Transformation Potential of Ras Isoforms Correlates with Activation of Phosphatidylinositol 3-Kinase but Not ERK*  

This article has been withdrawn by the authors. In Fig. 3A, lanes 4 and 5 were duplicated in the left ERK activity panel, which the authors state was due to an error during figure preparation. In Fig. 3D, lanes 2-5 of the AKT panel were duplicated in lanes 7-10 of the same panel. Because the original data for Fig. 3D could not be found, the authors state that they do not have a definitive means of verifying the data in question in the paper. Although the authors state that they believe the conclusions of the paper were correct, they have decided that the proper action is to withdraw this paper.
transformation and activation of ERK and PI3K. H-Ras was the most potent of the three in inducing transformation of both NIH3T3 fibroblasts and rat epithelial RIE-1 cells. While showing low activity in transformation, N-Ras and K-Ras were the stronger activators of the MAP kinase and Elk-1-dependent transcription. Interestingly the transformation potentials of Ras isoforms correlated with their abilities to activate PI3K in contrast to the widely accepted model that activation of the MAP kinase pathway plays the major role in Ras-induced transformation. Our results demonstrated the differential activities of the three Ras proteins in transformation and activation of downstream targets and also showed that activation of PI3K, but not MAP kinase, was a limiting factor in Ras-induced cellular transformation.

MATERIALS AND METHODS

Cell Culture and Transfection Conditions—NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum in a humidified atmosphere of 10% CO2. Human embryonic kidney (HEK) 293 and rat intestinal epithelial (RIE-1) cells were cultured in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2. PC12 cells were cultured in DMEM supplemented with 5% fetal bovine serum and 10% horse serum. Transfections of NIH3T3, HEK293, and PC12 cells were performed by LipofectAMINE method as suggested by the manufacturer (Invitrogen).

Plasmids—cDNAs for human H-ras, K-ras, and N-ras were obtained from Drs. Quilliam and Vojtek (28, 29). The H-ras cDNA and N-ras were amplified by PCR and subcloned into the EcoRI and BamHI sites of pCDNA3 (Invitrogen). K-ras cDNA was amplified by PCR and subcloned into the BamHI site of pCDNA3 (Invitrogen). Mutation of ras was created by the Quik Change site-directed mutagenesis (Stratagene). ras plasmids were confirmed by DNA sequencing. The plasmids of FLAG-p85 and HA-p110 were from Drs. Skolnik (New York University Medical Center) (30). Transformant (31) and Elk (32) are laboratory stocks.

Transformation Assay—Transformation was performed following standard protocols (33). Cells were plated in 6-well plates (30 mm) and cultured in DMEM supplemented with 10% fetal bovine serum (100 mm). ras DNA. Cells were transfected with 100 ng of p110 and p85 plasmids. Where indicated, varying amounts of H-ras12V, K-ras12V, and N-ras12V were co-transfected. 24 h after transfection, cells were stained by crystal violet as described previously (33). Activity of Ras transformation was measured as described previously (33). Activity of co-transfected β-galactosidase was also determined and used to normalize the luciferase activity.

Western Blot—pCDNA3-HA-Elk-1 plasmid (300 ng) (32) was transfected into NIH3T3 cells together with different Ras plasmids. Transfected cells were starved in serum-free medium for 24 h after transfection. Luciferase activity was measured as described previously (33). Activity of co-transfected β-galactosidase was also determined and used to normalize the luciferase activity.

RESULTS

Different Transforming Activities of H-, K-, and N-Ras in NIH3T3 and RIE-1 Cells—Functional differences between the three Ras isoforms, H-, K-, and N-Ras, have not been appreciated until recently (5, 25, 26, 40). Gene knock-out experiments clearly demonstrate that the three Ras genes have different biological functions. In addition, it has been shown that H-Ras is more potent than N-Ras in P13K activation (5, 27). In contrast, K-Ras is more potent than H-Ras in Raf activation. We observed that expression of the GTP-bound V12 mutant of the three Ras proteins under control of the human CMV promoter
produced dramatically different results with respect to cellular transformation in NIH3T3 cells. The focus formation assay in NIH3T3 cells indicated that H-RasV12 was a very potent inducer of transformation (Fig. 1, A and B), while K-RasV12 and N-RasV12 showed low transforming activity. The foci formed by H-RasV12-transformed cells were typical of Ras transformation: highly refractive with irregular edges (Fig. 2A). To exclude the possibility that the inability of K-Ras or N-Ras to transform NIH3T3 cells was due to different levels of expression, immunoblots with antibodies recognizing all isoforms of Ras were performed. The three Ras constructs showed similar levels of protein expression (Fig. 1C).

It has been shown that Ras can transform rat intestinal epithelial cells (34). We tested the three active RasV12 mutants in transformation of RIE-1 cells. Similar results were obtained showing that H-Ras is more potent in transformation than K-Ras and N-Ras in RIE-1 cells (data not shown). These results confirmed that the different transformation activities of different Ras isoforms were not restricted to fibroblasts but were also observed in epithelial cells.

Transforming Phenotypes of H-, K-, and N-Ras Stable Cell Lines—The focus formation assay described above was based on the ability of transiently transfected cells to grow on a confluent monolayer. To extend the results of transient transfections, we examined the transformation phenotype of stable Ras cell lines. Stable cell lines of NIH3T3 cells transfected with one of the three Ras constructs were selected by resistance to G418. The cells in H-RasV12-transfected colonies were highly refractive, typical of transformation (Fig. 2, A and B). In contrast, the majority of cells in K-RasV12-transfected colonies were flatter and did not exhibit features of transformation (Fig. 2A). Cells in N-RasV12-transfected colonies showed morphology significantly different from those of vector-transfected cells (Fig. 2B). The N-Ras-transfected cells showed a very regular pattern of cell type and did not grow in soft agar under similar conditions (Fig. 2C). In contrast, the K-Ras- or N-Ras-transfected clones exhibited less transformed phenotype and did not grow in soft agar under similar conditions (Fig. 2C). The expression levels of Ras were similar in these stable cell lines (Fig. 2D). These results further confirmed that H-Ras was more potent in transformation than K-RasV12 or N-RasV12 under our assay conditions.

Activation of ERK1 and PI3K by RasV12 Isoforms—One of the major downstream effectors of activated Ras is the Raf-MEK-ERK kinase pathway (3, 4, 8). To test whether the low transforming activity of K-RasV12 and N-RasV12 correlated with their inability to activate the ERK pathway, we determined ERK activation by Ras. Myc-tagged ERK1 was co-transfected with increasing amounts of a Ras plasmid, and the ERK kinase activity was determined. H-RasV12 stimulated ERK in a dose-dependent manner (Fig. 3A). Interestingly both K-RasV12 and N-RasV12 were very potent in activation of ERK (Fig. 3A). While maximal ERK activation levels achieved by the three Ras isoforms were similar at high concentrations of DNA, at low concentrations of DNA, K-RasV12 and N-RasV12 were more effective than H-RasV12 in ERK activation (Fig. 3A). These data suggested that although K-RasV12 and N-RasV12 effectively activate ERK1, they showed low transforming activity compared with H-RasV12. The low transforming ability of K-RasV12 and N-RasV12 was not due to a defect in ERK activation. These results also suggested that activation of ERK by oncogenic Ras was not sufficient to induce NIH3T3 cell transformation at least under these experimental conditions.

Ras has been shown to bind and activate PI3K (15, 41). The
effect of Ras expression on PI3K activity was determined. Whereas all three Ras proteins activated PI3K, H-RasV12 was the most potent PI3K activator (Fig. 3, B and C). It is noteworthy that activation of PI3K required the transfection of Ras DNA at higher doses than was required for ERK activation. In addition, the maximal level of activation of PI3K by K-RasV12 or N-RasV12 was still lower than that achieved by H-RasV12, while the maximal activation of ERK1 by the three RasV12 was comparable.

The lipid products of PI3K are known to bind pleckstrin homology domains existing in numerous signaling molecules (18). Binding of the PI3K products to the pleckstrin homology domain of phospholipid-dependent kinase 1 increases the kinase activity of phospholipid-dependent kinase 1, which can subsequently phosphorylate and activate AKT, which also contains a pleckstrin homology domain (21). We determined the activation of AKT by Ras (Fig. 3D). HA-AKT was co-transfected with Ras in NIH3T3 cells. The transfected AKT was immunoprecipitated and assayed for kinase activity using histone 2B as a substrate. H-RasV12 was the most potent activator of AKT, while K- and N-RasV12 displayed a much lower AKT activation. Our data showed that activation of AKT by different Ras isoforms correlated with the relative activation of PI3K.

We extended our studies to include different cell lines. Both ERK and PI3K activation were examined in HEK293 cells. HEK293 cells can be transfected with higher efficiency than NIH3T3 cells; therefore, less RasV12 DNA was needed to stimulate ERK activity. Again N-Ras was most potent in ERK activation, while H-Ras was the weakest ERK activator in HEK293 cells (Fig. 4A). However, high concentrations of H-RasV12 (200 ng) activated ERK1 to a degree (~500-fold) similar to that activated by K- or N-RasV12 (data not shown). As a control, EGF effectively activated ERK1 in HEK293 cells. PI3K activity was also determined in 293 cells in response to co-expression of the different Ras isoforms. Although all three Ras proteins were able to activate PI3K, H-RasV12 was the most potent PI3K activator (Fig. 4, B and C), similar to the results obtained with NIH3T3 cells (Fig. 3, B and C). These observations further confirmed that H-RasV12, K-RasV12, and N-RasV12 displayed different activities in stimulating ERK and PI3K. Our results also indicated a correlation between PI3K activation and transformation by Ras.
Fig. 3. Activation of ERK and PI3K in NIH3T3 cells. A, N-RasV12 and K-RasV12 are more potent than H-RasV12 in ERK1 activation. NIH3T3 cells were co-transfected with 200 ng of Myc-ERK1 and increasing concentrations of pCDNA3-rasV12 plasmid. 24 h after transfection, cells were starved in serum-free medium for 12 h. Myc-ERK1 was immunoprecipitated with anti-Myc antibody. ERK1 kinase activity was determined using GST-Elk-1 as a substrate (top panel). The amount of Myc-ERK1 in immunoprecipitates was determined by Western blot with anti-ERK antibody (middle panel). The anti-ERK Western blots of the left and right panels were performed separately. Therefore, the relative intensities of the left panel should be compared within the left panel but not with that of the right panel. The expression of Ras was determined by Western blot of cell lysates with anti-Ras antibodies, which recognize all three forms of Ras as indicated (bottom panel). Lanes 1 in the right and left panels are negative controls with no Myc-ERK1 transfection. All other lanes are samples transfected with Myc-ERK1. Lanes 2, lane 3 in the left panel, and lane 2 in the right panel are control transfections without ras DNA. ras DNA used in the left panel was 1 and 3 ng, respectively. Similarly ras DNA used in the right panel was 10, 50, and 200 ng, respectively. The results are representative of four independent experiments.

B, H-RasV12 is more potent than K-RasV12 and N-RasV12 in PI3K activation in NIH3T3 cells. NIH3T3 cells were transfected with FLAG-p85 (the regulatory subunit of PI3K) and HA-p110 (the catalytic subunit of PI3K) together with varying amounts of rasV12 plasmid. PI3K was
Activation of Elk-1-dependent Transcription and Elk-1 Phosphorylation—One of the downstream effects of the Ras-MAP kinase pathway is the phosphorylation and activation of the Elk-1 transcription factor (10). Expression of active Ras results in phosphorylation of Elk-1, which can be easily detected by anti-phospho-Elk antibody. Our results indicated that Elk-1 phosphorylation was stimulated by co-expression of H-RasV12, K-RasV12, or N-RasV12 (Fig. 5A). The abilities of the three Ras isoforms to stimulate Elk-1 phosphorylation generally correlated with their abilities to stimulate ERK activation. K-RasV12 and N-RasV12 were much more effective in inducing Elk-1 phosphorylation than H-RasV12 (Fig. 5A).
ternary complex factors (Elk-1 is a member of ternary complex factor) plays a critical role in the induction of the c-fos proto-oncogene (10). We therefore tested the effect of Ras on a c-Fos reporter, which expresses luciferase under the control of the c-fos promoter (37). Both K-RasV12 and N-RasV12 stimulated the c-Fos reporter more efficiently than H-RasV12 (Fig. 5B), although all three Ras isoforms stimulated the c-Fos reporter to a similar extent at high DNA doses (data not shown). Induction of c-Fos promoter qualitatively correlated with the phosphorylation of Elk-1.

AKT Cooperates with Ras in Transformation—To further characterize the correlation between the low transformation activity of K- and N-Ras and their reduced ability to activate PI3K and AKT, we examined the possibility that enhanced AKT activity might cooperate with Ras in cellular transformation. AKT is an important downstream target of PI3K and has been implicated in cellular transformation (16, 23, 24). The constitutively active AKT, when co-transfected with ras plasmids, enhanced the transforming activity of Ras (Fig. 6). The low quantity of AKT, K-RasV12, or N-RasV12 alone did not induce focus formation in NIH3T3 cells under our assay conditions. However, the combination of constitutively active AKT

Fig. 5. K-RasV12 and N-RasV12 are functional in transcription induction. A, K-RasV12 and N-RasV12 are more potent than H-RasV12 in stimulation of Elk-1 phosphorylation. NIH3T3 cells were transfected with 300 ng of Elk-1 plasmid except lane 1, which was not transfected with Elk-1. Various Ras plasmids were co-transfected as indicated. The transfected cells were starved in serum-free medium for 12 h before lysis and Western blot with anti-Elk-1 antibody (bottom panel) or anti-phospho-Elk-1 (pElk-1) antibody, which recognizes the phosphorylated and active Elk-1 (top panel). Lane 2, control with no ras. Lanes 3, 4, and 5 were co-transfected with 10, 50, and 200 ng of H-rasV12, respectively. Lanes 6, 7, and 8 were co-transfected with 10, 50, and 200 ng of K-rasV12, respectively. Lanes 9, 10, and 11 were co-transfected with 10, 50, and 200 ng of N-rasV12, respectively. Results shown are a representative of three similar experiments. B, activation of c-Fos reporter by Ras. NIH3T3 cells were transfected with a c-fos reporter, β-galactosidase control, and varying amounts of ras. 24 h after transfection, cells were starved in serum-free medium for 12 h. Cells were lysed, and luciferase activity was determined and normalized against the co-transfected β-galactosidase activity. Lane 1 is a control without Ras co-transfection. Lanes 2, 3, 4, and 5 were co-transfected with 1, 3, 10, and 50 ng of H-rasV12, respectively. Lanes 6, 7, 8, and 9 were co-transfected with 1, 3, 10, and 50 ng of K-rasV12, respectively. Lanes 10, 11, 12, and 13 were co-transfected with 1, 3, 10, and 50 ng of N-rasV12, respectively. C, expression levels of co-transfected Ras correspond to the bar graph of luciferase assay in B.

Fig. 6. Cooperation between AKT and Ras in transformation. Focus formation assay of NIH3T3 cells were performed by transfection with 100 ng of Ras plasmid alone (left column) or co-transfection with 200 ng of constitutively active AKT (right column). DNAs used for each transfection are indicated in the top left panel. Results shown are representatives of three independent experiments. MEK1* denotes a constitutively active MEK1 mutant.
with either K-RasV12 or N-RasV12 resulted in efficient transformation (Fig. 6). AKT also enhanced H-RasV12 in transformation (Fig. 6). Co-transfection of AKT with the constitutively active MEK1 also enhanced the transformation potential albeit the colonies were smaller. These data demonstrated that AKT had a critical role in cellular transformation and that the low transforming activities of K- or N-Ras were likely due to a low activation of PI3K.

**DISCUSSION**

Although it is generally accepted that H-, K-, and N-Ras have similar biochemical and biological functions, gene inactivation studies and in vitro characterization indicate that the three Ras proteins may have different functions or activities. In this study, we showed that H-RasV12 had the highest transformation potential in NIH3T3 cells. We were surprised to find that the three RasV12 mutants showed dramatic differences in their abilities to transform cells in culture given their high degree of sequence identity. These results were confirmed in RIE-1 cells. The different transformation potentials of the three Ras proteins might be due to a difference in interaction/activation of downstream targets. We tested the interaction of several known downstream Ras targets. Our data showed that the V12 mutants of all three Ras proteins interacted similarly with C-Raf, PI3K, and Ral guanine nucleotide dissociation stimulator in a GTP-dependent manner in the yeast two-hybrid system (data not shown).

It has been shown that H-Ras activates PI3K better than K-Ras, while K-Ras activates Raf better than H-Ras (5, 27). Our data are completely consistent with the previously reported observations. We further extended previous observations and measurements in the following aspects. We investigated transformation activities of the three Ras isoforms and their abilities to activate MAP kinase cascades. Our data were consistent with published results (23).

Ras is known to directly interact with downstream targets to transmit its signal. Paradigmatically, N-terminal 86 residues including the effector domain (residues 30–42) are absolutely conserved among the three Ras proteins. This effector domain of Ras is believed to mediate the interaction between Ras and its downstream targets. Therefore, it is reasonable to assume that the differential activities of Ras isoforms are due to the C-terminal variable domain. Previous studies indicate that the C-terminal domain of Ras plays a role in transformation (42, 43). Interestingly the C-terminal domains of Drosophila and Caenorhabditis elegans Ras are more similar to K-Ras than H-Ras. It should be noted that all the experiments described in this report used mutationally activated Ras. The functions of endogenous Ras genes will certainly depend on the relative expression of Ras proteins in different tissues.

H-RasV12 showed a weaker ability to activate co-transfected ERK1 than K-RasV12 or N-RasV12 in both NIH3T3 and HEK293 cells at low DNA concentrations. However, H-Ras activated ERK to the same extent as K-RasV12 or N-RasV12 at higher concentrations. The same did not hold for PI3K activation. Even at high concentration, neither K-RasV12 nor N-RasV12 activated PI3K to the same extent as H-RasV12. Furthermore activation of PI3K required a much higher concentration of Ras DNA than was required for ERK activation. Another noticeable difference between ERK and PI3K activation by Ras was the magnitude of activation. The maximal PI3K activation by Ras was ~10-fold above basal, yet ERK was activated as much as 500-fold. This difference could be attributed to a higher basal activity of PI3K or the amplification effect of the Raf-MEK-ERK kinase cascade. Alternatively Ras might be a more potent activator of ERK than PI3K. Nevertheless the above observations were consistent with the model that activation of PI3K, but not ERK, was the limiting factor in Ras transformation.

Constitutive activation of MEK-ERK has been shown to induce transformation in NIH3T3 cells (11, 12), and therefore, activation of ERK is generally thought to mediate the transforming potential of Ras. It is worthy to note that focus formation by constitutively active MEK was achieved with stable cell lines expressing the constitutively active MEK (12). The constitutively active MEK failed to induce foci under the conventional focus formation assay conditions used for Ras and other oncogenes. Point mutants of Ras that fail to activate ERK are also defective in inducing transformation (12). These observations suggest that ERK plays an important role in NIH3T3 transformation. Results in this study showed that both K-RasV12 and N-RasV12 were more effective than H-RasV12 in ERK activation (Fig. 6). This suggested that H-Ras might be a more potent activator of ERK than PI3K. Therefore activation of ERK alone might not be sufficient to induce cellular transformation. It is possible that factors of Ras could be involved in the transformation of NIH3T3 cells.

It is worth noting that mutation of K- and N-Ras is more frequent than mutation of H-Ras in human cancers. How can our results be reconciled with existing data for an important role of K- and N-Ras in human cancer? Another puzzling question is how mutation in B-Raf, which is frequently found in human cancers and only activates the ERK pathway (14), contributes to human cancer. The NIH3T3 and RIE-1 transformation assay is based on expression of a single transforming gene. However, human cancers arise from multiple genetic alterations. For example, mutation in the PTEN tumor suppressor gene occurs in ~50% of human cancers (45–47). PTEN mutation results in constitutive activation of the AKT pathway, which provides a major signal to stimulate cell growth and inhibit apoptosis (48–50). Furthermore genetic alterations resulting in suppression of apoptosis, such as mutations in Bcl-2 family members (some are targets of AKT) (51, 52), may also substitute for the requirement of PI3K activation in cancer growth. In PTEN mutant cells, no additional genetic change is needed to activate AKT; therefore, activation of the ERK pathway by mutations, such as K-Ras, N-Ras, and B-Raf, may be sufficient to cause tumorigenesis. This notion is consistent with the high potential of K- and N-Ras to activate the ERK path-
way and the high frequency of K- and N-Ras mutations found in human cancers.

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