

The Death Effector Domain of PEA-15 Is Involved in Its Regulation of Integrin Activation*

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Increased integrin ligand binding affinity (activation) is triggered by intracellular signaling events. A Ras-initiated mitogen-activated protein kinase pathway suppresses integrin activation in fibroblasts. We used expression cloning to isolate cDNAs that prevent Ras suppression of integrin activation. Here, we report that PEA-15, a small death effector domain (DED)-containing protein, blocks Ras suppression. PEA-15 does not block the capacity of Ras to activate the ERK mitogen-activated protein kinase pathway. Instead, it inhibits suppression via a pathway blocked by a dominant-negative form of the distinct small GTPase, R-Ras. Heretofore, all known DEDs functioned in the regulation of apoptosis. In contrast, the DED of PEA-15 is essential for its capacity to reverse suppression of integrin activation. Thus, certain DED-containing proteins can regulate integrin activation as opposed to apoptotic protease cascades.

Integrins are transmembrane heterodimers that mediate cell-cell and cell-extracellular matrix adhesion (1). The affinity of some integrins for ligand is regulated by “inside-out” cell signaling cascades (2, 3). Regulation of integrin affinity for ligand (activation) is important in cell migration (4), fibronectin matrix assembly (5), platelet aggregation in hemostasis and thrombosis (6), and morphogenesis (7, 8). This cellular regulation of integrin activation is energy-dependent, cell type-specific, and is mediated through integrin cytoplasmic domains (9).

In fibroblastic cells, activation of the small GTP-binding protein Ha-Ras or its effector kinase, c-Raf-1, initiates a signaling pathway that blocks integrin activation (10). This suppressor activity correlates with the activation of the ERK MAP¹ kinase pathway and does not require mRNA transcription or

protein synthesis. The downstream effectors or regulators of this integrin suppression pathway remain to be identified.

Integrin activation is readily measured by the binding of activation-dependent ligands, which can be used as a selective marker in expression cloning schemes. One such scheme (10, 11) uses a Chinese hamster ovary cell line ($\alpha\beta\text{py}$ cells) stably expressing a chimeric integrin ($\alpha_{\text{IIB}}\alpha_{6A}\beta_3\beta_1$) that contains the extracellular and transmembrane domains of $\alpha_{\text{IIB}}\beta_3$ fused to the cytoplasmic domains of $\alpha_{6A}\beta_1$. This chimeric integrin has the ligand binding properties of $\alpha_{\text{IIB}}\beta_3$, and its activation state is regulated through the $\alpha_{6A}\beta_1$ cytoplasmic domains. Consequently, flow cytometry (FACS) can be used to assess the activation state of the chimeric integrin by measuring the binding of fibrinogen or the ligand-mimetic monoclonal antibody, PAC1. To elucidate Ras-induced integrin suppression, we modified this scheme to identify proteins that prevent Ras suppression. Specifically, we used Ras to suppress integrin activation in $\alpha\beta\text{py}$ cells and isolated co-transfected cDNAs that blocked this suppression. Here we report that PEA-15 (phosphoprotein enriched in astrocytes), a small death effector domain (DED)-containing protein, blocks Ras suppression downstream of MAP kinase via a pathway blocked by a dominant interfering mutant of a distinct small GTPase, R-Ras.

EXPERIMENTAL PROCEDURES

Cell Culture— $\alpha\beta\text{py}$ cells are a CHO cell line that expresses the polyoma large T antigen and a constitutively active recombinant chimeric integrin ($\alpha_{\text{IIB}}\alpha_{6A}\beta_3\beta_1$) (12). $\alpha\beta\text{py}$ cells were maintained in Dulbecco's modified Eagle's medium (BioWhitaker, Walkersville, MD) supplemented with 10% fetal calf serum (BioWhitaker), 1% non-essential amino acids (Life Technologies, Inc.), 1% glutamine (Sigma), 1% penicillin and streptomycin (Sigma), and 700 $\mu\text{g}/\text{ml}$ G418 (Life Technologies, Inc.).

Antibodies, Reagents, and cDNA Constructs—The activation-specific anti- $\alpha_{\text{IIB}}\beta_3$ monoclonal antibody PAC1 (13) was generously provided by Dr. S. Shattil (Scripps Research Institute). The anti- $\alpha_{\text{IIB}}\beta_3$ monoclonal antibody anti-LIBS6 has been described previously (14). The anti-Tac antibody, 7G7B6, was obtained from the American Tissue Culture Collection (Rockville, MD). 7G7B6 was biotinylated with biotin-*N*-hydroxysuccinimide (Sigma) according to the manufacturer's instructions. The mouse monoclonal anti-HA antibody (12CA5) was produced in our laboratory (15). The $\alpha_{\text{IIB}}\beta_3$ -specific peptide inhibitor Ro43-5054 (16) was a generous gift from B. Steiner (Hoffmann-La Roche, Basel). The CHO-K1 oligo(dT)-primed library is directionally cloned into pcDNA1 and was obtained from Invitrogen (San Diego, CA). The library is reported to contain 1.8×10^7 primary recombinants. pDCR-Ha-RasG12V was a gift from Dr. M. H. Wigler (Cold Spring Harbor Laboratory). Tac- $\alpha 5$ (17) was generously provided by Drs. S. LaFlamme and K. Yamada (National Institutes of Health). HA-Erk2 was described previously (18). Dr. G. Bokoch kindly provided pCMV5-Ha-RasT17N. pcDNA3-R-RasG38V and pcDNA3-R-RasT43N (19) were gifts from Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA) with permission from Dr. A. Hall (University of London). pCHA-MEK2 222/226D was provided by Dr. M. Weber (University of Virginia).

Expression Cloning—Expression cloning was done using $\alpha\beta\text{py}$ cells. $\alpha\beta\text{py}$ cells were divided into 18 subconfluent 100-mm plates and co-transfected with Tac- $\alpha 5$ (2 $\mu\text{g}/\text{plate}$), Ha-RasG12V (3 $\mu\text{g}/\text{plate}$) and a CHO-K1 library (4 $\mu\text{g}/\text{plate}$) using LipofectAmineTM (Life Technologies, Inc.). 48 h after transfection, cells were collected and stained for FACS analysis with antibodies PAC1 and 7G7B6 as described previously (9). Cells that bound high levels of both PAC1 and 7G7B6 were collected by fluorescence-activated cell sorting (FACSTAR, Becton Dickinson). Plasmid DNA was extracted from collected cells by Hirt Supernatant (20).

untranslated region; AI, activation index; DD, death domain; FACS, fluorescence-activated cell sorter; CHO, Chinese hamster ovary; HA, hemagglutinin.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF080001.

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¹ The abbreviations used are: MAP, mitogen-activated protein; DED, death effector domain; PCR, polymerase chain reaction; 3'-UTR, 3'-

This plasmid DNA was used to transform *Escherichia coli* MC1061/P3 cells. Bacterial colonies were grown, stored, and pooled into groups of 16 for plasmid purification (Qiagen, Chatsworth, CA) and analysis. To isolate single cDNAs that reverse Ras suppression of PAC1 binding, groups of cDNAs were transfected into $\alpha\beta\text{py}$ cells along with Ha-RasG12V and Tac- $\alpha 5$. Transfectants were screened by two-color flow cytometry (FACScalibur, Becton Dickinson) as described above. A group containing cDNAs that reverse Ras suppression was identified and divided into groups of four for further screening. Positive groups were finally screened as single cDNAs.

Construction of PEA-15 Mutants—An HA-tagged PEA-15 lacking the 3'-UTR was created by PCR of CHO PEA-15-pcDNA1 clone using pfu polymerase (New England Biolabs). The amplified product was subcloned into the *Bam*HI/*Eco*RI sites of pcDNA3. HA-tagged DED and C-terminal domains of PEA-15 were similarly constructed by PCR. PEA-15(D74A) was constructed using the Quickchange kit (Stratagene). DD-PEA-15 was constructed by splice-overlap PCR with pcDNA3-FADD and CHO PEA-15-pcDNA1 as templates. The insert was subcloned into the *Bam*HI/*Eco*RI sites of pcDNA3. Mutations were verified by sequencing.

Flow Cytometry—Analytical two-color flow cytometry was done as described (9). In transiently transfected $\alpha\beta\text{py}$ cells, PAC1 binding was determined for transfected cells (cells positive for the co-transfected Tac- $\alpha 5$ as measured by 7G7B6 binding). Integrin activation was quantitated as an activation index (AI) defined as $100 \times (F - F_r) / (F_{\text{LIBS6}} - F_r)$ in which F is the median fluorescence intensity of PAC1 binding; F_r is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor (Ro43-5054, 1 μM); and F_{LIBS6} is the median fluorescence intensity in the presence of anti-LIBS6 (2 μM). From this we calculated the percent inhibition as $100 \times (AI - AI_s) / AI$, in which AI is the activation index of control cells and AI_s is the activation index in the presence of a transfected suppressing cDNA.

Measurement of ERK Activity—For ERK kinase assays, $\alpha\beta\text{py}$ cells were transfected with HA-ERK2 (2 μg) along with test cDNA such as pcDNA3-PEA15 (3 μg) using LipofectAmineTM (20 μl /plate, Life Technologies, Inc.). In instances where more than one test plasmid is used, the amount of DNA transfected was standardized by addition of pcDNA1 control vector. Cells were lysed 48 h after transfection in ice-cold M2 buffer (0.5% Nonidet P-40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM sodium phosphate, 20 mM sodium pyrophosphate, 3 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 10 $\mu\text{g}/\text{ml}$ each of leupeptin and aprotinin). ERK2 activity was measured by an immune-complex assay (from 100 μg of cell lysate protein) using myelin basic protein as a substrate (18). ERK2 activity was determined by autoradiography.

RESULTS AND DISCUSSION

Expression Cloning of PEA-15 by Prevention of Ha-Ras Suppression of Integrin Activation—To elucidate the mechanism of Ras-mediated suppression of integrin activation, we used an expression cloning strategy to identify proteins that prevent Ras suppression (Fig. 1A). $\alpha\beta\text{py}$ cells were co-transfected with activated Ha-Ras and a CHO cell cDNA library. We used flow cytometry to isolate cells that still bound the activation-specific anti- $\alpha_{\text{IIb}}\beta_3$ antibody, PAC-1, despite transfection with activated Ha-Ras (Fig. 1B, left panel, box). Seventy-nine cDNAs were recovered from isolated cells. One of these cDNAs, R36, restored PAC1 binding in cells transfected with activated Ras (Fig. 1B, middle panel). Indeed, R36 transfection resulted in FACS profiles similar to those observed in the absence of Ras suppression (Fig. 1B, right panel). Ras expression levels in the PEA-15 transfected cells remained comparable to cells transfected with Ras alone (Fig. 1C). Thus, the reversal of Ras suppression was not due to a loss of Ras expression.

R36 contained 1,700 base pairs encoding an open reading frame of 130 amino acids (GenBankTM accession number AF080001). A BLAST data base search indicated that the 130-amino acid sequence is 99% identical to mouse phosphoprotein enriched in astrocytes (PEA-15, Fig. 2A) (21). The first 80 amino acids of PEA-15 correspond to the canonical DED sequence found in proteins that regulate apoptotic signaling pathways (22–24) (Fig. 2, A and B). In fact, the PEA-15 DED is

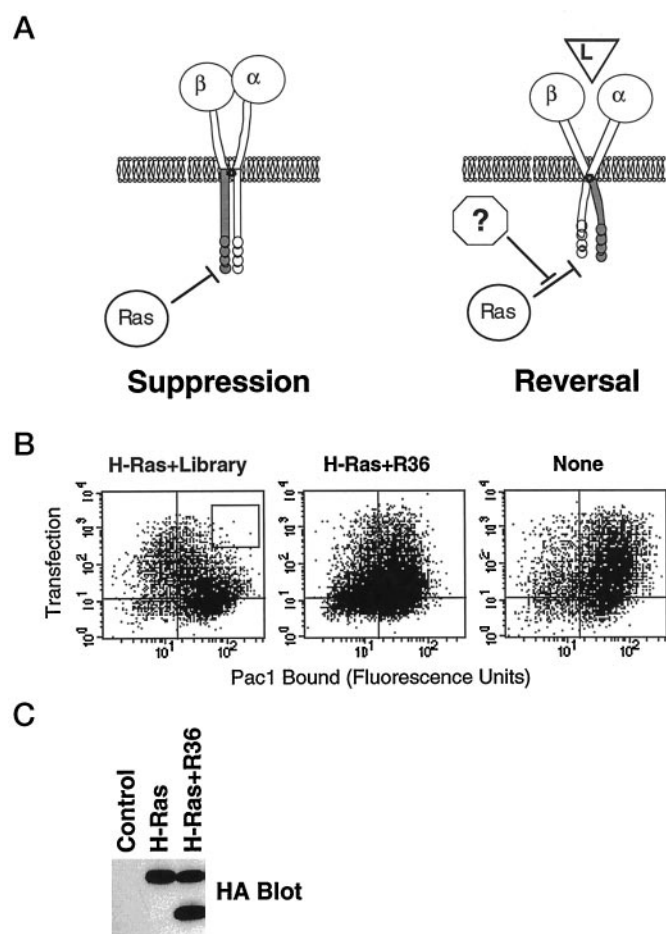


FIG. 1. PEA-15 reverses Ras suppression of integrin activation. A, depicted are the integrin α and β subunits. Ras initiates a MAP kinase pathway that inhibits integrin ligand binding (left panel). We used an expression cloning strategy to isolate cDNAs that encode proteins (?) that prevent Ras suppression of integrin ligand binding (right panel). Ligand (L) is shown as a triangle. These proteins might work by blocking a Ras-initiated pathway or activating a competing pathway. In our cloning strategy the ligand was the activation-dependent antibody, PAC1. B, $\alpha\beta\text{py}$ cells were co-transfected with cDNA encoding Tac- $\alpha 5$ (2 μg) alone (None) or in combination with Ha-RasG12V (3 μg) and pcDNA1-CHO library (4 μg) or Ha-RasG12V (3 μg) and clone R36 (4 μg) as indicated. After 48 h, the cells were stained for Tac expression (ordinate) and PAC1 binding (abscissa). C, immunoblot with 12CA5 (anti-HA) antibody. Cells were co-transfected as in B.

more similar to that of FADD than that of the viral DED-containing protein MC159 (Fig. 2B). The remaining 50 amino acids contain a serine (Ser-104) that is phosphorylated by protein kinase C (25) and a serine (Ser-116) phosphorylated by calcium calmodulin kinase II (26). No function has yet been ascribed to PEA-15.

The sequence of R36 also contained a predicted 1190-base pair 3'-UTR containing a polyadenylation signal and poly(A) tract. The final 1050 bases of this region are 70% identical to MAT1, a transforming cDNA isolated from a lithium-induced mouse mammary tumor (27). To determine whether the 3'-UTR is necessary for the reversal of Ha-Ras integrin suppression, we tested a construct without this sequence and found that it functioned like the full-length cDNA (data not shown).

PEA-15 Reversal of Ras Suppression Requires the DED—More than half of the PEA-15 protein consists of a conserved DED (Fig. 2, A and B). This domain, to date, is associated with proteins involved in apoptosis (28–31). To determine if the DED of PEA-15 is necessary or sufficient for PEA-15 reversal of Ras suppression, we overexpressed mutant forms of PEA-15 in

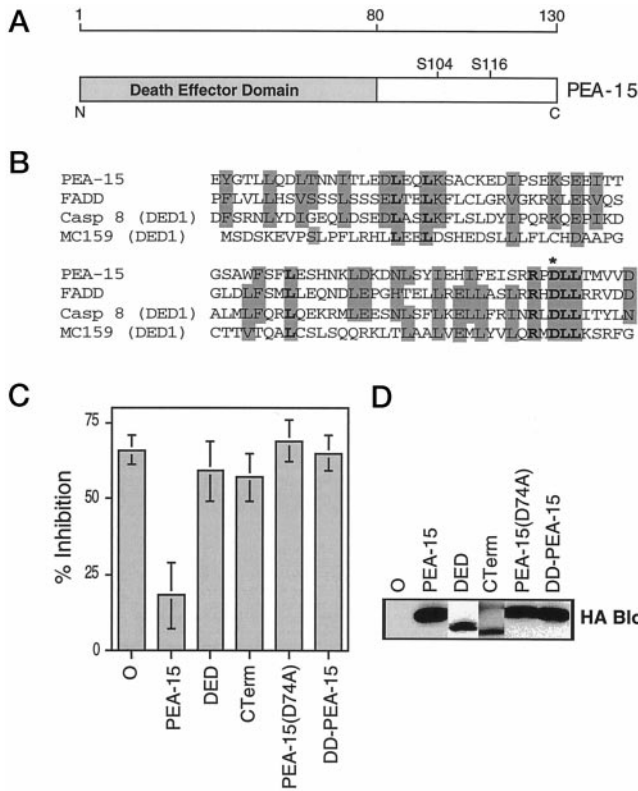


FIG. 2. PEA-15 reversal of Ras suppression requires the DED. A, the structure of PEA-15 is depicted. CHO PEA-15 contains 130 amino acids. Amino acids 1 to 80 constitute the death effector domain. Serine 104 is a protein kinase C site phosphorylated in astrocytes (25). Serine 116 is a calcium calmodulin kinase II site phosphorylated in astrocytes (43). B, amino acid sequence alignment of the DED of PEA-15 with DEDs of FADD, caspase 8, and MC159. Similar residues found at the same position in three or more of the DEDs are shaded. Residues conserved in all four DEDs are in bold type. The aspartate found in the highly conserved RxDLL motif is marked with an asterisk. Note that the DED of PEA-15 is 46% similar to that of FADD. C, $\alpha\beta$ cells were co-transfected with expression vectors encoding Ha-RasG12V (3 μ g) in combination with PEA-15 (4 μ g), DED (8 μ g), C-Term (8 μ g), PEA-15(D74A) (4 μ g), DD-PEA-15 (4 μ g), or vector lacking insert (8 μ g). After 48 h, integrin activation was assayed by PAC1 binding. Shown is the mean percentage inhibition \pm S.D. of at least three independent experiments. D, immunoblot using anti-HA antibody 12CA5. Expression levels of PEA-15 and its mutants from the experiments in C are similar.

$\alpha\beta$ cells in this assay. Overexpression of only the DED of PEA-15 did not reverse Ras suppression (Fig. 2C). It is therefore not sufficient for this function. Mutants of PEA-15 lacking the DED (C-Term) were also unable to reverse Ras suppression (Fig. 2C). Furthermore, a conserved aspartate is present in a RxDLL sequence in all DEDs (32) (asterisk in Fig. 2B). Mutation of this aspartate (D74A) prevented PEA-15 reversal of Ras suppression (Fig. 2C). The structure of the DED is similar to that of the death domain (DD) of FADD (33). Substitution of the DD of FADD for the DED of PEA-15 yielded a chimeric molecule incapable of reversing Ras suppression (Fig. 2C). In all cases, mutant PEA-15 constructs were expressed (Fig. 2D). The DED of PEA-15 is therefore necessary, but not sufficient, for reversal of Ras suppression. Additionally, substitution of the DED of PEA-15 with the DED of FADD resulted in a chimeric protein that induced apoptosis (data not shown). This indicates that the DED of PEA-15 is functionally distinct from that of FADD and contains primary sequence information required for PEA-15 function. Thus, our studies define a new function for DEDs.

PEA-15 Does Not Block Ras Activation of ERK—Ras sup-

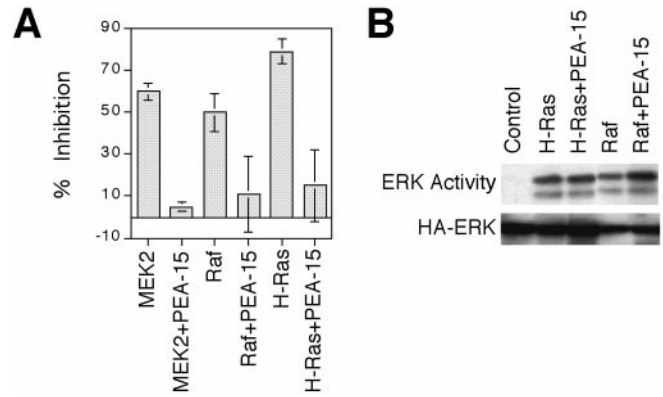


FIG. 3. PEA-15 rescues Ras, Raf, and MEK2 suppression but does not interfere with ERK2 activation. A, $\alpha\beta$ cells were co-transfected with expression vectors encoding 3 μ g of MEK2(222/226D), RafCAAX, or Ha-RasG12V in combination with PEA-15 (4 μ g) or vector lacking an insert (4 μ g). After 48 h, integrin activity was assayed by PAC1 binding. Shown is the mean percentage inhibition \pm S.D. of at least three independent experiments. B, $\alpha\beta$ cells were co-transfected with HA-ERK2 (2 μ g), and expression vectors encoding 3 μ g of Ha-RasG12V or RafCAAX in combination with PEA-15 (3 μ g) or vector lacking an insert (3 μ g). As a control, cells transfected with only vector lacking an insert were also assayed. The transfected ERK2 was immunoprecipitated and incubated with myelin basic protein to determine activity. Top, relative activity of ERK2. Bottom, immunoblots using anti-HA antibody, 12CA5. The amount of ERK2 expressed in the experiments is comparable.

presses integrin activation by activating a MAP kinase pathway (10). When activated Ras (RasG12V) and PEA-15 were co-expressed, the integrins were not suppressed although the activated variant of Ras was present. Consequently, we assessed the effect of PEA-15 on other activated components of the MAP kinase pathway. Exchange factor mediated GTP loading, and activation of Ras initiates the MAP kinase pathway (34, 35). Active Ras recruits and thus activates Raf kinase. We found that PEA-15 could reverse suppression initiated by an activated Raf (RafCAAX, Fig. 3A), indicating that its site of action is distal to Raf activation. Raf phosphorylates and activates MEK, which in turn activates ERK. PEA-15 also rescued suppression mediated by activated MEK (MEK2 222/226D, Fig. 3A) and did not block ERK activation (Fig. 3B). This suggests that PEA-15 function is distal to ERK.

PEA-15 Reverses Suppression by a Mechanism Sensitive to Dominant-Negative R-Ras—PEA-15 might reverse Ras suppression by activating effectors that oppose Ras signaling to integrins. R-Ras is a Ras-related GTP-binding protein that activates integrins (19) and reverses Ha-Ras suppression of integrin activation.² Like PEA-15, R-Ras does not affect Ha-Ras activation of Erk. Consequently, we asked if the capacity of PEA-15 to reverse suppression depends on R-Ras. A dominant-negative R-Ras (R-RasT43N) blocked the ability of PEA-15 to reverse suppression by activated Ha-Ras (Fig. 4A). Cells expressing the dominant-negative R-RasT43N had moderately reduced expression of PEA-15 and Ha-Ras (Fig. 4B); however Ha-Ras expression levels remained sufficient to suppress integrin activity (Fig. 4A). Thus, the reduced expression levels of Ha-Ras do not account for the effects of dominant-negative R-Ras. Furthermore, activated R-Ras still reversed suppression when co-expressed with the dominant-negative R-RasT43N (Fig. 4A). Therefore, the effect of R-RasT43N was upstream of R-Ras. These results indicate that PEA-15 inhibition of Ha-Ras suppression is impaired by expression of a dominant-negative R-Ras construct.

² T. Sethi, M. Ginsberg, J. Downward, and P. Hughes, submitted for publication.

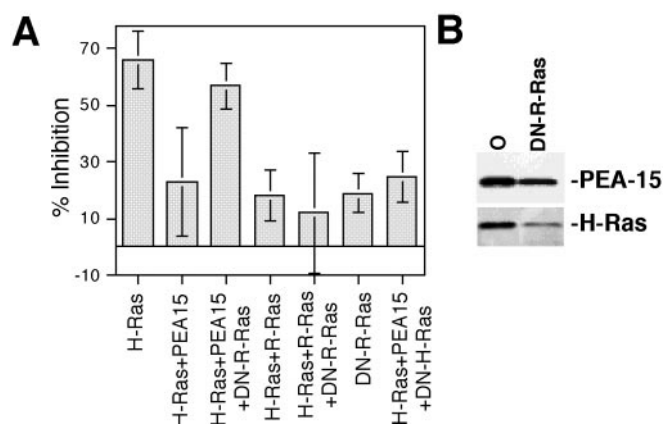


FIG. 4. PEA-15 rescues Ras suppression by an R-Ras-dependent mechanism. *A*, $\alpha\beta\gamma$ cells were co-transfected with Tac- $\alpha 5$ (2 μg), and the indicated combinations of Ha-RasG12V (3 μg), PEA-15 (3 μg), R-RasT43N (*DN-R-Ras*, 3 μg), R-RasVM8 (*R-Ras*, 2 μg), and Ha-RasT17N (*DN-H-Ras*, 2 μg). Total amounts of transfected plasmid were adjusted to 11 μg by addition of appropriate amounts of control vector lacking an insert. After 48 h, integrin activation was determined by PAC1 binding. Depicted is the mean percentage inhibition \pm S.D. of four independent experiments. *B*, immunoblot using anti-HA antibody 12CA5. Expression levels of PEA-15 from the experiments in *A* containing Ha-Ras + PEA-15 (0, *top*) and Ha-Ras + PEA-15 + DN-R-Ras (*DN-R-Ras*, *top*) are similar, as are the levels of Ha-Ras (*bottom*).

Because R-Ras and Ha-Ras are similar (36), they may share some of the same guanine-nucleotide exchange factors (37), but R-Ras is also regulated by effectors and activators distinct from those that control Ha-Ras (38). The dominant-negative form of R-Ras we used probably sequesters GEFs (39). Consistent with this site of action, DN-R-Ras failed to affect rescue mediated by an activated variant of R-Ras (G38V). Furthermore, a DN-Ha-Ras construct did not affect PEA-15 reversal of suppression. Hence, the DN-R-Ras acts by blocking events specific for R-Ras and not ones common to both Ha-Ras and R-Ras. Similarly, dominant-negative constructs of the small GTP-binding proteins Cdc42, Rac, and Rho did not prevent PEA-15 reversal of Ras suppression (data not shown), further suggesting that the effect is R-Ras-specific. R-Ras activates integrins (19) and reverses Ha-Ras suppression of integrin activation (Fig. 4A). Therefore, PEA-15 may reverse Ras suppression via an R-Ras-dependent mechanism. The proteins involved in R-Ras regulation remain unclear (40). However, our data suggest that PEA-15 could be a novel upstream regulator of R-Ras activity. Alternatively, the effects of the dominant-negative R-Ras construct may be due to interference with the closely related protein TC21/R-Ras2 (41, 42). It will be interesting to analyze the potential interplay between PEA-15 and R-Ras.

In summary, we used an expression cloning scheme to identify proteins that prevent Ha-Ras suppression of integrin activation. We report that the DED-containing protein, PEA-15, blocks Ras suppression. PEA-15 does not inhibit Ras activation of the ERK MAP kinase pathway, but rather blocks Ras suppression via a pathway inhibited by a dominant-negative form of R-Ras. Finally, the DED of PEA-15 is necessary, but not sufficient, for the reversal of Ras suppression. Hence, these data provide evidence that DED-containing proteins can regulate integrin activation as well as apoptosis. Moreover, we have identified PEA-15 as a novel regulator of inside-out integrin signaling pathways.

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