The RasGAP N-terminal fragment generated by caspase cleavage protects cells in a Ras/PI3K/Akt-dependent manner that does not rely on NFkB activation.

Running title: Anti-apoptotic mechanisms of the RasGAP N-terminal fragment.

Jiang-Yan Yang§ and Christian Widmann§*

§ Institut de biologie cellulaire et de morphologie (IBCM), Université de Lausanne, Switzerland and *Département de médecine interne, centre hospitalier universitaire vaudois (CHUV), Lausanne

Address correspondence to: Christian Widmann, IBCM, Bugnon 9, 1005 Lausanne, Switzerland
Phone: +41 21 692 5123
FAX: +41 21 692 5255
E-mail: Christian.Widmann@ibcm.unil.ch
Abstract.

RasGAP, a regulator of Ras GTPase family members, is cleaved at low levels of caspase activity into an N-terminal fragment (fragment N) that generates potent anti-apoptotic signals. At higher levels of caspase activity, fragment N is further cleaved into two fragments that strongly potentiate apoptosis. RasGAP could thus function as a sensor of caspase activity to determine whether a cell should survive or not. Here we show that fragment N protects cells by activating the Ras, PI3K, and Akt pathway. Surprisingly, even though NFkB can be activated by Akt, it plays no role in the anti-apoptotic functions of fragment N. This indicates that Akt effectors are differentially regulated when fragment N is generated.
Introduction.

Most, if not all, apoptotic responses rely on the activation of caspases, a family of cysteine-proteases that selectively cleave their substrates after aspartic residues (1;2). The execution phase of apoptosis is triggered when the caspase substrates in a cell are cleaved. Dozens of caspase substrates have been identified and the list is growing steadily (3;4). Once cleaved, caspase substrates mediate the biochemical and morphological events observed during apoptosis, such as amplification of the activation of caspases, DNA fragmentation, nuclear breakdown, etc. In some cases, however, caspase activation does not result in cell death but may in fact participate in other cellular responses, such as the regulation of cell differentiation (5-7).

We have recently demonstrated that RasGAP, a regulator of Ras and Rho GTP-binding proteins, is an unconventional caspase substrate because it can induce both anti- and pro-apoptotic signals depending on the extent of its cleavage by caspases (8). At low levels of caspase activity, RasGAP is cleaved at position 455 generating an amino-terminal fragment (fragment N) and a carboxy-terminal fragment (fragment C). Fragment C alone can induce apoptosis but this response is completely inhibited by fragment N. Fragment N appears to be a general blocker of apoptosis downstream of caspase activation since it inhibits caspase 9-induced cell death. How fragment N mediates its protective effects is unknown. At higher levels of caspase activity, the ability of fragment N to counteract apoptosis is suppressed when it is cleaved at position 157. This latter cleavage event generates two fragments that, in contrast to fragment N, potently sensitize cells towards apoptosis. RasGAP could thus be viewed as an "apoptostat" in the sense that it can allow the cell to determine when caspases have been mildly activated to fulfill functions other than apoptosis or when caspases are strongly activated to mediate apoptosis.

In the present study we have characterized the molecular mechanisms underlying the protective effects of fragment N. Our results show that fragment N inhibits apoptosis in a Ras-PI3K-Akt-dependent manner that, surprisingly, does not rely on NFkB activation.
Experimental procedures.

Cell lines.

HeLa cells were maintained in RPMI 1640 containing 10% newborn calf serum (NBCS) (GIBCO/BRL) at 37°C and 5% CO₂. Cells were transfected using lipofectamine 2000 (GIBCO/BRL) as described (9). The total amount of DNA was kept constant using empty vectors when required.

Chemicals and antibodies.

The anti-Ras mouse monoclonal IgG 2αK antibody (clone RAS10) and the anti-ERK1/2 rabbit polyclonal IgG antibody were from Upstate Biotechnology (catalog #05-516 and #06-182, respectively). The anti-phospho serine 473-Akt rabbit polyclonal IgG antibody was from Cell Signaling Technology (catalog #9271). The rabbit polyclonal IgG antibody recognizing Akt1/2 was from Santa-Cruz Biotechnology (catalog #SC-8312). The anti-PI3-Kinase p85 antibody used for the in vitro PI3-Kinase assay was from Upstate Biotechnology (catalog #06-195). The monoclonal antibody specific for the HA tag was purchased as ascites from BabCO (#MMS-101R). This antibody was adsorbed on HeLa cell lysates to decrease non-specific binding as previous described (8).

