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The Small GTPase Rac Suppresses Apoptosis Caused by Serum Deprivation in Fibroblasts

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Abstract

Background: The small GTPase Rac1 is a key signaling protein that mediates a number of important physiologic functions including the organization of the actin cytoskeleton, lipid metabolism, and gene transcription. Rac1 has also been implicated in oncogenic transformation. Expression of constitutively active Rac1 in Rat1 fibroblasts elicits serum- and anchorage-independent growth and causes tumorigenicity in nude mice. The signaling pathways that mediate the role of Rac in cell transformation remain to be identified. Here, we study the role of Rac in cell survival in the absence of serum.

Materials and Methods: The cell lines used in this study are Rat1 fibroblasts that express constitutively active or dominant negative mutants of Rac1. We used long-term video time-lapse microscopy to analyze the effects of these Rac1 mutants on mitogenicity and apoptosis.

Results: We show that the increase in viability, which is stimulated by Rac1 in the absence of serum, is predominantly caused by an inhibition of apoptosis, with a minor increase in cell division. We also show that Rac1-stimulated cell viability in serum-starved cells is inhibited by chemical inhibition of phosphatidylinositol 3-kinase.

Conclusions: Our observations indicate a role for Rac1 in survival signaling, possibly via activation of phosphatidylinositol 3-kinase. We propose that Rac1-stimulated cell survival may contribute to the role of Rac1 in serum-independent growth and cell transformation.

Introduction

Members of the Rho family of small GTP-binding proteins control a large number of functions including the organization of the actin cytoskeleton and the regulation of lipid metabolism, gene transcription, and vesicle trafficking (1,2). Rho family GTPases, like the closely related Ras proteins, are thought to function as switches, which are "on" in the GTP-bound and "off" in the GDP-bound state. Rho GTPases are activated by growth factors, cytokines and integrins, and transmit these signals to downstream effectors. These switches are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs); see Boguski and McCormick (3) for a review. The unique properties of the Ras GTPases have made it possible to establish specifically acting dominant negative and constitutively active mutants. Dominant negative mutants have a lower nucleotide affinity (in particular for GTP) and a higher affinity to their respective GEFs, thereby blocking exchange factor access of the endogenous GTPases and inhibiting their activation. Constitutively active mutants have impaired GTP hydrolysis and cannot be down-regulated.

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Rho family GTPases also play an important role in the aberrant growth and invasive properties of a number of transformed cells (4–9). We and others have shown that, in fibroblasts, expression of the constitutively active Rac1V12 mutant is sufficient to induce serum- and anchorage-independent growth and is tumorigenic in nude mice, and conversely, that expression of dominant negative Rac1N17 inhibits the serum- and anchorage-independent growth of Ras-transformed Rat1 cells (10–12). Ras-induced activation of Rac is mediated in part by phosphatidylinositol 3-kinase (PI3K) (13). The PI3K-Rac branch of Ras signaling appears to function largely independently from the Raf/MEK/ERK and the RalGDS/Ral pathways (10,13,14), and it is likely that these three pathways deliver distinct contributions to Ras transformation. The signaling pathways that act downstream of Rac in the control of cell proliferation largely remain to be elucidated (2,5). One possible mediator is the transcription factor NF-κB. Expression of constitutively active Rac has been shown to stimulate NF-κB (15,16). NF-κB is required for transformation by oncogenic Ras (17) and Rac is necessary for Ras-induced NF-κB activation (15).

The acquisition of resistance to apoptosis is an essential step in tumor progression (18). PI3K is a key signaling molecule that has been implicated in the control of cell survival in a number of different cell types. Because Rac1 has been shown to function

downstream of PI3K (13,19–21), we reasoned that activation of Rac1 may contribute to cell transformation at least in part because it activates signaling pathways that promote cell survival. Reports in the literature on the role of Rac in cell survival are highly controversial, however. For instance, studies in hematopoietic cells have shown that expression of constitutively active Rac in some conditions can induce apoptosis (22–24), but in other conditions it enhances cell survival (25). A similar situation holds for fibroblasts (26–28).

