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Partial Cleavage of RasGAP by Caspases Is Required for Cell Survival in Mild Stress Conditions†

Jiang-Yan Yang,[1] David Michod,[1] Joël Walicki,[1] Brona M. Murphy,[2] Shailaja Kasibhatla,[3] Seamus J. Martin,[2] and Christian Widmann[1]*

Department of Cellular Biology, Biology and Medicine Faculty, Lausanne University, Lausanne, Switzerland[1]; Molecular Cell Biology Laboratory, Department of Genetics, The Smurfit Institute, Trinity College, Dublin, Ireland[2]; and Maxim Pharmaceuticals, San Diego, California[3]

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Tight control of apoptosis is required for proper development and maintenance of homeostasis in multicellular organisms. Cells can protect themselves from potentially lethal stimuli by expressing antiapoptotic factors, such as inhibitors of apoptosis, FLICE (caspase 8)-inhibitory proteins, and members of the Bcl2 family. Here, we describe a mechanism that allows cells to survive once executioner caspases have been activated. This mechanism relies on the partial cleavage of RasGAP by caspase 3 into an amino-terminal fragment called fragment N. Generation of this fragment leads to the activation of the antiapoptotic Akt kinase, preventing further amplification of caspase activity. Partial cleavage of RasGAP is required for cell survival under stress conditions because cells expressing an uncleavable RasGAP mutant cannot activate Akt, cannot prevent amplification of caspase 3 activity, and eventually undergo apoptosis. Executioner caspases therefore control the extent of their own activation by a feedback regulatory mechanism initiated by the partial cleavage of RasGAP that is crucial for cell survival under adverse conditions.

Members of the caspase family play crucial roles in the regulation of apoptosis. When cells are subjected to apoptotic stimuli, upstream caspases (e.g., caspase 8 or caspase 9) are stimulated to cleave downstream or executioner caspases (e.g., caspase 3) (5, 37). The executioner caspases in turn target a variety of substrates that, once cleaved, activate a series of cellular processes that culminate in the dismantling of the cell (39, 41).

Until recently, it was assumed that activation of caspases would inexorably engage cells on the road leading to cell death (31, 40). While this is certainly true in most cases, recent evidence indicates that there are situations in which upstream or executioner caspases are activated to fulfill functions other than the induction of apoptosis (reviewed in references 2 and 30). For example, caspase 3 is required for skeletal-muscle differentiation (15) and caspase 8 is essential for lymphocyte activation and homeostasis (1, 25, 35). The physiological importance of these nonapoptotic functions of caspases is supported by the observation that some mutations in caspase 8 in humans are associated with defects in lymphocyte activation leading to immunodeficiency (8).

A critical question raised by the demonstration that upstream and executioner caspases participate in nonapoptotic cellular functions is how cells survive after activating their caspases. Several antiapoptotic factors have been described, including FLICE (caspase 8)-inhibitory proteins (FLIPs), Bcl2 family members, and inhibitor of apoptosis proteins (IAPs) (20, 24, 28, 32). It appears, however, that only the last can directly inhibit executioner caspases (28). Cells, therefore, may express basal levels of IAPs to confer constitutive resistance to low levels of caspase activation. On the other hand, expression of IAPs and other antiapoptotic factors may be induced following caspase activation to regulate cell death in a more dynamic manner. The observations that caspases are required for neuroprotection following preconditioning (29) and to counteract tumor necrosis factor toxicity (7, 44) support the latter hypothesis.

RasGAP, a regulator of Ras and Rho GTP-binding proteins, is a caspase substrate that bears two cleavage sites used sequentially as caspase activity increases in cells (47, 50, 53) (Fig. 1A). It has been shown that fragment N, one of the fragments generated by the partial cleavage of RasGAP, inhibits apoptosis when overexpressed in cells. This has led to the hypothesis that mild activation of caspases, rather than promoting apoptosis, might generate a protective response (53). Here, we demonstrate that low-stress conditions can activate executioner caspases without inducing cell death. Cell survival under these conditions cannot occur if the caspase 3-mediated cleavage of RasGAP into fragment N is prevented. Cells can therefore generate an antiapoptotic response to the moderate activation of executioner caspases.

MATERIALS AND METHODS

Plasmids. HA-GAP.dn3 encodes the full-length human RasGAP protein bearing a hemagglutinin (HA) tag (MGYPYDVPDYAS) at the amino-terminal end (53). The extension .dn3 indicates that the backbone plasmid is pcDNA3 (Invitrogen). Plasmid HA-D455A.dn3 encodes a RasGAP mutant that cannot be cleaved at position 455 (53). The plasmid bearing the uncleavable form of fragment N used for the production of fragment N-encoding lentivirus (N-D157A.lti) has been generated by subcloning the BamHI-XhoI fragment of N-D157A.dn3 (55) in TRIP-PGK-ATGm-MCS-WHV opened with the same enzymes. The plasmid used for the generation of Bcl2-encoding lentivirus (SIN-PGK-hBcl2-WHV) was described previously (13). The plasmid encoding the dominant-negative kinase dead mutant of Akt [HA-Akt1(K179M).cmv; previ-

* Corresponding author. Mailing address: IBCM, Bugnon 9, 1005 Lausanne, Switzerland. Phone: 41 21 692 5123. Fax: 41 21 692 5255. E-mail: Christian.Widmann@ibcm.unil.ch.

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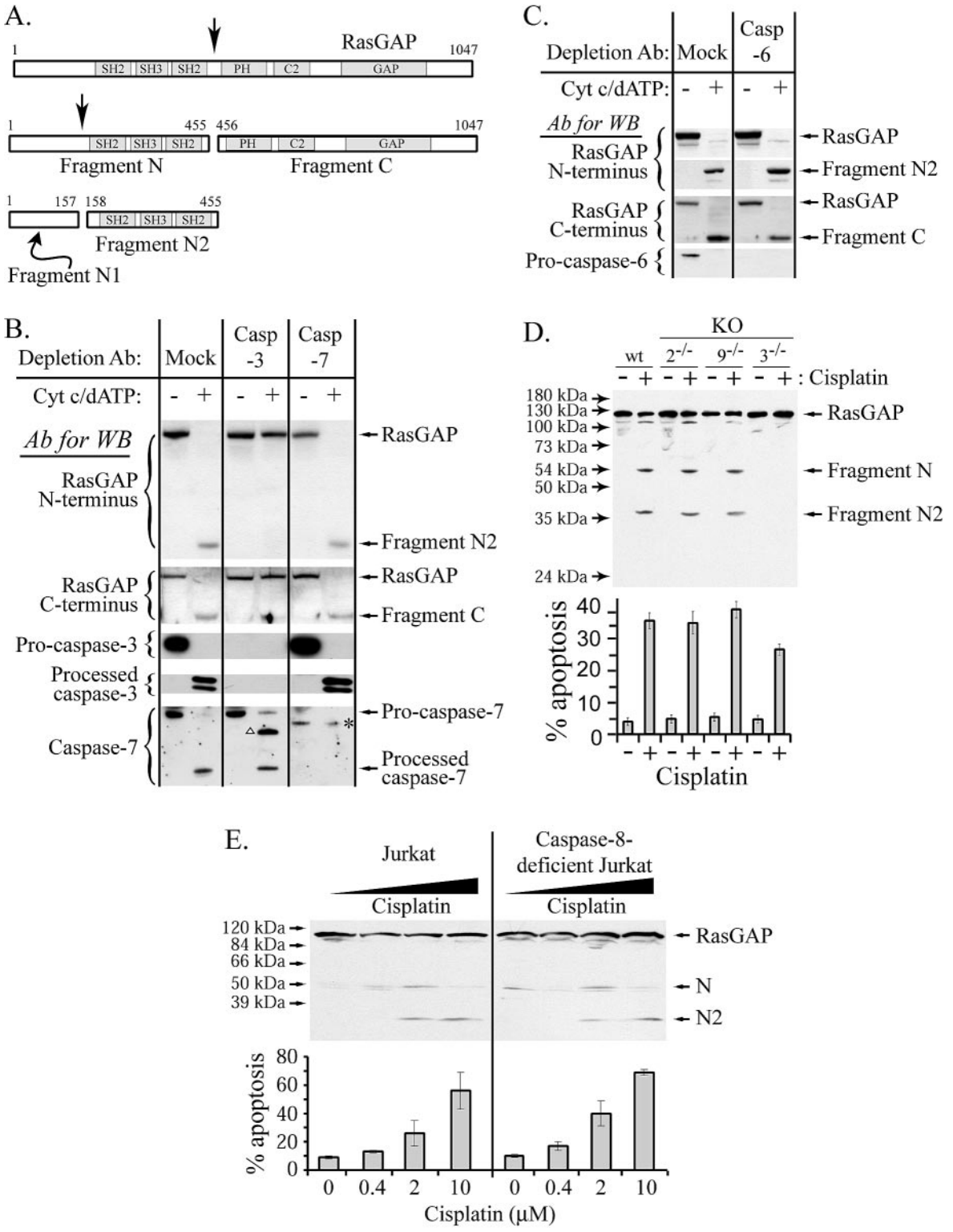


FIG. 1. RasGAP is cleaved by caspase 3 in cells. (A) Schematic representation of RasGAP cleavage by caspases. RasGAP bears two caspase cleavage sites (indicated by the arrows). When targeted by caspases, RasGAP is cleaved into fragment N and fragment C. Fragment N can be further cleaved into fragments N1 and N2. Note that the cleavage at positions 455 and 456 is mandatory for cleavage at positions 157 and 158 to occur (53). PH, plekstrin homology domain; C2, Ca^{2+}-dependent phospholipid binding domain; GAP, GTPase-activating protein domain. (B and C) Cell extracts depleted from the indicated caspases (Casp) were incubated (+) or not (−) with cytochrome (Cyt) *c* and dATP as indicated. The presence of RasGAP and its cleavage fragments was then visualized by Western blotting using N- and C-terminal RasGAP-specific antibodies (Ab). The efficiency of caspase depletion was checked with antibodies recognizing the different caspases. The white triangle indicates a partially processed form of caspase 7 resulting from slower processing due to the absence of the caspase 3 → caspase 9 feedback loop (38). The asterisk

ously called Akt-DN.cmv] was described earlier (55). The plasmid encoding green fluorescent protein (GFP) (pEGFP-C1) was from Clontech.

Electroporation. The total quantity of DNA used in the electroporation procedure was kept to 30 μg (appropriate amounts of pcDNA3 were added when required). Plasmids were diluted in 60 μl of H_2O, mixed with 300 μl of Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (NBCS) (2×10^6 cells) and electroporated at a voltage of 200 V and a capacitance of 1,050 μF. In the experiment (see Fig. 7H and I), 3 μg of pEGFP-C1, together with 13.5 μg of HA-Akt1(K179M), 13.5 μg of N-D157A.dn3, or a combination of both, was used. The electroporated cells were incubated in their culture medium for an additional 24-h period before being analyzed.

Cell lines. RasGAP$^{+/+}$ mouse embryonic fibroblasts (MEFs) (clone 12.78), RasGAP $^{-/+}$ MEFs (clone 12.37), and RasGAP$^{-/-}$ MEFs (clone 12.64), as well as MEFs lacking caspase 2, 3, or 9, were maintained in DMEM containing 10% NBCS (catalog no. 26010-074; Invitrogen) at 37°C and 5% CO_2. Jurkat cells were cultured in RPMI 1640 (catalog no. R8758; Sigma)–10% NBCS. U2OS cells were maintained in DMEM–15% fetal calf serum (FCS). HCT116 cells were cultured in RPMI 1640–10% FCS. MEFs expressing various forms of RasGAP were obtained after electroporation of 27 μg of RasGAP.dn3 or D455A.dn3 together with 3 μg of PTK.hyg (a selection vector that confers hygromycin resistance on mammalian cells). The cells were then selected in 0.1-mg/ml hygromycin B (catalog no. 400051; Calbiochem) for 2 to 4 weeks. About 40 clones per plasmid were isolated from two independent electroporations and analyzed for RasGAP expression levels. Only the clones with RasGAP expression levels similar to that of control RasGAP$^{+/+}$ MEFs were kept for further analysis. Unless otherwise indicated, all the experiments described in this paper were repeated at least three times with similar results and involved all the clones (see Fig. 4A). Giemsa staining of colonies was performed as described previously (51).

Chemicals and antibodies. The anti-phospho-p44/p42 ERK MAP kinase (MAPK) (T202/Y204) E10 monoclonal antibody was from Cell Signaling Technology (catalog no. 9106). The antibody directed at all forms of ERK MAPKs was from Upstate (catalog no. 06-182). The anti-phosphoserine 473-Akt rabbit polyclonal immunoglobulin G (IgG) antibody was from Cell Signaling Technology (catalog no. 9271). The rabbit polyclonal IgG antibody recognizing Akt1/2 was from Santa-Cruz Biotechnology (catalog no. SC-8312). The anti-RasGAP antibody directed at the Src homology (SH) domains of RasGAP has been described before (42). The anti-RasGAP antibody recognizing the C-terminal end of the protein was from Alexis (catalog no. ALX-210-781). Stimulation of Fas was performed using a hexameric form of a fusion protein between the Fas ligand and the Fc portion of IgG1 (19) and was a generous gift from Pascal Schneider, University of Lausanne, Lausanne, Switzerland. For simplicity, this fusion protein is called FasL throughout this paper. Staurosporine and cisplatin were from Roche Diagnostics GmbH and Sigma (catalog no. 1055682 and P4394, respectively). Doxorubicin (Adriamycin) hydrochloride was from Sigma (catalog no. 44583). The antibodies specific for the active form of caspase 3 were from Cell Signaling Technology (catalog no. 96613). The rabbit anti-mouse ICAD 312-331 antibody was from BD Biosciences (catalog no. 550736). The fodrin antibody was from Signet Laboratories (Dehma, Mass.; catalog no. 9702 1IC 1478). The PARP antibody was from Cell Signaling Technology (catalog no. 9546S). The caspase inhibitor MX1013 was a kind gift from Maxim Pharmaceuticals (San Diego, Calif.).

Apoptosis assay. Apoptosis was determined by scoring cells displaying pycnotic nuclei (visualized with Hoechst 33342) (53).

Western blot analysis. Cells were lysed in monoQ-c buffer (53). Western blotting was performed as described previously (49) using a homemade enhanced chemiluminescence reagent (53). To improve the signal in some experiments, an enhanced chemiluminescence solution (Super Signal west Femto Maximum Sensitivity Substrate from Pierce [catalog no. 34095]) was mixed with the homemade reagent.

In vitro RasGAP cleavage assays. Preparation of cell extracts, depletion of caspases from these extracts, and activation of caspases in the extracts were performed as described previously (38).

Caspase 3 production. Active caspase 3 was produced in BL21(DE3)pLysS (Promega) containing the Casp3.rst plasmid encoding the six-His-tagged version of active hamster caspase 3. The bacteria were cultured overnight at 37°C in 500 ml of LB-amp (0.8% peptone, 0.8% yeast extract, 3.6 mM NaOH, 0.34 M NaCl, 100 μg of ampicillin/ml). They were then diluted in 1.5 liters of the same medium and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h at 37°C. The bacteria were pelleted at 6,000 × *g* and lysed by sonication in 40 ml of buffer A (20 mM HEPES, 10% TX-100, 100 μM phenylmethylsulfonyl fluoride, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% β-mercaptoethanol, and the protease inhibitor cocktail from Roche [catalog no. 1873580]) containing 2 mg of lysozyme/ml. The purification of active caspase 3 was performed by incubating the cell lysates for 2 h with 0.5 ml of a 1:1 slurry of Ni-nitrilotriacetic acid beads (QIAGEN). The beads were then harvested by centrifugation and transferred to Eppendorf tubes and washed three times with 1 ml of buffer A and two times with 1 ml of buffer A containing 10 mM imidazole. The beads were then incubated for 10 min with 600 μl of buffer A containing 50 mM imidazole, and the supernatant was harvested. This step was repeated once, and the two supernatants were pooled. The imidazole was eliminated by exclusion-diffusion chromatography using G25 Sephadex beads. The resulting purified caspase 3 was kept at −20°C in 50% glycerol, 50 mM HEPES, 2 mM EDTA, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 5% sucrose, 0.2 mM dithiothreitol.

Measurement of caspase 3 activity. Caspase 3 activity was determined in 3 ml of caspase 3 buffer (100 mM HEPES, 1% sucrose, 0.1% CHAPS, 2 mM dithiothreitol) in the presence of 5 μM fluorogenic caspase 3 substrate AFC 138 (Enzyme System Product). After a 1-h incubation at 37°C, the extent of the cleavage of AFC 138 was measured using a Photon Technology International fluorimeter (excitation, 400 nm; emission, 505 nm).

Lentivirus. Recombinant lentivirus was produced as described previously (12). Briefly, 293T cells were cotransfected using the calcium phosphate DNA precipitation method (23) with 10 μg of the lentiviral vector containing the cDNA of interest (e.g., N-D157A.lti), 2.5 μg of the envelope protein-coding plasmid (pMD.G), and 7.5 μg of the packaging construct (pCMVΔR8.91). Two days after the transfection, the virus-containing medium was harvested. To determine how much of the virus preparation was needed to infect 100% of the MEFs, subconfluent wild-type MEFs seeded in six-well plates were cultured overnight with various volumes of fragment N- or Bcl2-encoding recombinant virus. After removal of the virus solution, the cells were maintained for two more days before fixation and immunocytochemical staining with antibodies directed at the protein expressed by the lentivirus. The lowest volumes of the lentiviral preparations required to infect 100% of the cells were chosen for further experiments.

Image acquisition of wounds in cell layers. Cells were grown to confluency, wounds were generated, and the cells were fixed as described previously (26, 36). After fixation, the cells were mounted in Vectashield (catalog no. H-1000; Vector Laboratories, Burlingame, Calif.). Pictures of the wounds were taken at room temperature with a Zeiss Axioplan 2 imaging microscope equipped with a Plan-Neofluar 10×/0.30 ∞/− lens and a Zeiss AxioCam HRC camera using the Zeiss AxioVision acquisition software.

RESULTS

Caspase 3 is the limiting protease that cleaves RasGAP in cells. RasGAP is cleaved by purified caspase 3 and caspase 7 but only poorly by purified caspase 6 or caspase 8 (47). However, these in vitro experiments cannot determine the contri-

indicates a nonspecific band. Only depletion of caspase 3 abolished the ability of the cytochrome *c*-dATP-stimulated extracts to cleave RasGAP. (D) MEFs derived from control mice or from the indicated caspase knockout (KO) mice were left untreated or incubated with 50 μM cisplatin (wild-type [wt] and caspase $2^{-/-}$ MEFs) or 100 μM cisplatin (caspase $9^{-/-}$ and caspase $3^{-/-}$ MEFs). After 24 h, the extent of apoptosis was assessed, and the cells were then lysed. The presence of RasGAP and its cleavage fragments was visualized by Western blotting using an N-terminal RasGAP-specific antibody. The extent of apoptosis for each treatment is indicated below the Western blot and corresponds to the mean ± standard deviation ($n = 6$ to 8). (E) Control Jurkat cells or Jurkat cells lacking functional caspase 8 were incubated with increasing concentrations of cisplatin (0, 0.4, 2, and 10 μM) for 24 h. The cleavage of RasGAP and the measure of apoptosis was then analyzed as described for panel D. The absence of caspase 8 did not prevent the cleavage of RasGAP in cells undergoing apoptosis.

bution of a given caspase to the cleavage of RasGAP upon activation of caspase cascades initiated by physiological stimuli. A series of experiments were therefore performed to assess the roles of individual caspases in the cleavage of RasGAP. We first determined which executioner caspases were required for RasGAP cleavage. Executioner caspases comprise caspases 3, 6, and 7, all of which can be activated, directly or indirectly, by caspase 9 in response to cytochrome *c* release from mitochondria. The mitochondrion-dependent caspase cascade can be recapitulated in vitro using cell extracts stimulated with cytochrome *c* and dATP (38). Stimulation of this pathway induces the complete cleavage of RasGAP as assessed by the disappearance of full-length RasGAP and the appearance of fragment N2 and fragment C (Fig. 1B and C). Selective depletion of executioner caspases from the cell extracts revealed that caspase 3 is the only executioner caspase required to efficiently cleave RasGAP in the mitochondrial caspase cascade pathway (Fig. 1B and C). We then determined if caspase 3 was also required to cleave RasGAP in living cells and whether there was an upstream caspase (i.e., caspase 2, 8, or 9) that participated in the processing of RasGAP. Control MEFs or MEFs lacking caspase 2, 9, or 3 were incubated with cisplatin concentrations that led to similar apoptotic responses (Fig. 1D, bottom). Under these conditions, only cells lacking caspase 3 were unable to process RasGAP into fragments N and N2 (Fig. 1D). To assess the contribution of caspase 8 to the cleavage of RasGAP, control Jurkat cells or Jurkat cells lacking functional caspase 8 were stimulated with increasing concentrations of cisplatin. The two cell types displayed similar apoptotic responses and similar patterns of RasGAP cleavage in response to cisplatin (Fig. 1E). These data indicate that caspase 3 is the only caspase whose presence is critically required for the cleavage of RasGAP in cells subjected to stress stimuli.

RasGAP is a caspase 3 substrate cleaved in nonapoptotic cells. If RasGAP is cleaved by caspase 3 into fragment N to generate a survival pathway, it is anticipated that this cleavage should be observed in nondying cells and in the absence of significant cleavage of caspase substrates involved in the dismantling of the cells, such as ICAD (14, 34). We therefore analyzed the cleavage of RasGAP and ICAD in cells stimulated with the apoptotic inducer cisplatin. Figure 2A shows that the formation of fragment N could occur in situations (i.e., at cisplatin concentrations below 2.5 μM) that did not lead to detectable ICAD cleavage or induction of apoptosis (even on a long-term basis) (Fig. 3; also see Fig. 5). The exposure time allowing the detection of active caspase 3 in apoptotic cells by Western blot analysis was insufficient to reveal activation of caspase 3 in cells incubated with low cisplatin concentrations (Fig. 2A, top, compare the first three lanes with the last three lanes). Longer exposure, however, revealed that low-stress conditions (e.g., those induced by 0.5 μM cisplatin) induced very mild caspase 3 activity (see Fig. 7A, in which short and long exposures of the same Western blot are depicted).

To determine whether the partial cleavage of RasGAP into fragment N precedes the cleavage of other caspase substrates in different cell types in response to various stress and apoptotic stimuli, Jurkat cells, MEFs, and U2OS cells were incubated with increasing doses of FasL, cisplatin, UV, and doxorubicin. Figure 2B shows that in each case generation of fragment N occurred before the appearance of fodrin and PARP fragments or formation of fragment N2. This indicates that the cleavage of RasGAP at position 455 is a very early event in stressed or apoptotic cells.

Long-term incubation of cells with low cisplatin concentrations also resulted in fragment N formation but not in further processing of fragment N into fragments N1 and N2 (Fig. 3A). Under these conditions, caspase 3 activity could be detected, but only after maximal exposure of the Western blots (Fig. 2 and 3; also see Fig. 7A). This weak caspase activation was nevertheless required for the production of fragment N, since RasGAP was not cleaved in cells incubated with low cisplatin concentrations in the presence of general caspase inhibitors, such as MX1013 (21, 56) (Fig. 3B) or z-VAD-fmk (53). Fragment N was further cleaved into fragments N2 and N1 (the latter is not recognized by the antibody used here) when cisplatin concentrations reached ≥10 μM, conditions that also induced strong activation of caspase 3, the cleavage of ICAD, and apoptosis (Fig. 2A). The weak caspase 3 activity detected in extracts from cells stimulated with low cisplatin concentrations could result from a low level of caspase 3 activity in many surviving cells or a high level of caspase 3 activity in a small number of cells undergoing apoptosis. The observation that 0.5 μM cisplatin did not increase the basal apoptotic rate (Fig. 2A), however, supports the first possibility. Two further approaches were used to confirm that these low cisplatin concentrations do not induce cell death. First, we followed the fates of individual cells by video microscopy recording. Figure 3C shows that there were no more cells dying in response to 0.5 μM cisplatin than in the absence of the genotoxin. Second, we assessed the potential of untreated cells and cells incubated with 0.5 μM cisplatin to form clones. As 0.5 μM cisplatin did not decrease the ability of cells to generate clones (see Fig. 5D and E), it can be concluded that the viability of the cells is not affected by low cisplatin concentrations. Altogether, these data indicate that the first cleavage of RasGAP, although caspase dependent, occurs in nonapoptotic cells able to proliferate and generate clones and in the absence of the cleavage of caspase substrates that participate in the destructive phase of apoptosis.

RasGAP cleavage into fragment N is required for cell survival under adverse conditions. To investigate specifically the functional role of RasGAP cleavage by caspases, we generated MEFs derived from RasGAP knockout mice in which the wild-type RasGAP cDNA or a mutant that cannot be cleaved at position 455 (mutant D455A), was reexpressed at endogenous levels (Fig. 4A; compare the third lane with subsequent lanes). In response to increasing concentrations of cisplatin, cells expressing the wild-type RasGAP protein generated fragment N and then fragments N2 and N1 (N1 is not recognized by the antibody used here) (Fig. 4B). As expected, because the first cleavage of RasGAP at position 455 is required for the second cleavage to occur (53), cells expressing mutant D455A did not display any processing of RasGAP in response to cisplatin (Fig. 4B).

We next determined whether the mutation in the caspase cleavage site affected the function of the full-length RasGAP protein. RasGAP shortens the activation of Ras-dependent pathways by stimulating the intrinsic GTPase activity of Ras. Hence, MEFs lacking RasGAP generate stronger and more prolonged ERK MAPK activation in response to growth factors than in control cells (26, 43). RasGAP is also required for

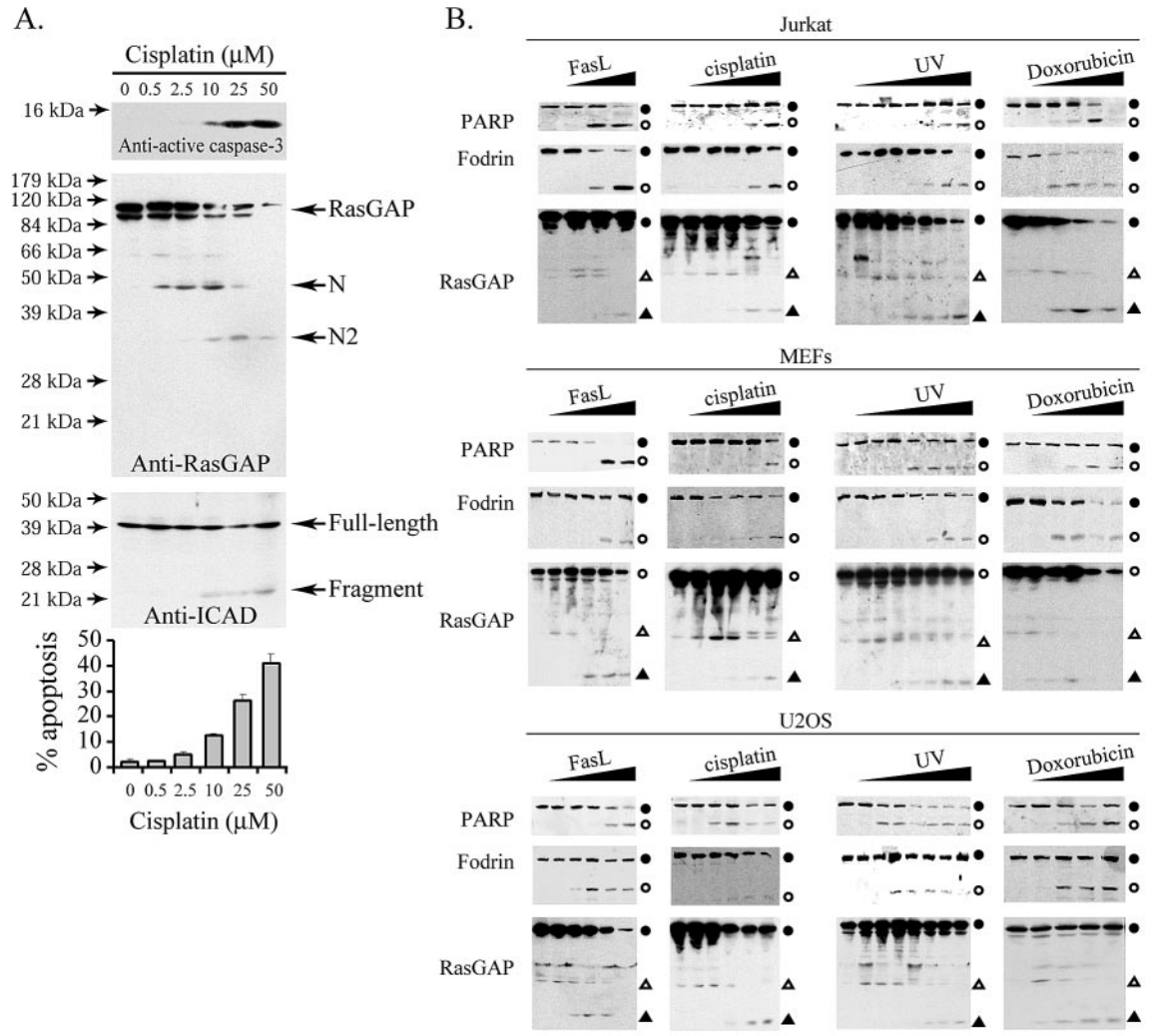


FIG. 2. RasGAP is a very early caspase substrate. (A) Wild-type MEFs were incubated for 24 h with increasing concentrations of cisplatin (0, 0.5, 2.5, 10, 25, and 50 μM). The cells were then lysed, and active caspase 3, RasGAP and its cleavage fragments, and ICAD and its cleavage fragments were visualized by Western blotting. Note that the short exposure used here for the Western blot probed with active caspase 3-specific antibodies allows the detection of only strong activation of the caspase. The anti-ICAD antibody recognizes amino acids 312 to 331 of ICAD, while the anti-RasGAP antibody is directed at the SH domains of RasGAP. The extent of apoptosis for each treatment is indicated below the Western blot and corresponds to the mean ± standard deviation of three independent experiments. RasGAP cleavage into fragment N occurred in the absence of apoptosis, while further processing of fragment N occurred when the cells underwent apoptosis and ICAD was cleaved. (B) Jurkat, MEF, and U2OS cells were incubated for 24 h with increasing doses of the indicated stimuli. The cells were then lysed, and cleavage of PARP, Fodrin, and RasGAP was assessed by Western blot analysis. Closed circles, full-length protein; open circles, cleaved fragments of either PARP or Fodrin; open triangle, fragment N; closed triangle, fragment N2.

normal cell polarization and cell migration, and this involves the regulation of both Ras and Rho (26). Consequently, MEFs lacking RasGAP have a reduced capacity to fill and heal wounds (26). We therefore assessed the capacities of the clones expressing the wild-type and the cleavage-resistant forms of RasGAP to control ERK activation in response to serum and their abilities to migrate into wounds. The wild-type and the cleavage-resistant forms of RasGAP were similarly able to rescue the impaired control of ERK activity observed in RasGAP$^{-/-}$ cells and to restore the wound-healing capacities of these cells (Fig. 4C and D). Moreover, there was no difference among the expansion rates in the clones expressing the various forms of RasGAP (Fig. 4E). When these three criteria (regulation of ERK activation, wound healing, and clonal expansion) were considered, the RasGAP$^{-/-}$ clones, expressing the wild-type or the cleavage-resistant form of RasGAP, were in fact indistinguishable from RasGAP$^{+/+}$ cells (Fig. 4C to E). The absence of the D455 caspase cleavage site, therefore, does not seem to affect the function of full-length RasGAP.

We then assessed cell survival in the presence of mild stresses when RasGAP cleavage was abolished. In contrast to the control RasGAP$^{+/+}$ cells or the clones expressing the wild-type RasGAP, the clones expressing the RasGAP mutant that cannot be cleaved at position 455 underwent apoptosis in response to 0.5 μM cisplatin (Fig. 5A; also see Video S1 in the supplemental material). RasGAP$^{-/-}$ cells also underwent apo-

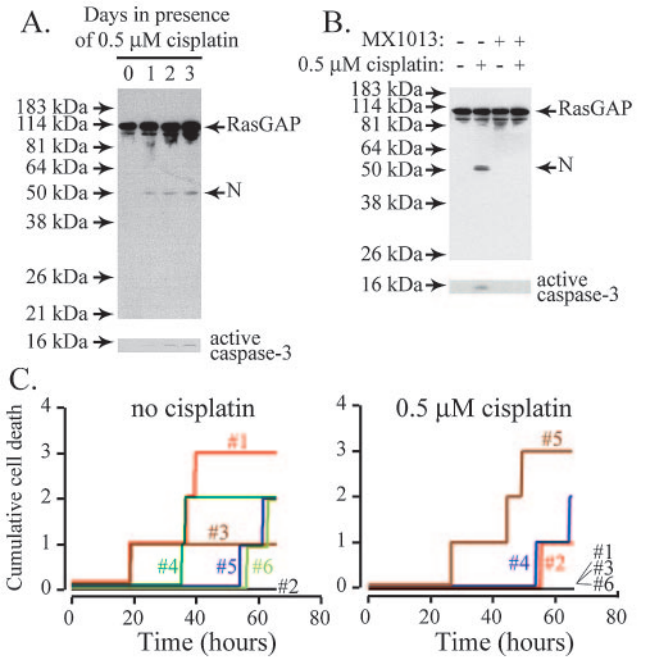


FIG. 3. RasGAP cleavage into fragment N is mediated by caspases in nonapoptotic cells. (A) Wild-type MEFs were incubated for the indicated periods with 0.5 μM cisplatin. Apoptosis, activation of caspase 3, and the extent of cleavage of RasGAP were assessed as described for Fig. 1. The blot corresponding to active caspase 3 was exposed maximally in order to visualize the weak activation of the caspase. (B) Wild-type MEFs were left untreated (−) or stimulated (+) for 2 days with 0.5 μM cisplatin in the absence (−) or in the presence (+) of 10 μM caspase inhibitor (MX1013). The activation of caspase 3 and the presence of fragment N were assessed as described for panel A. (C) Images of wild-type MEFs incubated in control medium or in medium containing 0.5 μM cisplatin were taken every 10 min for 65.8 h. The fates of individual cells were then analyzed, and the cumulative number of cells undergoing apoptosis at a given time was calculated. Six different recordings were performed for each condition (the numbers of each recording are indicated on the graph; the lines are slightly offset for clarity). The numbers of cells in the microscope field at the beginning and at the end of the experiments were not statistically different between cultures incubated in control medium and cultures incubated with 0.5 μM cisplatin (initial numbers, 32 ± 13 and 32 ± 8, respectively; final numbers, 417 ± 131 and 296 ± 141, respectively), nor were the cumulated numbers of apoptotic cells at the end of the recording (1.7 ± 1.0 and 1.4 ± 1.6, respectively).

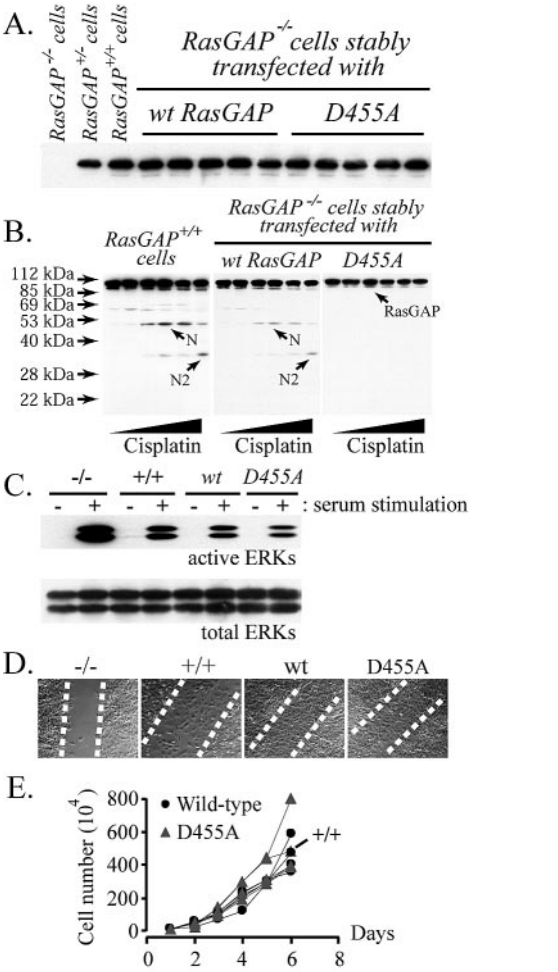


FIG. 4. Characterization of cells expressing wild-type (wt) and caspase-resistant forms of RasGAP. (A) MEFs derived from RasGAP$^{-/-}$, RasGAP$^{-/+}$, and RasGAP$^{+/+}$ mice, as well as RasGAP$^{-/-}$ MEFs stably transfected with the indicated RasGAP forms (five independent clones of each) were analyzed for their RasGAP expression levels by Western blotting using a RasGAP-specific antibody. (B to E) RasGAP$^{-/-}$ MEFs, RasGAP$^{+/+}$ MEFs, or representative RasGAP$^{-/-}$ MEF clones stably expressing a wild-type RasGAP-encoding plasmid or a plasmid encoding the RasGAP mutant that cannot be cleaved at position 455 (D455A) were subjected to increasing concentrations of cisplatin (0, 0.5, 5, 10, 25, and 50 μM) for 24 h (B) (the presence of RasGAP and RasGAP-derived fragments was detected by Western blotting using an antibody directed at the SH domains of RasGAP); starved for 24 h in DMEM lacking serum and stimulated for 15 min with 15% NBCS (C) (the cells were then lysed and the extents of ERK activation and total ERK expression were evaluated by Western blotting); grown to confluency and starved for 24 h in medium lacking serum, and a wound was made with a tip as described previously (26) (D) (pictures of the wounds were taken after 48 h; the dotted lines indicate the edges of the wound); or seeded in six-well plates (10,000 cells per well) (the cells in the wells were counted after the indicated periods in culture) (E).

ptosis under these conditions. The increased susceptibility to apoptosis under mild stress conditions correlated with the inability of the RasGAP D455A-expressing cells to expand (Fig. 5B) or to form colonies (Fig. 5D and E) in the presence of 0.5 μM cisplatin. Since the mutations removing the caspase cleavage sites in RasGAP did not alter the ability of the cells to expand under control conditions (Fig. 4E), these results demonstrate that generation of fragment N gives a selective survival advantage only in cells facing stressful conditions. Cells unable to generate fragment N also underwent apoptosis when subjected to low concentrations of FasL (Fig. 5C), indicating that cleavage of RasGAP into fragment N is a general means for cells to survive adverse conditions.

Fragment N is not a direct inhibitor of caspase 3. To assess whether fragment N could directly inhibit caspase 3, purified caspase 3 and a fluorescent caspase 3 substrate were incubated in vitro with increasing concentrations of recombinant fragment N. A caspase-resistant mutant of fragment N [fragment N(D157A)] was used to prevent its processing by caspase 3. In order to detect potential modulation of caspase 3 activity by fragment N, a nonsaturating dose of caspase 3 was used (Fig. 6A). As shown in Fig. 6B, recombinant fragment N was unable

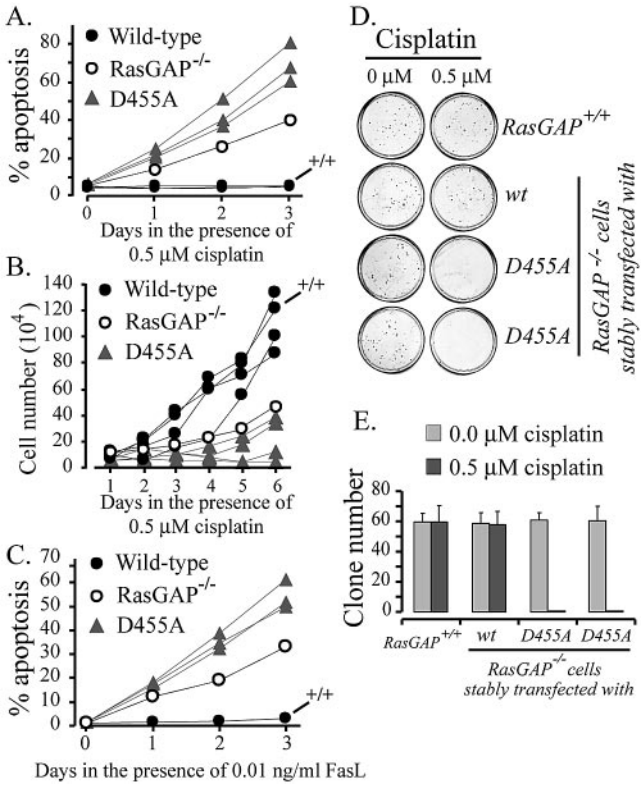


FIG. 5. Cleavage of RasGAP at position 455 is required for long-term survival under adverse conditions. (A) MEFs derived from RasGAP$^{-/-}$ and RasGAP$^{+/+}$ mice (indicated by +/+) and RasGAP$^{-/-}$ clones stably expressing plasmids encoding either wild-type RasGAP (two independent clones) or the uncleavable D455A mutant (three independent clones) were incubated with 0.5 μM cisplatin for the indicated periods, and the extent of apoptosis was assessed. (B) MEFs derived from RasGAP$^{-/-}$ and RasGAP$^{+/+}$ mice (indicated by +/+) and RasGAP$^{-/-}$ clones stably expressing plasmids encoding either wild-type RasGAP (three independent clones) or the uncleavable D455A mutant (four independent clones) were seeded in six-well plates (10,000 cells per well) and incubated with 0.5 μM cisplatin for the indicated periods, and the cells in the wells were then counted. (C) The cells described in the legend to panel A were incubated with 0.01 ng of FasL/ml for the indicated periods, and the extent of apoptosis was assessed. (D and E) One hundred wild-type cells (top row), RasGAP$^{-/-}$ cells stably expressing plasmids encoding either wild-type RasGAP (second row), or the uncleavable D455A mutant (two independent clones; bottom rows) were plated in 10-cm dishes and incubated for 8 days in the absence (left lane) or in the presence (right lane) of 0.5 μM cisplatin. The colonies were then stained with Giemsa dye (D) and counted. The quantitative results are presented in panel E (mean plus standard deviation; $n = 4$).

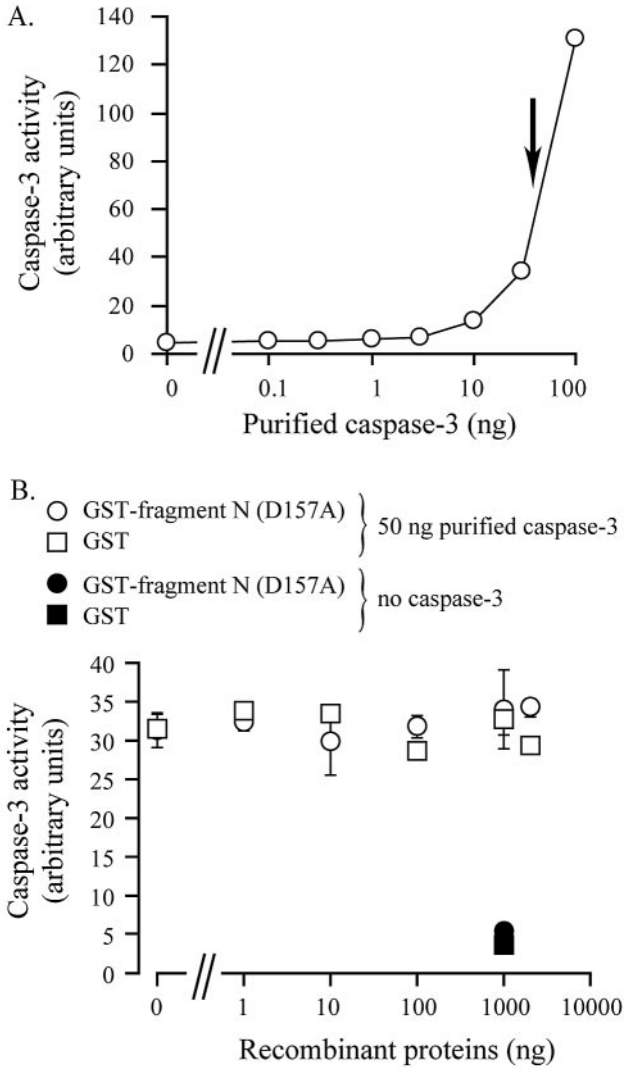
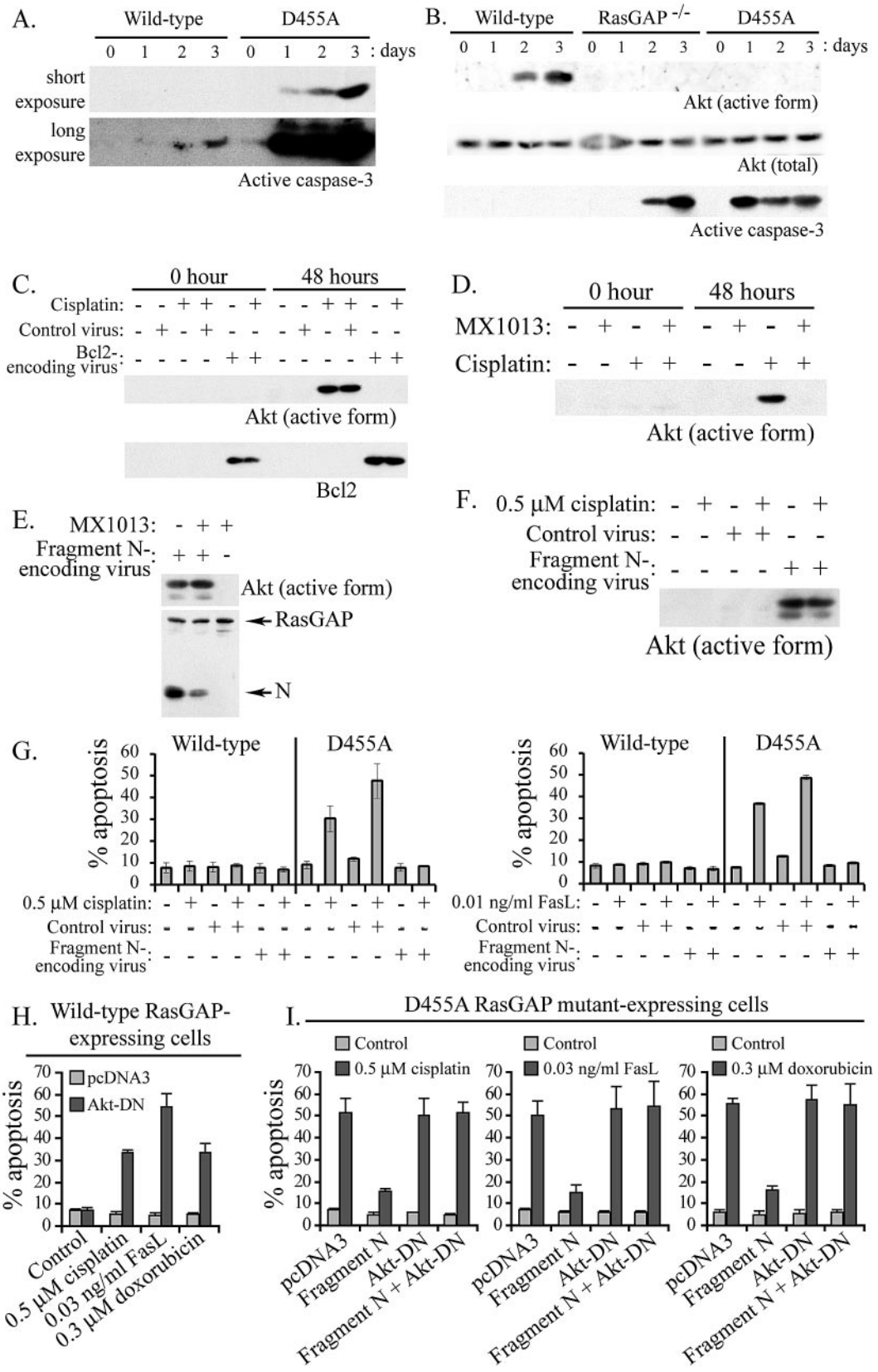


FIG. 6. Fragment N does not directly inhibit caspase 3. (A) The indicated amounts of purified caspase 3 were mixed with the fluorogenic caspase 3 substrate AFC138 for 1 h at 37°C. Cleavage of AFC138 was then monitored as described in Materials and Methods. (B) The indicated amounts of GST or GST-fragment N (D157A) were incubated in the presence or in the absence of a nonsaturating dose of purified caspase 3 (arrow in panel A). Caspase activity was then measured as described for panel A. The results correspond to the mean ± standard deviation of three independent determinations.

to modulate caspase 3 activity, indicating that its inhibition of caspase activity in cells is indirect.

Fragment N allows cell survival by activating Akt. The increased sensitivity to apoptosis of cells that cannot generate fragment N in response to low cisplatin concentrations was correlated with amplified caspase 3 activation (Fig. 7A) and a lack of activation of the antiapoptotic Akt kinase (Fig. 7B). Cells lacking RasGAP were also unable to activate Akt in response to mild stress, and this correlated with amplified caspase 3 activation (Fig. 7B) and increased sensitivity to apoptosis (Fig. 5A). The activation of Akt in wild-type RasGAP-expressing cells stimulated with 0.5 μM cisplatin was dependent on caspase activation, since it could be blocked by Bcl2 overexpression (Fig. 7C) or by a caspase inhibitor (Fig. 7D). The caspase inhibitor was unable to prevent Akt activation in cells expressing fragment N (Fig. 7E), indicating that it is by inducing the cleavage of RasGAP into fragment N that caspases are able to induce Akt activity. Ectopic expression of fragment N in cells expressing the uncleavable form of RasGAP allowed them to activate Akt (Fig. 7F) and to survive under various mild stress conditions (Fig. 7G), demonstrating that of the two fragments generated following the cleavage of RasGAP at position 455 (Fig. 1A), only fragment N is required for the induction of the survival pathway. Finally, a dominant-



negative form of Akt suppressed the ability of wild-type RasGAP-expressing cells to survive in the presence of low concentrations of cisplatin, FasL, and doxorubicin (Fig. 7H) and abolished the ability of ectopically expressed fragment N to protect cells expressing the uncleavable D455A RasGAP mutant in response to these mild stresses (Fig. 7I). Altogether, these data indicate that cells failing to cleave RasGAP into fragment N under adverse conditions cannot keep in check the activation of downstream caspases in response to mild insults because they cannot activate the antiapoptotic Akt pathway.

Ectopic expression of Fragment N in cells activates a survival pathway that is dependent on Ras activation (55). As Ras activation can lead to stimulation of the ERK MAPK pathway that can, under certain conditions, promote cell survival (22, 52), we assessed whether the ERKs could also play a role in protecting cells following RasGAP cleavage at position 455. Wild-type RasGAP-expressing cells, cells lacking RasGAP, or cells expressing the uncleavable mutant of RasGAP stimulated with low cisplatin concentrations for several days did not, however, display noticeable activation of the ERK MAPK pathway (Fig. 8A). In contrast, but only in wild-type RasGAP-expressing cells, Akt was strongly activated upon exposure to mild stress (Fig. 8A). The cell lines tested activated the ERKs and Akt in similar manners in response to serum stimulation (Fig. 8B). Their inability to activate the ERKs and, for those unable to cleave RasGAP, to stimulate Akt in response to a mild stress is therefore not a consequence of nonfunctional ERK and Akt pathways. These results suggest that ERK activation is not required to promote cell survival after the partial cleavage of RasGAP into fragment N.

Since cells expressing the D455A mutant of RasGAP more readily activate caspases than wild-type RasGAP-expressing cells, it could be argued that the D455A mutant, as a full-length protein, lacks a death-protecting activity found in the wild-type protein. However, overexpression of wild-type RasGAP or the D455A mutant in HCT116 cells (Fig. 9) and MEFs (data not shown) did not alter their sensitivity to apoptosis induced by

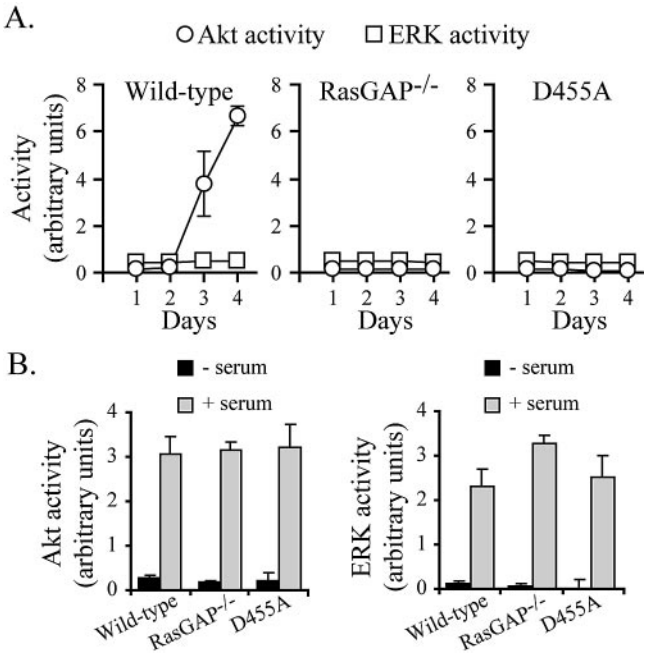


FIG. 8. Absence of long-term ERK activation in cells subjected to mild stress. (A) MEFs derived from RasGAP$^{-/-}$ and the corresponding clones stably expressing a plasmid encoding the wild-type RasGAP or a plasmid encoding the uncleavable D455A RasGAP mutant were starved for 48 h in DMEM–0.2% NBCS and then incubated with 0.5 μM cisplatin in the same medium for the indicated periods. The extents of ERK and Akt activation were assessed on Western blots following quantitation of the intensities of the bands corresponding to the activated phospho forms of the kinases. The results correspond to the mean ± standard deviation of triplicate measurements. (B) The cells described in the legend to panel A were starved for 48 h in DMEM–0.2% NBCS and then left untreated or stimulated for 15 min with 15% NBCS. Akt and ERK activities were assessed as described above.

FIG. 7. Fragment N activates a survival Akt-dependent pathway following caspase activation. (A) MEFs derived from RasGAP$^{-/-}$ clones stably expressing a plasmid encoding the wild-type RasGAP or a plasmid encoding the uncleavable D455A RasGAP mutant were incubated with 0.5 μM cisplatin for the indicated periods. The levels of active caspase 3 were then visualized by Western blot analysis. Note that two exposure times were used to assess the difference in the stimulation of the caspase between wild-type cells and D455A mutant-expressing cells. (B) MEFs derived from RasGAP$^{-/-}$ and the corresponding clones stably expressing a plasmid encoding the wild-type RasGAP or a plasmid encoding the uncleavable D455A RasGAP mutant were incubated for 48 h in DMEM–0.2% FCS and then stimulated with 0.5 μM cisplatin for the indicated periods. The levels of active caspase 3, active Akt, and total Akt were then visualized by Western blot analysis. (C) Wild-type RasGAP-expressing MEFs infected with an empty lentivirus or with a lentivirus encoding Bcl2 were incubated for 48 h in DMEM–0.2% FCS and then left untreated (−) or stimulated with 0.5 μM cisplatin (+) for an additional 48-h period. The levels of active Akt and total Bcl2 were then assessed by Western blot analysis. (D) Wild-type RasGAP-expressing MEFs were incubated for 48 h in DMEM–0.2% FCS and then left untreated or stimulated with 0.5 μM cisplatin for 48 h in the absence or in the presence of 10 μM specific caspase inhibitor MX1013. The level of active Akt was then assessed by Western blot analysis. (E) Wild-type RasGAP-expressing MEFs were infected with an empty lentivirus or with a lentivirus encoding fragment N. The cells were then incubated in DMEM–0.2% FCS, and 48 h later they were either left untreated or incubated with 10 μM MX1013 for an additional 24-h period. The levels of active Akt, RasGAP, and fragment N were then assessed by Western blot analysis. (F) Cells derived from RasGAP$^{-/-}$ MEFs stably expressing a plasmid encoding the uncleavable D455A RasGAP mutant were infected with an empty lentivirus or with a lentivirus encoding fragment N, as described for panel D. The cells were then incubated in DMEM–0.2% FCS, and 48 h later they were either left untreated or incubated with 0.5 μM cisplatin for an additional 48 h. The level of active Akt was then assessed by Western blot analysis. (G) The cells described in the legend to panel A were infected with an empty lentivirus or with a lentivirus encoding fragment N, as described for panel D. Twenty-four hours later, the cells were left untreated or stimulated for an additional 48 h with 0.5 μM cisplatin (left) or 0.01 ng of FasL/ml (right), and apoptosis was then measured. (H) Control MEFs were electroporated with pcDNA3 or with a plasmid encoding a dominant-negative form of Akt (Akt-DN) in the presence of a GFP-encoding plasmid and incubated for 24 h with the indicated compounds, and the extent of apoptosis among green cells was then assessed. The results correspond to the mean ± standard deviation of triplicate measurements. (I) Cells derived from RasGAP$^{-/-}$ MEFs stably expressing a plasmid encoding the uncleavable D455A RasGAP mutant were electroporated with the indicated combinations of plasmids encoding fragment N and Akt-DN (in the presence of a GFP-encoding plasmid) and were incubated for 24 h with the indicated compounds. The extent of apoptosis among the green cells was then assessed. The results correspond to the mean ± standard deviation of triplicate measurements.

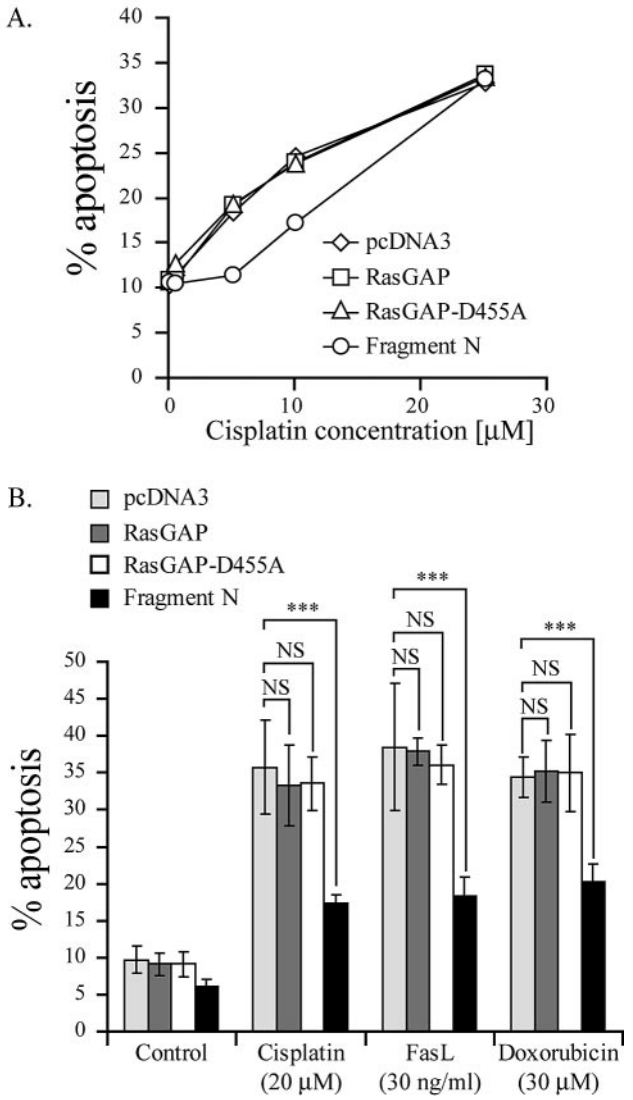


FIG. 9. Ectopic expression of full-length RasGAP constructs does not protect cells from apoptosis. (A) HCT116 cells transfected with plasmids encoding the indicated constructs were incubated with the indicated concentrations of cisplatin for 24 h. The extent of apoptosis was then assessed. (B) HCT116 cells were transfected as described for panel A and incubated for 24 h with the indicated compounds. The extent of apoptosis was then assessed. The results correspond to the mean ± standard deviation of triplicate measurements (*t* test analysis: ***, $P < 0.001$; NS, not significant).

cisplatin, FasL, or doxorubicin (Fig. 9). In contrast, overexpression of fragment N in these cells increased their resistance to these apoptotic stimuli (Fig. 9). Consistent with previous results obtained in HeLa cells (54), these results indicate that full-length RasGAP proteins, whether caspase resistant or not, play no major role in the regulation of apoptosis. Only following its cleavage into fragments does RasGAP acquire apoptosis-regulatory properties.

DISCUSSION

Accumulating evidence shows that under some circumstances upstream and executioner caspases are activated without causing cell death (1, 6, 8–10, 15, 25). In these situations, caspases must be only minimally stimulated so as to cleave only a subset of caspase substrates. In particular, caspase substrates, such as ICAD, that are actively involved in the destruction phase of apoptosis when targeted by caspases must be left uncleaved. Our data provide a molecular basis for cell survival in the presence of activated caspases. We have demonstrated that mild stresses generate very low levels of caspase activation that induce the first cleavage of RasGAP into fragment N but that are insufficient to cause significant cleavage of ICAD and to induce apoptosis. We have shown that fragment N prevents an amplification cascade that leads to massive caspase 3 activation. Therefore mild stresses do not cause apoptosis because fragment N is generated and because fragment N maintains the activation of caspase 3 to minimal levels. Consequently, it is the caspases themselves that prevent their massive activation by cleaving RasGAP into fragment N.

Cells expressing a RasGAP mutant bearing a single point mutation abolishing its cleavage by caspases are killed by mild stresses that would otherwise not affect the viability of wild-type cells. This indicates that even if other antiapoptotic fragments are generated by caspases, they cannot compensate for the lack of RasGAP cleavage. Remarkably, therefore, the negative feedback control of caspases following their mild activation cannot operate if only one protein among the 280 described caspase substrates is not cleaved (16).

Fragment N is not a direct caspase inhibitor but rather protects cells by activating Akt, a well-known antiapoptotic kinase (55). An amino-terminal moiety of RasGAP, corresponding to fragment N plus the plekstrin homology domain of fragment C, can in fact interact directly with Akt (57). It is therefore likely that the binding of fragment N to Akt modulates the activity of the kinase. However, the exact mechanisms underlying the regulation of Akt by fragment N remain to be characterized.

Akt promotes cell survival by inactivating proapoptotic molecules, such as Bad, or by stimulating the mTOR or the NF-κB pathways (4, 48). However, fragment N, while protecting cells in an Akt-dependent manner, does not do so by stimulating NF-κB activity (55). This could indicate that NF-κB activation may not always participate in cell protection. Indeed, there are situations in which activation of NF-κB is detrimental to the cell. For example, NF-κB is required for p53-mediated apoptosis (33) and cytokine-induced apoptosis in β cells of the pancreas (3, 17). Therefore, activating Akt in a way that precludes NF-κB stimulation may be crucial for fragment N to be a general inhibitor of apoptosis.

It is unknown which effector proteins activated or induced by fragment N in an Akt-dependent manner mediate its protective function. Several inhibitors of caspases, such as cellular FLIPs and members of the IAP family, are potential candidates. FLIPs specifically prevent the activation of caspase 8 in response to Fas ligand or tumor necrosis factor but appear not to be involved in the regulation of other caspases (27). Consequently, it would not be anticipated that FLIPs protect cells once executioner caspases are activated. The IAP family of proteins can directly inhibit upstream and downstream caspases, including caspase 3 (11, 28). As these proteins seem to be the only endogenous inhibitors that can block apoptosis once caspases have been activated, they may be responsible for

the protective effect induced by the formation of fragment N. However, the expression of many IAPs depends on NF-κB activity (11, 46), and expression of at least one IAP family member, cIAP-2, does not rely on phosphatidylinositol 3-kinase activity (45). In contrast, fragment N-induced protection depends on phosphatidylinositol 3-kinase but not on NF-κB (55), which raises the question of whether protection by fragment N may be mediated by proteins other than the IAPs.

RasGAP knockout mice die in utero as a result of abnormal development of the circulatory system (18). The RasGAP$^{-/-}$ embryos also display extensive neuronal death (18). Our data provide a plausible explanation for this phenotype: RasGAP$^{-/-}$ cells are more sensitive to apoptosis because they do not have the possibility of producing fragment N and consequently cannot activate the survival Akt pathway. RasGAP$^{-/-}$ cells are, however, not the ideal system to specifically assess the role of RasGAP cleavage because the full-length RasGAP may also regulate cell survival independently of its caspase cleavage fragments. In this context, it should be noted that RasGAP$^{-/-}$ cells are slightly less susceptible to mild stress-induced apoptosis than cells expressing an uncleavable form of RasGAP (Fig. 5). Because Ras activation may stimulate survival pathways (55), it is possible that the higher basal Ras activity in RasGAP$^{-/-}$ cells than in cells expressing full-length RasGAP (Fig. 4C) (43) gives them a slight survival advantage over cells expressing an uncleavable form of full-length RasGAP. However, the observation that overexpression of fragment N, but not full-length RasGAP, renders cells more resistant to apoptosis (54) further indicates that RasGAP induces a protective pathway only when it is cleaved at position 455.

Our results indicate that caspase 3 controls the level of its own activation by targeting RasGAP. In the absence of this safeguard mechanism, low caspase 3 activities are amplified inexorably until apoptosis is induced. There is therefore no constitutive cellular signal to protect cells such as MEFs from untimely caspase 3 activation. Rather, it is the caspases themselves that turn on a survival pathway. Presumably, this mechanism has arisen to protect cells against caspase activation occurring inopportunely or for other purposes than apoptosis.

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