Oncogenic Ras Signals through Activation of Both Phosphoinositide 3-Kinase and Rac1 to Induce c-Jun NH$_2$-Terminal Kinase–Mediated, Caspase-Independent Cell Death

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Abstract

Cells avert the development of malignancy in response to deregulated oncogene expression by activating a regulated cell death pathway. However, the molecular mechanism underlying this oncogene-induced cellular death process remains unclear. Here, we show that retroviral expression of oncogenic H-ras induced cell death in a caspase-independent manner in normal cells. Inhibition of c-Jun NH$_2$-terminal kinase (JNK) by pretreatment with SP600125 or a dominant-negative form of JNK blocked cell death. Rac1 and phosphoinositide 3-kinase (PI3K) were activated in cells overexpressing oncogenic H-ras. Inhibition of Rac1 with RacN17, a dominant-negative form of Rac1, attenuated oncogenic H-ras–induced JNK activation and subsequent cell death. Interestingly, inhibition of PI3K with LY294002 or by small interfering RNA–mediated knockdown of PI3K p85 or p110 subunits also clearly attenuated JNK activation and cell death. No cross talk was observed between Rac1 and PI3K, indicating that these pathways operate in parallel. Our findings show that JNK is necessary for oncogenic H-ras–induced, caspase-independent cell death, and that both PI3K and Rac1 activities are required for JNK activation and cell death. Determining the molecular mechanisms that mediate cell death responses to deregulated oncogenes provides a more refined understanding of cellular disposal processes in normal cells and increases our appreciation of these events as a mechanism for protecting against malignant progression. (Mol Cancer Res 2009;7(9):1534–42)

Introduction

Oncoproteins of the Ras family have been extensively studied due, in part, to their involvement in human cancer. Ras oncoproteins have generally been associated with the initiation of cell proliferation and suppression of apoptosis, consistent with the involvement of Ras activation as a signal transduction intermediate in a number of pathways (1–4). However, under certain circumstances, oncogenic Ras can initiate cell death (2–5), extending the functional range of this protein superfamily. Induction of cell death by oncogenic Ras is important in preventing the development of abnormalities and malignancies and is thus an important mechanism for tumor suppression. Despite the shown involvement of oncogenic Ras in the induction of some cell death processes, relatively little is known about the molecular and biochemical basis for oncogenic Ras–induced cell death.

c-Jun NH$_2$-terminal kinases (JNK) represent a subgroup of mitogen-activated protein kinases (MAPK) that are activated primarily by cytokines and exposure to environmental stress. Numerous reports have provided evidence that JNK can function as a proapoptotic kinase in response to a variety of stimuli, including tumor necrosis factor (6), UV irradiation (7), cytokines (8), ceramide (9), and chemotherapeutic drugs (10). In these studies, the JNK pathway has been shown to activate caspases and may also target other factors that have been implicated in the regulation of apoptosis, including p53, Bcl-2, and Bax (11–13). Moreover, recent reports have shown that the JNK pathway is involved in caspase-independent cell death in response to certain stimuli, such as oxidative stress and chemical hypoxia (10, 14).

The ras-like GTPase Rac1 can synergize with the serine/threonine kinase Raf to promote activation of extracellular signal–regulated kinase (ERK; ref. 15) and also acts as an intermediate between H-ras and MAPK kinase in the signaling cascade leading from growth factor receptors to MAPK activation (16, 17). Rac1 is also a downstream effector of phosphinositide 3-kinase (PI3K) and has been identified as a regulator of the JNK and p38 MAPK pathways (18, 19). Interestingly, Rac1 has been shown to mediate apoptotic cell death in a wide range of cell types in response to diverse apoptotic stimuli (20, 21). In apoptotic cell death, Rac1 is thought to mainly regulate the activation of the JNK and/or p38 MAPK pathways (20, 22, 23).
PI3K is a well-known downstream effector of activated Ras (24) that controls cell motility and other cellular responses in both physiologic and pathophysiologic states (25, 26). Among the functions of PI3K that have been clearly established by earlier studies is a cell survival–promoting ability. Recent studies have indicated a contrasting role for PI3K, providing several lines of evidence to support the involvement of PI3K in promoting apoptotic cell death (27-30). For example, PI3K has been implicated in the induction of apoptosis in response to endoplasmic reticulum stress (27), IFN-α (28), and amyloid β/ glutamate (29) and is involved in calcium-mediated cell death (30). These findings indicate that PI3K activates two functionally opposing pathways, depending on the cell type, nature of the stimulus, and extent of cross talk with other signaling pathways.

