Endogenous c-N-Ras Provides a Steady-state Anti-apoptotic Signal*

Received for publication, January 12, 2000, and in revised form, March 20, 2000 Published, JBC Papers in Press, April 20, 2000, DOI 10.1074/jbc.M000250200

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We report that c-N-Ras possesses an isoform-specific, functional role in cell survival under steady-state conditions. This function includes protection from programmed cell death by serum deprivation or upon treatment with apoptosis-inducing agents. The data demonstrate that c-N-Ras may play a functional role in the regulation of steady-state phosphorylated Akt and serine 136-phosphorylated Bad (Ser136-pBad). Immortalized N-Ras knockout fibroblasts possess nearly undetectable levels of steady-state Ser136-pBad. In contrast, wild-type control cells and the N-Ras knockout cells ectopically expressing c-N-Ras at control levels maintained easily detectable levels of Ser136-pBad both at steady-state and following treatment with tumor necrosis factor α. Similar results were seen with Ser112-pBad. These differences did not arise from differences in total Bad protein levels. These data correlate with the observation that the N-Ras knockout cells exhibit a heightened susceptibility to the induction of apoptosis. Ectopic expression of c-N-Ras in the N-Ras knockout cells at endogenous levels, compared with control cells, significantly rescues the apoptotically sensitive phenotype. Elevated expression of either c-Kirsten A-Ras or c-Kirsten B-Ras did not reverse the apoptotic sensitivity of the N-Ras knockout cells or result in increased levels of either phospho-Akt or phospho-Bad. Our results indicate that, at steady state, c-N-Ras possesses an isoform-specific, functional role in cell survival.

There are four mammalian Ras isoforms: Harvey (Ha),1 N, and two splice variants of the Kirsten gene, Kirsten A (K(A)) and Kirsten B (K(B)). All four proteins are highly homologous except for the C terminus, where they share no sequence similarity. Ras GDP, the active form, interacts with diverse targets within the cell. Amino acids 32–40 and 60–72 comprise the switch 1 and switch 2 regions, respectively, which are identical in all isoforms (1, 2). When Ras binds GTP, both regions undergo conformational changes to form the effector binding pocket (3). Distinct Ras isoform functions are now becoming apparent. Transformation of C3H10T1/2 fibroblasts by expression of oncogenic G12V-Ha-Ras at endogenous levels requires the cooperation with cellular N-Ras (4). In vitro assays also suggest differences in Ras isoform-dependent activation of phosphatidylinositol (PI) 3-kinase and Raf-1 (5).

Most of the biochemical effectors of Ras have been identified by in vitro binding assays and yeast two-hybrid screening and include Raf kinases (6–10), mitogen-activated protein kinase-extracellular signal-regulated kinase, redox-sensitive (ERK), and Raf-1 (31). Most of the biochemical effectors of Ras have been identified in Ras immunoprecipitates from cells not ectopically expressing either Ras or the putative target protein. The most well characterized Ras-dependent signaling pathways are the Raf-1/mitogen-activated protein (MAP) kinase-extracellular signal-regulated kinase pathway (MEK1)/MAP kinase pathway and the PI 3-kinase/Akt pathway. In the Raf-1/MEK-1/MEK1 pathway, Ras-GTP recruits and participates in the activation of Raf-1, which then phosphorylates and activates MEK-1 (19, 20). Phosphorylated MEK-1, a dual specificity kinase, phosphorylates and activates p42 and p44 MAP kinases (3, 18, 21). Activated MAP kinase translocates to the nucleus and phosphorylates and activates several transcription factors including Elk-1 and Ets-2 (3, 22, 23). Activated MAP kinase also phosphorylates and activates p70S6K (24, 25).

Ras is also thought to bind and activate PI 3-kinase, causing an increase in the production of 3-phosphorylated phosphatidylinositol lipids (16, 26). Phosphatidylinositol 3,4,5-trisphosphate binds to protein kinase B/Akt directly, which then allows for its activation through phosphorylation. In vivo studies also confirm this activation of PI 3-kinase by c-N-Ras (16). PI 3-kinase is activated by growth factors and growth factor receptor stimulation and is thought to play a role in cell survival.

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Apoptosis, also known as programmed cell death, is an ordered disassembly of a cell, characterized by specific cellular and phenotypic changes including cell shrinkage, membrane blebbing, and DNA degradation (37, 38). The role of Ras in apoptosis has focused on the effect of ectopically expressed, oncopgenic Ras proteins and changes in apoptosis following treatment with various stimuli including tumor necrosis factor α (TNFα), Fas, and withdrawal of serum or growth factors. The reports of these studies are conflicting, in some cases suggesting that oncopgenic Ras inhibits apoptosis (39–41). In other instances, oncogenic Ras expression enhances apoptosis (42–46). The role of endogenous, cellular Ras isoforms in apoptosis has not yet been examined. We have found that endogenous c-N-Ras provides a steady-state survival or antiapoptotic signal. This antiapoptotic signal appears to be generated, at least in part, through regulation of basal phospho-Bad levels. Neither c-K(A)- nor c-K(B)-Ras can substitute for this c-N-Ras survival function.

**EXPERIMENTAL PROCEDURES**

**Antibodies**

Bad polyclonal, phosphospecific Bad polyclonal (Ser112 and Ser155), Akt polyclonal, and Ser637 phospho-Akt polyclonal antibodies were from New England Biolabs. Phospho-MAP kinase monoclonal, anti-N-Ras monoclonal, anti-ERK2 polyclonal, anti-K(A)-Ras polyclonal, and anti-K(B)-Ras polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG monoclonal antibody was from Eastman Kodak Co. Hamster anti-mouse Fas receptor antibody (clone Jo2) (for activation of the Fas receptor) was from Pharmingen (San Diego, CA). Anti-Fas/CD95 antibody (used for Western analysis of Fas receptor) was from Transduction Laboratories. Anti-p55 TNF receptor I was from Biodesign International. Anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) was from Transduction Laboratories, and goat anti-mouse-HRP was from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

**Cell Culture**

N-Ras knockout (N−/−), heterozygote (N+/−), and control N+/+ mouse embryos fibroblasts (MEFs) were a generous gift from R. Kucherlapati (Albert Einstein College of Medicine) (47). K-Ras knockout and control K+/+ MEFs were a generous gift from T. Jacks (Howard Hughes Medical Institute, Massachusetts Institute of Technology) (48). MEFs were immortalized by a modification of the 3T3 protocol (49). The MEFs were passaged 1.3 every 7 days until they developed a fibroblast morphology. To avoid any cell-species arising from immortalization, multiple, independently isolated cell lines were used throughout these studies. Cells were grown in complete medium consisting of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Atlanta Biologicals), 1 mM glutamine, 1% penicillin/streptomycin (Life Technologies), and 0.5 mM sodium pyruvate, 25 μM 2-mercaptoethanol, and 0.1% nonessential amino acids and 0.1% glucose (Sigma). Cells were kept in complete medium in all experiments unless otherwise stated. MEFs were grown in complete medium with additional serum to a final concentration of 20%. Serum starvation was performed by rinsing cells with complete medium with no serum, and 0.5% charcoal-stripped serum (CS) was added. The medium was exchanged every 2 days. The experiments were performed in a 5% CO2 humidified incubator at 37 °C.

**Preparation of Cell Lysates**

N-Ras knockout cells stably expressing wild-type c-N-Ras (N−/− wtN) cells were harvested, and lysates were prepared as described in p21 buffer containing 1% saponin followed by centrifugation and resuspension of the pellet in p21 buffer containing 1% CHAPS (U.S. Biochemical Corp.). The pellets were centrifuged at 13,000 × g, and the supernatant was retained, and protein concentration was determined by the method of Bradford (50). 100 μg of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. For Western blot analysis, the gel was transferred to PVDF (Hybond P; Amersham Pharmacia Biotech). The membrane was blocked with anti-N-Ras monoclonal antibody (Santa Cruz Biotechnology) and developed using HRP-coupled goat anti-mouse secondary antibody and standard ECL techniques. The standard is histidine-tagged, recombinant N-Ras and runs at approximately 30 kDa.

**Pharmacological Treatments**

Recombinant murine TNFα (Calbiochem) was dissolved in 0.2-μm filtered PBS containing 0.1% bovine serum albumin (Sigma) and stored in aliquots at −80 °C. We have found that the TNFα potency varied with the number of freeze/thaw cycles. In general, each aliquot was used only twice. Activation of the Fas receptor was achieved by incubation of the cells for the times indicated in complete medium containing 1 μg/ml murine anti-Fas receptor (Pharmingen, clone Jo2, form NA/LE) and 0.5 μg/ml recombinant protein G (Sigma). Staurosporine (Sigma) was dissolved in Me2SO and used at 75–100 nm.

