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The P34G Mutation Reduces the Transforming Activity of K-Ras and N-Ras in NIH 3T3 Cells but Not of H-Ras*

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activates H-Ras and R-Ras *in vivo*, but not N-Ras or K-Ras 4B (5), or Sos induces GDP/GTP exchange on the three Ras isoforms in the hierarchy H-Ras > N-Ras > K-Ras (6). In addition to this, N-Ras and H-Ras proteins can be reversibly palmitoylated at one (Cys-181) or two (Cys-181 and Cys-184) cysteine residues, respectively, whereas K-Ras possesses a polybasic domain close to the C-terminal end, and these variations may contribute to differences in trafficking (7), membrane association, and effector pathway engagement between the three Ras homologues (5). Indeed, recently the activation of H-Ras and N-Ras proteins (but not K-Ras) in the Golgi apparatus and in the endoplasmic reticulum (8, 9) has been described, depending

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activate protein kinase; ERK, extracellular signal-regulated kinase; p-ERK, phospho-ERK; MEKK1, MAPK/ERK kinase 1; HA, hemagglutinin; PLD, phospholipase D; RBD, Ras-binding domain; TRITC, tetramethylrhodamine isothiocyanate; GST, glutathione *S*-transferase; GDS, guanine nucleotide dissociation stimulator; PtdBut, phosphatidylbutanol.

on Ras-GRP and Ras-GRF proteins, respectively (10, 11). The functional specificity in signaling by the three homologues is also evidenced by the differences in the relative ability of H-Ras *versus* K-Ras to activate the Raf and PI3K effector pathways (12, 13). It has been suggested that K-Ras activates Rac more efficiently than H-Ras (14), or that H-Ras and K-Ras induce higher activation of NF-κB than N-Ras (15). Furthermore, we found recently that the cyclopentenone 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 binds to and activates H-Ras through the formation of a covalent adduct with this isoform that does not occur with N-Ras or K-Ras 4B (16). However, *in vitro* binding assays have failed to detect large affinity differences for each type of Ras protein and any of the known putative downstream targets, with the exception of Sur8, a conserved Ras-binding protein with multiple leucine-rich repeats (17, 18). Sur8 specifically binds K-Ras and N-Ras but not H-Ras *in vitro* (17) and displays a synergistic effect on the Ras-Raf-MEK-Erk pathway probably by the stabilization of a ternary complex with Ras and Raf-1 (19). Different *in vitro* binding protein studies have demonstrated that specific point mutations at the effector domain of Ras can reduce the interaction with Sur8 (P34G mutation), with Raf-1 (E37G and Y40C mutations), with Ral-GDS (T35S and Y40C mutations), or with PI3K (T35S and E37G mutations) (17, 20). This suggests a key role for these residues in how they affect the ability of Ras to interact with these effector proteins.

Based on these observations, we hypothesized that the codon 34 of Ras may play a different role in H-, K-, and N-Ras, thereby providing a distinct signaling capacity to each protein. Thus, in the present study we analyzed how ment of this residue, as in the P34G mutation, the signaling ability and transforming pote types of Ras proteins. We demonstrate t mutant shows the same transformi blasts as that of the hyperact P34G mutation dramatical N- and K-Ras4B proteins. F showing that, independent of distinct biological behavior amo is dictated by their specific ability al-GDS/Ral A/PLD and the Ras/Rac pathways.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and Antibodies—NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Invitrogen). COS1 and the human 293T (kidney keratinocyte) cell lines were maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen). Transient transfections in COS1 and 293T cells were performed with the LipofectAMINE reagent (Invitrogen). Assays were done 48 h after transfection. NIH 3T3 fibroblasts were transfected (transient or stable) by the calcium phosphate precipitation technique (21). Morphologically transformed foci were scored after 2–3 weeks in culture (21). Transfected cells were also selected in medium supplemented, as appropriate, with Geneticin 750 μg/ml (Invitrogen). EGF was purchased from Sigma-Aldrich. Monoclonal antibodies to phospho-MAPK (p-ERK1/p-ERK2) and phospho-AKT proteins were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antisera to MAPK (ERK1/ERK2) and AKT came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); monoclonal anti-p85 was from Upstate Biotechnology (Lake Placid, NY); anti-Ral and anti-Rac1 monoclonal antibodies came from BD Transduction (Lexington, KY); and anti-HA and anti-AU5 monoclonal antibodies came from Berkeley Antibody Co. (Berkeley, CA).

DNA Constructs—The plasmids pCEFL-KZ-HA, pCEFL-KZ-AU5, pCEFL-KZ-AU5-H-Ras wt, pCEFL-KZ-AU5-K-Ras 4B wt, pCEFL-KZ-AU5-N-Ras wt, pCEFL-KZ-AU5-H-RasV12, pCEFL-KZ-AU5-K-RasV-12, pCEFL-KZ-AU5-N-RasV12, pGal4-Luc, and pCDNAIII-Gal4-Elk-1 were previously described (21). The effector dominant mutants V12G34, V12S35, V12G37, and V12C40 of H-Ras, K-Ras4B, and N-Ras were obtained by PCR from pCEFL-KZ-AU5-H-RasV12, pCEFL-KZ-AU5-K-RasV12, and pCEFL-KZ-AU5-N-RasV12, using the specific primers and providing sites BglII and NotI at the 5′ and 3′ ends, respectively. The amplified products were then subcloned into BglII and NotI sites of pCEFL-KZ-AU5. The sequences of the oligonucleotides utilized are available upon request.

Immunocytochemistry—Cells on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min and permeabilized with 0.2% Triton X-100 for 10 min. To detect F-actin, cells were stained with tetramethylrhodamine B isothiocyanate-labeled phalloidin (TRITC-phalloidin, 10 μg/ml, Sigma) for 1 h. The nuclei were stained with Hoechst-33258 (H-33258, Riedel-de Haën).

Bacterial Expression of Fusion Proteins—The plasmids pGEX-RBD, pGEX-RBD-Ral-GDS, pGEX-Ral-BD, and pGEX-PAK-CRIB containing the Raf Ras-binding domain, the Ral-GDS Ras-binding domain, the Ral A-binding domain, and the Rac1-binding domain, respectively, fused to glutathione *S*-transferase (GST) were kindly provided by D. Shalloway, J. L. Bos, and J. G. Collard. All GST fusion proteins were purified (from *Escherichia coli* BL21(DE3) harboring these plasmids) following a method previously described (21).

In Vitro Binding Assays—Transfected cells were lysed in cold lysis buffer containing 25 mM HEPES, pH 7.5; 1% Triton X-100; 150 mM NaCl; 10 mM $MgCl_2$; 1 mM sodium orthovanadate (Na_3VO_4); 25 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml each of leupeptin, aprotinin, and pepstatin A, and trypsin inhibitor. Nucleus-free supernatants were incubated with the corresponding GST fusion protein on glutathione-Sepharose beads and analyzed as previously described (21).

Kinase Assays—MAPK tivity, in cells transfected with an epitope-tagged MAPK K2 and HA-ERK1, referred to herein as HA-ERK), w s previously described (21), using myelin basi a substrate. AKT assays, in cells transfec AKT (HA-AKT), were carried out follo viously (16), using histone H2B PI3K activity, the enzyme was with anti-p85α following the precipitate was resuspended DTA; 100 mM NaCl; 1 mM dithio- sulfonyl fluoride; 2 μg/ml aprotinin; 10 ml 1-chloro-3-tosylamido-7-amino-2-hep- mM $NaVO_4$; 10 mM Na_2MoO_4; and 10 nM okadaic of protein were used to measure the activity of PI3K hatidylinositol (20 μg) and 50 μM [γ-^{32}P]ATP as sub- 22). In all *in vitro* kinase assays, some plaques were stimu- for 10 min with EGF (100 ng/ml) and after PAGE or TLC, the amount of phosphorylated substrates (myelin basic protein, histone H2B, or lipids) was evaluated using a Fuji BAS1000 detector.

