Proteolytic cleavage of Ras GTPase-activating protein during apoptosis

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Abstract

p120-ras GTPase-activating protein (rasGAP) associates with Ras and negatively regulates Ras signaling by stimulating the intrinsic rate of Ras GTPase activity. rasGAP also associates with other cellular signaling proteins which suggest that rasGAP may play a role in coordinating other signal transduction pathways. Disruption of rasGAP in vivo results in extensive apoptosis. Fas-mediated apoptosis results in the activation of caspases that cleave cellular substrates which are important for maintaining cytoplasmic and nuclear integrity. We show here that rasGAP is proteolytically cleaved by caspases early in Fas-induced apoptosis of Jurkat cells. rasGAP was also cleaved by DNA-damaging chemotherapeutic agents and TNF-related apoptosis inducing ligand (TRAIL), also known as Apo2L. Based on the size of the products generated by cleavage of deletion mutants of rasGAP we predict that cleavage of rasGAP occurs in the hydrophobic region and between the SH2(2) and ras-p21 interacting domain which would leave an intact ras-p21 interacting domain. Interestingly, cleavage of rasGAP in vitro enhanced rasGAP hydrolysis activity. Our results demonstrate that diverse apoptotic stimuli cause caspase-mediated cleavage of rasGAP early in apoptosis.

Keywords: programmed cell death; caspase; Ras; Fas

Abbreviations: TNF, tumor necrosis factor; GTP, guanosine triphosphate; GAPs, GTPase-activating proteins; NF1, type 1 neurofibromatosis; ICE, interleukin-1β converting enzyme; PARP, poly-ADP-ribose polymerase; TRAIL, TNF-related apoptosis inducing ligand; FAK, focal adhesion kinase

Introduction

Apoptosis is a physiological process that is important for the elimination of transformed cells, for organization of develop-ing tissues and for immune regulation (reviewed in Steller, 1995). Fas/Apo-1 is a member of the tumor necrosis factor (TNF) receptor family that induces apoptosis in Fas ligand-expressing cells (Itoh et al, 1991). Fas has been shown to play an important role in modulation of the immune response. A recent study has demonstrated that activation of the Fas pathway induces activation of the Ras signaling pathway and that inhibition of Ras inhibits Fas-induced apoptosis (Gulbins et al, 1995). Additional support for a role Ras activation in Fas-induced apoptosis comes from a study which showed that Fas receptor triggering causes Ras-dependent activation of the small G-proteins Rac 1 and Rac 2 and inhibition of Ras, Rac 1 or Rac 2 inhibits Fas-induced apoptosis (Gulbins et al, 1996).

Ras is activated by the exchange of bound guanosine 5′-diphosphate (GDP) for guanosine triphosphate (GTP) (reviewed in McCormick, 1994). In the GTP-bound active state, Ras-GTP interacts with and activates downstream targets. GTPase-activating proteins (GAPs) inactivate Ras by accelerating the slow intrinsic rate of GTP hydrolysis (Trahey et al, 1988; Martin et al, 1990). Five mammalian GAP's for Ras have been described which include p120-rasGAP (rasGAP), the prototype of this class and neurofibromin, the product of the type I neurofibromatosis (NF1) gene (reviewed in Bollag and McCormick, 1991). Recently, Henkemeyer et al, demonstrated that disruption of rasGAP in vivo results in massive neuronal cell death (Henkemeyer et al, 1995). Their findings support previous observations that, without inhibiting Ras signaling, neural-derived PC12 cells make an abortive attempt to enter the cell cycle and undergo apoptosis (Ferrari and Greene, 1994). It is also possible that rasGAP may regulate specific apoptosis signaling pathways. For example, it was recently shown that R-Ras, a Ras-related protein which promotes apoptosis in some cells interacts with Bcl-2 (Wang et al, 1995). Although the mechanism by which inactivation of rasGAP promotes apoptosis is as yet unknown, the study by Henkenmeyer et al (1995) supports a role rasGAP in controlling apoptotic signaling pathways in vivo.

