The RasGAP N-terminal Fragment Generated by Caspase Cleavage Protects Cells in a Ras/PI3K/Akt-dependent Manner That Does Not Rely on NFκB Activation

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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-Akt pathway. Surprisingly, even though nuclear factorκB (NFκB) 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.

Most, if not all, apoptotic responses rely on the activation of caspasases, 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 N-terminal fragment (fragment N) and a C-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 because 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 toward 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 NFκB activation.

EXPERIMENTAL PROCEDURES

Cell Lines—HeLa cells were maintained in RPMI 1640 containing 10% newborn calf serum (Invitrogen) at 37 °C and 5% CO2. Cells were transfected using LipofectAMINE 2000 (Invitrogen) 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 nos. 05-516 and 06-182, respectively). The anti-phospho serine 473-Akt rabbit polyclonal IgG antibody was from Cell Signaling Technology (catalog no. 9279). The rabbit polyclonal IgG antibody recognizing Akt1/2 was from Santa Cruz Biotechnology (catalog no. SC-8312). The anti-PI3K p85 antibody used for the in vitro PI3K assay was from Upstate Biotechnology (catalog no. 06-195). The monoclonal antibody specific for the HA tag was purchased as ascites from Babco (catalog no. M18-101B). This antibody was adsorbed on HeLa cell lysates to decrease nonspecific binding as previously 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 the constitutive active form of Ras bearing the mutations Thr35Ser, Gly37Cys, respectively. SH2-p85.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 Ser19→Ala dominant negative Ras mutant. N19Rho.dn3 encodes a Myc-tagged form of the Ser19→Ala dominant negative Rho mutant. RasN17 encodes the constitutively active Gly12→Val form of the protein. V12S35.sg5, V12G37.sg5, and V12C40.sg5 are pSG5-derived plasmids encoding the constitutively active form of Ras bearing the mutations Thr35→Ser, Gln37→Gly, and Tyr14→Cys, respectively. SH2-p85.dn3 encodes the SH2 domain of p85α (amino acids 21–140) bearing the Myc tag at its N terminus.
p110-CAAX encodes the membrane targeted, constitutively active form of the catalytic subunit of PI3Kα. Akt-DN.cmv encodes the dominant negative kinase-inactive mutant of Akt bearing a HA tag at its N terminus. myr-Akt.cmv 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. MEKK1.dn3 has been described previously (10). IxBoAN2 encodes a mutant of IxBα that cannot be phosphorylated by IxK proteins and degraded by the proteasome and therefore functions as an inhibitor of NFkB. IxK2.vev encodes the wild-type IxK2 protein. Raf-RBD.pex (also called pGEX 2T-RBD) encodes a fusion protein between GST and amino acids 51-131 of the Ras binding domain of Raf1.

**Apoptosis Assay—** Apoptosis was determined by scoring transfected cells that failed to express the expression of GFP displaying pyknotic nuclei (visualized with Hoechst 33342), as described previously (8).

**In Vitro PI3K Assay—** HeLa cells were starved for 16 h and then lysed in RIPA buffer (50 mM Tris/HCl, pH 7.2, 500 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 100 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet per ml of a complete protease inhibitor mixture (Roche, catalog no. 1.697.498)). 500 μg of total protein were then incubated with a 1:100 dilution of an anti-PI3K antibody in the presence of 40 μg of the catalytic subunit of PI3K encoding GST and amino acids 51-131 of the Ras binding domain of Raf1. Western blotting was performed as described (11) using a homedesigned previously (8). For the Western blot analysis using anti-phospho-PI3K assay. Akt kinase activity was measured using a kit from Upstate Biotechnology (catalog no. 06-195). 20 μl of 8 N HCl. The lipids were extracted by adding 160 μl of CHCl3/MeOH (1:1) and centrifuged for 10 min at 15,000 × g. Fifty μl of the lower organic phase were harvested and loaded onto a TLC plate (silica gel 60, glass support, 20 × 20 cm, Sigma catalog no. 2.92794) that 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 h and then visualized by autoradiography.