In this study we investigated the role of Rac in the survival of Rat1 fibroblasts, a cell line that we have previously used to characterize the effect of constitutively active and dominant negative Rac1 mutants on cell proliferation. To differentiate between the effect of these Rac1 mutants on cell death and mitogenesis, we used time-lapse video microscopy, a technique that makes it possible to quantify cell death and cell division rates (29). We show that constitutively active Rac1 enhances cell proliferation in the serum-free conditions predominantly by inhibiting cell death.

Materials and Methods

Cell Lines and Viability Assay

Generation, properties, and growth conditions of Rat-1 fibroblast cell lines, expressing vector control (V-5-5 and V-5-8), Rac1V12 (Rac1V12-2a, -6a and 14a), or Rac1N17 (Rac1N17-2a, -7 and -12), have been previously described (10). These lines express the mutant proteins of the tetracyclin-repressible promoter. Prior to analysis, cells were transferred for 24 hr to fresh medium lacking tetracyclin to allow full expression of the Rac mutants. Cell viability was measured using the SRB assay essentially as previously described (30). Briefly, cells were seeded at 10^4 cells/well in a 96-well microtiter plate. To achieve reproducibility, seven wells per cell line and two plates for each time point—0, 2, or 4 days—were tested. After allowing a few hours for attachment, medium was changed and treatments were started. At this point the time 0 plate was fixed to provide for an internal control to use for normalization of data. At the desired time point, cells were fixed with a solution of 10% trichloroacetic acid. Plates were incubated at 4°C for 60 min, then stained for 30 min at room temperature (RT) with 0.2% SRB dissolved in 1% acetic acid and let air dry. The bound dye was solubilized in 200 μl of 10 mM unbuffered Tris base for 5 min on a shaker at low speed and the optical density was read at 515 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader.

TUNEL Assay

Apoptotic cells were detected using the ApopTag In Situ Apoptosis Detection kit (Intergen Company, Purchase, NY, USA). Cells were plated on coverslips and were washed twice after treatment with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 15 minutes at RT. They were washed twice for 2 min with PBS containing 0.1% Triton X-100 and subsequently processed for staining as recommended by the supplier of the kit. Stained cells were visualized with an axiovert 100 microscope (Thorwood, NY, USA), using a 40× (0.75 NA) neofluar objective and pictures were captured with a cooled CCD camera.

Time-Lapse Video Microscopy

Time-lapse video microscopy was carried out using an axiovert 100 microscope provided with a 10× (0.25 NA) achrostigmat objective. The microscope was encased in a plexiglass chamber kept at 37°C by a thermostat unit and provided with CO_2 (5%) flow directed to a smaller chamber that contained the sample dish. Images from a COHU videocamera (COHU, Inc., Danville, CA, USA) were acquired with a TLC1800 time-lapse video recorder (GYYR, Odetics, Anaheim, CA, USA). Induced cells were seeded in a 3-cm dish. After attachment, cells were deprived of serum, and analysis was started 6 hr after starvation. Cell division and apoptotic cell death events were scored essentially as described by Harrington et al. (24).

Antibodies and Immunologic Techniques

Monoclonal c-Myc (Ab-2) antibodies and anti-JNK1 peptide antiserum were purchased from Calbiochem (Oncogene Research Products, San Diego, CA, USA) and from Santa Cruz Biotechnology, Santa Cruz, CA, USA, respectively. Precipitation of total cellular JNK was conducted using recombinant GST-Jun bound to glutathione sepharose-4B beads. Kinase assays were performed as described by Beltman et al. (31). Briefly, the immune complexes were incubated in kinase buffer, containing 10 mM ATP, 100 μCi/ml [γ-^{32}P] ATP, and 100 μg/ml GST-c-Jun, the latter added only for the JNK1 test. Kinase reactions took place at 30°C for 30 min and were stopped by adding SDS-PAGE sample buffer and heated for 10 min. JNK-GST–c-Jun samples were resolved on 12% SDS-PAGE gels. The amount of ^{32}P incorporated in GST–c-Jun was counted using an AMBIS4000 scanner (Scanalytic, Inc. Fairfax, VA, USA).