It is clear that mammalian cysteine proteases (now designated caspases) related to the Caenorhabditis elegans cell death gene CED-3 are the effectors of the apoptotic signaling pathway triggered by members of the tumor necrosis factor (TNF) family (reviewed in Dixit, 1996). Activation of interleukin-1β (ICE)-related proteases by Fas results in the cleavage of substrates that are thought to play a role in maintaining cytoplasmic and nuclear integrity such as lamin A, poly-ADP-ribose polymerase (PARP), α-Fodrin, sterol regulatory element-binding proteins, the 70-kDa protein component of the U1 small nuclear ribonucleo-protein, the retinoblastoma tumor suppresser gene product, product kinase C δ, DNA-dependent protein kinase and the

GDP dissociation inhibitor D4-GDI (Song *et al*, 1996; Na *et al*, 1996; Waterhouse *et al*, 1996; Casciola-Rosen, 1996; Emoto *et al*, 1995; Wang *et al*, 1996; Brancolini *et al*, 1995; Lazebnik *et al*, 1994).

We have recently shown that apoptosis induced by Fas and TNF-related apoptosis inducing ligand (TRAIL), also known as Apo2L, cause caspase-mediated cleavage of focal adhesion kinase (FAK) in both suspension and adherent cell lines (Wen *et al*, 1997). Here we show that activation of the Fas pathway also induces caspase-mediated cleavage of p120/rasGAP (rasGAP). Cleavage of rasGAP occurred early in apoptosis at approximately the same time as PARP and FAK cleavage. We also observed that rasGAP is cleaved by other apoptotic stimuli such as TRAIL and doxorubicin. Cleavage of rasGAP *in vitro* enhanced rasGAP hydrolysis activity suggesting that cleavage of rasGAP during apoptosis *in vivo* may down-regulate Ras activity.

Results and Discussion

Fas-induced cleavage of rasGAP

Disruption of rasGAP causes apoptosis *in vivo* (Henke-meyer *et al*, 1995). We hypothesized, therefore, that rasGAP may be cleaved during apoptosis. We observed that rasGAP is cleaved beginning at 1 h after the addition of a crosslinking activating Fas antibody, a time that coincides with PARP and FAK cleavage in Jurkat cells (Figure 1A) (Wen *et al*, 1997). Ras GAP was cleaved into two fragments of approximately 55 kD and 37 kDa (Figure 1A). The 55 kD fragment (I) was observed at 1 h after Fas activation followed at 2 h by the appearance of the 37 kD fragment (II). The commercial antibody that was used to detect rasGAP cleavage *in vivo* is a monoclonal antibody but its epitope is unknown. The SH3 domain of rasGAP, however, is the most antigenic and our *in vitro* cleavage data suggests that the antibody recognizes the SH3 domain. Cleavage of rasGAP was almost completely inhibited by the tetrapeptide caspase inhibitor DEVD-CHO (40 μM) and to a lesser extent by YVAD-CHO (40 μM) (Figure 1A). Activation of the Fas pathway in other cell lines such as H460 non-small cell lung cancer cells and MCF-7 breast cancer cells also induced cleavage of rasGAP (data not shown). Additionally, other stimuli which induce caspase-mediated apoptosis such as the chemotherapeutic drug doxorubicin and TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo2L, induced rasGAP cleavage (Figure 1B). Taxol, however, did not induce rasGAP cleavage (Figure 1B). Doxorubicin, taxol and TRAIL/Apo2L decreased H460 viability by more than 80% (data not shown). We also examined if the rasGAP-associated protein p62 was cleaved during apoptosis but Fas-induced apoptosis did not induce cleavage of p62 (data not shown). These data show that cleavage of rasGAP is mediated by a DEVD-inhibitable caspase(s) such as caspase-3 and caspase-7 and that cleavage of rasGAP is induced by diverse apoptotic stimuli and in different cell lines.
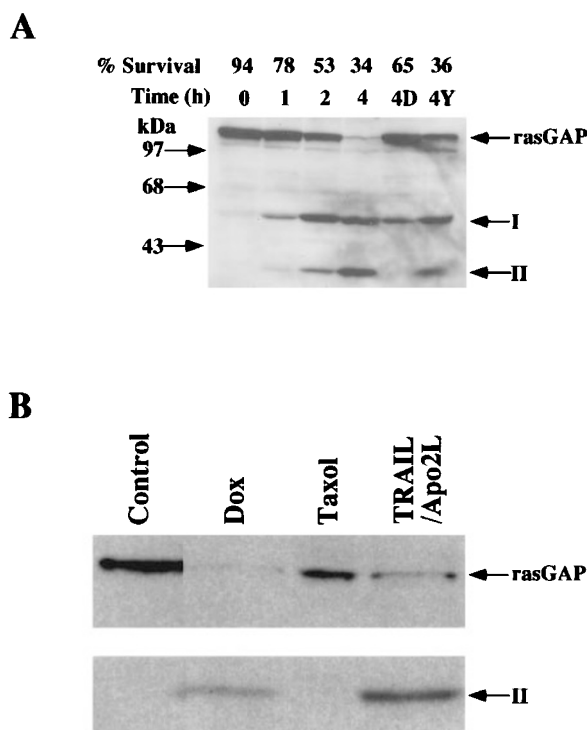