Plasmids.

The eukaryotic expression vector pcDNA3 is from Invitrogen. The dn3 and cmv extensions in some of the names of the plasmids used in this study indicate that the backbone vector is pcDNA3 and pCMV4/5, respectively. GFP-GAPC encodes a fusion protein between GFP and fragment C of RasGAP (amino acids 456-1047) bearing a HA tag at the carboxy-terminal end. N-D157A.dn3 encodes the HA-tagged version (at the N-terminus) of a caspase resistant form of fragment N (RasGAP sequences 1-455). These plasmids have been described previously (8). N17Ras.cmv is a pCMV5 derived plasmid encoding the S17->N dominant negative Ras mutant. N19Rho.dn3 encodes a Myc tagged form of the S19->N dominant negative Rho mutant. Ras.dn3 encodes human
c-Ha-Ras-1 and \textit{V12Ras.dn3} corresponds to the constitutively active G12->V form of the protein. \textit{V12S35.sg5}, \textit{V12G37.sg5}, and \textit{V12C40.sg5} are pSG5-derived plasmids encoding the constitutively active form of Ras bearing the mutations T35->S, E37->G and Y40->C, respectively. \textit{SH2-p85.dn3} encodes the SH2 domain of p85\(\alpha\) (amino acids 21-140) bearing the myc tag at its N-terminus. \textit{p110-CAAX} encodes the membrane targeted, constitutively active form of the catalytic subunit of PI3K\(\alpha\). \textit{Akt-DN.cm}v encodes the dominant-negative kinase-inactive mutant of Akt bearing an HA tag at its N-terminus. \textit{myr-Akt.cm}v encodes a constitutively active form of Akt that bears a Src myristoylation sequence at its N-terminus and an HA tag at its C-terminus. \textit{MEKK1.dn3} has been described previously (10). \textit{I\(\kappa\)B\(\alpha\)ΔN2} encodes a mutant of I\(\kappa\)B\(\alpha\) that cannot be phosphorylated by IKKs and degraded by the proteasome, and therefore functions as an inhibitor of NF\(\kappa\)B. \textit{IKK2.vsv} encodes the wild-type IKK2 protein. \textit{Raf-RBD.pgx} (also called pGEX 2T-RBD) encodes a fusion protein between glutathione-S-transferase and amino acids 51-131 of the Ras binding domain of Raf1. \textit{MEKK1.dn3} has been described before (10).

**Apoptosis assay.**

Apoptosis was determined by scoring transfected cells (as assessed by the expression of GFP) displaying pycnotic nuclei (visualized with Hoechst 33342), as described previously (8).

**In vitro PI3K assays.**

HeLa cells were starved for 16 hours, then lysed in RIPA buffer (50 mM Tris/HCl, pH 7.2, 500 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 100 \(\mu\)M Na\(_3\)VO\(_4\), 1 mM PMSF, 1 tablet per 50 ml of complete protease inhibitor cocktail [Roche, #1.697.498]). Five hundred \(\mu\)g of total protein were then incubated with a 1:100 dilution of an anti-PI3K antibody in the presence of 40 \(\mu\)l of a 1:1 slurry of protein-Sepharose A beads (Amersham Pharmacia Biotech AB #17-0974-01) with gentle rocking overnight at 4\(^\circ\)C. The beads were washed three times with the kinase buffer A
(1X TBS [50 mM Tris pH 7.5, 150 mM NaCl], 1% NP40, 100 µM Na₃VO₄), three times with kinase buffer B (100 mM Tris-HCl (pH 7.5), 500 mM LiCl₂, 100 µM Na₃VO₄), three times with kinase buffer C (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA 100 µM Na₃VO₄). The beads were then resuspended in 50 µl of kinase buffer C without Na₃VO₄. Ten µl of 100 mM MgCl₂, 10 µl of phosphatidylinositol (2 mg/ml in Tris-HCl (pH 7.5), 1 mM EGTA, together with 10 µl of 440 µM ATP containing 20 µCi of ³²P-ATP. This mixture was then incubated for 10 minutes at room temperature. The reaction was terminated with the addition of 20 µl of 8 N HCl. The lipids were extracted by adding 160 µl of CHCl₃:MeOH (1:1), and centrifuged for 10 minutes at 14’000 rpm. Fifty µl of the lower organic phase were harvested and loaded onto a TLC plate (Silica Gel 60, glass support, 20x20 cm, Sigma #Z 29 2974), which had been pretreated with 1% potassium oxalate in H₂O:MeOH (3:2). The plates were then developed in CHCl₃:CH₃OH: H₂O: NH₄OH (60:47:11.3:2) for 2 hours, and then visualized by autoradiography.