Luciferase Assays NF-kB promoter activity was assayed using the NF-κB–dependent E-selectin–promoter (32). As an internal control, this reporter construct was cotransfected with a plasmid expressing renilla luciferase downstream of the CMV promoter (pRL-CMV, Promega, Madison, WI, USA). Cells were plated in 24-well dishes and were 40% confluent on the day of transfection. Reporter (250 ng) and control (12.5 ng) plasmids were mixed with GenePorter reagent (Gene Therapy Systems, San Diego, CA, USA) at a ratio of 1:5 and added to the cells in the absence of serum for 3.5 hr. From the time

of transfection onward, cells were kept in tetracyclin-free medium to fully induce the Rac1 transgenes. After overnight recovery, cells were either serum starved or kept in the presence of serum. Cells were harvested and assayed for NF-kB activity 24 hr later by measuring the level of reporter and control luciferase activity using the Dual Luciferase Reporter Assay (Promega, Madison, WI, USA).

Results

Rac1V12 Provides Survival Activity in the Absence of Serum

To investigate the role of Rac in survival signaling, we tested Rat1 fibroblast cell lines expressing a constitutively active allele of Rac, Rac1V12, or a dominant interfering mutant, Rac1N17, for their viability upon serum withdrawal. Cells were deprived of serum for 4 days and tested for viability using the sulforhodamine B (SRB) assay (30). Figure 1 shows that control cells did not proliferate in the absence of serum and their growth was slightly reduced. In contrast, Rac1V12-expressing cells showed cell proliferation in the absence of serum, whereas Rac1N17-expressing cells showed a significant decrease in cell growth in comparison to controls. Observation by phase microscopy of Rac1N17-expressing cells revealed that dying cells rounded up and fragmented, suggesting the induction of cell death by apoptosis (data not shown). The Rac1V12 cultures showed a significantly smaller number of dying cells.

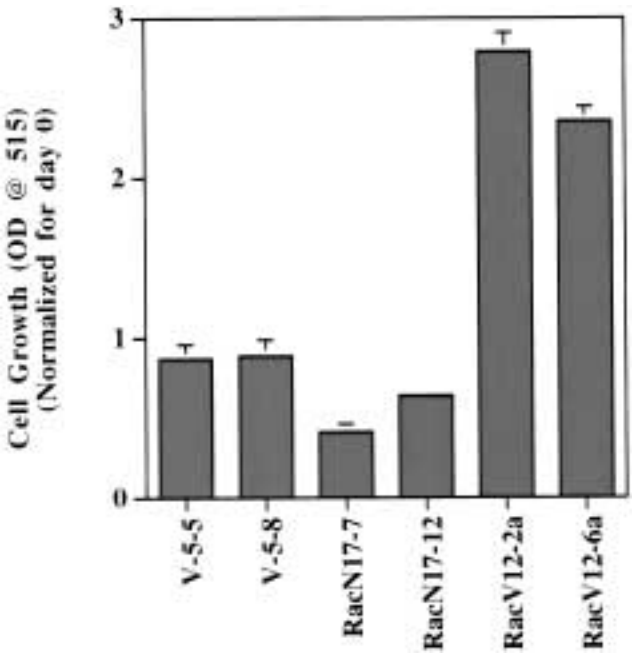

Fig. 1. Proliferation of cell lines expressing different Rac mutants in the absence of serum. Cells were induced for the expression of the Rac mutants, deprived of serum for 4 days and then assayed for SRB staining. The values of two independent clones for each cell line are shown. V-5-5 and V-5-8 are clones expressing the vector plasmid control. Each sample value represents the mean derived from 14 independent wells and normalized for the value at time 0. Standard deviations (SD) are indicated by the vertical bars; for the RacN17-12 line the SD value is very small and is contained within the data bar. Data are representative of three independent experiments.