Figure 1 Cleavage of rasGAP during apoptosis. In (**A**) Jurkat cells were treated with the CH-11 anti-Fas mAB (100 ng/ml) and harvested for immunoblot analysis at the times shown. The blot was probed with a commercial anti-rasGAP mAb (Santa Cruz Biotechnology, Santa Cruz, CA). Intact rasGAP migrates at 115–120 kDa. A rasGAP cleavage product of approximately 55 kDa (I) appears 1 h after the addition of Fas which is followed by the appearance of a second cleavage product of approximately 37 kDa (II) at 2 h. The caspase inhibitors DEVD-CHO (40 μM) (lane 4D) and YVAD-CHO (40 μM) (lane 4Y) were added to Jurkat cells 1 prior to anti-Fas. In (**B**) H460 non-small cell lung cancer cells were treated with TRAIL/Apo2L (100 ng/ml), doxorubicin (Dox) (2 μM) or taxol (100 nm) and harvested for immunoblot analysis with an anti rasGAP mAB 48 h later

rasGAP is cleaved *in vitro* by caspase-3 and caspase-7

In order to determine which caspase(s) mediates rasGAP cleavage we examined the sensitivity of rasGAP to different caspases *in vitro*. Incubation of purified rasGAP with 100 ng of caspase-3 or caspase-7 resulted in complete cleavage of rasGAP into the 37 kDa fragment with only a small amount of the 55 kDa fragment (Figure 2A). Caspase-6 and caspase-8 alone had limited activity against rasGAP but they completely cleaved rasGAP in the presence of naive extract (Figure 2A and B). These data are in agreement with previous studies which show that caspase-6 and caspase-8 activate other caspases in naive extract such as caspase-3 and caspase-7 (Srinivasula *et al*, 1996; Chinnaiyan *et al*, 1996).

We then compared the sensitivity of rasGAP to caspase-3 *versus* caspase-7 *in vitro*. We have previously shown that FAK is more sensitive to cleavage by caspase-7 *in vitro* (Wen *et al*, 1997). These purified caspases are equally active against a defined substrate (see Materials and Methods). Incubation of purified rasGAP with increasing

