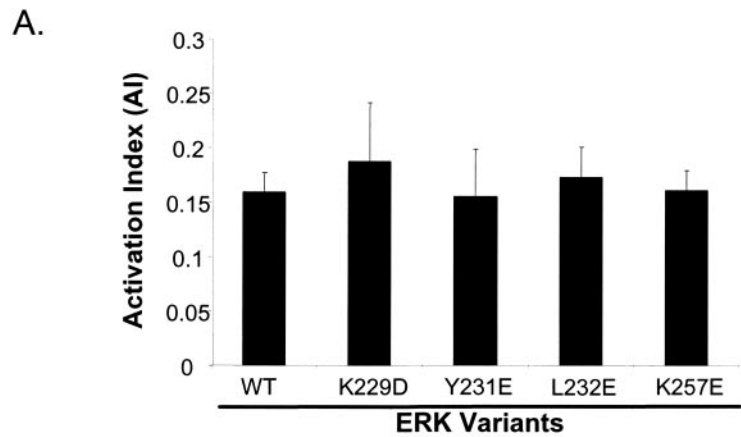
tween ERK1 and ERK2. To map the regions of ERK1/2 involved in PEA-15 binding, we employed a number of existing ERK/p38 chimeras (Fig. 3*A*) used previously to identify domains important for interacting with upstream kinases, target substrates, and phosphatases (12, 13, 21). The three-dimensional structures of ERK1/2 and p38 are similar (22), and the chimeras contain selected intact structural subdomains from each enzyme. Binding to PEA-15 was assessed using lysates from CHO cells transfected with HA-tagged ERK/p38 chimeras incubated with immobilized GST-PEA-15 (Fig. 3, *B* and *C*). A chimera possessing the first four subdomains and the C-terminal loop of p38, which form the N-terminal half of the protein, retained the ability to bind PEA-15 (chimera PIVECTP in Fig. 3*B*). In contrast, studies of C-terminal chimeras of ERK1 and p38 indicated that only a chimera containing subdomains X and XI of ERK1 (chimera EXIP in Fig. 3*C*) retained binding activity. Conversely, insertion of subdomains X and XI of p38 into ERK1 abolished binding (chimera EVIIPXIE in Fig. 3*C*). These data indicate that regions localized within the C-terminal domain of ERK, subdomains X and XI in particular, are required for PEA-15 binding.

ERK1/2 have a characteristic protein kinase structure with an N-terminal domain rich in  $\beta$ -sheet and a C-terminal domain that is mostly  $\alpha$ -helical (23, 24). Structural features unique to MAPK are an insertion of  $\sim 30$  residues between subdomains X and XI and a C-terminal extension that folds back over the N-terminal domain such that the N and C termini are in close proximity. Subdomains X and XI of ERK1/2 encompass the  $\alpha$ G helix and the  $\alpha$ 1L14- $\alpha$ 2L14 loop of the MAPK insert, which form a distinct, surface exposed region of the protein situated below the phosphorylation lip (Fig. 4*A*). Furthermore, this region displays considerable sequence and structural diversity among MAPK family members (23, 25, 26) yet is highly conserved between ERK1 and ERK2 (Fig. 4*B*). Thus, this diversity could account for the failure of p38 and c-Jun N-terminal kinase to bind to PEA-15. A series of point mutations were introduced into the  $\alpha$ G helix and MAPK insert of ERK2 to further investigate the importance of this region for the interaction with PEA-15. Mutations at five positions, Lys<sup>229</sup>, Tyr<sup>231</sup>, Leu<sup>232</sup>, Lys<sup>257</sup>, and Arg<sup>259</sup>, substantially reduced or abrogated binding to PEA-15 (Fig. 5*A*). These residues are located at the start of the  $\alpha$ G helix and in the  $\alpha$ 2L14 helix of the MAPK insert (Fig. 5*B*). Alteration of residues in the  $\alpha$ 1L14 helix (Asn<sup>251</sup>), in the  $\alpha$ 1L14- $\alpha$ 2L14 loop (Ile<sup>254</sup> and Leu<sup>256</sup>), and at the end of the  $\alpha$ 2L14 helix (Asn<sup>260</sup>, Leu<sup>263</sup>, and His<sup>267</sup>) had no effect on binding to PEA-15 (Fig. 5*A* and data not shown). This localizes part of the PEA-15 binding site to the loop preceding and start of the  $\alpha$ G helix and to the beginning of the  $\alpha$ 2L14 helix within the MAPK insert (Fig. 5*B*). An essential role for this region of ERK2 has also recently been demonstrated for the interaction with its upstream activators, MEK1/2 (27).