**Cloning and Transfections**

N-Ras knockout cells stably expressing wild-type c-N-Ras (N−/− wtN cell lines) were generated by transfection of N−/− cells using Lipofectamine Plus (Life Technologies) with c-N-Ras/pBWI3 (a gift from Angel Pellicer, New York University), which has the c-N-Ras gene under the control of the thymidine kinase promoter, and selection in G418 (Fisher). Stable clones were maintained in complete medium containing 200 μg/ml G418. N-Ras knockout cells stably expressing Bel-2-FLAG (a gift from Alex Almasan, Cleveland Clinic Foundation) were generated by the same protocol. K(A)-Ras was cloned by polymerase chain reaction (Expand High Fidelity PCR System; Roche Molecular Biochemicals) from a bacterial expression vector containing the sequence of c-K(A)-Ras (gift from Berthe Willumsen, University of Copenhagen). Primers corresponding to the N-terminal region of c-K(A)-Ras (forward, 5′-AGGCTTCCGGGCGCGGGGATCATCATGACGGAAT-3′) and the reverse complement of the C-terminal region of c-K(A)-Ras (reverse, 5′-AGTCATGGTCACGGCTCCTCTAGATTTAATTACAGCAGAT-3′) were prepared by Life Technologies, Inc. Following the polymerase chain reaction, the product was ligated into pTargetP (Promega) containing a cytomegalovirus enhancer and promoter and the ligation product used to transform JM109-competent Escherichia coli cells. Colonies were selected on LB plates containing 100 μg/ml ampicillin (U.S. Biochemical Corp.) and screened for the presence and direction of the transgene by restriction digest. Positive, forward clones were used to transfect N-Ras knockout fibroblasts by the method described above. A similar procedure was used to clone c-K(B)-Ras from GI12V-KB-Ras/Zip (gift from J. Gibbs, Merck) where the forward N-terminal primer was extended beyond the 12th codon to back-mutate the valine 12 to the wild-type glycine (5′-ACACCATGACTGAATTAATACCTTGATCGGACGCTGGTGTCGTTACCC-3′). The reverse complement or the C-terminal region of c-K(B)-Ras was used for the reverse primer (3′-AGATCTCCATGCGTGATTTGACCGTACTTACACTAATTACACACTTG-5′). The resulting c-K(B)-Ras/pTargetP was transfected into N-Ras knockout cells as described. Prior to transfections, the c-K(A)- and c-K(B)-Ras plasmids were sequenced to confirm their identity with the sequences of mouse c-K(A)- or c-K(B)-Ras in the GenBank database. All transfected clones were tested for the presence and level of expression of the transgene by Western analysis.

**Preparation of Cell Lysates**

All lysis buffers contained the following phosphatase inhibitors: 30 mM β-glycerophosphate, 5 mM p-nitrophenol phosphate, 1 mM each of phosphoserine and phosphothreonine, 0.2 mM phosphotyrosine, 100 μM sodium vanadate, and the following protease inhibitors: 50 μg/ml each of aprotinin and leupeptin, 25 μg/ml pepstatin A, and 1 μM phenylmethanesulfonyl fluoride. For Western analysis of Ras expression, serine 473-phospho-Akt (pAkt) levels, total Akt levels, and phospho-MAP kinase (pMAPK) levels, cells were harvested by scraping into PBS, and the resulting cell pellet was resuspended in p21 buffer (20 mM MOPS, 5 mM MgCl2, 0.1 mM EDTA, 200 mM sucrose, pH 7.4) containing 1%
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CHAPS (U.S. Biochemical Corp.) and incubated for 20 min on ice. The lysate was centrifuged again at 13,000 \( \times \) g, and the supernatant was retained. Protein concentration was determined by the method of Bradford (50). For Western analysis of total Bad or phospho-Bad levels, cells were harvested by trypsinization, combined with their medium, and centrifuged at 1000 \( \times \) g for 10 min. The cells were washed once in Tris-buffered saline (TBS; 20 mM Tris, 140 mM NaCl, pH 7.4) and solubilized in TBS containing 1% Nonidet P-40 (Igepal, Sigma) and phosphatase and protease inhibitors as described. After 20 min on ice, the lysate was centrifuged at 13,000 \( \times \) g, and the supernatant was retained for protein measurements and Western analysis.

**Western Analysis**

Lysates containing equal amounts of protein were loaded onto SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) (Hybond P; Amersham Pharmacia Biotech). Blocking was performed in 5% nonfat milk containing 5% newborn calf serum (Life Technologies). Blots were incubated with primary antibodies for 2–3 h at room temperature or overnight at 4°C. Following electrophoresis, the proteins were transferred to PVDF, and the blot was developed with antiphospho-MAP kinase monoclonal antibody and goat anti-mouse-HRP secondary antibody. Detection was performed as in Fig. 1. 20 \( \mu \)g of protein was loaded in each lane of a 10% SDS-polyacrylamide gel.

**Fig. 2. Signaling in the N-Ras knockout cells.** A, pMAPK levels in N-Ras knockout cells. Treatment of N-Ras knockout (N/0/) control N/+/, and N-Ras knockout cells ectopically expressing c-N-Ras at endogenous levels (N/−−/−−wtN) in medium containing 10% fetal bovine serum. Untreated or cells treated with TNF \( \alpha \) at 1 ng/ml in the presence of 2.5 \( \mu \)g/ml cycloheximide for 1 and 4 h were harvested by scraping in ice-cold PBS, and lysates were prepared in p21 buffer containing 1% CHAPS as described. Protein concentration was determined as described in the legend to Fig. 1. 20 \( \mu \)g of protein was loaded in each lane of a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to PVDF, and the blot was developed with antiphospho-MAP kinase monoclonal antibody and goat anti-mouse-HRP secondary antibody. Detection was performed using standard ECL techniques (Amersham Pharmacia Biotech). The results are representative of three separate experiments. B, top, pAkT levels in the N-Ras knockout cells. N-Ras knockout, control, N−−/−−/−−wtN recombinant cells left untreated (t = 0) or treated with TNF \( \alpha \) and cycloheximide, as in A, were harvested at the indicated times, and lysates were prepared in p21 buffer containing 1% CHAPS as described. 50 \( \mu \)g of protein was loaded in each lane of a 10% SDS-polyacrylamide gel. Following electrophoresis, the gel was transferred to PVDF and immunoblotted with Ser173-pAkT rabbit polyclonal antibody (New England Biolabs) and developed using anti-rabbit-HRP secondary antibody (Transduction Laboratories). Detection was performed with ECL-Plus (Amersham Pharmacia Biotech) and a Molecular Dynamics Storm Imager set on chemiluminescence. The results are representative of two separate experiments. Lower panel, total Akt levels. N-Ras knockout, control, and N−−/−−/−−wtN reconstituted cells were untreated or treated with TNF \( \alpha \) at 1 ng/ml in the presence of cycloheximide, as in A, and harvested at the indicated times, and lysates were prepared as described. Above 50 \( \mu \)g of protein was loaded in each lane of a 10% SDS-polyacrylamide gel, and following electrophoresis proteins were transferred to PVDF and immunoblotted with Akt rabbit polyclonal antibody (New England Biolabs) and developed using anti-rabbit-HRP secondary antibody (Transduction Laboratories). Detection was performed with ECL (Amersham Pharmacia Biotech) and exposure to film (Hyperfilm ECL; Amersham Pharmacia Biotech). The results are representative of three separate experiments. C, pBad levels in N-Ras knockout cells. N-Ras knockout, control, and N-Ras knockout cells ectopically expressing c-N-Ras (N−−/−−/−−wtN) were left untreated or treated with TNF \( \alpha \) and cycloheximide as described for A. At the indicated times, cells were harvested by trypsinization and washed in cold PBS, and lysates were made in TBS, 1% Nonidet P-40 as described under “Experimental Procedures.” Protein concentrations were determined as in Fig. 1. 150 \( \mu \)g of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. Following electrophoresis, the gel was transferred to PVDF membrane and immunoblotted with Ser136-pBad polyclonal antibody (New England Biolabs). The membrane was developed with anti-rabbit secondary (Transduction Laboratories), and detection was performed with ECL-Plus and imaging with a Molecular Dynamics Storm Imager as in B. The results are representative of four separate experiments. D, total Bad levels in N-Ras knockout cells. N-Ras knockout, control N/+/, and N−−/−−/−−wtN reconstituted cells were untreated or treated with TNF \( \alpha \) at 1 ng/ml in the presence of 2.5 \( \mu \)g/ml cycloheximide. At the indicated times, lysates were prepared as in C. 150 \( \mu \)g of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. The gel was transferred to PVDF and blotted using anti-Bad polyclonal antibody (New England Biolabs) and anti-rabbit secondary antibody. Detection was performed as in C. The results are representative of three separate experiments.
FIG. 3. N-Ras knockout cells are highly sensitive to the induction of apoptosis. A, TNFα treatment of immortalized cells. N-Ras knockout, control N+/+, and N-Ras knockout fibroblasts ectopically expressing c-N-Ras (N−/−wtN) were left untreated or treated for 4.5 h with 1 ng/ml TNFα in the presence of 2.5 μg/ml cycloheximide. At the indicated times, the cells were harvested by trypsinization and
followed by washing in TBS, 0.1% Tween. The cells were incubated with either goat anti-mouse horseradish peroxidase (HRP) (Kirkegaard and Perry Laboratories) or anti-rabbit HRP (Transduction Laboratories). After washing, the blots were developed, as indicated, with ECL (Amersham Pharmacia Biotech) and exposure to film (Hyperfilm ECL; Amersham Pharmacia Biotech) or with ECL-Plus (Amersham Pharmacia Biotech) and detection with a Molecular Dynamics Storm Imager.

Apoptosis Assays

TUNEL Analysis—Untreated or cells treated for the indicated times were harvested by trypsinization and combined with their medium (to allow recovery of all nuclei) and centrifuged, and washed once in cold PBS. The pellet layers were resuspended in 1% paraformaldehyde (EM Science) and PBS and incubated for 20 min. The fixed cells were centrifuged and washed once with PBS and resuspended in cold 7% ethanol. TUNEL analysis was performed by fluorescence-activated cell sorting using the APO-BRDU flow cytometry kit for apoptosis according to the manufacturer’s directions (Phoenix Flow; Pharmingen).

Cell Death ELISA—Untreated or treated cells in 12-well cluster plates were resuspended in their medium and centrifuged at 500 × g for 5 min. The cell pellet was resuspended in 200 μl of lysis buffer supplied by the manufacturer (Cell Death Detection ELISA Plus kit; Roche Molecular Biochemicals). 20-μl aliquots were used in the analysis that measures the appearance and relative amounts of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes) with detection by ELISA plate reader at 405 nm, according to the manufacturer’s instructions. Incubation was performed overnight at 4 °C instead of 2–3 h at room temperature as suggested by the manufacturer. The reading from the negative control (buffer only) supplied by the manufacturer was subtracted from all sample values.

RESULTS AND DISCUSSION

Ras Signaling in N-Ras Knockout Cells—Expression of c-N-Ras is absent in all immortalized N-Ras knockout cell lines (N−/−) (Fig. 1, top). The expression levels of c-N-Ras in the N-Ras knockout cells ectopically expressing c-N-Ras (N−/−-wtN) are similar to that observed in the control N+/+ cells (Fig. 1, bottom). All cell lines, except K(i)-Ras knockout cells, express K(i)-Ras knockout cells (see below), and none express detectable levels of Ha-Ras (data not shown).

Since the N-Ras knockout cells express only c-K(A)- and c-K(B)-Ras, they present a unique system to examine signaling systems that might specifically require c-N-Ras. We chose to test for changes in either phospho-MAP kinase or phospho-Akt levels, since each of these is regulated through a distinct Ras signaling pathway (Raf-1 and PI 3-kinase, respectively). Differences between N-Ras knockout cells and control cells in the level of activated MAP kinase or Akt were examined both at steady state and following agonist stimulation. We examined phospho-MAP kinase (p42 and p44) levels under steady-state growth and following treatment with TNFα in the presence of cycloheximide (Fig. 2A). The N-Ras knockout cells, control N+/+ cells, and N-Ras knockout cells stably expressing c-N-Ras at control levels possessed similar levels of phosphorylated MAP kinase at steady state and following treatment with TNFα. There was a small increase in the level of phospho-MAP kinase at 1 h that decreased to steady-state levels after 4 h. This is consistent with the report that both Jun N-terminal kinases and extracellular signal-related kinases (ERKs) are activated in a Ras-dependent manner following Fas ligation in SHEP cells (51). Recently, two groups reported that phosphorylation of Bad on serine 112 is regulated by the MAP kinase pathway (31, 52). The results from these studies suggested that the MAP kinase pathway is necessary for Ser112 phosphorylation and inactivation of proapoptotic Bad, similar to Ser136 phosphorylation of Bad by Akt (32–34, 53, 54). Our data suggest that the MAP kinase pathway is unaffected by the absence of c-N-Ras. While our laboratory has demonstrated that c-N-Ras preferentially binds to Raf-1 in G12V-Ha-Ras-transformed C3H10T1/2 fibroblasts, it is possible that as a result of continuous culturing of the N-Ras knockout fibroblasts in serum-containing medium, these cells may have adapted alternative mechanisms that lead to MAP kinase activation. Unlike MAP kinase, Akt can be activated by a Ras/PI3-kinase-dependent pathway (3, 18). Our results demonstrate that, at steady state, the N-Ras knockout cells possess minimal levels of pAkt in contrast to control cells (Fig. 2B, upper panel). Ectopic expression of c-N-Ras in the N-Ras knockout cells significantly restores the level of pAkt to levels comparable with those observed in the control cells. The differences observed in pAkt are not a result of differences in the total amount of Akt (Fig. 2B, bottom panel). The N-Ras knockout cells, control N+/+ cells, and the N-Ras knockout cells ectopically expressing c-N-Ras (N−/−-wtN) possess similar levels of total Akt protein both at steady state and following treatment with TNFα. This implies that activation of the c-N-Ras/PI3-kinase/Akt pathway may be impaired in N-Ras knockout cells.

c-N-Ras Function Influences Steady-state Levels of Phosphorylated Bad (pBad)—Bad can be phosphorylated on position 136 by Akt (32–34, 53), which can itself be activated by a Ras-dependent PI 3-kinase pathway (55). Phosphorylation of Bad on serine 112 and/or 136 results in the sequestering of pBad by cytosolic 14–3–3, allowing an increase in free, antiapoptotic Bcl-2 and Bcl-xL (37, 56). c-N-Ras could provide a steady-state combined with their medium, centrifuged, and washed once in cold PBS. The cells were fixed in 1% paraformaldehyde in PBS and incubated on ice for 15 min. The fixed cells were centrifuged and washed with PBS and resuspended in cold 7% ethanol. TUNEL analysis was performed by fluorescence-activated cell sorting using the APO-BRDU flow cytometry kit for apoptosis according to the manufacturer’s directions (Phoenix Flow; Pharmingen).
FIG. 4. Reversal of the apoptotic sensitivity of N-Ras knockout cells is specific for the c-N-Ras isoform. A, Western analysis of c-K(B)-Ras levels in N-Ras knockout and control N+/+ cells. Cells were harvested by scraping in ice-cold PBS, and lysates were prepared in p21 buffer containing 1% CHAPS as described. Protein concentration was determined as in Fig. 1. 100 μg of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to PVDF, the blot was cut, the upper half was incubated with anti-ERK2 polyclonal antibody, and the bottom half was blotted with anti-K(B)-Ras polyclonal antibody. Both halves were developed with anti-rabbit HRP secondary antibody, and detection was with standard ECL techniques. The first lane is 25 ng of bacterially expressed c-K(B)-Ras. The results are representative of two separate experiments. B, Western analysis of c-K(A)-Ras levels in the N-Ras knockout and control N+/+ cells.

C

D

E

FIG. 4. Reversal of the apoptotic sensitivity of N-Ras knockout cells is specific for the c-N-Ras isoform. A, Western analysis of c-K(B)-Ras levels in N-Ras knockout and control N+/+ cells. Cells were harvested by scraping in ice-cold PBS, and lysates were prepared in p21 buffer containing 1% CHAPS as described. Protein concentration was determined as in Fig. 1. 100 μg of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to PVDF, the blot was cut, the upper half was incubated with anti-ERK2 polyclonal antibody, and the bottom half was blotted with anti-K(B)-Ras polyclonal antibody. Both halves were developed with anti-rabbit HRP secondary antibody, and detection was with standard ECL techniques. The first lane is 25 ng of bacterially expressed c-K(B)-Ras. The results are representative of two separate experiments. B, Western analysis of c-K(A)-Ras levels in the N-Ras knockout and control N+/+ cells.
state, survival signal through its regulation of basal Akt activity. In view of the differences observed in steady-state pAkt levels between N-Ras knockout and control cells, we examined the levels of pBad. In contrast to control N+/+ cells, the levels of Ser112-pBad were barely detectable in the N-Ras knockout cells and did not change upon treatment with TNFα (Fig. 2C) or Fas receptor ligation (data not shown). Stable expression of c-N-Ras in the N-Ras knockout cells restored the levels of Ser112-pBad to nearly control levels. Similar results were observed with Ser112 phosphorylation of Bad (data not shown). To be certain the differences observed in the levels of Ser112-pBad did not result from changes in Bad expression, parallel samples were analyzed for total Bad (Fig. 2D). The results demonstrate that there are no differences in the level of total Bad between N-Ras knockout, control, or N-Ras knockout cells ectopically expressing c-N-Ras. This implies that the differences in pBad levels arise from differences in basal “tonic” signaling by a c-N-Ras/Akt-dependent pathway.

N-Ras Knockout Cells Possess Heightened Susceptibility to Undergo Apoptosis—One of the cell’s protective mechanisms against apoptosis is the phosphorylation of the proapoptotic Bcl-2 family member Bad (37, 57). The decreased steady-state levels of pBad in the N-Ras knockout cells could imply that they are more susceptible to apoptotic agents. We therefore examined the sensitivity of the N-Ras knockout cells to the induction of apoptosis by treatment with apoptotic agonists or serum starvation. Treatment of N-Ras knockout cells with 1 ng/ml murine TNFα in the presence of cycloheximide results in the rapid onset of apoptosis, 40–50% by 4.5 h, as measured by a TUNEL assay (Fig. 3A). Reconstitution of N-Ras knockout cells by expression of c-N-Ras at endogenous levels (N/−/−–wtN3 or N/−/−–wtN8, Fig. 1, bottom) results in a significant resistance to TNFα treatment, more similar to control cells (Fig. 3A). Similar results were obtained by cell counting and by using the Cell Death Detection ELISA Plus assay (Roche Molecular Biochemicals) (data not shown).

To be certain that the differences in apoptotic sensitivity of the N-Ras knockout and control N+/+ fibroblasts were not simply a result of immortalization, we tested the sensitivity of the MEFs to treatment with cycloheximide and TNFα (Fig. 3B). Both the N-Ras knockout and control MEFs demonstrated some sensitivity to the presence of 2.5 μg/ml cycloheximide alone as measured by the Cell Death Detection ELISA assay. Higher absorbance values reflect increased levels of cytoplasmic histone-associated DNA fragments, which is a measure of the relative degree of apoptosis. The N-Ras knockout MEFs demonstrated significant sensitivity to the addition of TNFα at 1 ng/ml. In contrast, the control MEFs were not sensitive to the addition of TNFα above that observed with cycloheximide alone. This implies that the differences seen in the immortalized cell lines are reflective of similar sensitivity observed in the MEFs.

Treatment of the N-Ras knockout, control, and N/−/−–wtN reconstituted cells with activating anti-Fas antibody resulted in similar findings as observed with TNFα treatment (Fig. 3C). The N-Ras knockout cells demonstrate 25% apoptosis by 8 h of treatment with anti-Fas antibody and soluble protein G, which is reversed by ectopic expression of c-N-Ras at endogenous levels (N/−/−–wtN3 or N/−/−–wtN8, Fig. 3C). In both instances, we did not detect significant differences in the level of either p55 TNF receptor I or CD95/Fas receptor in the established knockout cell lines compared with control cell lines (data not shown).

Serum starvation also led to enhanced cell death by apoptosis of N-Ras knockout cells compared with control N+/+ cells with the restored N/−/−–wtN cells again displaying significant, although partial, reversion (Fig. 3D). Here withdrawal of serum to induce apoptosis takes longer, 40% cell death by 48 h, which is not unlike the results seen with IL-3 withdrawal from pro-B lymphocytes (40). These data suggest that the absence of c-N-Ras function in the N-Ras knockout cell lines renders them more apoptotically sensitive, possibly through altered levels of pAkt and pBad. The observations that multiple N-Ras knockout cell lines are more sensitive to a variety of apoptotic inducers suggest that c-N-Ras functions in a global fashion in providing a steady-state survival signal.

Since there are very noticeable differences in the steady-state levels of pBad in the presence versus the absence of c-N-Ras (Fig. 2C), we tested whether the stable expression of Bel-2 would protect N-Ras knockout cells from TNFα-induced apoptosis. Stable transfectants of N/−/− cells with a FLAG-tagged Bel-2 renders all clones resistant to TNFα-induced apoptosis (Fig. 3E). It seems likely that the overexpression of Bel-2 compensates for the higher levels of unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells. Shifting to a higher steady-state level of Bel-2 by overexpression presumably alters the ratio of Bel-2 to unphosphorylated Bad in the parental N-Ras knockout cells.

Neither c-K(A)- nor c-K(B)-Ras Substitutes for c-N-Ras in Providing a Steady-state Survival Function—We set out to test whether the restoration of the control N+/+ cell phenotype was specific for c-N-Ras. The levels of c-K(A)- and c-K(B)-Ras were examined in all cell lines. Both c-K(A)- and c-K(B)-Ras appear to be up-regulated in the N-Ras knockout cell lines compared with control N+/+ cells (Fig. 4, A and B; the levels of MAP kinase proteins are shown as a control for protein loading). This up-regulation may be a consequence of the immortalization process and/or the continuous culturing on the N-Ras knockout cells in serum-containing medium. The elevated lev-

Cells were harvested, and 100 μg of protein was loaded in each lane of a 13% SDS-polyacrylamide gel. Following electrophoresis and transfer, the blot was incubated with anti-K(A)-Ras polyclonal antibody and developed with anti-rabbit HRP and standard ECL techniques. The exposure was for 15 s except for the last lane, which was exposed for 5 min. Equal protein loading was confirmed with anti-ERK2 blotting as in A (data not shown). The standard is 25 ng of bacterially expressed c-K(A)-Ras protein. The results are representative of three separate experiments. C, TUNEL analysis of untreated and TNFα-treated N-Ras knockout fibroblasts stably transfected with c-K(A)-Ras. c-K(A)-Ras was cloned into pTargeT vector (Promega), the resulting c-K(A)-Ras/pTargeT was transfected into N-Ras knockout cells, and stable clones were selected in G418 as described under “Experimental Procedures.” N-Ras knockout, control N+/+, and N/−/−–(−)wtK(A)-Ras clones were untreated or treated with 1 ng/ml TNFα in the presence of 2.5 μg/ml cycloheximide. Untreated cells and cells treated for 3.5 h were harvested, and their media were collected. The cells were fixed, and TUNEL analysis was performed as described in the legend to Fig. 3A. The values for the untreated cells were less than 3% and are not shown in the figure. The results are representative of three separate experiments. D, K-Ras knockout cells are insensitive to the induction of apoptosis. TUNEL analysis of cycloheximide-treated and TNFα plus cycloheximide-treated K-Ras knockout and control K+/+ cells was performed. K-Ras knockout cells (K/−/−) and control K+/+ cells were treated with 2.5 μg/ml cycloheximide alone or in combination with 10 ng/ml TNFα for 24 h. Following the incubation, the cells, along with their medium, were harvested and fixed, and TUNEL analysis was performed as described in the legend to Fig. 3A. The results are representative of three separate experiments. E, measurement of apoptosis in K-Ras knockout and control MEFs. K-Ras knockout (K/−/−) and control K+/+ MEFs were plated in 12-well cluster plates and treated with TNFα at 10 ng/ml in the presence of 2.5 μg/ml cycloheximide or with cycloheximide alone for 6 h. The cells were harvested and lysed as described in the legend to Fig. 3B. The level of apoptosis was measured using the Cell Death Detection ELISA Plus kit as described in the legend to Fig. 3B. The absorbance values obtained by treatment with cycloheximide alone were subtracted from the TNFα-treated sample values. The assay was performed in triplicate.
els of c-K-Ras proteins may have been necessary for these cells to survive in the absence of c-N-Ras. Overexpression of the K-Ras gene products did not result in (a) protection from apoptotic agents or (b) restoration of the either basal pAkt or pBad levels. These biological events and biochemical properties were only restored by the ectopic expression of c-N-Ras. Ectopic expression of additional c-K(A)-Ras into N-Ras knockout cells did not reverse their apoptotic sensitivity (Fig. 4C). None of the stable c-K(A)-Ras-expressing clones were protected from TNFα-induced apoptosis. Similar results are seen with ectopic expression of c-K(B)-Ras (data not shown). Studies with K-Ras knockout cells support the results with overexpression of c-K(A)- and c-K(B)-Ras in the N-Ras knockout cells. K-Ras knockout cells do not express either c-K(A)- or c-K(B)-Ras; nor do they express detectable levels of Ha-Ras (data not shown). They provide a system to study the function of c-N-Ras alone. Treatment of immortalized K-Ras knockout cells with cycloheximide and TNFα at 10 ng/ml (10-fold higher concentration than that used with the N-Ras knockout cells) for 24 h did not cause an increase in apoptosis above that observed with cycloheximide alone (Fig. 4D). Cycloheximide alone caused some apoptosis that probably results from the extended incubation time (24 h rather than the 4-h incubation time with the N-Ras knockout cells). In contrast, the K(+/+) control cells demonstrated a high level of apoptosis in response to TNFα treatment that is above the level observed with cycloheximide alone (Fig. 4D). Similar results were observed with treatment of the K(−/−) and K(+/+) cells with 75 nM staurosporine (data not shown). We also tested the sensitivity of the K-Ras knockout and control K(+/+) MEFs and found that they responded in a similar fashion to the immortalized cell lines. The K(+/+) MEFs demonstrated higher apoptosis than the K-Ras knockout MEFs after 6 h of treatment with cycloheximide and TNFα at 10 ng/ml (Fig. 17). Cycloheximide had a significant effect in both K-Ras knockout and control K(+/+) MEFs after 24 h of treatment (data not shown). The data with the K-Ras knockout MEFs and immortalized K-Ras knockout fibroblasts, both of which express only c-N-Ras, support the idea that c-N-Ras, but not c-K-Ras, possesses a steady-state survival function. We interpret these results to suggest that c-N-Ras specifically acts to provide a steady-state survival signal through its regulation of steady-state pAkt and pBad.

Our results indicate that, unlike cells that express c-N-Ras, steady-state, exponentially growing N-Ras knockout cells possess very little pBad (Fig. 2C). This implies that the steady-state balance between pro- and antiapoptotic Bcl-2 family proteins may be significantly different for N-Ras knockout cells compared with control N(+/+) cells. It could be postulated that it is this difference that makes N-Ras knockout cells poised to undergo apoptosis given any death-promoting stimulus. The reversal of sensitivity to apoptotic stimuli by expression of Bcl-2 suggests that Bcl-2 compensates for higher levels of unphosphorylated Bad in N-Ras knockout cells. If one of the functions of c-N-Ras is to provide a steady-state signal through PI 3-kinase to maintain basal Akt activity and pBad levels, then the absence of c-N-Ras could result in an altered ratio of Bcl-2 or Bcl-χ, to Bad. It is apparent that c-N-Ras plays a role in “setting and maintaining” the position of the pBad/Bcl-2 or Bcl-XL “ rheostat” has been suggested for Bax/Bcl-2 (57–59). In view of the different expression levels of c-K(A)- and c-K(B)-Ras, our data also specifically link c-N-Ras, but not c-K-Ras, function to the control of pBad levels and the biological end point of cell survival. We could not, however, mimic the apoptotic sensitivity of the N-Ras knockout fibroblasts by long-term treatment of control N(+/+) cells with PI 3-kinase inhibitors (data not shown), suggesting that the mechanism through which c-N-Ras provides its antiapoptotic function goes beyond just the regulation of steady-state phospho-Bad levels.

Between 2 and 8% of the cellular Ras is GTP-bound in serum-deprived cells (60–63). Serum withdrawal induces significant apoptosis in the N-Ras knockout fibroblasts compared with control cells and N-Ras knockout fibroblasts ectopically expressing c-N-Ras at control levels (Fig. 3D). At 48 h following serum withdrawal, there was less than 10% apoptosis in the control cells and the N-Ras knockout cells ectopically expressing c-N-Ras (N(+/−)/wtN). TUNEL analysis revealed nearly 40% apoptosis in the N-Ras knockouts at 48 h following serum starvation. These data imply that even under conditions of serum deprivation the small amount of c-N-Ras-GTP that is likely to be present in control and reconstituted N(+/−)/wtN cells may be sufficient to maintain survival in the absence of serum.

Acknowledgments—We thank Joseph DiDonato, Martha K. Cathcart, Mark Hamilton, and Thomas L. Brown for critical review of the manuscript. We also thank Andrew C. Larner, Maria Karasirides, Mark Hamilton, and Anna Gamero for helpful discussions.

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J. Biol. Chem. 2000, 275:19315-19323.
doi: 10.1074/jbc.M000250200 originally published online April 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000250200

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