To confirm whether cells died by apoptosis, we performed the terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end-labeling (TUNEL) assay, which reveals DNA breakage. We observed an increase in TUNEL staining in Rac1N17-expressing versus control cells, whereas TUNEL staining in Rac1V12 expressing cells was less marked (Fig. 2). Thus this assay confirmed that serum-starvation induced cell death by apoptosis. The TUNEL assay, however, was not quantitative in our hands. First, it only stains cells that remain adherent to the dish and the majority of dying cells in these cultures detached from the coverslip during processing. Second, apoptotic cells are subject to rapid phagocytosis in fibroblast cultures, making it difficult to unambiguously score apoptotic cells.

Rac1V12 Protects Against Serum Starvation-Induced Apoptosis

To quantify the responses of the different cell lines to serum withdrawal, we used time-lapse video microscopy, which records cellular events over extended time periods and allows simultaneous monitoring of cell death and division rates (29). It is important to distinguish between these parameters, because the final cell number after serum starvation may be determined by either reduced cell death or increased cell division. As indicated by the sulforhodamine B (SRB) assay (Fig. 3A), the growth properties of the various cell lines after 2 days in the absence of serum were qualitatively similar to those over a 4-day period (Fig. 1). We therefore chose the former time period for time-lapse analysis. The respective levels of cell viability in the three sets of cell lines, as monitored for 52 hr following serum withdrawal (Fig. 3B), indicated strong agreement between the time-lapse and SRB assays. The Rac1V12 culture showed more live cells than the control culture, whereas the Rac1N17 cells showed a lower number than control over the first 20 hr, although this difference diminished at later time points (Fig. 3B).

The cell death rate of Rac1V12-expressing cells was much lower than for control cells. The cell death rate of Rac1N17 cells also appeared to be somewhat lower than that of controls (Fig. 3C). Figure 3D shows that Rac1V12 and control cells had identical cumulative cell divisions for the first 20 hr. However, there was a small increase in cell division rate over control values for the remainder of the observation time. On the other hand, during the first 20 hr in the absence of serum, the cell division numbers of Rac1N17 cells were significantly lower than those of control cells (Fig. 3D). Together, these data show that the increase in the number of surviving cells observed in the Rac1V12 line was mainly caused by a reduction in cell death, with a minor contribution from an increase in cell division.

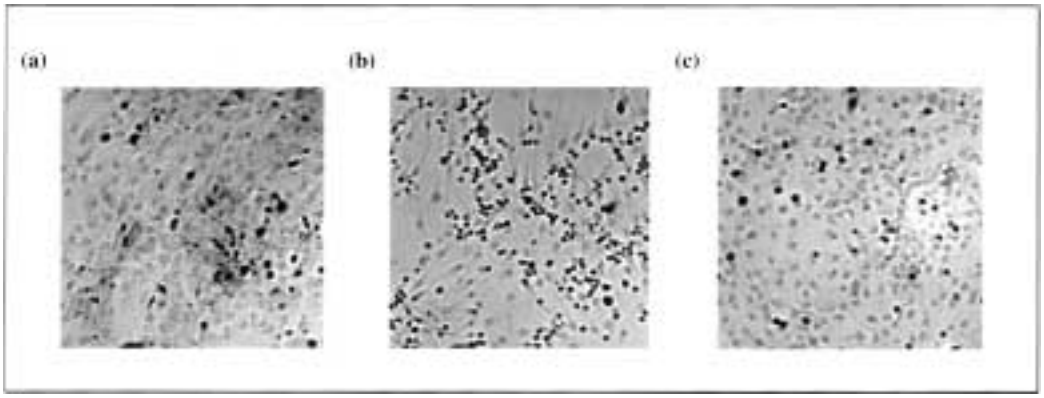

Fig. 2. TUNEL assay. Phase-contrast micrographs of **(A)** control cells, **(B)** Rac1N17 cells, and **(C)** Rac1V12 cells. After 4 days in the absence of serum, cells were processed for direct immunoperoxidase detection of digoxigenin-labeled genomic DNA, as described in Materials and Methods.