**Western Blot Analysis—** Cells were lysed in mono Q-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 a home-made ECL solution (SuperSignal® West Femto maximum sensitivity substrate from Pierce, catalog no. 34095) was mixed with the homemade reagent.

**Affinity Precipitation of GTP-bound Cellular Ras—** The fusion protein encoding GST and amino acids 51-131 of c-Raf1 was isolated as described previously (12). Briefly, Raf-RBD.pex-transformed bacteria were incubated with isopropyl-β-D-thiogalactopyranoside for 2 h at 37 °C and sonicated on ice six times for 1 min in phosphate-buffered saline containing 0.5 mM dithiothreitol, 0.1 mM aprotinin, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Triton X-100 was added to a final concentration of 1% and, after gently stirring for 30 min 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 Ras-binding domain of Ras (RBD-Raf) was thawed and incubated with glutathione-agarose beads at room temperature for 30 min. 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% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.1 mM aprotinin, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride at 4 °C. The lysate were centrifuged for 10 min at 12,000 × g in a Eppendorf-centrifuge to remove nuclei. GST-RBD precoupled to glutathione-agarose beads in RIPA buffer was added to the lysates and incubated with gentle rocking at 4 °C for 60 min. Beads were collected by centrifugation, washed three times with RIPA buffer, and resuspended in sample buffer (10% glycerol, 60 mM Tris, pH 8.6, 2% SDS, 300 mM β-mercaptoethanol). The protein samples were separated on a 15% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose mem

**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 toward apoptosis (8). Therefore, to specifically study the anti-apoptotic function of fragment N in the present study we have used a mutant of fragment N (1 μg) and fragment C (4 μg), alone or in combination. After a 24 h recovery period, the cells were starved for 16 h and lysed in RIPA buffer. The amount of active Ras was visualized as described under “Experimental Procedures.” 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. 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 4 μg of the fragment C-encoding plasmid and 1 μg of the plasmid encoding an HA-tagged uncleavable form of fragment N (C + N) in the presence or 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 mean ± S.D. of five independent experiments performed in duplicate. B. HeLa cells were transfected with pcDNA3 or with 2 μg of a plasmid encoding the constructively active V12Ras mutant in the presence or 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 h recovery period, the cells were starved for 16 h and lysed in RIPA buffer. The amount of active Ras was visualized as described under “Experimental Procedures.” 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.

**NFkB Assay—** NFkB activity was measured using a luciferase reporter assay, as described previously (13).
using phospho-ERK-specific antibodies on lysates from HeLa cells transfected with V12Ras in the presence or absence of N19Rho demonstrated that N19Rho prevented the ability of V12Ras to induce ERK phosphorylation (Fig. 1B). This demonstrates that N19Rho functions as a specific Rho inhibitor in our experimental system. The data presented in Fig. 1, A 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 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). Fig. 1C shows that fragment N activates Ras either in the presence or 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 Fig. 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 (G12V/T35S Ras for the ERK MAPK pathway, G12V/Y40C for PI3K-dependent pathways, and G12V/E37G for Ral-GDS-dependent pathways) (14, 17) were tested for their ability to block fragment C-induced apoptosis. G12V/Y40C Ras was the only mutant that inhibited fragment C-induced apoptosis with the same potency as activated Ras (V12Ras) (Fig. 2). G12V/T35S partially blocked apoptosis but only in conditions leading to the highest expression levels. G12V/E37G Ras had no protective effect. These results suggest that the activation of PI3K-dependent pathways could mediate fragment N-induced anti-apoptotic functions.

**The Protective Function of Fragment N Is PI3K-dependent**—Fig. 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, which functions as a dominant negative mutant, totally blocked the ability of fragment N to inhibit fragment C-induced apoptosis (Fig. 3B). Moreover, a constitutively active form of PI3K, p110CAAX, mimicked the ability of fragment N to inhibit fragment C-induced apoptosis (Fig. 3C). Activation of PI3K is thus necessary and sufficient to mediate fragment N-induced protection.
HeLa cells were transfected with fragment C and/or fragment H9260 signaling tant of Akt (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 described in the Fig. 1 legend. D, 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 (C + N) 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 described in the Fig. 1A legend. 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.

**Activation of Akt Is Required for Fragment N to Mediate Its Protective Effects**—The activation of Akt has been shown to promote cell survival in many systems (18). Because 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 (Fig. 4A) or a Western blot analysis using antibodies that recognize the activated form of Akt (Fig. 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 (Fig. 4C). The addition of the src myristoylation sequence to Akt renders it constitutively active (18). This Akt construct mimicked the protective function of fragment N (Fig. 4D). Akt activation is thus necessary and sufficient to mediate fragment N-induced protection.

**NFκB Is Not Involved in Fragment N-induced Survival Signaling**—Different mechanisms can be used by Akt to protect cells from apoptosis, including the activation of NFκB, the inhibition of caspase 9, the inhibition of Bad, and the inhibition of specific transcription factors (Forkhead, Nur77) (18, 19). We determined whether NFκB could be involved in the Akt-dependent protection mediated by fragment N. Fig. 5A shows that fragment N weakly stimulated NFκB activity (about 3-fold over basal; in four other independent experiments fragment N stimulated NFκB by only 1.04–1.8-fold). In comparison, MEKK1, a potent activator of NFκB when overexpressed in cells (13, 20, 21), was about 10 times more effective than fragment N in stimulating NFκB (Fig. 5A). To determine whether the weak activation of NFκB 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 NFκB inhibitor IκBαΔN2. As expected, IκBαΔN2 strongly reduced NFκB activity (Fig. 5B, upper panel). However, the inhibitor did not affect the ability of fragment N to block fragment C-induced apoptosis (Fig. 5B, lower panel). It could be argued that, in HeLa cells, the NFκB pathway is not able to induce protective signals. This is not the case, because blocking NFκB activity with IκBαΔN2 induces cell death in tumor necrosis factor α-treated HeLa cells (Fig. 5C), and because activating NFκB by overexpression of IκBαΔN2 protects cells from fragment C-induced apoptosis (Fig. 5D). This demonstrates that NFκB can protect HeLa cells from apoptosis, including the apoptotic response induced by fragment C (see also Ref. 22). Therefore, despite a functional anti-apoptotic NFκB pathway in HeLa cells, the protection induced by fragment N, which is Akt-dependent, does not operate through the activation of NFκB.
Antisense Mechanisms of the RasGAP N-terminal Fragment

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 and 0.5 μg of prLUC, the luciferase NFκB reporter gene-encoding plasmid. NFκB activity is expressed as a fold increase over basal (mean ± S.E. of duplicate determinations). This experiment has been repeated four times with similar results.

The Ras-PI3K-Akt Pathway Is Used by Fragment N to Inhibit Apoptosis Mediated by Different Stimuli—Fig. 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 other than fragment C, and this also occurs via the Ras-PI3K-Akt pathway.

DISCUSSION

The 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 the 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 blast transformation of these cells (6). Similarly, the activation of caspase 8 is required for CD95-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 a 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 mechanism 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 borne by fragment N is involved because a monoclonal antibody specific for the SH3 domain of RasGAP inhibits downstream signals initiated by oncogenic Ras. (23, 24). However, because 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, p190RhoGAP, 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, although Akt activity was stimulated by fragment N, this did not result in a 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 Nf-xB. G3BP2, a close relative to the RasGAP SH3-binding protein G3BP1, sequesters 1xBa/Nf-xB complexes in the cytoplasm, thereby preventing Nf-xB 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 Nf-xB from reaching the nucleus even when Akt is activated.

Because fragment N does not use Nf-xB 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 136,2 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 Nf-xB to exert its transcriptional activity. This indicates that effector proteins of the Ras-Pi3K-Akt pathway can be differently modulated in the presence or absence of RasGAP caspase cleavage fragments. Fragment N adds an additional level of complexity in the way the Ras-Pi3K-Akt pathway is modulated.

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