**In vitro Akt kinase assay.**

HeLa cells were starved and lysed as described for the in vitro PI3K assay. Akt kinase activity was measured using a kit from Upstate Biotechnology (#06-195) in accordance with the manufacturer’s instructions.

**Western blot analysis.**

Cells were lysed in monoQ-c buffer, as described previously (8). For the Western blot analysis using anti-phospho Akt or anti-phospho-ERK antibodies, the cells were lysed in RIPA buffer. Western blotting was performed as described (11) using an home-made ECL reagent (8). To improve the signal in some experiments, an enhanced ECL solution (Super Signal® west Femto Maximum Sensitivity Substrate from PIERCE [cat #34095]) was mixed with the home-made reagent.

**Affinity-precipitation of GTP-bound cellular Ras.**

The fusion protein encoding glutathione-S-transferase and amino acids 51-131
of c-Raf1 was isolated as previously described (12). Briefly, Raf-RBD.pgx transformed bacteria were incubated with IPTG for 2 hours at 37°C and sonicated on ice 6 times for 1 minute in PBS containing 0.5 mM DTT, 0.1 µM aprotinin, 1 µM leupeptin and 1 mM PMSF. Triton-X100 was added to a final concentration of 1% and, after gently stirring for 30 minutes at 4°C, glycerol was added to a final concentration of 10%. The lysate was aliquoted and stored at -80°C until used (but no more than 4 weeks). The desired amount of crude GST-RBD was thawed and incubated with glutathione-agarose beads at room temperature for 30 minutes. The beads were isolated by centrifugation and washed three times with RIPA buffer. Cells from a 10 cm dish were lysed and scraped in 1 ml of RIPA buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 % DOC, 1% NP40, 0.1% SDS, 0.1 µM aprotinin, 1 µM leupeptin and 1 mM PMSF at 4°C. Lysates were centrifuged for 8 minutes at 1’200 g in an Eppendorf centrifuge to remove nuclei. GST-RBD precoupled to glutathione–agarose beads in RIPA buffer, was added and the lysates were incubated at 4°C for 30 minutes. Beads were collected by centrifugation, washed 3 times with RIPA buffer and resuspended in sample buffer (10 % glycerol, 60 mM Tris, pH 6.8, 2 % SDS, 300 mM β-mercaptoethanol). The protein sample were separated on a 15% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane for Western blotting analysis using a Ras-specific monoclonal antibody.

**NFκB assay.**

NFκB activity was measured using a luciferase reporting assay, as described previously (13).
RESULTS

The cleavage of RasGAP by caspases at position 455 occurs at very low caspase activity (8). The resulting N-terminal fragment generates potent anti-apoptotic signals. However, as caspase activity increases, fragment N is cleaved at position 157 (8). The fragments resulting from this cleavage event strongly sensitize cells towards apoptosis (8). Therefore, in order to specifically study the anti-apoptotic function of fragment N in the present study, we have used a mutant of fragment N bearing an aspartate to alanine substitution at position 457 that cannot be further cleaved by caspases (8).

Ras, but not Rho, is required for fragment N-induced cell protection.