A

B
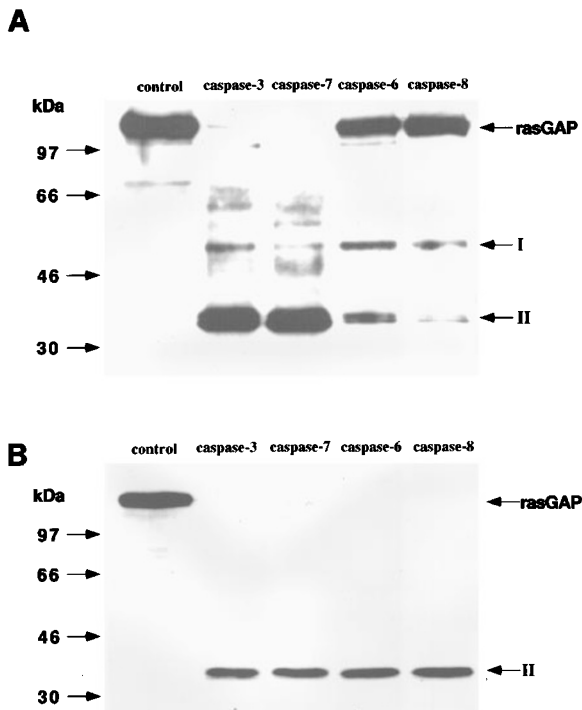
Figure 2 Caspase-3 (CPP32, YAMA, Apopain) and caspase-7 (Mch3, ICE-LAP3, CMH-1) mediate cleavage of rasGAP *in vitro*. 200 ng of purified rasGAP was incubated with 200 ng of caspase-3, caspase-7, or caspase-6 or 400 ng of caspase-8 in the absence (**A**) or presence (**B**) of Jurkat cell extract (50 μg) for 1 h at 37°C. The samples were then processed for immunoblot analysis with the rasGAP commercial mAB. Caspase-3 and caspase-7 generate predominantly the 37 kDa second rasGAP cleavage product (II) (**A** and **B**) and caspase-6 and caspase-8 cleave rasGAP completely only in the presence of naive extract (**B**)

concentrations of caspase-3 resulted in the generation of the 55 kDa and 37 kDa fragments and a small amount of a newly observed fragment of approximately 100 kDa (Figure 3). Caspase-7 generated predominantly the 55 kDa fragment with less of the 37 kDa fragment than caspase-3 and no 100 kDa fragment (Figure 3). These data suggest the cleavage of rasGAP, unlike FAK cleavage, is more sensitive to caspase-3 than caspase-7 and also that the caspase-3 likely generates the 37 kDa fragment *in vivo*.

Mapping of the rasGAP cleavage sites

We have observed that rasGAP is cleaved into two fragments *in vivo* and that cleavage is inhibited by the tetrapeptide DEVD but the epitope recognized by the commercial mAB is unknown. DEVD-inhibitable caspases such as caspase-3 and caspase-7 preferentially cleave substrates at DXXD (Nicholson *et al*, 1995; Talanian *et al*, 1997). In order to better define the rasGAP cleavage sites we used an antibody that recognizes a KT3 epitope tag which is present on the carboxyl terminal end of the purified rasGAP. Incubation of purified full length rasGAP with caspase-3 followed by immunoblotting with the anti-KT3 antibody detected a fragment of approximately 66 kDa (Figure 4A). A small amount of a 97 kDa
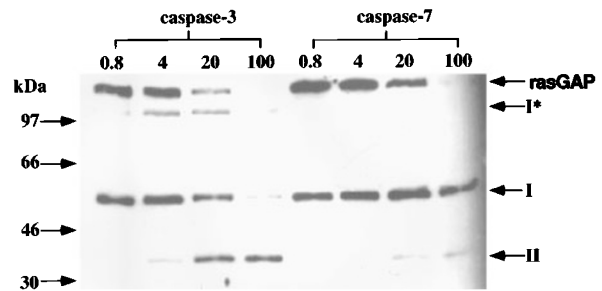
Figure 3 The 37 kDa rasGAP cleavage product (II) is preferentially generated by caspase-3 *in vitro*. 200 ng of purified rasGAP was incubated with the indicated amounts of purified caspase-3 or caspase-7 for 1 h at 37°C and then processed for immunoblot analysis with the rasGAP mAb. Caspase-3 generates more of the second rasGAP cleavage product (II) then caspase-7 at the same caspase concentrations. Caspase-3 also generates a new cleavage product of approximately 100 kDa (I*)