JNK Activation Does Not Correlate With Apoptosis Caused by Serum Deprivation in Rat-1 Fibroblasts

Rac has been shown in a variety of systems to activate the MAPK pathway that leads to the activation of Jun kinases (JNKs) (33–35). Because JNK activation has been implicated in the regulation of cell survival (36), we investigated JNK activity in cell extracts from the different lines after four days of serum deprivation. When JNK1 was specifically tested by immunoprecipitation with JNK1 antiserum in Rac1-V12–expressing cells versus controls, only a minor increase in JNK1 activation was found (Fig. 4A). This is in contrast to the 5- to 30-fold activation levels frequently observed in transient transfection studies (33–35). Furthermore, no significant difference among the cell lines was observed in total JNK activity, that is precipitable by GST-Jun from cell lysates (Fig. 4B). These data indicate that JNK activation by Rac1 is unlikely to mediate Rac1-stimulated cell survival.

Cell Survival of Serum-Starved Rat-1 Fibroblasts Does Not Correlate With NF-κB Activation

NF-κB plays a central role in cell survival signaling (37–39). In most experimental conditions, activation of NF-κB stimulates cell survival (38,39). In some

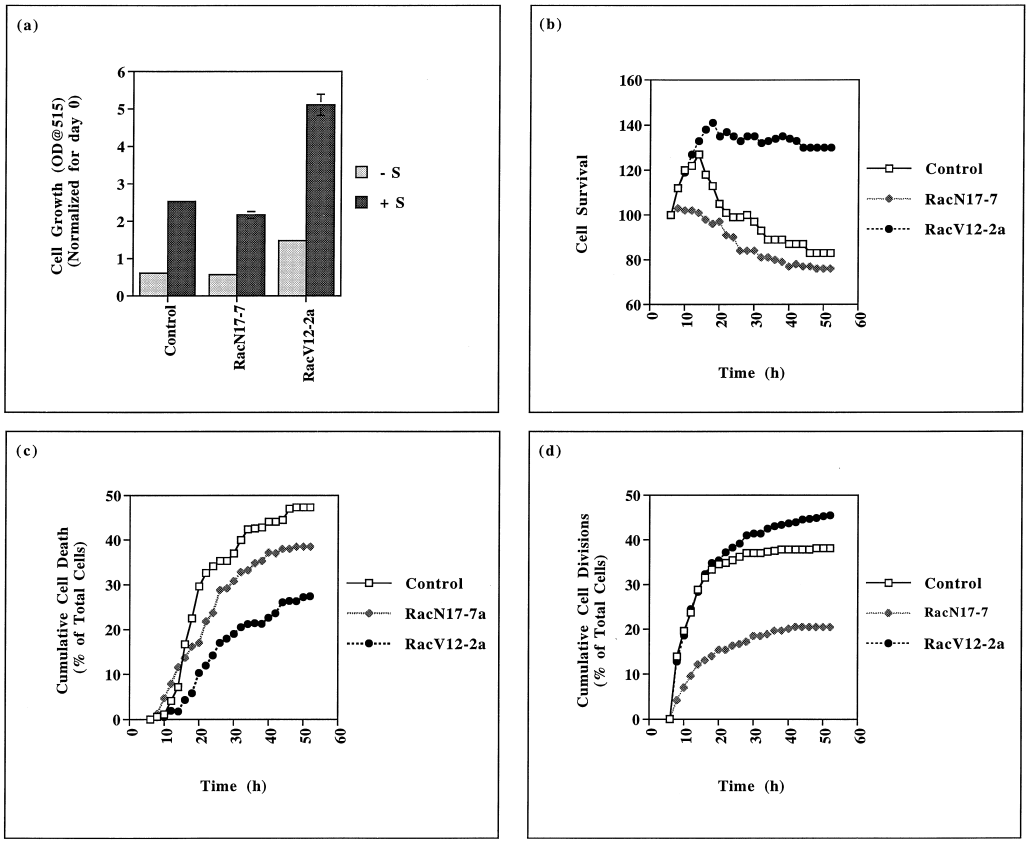

Fig. 3. Analysis of cell death and cell division rates of cell lines expressing different Rac mutants. **(A)** SRB assay after 2 days of serum withdrawal **(−S)**, or in the presence of serum **(+S)**, were conducted as described in the legend to Fig. 1. **(B)**, **(C)**, and **(D)** Analysis of time-lapse video microscopy data. After induction of Rac expression, the different cell lines were deprived of serum and monitored for 52 hr by time-lapse video microscopy. **(B)** Live cell numbers, calculated as percent of the number of cells in each field at the beginning of the observation, **(C)** cumulative number of dying cells, and **(D)** cumulative number of mitosis. For each cell line, a field of about 150 cells was examined and images were acquired at a rate of 1 frame per 8 seconds. Each point represents cumulative data over a 2-hr period. Data are representative of two independent experiments.

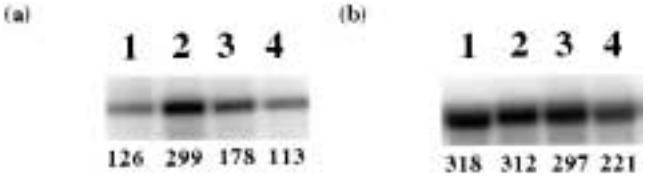

Fig. 4. JNK kinase activity in mutant Rac1-expressing cell lines after serum deprivation. (A) GST-Jun phosphorylation by JNK1-immunocomplexes and **(B)** by total JNK complexes. Proteins (300 μg) from lysates of cells deprived of serum for 4 days, were immunoprecipitated with JNK1 antibody in **(A)** or affinity complexed with GST-Jun in **(B)**. Lanes are as follows: 1, control samples; 2 and 3, two independent Rac1V12 samples; and 4, Rac1N17 samples. Numbers under each lane indicate the cpm values of ^{32}P incorporated in each band. Data are representative of two independent experiments.

settings however, including serum starvation, NF-κB activation has been shown to mediate cell death (39). Transient expression of constitutively active Rac1 has been shown to stimulate NF-κB in a number of different cell lines (15,16). We therefore determined whether expression of constitutively active Rac1, at the relatively low levels that we achieved in the stable cell lines, can stimulate NF-κB. To investigate this, we used a luciferase reporter construct driven by the E-selectin promoter that is dependent on NF-κB activation (32). Figure 5 shows enhanced E-selectin reporter activity in Rac1-V12–expressing cells compared to vector control cells. Interestingly, however, expression of Rac1-N17 stimulates the activity of NF-κB to a larger extent than Rac1-V12.

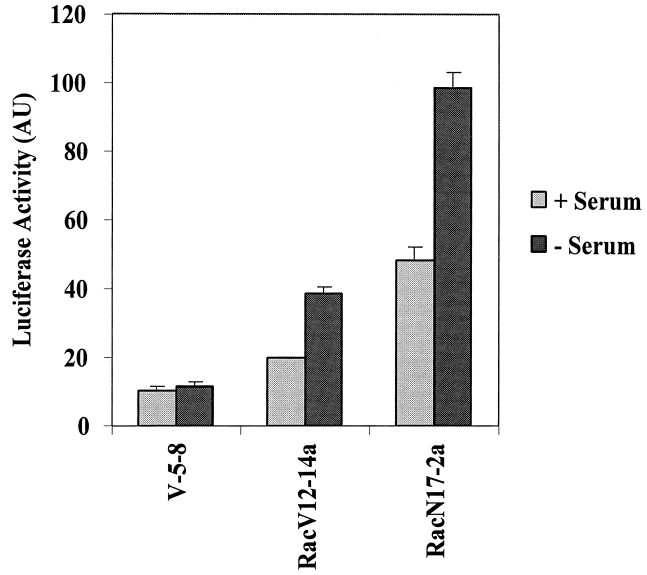

Fig. 5. Transcriptional activity of NF-κB is stimulated by expression of either Rac1V12 or Rac1N17. Mutant Rac1-expressing cell lines were transfected with the NF-κB–dependent E-selectin luciferase reporter and subsequently maintained in the presence or absence of serum as described in Materials and Methods. Data shown are the mean ± SEM of four experiments.

In addition, the NF-κB activity that is stimulated by the Rac1 mutants is strongly enhanced by serum deprivation. These results are consistent with a pro-apoptotic role of NF-κB in serum starvation conditions and suggest that the Rac1-stimulated cell survival is not mediated by NF-κB activation.

Rac1-Stimulated Cell Survival Is PI3K Dependent

Whereas a large volume of data supports the view that Rac1 functions downstream of PI3K (13,19–21), a number of biological functions that are regulated by Rac1 have been shown to be inhibited by pharmacologic inhibitors of PI3K (8,25,40). We therefore tested the role of PI3K in Rac1-V12–stimulated cell survival in the absence of serum using the PI3K-specific inhibitor LY259002 (41). PI3K inhibition significantly reduced cell survival that is stimulated by Rac1-V12 (Fig. 6), suggesting that the Rac1-activated survival signals are mediated by PI3K.

Discussion

Oncogenic transformation may involve deregulation of cell division as well as increased resistance to cell death. Because these two functions are not necessarily coupled and because activation of Rac1 has been shown to enhance mitogenicity (10,35), we addressed the potential role of Rac1 in cell survival. We chose to use a panel of Rat1 fibroblasts that express mutant alleles of Rac1, because the growth properties of these cell lines have previously been extensively characterized (10). When deprived of mitogenic signals in vitro, most nontransformed cells arrest in the G1 phase of the cell cycle for a limited period of time. Rat1 fibroblasts do not show appreciable loss of viability for up to twelve hours of

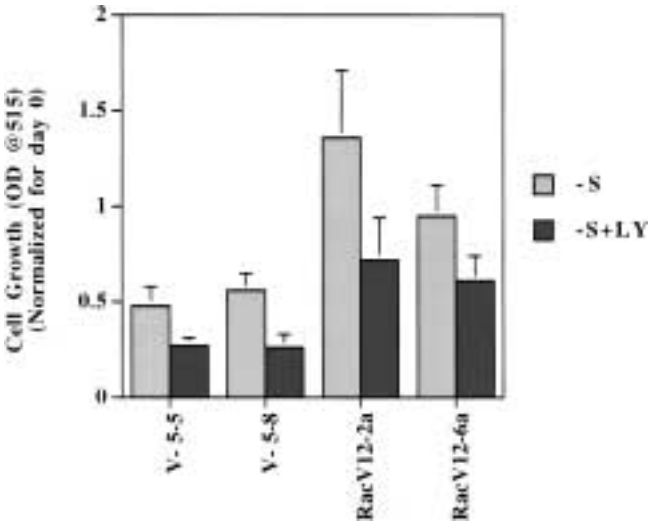

Fig. 6. Requirement of PI3K activity in Rac1-mediated survival. Cells were deprived of serum in the absence (−S) or presence (−S + LY) of LY259002 (10μM) for 4 days. Cell viability was assayed by SRB staining.

serum deprivation (as shown in Fig. 3B). However, as we demonstrated here, after prolonged culture in the absence of serum, control cells started to die, whereas Rac1V12-expressing cells showed significant protection against apoptosis.

Our study also confirms a role for Rac in the control of mitogenicity. In serum starvation conditions, expression of Rac1V12 only leads to a minor increase in cell division rate, but inhibition of the Rac pathway by expression of the Rac1N17 mutant caused a strong reduction in cell division. These results are in agreement with earlier observations, showing that Rac1V12 enhances growth in low serum (10) and stimulates DNA synthesis (35).

The involvement of Rac1 in protection against apoptotic signals may represent an important aspect of the role of Rac1 in transformation. It is interesting to note that in focus formation assays, expression of Rac1V12 on its own is poorly transforming (10,12, 42). However, Rac1 strongly cooperates with activated Raf to transform cells. Although Raf is strongly mitogenic, it does not appear to significantly contribute to cell survival (43–45). It is therefore tempting to speculate that the survival activity of Rac1 is crucial for this cooperative effect.

The observation that Rac1 provides a survival signal under serum starvation conditions is in line with recent observations that expression of constitutively active Rac1V12 provides some level of protection against serum starvation in MycER-expressing Rat1 fibroblasts (27) and that Rac1V12 enhances cell survival in REF-52 fibroblasts that express high levels of oncogenic HRas (28). Activated Rac1 has also been shown to suppress apoptosis induced by interleukin-3 withdrawal in hematopoietic BaF3 cells, although in this system Rac1-stimulated cell survival is strictly dependent on the presence of serum (25).

It should be noted, however, that in other cell systems and conditions, Rac appears to be actively involved in relaying pro-apoptotic signals, including ceramide-, Fas-, and cytotoxic T lymphocyte (CTL)-induced cell killing (24,46). Interestingly, ceramide-, Fas-, and CTL-induced apoptosis is rapid and mediated by direct activation of pro-apoptotic signaling pathways that may involve reorganization of the actin cytoskeleton (24). In addition, Rac activation has been shown to be necessary for nerve growth factor withdrawal–induced cell death in rat sympathetic neurons (47).

The signaling pathways that mediate the stimulation of cell survival downstream of Rac1 largely remain to be identified. Our observations that inhibition of PI3K interferes with Rac1-mediated protection against serum starvation suggests a role for PI3K in this Rac1-stimulated survival function. This is somewhat surprising, because Rac1 is thought to function downstream of PI3K (13,19–21). Our findings, however, are in line with other reports that suggest a role for PI3K in Rac-stimulated functions, including invasion, cell spreading, and survival (8,25,40). These observations may reflect the existence of a positive feedback loop that involves PI3K and Rac. Alternatively, Rac may stimulate the production of autocrine factors that in turn bind to receptors that cause the activation of PI3K. Our preliminary results, however, failed to detect any enhancement of the viability of control cells by the addition of conditioned medium from Rac1V12-expressing cells (data not shown). The signaling elements that act downstream of PI3K in the Rac-controlled cell survival remain to be identified. However, because PKB plays a prominent role in PI3K-mediated cell survival (48), it is also likely to be important for the protection against serum starvation that is stimulated by Rac.

NF-κB also is a key control element in cell survival signaling that in some conditions mediates survival signaling, but in other conditions has pro-apoptotic effects (39). We showed that although expression of constitutively active Rac1 in Rat1 fibroblasts can stimulate NF-κB, this activity does not correlate with cell survival; rather, it is strongly enhanced by serum deprivation. Similar observations have been made in other cell lines (49,50). Interestingly, inhibition of endogenous Rac1 activity by expression of Rac1N17 also activates NF-κB, possibly reflecting a stress-induced response. Together, these results strongly suggest that the Rac1-stimulated cell survival in serum starvation conditions is not mediated by NF-κB activation.

Rac has also been shown to signal through the JNK pathway, which under certain circumstances appears to mediate apoptosis (51–54). Measurement of JNK activity in the cell system studied in this paper indicated only marginal activation of JNK1 by Rac1V12, suggesting that Rac-stimulated survival signals are also likely to be independent of the JNK pathway.

To identify other factors that may contribute to Rac-controlled cell survival, we tested expression levels of a number of signaling molecules that have been implicated in the regulation of cell death. However, we could not detect any significant changes in expression levels of either anti-apoptotic (Bcl-2 and Bcl-X) or pro-apoptotic (Bax and Bad) molecules in Rac1V12-expressing cells versus controls (data not shown).

Further studies will be needed to identify the Rac-controlled signaling pathways that regulate cell survival. Characterization of these pathways also may shed new light on the tissue specificity of the role of Rac in cell survival and will contribute to the understanding of how survival and mitogenic signals are integrated in the cell.

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