Since RasGAP is a regulator of Ras and Rho (14), we assessed whether these small GTP-binding proteins are required for the protective effects mediated by fragment N. As shown in Figure 1A, a dominant negative mutant of Ras (N17Ras), but not a dominant negative mutant of Rho (N19Rho), totally blocked the ability of fragment N to inhibit fragment C-induced apoptosis. Rho has recently been shown to be required for the ability of V12Ras, a constitutively active form of Ras, to activate the ERK MAPK (15). Western blot analysis using phospho-ERKs specific antibodies on lysates from HeLa cells transfected with V12Ras in the presence or in the absence of N19Rho demonstrated that N19Rho prevented the ability of V12Ras to induce ERK phosphorylation (Figure 1B). This demonstrates that N19Rho functions as a specific Rho inhibitor in our experimental system. The data presented in Figure 1A and B indicate that blocking Ras, but not Rho, prevents fragment N from being anti-apoptotic.

A pull-down assay employing the Ras binding domain of Raf (RBD) fused to GST was used to determine whether fragment N can activate Ras. GST-RBD only binds to the active GTP-bound form of Ras and the amount of active Ras bound to GST-RBD can be measured by quantitative Western blot analysis using Ras-specific antibodies (12). Figure 1C shows that fragment N activates Ras either in the presence or in the absence of fragment C (fragment C alone had no significant effect on Ras activity by itself).
Together with the observation that an active form of Ras, V12Ras, can mimic fragment N-induced inhibition of apoptosis (8), the results presented in Figure 1 demonstrate that Ras activation is necessary and sufficient to mediate fragment N-induced protection.

Several pathways are activated by Ras, some of them with known anti-apoptotic functions (16). To assess which Ras-dependent pathways could be involved in the protective effect mediated by fragment N, constitutively activated Ras mutants that preferentially activate a subset of the Ras effector pathways (V12S35 Ras for the ERK MAPK pathway, V12C40 for PI3K-dependent pathways, and V12G37 for RalGDS-dependent pathways) (14;17) were tested for their ability to block fragment C-induced apoptosis. V12C40Ras was the only mutant that inhibited fragment C-induced apoptosis with the same potency as activated Ras (V12Ras) (Figure 2). V12S35S partially blocked apoptosis but only in conditions leading to the highest expression levels. V12G37Ras had no protective effect. These results suggest that activation of PI3K-dependent pathways could mediate fragment N-induced anti-apoptotic functions.

The protective function of fragment N is PI3K-dependent.

Figure 3A shows that fragment N induces PI3K activation in the absence or in the presence of fragment C. The SH2 domain of the p85 regulatory PI3K subunit - that functions as a dominant negative mutant - totally blocked the ability of fragment N to inhibit fragment C-induced apoptosis (Figure 3B). Moreover, a constitutively active form of PI3K, p110CAAX, mimicked the ability of fragment N to inhibit fragment C-induced apoptosis (Figure 3C). Activation of PI3K is thus necessary and sufficient to mediate fragment N-induced protection.

Activation of Akt is required for fragment N to mediate its protective effects.

Activation of Akt has been shown to promote cell survival in many systems (18). Since Akt can be activated by PI3K we determined whether it could participate in the anti-apoptotic response induced by fragment N. Using an in vitro Akt kinase assay (Figure 4A) or a Western blot analysis using antibodies recognizing the activated form of Akt (Figure 4B), we observed that fragment N led to Akt activation, whether or not
fragment C was present. A kinase-dead Akt mutant inhibited, in a dose-dependent manner, the ability of fragment N to block fragment C-induced apoptosis (Figure 4C). Addition of the src myristoylation sequence to Akt renders it constitutively active (18). This Akt construct mimicked the protective function of fragment N (Figure 4D). Akt activation is thus necessary and sufficient to mediate fragment N-induced protection.