A

B

C
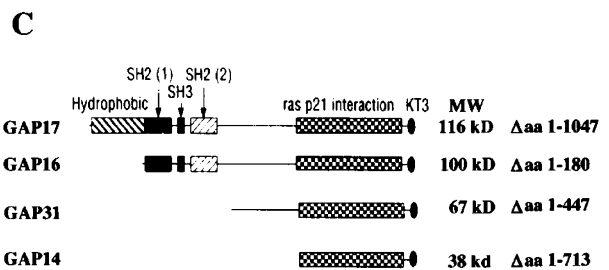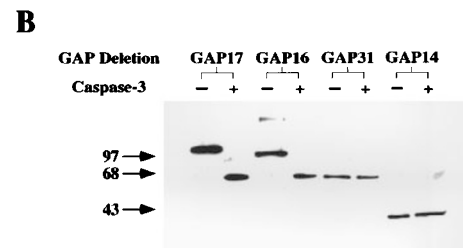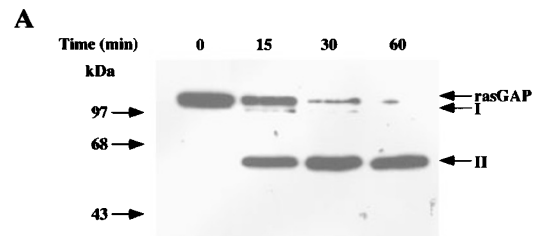
Figure 4 Mapping of rasGAP cleavage sites. In (**A**) 200 ng of purified rasGAP was incubated with caspase-3 (100 ng) for the indicated times at 37°C and then processed for immunoblot analysis with an anti-KT3 antibody. KT3 is an epitope tag at the carboxyl terminus of rasGAP. The αKT3 antibody detects cleavage products of approximately 97 kDa (I) and 66 kDa (II). In (**B**) deletion mutants of rasGAP were incubated with caspase-3 as described in (**A**) and then processed for immunoblot analysis with the anti-KT3 antibody. In (**C**) a description of the different deletion mutants is shown

fragment was also detected at 30′ after the addition of caspase-3. To more precisely map the putative cleavage sites in rasGAP we examined the pattern of rasGAP cleavage

using deletion mutants of rasGAP (Martin *et al*, 1992) which also contain the KT3 epitope at the carboxyl terminus (Figure 4B and C). Cleavage of GAP16 generated a fragment which migrated at the same position as cleavage of full length rasGAP (Figure 4B and C). The migration of GAP 31 and GAP14 was unaffected by cleavage with caspase-3 (Figure 4B and C). On the basis of the size of these rasGAP cleavage products we predict that the first cleavage occurs after the P1 at amino acid 157 in the sequence DEGD/S (I) and the second cleavage occurs after the P1 at amino acid 459 in the sequence DTVD/G (II) (see Figure 5 for schematic of rasGAP cleavage). Cleavage of rasGAP at these sites would, therefore, not disrupt GAP14 and it would cleave only 12 amino acids off GAP31 which accounts for cleaved GAP 14 and GAP31 migrating at the same MW as uncleaved GAP14 and GAP31. The cleavage site after the aspartate at amino acid 157 is in the hydrophobic region of rasGAP and the cleavage site after the aspartate at amino acid 459 is between the second SH2 domain of rasGAP and the ras-p21 interacting domain (Figure 5). This pattern of cleavage would leave an intact ras p21-interacting domain and map the epitope recognized by the commercial antibody to the SH3 domain in rasGAP.

## Cleavage of rasGAP by caspase-3 *in vitro* enhances rasGAP hydrolysis activity

We then examined if cleavage of rasGAP affects rasGAP hydrolysis activity, i.e., the conversion of rasGTP to rasGDP. rasGAP activity was difficult to quantify in apoptotic Jurkat cell extracts so that we examined the effect of caspase-3-mediated cleavage of rasGAP on rasGAP activity *in vitro*. Increasing doses of purified rasGAP were incubated with caspase-3 and then followed by a filter binding rasGAP activity assay. One half of the sample was analyzed by immunoblotting with a rasGAP mAB and confirmed that rasGAP is cleaved (data not shown). At the lowest concentration of rasGAP (125 ng) cleaved rasGAP converted 92% of Ras-GTP to Ras-GDP but uncleaved rasGAP only converted 71% of Ras-GTP to Ras-GDP (Figure 6). There was no significant difference in rasGAP activity between cleaved and uncleaved rasGAP with higher concentrations of rasGAP (Figure 6). Approximately 60% of Ras-GTP is converted to Ras-GDP at 10′ in the absence of

rasGAP which reflects the intrinsic rate of Ras hydrolysis (Figure 6). Our data suggests that cleaved rasGAP is more active than uncleaved rasGAP in converting active Ras to inactive Ras. We predict that cleavage of rasGAP generates an intact ras p21-interacting domain (see Figure 5) but isolates this domain from the remainder of rasGAP. It is possible, therefore, that the other domains in rasGAP suppress rasGAP activity in dividing cells. Cleavage of rasGAP in apoptotic cells may inhibit Ras activity and thereby downregulate signals that promote cell growth.

rasGAP is an important negative regulator of Ras activity and we have observed that cleavage of rasGAP enhances the hydrolysis of active Ras to inactive Ras *in vitro*. Recent studies also suggest that rasGAP possesses other important biological functions. rasGAP, for example, associates through its SH2 domains with autophosphorylated receptors and two intracellular proteins, p62 and p190 (Ellis *et al*, 1990; Kazlauskas *et al*, 1990; Moran *et al*, 1991). p190 contains a rasGAP domain that is specific for Rho-related GTPases, which regulate the formation of actin stress fibers and focal adhesions (Settleman *et al*, 1992; Ridley and Hall, 1992). A recent study described a new
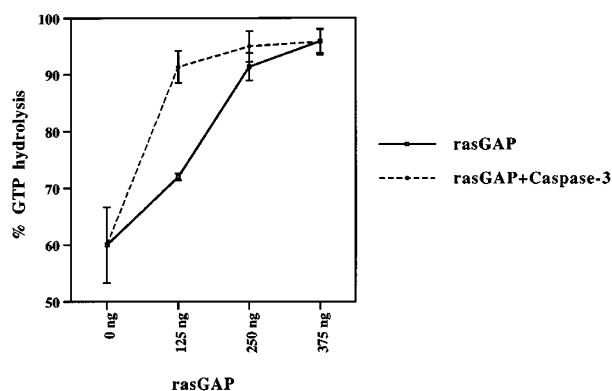
Figure 6 Cleavage of rasGAP *in vitro* enhances rasGAP activity. Varying concentrations of rasGAP were incubated with caspase-3 (200 ng) for 10 min followed by analysis of rasGAP activity with a filter binding assay. % GTP hydrolysis was calculated as 1-(GTP at 10 min/GTP at 0 min) × 100. The point at 0 ng reflects the intrinsic rate of GTP hydrolysis. Data is presented as the mean of triplicate samples from three experiments ± S.D.
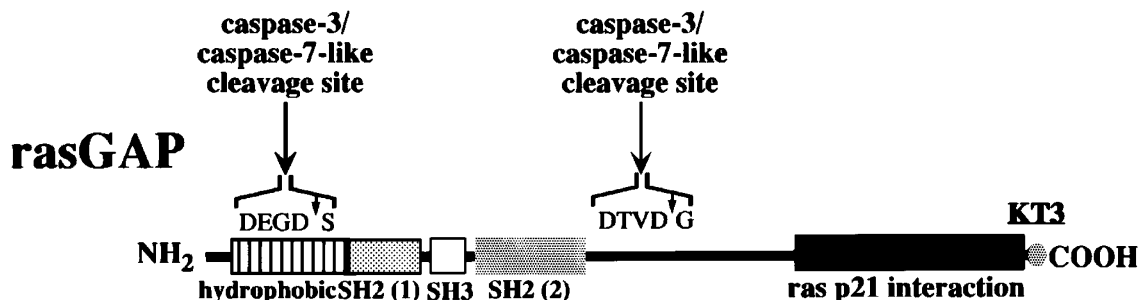
Figure 5 Predicted cleavage sites in rasGAP. A schematic of rasGAP with the predicted cleavage sites in its hydrophobic domain and between the SH2 (2) and ras p21-interacting domains are shown

function for rasGAP demonstrating that rasGAP inhibits GDP/GTP exchange factor (GEF) activity *in vitro* (Giglione *et al*, 1997). GEF plays an important role in modulating GDP/GTP exchange on Ha-Ras. These studies in addition to the recent observation that disruption of rasGAP results in neuronal apoptosis *in vivo* suggest that rasGAP may play a key role in coordinating signal transduction pathways that regulate cell survival.

Apoptosis results in the activation of caspases and the disruption of many cellular substrates that are important for maintaining nuclear and cytoplasmic integrity. We show here that rasGAP is cleaved by caspases early in Fas-induced apoptosis of Jurkat cells. Moreover, we observed that rasGAP was cleaved by Fas in other cell lines and by other apoptotic stimuli such as chemotherapy and TRAIL. Gulbins *et al*, recently demonstrated that Fas-induced apoptosis of Jurkat cells may be mediated by a Ras signaling pathway but they did not detect a change in rasGAP activity minutes after the addition of Fas (Gulbins *et al*, 1995). We observed sequential cleavage of rasGAP in Fas-treated Jurkat cells which we preliminary mapped, based on the size of the cleavage products and the location of the DXXD motifs, to the hydrophobic region and the region between the SH2(2) and ras-p21 interacting domain in rasGAP. Cleavage of rasGAP in its hydrophobic region is detected first followed by the cleavage between the SH2(2) and ras-p21-interacting domain. This pattern of cleavage leaves an intact ras-p21 interacting but cleaved rasGAP is more active *in vitro* than uncleaved rasGAP in catalyzing the hydrolysis of GTP-Ras to GDP-Ras. We do not yet know the functional significance of rasGAP cleavage *in vivo* but our observation that rasGAP is cleaved early in apoptosis by diverse apoptotic stimuli and that cleavage of rasGAP enhances hydrolysis of Ras coupled with a recent study which shows that disruption of rasGAP causes apoptosis *in vivo* suggest that cleavage of rasGAP may play a role in the activation of apoptotic signaling pathways and the inactivation of growth stimulatory pathways. It will be important to mutate the cleavage sites in rasGAP and then examine the effect on apoptosis *in vivo*.

Materials and Methods

Cell culture

The Jurkat T cell line and H460 lung cancer cell lines were purchased from ATCC (Rockville, MD). Jurkat cells were cultured in RPMI and H460 cells in DMEM with 10% FCS supplemented with L-glutamine, penicillin and streptomycin.

Cell death reagents and assays

Anti-Fas (IgM) mAb, clone CH-11, was purchased from Medical and Biological Laboratories (Nagoya, Japan) and used at a concentration of 100 ng/ml. TRAIL (Apo2L) was a gift from Dr. Avi Ashkenazi, Genentech, South San Francisco. Doxorubicin and taxol were provided by Dr. Branimir Sikic, Stanford University. Cell death was determined with the ApoAlert Annexin V Apoptosis Kit from Clontech (Palo Alto, CA) according to the manufacturer's protocol. After the cells were incubated with Annexin V-FITC (1 μg/ml) they were analyzed by FACS analysis. The tetrapeptide caspase inhibitors DEVD-CHO and YVAD-CHO were purchased from BACHEM (Torrance, CA). For *in vivo* studies with the caspase inhibitors, DEVD-CHO (200 μM) or YVAD-CHO (200 μM) were added to cells 1 h prior to the addition of the anti-Fas mAb.

Antibodies and Western blot analysis

An anti-rasGAP mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-KT3 antibody is directed against the epitope tag at the carboxyl terminus of purified rasGAP (Martin *et al*, 1990). Purified rasGAP and deletion mutants of rasGAP were prepared as described (Martin *et al*, 1992). For immunoblot analysis, Jurkat cells were treated with the CH-11 anti-Fas mAb and then processed for immunoblotting as described previously (Wen *et al*, 1997).

Preparation of naive extracts

Jurkat cells were washed with ice-cold PBS, and then resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5, 20 mM KCl, 1 mM DTT, and 0.1 mM PMSF) and left on ice for 30 min. Cells were then lysed with 20 strokes of a Dounce homogenizer (type B pestle) and centrifuged at maximum speed for 20 min at 4°C. Supernatants (naive and apoptotic extracts) were divided into small aliquots and stored at −80°C until use.

In vitro cleavage with purified caspases

One μg of purified Ras GAP was incubated with the indicated amount of purified caspases in the presence or absence of naive Jurkat cellular extract and placed in cleavage buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl$_2$, and 1 mM DTT) at 37°C for 1 h, separated on a 10% SDS gel, and immunoblotted with the rasGAP mAB from Santa Cruz or the αKT3 antibody. The purified caspases were provided by Guy Salvesen, Burnham Institute (La Jolla, CA). The activity of the caspases was determined by Dr. Salvesen with an affinity labeled Ac-Asp-Glu-Val-fluromethyl ketone (Ac-DEVD-fmk) (a gift of Joe Krebbs, IDUN Pharmaceuticals). The caspases were titrated with a known amount of the affinity reagent, followed by analysis of residual activity using the caspase substrate Ac-DEVD-p-Nitroanailide (Alexis Corp., San Diego, CA).

rasGAP activity assay

A filter-binding rasGAP assay was performed essentially as described but with some modifications (Bollag and McCormick, 1995). Purified rasGAP (125 – 375 ng) was incubated in the presence or absence of caspase-3 (200 ng) in cleavage buffer at 37°C for 1 h. One-half of both samples was used for Western blot analysis with the αKT3 antibody and confirmed that rasGAP was cleaved. The other half of the cleaved and uncleaved rasGAP samples was combined with the Ras-GTP loading reaction containing 1 μl of purified K-Ras (1 μg), 10 μL of GTP loading buffer (1 mM EDTA, 20 mM HEPES (pH 7.3), 2 mM DTT and 1 μl of γ-^{32}P-GTP and the mixture was incubated at RT for 10 min. The reactions was then divided equally for three GTP-hydrolysis reactions which contained: (1) 10 μl of cleaved rasGAP plus 40 μl of buffer B (20 mM HEPES (pH 7.3), 2 mM MgCl$_2$, 2 mM DTT) and 4 μl of the Ras-GTP loading mix; or (2) 10 μl of uncleaved rasGAP plus 40 μl of buffer B and 4 μl of Ras-GTP loading mix; or (3) 10 μl of cleavage buffer plus 40 μl of buffer B and 4 μl of Ras-GTP loading mix. The samples were incubated at room temperature for 10 min. Samples

were diluted in 1 ml of wash buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM MgCl$_2$), placed on 0.45 μM nitrocellulose filters (Amersham, Arlington Heights, IL) and washed two times with 7 ml of wash buffer. The samples were quantitated by Cerenkov counting.

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