**ERK2 Mutants That Fail to Bind PEA-15 Block PEA-15 Reversal of Integrin Suppression**—As described above, we identified several point mutations in ERK2 that blocked binding to PEA-15. Transfection of cells with wild-type ERK2 or any of the mutants failed to suppress or enhance the binding of 3F<sub>n</sub>(9–11) to CHO cells, although all of the mutants were well expressed (Fig. 6). Furthermore, when these mutants were co-transfected with H-Ras(G12V), they failed to enhance or reverse suppression (Fig. 7). Moreover, each of the mutants was phosphorylated to a similar extent to wild-type ERK2 and did not interfere with the phosphorylation of endogenous ERK1/2 (data not shown). Thus, expression of these ERK2 mutants neither suppressed integrin activation; nor did they influence the ability of activated H-Ras to suppress it.

We then examined the effect of these ERK2 mutants on the

**FIG. 6. Expression of ERK2 mutants has no effect on integrin activation.** A, CHO cells were transiently transfected with expression vectors encoding GFP as transfection reporter and the indicated ERK2 variants. Cells were harvested and analyzed by two-color FACS for transfected cells (GFP+) and 3Fn-(9–11) binding. Shown on the ordinate is mean activation index  $\pm$  S.E. of three independent experiments. A vector-transfected cell had the same activation index as that transfected with wild type ERK2 (data not shown). B, expression of the ERK2 variants was assessed by immunoblotting with anti-ERK2.



**FIG. 7. Expression of ERK2 mutants has no effect on H-Ras(G12V)-dependent integrin suppression.** A, CHO cells were transiently transfected with expression vectors encoding GFP as transfection reporter, H-Ras(G12V), and the indicated ERK2 variants. Cells were harvested and analyzed by two-color FACS for GFP expression and 3Fn-(9–11) binding. Shown on the ordinate is mean percentage suppression of integrin activation  $\pm$  S.E. from three independent experiments. Transfection with wild-type or mutant ERK2 variants had little effect on the ability of H-Ras(G12V) to induce suppression of integrin activation. A vector-transfected cell, co-transfected with H-Ras(G12V), exhibited the same activation index as the cell transfected with wild type ERK2 and H-Ras(G12V) (data not shown). B, ERK2 expression was assessed by immunoblotting with anti-ERK2. Each of the mutants was well expressed.

capacity of PEA-15 to regulate activation of integrin  $\alpha_5\beta_1$ . Expression of the mutants that failed to bind PEA-15 (ERK2 Y231E, L232E, or K257E) completely prevented PEA-15 reversal of H-Ras suppression (Fig. 8A). In contrast, ERK2 bearing mutations that weakly inhibited PEA-15 binding (e.g. K229E) only partially reversed the effect of PEA-15. Expression of wild-type ERK2 or of a mutant with intact PEA-15 binding did

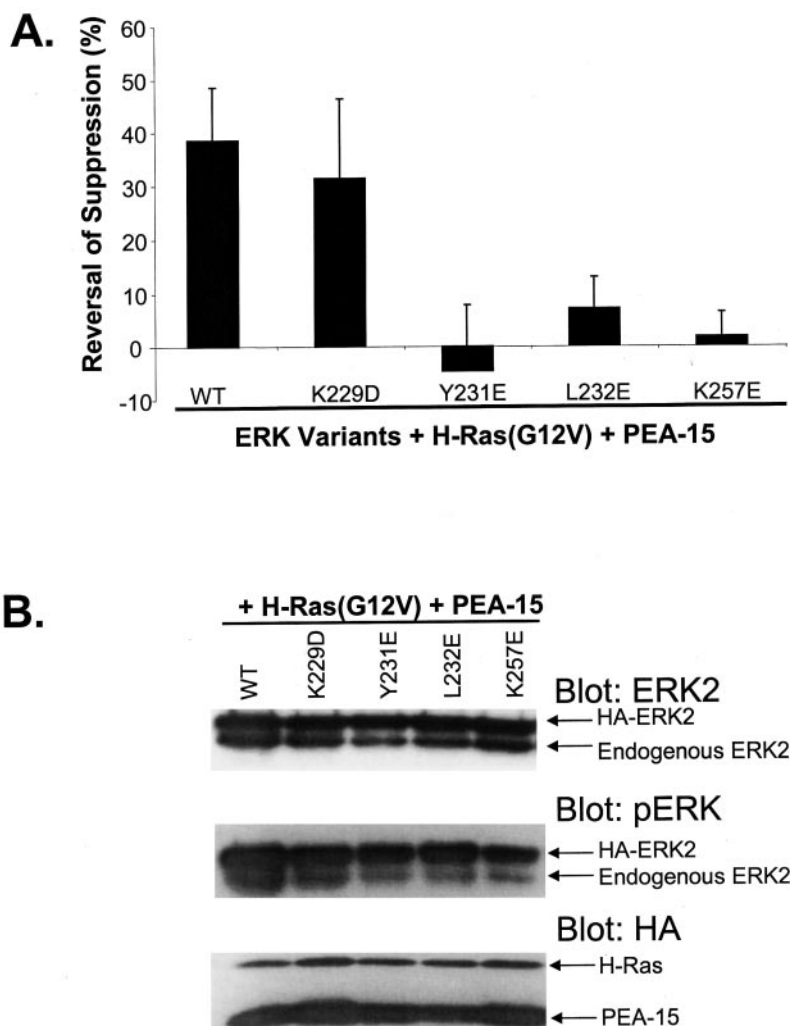
not modify the effects of PEA-15 on integrin activation. Importantly, none of the ERK2 mutants changed the expression of H-Ras(G12V), endogenous ERK1/2, or PEA-15 (Fig. 8B). In addition, these mutants were phosphorylated and did not consistently alter the phosphorylation of endogenous ERK2 in the cells co-transfected with H-Ras(G12V) and PEA-15 (Fig. 8B). Thus, expression of PEA-15 binding-defective mutants of ERK2 abolished the capacity of PEA-15 to reverse suppression of integrin activation, confirming that PEA-15 and ERK1/2 interact directly to modulate integrin function.

**Membrane-targeted ERK1/2 Suppresses Integrin Activation**—PEA-15 binding to ERK1/2 prevents the nuclear localization of the activated kinases (10), suggesting that PEA-15 might reverse integrin suppression by changing the cellular localization of ERK1/2. To test this idea, we examined the effects of enforcing a membrane localization of ERK1/2 on integrin activity. Transfection of cells with a chimera of the membrane-targeting sequence of H-Ras with ERK1 (ERK1-CAAX) resulted in profound suppression of integrin activation (Fig. 9A) and membrane localization of the ERK1 (Fig. 9C). Membrane localization was responsible for this effect, since a Ser substitution for the Cys of the CAAX box (ERK1-SAAX) was neither membrane-localized (Fig. 9C) nor suppressive (Fig. 9A). Integrin suppression required the kinase function of ERK1, since an ERK1(K71R)-CAAX construct, which lacks kinase activity, was much less suppressive (Fig. 9A) although it was membrane-localized (Fig. 9C). All three constructs were expressed at similar levels (Fig. 9B), and a corresponding series of ERK2 constructs showed identical effects on integrins to the ERK1 constructs (data not shown). Thus, membrane localization or ERK1/2 leads to suppression of integrin activation.

**PEA-15 Fails to Reverse Suppression Mediated by ERK1-CAAX**—Because enforced membrane localization of ERK1/2 led to suppression of integrin activation and PEA-15 changes the cellular localization of ERK 1/2, PEA-15 might reverse suppression by displacing ERK1/2 from the membrane. We were unable to detect enrichment of activated ERK1/2 in our bulk membrane preparations. However, we reasoned that if the capacity of PEA-15 to reverse suppression depended on its ability to bind and relocalize ERK1/2, then PEA-15 should fail

**FIG. 8. Expression of ERK2 mutants that do not bind PEA-15 abolishes PEA-15 reversal of integrin suppression.**

**A.** CHO cells were transiently transfected with expression vectors encoding GFP as transfection reporter, H-Ras(G12V), PEA-15, and the indicated ERK2 variants. Cells were harvested and analyzed by two-color FACS for GFP expression and 3Fn(9–11) binding. Shown on the *ordinate* is mean percentage reversal of suppression  $\pm$  S.E. from three independent experiments. ERK2 mutants that are lacking PEA-15 binding function (Y231E, L232E, and K257E) eliminated PEA-15 reversal of suppression. ERK2 mutants that exhibited a partial decrease in PEA-15 binding (K229D and R259E) showed decreased PEA-15-mediated reversal of integrin binding. **B.** cell lysates from the experiment described in **A** were fractionated by SDS-PAGE, and immunoblots were probed for ERK2, phospho-ERK1/2 (*pERK*), and the HA tag (*HA*). Note that phospho-ERK staining correlated with ERK expression of endogenous and transfected ERK2. The ERK2 mutations that block reversal (i.e. result in increased suppression of integrin activation) do not increase the phosphorylation of endogenous ERK2. The *lower panel* illustrates similar H-Ras(G12V) and PEA-15 expression in each cell lysate.



to reverse suppression mediated by membrane-tethered ERK1/2. Indeed, co-transfection of PEA-15 with ERK1-CAAX did not reverse suppression of integrin activation (Fig. 10A). In sharp contrast, PEA-15 completely reversed suppression mediated by membrane-tethered Raf-CAAX (Fig. 10A) although PEA-15 was equally well expressed in both transfections (Fig. 10B). The ERK1-CAAX bound PEA-15 to a similar extent as wild type ERK1 (Fig. 10C). Thus, when ERK is membrane-tethered, PEA-15 fails to reverse suppression of integrin activation.

#### DISCUSSION

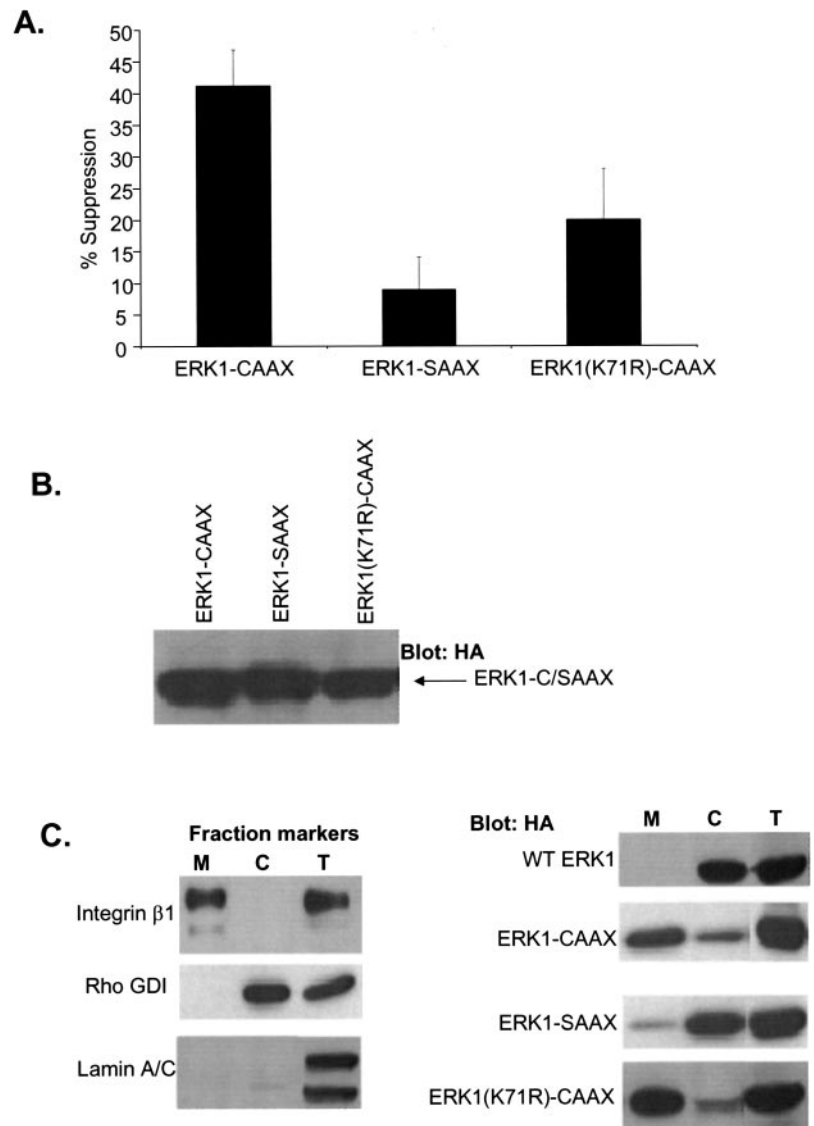
Rapid modulation of ligand-binding affinity (activation) is a central property of the integrin family of adhesion receptors and has important functional consequences in development, homeostasis, the immune response, and maintenance of tissue integrity (1). The Ras family of small GTP-binding proteins and their downstream effectors are key players in regulating integrin activation. Activated H-Ras can suppress integrin activation via its downstream effector kinase, Raf-1, to control cell morphology, migration, and assembly of the extracellular matrix (4). This suppression could be mediated by the ERK1/2 MAPK pathway; however, bulk ERK1/2 activation does not correlate with suppression (6). The small DED-containing protein, PEA-15, reverses H-Ras suppression of integrin activation and binds to ERK1/2, altering the subcellular localization of ERK1/2 and consequently the outcome of the ERK1/2 signaling cascade (8, 10). Here we demonstrate that the ability of PEA-15 to reverse suppression lies in its capacity to bind ERK1/2,

indicating that ERK1/2 function is required for suppression. Our results further suggest that reversal of suppression by PEA-15 is due to inhibition of an ERK1/2 function, possibly by preventing access to membrane-associated substrates, rather than the acquisition of integrin-activating functions by the PEA-15-ERK1/2 complex.

The recently determined three-dimensional structure and mutational analysis of PEA-15 indicated the presence of two distinct binding sites for ERK, an acidic patch within the DED and a positively charged region within the C-terminal tail (11). Point mutations of individual residues in either of these regions of PEA-15 are sufficient to abrogate ERK1/2 binding and resulted in a loss of the capacity of PEA-15 to reverse H-Ras/Raf-1-dependent integrin suppression. Mutations in the C-terminal tail that block ERK1/2 binding (I121R, L123R, and K129E) also block PEA-15 reversal of integrin suppression, as previously observed for the D74A mutant in the DED (8). Mutations within PEA-15 that exhibited reduced ERK1/2 binding retained partial ability to reverse H-Ras suppression (K122E) or fully reversed suppression (E18R). These results correlate with the relative ability of these mutants to inhibit the ERK-stimulated transcription of Elk-1 (10, 11) and reinforce the functional importance of both the DED and C-terminal tail of PEA-15.

An analysis of ERK/p38 chimeras and ERK1/2 mutants pinpointed residues within the  $\alpha$ G helix and MAPK insert as critical for both PEA-15 binding and reversal of integrin suppression. Mutations at five positions, Lys<sup>229</sup>, Tyr<sup>231</sup>, Leu<sup>232</sup>,

**FIG. 9. Expression of membrane-targeted ERK1 induces integrin suppression.** *A*, CHO cells were transfected with plasmids encoding membrane-targeted ERK1-CAAX, kinase-dead ERK1 (K71R)-CAAX, and nontargeted ERK1-SAAX. After 48 h, the cells were suspended, and 3Fn(9–11) binding and percentage suppression were assessed as described under “Materials and Methods.” Membrane targeting of ERK1 induces suppression, whereas nontargeted SAAX construct did not. This suppression is dependent on the kinase activity of ERK1, since the kinase-dead K71R ERK1-CAAX exhibits diminished ability to suppress. *B*, lysates from each transfection were fractionated by SDS-PAGE, and expression of each ERK construct was analyzed by immunoblotting with anti-HA. *C*, cells from each transfection were disrupted in a Dounce homogenizer, and the resulting lysate was subjected to differential centrifugation as described under “Materials and Methods.” Total lysate, membrane, and cytosolic fractions were analyzed by immunoblotting. The identity and purity of the subcellular fractions were assessed by blotting with a membrane marker (integrin  $\beta_1$ ), cytosolic marker (RhoGDI), and nuclear marker (lamin A/C). In addition, each fraction was blotted with anti-HA to ascertain the subcellular location of each transfected construct. ERK1-CAAX and ERK1 (K71R)-CAAX were enriched in the membrane, whereas ERK1-SAAX was primarily in the cytosol. Representative images from one experiment; lanes were rearranged for clarity.

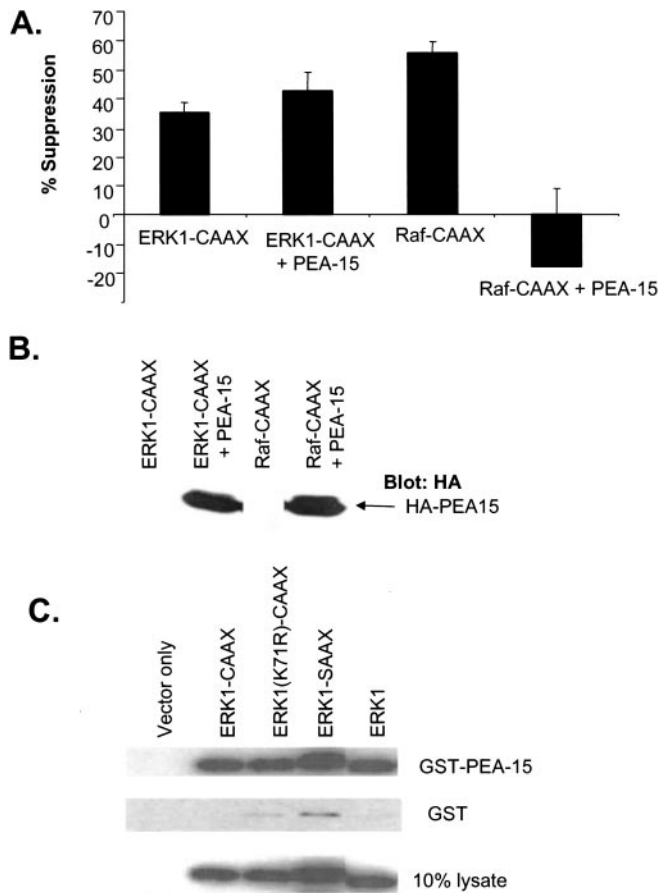


Lys<sup>257</sup>, and Arg<sup>259</sup>, substantially reduced or abrogated binding to PEA-15. Cellular expression of each of these ERK2 mutants blocked the ability of PEA-15 to reverse suppression of integrin activation. In the crystal structure of unphosphorylated ERK2, the activation loop extends from the active site and folds down upon the C-terminal domain, making contacts with residues from both the N terminus of helix  $\alpha$ G and the MAPK insert (24). Although the mutations described here are located on the same surface of ERK2 as the active site, and residues Tyr<sup>231</sup> and Leu<sup>232</sup> are in close proximity to the activation loop, they have no adverse effect on ERK2 activation. Each of the mutants was expressed at level comparable with wild-type ERK2 and was phosphorylated to a similar extent, indicating that the mutants were correctly folded and able to serve as a substrate for MEK1/2. Importantly, these mutants blocked the effect of PEA-15 in the presence of endogenous wild-type ERK1/2. This implies that the mechanism of action of PEA-15 on integrins involves loss of ERK1/2 function rather than a novel function acquired by the PEA-15-ERK1/2 complex.

Several regions of MAPKs have been implicated in interactions with their substrates, activators, and regulators. A common docking groove has been identified in the C-terminal domain that functions as a general binding site (28–31) for many proteins that contain a conserved docking site sequence (32). This docking groove contains an acidic patch termed the CD

domain (29) and adjacent hydrophobic patches (28) that accommodate the basic and hydrophobic motifs of the docking site sequences. However, this docking groove does not appear to play a role in the interaction with PEA-15, since ERK1/2 mutants that have been shown to abrogate binding by substrates and activators of ERK1/2 (T157E, T158D, D316N, and D319N) (29, 30) had no effect on PEA-15 binding (data not shown). These findings are consistent with the absence of a conserved docking site sequence within PEA-15. In contrast, recognition of the  $\alpha$ G helix and MAPK insert of ERK1/2 by PEA-15 and MEK1/2 (27) highlights this region as a potential second common docking site. In addition to MAPKs, insertions in the cyclin-dependent kinases and glycogen synthase kinase-3. X-ray crystal structures of cyclin-dependent kinase 2 and glycogen synthase kinase-3 in complex with their regulatory factors have also recently demonstrated the functional importance of this region for these kinase families (33–35). Thus, the insertion in MAPKs and other structurally related serine/threonine kinases may act as a common docking site for a variety of regulatory proteins.

The dominant feature of the ERK1/2 binding surface of PEA-15 is the presence of two loci of opposite charge. The binding epitope within the PEA-15 DED is predominantly negatively charged, whereas the binding surface in the C-



**FIG. 10. PEA-15 fails to reverse integrin suppression induced by ERK1-CAAX.** *A*, CHO cells were transfected with plasmids encoding membrane-targeted ERK1-CAAX or Raf-CAAX alone or in conjunction with wild-type PEA-15. After 48 h, the cells were suspended, and 3Fn-(9–11) binding and percentage suppression were estimated as described under “Materials and Methods.” Wild-type PEA-15 reversed Raf-CAAX-induced suppression but not ERK1-CAAX-induced suppression. *B*, immunoblotting of total cell lysate with anti-HA shows comparable expression of HA-PEA-15 in cells co-transfected with either ERK1-CAAX or Raf-CAAX. *C*, GST-PEA-15 pull-down assay was carried out with total cell lysate from each transfection. Immunoblotting of the pull-down with anti-HA indicates comparable binding of ERK1-CAAX, ERK1-SAAX, ERK1(K71R)-CAAX, and wild type ERK1 to GST-PEA-15.

terminal tail is composed of hydrophobic and positively charged residues (11). Thus, it is tempting to speculate that the DED of PEA-15 may contact the positively charged ERK1/2 binding surface provided by helix  $\alpha$ G and the MAPK insert. However, this region is not sufficient for PEA-15 binding, because a chimera containing subdomains X and XI of ERK1/2 in p38 is unable to bind PEA-15 (data not shown). This suggests that there are additional binding determinants required for PEA-15 binding. The tail of PEA-15 is also essential for ERK1/2 interaction and probably interacts with ERK1/2 at a second site, which may be discontinuous with the binding surface of the PEA-15 DED. The tail most likely contacts a negatively charged and/or hydrophobic surface given the deleterious effect of the PEA-15 mutations at Lys<sup>122</sup>, Lys<sup>128</sup>, and Lys<sup>129</sup> on ERK1/2 binding.

In summary, we have shown that the capacity of PEA-15 to regulate integrin activation depends upon its binding to ERK1/2. In addition, we identified ERK1/2 mutants that are deficient for PEA-15 binding and prevent PEA-15 reversal of integrin suppression. These observations suggest that reversal of suppression is due to inhibition of an ERK1/2 function rather than the acquisition of integrin-activating functions

by the PEA-15-ERK1/2 complex. Our data indicate that ERK1/2 function is critical for H-Ras/Raf-1 suppression of integrin activation. Whereas PEA-15 does not block ERK1/2 activation or its ability to phosphorylate several substrates (10), it does change the subcellular localization of ERK1/2 (10). It is plausible to consider a mechanism in which ERK1/2 is unavailable to phosphorylate critical substrates required for integrin suppression due to the interaction between ERK1/2 and PEA-15. PEA-15 may delocalize ERK1/2, blocking access of ERK1/2 to a location necessary for phosphorylation of substrates required for integrin suppression. For example, PEA-15 binding to ERK1/2 may remove ERK1/2 from a membrane-associated site such as focal adhesions (36) or an association with  $\beta$ -arrestin (37). The results described here suggest that ERK1/2 substrates at such sites will be critical for the suppression of integrin activation.

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