**NFkB is not involved in fragment N-induced survival signaling.**

Different mechanisms can be used by Akt to protect cells from apoptosis, including activation of NFkB, inhibition of caspase 9, inhibition of Bad, and inhibition of specific transcription factors (Forkhead, Nur77) (18;19). We determined whether NFkB could be involved in the Akt-dependent protection mediated by fragment N. Figure 5A shows that fragment N weakly stimulated NFkB activity (about 3 fold over basal; in four other independent experiments fragment N stimulated NFkB by only 1.04-1.8 fold). In comparison, MEKK1, a potent activator of NFkB when over-expressed in cells (13;20;21), was about 10 times more effective than fragment N in stimulating NFkB (Figure 5A). To determine if the weak activation of NFkB induced by fragment N is required for its ability to block fragment C-induced apoptosis, HeLa cells were transfected with fragment C and/or fragment N in the presence of increasing amounts of a plasmid encoding the NFkB inhibitor IkBαΔN2. As expected, IkBαΔN2 strongly reduced NFkB activity (Figure 5B, upper panel). However, the inhibitor did not affect the ability of fragment N to block fragment C-induced apoptosis (Figure 5B, lower panel). It could be argued that, in HeLa cells, the NFkB pathway is not able to induce protective signals. This is not the case, since blocking NFkB activity with IkBαΔN2 induces cell death in TNFα-treated HeLa cells (Figure 5C) and since activating NFκB by overexpression of IKK2 protected cells from fragment C-induced apoptosis (Figure 5D). This demonstrates that NFκB can protect HeLa cells from apoptosis, including the apoptotic response induced by fragment C (see also (22)). Therefore, despite a functional anti-apoptotic NFkB pathway in HeLa cells, the protection induced by fragment N, which is Akt-dependent, does not operate through the activation of NFkB.
Fragment N specifically blocks Akt-induced NFκB activation.

The observation that fragment N stimulates Akt without a concomitant NFκB activation suggests that fragment N is able to inhibit the ability of Akt to activate NFκB. To test this hypothesis, HeLa cells were transfected with a plasmid encoding a constitutively active form of Akt (myr-Akt) in the presence of increasing quantities of a fragment N-encoding plasmid. Figure 6 shows that fragment N was able to inhibit, in a dose-dependent manner, the activation of NFκB induced by Akt. This inhibitory effect was specific for Akt since activation of NFκB mediated by MEKK1 was not decreased by fragment N. Western blot experiments showed that the levels of expression of myr-Akt or MEKK1 were unaffected by the increased expression of fragment N (data not shown). This result indicates therefore that fragment N does not have a broad NFκB inhibitory effect.

The Ras-PI3K-Akt pathway is used by fragment N to inhibit apoptosis mediated by different stimuli.

Figure 7 shows that dominant negative forms of Ras, PI3K and Akt inhibited the ability of fragment N to block apoptosis induced by low doses of caspase 9. Therefore, fragment N inhibits apoptosis mediated by apoptotic inducers others than fragment C and this also occurs via the Ras-PI3K-Akt pathway.
DISCUSSION

Activation of caspases in a cell generally results in apoptosis. There are, however, exceptions to this rule. For example, caspases stimulated by activated death receptors participate in the negative regulation of erythropoiesis by cleaving GATA-1, a transcription factor required for differentiation of mature erythroblasts and this occurs in the apparent absence of any apoptotic response (5). In peripheral blood lymphocytes, caspase 8 and caspase 3 are activated upon stimulation. Blocking caspase activity inhibits proliferation, major histocompatibility class II expression, and blastic transformation of these cells (6). Similarly, activation of caspase 8 is required for CD3-induced proliferation and IL2 production by human T cells (7). These data indicate that caspase activation occurs in viable cells and this appears to be involved in physiological responses different from apoptosis. A corollary to the above observations is that specific protective pathways must be activated in viable cells having activated their caspases to fulfill functions different than apoptosis. These protective pathways need now to be characterized.

Caspase activation with no associated death response is not only observed in cells from the hematopoietic lineage. We have recently shown that in mild stress conditions, HeLa cells displayed increased caspase 3-like activity. Despite this caspase activation, the cells did not undergo apoptosis. RasGAP is cleaved in these conditions and this generates an N-terminal fragment (fragment N) with potent anti-apoptotic functions (8). HeLa cells are protected from cell death as long as fragment N is not further cleaved, which abrogates its anti-apoptotic functions and generates two new pro-apoptotic RasGAP N-terminal peptides (fragments N1 and N2). The second cleavage of RasGAP only occurs at high levels of caspase activity (8). The first cleavage of RasGAP into fragment N could thus represent an important safeguard mechanisms to protect cells from apoptosis resulting from caspase activation induced by mild stress or in physiological responses needing some kind of caspase activation.