PLD Activity Assay—PLD activation was measured by its transphosphatidylating activity in the presence of 1-butanol to generate PtdBut (23). NIH 3T3 fibroblasts or COS1 and 293T cells were grown in multiwell plates and labeled for 48 h in the presence of 1 μCi/ml of [^{14}C]g-lycerol (148 mCi/mmol). To assay PLD activity, labeling medium was discarded and cells were washed with TD buffer (137 mM NaCl, 5 mM KCl, 1 mM $NaHPO_4$, 20 mM Tris/HCl, pH 7.4) and incubated for 30 min in serum-free DMEM supplemented with 0.5% 1-butanol. Medium was again removed, cells were then scraped in 1 ml of methanol, and plates were washed once with 0.5 ml of methanol. The two methanol samples were collected and mixed with 2 ml of chloroform and 1 ml of water. The organic phases were dried in a 37 °C heating block under a nitrogen stream. TLC using Silica Gel 60A plates (Whatman; LK6D) separated the lipids. The plates were developed with the upper phase of a mixture of ethyl acetate/iso-octane/acetic acid/water (90:50:20:100, v/v), visualized, and quantified using an electronic-autoradiography system (Instant Imager, Packard). Counts in PtdBut were normalized for the radioactivity incorporated into total lipids.

Reporter Gene Analysis—NIH 3T3 fibroblasts were transfected with 0.6 μg of constructs encoding for each Ras mutant, together with 16 ng of pCDNAIII-Gal4-Elk-1, 0.1 μg of pRL-TK (a plasmid expressing the enzyme *Renilla* luciferase), and 0.3 μg of the reporter plasmid (pGal4-Luc). 18 h after transfection, NIH 3T3 fibroblasts were placed in medium containing 0.1% calf serum, and 18 h later stimulated for 8 h with EGF (100 ng/ml). The assays were performed as described previously (21).

RESULTS

P34G Mutation Abolishes the Transforming Activity of N-Ras and K-Ras4B but Not of H-Ras—The interaction of Ras with its effectors is mediated by the effector-binding loop, which spans residues 32–40. Despite the fact that the amino acid sequence corresponding to effector binding is identical between the Ras

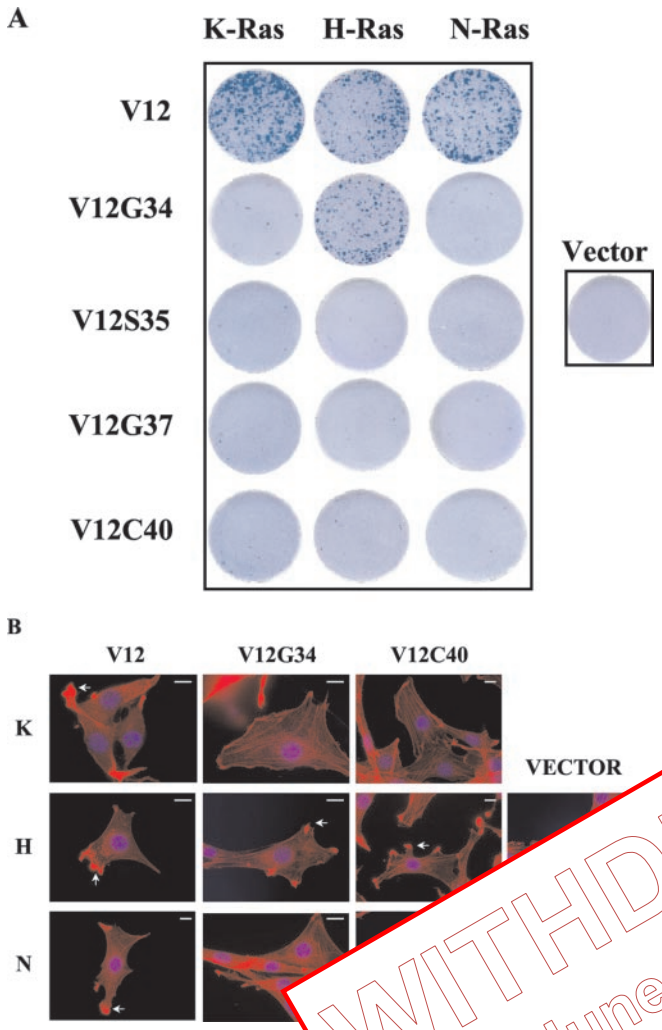

FIG. 1. **Biological properties** activity of Ras mutants. NIH 3T3 ng of each type of Ras (H-, N-, o hyperactive form (*V12*) or the effect *V12S35*, *V12G37*, and *V12C40*) in pCE the cells were transfected with the vect days the dishes were stained with Giemsa All plasmids DNAs produced similar numbers of marker-selectable colonies. Results presented correspond to a representative experiment, and similar results were obtained in four additional, separate experiments performed in triplicate. *B*, effects of overexpressed Ras mutants on actin organization. NIH 3T3 clones overexpressing different Ras mutants (*V12*, *V12G34*, and *V12C40*) or transfected only with the vector (pCEFL-KZ-AU5), as negative control, were serum-starved for 18 h and then analyzed. Actin was detected with TRITC-phalloidin, and the nuclei were stained by H-33258. The *arrows* indicate membrane ruffles.

proteins, several studies have demonstrated that the three Ras isoforms differentially activate Raf-1 and PI3K (12, 13). To ascertain possible biological differences between the three Ras isoforms, we tested the ability of mammalian expression vectors driving expression of each isoform (with different effector-binding loop mutations) to induce transforming foci after transfection into NIH 3T3 fibroblasts. To this end, NIH 3T3 cells were transfected with the hyperactive form (V12), or with the effector dominant mutants V12C40, V12G37, V12S35 (which only activate PI3K, Ral-GDS, and Raf-1, respectively) (20), and V12G34 (showing reduced binding affinity to Sur8) (17) of the three Ras isoforms (H-, N-, and K-Ras4B). We observed that overexpression of the transfected, full-length *ras V12* genes consistently and reproducibly displayed transforming activity, whereas this biological effect was not detected with the *ras V12C40*, *V12G37*, or *V12S35* mutants (Fig. 1*A*). The difference in transforming potency among the Ras isoforms was further manifested using the effector dominant mutant V12G34. As shown in Fig. 1*A*, both the N-Ras V12G34 and the K-Ras4B V12G34 mutants failed to cause transformation in NIH 3T3 fibroblasts when compared with their hyperactive forms (N-Ras V12 and K-Ras4B V12). In sharp contrast, transfection with H-Ras V12G34 resulted consistently in a number of transformed foci that was similar to that produced by H-Ras V12 (Fig. 1*A*). Furthermore, actin stress fibers were dramatically disrupted, and cells exhibited a high number of membrane ruffles and lamellipodia, when H-Ras V12G34 or any Ras V12 was overexpressed in NIH 3T3 fibroblasts (Fig. 1*B*). However, both the N-Ras V12G34- and the K-Ras4B V12G34-expressing cells (Fig. 1*B*) did not display membrane ruffles and exhibited a network of actin stress-fiber phenotypes that were similar to that of the negative control (NIH 3T3 fibroblasts transfected with the vector) and very close to that originated by any Ras V12C40 (Fig. 1*B*) (24). According to results already published, the different Ras V12C40 isoforms induced membrane ruffling and lamellipodia, although more weakly than the corresponding Ras V12 (20). Taken together, all these results suggest that the H-Ras V12G34 retained the ability to cause morphological transformation of NIH 3T3 fibroblasts, whereas both the N-Ras and K-Ras4B V12G34 mutants were defective.

Capacity of the Ras Proteins to Activate the PI3K/AKT Signaling Pathway Is Impaired by the P34G Mutation—To evaluate the Ras/Raf/MEK/ERK pathway activation, COS1 cells were transfected with the different Ras mutants (V12, V12G37, and V12C40), or with the vector as control, and the ERK1/2 phosphorylation was analyzed by immunoblotting. As shown in Fig. 2*A*, although the Ras V12 mutants induced high levels of ERK1/2 phosphorylation, the overexpression of any Ras V12G34 led to a significant reduction of activated ERK1/ERK2. In all Ras isoforms, the phospho-ERK levels induced by the Ras V12G34 mutants were lower than that generated by their corresponding Ras V12 or Ras V12S35 mutants and higher than that induced by the other Ras effector mutants (V12G37 and V12C40) (Fig. 2*A*). However, H-Ras V12G34 induced slightly higher ERK activation than N- and K-Ras4B V12G4 and lower than its corresponding H-Ras V12S35 (-fold change, 3.4 *versus* 4.3) (Fig. 2*A*). Same results were obtained using 293T or NIH 3T3 cell lines, transiently or stably transfected, respectively (data not shown). Likewise, and using an *in vitro* kinase assay, we observed a sharp reduction of the ERK2 activity induced by all Ras V12G34 mutants in comparison with the values from the corresponding Ras V12 (Fig. 2*B*). Again, the ERK2 activity induced by the H-Ras V12G34 mutant was higher than the values generated by the N-Ras V12G34 and the K-Ras4B V12G4 mutants (Fig. 2*B*).