In this study, we investigated the molecular mechanism of oncogenic H-ras–induced cell death. We show that the JNK pathway is essential for this H-ras–induced cellular disposal process, and show that activation of both Rac1 and PI3K is required for JNK activation and subsequent cell death.

Results
Oncogenic Ras Induces Caspase-Independent Cell Death in Normal Cells

In response to deregulated oncogene expression, cells activate cellular disposal programs, such as programmed cell death, which prevent the development of malignancies. To investigate whether the Ras oncogene induces cell death in normal fibroblasts, we infected Rat2 cells with a MFG retroviral expression vector for Ras-family genes (MFG-H-ras, MFG-K-ras, MFG-N-ras, and MFG-R-ras) or the oncogenic mutant forms of H-ras (MFG-H-rasV12) and K-ras (MFG-K-rasV12), and analyzed induction of cell death by flow cytometry analysis. As shown in Fig. 1A, overexpression of wild-type H-ras or K-ras induced significant cell death in Rat2 cells. Infection with the oncogenic forms of H-ras (MFG-H-rasV12) or K-ras (MFG-K-rasV12) induced cell death at an even higher rate. By contrast, Rat2 cells overexpressing N-ras or R-ras showed a relatively low level of cell death: more than 30% of Rat2 cells underwent cell death by 72 hours in response to H-rasV12 expression (Supplementary Fig. S1A). Inhibition of Ras effector activation with ectopic expression of dominant-negative form of Ras (RasN17) significantly attenuated oncogenic H-ras–induced cell death in Rat2 fibroblasts (Supplementary Fig. S1B). Oncogenic H-rasV12 and K-rasV12 also induced cell death in two different normal fibroblasts cell lines (NIH3T3 and WI38) and in primary fibroblasts and astrocytes (Fig. 1C). To determine whether caspase activity is required for oncogenic H-ras– and K-ras–induced cell death, we looked for evidence of caspase processing and investigated the effects of caspase inhibition. Pretreatment of H-rasV12– or K-rasV12–overexpressing cells with the broad-spectrum caspase inhibitor z-VAD-fmk did not attenuate oncogenic Ras–induced cell death (Fig. 1C). Furthermore, Western blot analyses failed to detect the cleavage of procaspases to active caspases (Fig. 1D). These results indicate that oncogenic Ras–induced cell death occurs in a caspase-independent fashion.

JNK Is Required for Oncogenic H-ras–Induced Cell Death

MAPK is a well-known downstream effector of Ras that has been implicated in diverse Ras-induced cellular events (1, 31). To investigate the potential involvement of MAPK in oncogenic Ras–induced cell death, we infected cells with an expression construct for H-rasV12 and analyzed the activation status of ERK, JNK, and p38 MAPK by immunoblot analysis using antibodies specific to the phosphorylated forms of these kinases. As expected, overexpression of H-rasV12 resulted in marked increases in the phosphorylated forms of all three MAPKs, indicating that these kinases were activated in response to H-rasV12 (Fig. 2A). To determine whether activation of MAPK was required for oncogenic H-ras–induced cell death, we pretreated cells with specific chemical inhibitors of MAPKs or with dominant-negative forms of MAPK and then infected them with H-rasV12. As shown in Fig. 2B, pretreatment with SP600125, a JNK-specific inhibitor, clearly attenuated H-rasV12–induced cell death. However, pretreatment with PD98059, a MAPK kinase inhibitor, did not. A dominant-negative form of JNK2 (pCDNA3.1-Flag-DN-JNK2) also selectively inhibited H-rasV12–induced cell death (Fig. 2C). Moreover, targeted silencing of JNK1 or JNK2 using small interfering RNA (siRNA) significantly inhibited H-rasV12–induced cell death (Fig. 2D). These results indicate that activation of JNK1 and JNK2 is required for the induction of cell death by oncogenic H-ras expression.

Rac1 Is Involved in Oncogenic H-ras–Induced JNK Activation and Cell Death

Rac1 has been implicated in several cellular responses to various stress stimuli. To determine whether Rac1 is involved in oncogenic Ras–induced JNK activation and cell death, we examined Rac1 activation and evaluated the effects of Rac1 inhibition in Rat2 cells infected with H-rasV12. In cells overexpressing oncogenic H ras, the levels of PAK-bound Rac1 were markedly increased, indicating activation of Rac1 (Fig. 3A; Supplementary Fig. S2A).

Inhibition of Rac1 by ectopic expression of RacN17 (MFG-HA-RacN17), a dominant-negative form of Rac1, significantly attenuated H-rasV12–induced JNK activation (Fig. 3B) and cell death (Fig. 3C). Moreover, siRNA-mediated knockdown of Rac1 also partially inhibited oncogenic H-rasV12–induced cell death (Fig. 3C). In addition, RacN17 partially attenuated JNK activation (Supplementary Fig. S2B) and cell death (Supplementary Fig. S2C) in cells overexpressing oncogenic K-ras. These results support the idea that Rac1 acts as an upstream regulator of JNK in oncogenic ras-induced cell death.

PI3K Is Involved in Oncogenic H-ras–Induced JNK-Mediated Cell Death

PI3K is a well-characterized effector of Ras function that is known to increase cell survival under various cellular stress conditions. Paradoxically, it has also recently been shown to have an apoptosis-promoting role under certain conditions. To establish whether PI3K is involved in the cell death response to oncogenic H-ras, we assessed PI3K activation in Rat2 cells infected with H-rasV12 and assessed the effects of PI3K inhibition on H-rasV12–induced cell death. Consistent with a role for PI3K in this process, overexpression of H-rasV12 or K-ras induced an increase in PI3K activity (Fig. 4A; Supplementary Fig. S3A). Moreover, inhibition of PI3K by pretreatment with LY294002 resulted in a marked decrease in

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H-rasV12–induced cell death in three different normal fibroblasts and primary astrocytes (Fig. 4B). In agreement with this, inhibition of PI3K by siRNA-mediated knockdown of the p85 or p110 subunit of PI3K suppressed cell death in response to oncogenic H-ras (Fig. 4C). Treatment with LY294002 or siRNA against p85 or p110 also clearly attenuated H-rasV12–induced JNK activation (Fig. 4D). In addition, pretreatment with LY294002 also significantly attenuated oncogenic K-ras–induced JNK activation (Supplementary Fig. S3B) and cell death (Supplementary Fig. S3C). Collectively, these results

**FIGURE 2.** JNK is required for oncogenic H-ras–induced cell death. **A.** Rat2 cells were infected with retroviral MFG or MFG-H-rasV12. After 48 h, cell lysates were subjected to Western blot analysis with the indicated antibodies. **B.** Rat2 cells were infected with retroviral MFG, MFG-H-rasV12, or MFG-K-rasV12 and then treated with PD98059 (25 μmol/L), SB203580 (10 μmol/L), or SP600125 (10 μmol/L). After 72 h, cell death was determined by flow cytometric analysis after propidium iodide staining. *, P < 0.01, versus control. **C.** Cells were infected retroviral MFG or MFG-H-rasV12 in the presence or absence of mock or dominant-negative form of ERK2 (pCEP4-DN-ERK), p38 MAPK (PCMV-Flag-DN-p38), or JNK2 (pCDNA3.1-Flag-DN-JNK2). After 72 h, cell death was determined by flow cytometric analysis. *, P < 0.01, versus control. **D.** Cells were infected retroviral MFG or MFG-H-rasV12 in the presence or absence of si-JNK1 or si-JNK2. After 72 h, cell death was determined by flow cytometric analysis. *, P < 0.05, versus control.

**FIGURE 1.** Oncogenic Ras induces caspase-independent cell death in normal fibroblasts. **A.** Rat2 cells were infected with retroviral MFG, MFG-H-rasV12, MFG-K-rasV12, MFG-H-ras, MFG-K-ras, MFG-R-ras, and MFG-N-ras (1 × 10⁵ cfu/mL). Top, after 72 h, cell lysates were subjected to Western blot analysis with anti–Pan-Ras and anti–β-actin antibodies. β-Actin was used as a loading control. Bottom, cells were harvested and stained with propidium iodide. Cell death was determined by flow cytometric analysis. Columns, mean from three independent experiments; bars, SE. **B.** NIH3T3, WI38, primary astrocytes, and primary fibroblasts were infected with retroviral MFG, MFG-H-rasV12, or MFG-K-rasV12 (1 × 10⁵ cfu/mL). Top, after 72 h, cell lysates were subjected to Western blot analysis with anti–Pan-Ras and anti–β-actin antibodies. β-Actin was used as a loading control. Bottom, cells were harvested and stained with propidium iodide. Cell death was determined by flow cytometric analysis. Columns, mean from three independent experiments; bars, SE. **C.** Rat2 cells were infected with retroviral MFG, MFG-H-rasV12, or MFG-K-rasV12 in the presence or absence of z-VAD-fmk (30 μmol/L). Cells were harvested at 72 h and cell death was determined by flow cytometric analysis. Columns, mean from three independent experiments; bars, SE. **D.** H-ras, caspase-8, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP) were detected by Western blot analysis with total cell lysates. β-Actin was used as the loading control. The data represent a typical experiment conducted three times with similar results.
indicate that activation of PI3K is necessary for the activation of JNK and subsequent cell death caused by overexpression of oncogenic ras. Inhibition of PI3K also attenuated H-ras\(^{V12}\)–induced Akt activation (Supplementary Fig. S4A). However, inhibition of Akt by siRNA targeting did not affect H-ras\(^{V12}\)–induced cell death (Supplementary Fig. S4B), suggesting that the PI3K/Akt pathway is not involved in this process.

Both PI3K and Rac1 Activities Are Critical for the Induction of Cell Death in Cells Overexpressing Oncogenic H-ras

To confirm the hypothesis that two separate pathways are involved in mediating oncogenic H-ras–induced cell death in normal fibroblasts, we analyzed the levels of cell death in H-ras\(^{V12}\)–overexpressing cells transfected with a p110-specific siRNA to inhibit PI3K and with an expression construct for the dominant-negative inhibitor of Rac1, RacN17. As shown in Fig. 5A, cell death was attenuated in cells simultaneously transfected with p110-siRNA and RacN17 to a greater extent than in cells treated with either reagent alone. Similar results were obtained by simultaneously inhibiting PI3K and Rac1 with p85-siRNA and Rac1-siRNA (Fig. 5B). These results indicate that both PI3K and Rac1 activities are critical for the induction of cell death in cells overexpressing oncogenic H-ras.

Consistent with the idea that the Rac1 and PI3K pathways act in parallel to contribute to oncogenic H-ras–induced cell death,
Rac1 activity in H-rasV12–overexpressing cells was unaffected by inhibition of PI3K (Supplementary Fig. S5A), and PI3K activity was not affected by transfection with RacN17 (Supplementary Fig. S5B).

**Discussion**

To avert the development of malignancy, mammalian cells have evolved mechanisms that activate cellular disposal programs, such as programmed cell death, in response to...
deregulated oncogene expression. However, certain molecular details of the regulatory pathways that are induced by oncogenes and ultimately lead to cellular destruction have not yet been clearly defined. In this study, we investigated the signaling pathways affected by oncogenic H-ras that are involved in the subsequent regulated cell death process. We show that JNK activation is essential for oncogenic H-ras–induced cell death, and further show that both Rac1 and PI3K activities are required for JNK activation and subsequent cell death. We suggest that the cell death response to oncogenic Ras is important for preventing the development of abnormalities and malignancies in normal cells, and as such is an important mechanism for suppressing tumor initiation.

Although oncoproteins of the Ras family are perhaps best known for their role in initiating cell proliferation and suppressing apoptosis, oncogenic Ras can, under certain conditions, initiate cell death (2-5). In this study, we provided further evidence that overexpression of oncogenic H-ras or K-ras triggers cell death in normal cells through a caspase-independent mechanism. Recent reports have indicated that the JNK pathway is involved in caspase-independent cell death in response to certain stimuli, such as oxidative stress and chemical hypoxia (10, 14). These observations extend the results of previous studies, which have shown that JNK can function as a proapoptotic kinase in response to a variety of stimuli (6-10). Consistent with these findings, we also found that JNK has an important role in caspase-independent cell death in response to oncogenic Ras expression in normal fibroblasts. Inhibition of JNK by pretreatment with a JNK-specific inhibitor or using a dominant-negative form of JNK clearly attenuated oncogenic H-ras–induced, caspase-independent cell death.

Evidence for extensive cooperation and cross talk between GTPases and other signaling pathways is well documented. Rac1, one of the best-characterized small GTPases, has been implicated in several cellular responses to a variety of cellular stresses. In this study, we found that Rac1 is also involved in JNK activation and cell death in cells overexpressing oncogenic Ras. Levels of PAK-bound Rac1 were markedly increased in response to oncogenic H-ras, and inhibition of Rac1 by ectopic expression of a dominant-negative form of Rac1 significantly attenuated JNK activation and cell death. These results support the idea that Rac1 acts as an upstream regulator of JNK in the cell death response to oncogenic H-ras expression, and are in agreement with those of recent studies showing that Rac1 is involved in the JNK- and p38 MAPK–mediated cell death induced by UV (32) and ionizing irradiation (33).

Several reports have shown that the PI3K pathway is involved in cytoprotective responses to diverse stimuli (25, 26). Interestingly, our study showed an opposing role for PI3K, showing that activation of PI3K is required for the cell death response to oncogenic Ras. PI3K was activated in cells overexpressing oncogenic Ras, and inhibition of PI3K with a chemical inhibitor or using siRNAs against PI3K subunits effectively attenuated oncogenic H-ras–induced JNK activation and cell death in three different fibroblasts. These results indicate that PI3K is responsible for the JNK-mediated cell death response to oncogenic H-ras. Our findings are in agreement with recent reports that implicate PI3K as a positive regulator of the cell death response to a number of stimuli, including ER stress (27), IFN-α (28), and amyloid β/glutamate (29), as well as in calcium-mediated cell death (30). Taken together, these results indicate that PI3K triggers functionally opposing pathways, depending on the nature of the stimulus and the extent of cross talk with other signaling pathways. In this study, the absence of cross talk between the PI3K and Rac1 pathways was shown.

FIGURE 5. Both PI3K and Rac1 activities are critical for the induction of cell death in cells overexpressing oncogenic H-ras. A. Rat2 cells were infected with retroviral MFG or MFG-H-rasV12 in the presence or absence of si-p110 and/or MFG-RacN17 or si-p85 and/or si-Rac1. After 72 h, cell death was determined by flow cytometric analysis. Columns, mean from three independent experiments; bars, SE. *, $P < 0.05; **, $P < 0.001, versus control. B. Rat2 cells were infected with retroviral MFG or MFG-H-rasV12 in the presence or absence of si-p85 and/or si-Rac1. After 72 h, cell death was determined by flow cytometric analysis. Columns, mean from three independent experiments; bars, SE. *, $P < 0.05; **, $P < 0.001, versus control. C. A model for the cell death in response to oncogenic H-ras expression. Activation of PI3K and Rac1 is critically required for JNK-mediated cell death.
by the finding that Rac1 activity was not affected by inhibition of PI3K (and vice versa) during oncogenic H-ras–induced cell death. These results also indicate that activation of both PI3K and Rac1 is required for activation of JNK and subsequent induction of cell death in cells overexpressing oncogenic H-ras.

It has been shown that PI3K and JNK lethality is linked to a toxic form of autophagy (34, 35). In this study, we also found that oncogenic H-ras (H-rasV12) induced a marked LC3-positive vacuole formation (data not shown). Moreover, inhibition of LC3-positive vacuole formation with chemical inhibitor, 3-methyladenine, or siRNA of Beclin1 effectively attenuated oncogenic H-ras–induced cell death in Rat2 fibroblasts (data not shown), indicating that autophagic vacuole formation may be involved in the cell death in response to oncogenic H-ras. The precise mechanisms by which involvement of autophagy in oncogenic H-ras–induced cell death occurs remain to be elucidated.

In summary, we show here that overexpression of oncogenic Ras (H-rasV12 and K-rasV12) triggered caspase-independent cell death in normal fibroblasts. We further showed that JNK activation is essential for oncogenic H-ras–induced cell death, and that activation of both Rac1 and PI3K is required for JNK activation and the subsequent cell death response to oncogenic Ras. These results indicate that two different signaling pathways converge on JNK in response to oncogenic H-ras overexpression to promote JNK-dependent cell death (Fig. 5C). Determining the molecular mechanisms involved in the cell death response to deregulated oncogenes will increase our understanding of cellular disposal processes in normal cells and increase our appreciation of these events as a mechanism of cells to protect against malignant progression.

Materials and Methods

Cell Culture

Rat2, WI38, and NIH3T3 cells were obtained from American Type Culture Collection. Rat2 cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (Life Technologies, Inc.), penicillin (1,000 units/mL), and streptomycin (1,000 μg/mL) in a humidified 5% CO2 atmosphere. WI38 and NIH3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies), penicillin (1,000 units/mL), and streptomycin (1,000 μg/mL) in a humidified 5% CO2 atmosphere.

Production of Retrovirus Contained Ras-Family Genes

To generate MFG-H-rasV12, MFG-K-rasV12, MFG-H-ras, MFG-K-ras, MFG-R-ras, and MFG-N-ras, PCR fragments produced against pSPORT-H-rasV12, pSPORT-K-rasV12, pSPORT-H-ras, pSPORT-K-ras, pSPORT-R-ras, and pSPORT-N-ras as templates were cloned into MFG retroviral vector using Ncol and BamHI sites. For retrovirus production, a modified 293T cell line was cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 2 mmol/L GlutaMAX (Invitrogen), 50 units/mL penicillin-streptomycin, 1 μg/mL tetracyclin, 2 μg/mL puromycin, and 0.6 μg/mL G418 sulfate (Calbiochem) and transfected with plasmid containing Ras-family genes using the Lipofectamine 2000 reagent (Invitrogen). The virus-containing supernatant was filtered through 0.45-μm filter unit (Milipore Corp.) and stored at −80°C. To express Ras-family genes, cells were infected with retrovirus containing Ras-family genes or mock retrovirus generated using MFG control vector with the indicated volumes for 3 h.

Chemical Reagents and Antibodies

Polyclonal antibodies to phospho-ERK, p38, p110 subunit of PI3K, and Ras were purchased from Santa Cruz. Polyclonal antibody to p85 was purchased from Upstate. Pak-conjugated agarose were purchased from Upstate Biotechnology, Inc. Anti-Rac1 was purchased from BD Pharmingen. β-Actin and DMSO were purchased from Sigma. Polyclonal antibodies to ERK, JNK, phospho-p38 MAPK, phospho-JNK, and phospho-Akt (473) were obtained from Cell Signaling Technology. PD98059, SB203580, SP600125, and LY294002 were obtained from Calbiochem.

siRNA Transfection

siRNAs against p85, p110, and Akt1 were purchased from Ambion. A control siRNA specific for green fluorescent protein (CCACCTACGTGACCCAG) was used as the negative control. siRNA duplexes (50 mmol/L) were introduced into cells using Lipofectamine 2000 (Invitrogen Corp.) by following the procedure recommended by the manufacturer.

PI3K Assay

Cells were harvested and lysed in 1-mL lysis buffer (25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% NP40, 100 μL Na3VO4, 1% aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Equal amounts of protein lysates (300 μg in 500 μL) were subjected to immunoprecipitation using anti-p85 antibodies (Upstate Biotechnology). The precipitates were washed twice with PBS/1% NP40, followed by PBS, 0.1 mol/L Tris-HCl (pH 7.5)/0.5 mol/L LiCl, and finally 25 mmol/L HEPES (pH 7.5)/100 mmol/L NaCl/1 mmol/L EDTA. The precipitates were then resuspended in 50 μL of presonicated phosphatidylinositol substrate and incubated for 10 min at room temperature. Each sample was labeled with 10-μCi [32P]ATP for 10 min at room temperature. Reactions were stopped by adding 100 μL of chloroform/methanol/HCl (50:100:1). Lipids were extracted with 200-μL chloroform, and after mixing and centrifugation, the lower “organic” phase was transferred to a new tube. The organic phase was washed once with 100 μL of methanol-1 mol/L HCl (1:1) and the upper phase was discarded. Finally the lipid fraction was resuspended in 20 μL of chloroform and applied to a TLC plate preimpregnated with 1% potassium oxalate. Phospholipids were resolved by TLC in freshly prepared chloroform/methanol/ammonia/water (43:38:5:7) for 45 min in a closed glass chamber at room temperature.

Rac1 Activity Assay

Briefly, cell lysates were immunoprecipitated with a PAK-agarose bead and washed thrice with lysis buffer, and then run on 12.5% SDS-PAGE and Western blotted using monoclonal anti-Rac1 antibody from BD Pharmingen. For all assays, cells were lysed 48 h after viral infection.
Quantification of Cell Death

Fluorescence-activated cell sorting analysis using propidium iodide staining detects cell death. For the cell death assessment, the cells were plated in a 60-mm dish at a cell density of 1 x 10^5 per dish and treated with H-ras virus the next day. At the indicated time points, cells were harvested by trypsinization, washed in PBS, and then incubated in propidium iodide (2.5 μg/mL) for 5 min at room temperature. Then, cells (10,000 per sample) were analyzed on a FACScan flow cytometer using CellQuest software.

Statistical Analysis

All experimental data are reported as the mean, and error bars represent the experimental SE. Statistical analysis was done using nonparametric Student’s t test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Mediated, Caspase-Independent Cell Death by Phosphoinositide 3-Kinase and Rac1 to Induce c-Jun NH₂-Terminal Kinase–Mediated, Caspase-Independent Cell Death

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