In the present study we have determined that the upstream anti-apoptotic pathway activated by fragment N employs the Ras, PI3K, and Akt proteins. These proteins are necessary and sufficient to mediate the protective function of fragment N because 1) the
corresponding dominant negative mutants totally abrogated the anti-apoptotic abilities of fragment N; 2) constitutively activated mutants of Ras, PI3K and Akt mimicked the protective effect of fragment N; and 3) fragment N induces the activation of Ras, PI3K and Akt.

How fragment N induces Ras activity is currently not understood. It is possible that the SH3 domain born by fragment N is involved since a monoclonal antibody specific for the SH3 domain of RasGAP inhibits downstream signals initiated by oncogenic Ras. (23;24). However, since these studies used constitutively active forms of Ras, it does not provide any information on how the SH3 domain of RasGAP could activate Ras. Several proteins interact with the N-terminal domain of RasGAP, such as the PDGF receptor, p190 RhoGAP, Huntingtin, Dok proteins, and Src (and other tyrosine kinases) (25-32). It is currently not known whether any of these is required for fragment N to stimulate Ras activity.

A major anti-apoptotic factor activated by the Ras-PI3K-Akt pathway is NFκB (33-35). Surprisingly, NFκB was not required for fragment N to protect cells despite the fact that the NFκB pathway can mediate anti-apoptotic responses in HeLa cells (e.g. upon TNFα stimulation). In fact, while Akt activity was stimulated by fragment N, this did not result in significant transcription of NFκB-driven reporter genes. This is due to the fact that fragment N inhibits the ability of activated Akt to stimulate NFκB. Fragment N, however, is not a general blocker of NFκB activation because it did not hamper MEKK1 from activating NFκB. Fragment N seems thus to inhibit only a subset of the signaling pathways that activate NFκB. G3BP2, a close relative to the RasGAP SH3-binding protein G3BP1, sequesters IκBα/NFκB complexes in the cytoplasm, thereby preventing NFκB to exert its transcriptional activity in the nucleus (36). An interesting hypothesis is that fragment N, which bears the SH3 domain of RasGAP, uses G3BP2 to prevent NFκB from reaching the nucleus even when Akt is activated.

Because fragment N does not use NFκB to mediate its protective functions, it must use alternative mechanisms. These could include inactivation by phosphorylation of pro-apoptotic proteins such as caspase 9, forkhead proteins or Bad (18). Indeed, fragment N seems to induce the phosphorylation of Bad on serine 136 (unpublished results),
indicating that Bad inactivation is a potential mechanism by which fragment N blocks apoptosis.

In summary, our findings indicate that fragment N utilizes the Ras-PI3K-Akt signaling pathway to protect cells from apoptosis. Despite stimulating Akt activity, fragment N does not allow the Akt effector NFκB to exert its transcriptional activity. This indicates that effector proteins of the Ras-PI3K-Akt pathway can be differently modulated in the presence or in the absence of RasGAP caspase cleavage fragments. Fragment N adds an additional level of complexity in the way the Ras-PI3K-Akt pathway is modulated.
Acknowledgements.

We thank Dr. Julian Downward for providing the plasmids encoding the V12S35, V12C40 and V12G37 Ras mutants. We thank Dr. Romano Regazzi for the gift of the N17Rho.dn3 plasmid. We thank Dr. Peter Vollenweider and Barbara Menard for helping us with the in vitro Akt and PI3K kinase assays and for providing the anti-phospho Akt antibody and the SH2-p85.gst plasmid from which SH2-p85.dn3 was derived. We thank Fabio Martinon and Dr. Jürg Tschopp for the generous gift of the IKK2.vsv plasmid and Dr. Johannes L. Bos for the kind gift of plasmid Raf-RBD.pgx. We also thank Dr. Peter Vollenweider, Dr. Mark Epping-Jordan and Dr. Matthias Peter for critical reading of the manuscript. This work is supported by grant 3100-055606 from the Swiss National Science Foundation and grants from the Botnar foundation (Lausanne).
Reference List