Ras can induce gene expression via the transcription factor Elk-1 (1), which lies down-stream of the Ras/ERK cascade. To confirm the results presented above, we carried out a reporter assay using NIH 3T3 fibroblasts cotransfected with Ras constructs, together with a chimeric Gal4-Elk1 transcription factor and the reporter plasmid TATA-Gal4-Luc. Fig. 2*C* shows the results obtained in a set of experiments in which we measured the induction of luciferase activity in starved conditions, using the stimulation with EGF (100 ng/ml, 8 h) as positive control. In accordance with the above ERK results, we have also detected reduction Gal4-Elk1 activation by the Ras V12G34 and Ras V12C40 mutants *versus* their corresponding hyperactive forms (Ras V12) (Fig. 2*C*).

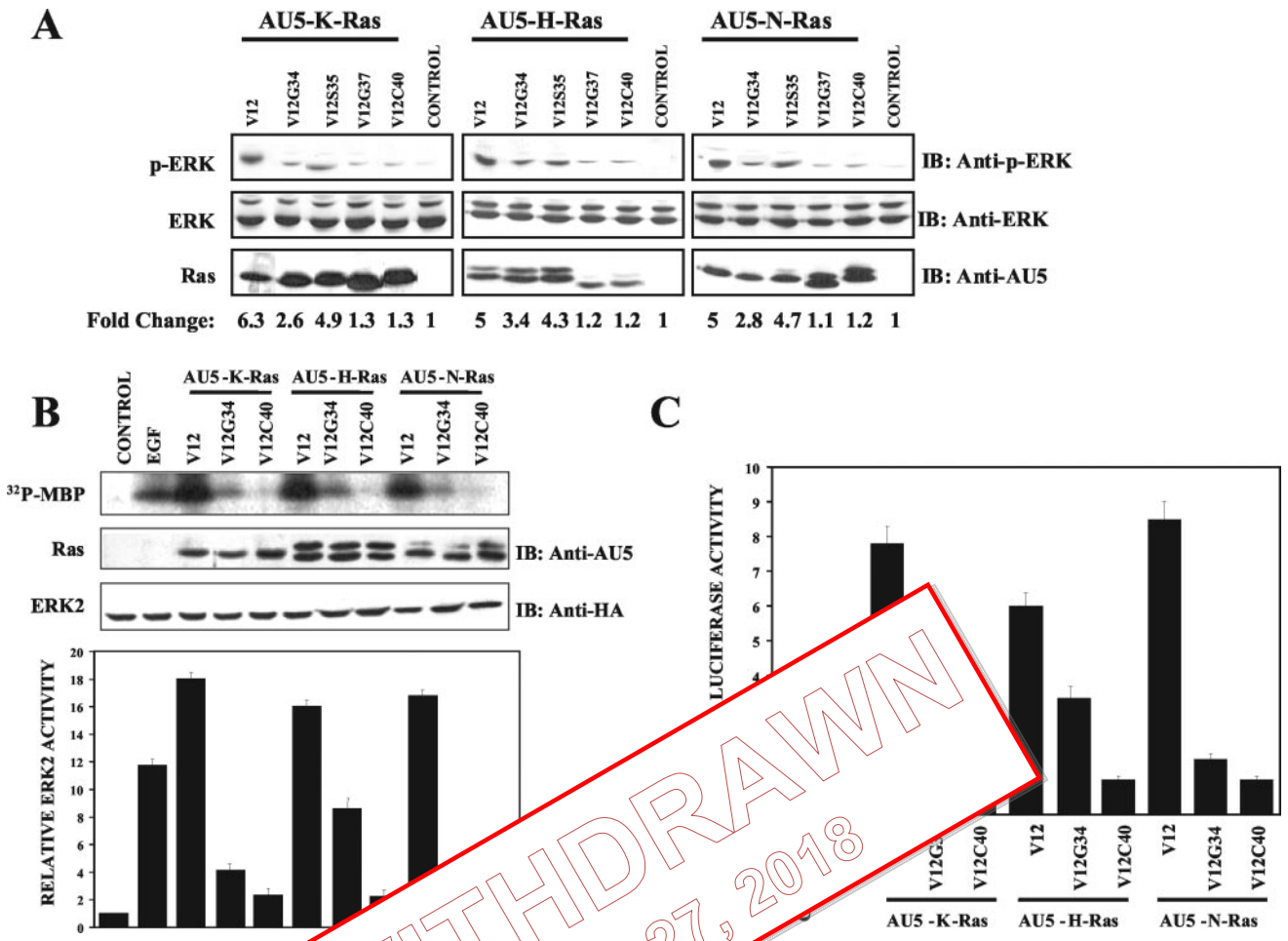

FIG. 2. **Relative activation o** 1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding t dominant mutants (*V12G34*, *V12S35*, *V12G37*, and *V12C40*) in pCEFL-KZ-AU5. As negative co ector (pCEFL-KZ-AU5). All cells were serum-starved for 18 h and then the levels of phosphorylation of E rmined using specific anti-phospho- and full antibodies. The -fold change of activation was estimated by densit values (given as arbitrary units) were normalized taking into consideration the ERK protein levels of each sample a oblot analysis. Results show a representative blot out of three. *B*, COS1 cells were cotransfected with 1 μg of ERK2 in pC μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyperactive form (*V12*) or the effector dominant mutants (*V1 2C40*) in pCEFL-KZ-AU5. As negative control the cells were transfected with the vector (pCEFL-KZ-AU5). All cells were serum- for 18 h and then analyzed, as positive control cells transfected with the vector were stimulated with EGF (100 ng/ml, 10 min). The *histo ams* represent the ERK2 relative activity, expressed as -fold induction of [^{32}P]myelin basic protein normalized with respect to control cells and correspond to the average and standard deviation of five separate assays. *C*, NIH 3T3 fibroblasts were cotransfected with the plasmid pcDNAIII-Gal4-Elk-1 encoding the Gal4-Elk1 fusion protein (containing the Gal4 DNA-binding domain and the activation domain of Elk1), the plasmid pGal4-Luc containing the *Photinus* luciferase gene controlled by six copies of a Gal4-responsive element and the plasmid pRL-TK containing the *Renilla* luciferase gene under control of the HSV-TK promoter region, together expression plasmids pCEFL-AU5 containing the indicated Ras constructs described above. Cells were serum-starved and assayed 24 h later for luciferase activity. As positive control cells transfected with the vector were stimulated with EGF (100 ng/ml, 8 h). The data represent *Photinus* luciferase activity normalized by the *Renilla* luciferase activity present in each cellular lysate, expressed as -fold induction with respect to control cells, and are the average and standard deviation of three separate assays performed in duplicate.

Similarly, the effect of P34G mutation was carried out on another Ras effector cascade, the PI3K/AKT signaling pathway. After transfection with different constructs (wt, V12, V12G34) of each Ras isoform, serum-starved COS1 cells were studied for PI3K activity using an *in vitro* kinase assay (Fig. 3*A*). In all cases, we detected lower PI3K activity in the Ras V12G34 transfectants than their corresponding Ras V12. However, the Ras V12G34 mutants induced higher PI3K activity than their wt versions (Fig. 3*A*). Likewise, and using an *in vitro* kinase assay, we observed a reduction of the AKT/PKB activity detected in all Ras V12G34 transfectants in comparison with the values obtained of the corresponding Ras V12 transfectants (Fig. 3*B*). Finally, COS1 cells were transiently transfected with the different Ras mutants (V12, V12G34, V12S35, V12G37, and V12C40), or with the vector as negative control, and AKT/PKB phosphorylation was analyzed by immunoblotting (Fig. 3*C*). Again, although Ras hyperactively (V12) induced high levels of AKT phosphorylation, the overexpression of any Ras V12G34 led to a significant reduction of activated AKT/PKB (Fig. 3*C*). The phospho-AKT levels induced by the Ras V12G34 mutants were lower than that generated by their corresponding Ras V12C40 mutants and only slightly higher than that induced by the other Ras effector mutants (V12S35 and V12G37) (Fig. 3*C*). The same results were obtained using NIH 3T3 and 293T cells (data not shown). All these results indicate that the P34G mutation diminished the capacity of Ras proteins to activate the Ras/ERK/Elk-1 and PI3K/AKT signaling pathways.

P34G Mutation in N-Ras and K-Ras4B Diminishes the Ral-GDS/Ral A Pathway Activation but Not in the Case of H-Ras—The Ral-GDS/Ral A pathway has been characterized as the third best understood Ras effector pathway (25). Again, COS1 cells were transfected with the different Ras mutants, or with the vector as negative control, and they were incubated overnight with serum-free medium. The activation of the Ral-GDS/

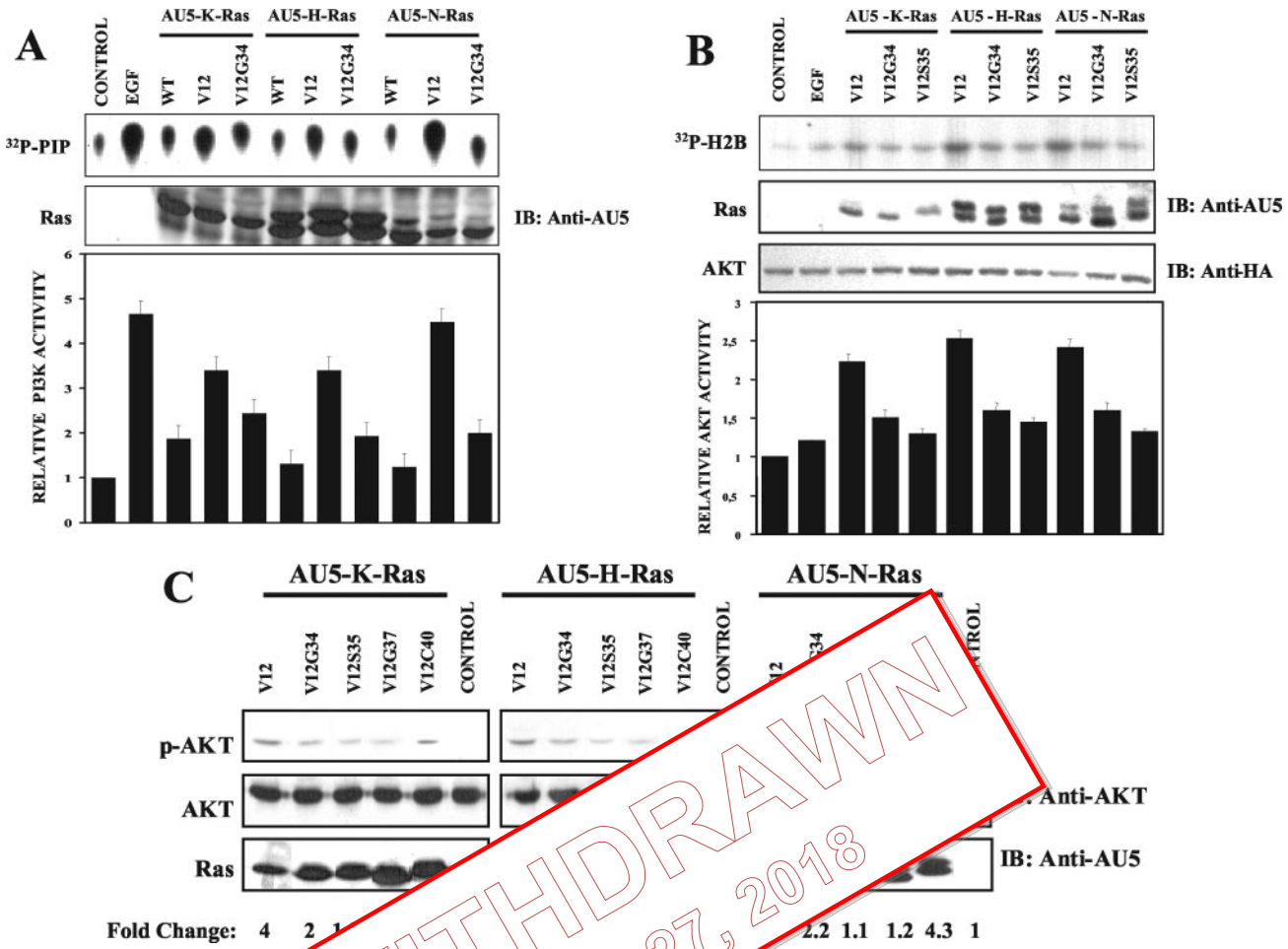

FIG. 3. **Relative activation o** COS1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), correspondin , or the effector dominant mutant (*V12G34*) in pCEFL-KZ-AU5. As negative control the cells were t 5). All cells were serum-starved for 18 h and then analyzed, and positive control cells transfected with the v 0 ng/ml, 10 min). *Histograms* represent the PI3K relative activity, expressed as -fold induction of [^{32}P]phosphat ized with respect to control cells, and correspond to the average and standard deviation of five separate assays. *B*, sfected with 1 μg of AKT in pCEFL-KZ-HA and 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyper the effector-dominant mutants (*V12G34* or *V1S35*) in pCEFL-KZ-AU5. Negative control cells were transfected with the vector AU5). All cells were serum-starved for 18 h and then analyzed, and positive control cells transfected with the vector were stimula EGF (100 ng/ml, 10 min). The *histograms* represent the AKT relative activity, expressed as -fold induction of [^{32}P]H2B normalized with re ect to control cells, and correspond to the average and standard deviation of six separate assays. *C*, COS1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyperactive form (*V12*) or the effector dominant mutants (*V12G34*, *V12S35*, *V12G37*, and *V12C40*) in pCEFL-KZ-AU5. As negative control the cells were transfected with the vector (pCEFL-KZ-AU5). All cells were serum-starved for 18 h, and then the levels of phosphorylation of AKT using specific anti-phospho- and full antibodies were determined. The -fold change of activation was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the AKT protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three.

Ral A pathway was analyzed by detection of Ral A-GTP levels using a non-radioactive method by precipitation with a GST fusion protein containing the Ral-binding domain of RIP1 (26). As shown in Fig. 4*A*, although all Ras V12 mutants induced high levels of Ral A-GTP, both the N-Ras V12G34 and the K-Ras4B V12G34 mutants led to a significant reduction of the activated Ral A pathway. In sharp contrast, H-Ras V12G34 induced Ral A-GTP levels similar to that of its hyperactive form (H-Ras V12). Moreover, the activation of Ral A, due to the V12G34 mutants of N-Ras and K-Ras4B, respectively, was of the same level of that of their corresponding V12S35 and V12C40 mutants (Fig. 4*B*), previously defined as being deficient in the induction of the Ral-GDS/Ral A pathway (20). Nevertheless, H-Ras V12G34 originated values of Ral A-GTP similar to those of H-Ras V12G37 (Fig. 4*B*), absolutely active on the Ral-GDS/Ral A pathway (20). Similar results were obtained using 293T or NIH 3T3 cell lines, transiently or stably transfected, respectively (data not shown). Thus, the P34G mutation reduces the capacity to activate the Ral-GDS/Ral A pathway only in the case of N-Ras and K-Ras4B, but not in the case of H-Ras.

P34G Mutation in N-Ras and K-Ras4B Reduces the in Vitro Binding Affinity to Ral-GDS, but Not in the Case of H-Ras—It has been demonstrated that Ras proteins interact through the effector domain with Raf (27), the p110α catalytic subunit of PI3K (24), and Ral-GDS (25). Therefore, specific point mutations at the effector domain of Ras can abolish the interaction with Raf-1 (E37G and Y40C mutations), with Ral-GDS (T35S and Y40C mutations), or with PI3K (T35S and E37G mutations) (2). The P34G mutation also maps at the effector loop of Ras proteins diminishing the binding affinity to Sur8 (17). In view of our results about the effect of the P34G mutation on the activation of Ral-GDS/Ral A pathway, we asked whether this Ras mutation could be responsible for a different Ral-GDS affinity with each Ras isoform. For this purpose, we carried out *in vitro* binding assays of the different Ras mutants with the

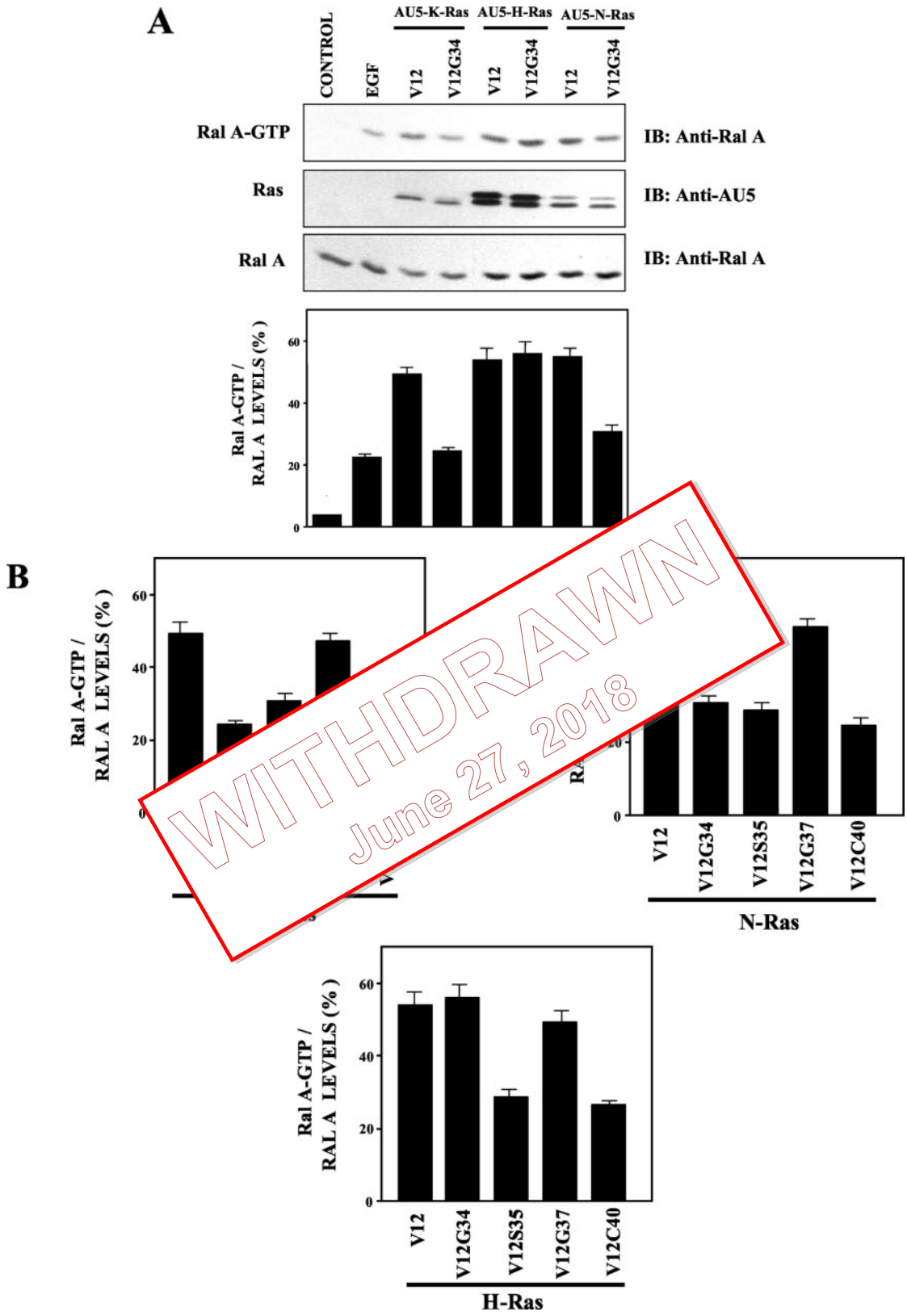

FIG. 4. **Relative activation of the Ral-GDS/Ral A pathway by Ras mutants.** COS1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyperactive form (*V12*) or the effector dominant mutants (*V12G34*, *V12S35*, *V12G37*, and *V12C40*) in pCEFL-KZ-AU5. As negative control the cells were transfected with the vector (pCEFL-KZ-AU5). *A*, all cells were serum-starved for 18 h and then analyzed, and positive control cells transfected with the vector were stimulated with EGF (100 ng/ml, 10 min). Ral A-GTP was recovered from cell lysates by binding to immobilized GST-Ral-BD and detected by immunoblotting with anti-Ral A monoclonal antibody (*upper panel*). The expression levels of the transfected AU5-Ras constructs and the endogenous Ral A were detected by immunoblotting of the cell extracts with the corresponding anti-AU5 and anti-Ral A monoclonal antibody, respectively (*middle* and *lower panels*). Results presented correspond to a representative experiment, and similar results were obtained in three additional, separate experiments. *Histograms* represent the Ral A-GTP levels normalized (by GelWoks Analyses) to Ral A levels and correspond to the average and standard deviation of four separate assays. *B*, histogram representation of four separate experiments performed as above and including the rest of effector dominant mutants. As in *A* the histograms represent the Ral A-GTP levels normalized (by GelWoks Analyses) to Ral A levels and correspond to the average and standard deviation of four separate assays.

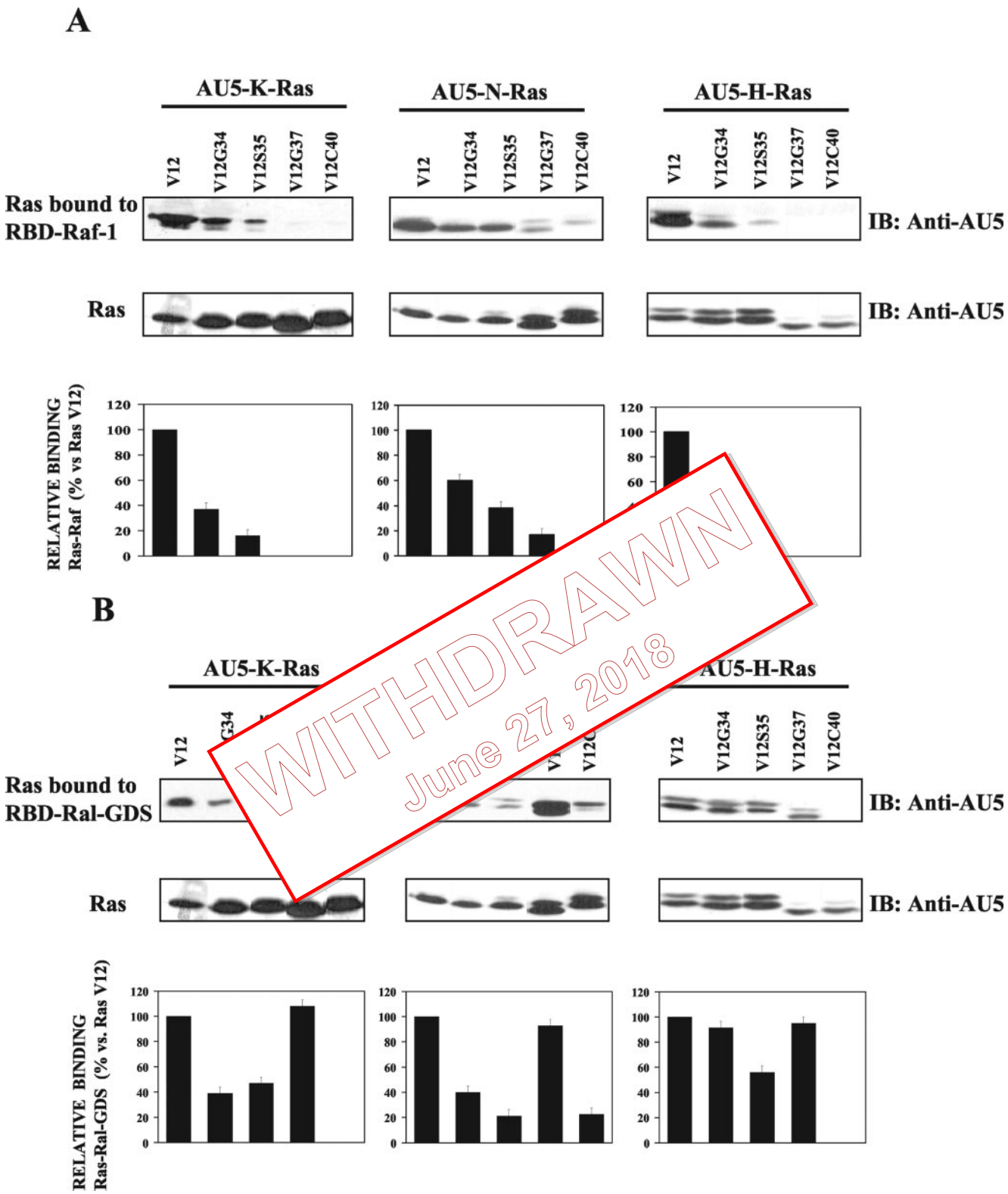

FIG. 5. ***In vitro* binding assay of Ras mutants to GST-RBD-Raf 1 and GST-RBD-Ral-GDS fusion proteins in cell-free extracts.** COS1 cell-free extracts, overexpressing the indicated AU5-Ras mutants, were incubated with 10 μg of GST-RBD-Raf 1 (*A*) and GST-RBD-Ral-GDS (*B*) proteins coupled to glutathione-Sepharose beads. After washing, cellular proteins bound to the beads were run in SDS alongside the corresponding cell extract and immunoblotted against monoclonal AU5 antibody. Results presented correspond to a representative experiment, and similar results were obtained in three additional, separate experiments. *Histograms* represent the relative binding affinity of each Ras effector dominant mutant to RBD-Raf 1 (*A*) and RBD-Ral-GDS (*B*), normalized (by GelWoks Analyses) to Ras levels and expressed as a percentage of each Ras V12-RBD binding, and correspond to the average and standard deviation of four separate assays.

Ras-binding domain (RBD) of Raf-1 and Ral-GDS. Thus, we expressed as GST fusion proteins either RBD of both Ras effector proteins. Cytoplasmic extracts from COS1 cells over-expressing different Ras mutants (V12, V12G34, V12S35, V12G37, and V12C40) were incubated with purified GST-RBD-Raf 1 or GST-RBD-Ral-GDS proteins coupled to glutathione-

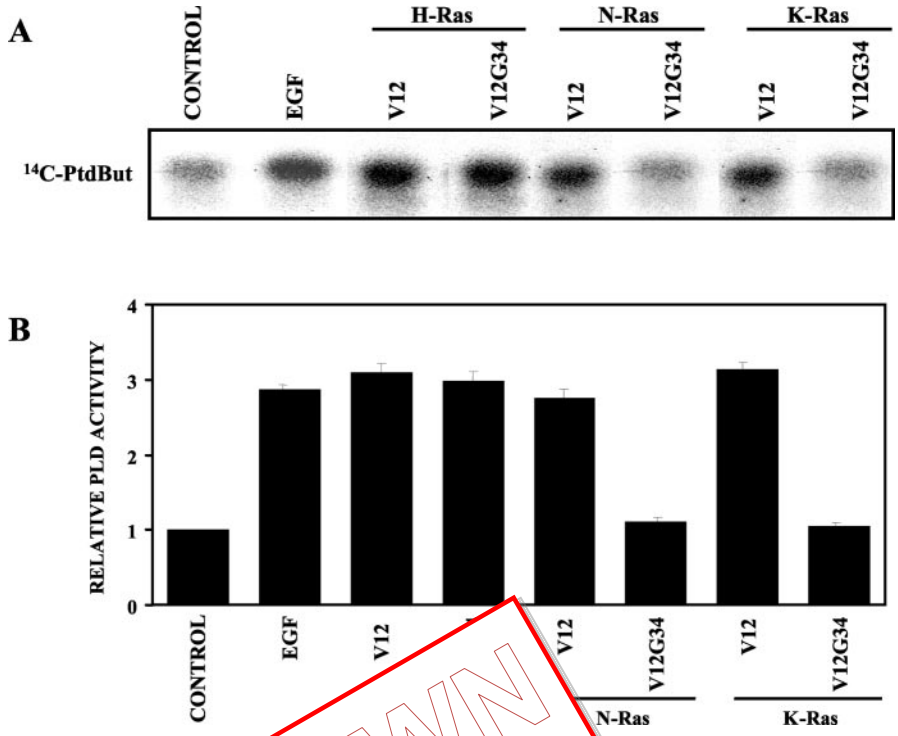

FIG. 6. **PLD activation by Ras mutants.** COS1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyperactive form (*V12*) or the effector dominant mutant (*V12G34*) in pCEFL-KZ-AU5. As negative control the cells were transfected with the vector (pCEFL-KZ-AU5). All cells were serum-starved for 18 h and then analyzed as positive control cells transfected with the vector were stimulated with EGF (100 ng/ml, 10 min). *A*, PLD activity was tested as described under "Experimental Procedures." Results presented correspond to a representative experiment, and similar results were obtained in four additional, separate experiments. *B*, the *histograms* represent the PLD relative activity, expressed as -fold induction of [^{14}C]PtdBut normalized with respect to control cells and are the average and standard deviation of five separate assays.

Sepharose beads, and the proteins bound to the beads were ... non-radioactive method by analyzed by immunoblotting (Fig. 5). Whereas all Ras V12G... ontaining the Rac-binding mutants showed reduced affinity to RBD-Raf 1 (Fig. 5... owing the same approach used H-Ras V12G34 displayed an unaffected bindi... though the P34G mutation did not RBD-Ral-GDS (Fig. 5*B*). Indeed, both the N-... binding affinity to Tiam1 (data not the K-Ras4B V12G34 mutants showed ... N-Ras V12G34 and the K-Ras4B V12G34 affinity to RBD-Ral-GDS, in sharp... d to promote activation of Rac, whereas the H-Ras with a RBD-Ral-GDS binding ... mutant retained the ability to activate this GTPase and H-Ras V12G37 (Fig. 5... 7). different levels of Ral-GDS/R... among the Ras V12G34 muta... of the N- and K-Ras4B V12G34 ... apacity to Ral-GDS.

P34G Mutation in N-Ras and ... uces Their Capacities to Activate PLD and Rac... Not in the Case of H-Ras—Because phospholipase D (PLD) acts downstream of Ral A (28), we tested whether the P34G mutation of Ras proteins affected PLD activation differently. Thus, COS1 cells were transfected with the different Ras mutants (V12 or V12G34), or with the vector as negative control, and they were incubated overnight with serum-free medium. As shown in Fig. 6, although all Ras V12 mutants induced high levels of PLD activity, both the N-Ras V12G34 and the K-Ras4B V12G34 mutants failed to activate PLD. By contrast, H-Ras V12G34 promoted similar levels of PLD activity to those of its hyperactive form (H-Ras V12) (Fig. 6). Same results were obtained using 293T or NIH 3T3 cell lines, transiently or stably transfected, respectively (data not shown). These results are in accordance with the possibility that the P34G mutation reduces the capacity to activate the Ral-GDS/Ral A/PLD pathway only in the case of N-Ras and K-Ras4B, but not in the case of H-Ras.

As it was denoted, NIH 3T3 fibroblasts overexpressing H-Ras V12G34 or any Ras V12 exhibited high number of membrane ruffles and lamellipodia (Fig. 1*B*), whereas N- or K-Ras4B V12G34-expressing NIH 3T3 cells did not display this cortical actin rearrangements. Given that Rac (another member of the Ras superfamily of GTPases) functions downstream of Ras in pathways that stimulate membrane ruffling (29) and growth transformation (30), we studied the effect of the P34G mutation on the Ras activation of Rac. The detection of Rac-

N-Ras V12G37 and K-Ras4B V12G37 Rescue the Transforming Activity of Their Corresponding V12G34 Mutants—In accordance with the results obtained, if our hypothesis, that the diminished transforming activity of N- and K-Ras4B V12G34 was due to the Ral-GDS/Ral A/PLD pathway, is correct, a complementation effect should be observed with the V12G37 mutants. To test this hypothesis we carried out an assay of transforming activity in NIH 3T3 fibroblasts. To this end, the N- and K-Ras4B effector dominant mutants (V12S35, V12G37, and V12C40) were transfected alone or in cotransfection with its corresponding V12G34 mutant (Fig. 8*A*). Focus formation was expressed as a percentage of the maximum focus-forming activity of each Ras V12 (Fig. 8*B*). Only the cotransfectants V12G34 plus V12G37 generated a statistically significant number of transforming foci (higher than the simple addition originated by each one), which partially rescued the transforming activity of each hyperactive (V12) mutant (Fig. 8*B*). Moreover, the ability to induce focus formation correlated with the efficiency of the corresponding cotransfectants in proliferating in semisolid agar (data not shown). These data suggest that both the N-Ras V12G34 and the K-Ras4B V12G34 mutants were unable to cause morphological transformation of NIH 3T3 fibroblasts due to the diminished activation of the Ral-GDS/Ral A/PLD pathway.

DISCUSSION

In this study, we have investigated the differential impact of the effector loop mutation P34G on the biological activity of the three Ras isoforms, in the context of a hyperactive mutation (G12V). Although this P34G mutation maps to a region with absolute homology among all Ras isoforms, we have observed

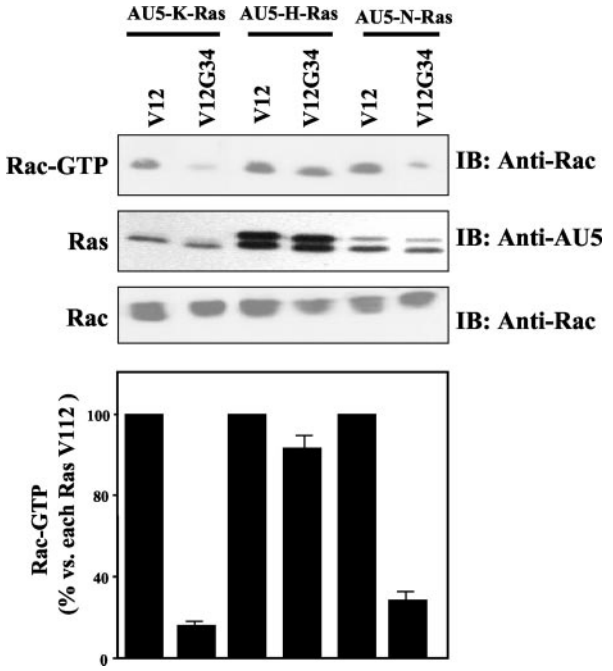

FIG. 7. **Relative Rac activation by Ras mutants.** COS1 cells were transfected with 1 μg of each type of Ras (H-, N-, or K-Ras4B), corresponding to the hyperactive form (*V12*) or the effector dominant mutant (*V12G34*) in pCEFL-KZ-AU5. As negative control, the cells were transfected with the vector (pCEFL-KZ-AU5). All cells were serum-starved for 18 h and then analyzed. Rac-GTP was recovered from cell lysates binding to immobilized GST-PAK-CRIB and detected by immunoblotting with anti-Rac monoclonal antibody (*upper panel*). The levels of the transfected AU5-Ras constructs and the were detected by immunoblotting of the cell ex... corresponding anti-AU5 and anti-Rac monoclo... (*middle* and *lower panel*). Results prese... ative experiment, and similar result... separate experiments. *Histogr...* malized (by GelWoks Analyses... centage of each Ras V12-induced... average and standard deviation o... were obtained using stably transfe...

different biological phenotypes and ... biochemical effects on the signaling pathways downstream of Ras. Thus, we found that, although the H-Ras V12G34 mutant retained the ability to cause morphological transformation of NIH 3T3 fibroblasts, both the N-Ras V12G34 and the K-Ras4B V12G34 mutants were completely devoid of transforming activity. Therefore, the actin cytoskeleton of NIH 3T3 fibroblasts overexpressing H-Ras V12G34 or any Ras V12 was identical, but not in the case of cells overexpressing N- or K-Ras4B V12G34, which showed an actin cytoskeleton typical of untransformed cells. According to a previous study (20), we found that all Ras V12S35 and V12G37 mutants failed to induce cortical actin rearrangements (ruffling or lamellipodia), whereas all Ras V12C40 mutants induced membrane ruffling but more weakly than their counterpart Ras V12. Likewise, we detected that only H-Ras V12G34 induced ruffling and lamellipodia, but not N- or K-Ras4B V12G34. Previously it has been described that H-Ras V12G34 produced transforming foci in 69BUR cells, but not in Rat2 cells (32, 33), and it was unable to induce differentiation of PC12 cells into neuronal cells (32, 34). However, this behavior is not unusual, because oncogenic versions of Ras cause cell transformation in some cells but growth inhibition in others, and they promote the differentiation of some cells yet block the differentiation of others (2). Thus, it is critical to compare the biological activity of V12G34 mutants of each Ras isoform into the same cellular background.

Ras proteins cause a diverse spectrum of cellular responses that might require multiple effectors. Thus, Ras inhibition of suspension-induced apoptosis (anoikis) is dependent on Ras activation of PI3K but not of Raf (35, 36). Conversely, Ras induction of senescence in primary fibroblasts appears to be facilitated by Raf activation alone (37). Frequently, the cooperative interaction of multiple effectors is required to originate specific actions of Ras, such as the cell cycle progression (38). Indeed, Ras inhibition of myoblast differentiation depends on several effectors, including Raf and Ral-GDS, as well as unidentified effectors (39).

Given that the transformed phenotype by oncogenic Ras requires multiple signaling pathways (20), we have analyzed the three main pathways downstream of Ras. In all Ras isoforms, the P34G mutation reduced the capacity to activate the Raf/MEK/ERK and the PI3K/AKT signaling pathways. However, the activation of these pathways was not abolished completely, we detected that cells overexpressing any Ras V12G34 showed higher activation of these pathways than the negative control or cells overexpressing Ras wt (data not shown). Indeed, the Ras V12G34 mutan... induced levels of phospho-ERK higher than their co... ling V12G37 and V12C40 mutants and slightly lo... in the case of H-Ras V12G34) than their ... 35 mutants. However, only in the c... the P34G mutation diminish t... -GDS/Ral A/PLD pathway, ... ted in its ability to induce ... nsequence of the different ..., specifically both the N-Ras ... V12G34 mutants showed very di... ding affinity to Ral-GDS-RBD in compar... counterparts Ras V12. In contrast, both H-Ras ... d H-Ras V12 displayed similar affinity to Ral-GDS-... This is the first description of a mutation in the effector loop of Ras with different impact on the *in vitro* affinity to an effector protein (Ral-GDS), depending of the Ras protein isoform. Because the effector loop is a domain with absolute homology among all Ras isoforms, the explanation could be the influence of the hypervariable C-terminal region of Ras, because it has been proposed for its different ability between K-Ras and H-Ras to activate *in vivo* Raf-1 and PI3K (12). The N-terminal 85 residues of all Ras isoforms are identical and contain the two switch regions that undergo changes on GTP binding; in addition the next 80 amino acids are 95% conserved, and the major differences between Ras proteins are confined to the hypervariable region between residues 166 and 185. However, the role of the hypervariable region in influencing the interaction of Ras with its effector protein *in vivo* has been related to different plasma location signals (7, 12), whereas here we observed differences of *in vitro* binding independently of the mechanisms of plasma attachment. On the other hand, although the structures of the RBD are similar, the thermodynamics of the interactions Ras/Raf-RBD and Ras/Ral-GDS-RBD are quite different (40), suggesting a different mode of binding that is not obvious from the three-dimensional structure (40), such as might occur with our case.

Probably, future works using a molecular dynamic approach by comparison of the three Ras V12G34 structures could discern a plausible and mechanistic explanation for these results. Furthermore, the P34G mutation reduces the *in vitro* binding affinity of Ras to Sur8 (17), and this protein displays a synergistic effect on the Ras-Raf-MEK-Erk pathway by stabilizing a ternary complex with Ras and Raf-1 (19). Notwithstanding, we have not detected any interaction (*in vitro* and *in vivo*) between

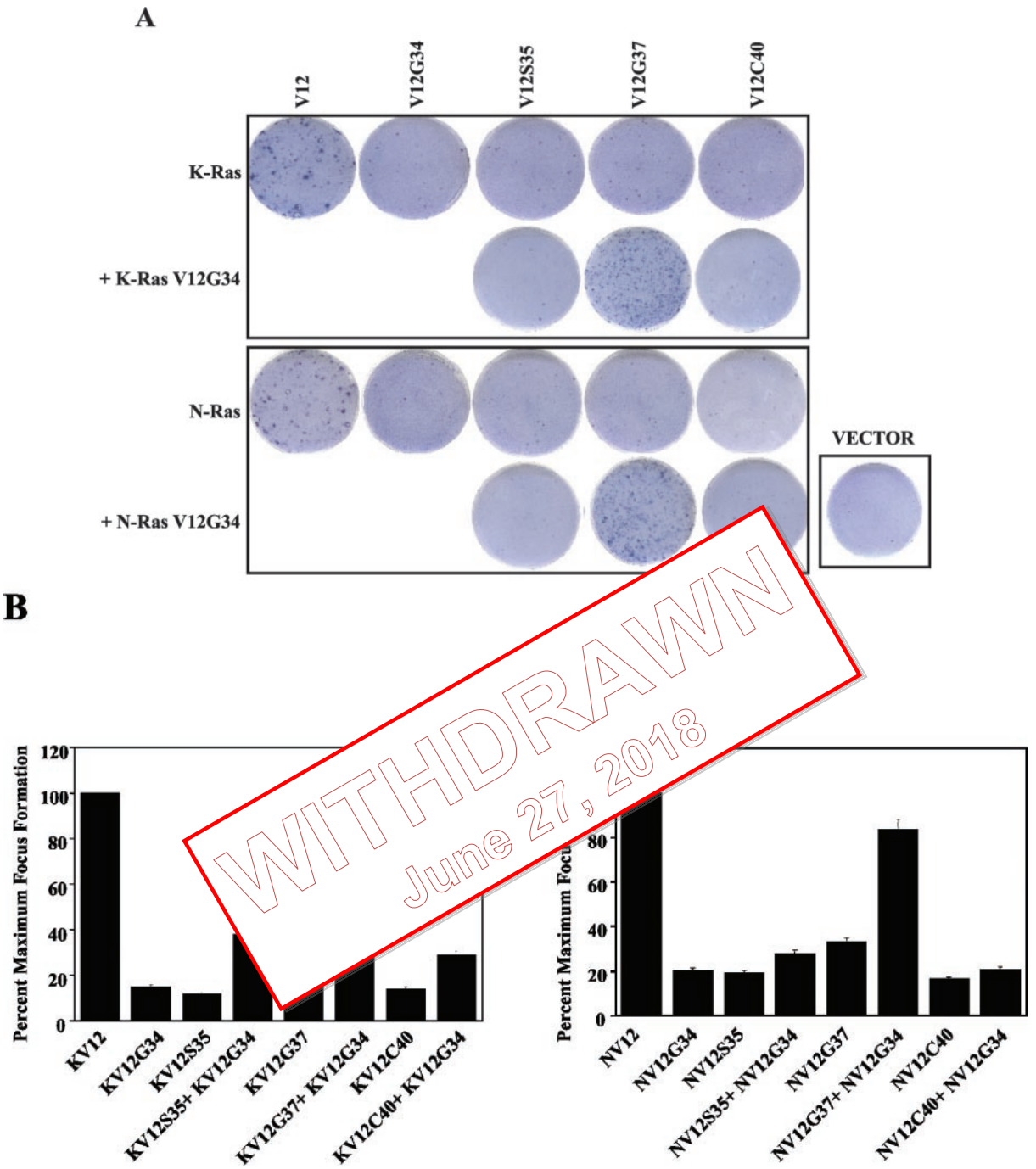

FIG. 8. **Focus formation assay in NIH 3T3 fibroblasts cotransfected with different N and K-Ras4B mutants.** *A*, NIH 3T3 fibroblasts were transfected with 25 ng of N- or K-Ras4B, corresponding to the hyperactive form (*V12*) or the effector dominant mutants (*V12G34*, *V12S35*, *V12G37*, and *V12C40*) in pCEFL-KZ-AU5 or cotransfected with the corresponding V12G34 plus V12S35, V12G34 plus V12G37, or V12G34 plus V12C40 (25 ng plus 25 ng, in all cases). As negative control the cells were transfected with the vector (pCEFL-KZ-AU5). After 14 days the dishes were stained with Giemsa to score the transformed foci. All plasmids DNAs produced similar numbers of marker-selectable colonies. *B*, focus formation was then quantitated and expressed as a percentage of each Ras V12-induced focus formation. *Histograms* correspond to the average and standard deviation of five separate assays performed in triplicate.

Sur8 and Ral-GDS[2] that could explain the different binding of Ras V12G34 mutants to this effector protein. Curiously, in all Ras isoforms, the P34G mutation reduced the *in vitro* affinity to Raf-RBD to a lesser extent than the T35S mutation, which has been proposed to affect the PI3K and Ral-GDS pathways but not the Raf pathway (20). When we analyzed the Raf/MEK/ERK activation, all Ras V12G34 mutants (especially N- and K-Ras4B V12G34) induce lower ERK activity than their corresponding V12S35 mutants. This result could be due to reduction of the stability of the Ras-Raf *in vivo* complex in the V12G34 mutants (by absence of ternary complex with Sur8) (19). In accord with this hypothesis we found that ectopic overexpression of Sur8 increased the amount of phospho-ERK induced by any Ras V12G34 to the same levels generated by Ras V12S35.[2] Given that Sur8 was described as a protein that specifically binds to N-Ras and K-Ras but not to H-Ras (17), the results could also be consistent with the idea that N- and

[2] J. L. Oliva, N. Zarich, N. Martínez, R. Jorge, A. Castrillo, M. Azañedo, S. García-Vargas, S. Gutiérrez-Eisman, A. Juarranz, L. Boscá, J. S. Gutkind, and J. M. Rojas, unpublished results.

K-Ras4B require interaction with Sur8 for their efficient transforming activity, whereas H-Ras does not in the biological system used in this study. However, some data appear to suggest that Sur8 also binds to H-Ras protein (19).[2]

Finally, we found that only the cotransfection of NIH 3T3 fibroblasts with N-Ras V12G34 and N-Ras V12G37, or K-Ras4B V12G34 and K-Ras4B V12G37, partially rescued the number of transforming foci induced by N-Ras V12 or K-Ras4B V12, respectively. This suggests that the N- and K-Ras4B V12G34 mutants were devoid of transforming activity due to the diminished activation of the Ral-GDS/Ral A/PLD pathway. The transformed phenotype by oncogenic Ras requires at least the activity of two Ras effectors pathways (20), for this reason the Ras V12G37 mutants (working in the Ral-GDS/Ral A pathway, but failing in both the Raf/MEK/ERK and the PI3K/AKT pathways) are unable to induce transforming foci as the Ras V12S35 and V12C40 mutants. Nevertheless, the activation of Raf/MEK/ERK pathway induced by each Ras V12G34 was higher than that induced by Ras V12G37 or V12C40, and similarly the activation of the PI3K/AKT pathway generated by each Ras V12G34 was also higher than that led by Ras V12G37 or V12S35. Probably, the transforming activity depends on a minimum of Ras downstream pathways activation. Thus, both the N-Ras V12G34 and the K-Ras4B V12G34 mutants generated some levels of PI3K/AKT and Raf/MEK/ERK activation, which, together the Ral-GDS/Ral A/PLD activity induced by their corresponding V12G37 mutants, were enough to produce transforming foci in the cotransfection assays. Indeed, H-R V12G34 would be able to induce transforming foci in N fibroblasts due to the perfect functionality of the R A/PLD pathway (as that originated by H-R high activity of the Raf/MEK/ERK pat activation of the PI3K/AKT pathw also detected H-Ras V12G34 the N-Ras V12G34 and th unable to activate this GTPa membrane ruffling (29) and g it has been indicated that Rac-GT H-Ras V12-transformed NIH 3T3 cells (, it is possible that the decreased PLD activat d with the P34G mutation, for N- and K-Ras 4B, mig flect impaired activation of Rac, as well as Ral, because Rac (like Ral) has been shown to be upstream of PLD (28, 30, 31). Although several studies support that Ras might activate Rac by PI3K-dependent (41) or Tiam1-dependent mechanisms (31), here we have not found significant differences between PI3K activity and Tiam1 affinity among Ras V12G34 mutants. We can only speculate at this moment about the reasons for the different behavior of the Ras V12G34 mutants on Rac activation. One explanation may reside in the perfect functionality of the Ral-GDS/Ral A/PLD pathway induced by H-Ras V12G34; *i.e.* that it did not occur with N-Ras/K-Ras V12G34 mutants. There is circumstantial evidence that Ral A may have a function in regulating the cytoskeleton through its interaction with RIP1, a GTPase-activating protein specific for the small GTPases Cdc42 and Rac (26). Furthermore, it has been denoted that Ras V12G37 mutants elicit the same Rac-GTP levels as does Ras V12 (31), suggesting that the Ral-GDS/Ral A pathway might also mediate Ras activation of Rac (1). Indeed, the activated Ras-related protein TC21 induces PI3K activity, but not the Ral-GDS/Ral A pathway, and, unlike Ras, TC21 does not activate Rac (42). Alternatively, the different Rac activation may also reflect the complex biological localization of Ras proteins. Consistent with this idea, it has been demonstrated that Ras proteins localized in plasma membrane, endoplasmic reticulum, and the Golgi apparatus can signal differently (8). Further work will allow us to answer this question and to get a comprehensive view of the different mechanisms that directly mediates Ras activation of Rac.

Although all Ras isoforms qualitatively activate the same effector pathways, we have shown here that there are marked differences in the sensitivity to an identical mutation in their highly conserved effector loop, which are likely independent of the type of plasma membrane attachment of each Ras. These differences may account for the selective activation of different Ras in specific human tumors. Therefore, we found that the cyclopentenone, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, binds to and activates H-Ras through the formation of a covalent adduct with the cysteine 184 of this isoform and that it does not occur with N-Ras or K-Ras 4B (16). Ras-dependent inputs can be activating or inhibitory signals that reach the cell cycle regulatory machinery using different downstream pathways (38). Therefore, Ras probably utilizes distinct effectors with different combinations of pathways (in some cases depending on cell context) to originate its multiple and diverse biological actions.

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The P34G Mutation Reduces the Transforming Activity of K-Ras and N-Ras in NIH 3T3 Cells but Not of H-Ras

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