Phospholipase Cε Suppresses Integrin Activation*

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Received for publication, December 19, 2005, and in revised form, August 7, 2006 Published, JBC Papers in Press, August 8, 2006, DOI 10.1074/jbc.M513471200

Phospholipase Cε (PLCe) is a newly described effector of the small GTP-binding protein H-Ras. Utilizing H-Ras effector mutants, we show that mutants H-Ras(G12V/E37G) and H-Ras(G12V/D38N) suppressed integrin activation in an ERK-independent manner. H-Ras(G12V/D38N) specifically activated the PLCe effector pathway and suppressed integrin activation. Inhibition of PLCe activation with a kinase-dead PLCe mutant prevented H-Ras(G12V/D38N) from suppressing integrin activation, and low level expression of H-Ras(G12V/D38N) could synergize with wild-type PLCe to suppress integrins. In addition, knockdown of endogenous PLCe with small interfering RNA blocked H-Ras(G12V/D38N)-mediated integrin suppression. Suppressing integrin function with the H-Ras(G12V/D38N) mutant reduced cell adhesion to von Willebrand factor and fibronectin; this reduction in cell adhesion was blocked by coexpression of the kinase-dead PLCe mutant. These results show that H-Ras suppresses integrin affinity via independent Raf and PLCe signaling pathways and demonstrate a new physiological function for PLCe in the regulation of integrin activation.

Integrins are heterodimeric glycoproteins that control cell-cell and cell-substratum adhesion and that regulate cell survival, proliferation, and migration (1). An essential feature of integrins is their ability to regulate the strength of ligand binding, a process termed affinity modulation (2). Various intracellular signals can induce a conformational change in the integrin heterodimer, activating or suppressing ligand binding. Members of the Ras family of small GTP-binding proteins have been shown to modulate integrin affinity (2).

Expression of a constitutively active mutant of H-Ras, H-Ras(G12V), in Chinese hamster ovary (CHO)5 cells suppresses integrin activation (3). In addition, activation of the H-Ras downstream effector Raf also suppresses integrin activation in CHO cells (3, 4). In contrast, R-Ras, a closely related member of the Ras superfamily, activates integrins and reverses H-Ras-mediated suppression of integrin affinity (4, 5). Activation of Raf by H-Ras leads to the phosphorylation and activation of ERK1/2. H-Ras-mediated suppression of integrin affinity can be reversed by expression of MAPK phosphatase-1 (MKP-1), which can dephosphorylate and inactivate ERK1/2 (3). However, studies using H-Ras/R-Ras chimeras have revealed that integrin affinity modulation does not precisely correlate with ERK1/2 activation (6, 7). Remarkably, although targeting of ERK1 to the plasma membrane has been shown to be sufficient to suppress integrins (8), inhibition of ERK1/2 activation with either MKP-3 or U0126 fails to affect integrin suppression by H-Ras (7). This discrepancy in the current data might therefore be explained by the demonstration of an alternative pathway for integrin suppression that does not rely on ERK1/2 activation.

Several effector pathways are activated by H-Ras in addition to Raf, including phosphatidylinositol 3-kinase and Raf guanine exchange factors (GEFs). Amino acid substitution mutants of H-Ras have been extensively utilized in dissecting its downstream effector pathways. The effector mutant T35S retains its ability to activate Raf, whereas mutants E37G and Y40C are Raf-independent and activate Raf GEFs and phosphatidylinositol 3-kinase, respectively (9, 10). These H-Ras mutants also display differential action upon other H-Ras effectors, including Rin-1, AF-6, protein kinase Cε, p120 GTPase-activating protein (GAP) (11–14), and, recently, phospholipase Cε (PLCe) (15–17).

Phosphoinositide-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP3). Diacylglycerol stimulates protein kinase C activation, and IP3 mobilizes intracellular Ca2+ (18). Three major classes of PI-PLC have previously been identified: PLCβ, PLCγ, and PLCβ (19). They contain an N-terminal pleckstrin homology (PH) domain, an EF-hand domain, catalytic X and Y domains, and the regulatory C2 domain. PLCγ contains another PH domain, which is split by two SH2 domains and one SH3 domain. These PI-PLC classes are activated by distinct phospholipase C; IP3, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; SH, Src homology; RA, H-Ras/Rap1-associating; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; GST, glutathione S-transferase; FN, fibronectin; PBS, phosphate-buffered saline; AI, activation index; vWF, von Willebrand factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

This work was supported in part by a Scottish Health Research and Education Trust project grant (to Y. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 These authors contributed equally to this work.
2 Supported by a British Heart Foundation junior fellowship.
3 Supported by a Medical Research Council Clinical Research Training Fellowship.
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5 The abbreviations used are: CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; GEFs, guanine exchange factors; GAP, GTPase-activating protein; PLCe, phospholipase Cε; PI-PLC, phosphoinositide-specific phospholipase C; IP3, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; SH, Src homology; RA, H-Ras/Rap1-associating; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; GST, glutathione S-transferase; FN, fibronectin; PBS, phosphate-buffered saline; AI, activation index; vWF, von Willebrand factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
signaling mechanisms (20). PLCβ is activated by the α subunit (Gα) or βγ subunits (Gβγ) of heterotrimeric G proteins. PLCγ is activated by tyrosine phosphorylation following binding to tyrosine kinases of receptor or non-receptor types through its SH2 domain. PLC8 is activated by the high molecular weight G protein Gαs and/or by an increase in the concentration of intracellular Ca2+.

Recently, a fourth class of PI-PLC was identified, PLCε (15–17, 21). PLCε shares the typical X, Y, and C2 domains with the other PI-PLC classes. PLCε also contains putative PH and EF-hand domains and is activated by Gβγ subunits (22). Furthermore, PLCε is unique in that it possesses two types of functional domains not seen in other classes. At its N terminus, PLCε possesses a CDC25 homology domain (a GEF domain for the Ras family of small G proteins), which exhibits GEF activity toward Rap1 and H-Ras (17, 23, 24). At its C terminus, PLCε possesses two H-Ras/Rap1-associating (RA) domains, RA1 and RA2. H-Ras binds to PLCε in a GTP-dependent manner through its RA2 domain to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate into the secondary messengers IP3 and diacylglycerol, suggesting that PLCε may be a downstream effector of H-Ras and Rap1 (15–17).

Despite the characterization of these domains within PLCε, its physiological function remains unknown. However, given the signaling attributes and wide tissue distribution of PLCε, it is likely that this protein has a critical role in mammalian physiology. We therefore examined whether H-Ras utilizes PLCε to suppress integrins in cells and thus modulate cell adhesion to the extracellular matrix.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies were obtained from the indicated sources: antibody PAC-1 (activation-specific αinβ3) from BD Biosciences (Oxford, UK); R-phycocerythrin-conjugated anti-Tac antibody (ACT-1) and all horseradish peroxidase-conjugated species-specific antibodies from Dako (Cambridgeshire, UK); fluorescein isothiocyanate-conjugated anti-mouse IgM from BIOSOURCE (Nivelles, Belgium); anti-hemagglutinin (HA) (Y-11), anti-Myc (9E10), anti-ERK2 (C-14), and anti-PLCe (V-20) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-Thr115 ERK1/2, anti-actin (AC-40), and anti-FLAG (M2) antibodies from Sigma (Dorset, UK); and anti-RaA antibody from BD Transduction Laboratories.

**DNA Constructs**—Tac-αc (the extracellular domain of the interleukin-2 receptor fused to the intracellular domain of αc integrin) was obtained from Susan E. LaFlamme (Center for Cell Biology and Cancer Research, Albany, NY), and pDCR-H-Ras (G12V and effector mutants) was obtained from Michael H. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Johannes L. Bos (University of Utrecht, Utrecht, The Netherlands) provided pMT2-RaA (wild-type, G23V, and S28N), which was subsequently subcloned into pcDNA3.1 (Invitrogen, Paisley, UK), and pGEX-RalBD. Jean de Gunzburg (INSERM U-528, Paris, France) provided pGEX-RalBD. Jean de Gunzburg (INSERM U-528, Paris, France)

**Cell Lines and Transfection**—The CHO(αβ-py) cell line stably expresses a chimeric integrin composed of α1bβ3α2β1 (3, 4). CHO-K1 cells were obtained from American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum, 1% l-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids. CHO(αβ-py) cells were also maintained in G418 antibiotic at 400 μg/ml (Invitrogen). Serum-free medium was used to quiesce cells.

Transient transfection of cells with plasmid DNA was performed with LipofectamineTM Plus reagent (Invitrogen) following the manufacturer’s instructions. Twenty-four hours after transfection, the medium containing DNA-Lipofectamine complexes was removed and replaced with fresh complete medium. For experiments when protein activity was to be assessed, the transfection medium was replaced with quiescent medium. Forty-eight hours after transfection, cells were either lysed for SDS-PAGE analyses or used for integrin affinity determination.

**Small Interfering RNA (siRNA) of PLCε**—Kelley et al. (25) have previously described rat PLCε-specific siRNA oligonucleotides that effectively knock down PLCε in rat cells and scrambled non-targeting controls. To assess the whether these siRNA oligonucleotides could be used in CHO cells, we amplified the corresponding regions of siRNAPLCε#3 and siRNAPLCε#5 from CHO cDNA by reverse transcription-PCR using primers based on consensus rat/mouse PLCε sequence. The respective 616- and 380-bp products were gel-purified and sequenced. We confirmed that the region targeted by siRNAPLCε#3 and siRNAPLCε#5 was 100% conserved in CHO cells. Thus, we used these siRNA oligonucleotides to knock down PLCε in CHO cells. Transient transfection of cells with siRNA was performed with Oligofectamine (Invitrogen) following the manufacturer’s instructions. Twenty-four hours after transfection, the medium containing siRNA-Oligofectamine complexes was removed and replaced with fresh complete medium. Cells were then transfected with plasmid constructs using Lipofectamine as indicated below. Seventy-two hours after siRNA transfection, cells were either lysed for RNA extraction or protein analysis or used for integrin affinity determination.

**Fibronectin Type III Repeat 9–11 Fragment**—The soluble type III repeats 9–11 of fibronectin (referred to as Fn9–11), which contain the RGD domain responsible for integrin binding, were expressed as a glutathione S-transferase (GST) fusion protein in Escherichia coli BL21 cells from the pGEX-4T2 vector and biotinylated as described previously (7, 26).

**Flow Cytometry**—Integrin affinity in transfected cells was analyzed by three-color flow cytometry. Cells were transfected with test DNA (as stated) together with 0.75 μg of Tac-αc transfection reporter construct. Single cell suspensions of trypanrzed cells were resuspended in a total volume of 50 μl

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containing either PAC-1 (5 μg/ml) or FN9–11 for 30 min at room temperature in 20 mM HEPES, 140 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 2 mg/ml glucose (pH 7.4). Internal controls containing either 5 mM EDTA or 100 μM MnCl₂ were performed for each sample. Cells were washed with cold phosphate-buffered saline (PBS) and incubated on ice with 50 μl of DMEM containing 1:25 fluorescein isothiocyanate-conjugated anti-mouse IgM (for PAC-1) or fluorescein isothiocyanate-conjugated streptavidin (for FN9–11) for 30 min in the dark. Cells were washed again and incubated on ice for an additional 30 min with 50 μl of DMEM containing 1:50 (v/v) R-phycocerythrin-conjugated anti-Tac antibody (ACT-1). Cells were finally washed and resuspended in cold PBS. Immediately prior to analysis on a FACSCalibur (BD Biosciences, Erembodegem, Belgium), TO-PRO-3 (Molecular Probes, Leiden, The Netherlands) at a final concentration of 1 μM (in PBS) was added to each sample.

PAC-1/FN9–11 binding was determined by gating for live and highly transfected cells (TO-PRO-3-negative and high Tac binding, respectively). To obtain numerical estimates of integrin activation, an integrin activation index (AI) was calculated, where AI = ((F₀₄₅ – F₄₅)/ (F₀₄₅ – F₄₅)) × 100 and percent inhibition = (AI₀ – AI)/AI₀ × 100. F₀₄₅ is the geometric mean fluorescence intensity of PAC-1/FN9–11 binding of the native integrin, F₄₅ is the mean fluorescence intensity of PAC-1/FN9–11 binding in the presence of 5 mM EDTA, and F₀₄₅ is the mean fluorescence intensity of PAC-1/FN9–11 binding in the presence of 100 μM Mn²⁺. AI₀ is the activation index with the control vector, and AI is the activation index with the DNA under testing.

**Gel Electrophoresis and Western Blotting** —Cell lysates were resuspended in Laemmli sample buffer, separated on 10–12% SDS-polyacrylamide gels, and transferred onto Hybond-C nitrocellulose (Amersham Biosciences, Buckinghamshire, UK). Immunoblotting was performed with appropriate antibodies diluted in 5% nonfat dried milk powder and detected by chemiluminescence (ECL, Amersham Biosciences) following the manufacturer’s instructions. Relative protein expression was quantified using ImageJ software.

**RNA Extraction and Reverse Transcription-PCR** —Total RNA was extracted from 1 x 10⁶ cells using an RNeasy kit (Qiagen Inc.) according to the manufacturer’s instructions. Contaminating DNA was removed by treatment with RQ1 DNase (Promega Corp.), and the RNA was quantified using a spectrophotometer and then stored at −80 °C. cDNAs were generated by reverse transcription of 400 ng of RNA using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer’s instructions and stored at −20 °C. All reagents for PCR were obtained from Promega Corp. The reaction contained 2.5 μl of 10× buffer, 0.25 μl of Taq polymerase, 1 μl of dNTP (10 mM), 0.5 μM forward and reverse primers, 17.25 μl of nuclease-free water, and 2 μl of cDNA. The PCR program was as follows: 95 °C for 1 min, 35 cycles at 95 °C for 45 s; 56 °C for 45 s; and 72 °C for 30 s; followed by 72 °C for 5 min. The primers used were PLCefor (GGCTACGTAGGAG-GATTGTCTTA), PLCrev (TTTCCCTGCACCTTCCACTTGC), β-actin-for (CCACCAACTGGACACATG), and β-actin-rev (GTCTCAAACATGATCTGGTGTCATC). PCR products were resolved on a 2% agarose gel, purified using a gel extraction kit (Qiagen Inc.), and sequenced on both strands (MWG Biotech).

**PLC Activity Assay** —CHO-K1 cells were maintained in inositol-free DMEM (ICN Biochemicals, Basingstoke) supplemented with dialyzed 10% (v/v) fetal bovine serum (Labtech International, East Sussex, UK). Cells were transfected as described above and quiesced 24 h prior to lysis in serum-free DMEM containing 5 μCi of myo-[³H]inositol (Amersham Biosciences). Cells were then incubated with 20 mM lithium chloride for 60 min, washed, and lysed with 500 μl of 0.5 M trichloroacetic acid. Lysates were clarified at 13,000 × g for 10 min, and the supernatant (400 μl) was neutralized by addition to a 50:50 mixture of 1,1,2-trichlorotrifluoroethane/tri-n-oc-tylamine (750 μl). The mixture was vortexed and separated by centrifugation at 13,000 × g for 5 min. The aqueous phase was collected (300 μl), diluted by the addition of 10 ml of ice-cold distilled H₂O, and loaded onto an AG 1-X8 200–400 mesh formate form column (Bio-Rad, Hertfordshire, UK). Following a column wash with 10 ml of 60 mM ammonium formate and 5 mM sodium tetraborate, [³H]IP₁₋₃ was eluted with 5 ml of 1.2 M ammonium formate and 0.1 M formic acid and measured by liquid scintillation counting.

**Cell Adhesion Assay** —Cells were transfected with test DNA, and CHO(αβ-py) cells were also transfected with 2 μg of pEGFP-C3. Cells were harvested after 48 h and resuspended at 1 x 10⁶ cells/ml in DMEM. 96-Well cell culture cluster plates were coated with 5 μg/ml von Willebrand factor (vWF) (CHO(αβ-py) cells) or 10 μg/ml fibronectin (CHO-K1 cells) in PBS for 60 min at 37 °C and blocked with 2% (w/v) bovine serum albumin in PBS for 60 min at room temperature. The cell suspension (200 μl) was added to each well and allowed to adhere for 15 min at 37 °C. Unattached cells were removed by gentle shaking for 3 x 10 s. Total cell adhesion was assessed by centrifugation of the plate at 1000 rpm for 5 min. For CHO(αβ-py) cells, adhesion was quantified on a fluorescence plate reader. For CHO-K1 cells, adhesion was quantified by staining adherent cells with methylene blue (0.4%) for 5 min, three washes with PBS, and elution of stain with 0.1 M HCl. The absorbance of each sample was read at 640 nm on an optical plate reader. Adhesion was expressed as a percentage compared with total cell adhesion with background cell adhesion to plastic.
Integrin Suppression via PLCε

**RESULTS**

**Integrin Suppression by H-Ras(G12V) Effector Mutants T3SS and E37G**—It has previously been reported that a constitutively active mutant of H-Ras (H-Ras(G12V)) suppresses integrin function (3). However, the signaling pathways downstream of H-Ras are still poorly understood. To examine the role of downstream effectors of H-Ras in integrin suppression, we utilized the CHO(αβ-py) cell line, a CHO cell line stably expressing a chimeric integrin that contains the extracellular and transmembrane domains of α1β3 fused to the cytoplasmic domains of α6β1β3. H-Ras(G12V) effector mutants were transfected into CHO(αβ-py) cells, and their effect upon integrin affinity modulation was assessed. The integrin AI was quantified from changes in the binding levels of the α1β3 ligand-mimetic monoclonal antibody PAC-1, detected by flow cytometry as described under “Experimental Procedures.”

Fig. 1A shows that the H-Ras(G12V) effector mutants E37G (25.3 ± 14.6%) and T3SS (23.7 ± 6.6%) both induced a significant reduction in the AI compared with control vectortransfected cells (70.6 ± 8.5%, p < 0.01). This was comparable with H-Ras(G12V)-induced reduction in the integrin AI (23.1 ± 4.3%).

In contrast, effector mutant Y40C (59.0 ± 15%) did not significantly reduce the AI in our system. Furthermore, we confirmed previous results (3) that inhibition of phosphatidylinositol 3-kinase activity with LY294002 (10 μM) does not affect H-Ras(G12V)-mediated integrin suppression in CHO(αβ-py) cells (data not shown), in agreement with the inability of H-Ras(G12V/Y40C) to suppress integrins. Each mutant was expressed at similar levels in the transfected CHO(αβ-py) cells (Fig. 1B). H-Ras(G12V) and the effector mutant T3SS both stimulated ERK1/2 phosphorylation as detected by anti-phospho-ERK1/2 antibody (clone MAPK-YT; 4.6 ± 0.6- and 3.2 ± 0.5-fold increase (mean ± S.E.) compared with vector, respectively; n = 3 independent experiments) (Fig. 1B). As expected, the effector mutants E37G and Y40C did not stimulate ERK1/2 phosphorylation above that in control transfected cells (0.9 ± 0.2- and 1.1 ± 0.2-fold change, respectively). Transfection of the H-Ras mutants did not alter the level of ERK2 expression in these transfected cells.

Fig. 1C shows the effect of H-Ras(G12V/E37G) expression in the CHO(αβ-py) cell assay. Expression of H-Ras(G12V/E37G) caused a marked reduction in PAC-1 binding, resulting in a leftward shift in the highly transfected cell population (Fig. 1C, upper quadrants). The untransfected/pro Poorly transfected cell population (Fig. 1C, lower quadrants) did not display any significant change in PAC-1 binding. PAC-1 binding to the chimeric integrin in CHO(αβ-py) cells was inhibited by EDTA (Fig. 1C, left panel). In contrast, H-Ras(G12V/E37G)-transfected cells in the presence of Mn2+ displayed a slight rightward shift in the whole cell population as a result of increased PAC-1 binding (Fig. 1C, right panel). The ability of Mn2+ to override H-Ras(G12V/E37G) suppression of PAC-1 binding by activating integrins indicates that the effect of H-Ras is not due to changes in integrin expression in this system, but rather suppression of integrin activity. These results suggest that H-Ras(G12V) mediates integrin suppression by two separate effector pathways: a Raf/ERK-dependent signaling pathway utilized by T3SS and a Raf/ERK-independent pathway used by E37G.

Integrin Suppression by H-Ras(G12V/E37G) Is Not Mediated by RaLa—The small GTP-binding protein RaLa is a downstream effector of H-Ras(G12V) that is activated by the effector mutant E37G (29). We therefore examined whether RaLa plays a role in the suppression of integrin affinity. CHO(αβ-py) cells were transfected with Myc-tagged wild-type RaLa, constitutively active RaLa(G23V), or dominant-negative RaLa(S28N) in the presence of either the control vector or H-Ras(G12V/E37G). Integrin suppression was measured by the percent change in the integrin AI from the control as described under “Experimental Procedures.” Cotransfection with either the RaLa(G23V) (41.6 ± 3.9%) or RaLa(S28N) (47.3 ± 3.3%) mutant had no significant effect on H-Ras(G12V/E37G)-mediated suppression (57.1 ± 0.5%) (Fig. 2A). Furthermore, cells cotransfected with either RaLa(G23V) or RaLa(S28N) and the control vector also had no significant effect on integrin affinity compared with control cells.

Ral activity was assessed in duplicate samples using the Ral-binding domain of RLIP76 fused to GST to pull down active Ral (28). Cells cultured in serum-free medium for 12 h prior to assay showed a low basal level of RaLa activity, which was
H-Ras(G12V/E37G)-mediated suppression is unaffected by RalA activity. A, CHO(αβ)-py) cells were cotransfected with H-Ras(G12V/E37G) (1 µg) and either constitutively active (G23V) or dominant-negative (S28N) Myc-tagged RalA (1 µg) and the reporter construct Tac-α5. Forty-eight hours post-transfection, cells were harvested and assessed for integrin affinity by flow cytometry. Percent inhibition was calculated in reference to the empty vector. The results shown are the means ± S.E. of three independent experiments. B, Ral activity was measured in lysates from CHO(αβ-py) cells transfected with H-Ras(G12V/E37G) and Myc-tagged RalA constructs as described for A. Active Ral bound to GST-fused RIP76 Ral-binding domain (RalBD)-agarose beads was probed with anti-RalA antibody (upper panel). Whole cell lysates were run on a 15% acrylamide gel to an extent where the Myc-tagged constructs could be distinguished from endogenous Ral and then probed with anti-RalA antibody to show equal expression of endogenous RalA (middle panel). Whole cell lysates were also probed with anti-HA antibody to show expression of the H-Ras(G12V/E37G) construct (lower panel).

The ability of the H-Ras(G12V/D38N) mutant to mediate integrin suppression suggested that PLCε may regulate integrin affinity. To investigate this, we used the kinase-dead mutant PLCε(H1433L). We found that expression of PLCε(H1433L) alone in CHO(αβ-py) cells had no significant effect on integrin activation (Fig. 4A). However, cotransfection of H-Ras(G12V/E37G) or H-Ras(G12V/D38N) with PLCε(H1433L) significantly blocked integrin suppression mediated by these H-Ras mutants (49.3 ± 3.0 to 10.1 ± 2.3% (p < 0.01) and 47.2 ± 2.9 to 7.2 ± 2.5% (p < 0.001), respectively) (Fig. 4A). Furthermore, integrin suppression mediated by H-Ras(G12V/T35S) was not affected by PLCε(H1433L) (data not shown). Western blot analysis demonstrated that the equal FLAG-tagged expression of PLCε mutants in all transfected cells and expression of PLCε(H1433L) did not alter H-Ras(G12V/D38N) expression (Fig. 4B).
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The potential cooperation between H-Ras(G12V/D38N) and wild-type PLCε was examined further. We found that low level expression of H-Ras(G12V/D38N) with 250 or 375 ng of plasmid DNA caused only modest integrin suppression (13.5 ± 8.5 and 14.1 ± 6.5%, respectively) (Fig. 4C). Wild-type PLCε (15 μg) alone had little effect on integrin activation status. However, cotransfection of H-Ras(G12V/D38N) (250 or 375 ng) with wild-type PLCε significantly enhanced integrin suppression (33.5 ± 9.2% (p < 0.05) and 43.1 ± 7.1% (p < 0.05), respectively). Fig. 4C (inset) shows the concentration response of H-Ras(G12V/D38N) to integrin suppression in the presence of 15 μg of wild-type PLCε. The synergy between wild-type PLCε and H-Ras(G12V/D38N) was clear at low amounts (250 or 375 ng) of H-Ras(G12V/D38N), but was lost upon increased expression (1 μg and above). Concentrations of H-Ras(G12V/D38N) above 1 μg of DNA were sufficient to cause maximal protein expression and integrin suppression such that expression of wild-type PLCε failed to further enhance integrin suppression. Western blot analysis confirmed that expression of wild-type PLCε did not affect H-Ras(G12V/D38N) expression (Fig. 4D).

To confirm that PLCε was functional in transfected cells, inositol phosphate production was measured (Fig. 4E). Consistent with previous results (15), wild-type PLCε alone was insufficient to stimulate IP3 production detectable by ion exchange. However, in cells cotransfected with H-Ras(G12V/D38N) and wild-type PLCε, an ~6-fold increase was observed (3751 ± 526 cpm) compared with control cells (516 ± 365 cpm; p < 0.001). Transfection of the kinase-dead PLCε(H1433L) mutant alone had no effect on IP3 production. Cotransfection of PLCε(H1433L) with H-Ras(G12V/D38N) significantly reduced IP3 production stimulated by H-Ras(G12V/D38N) (678 ± 371 versus 1990 ± 423 cpm, respectively; p < 0.01).

ERK1/2 phosphorylation was assessed to exclude the possibility

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FIGURE 4. PLCε(H1433L) reverses H-Ras(G12V/D38N)-mediated integrin suppression. A, CHO(αβ5-py) cells were cotransfected with H-Ras(G12V/E37G) or H-Ras(G12V/D38N) (1 μg) and PLCε(H1433L) (15 μg) together with the reporter construct Tac-α5. Forty-eight hours post-transfection, cells were harvested and assayed for integrin affinity by flow cytometry. Percent inhibition was calculated in reference to the empty vector. The results shown are the means ± S.E. of five independent experiments. *, p < 0.05. B, transfected cell lysates from A were analyzed by Western blotting to determine HA-H-Ras and FLAG-PLCε expression. C, CHO(αβ5-py) cells were cotransfected with increasing concentrations of H-Ras(G12V/D38N) DNA and wild-type (WT) PLCε (15 μg) together with the reporter construct Tac-α5. Integrin affinity was assessed by flow cytometry forty-eight hours post-transfection. Percent inhibition was calculated in reference to the empty vector. The results shown are the means ± S.E. of five independent experiments. The inset shows integrin suppression with a dose response of increasing concentrations of H-Ras(G12V/D38N) DNA from 0 to 1 μg of DNA with (C) or without (D) wild-type PLCε (15 μg). *, p < 0.05. D, transfected cell lysates from C were analyzed by Western blotting to determine HA-H-Ras, FLAG-PLCε, and actin expression. E, CHO(αβ5-py) cells were cotransfected with H-Ras(G12V/D38N) (0.5 μg) and either wild-type PLCε or PLCε(H1433L) (15 μg). Inositol phosphate production in the transfected cells was assessed. The results shown are the means ± S.E. of four to five independent experiments. *, p < 0.05. F, quiescent cell lysates from CHO(αβ5-py) cells cotransfected with H-Ras(G12V) (0.5 μg) and wild-type PLCε or PLCε(H1433L) (15 μg) were assessed for ERK1/2 phosphorylation (p-ERK) and H-Ras and PLCε expression. The PLCε constructs had no effect on phosphorylation of ERK1/2 by H-Ras(G12V). ERK2 levels were shown to be consistent between lysates.
that PLCe(H1433L) was competing H-Ras away from other effectors. H-Ras(G12V)-stimulated ERK1/2 phosphorylation was not altered by cotransfection with either wild-type PLCe or PLCe(H1433L) (1.13 ± 0.11- and 0.92 ± 0.14-fold change (mean ± S.E.)) compared with H-Ras(G12V) alone, n = four independent experiments) (Fig. 4F). We confirmed previously published results (3, 31) showing that either expression of PLCβ, CDC42 (a known activator of PLCβ), or protein kinase C isoforms α and δ or artificial stimulation of protein kinase C by the phorbol ester phorbol 12-myristate 13-acetate does not suppress integrin affinity (data not shown). Thus, the effect of PLCe in suppressing integrin affinity appears to be isotype-specific.

To confirm the data obtained with PLCе(H1433L), we sought to knock down endogenous PLCе in CHO(αβ-pty) cells and to investigate the effect on H-Ras(G12V/D38N)-mediated integrin suppression. siRNA oligonucleotides targeting conserved sequences from rat, human, and mouse PLCе have previously been described and shown to reduce PLCе expression by up to 97% (25). Our analysis of the surrounding regions of siRAPLCE#3 and siRAPLCE#5 showed that CHO PLCе is nearly identical between rat and hamster and that the two siRNA target sites of siRAPLCE#3 and siRAPLCE#5 are both completely conserved. Therefore, we transfected CHO(αβ-pty) cells with either the PLCе-targeting siRNA or a scrambled non-targeting oligonucleotide and assessed the effect on integrin suppression mediated by transient expression of H-Ras(G12V/D38N).

We found that transfection of the non-targeting siRNA oligonucleotide had no significant effect on H-Ras(G12V/D38N)-mediated integrin suppression (49.3 ± 3.1 and 48.6 ± 2.9%, respectively) (Fig. 5A). In contrast, transfection of CHO(αβ-pty) cells with the PLCе-targeting siRNA (siRAPLCE#5) significantly blocked H-Ras(G12V/D38N)-mediated integrin suppression (11.6 ± 2.9%; p < 0.05). As expected, transfection with siRAPLCE#5 markedly reduced PLCе mRNA levels as assessed by reverse transcription-PCR (Fig. 5B). Comparable levels of PLCе mRNA were observed in untransfected cells and cells transfected with non-targeting siRNA. Furthermore, we confirmed the knockdown of endogenous PLCе protein in CHO cells by Western blot analysis (Fig. 5B). To validate our results with siRAPLCE#5, we transfected CHO(αβ-pty) cells with siRAPLCE#3 and confirmed the knockdown of PLCе and the reversal of H-Ras(G12V/D38N)-mediated integrin suppression (data not shown). Notably, transfection of cells with siRNA oligonucleotides did not affect expression of H-Ras(G12V/D38N) or actin (Fig. 5, B and C). Taken together, these results indicate that endogenous PLCе can modulate integrin suppression mediated by H-Ras.

H-Ras Suppresses Integrin Affinity via Independent Raf and PLCe Signaling Pathways—Hughes et al. (3) have previously reported that a constitutively active mutant of H-Ras (H-Ras(G12V)) suppresses integrin function via Raf and have subsequently shown that H-Ras(G12V)-mediated suppression is not reversed by MKP-3 (7). Fig. 6A shows that MKP-3 reversed integrin suppression mediated by a constitutively active form of Raf (Raf BXB CAAX, where is A is an aliphatic amino acid) (AI of 32.7 ± 3.1 to 63.2 ± 4.1%; p < 0.05). As shown previously, cotransfection of MKP-3 had no significant effect on H-Ras(G12V)-mediated integrin suppression (AI of 29.9 ± 1.7 and 32.5 ± 2.6%, respectively). Cotransfection of MKP-3 blocked both Raf BXB CAAX- and H-Ras(G12V)-induced ERK2 phosphorylation (Fig. 6B). We therefore examined whether PLCе activation by H-Ras(G12V) mediated MAPK-independent integrin suppression. PLCе(H1433L) expression did not affect either Raf BXB CAAX- or H-Ras(G12V)-mediated integrin suppression (AI of 32.7 ± 3.1 to 27.1 ± 11.9% and 29.9 ± 1.7 to 32.2 ± 7.1%, respectively) or ERK1/2 phosphorylation. However, cotransfection of PLCе(H1433L) with MKP-3 blocked H-Ras(G12V)-mediated integrin suppression (AI of 62.0 ± 8.7% versus 32.2 ± 3.1%; p < 0.05). These results indicate that H-Ras suppresses integrin activation via independent Raf and PLCе pathways and that either pathway is sufficient to modulate H-Ras-mediated integrin suppression.
Modulating Rap1A Activity Does Not Affect Integrin Suppression—PLCζ contains a CDC25 homology domain that has been shown to display guanine nucleotide exchange activity toward Rap1 (32). Prior studies have indicated a role for Rap1 in integrin-mediated cell adhesion (33). We therefore examined the role of Rap1 in integrin suppression by H-Ras(G12V/D38N). We used a dominant-negative mutant of Rap1A with the amino acid substitution S17N, which restricts the protein to the GDP-bound state (34). Transfection of Rap1A(S17N) alone did not modulate integrin affinity in CHO(-py) cells (Fig. 7A). Cotransfection with Rap1A(S17N) did not affect the ability of H-Ras(G12V/D38N) to suppress integrins (42.9 ± 1.6 and 47.0 ± 0.3%, respectively). To confirm these data, we used a truncated Rap1GAP construct (residues 75–415) that has previously been shown to down-regulate Rap1 activity by catalyzing GTP hydrolysis (35). We found that transient expression of Rap1GAP alone or with H-Ras(G12V/D38N) had no significant effect on integrin suppression in CHO(αβ- py) cells (Fig. 7A). Cotransfection with Rap1A(S17N) did not affect the ability of H-Ras(G12V/D38N) to suppress integrins (42.9 ± 1.6 and 47.0 ± 0.3%, respectively). To confirm these data, we used a truncated Rap1GAP construct (residues 75–415) that has previously been shown to down-regulate Rap1 activity by catalyzing GTP hydrolysis (35). We found that transient expression of Rap1GAP alone or with H-Ras(G12V/D38N) had no significant effect on integrin suppression in CHO(αβ-py) cells (Fig. 7A). These results suggest that Rap1A signaling is not required for H-Ras-mediated integrin suppression through PLCζ.

PLCζ Contributes to H-Ras-mediated Reduction in Cell Adhesion—Modulation of integrin affinity is a central process in the control of cell adhesion to the extracellular matrix (2). To investigate whether PLCζ participates in H-Ras-mediated reduction in cell adhesion, we assessed adhesion of CHO(αβ-py) cells to vWF, a ligand for the chimeric α1bα6β3β1 integrin present in this stable cell line. Transient transfection of H-Ras(G12V/D38N), which mediates integrin suppression, produced a significant reduction in cell adhesion (46 ± 10%; p < 0.01) (Fig. 8A). Coexpression of H-Ras(G12V/D38N) with PLCζ(H1433L) significantly blocked this reduction in adhesion to vWF back to control cell levels of adhesion (120 ± 20%; p < 0.05). This effect on adhesion by PLCζ was not due to a reduction in expression of H-Ras(G12V/D38N) (Fig. 8B). Reversing H-Ras(G12V/D38N)-mediated integrin suppression by coex-
pression of PLCe(H1433L) therefore restores the cells’ ability to adhere to vWF.

We went on to investigate the role of PLCe in the suppression of endogenous integrins. It has previously been shown that a soluble fragment of fibronectin composed of type III repeats 9–11 (FN9–11) can bind to endogenous αβ integrins in CHO-K1 cells and that FN9–11 binding can be suppressed by activated Raf-1 signaling (6). We therefore investigated the effect of transfection of H-Ras(G12V/D38N) with or without PLCe(H1433L) on FN9–11 binding in CHO-K1 cells as described under “Experimental Procedures.” We found that transfection of H-Ras(G12V/D38N) significantly suppressed FN9–11 binding and that this was blocked by cotransfection of dominant-negative PLCe(H1433L) (46.2 ± 1.9 and 13.5 ± 2.1%, respectively) (Fig. 8, B and C). Notably, Mn2+ activated and EDTA suppressed FN9–11 binding similarly in CHO-K1 cells regardless of the DNA transfected, indicating that changes in FN9–11 binding are not due to changes in integrin expression. In parallel, we allowed CHO-K1 cells transfected with H-Ras(G12V/D38N) in the absence or presence of PLCe(H1433L) to adhere to fibronectin-coated plastic. In support of the FN9–11 binding data, H-Ras(G12V/D38N) reduced cell adhesion, which was blocked by PLCe(H1433L) (Fig. 8 D). Thus, PLCe modulates H-Ras suppression of endogenous αβ integrin binding to fibronectin.

**DISCUSSION**

Modulation of integrin affinity plays a central role in the regulation of integrin function (1, 2). We have shown for the first time that PLCe, a novel H-Ras effector, suppresses integrin affinity. The constitutively active mutant of H-Ras suppresses integrins via two independent H-Ras effector pathways: a Raf-dependent T35S pathway and a Raf-independent E37G/D38N pathway. This alternative mechanism of inte-
Integrin Suppression via PLCε

![Diagram of Raf-dependent and Raf-independent signaling pathways of integrin suppression]

Integrin suppression utilizes PLCε, as shown in Fig. 9. The suppression of integrins via PLCε also reduces cell adhesion to a vWF-coated surface and to the extracellular matrix component fibronectin. Our results thus identify a new physiological function for PLCε.

Hughes et al. (3) have previously shown that both H-Ras(G12V) and activated Raf can suppress the binding of the αIIbβ3 ligand-mimetic antibody PAC-1 to the stably expressed chimeric αIIbα6β3β1 integrin in CHO cells. Fluorescein isothiocyanate-labeled fibronectin binding to endogenous αIIbβ3 in CHO cells is also suppressed by H-Ras(G12V) expression, indicating that inside-out signaling can act upon a native integrin and be detected by a physiological integrin ligand. H-Ras(G12V/T35S) is a well established activator of the Raf effector arm of H-Ras (9) and can mimic many of the putative effects of H-Ras through the activation of the ERK1/2 signaling cascade (36). Integrin suppression by H-Ras(G12V/T35S) indicates that a Raf-dependent mechanism is capable of modulating integrin affinity, consistent with previously published data (3). However, the requirement of ERK1/2 activation for integrin affinity suppression is still unclear. Integrin suppression occurs in the absence of bulk ERK1/2 activation and is not reversed by coexpression of MKP-3 (Fig. 5A). Targeting ERK1 to the plasma membrane with the CAAX box of H-Ras leads to integrin suppression (8), suggesting that localized ERK1/2 activity can allow integrin suppression in the absence of bulk ERK activation. The MEK inhibitor U0126 fails to inhibit both H-Ras(G12V)- and activated Raf-mediated integrin suppression; whether ERK1-CAAX activation at the plasma membrane is sensitive to MEK inhibition remains to be tested. The ability of H-Ras(G12V) to suppress integrins via PLCε in a Raf-independent manner provides a mechanism to resolve the conflicting data relating to the need for ERK1/2 activation. This alternative H-Ras pathway can compensate for and maintain integrin suppression in the presence of either MEK inhibitors or MKP-3 coexpression.

PLCε has previously been identified as an H-Ras effector (15–17). Kelley et al. (15) showed that PLCε activity is stimulated by the constitutively active H-Ras(Q61L) mutant. PLCε activity is also stimulated by the effector mutant H-Ras(Q61L/E37G), implying that the H-Ras(E37G) mutant may not solely be an activator of Raf effectors. In contrast, the H-Ras effector mutants T35S and Y40C fail to significantly increase PLCε activity compared with the control. However, the H-Ras(Q61L/D38N) mutant increases inositol production by 50–60% (15), implicating this mutant as an activator of PLCε along with E37G. The D38N mutation within the H-Ras effector domain is known to abrogate Raf binding and activation in vitro and displays minimal transformation potency in NIH/3T3 cells (37). Therefore, the ability of H-Ras(G12V/D38N) to selectively activate PLCε allowed us to specifically examine this pathway in isolation of other effector pathways in our system, viz. Raf, Raf effectors, and phosphatidylinositol 3-kinase.

We have shown that H-Ras(G12V/D38N) induces a level of integrin suppression similar to that induced by H-Ras(G12V/E37G) (Fig. 3), indicating that the PLCε effector pathway can suppress integrin affinity. Unfortunately, no constitutively active mutant of PLCε is available to test whether overexpression of active PLCε would activate integrin affinity. Furthermore, although physiological agonists of PLCε have been described (25), we (4, 6, 7) and others (5) have demonstrated previously that detection of integrin suppression requires sustained rather than transient activation of H-Ras. However, the ability of kinase-dead PLCε(H1433L) and endogenous PLCε knockdown to block H-Ras(G12V/D38N)-mediated integrin suppression (Fig. 4A) provides good evidence that PLCε catalytic signaling modulates integrin affinity. Our demonstration that PLCε(H1433L) does not affect H-Ras(G12V)-stimulated ERK phosphorylation or integrin suppression (Fig. 6) even at high ratios (15:1) suggests that this dominant-negative mutant is not acting through sequestration of H-Ras. Furthermore, our demonstration in Fig. 6A that coexpression of both PLCε and MKP-3 is needed to reverse integrin suppression caused by H-Ras(G12V) is important. It shows that blocking either effector pathway individually is insufficient to fully prevent integrin suppression and provides strong evidence for two independent pathways of H-Ras-mediated integrin suppression.

In platelets, PLC stimulation is essential for integrin affinity modulation of αIIbβ3 during platelet activation, and inhibition of PLC activity with the inhibitor U73122 suppresses adhesion of BaF3 cells to fibronectin (38, 39). Integrin activation is prevented pharmacologically by inhibiting the second messengers from PLC activation, viz. intracellular calcium and protein kinase C activation (40). However, we confirmed previously published results (3) showing that PLCβ and protein kinase C do not suppress integrin affinity in the CHO(αβ-py) system (data not shown). Furthermore, CDC42 expression, a known activator of PLCβ in the CHO(αβ-py) system, fails to suppress integrins (3, 31). Expression of protein kinase C isoforms or
The novel function of PLCε to modulate integrin affinity allows the H-Ras integrin suppression pathway to occur in a Raf/ERK-independent manner. Ras activation is a focal point for growth factor signaling and is associated with cell cycle control and proliferation (52). Activation of ERK1/2 via Raf is central to the control of cyclins and cyclin-dependent kinases (27). H-Ras-mediated integrin suppression via the PLCε pathway could therefore avoid inappropriate signaling to the cell cycle machinery. In summary, this study has demonstrated a novel and physiologically relevant function of PLCε in suppressing integrin affinity modulation. PLCε, a novel H-Ras effector, modulates integrin affinity via the effector mutants E37G and D38N in a Raf-independent manner.

Acknowledgment—We thank S. Johnston for expert technical help.

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