Figure Legends

Fig. 1: **Ras, but not Rho, is activated by fragment N and required to mediate its anti-apoptotic function.**

A. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with either empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid (C), or 4µg of the fragment C-encoding plasmid and 1 µg of the plasmid encoding an HA-tagged uncleavable form of fragment N (N+C), in the presence or in the absence of 2 µg of a plasmid encoding the dominant negative N17Ras mutant or 2 µg of a plasmid encoding the dominant negative N19Rho mutant. The number of transfected cells undergoing apoptosis was then scored and expressed as the means ± standard deviation of five independent experiments performed in duplicate.

B. HeLa cells were transfected with pcDNA3 or with 2 µg of a plasmid encoding the constitutively active V12Ras mutant, in the presence or in the absence of 2 µg of a plasmid encoding the dominant negative N19Rho mutant. The extent of ERK activation was assessed by western blot analysis using a specific anti-phospho-ERK antibody. This blot is a representative example of two independent experiments.

C. HeLa cells were transfected with a Ras-encoding plasmid (2 µg) together with plasmids encoding fragment N (1 µg) and fragment C (4 µg), alone or in combination. After a 24 hours recovery period, the cells were starved for 16 hours and lysed in RIPA buffer. The amount of active Ras was visualized as described in the “experimental procedures” section. Western blot analysis of the whole cell lysates showed that Ras expression levels were similar in the different samples (data not shown). This figure is representative of six independent experiments.

Fig. 2: **Ras mutants that are not impaired in PI3K activation mediate strong protection against fragment C-induced apoptosis.**

Yang et al. 2001
HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3) or 4 µg of the fragment C-encoding plasmid (C) in the presence of increasing quantities of various constitutively active Ras mutants (V12Ras, V12S35Ras, V12G37Ras, V12C40Ras). The number of apoptotic cells was then scored as described in figure 1A and expressed as the mean ± SEM of duplicate determinations. This figure is representative of three independent experiments.

Fig. 3: **PI3K activation is necessary and sufficient for fragment N to mediate its anti-apoptotic functions.**

A. HeLa cells were transfected with empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid (C), 1 µg of the fragment N-encoding plasmid (N), or a combination of the two latter plasmids (N+C). The cells were then washed twice with PBS and starved for 16 hours. PI3K activity was measured by using an *in vitro* kinase assay, as described in the “experimental procedures” section. The data are expressed as the mean ± SEM of duplicate determinations. This figure is representative of three independent experiments.

B. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid (C), 1 µg of the fragment N-encoding plasmid (N), or a combination of the two latter plasmids (N+C), in the presence of increasing quantities of a plasmid encoding the isolated SH2 domain of PI3Kα (that functions as dominant negative mutant for PI3Ks). The number of apoptotic cells was then scored as above.

C. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3) or 4 µg of the fragment C-encoding plasmid (C), in the presence of increasing quantities of a constitutively active mutant of PI3K (p110CAAX)-encoding plasmid. The number of apoptotic cells was then scored as above.

The figures presented in panels A, B, and C are representative of three independent experiments.
Fig. 4: Akt activation is necessary and sufficient for fragment N to mediate its anti-apoptotic functions.

A. and B. HeLa cells were transfected, starved and lysed as described in figure 3A. The Akt activity was measured either by using an in vitro Akt kinase assay (as described in the “experimental procedures” section) (panel A) or by monitoring the extent of phosphorylation of Akt at the activation site (serine 473) (panel B). In panel A, the data are expressed as percentage of the maximal response (mean ± SEM of 2 independent experiments performed in duplicate). The blot shown in panel B is a representative example of 4 independent experiments.

C. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid (C), 1 µg of the fragment N-encoding plasmid (N), or a combination of the two latter plasmids (N+C), in the presence of increasing quantities of a plasmid encoding a dominant negative mutant of Akt. The number of apoptotic cells was then scored as in Figure 3A.

D. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3) or 4 µg of the fragment C-encoding plasmid (C), in the presence of increasing quantities of a constitutively active mutant of Akt (myr-Akt)-encoding plasmid. The number of apoptotic cells was then scored as above.

Fig. 5. NFκB is not involved in fragment N-induced survival signaling despite being functional in HeLa cell.

A. HeLa cells were transfected with empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid, 1 µg of the fragment N-encoding plasmid, or 2 µg of an MEKK1-encoding plasmid, in the presence of a luciferase NFκB reporter gene encoding plasmid (prLUC). NFκB activity is expressed as fold increase over basal (mean ± SEM of duplicate determinations). This experiment has been repeated four times with similar results.

B. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid (C), 1 µg of the
fragment N-encoding plasmid (N), or a combination of the two latter plasmids (N+C), in the presence of increasing quantities of a plasmid encoding the inhibitor of NFκB (IκBΔN2) and in the presence of prLUC, the luciferase NFκB reporter gene encoding plasmid. The number of apoptotic cells was scored as described in figure 3A. The NFκB activity was measured as above. This figure iC. s representative of three independent experiments.

D. HeLa cells were transfected with 1µg of a GFP-expression plasmid together with increasing quantities of an NFκB inhibitor (IκBΔN2)-encoding plasmid. The cells were then treated with TNFα (0.3 nM) for 18-24 hours. The number of apoptotic cells was then scored as described in Figure 3A. This experiment has been repeated twice with similar results.

E. HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector (pcDNA3), 4 µg of the fragment C-encoding plasmid, 1 µg of the fragment N-encoding plasmid, 2 µg of an IKK2-encoding plasmid in the indicated combinations. The number of apoptotic cells was then scored as described in figure 3A. This figure is representative of two independent experiments.

Fig. 6: **Fragment N blocks Akt-induced NFκB activation, but has no effect on MEKK1-induced NFκB activation.**

HeLa cells were transfected with pcDNA3, with 1 µg of a MEKK1-encoding plasmid or with 1 µg of a constitutively active mutant of Akt (myr-Akt)-encoding plasmid, together with increasing quantities of a fragment N-encoding plasmid and 0.5 µg of prLUC, the luciferase NFκB reporter gene encoding plasmid. NFκB activity is expressed as fold increase over basal (mean ± SEM of duplicate determinations). This experiment has been repeated four times with similar results.

Fig. 7: **Ras, PI3K and Akt are required for fragment N to block apoptosis induced by low doses of caspase 9.**

HeLa cells were transfected with 1 µg of a GFP-expression plasmid together with an empty vector, 2 µg of the caspase 9-encoding plasmid, 1 µg of the fragment N-encoding
plasmid, 2 µg of the N17Ras-encoding plasmid, 2 µg of the SH2-p85-encoding plasmid, 2 µg of the Akt-DN-encoding plasmid, in the indicated combinations. The number of apoptotic cells was then scored as described in Figure 3A. This experiment has been repeated twice with identical results.
A. PI3K activity (fold over basal) for different conditions:
- pcDNA3
- C
- N
- N +

B. Graph showing the percentage of apoptosis with varying concentrations of
μg SH2-p85α-encoding plasmid:
- pcDNA3
- C
- C + N

C. Graph showing the percentage of apoptosis with varying concentrations of
μg p110CAAX-encoding plasmid:
- pcDNA3
- C
A. NFkB activity (fold over basal) for pcDNA3, N, C, and MEKK1.

B. Graph showing NFkB activity (% maximal response) for pcDNA3, C, and C + N.

C. Graph showing % apoptosis with varying µg of IkBαΔN2-encoding plasmid with and without TNFα.

D. Bar graph showing % apoptosis for pcDNA3, N, C, N + C, IKK2, and IKK2 + C.
The RasGAP N-terminal fragment generated by caspase cleavage protects cells in a Ras/PI3K/Akt-dependent manner that does not rely on NFkB activation

Jiang-Yan Yang and Christian Widmann

*J. Biol. Chem. published online February 14, 2002*

Access the most updated version of this article at doi: 10.1074/jbc.M111540200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts