

Caspase-mediated Cleavage of the TIAM1 Guanine Nucleotide Exchange Factor during Apoptosis¹

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Abstract

Rho family GTPases Rac and Cdc42 are pivotal regulators of apoptosis in multiple cell types. However, little is known about the mechanism by which these GTPases are regulated in response to apoptotic stimuli. Here, we demonstrate that TIAM1, a Rac-specific guanine nucleotide exchange factor, is cleaved by caspases during apoptosis. TIAM1 cleavage occurs in multiple cell lines in response to diverse apoptotic stimuli such as ceramide, Fas, and serum deprivation. Processing occurs at residue 993 of TIAM1 and removes the NH₂-terminal of TIAM1's two pleckstrin homology domains, leaving a stable fragment containing the Dbl homology and COOH-terminal pleckstrin homology domains. This leads to functional inactivation of TIAM1, as determined by failure of the cleavage product to stimulate GTP loading of Rac *in vivo*. Furthermore, this product is defective in signaling to two independent Rac effectors, c-Jun NH₂-terminal kinase and serum response factor. Finally, we demonstrate that in cells treated with ceramide, cleavage of TIAM1 coincided with the inactivation of endogenous Rac. These results reveal a novel mechanism for regulating guanine nucleotide exchange factor activity and GTPase-mediated signaling pathways.

Introduction

Rho family GTPases, members of the Ras superfamily of G proteins, govern multiple cellular processes, including pro-

liferation, transformation, cell motility, and apoptosis. Numerous studies reveal that the Rho GTPases Rac and Cdc42 can either promote or inhibit apoptosis, depending on the cell type and apoptotic stimulus examined. For example, in fibroblasts and some hematopoietic cells, constitutively active Rac protects cells from apoptosis induced by Ras or factor withdrawal (1–3). In contrast, activated Rac and Cdc42 can induce apoptosis in Jurkat T lymphocytes, thymocytes, peripheral T cells, and neuronal cell lines (4–10).

Most forms of apoptosis require the activation of a family of proteases termed caspases (11). A growing number of cellular proteins have been identified as caspase substrates, and in some cases, cleavage has been shown to modulate their biochemical activity, contributing to various aspects of the apoptotic program. For example, two caspase substrates are the protein kinases PAK2 and MEKK,³ both of which are effectors of Cdc42 and Rac. In both kinases, caspase processing results in removal of an NH₂-terminal autoinhibitory domain, leading to activation of the COOH-terminal kinase domain (12, 13). For PAK2, this allows phosphorylation of substrates that cause membrane blebbing in Jurkat cells (12). For MEKK, this promotes activation of the p38 and JNK mitogen-activated protein kinases as well as additional caspases (13).

Despite the clear role of Rho GTPases in regulating cell survival, little is known about how they are regulated in response to apoptotic stimuli. Like other G proteins, Rho GTPases are regulated predominantly through the coordinated activities of GEFs and GTPase-activating proteins (GAPs). To date, most studies have focused on GEF regulation as a means of GTPase activation. A recurrent theme in GEF activation is relocalization to the plasma membrane upon agonist stimulation. This was first demonstrated for mSOS, an activator of the p21^{ras} proto-oncogene. mSOS, through its association with the Grb2 adaptor protein, is recruited to growth factor receptors at the plasma membrane upon agonist stimulation (14–16). This allows juxtaposition of mSOS with its substrate, resulting in GTP loading of p21^{ras}. Similarly, membrane localization of TIAM1, a Rac-specific GEF, regulates its activity (17, 18). However, in this case, relocalization is mediated by a PH domain in TIAM1, which binds to phosphatidylinositol-3,4,5-trisphosphate at the plasma membrane (19).

Given the role of Rho family GTPases in apoptosis, we examined whether caspase-mediated processing of GEFs

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³ The abbreviations used are: MEKK, mitogen-activated protein kinase kinase; PH, pleckstrin homology; DH, Dbl homology; JNK, c-Jun NH₂-terminal kinase; SRF, serum response factor; GEF, guanine nucleotide exchange factor; FL, full-length; DEVD, Asp-Glu-Val-Asp; HA, hemagglutinin; GST, glutathione S-transferase; PBD, p21-binding domain; SRE, serum response element; FBS, fetal bovine serum; pen/strep, penicillin (100 units/ml), and streptomycin (0.1 mg/ml); SF, serum-free.

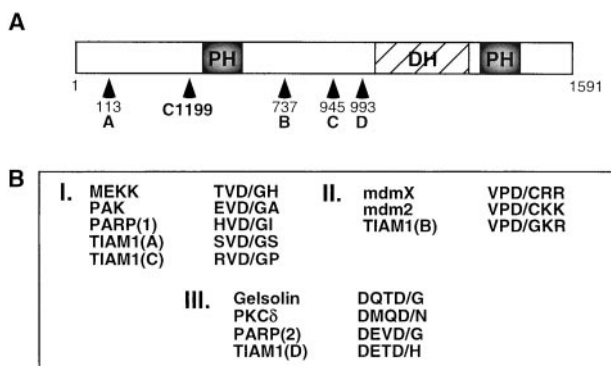


Fig. 1. Structure and potential caspase cleavage sites in TIAM1. **A**, domain structure of murine TIAM1 (1591 amino acids). PH, PH domain; DH, DH domain. Arrowheads indicate the positions of potential caspase sites A–D; numbers indicate the aspartate residue after which the caspase would cleave. The C1199 construct, which expresses the COOH-terminal 1199 amino acids of TIAM1, was used in subsequent experiments. **B**, consensus caspase motifs within TIAM1 and known caspase substrates. Three types of caspase recognition motifs have been described; sites A–D in TIAM1 fall into the three groups as indicated.

might serve as a mechanism for regulating their activity. Sequence analysis revealed the presence of multiple potential caspase cleavage sites in TIAM1. TIAM1 was originally identified as an oncogene, based on its ability to induce invasiveness and metastasis in T lymphoma cells (20). In addition to its Rac-specific GEF domain (termed the DH domain), it contains two PH domains. Structure/function analysis has revealed that the NH₂-terminal PH domain is required for TIAM1's ability to induce Rac-dependent processes, such as membrane ruffling, cell motility, and adhesion (17, 18, 21, 22). Cleavage at the potential caspase sites was predicted to remove the NH₂-terminal PH domain, which led us to hypothesize that processing of TIAM1 might occur during apoptosis and lead to its functional inactivation.

Here, we provide evidence that TIAM1 is in fact cleaved in response to diverse apoptotic signals *in vivo*. This cleavage occurs after residue 993, removing the NH₂-terminal PH domain of TIAM1 and leaving a stable COOH-terminal fragment that includes the tandem DH and COOH-terminal PH domains. In contrast to the FL TIAM1 protein, this cleavage product fails to localize to the plasma membrane. As a result, we demonstrate that it is defective in signaling to Rac and its downstream effectors. Finally, in HMN1 motor neuron cells induced to undergo apoptosis with ceramide, cleavage of TIAM1 coincided with the inactivation of endogenous Rac. This represents, to our knowledge, the first report that a GEF is cleaved during apoptosis and suggests a novel, irreversible mechanism for regulating Rac-dependent signaling pathways.

Results

Caspase-mediated Cleavage of TIAM1 in Multiple Apoptotic Systems. TIAM1 contains four potential caspase cleavage sites that are conserved between mouse and humans (Fig. 1). This led us to examine whether TIAM1 was cleaved in cells undergoing apoptosis. Cleavage of the en-

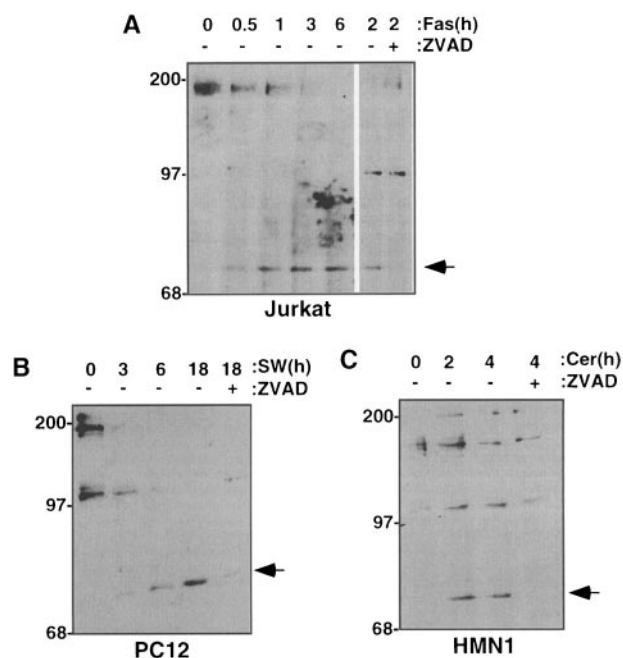


Fig. 2. TIAM1 is cleaved in a zVAD-inhibitable manner in three distinct apoptotic systems. Cells were exposed to their respective apoptotic stimuli for the indicated times. Cells were harvested, immunoprecipitated with antibody against the COOH terminus of TIAM1, and then immunoblotted with the same antibody. **A**, Jurkat cells were treated with antibodies against Fas (100 ng/ml); **B**, PC12 cells were subjected to serum withdrawal; **C**, HMN1 cells were treated with C2-ceramide (50 μ M). Where indicated, zVAD was added to a final concentration of 50 μ M, which is sufficient to block apoptosis in all three cell types. Arrowhead, M_r 75,000 cleavage product.

dogenous TIAM1 protein was monitored in three different cell types after exposure to distinct apoptotic stimuli: Fas stimulation of human Jurkat T lymphocytes, serum withdrawal of rat PC12 cells, and ceramide treatment of murine HMN1 neuroblastoma motor neurons.

To determine whether TIAM1 was cleaved in any of these systems, immunoprecipitations were performed using antibodies directed against the COOH terminus of TIAM1, followed by immunoblotting with the same antibody. In growing Jurkat cells, TIAM1 migrated at approximately M_r 200,000, as demonstrated previously for the FL protein (22). However, on treatment with cross-linking antibodies against Fas, this band gradually disappeared, concomitant with the appearance of an immunoreactive peptide of approximately M_r 75,000 (Fig. 2). The M_r 75,000 peptide was observed as early as 0.5 h after stimulation and persisted for at least 6 h, by which time the FL protein had disappeared. The initial appearance of the M_r 75,000 product preceded cell death, which began approximately 4 h after Fas cross-linking (23). Neither the M_r 200,000 nor the M_r 75,000 peptide was observed in control immunoprecipitations using nonimmune antibody (data not shown). In some experiments, a nonspecific band of M_r 120,000 was observed in anti-TIAM1 immunoprecipitations (Fig. 2). This band was not seen consistently, nor was it competed away by preincubation of the TIAM1 antibody with antigenic peptide (data not shown).

Similarly, during apoptosis induced by serum withdrawal of PC12 cells, the M_r 200,000 TIAM1 peptide disappeared, and a M_r 75,000 peptide was generated (Fig. 2B). The M_r 75,000 band was maximal 18 h after serum withdrawal, at which time 50% of the cells were apoptotic (data not shown; Ref. 24). A M_r 75,000 product was also observed in HMN1 cells on ceramide treatment. This peptide was observed at 2 and 4 h after the addition of the drug, when 25% and 75% of the cells were apoptotic, respectively (data not shown). In HMN1 cells, it was more difficult to consistently observe the FL protein, and additional background bands were seen (Fig. 2C). Nevertheless, the M_r 75,000 band was consistently observed only in the presence of ceramide and not in its absence. Together, these results suggest that cleavage of TIAM1 is a common early event in cells undergoing apoptosis.

Short peptides modeled after caspase recognition sequences potentially inhibit many forms of apoptosis (11, 25). To assess whether the observed cleavage of TIAM1 was mediated by caspase-type proteases, cells were treated with zVAD-fmk, a broad specificity caspase inhibitor, before the induction of apoptosis. As seen in Fig. 2, A–C, zVAD-fmk abrogated or largely inhibited production of the M_r 75,000 truncated TIAM1 product in all three cell types. Thus, apoptosis-induced processing of TIAM1 requires caspase activation.

TIAM1 Is Cleaved by a DEVD-inhibitable Caspase *in Vitro*. To demonstrate that TIAM1 serves as a substrate for caspases, *in vitro* protease reactions were performed. For these experiments an NH_2 -terminal-truncated TIAM1 construct termed C1199, which retains the three COOH-terminal potential caspase sites, was used (see Fig. 1). This construct has been widely used because it is more stable than the FL protein (18, 21, 22, 26). *In vitro* translated, [^{35}S]methionine-labeled C1199 protein was incubated with extracts prepared from either untreated or Fas-stimulated Jurkat cells, followed by SDS-PAGE and autoradiography. Whereas untreated C1199 protein migrated at approximately M_r 140,000, exposure to extracts from Fas-stimulated Jurkats cells resulted in the disappearance of this protein and the production of a M_r 75,000 band (Fig. 3, left panel). The appearance of this product was specific because incubation of C1199 with extracts of untreated Jurkat cells had no effect. A peptide of M_r 115,000 was also observed in the presence of Fas-stimulated extracts, suggesting that a second caspase site in TIAM1 is recognized *in vitro*. Such a band was not observed *in vivo*. It is possible that this species is a short-lived intermediate *in vivo* but can only be detected in *in vitro* reactions because the levels of *in vitro* translated protein are much higher than those of endogenous TIAM1.

To further establish that processing of TIAM1 is mediated by a protease activated during apoptosis, we made use of a Jurkat clone (I2.1) that is resistant to Fas-induced death (27). This mutant cell line is functionally defective in the Fas-associated death domain adaptor protein and fails to activate caspases on Fas ligation (28). When C1199 protein was incubated with extracts from Fas-treated I2.1 cells, no processing to the M_r 75,000 form was observed (Fig. 3), confirm-

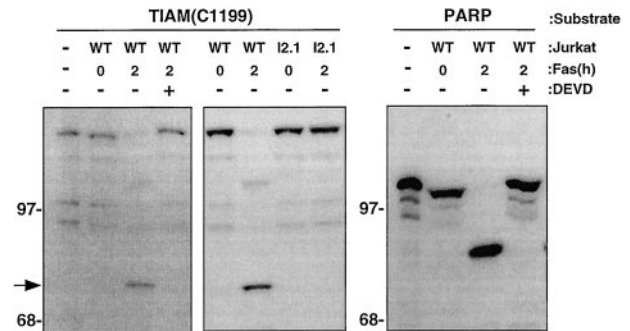


Fig. 3. TIAM1 is cleaved by a caspase-3 family caspase *in vitro*. TIAM1 C1199 was *in vitro* transcribed and translated in the presence of [^{35}S]methionine. The *in vitro* translated product was either left untreated (–) or incubated with extracts prepared from control Jurkat cells (0) or cells that had been stimulated with anti-Fas for 2 h (2). Extracts were prepared from wild-type Jurkat cells (WT) or I2.1, a Jurkat clone that is deficient in Fas-associated death domain. DEVD-CHO (100 nM) was added where indicated. Poly(ADP-ribose) polymerase, a defined caspase substrate, was used as a positive control. Reactions were resolved by SDS-PAGE and visualized by autoradiography. Arrow, TIAM1 cleavage peptide produced *in vitro*.

ing that TIAM1 is indeed processed by a caspase during apoptosis.

We next sought to determine which caspase family might be responsible for generation of the M_r 75,000 TIAM1 product. Caspases can be categorized into two subclasses based on their substrate specificity and sensitivity to various peptide inhibitors: (a) the caspase-1 (ICE)-related proteases; and (b) the caspase-3 (CPP32)-related proteases (11, 25). The latter but not the former family is specifically inhibited by DEVD-like peptides. As shown in Fig. 3, inclusion of the DEVD inhibitor in the protease reaction completely blocked conversion of C1199 to the M_r 75,000 form. Control reactions were performed in parallel with the well-characterized caspase substrate poly(ADP-ribose) polymerase (29) and confirmed that the extracts and peptide inhibitor functioned as expected (Fig. 3). This result indicates that TIAM1 is a substrate for a caspase-3 family protease.

Mapping of the Caspase Cleavage Site in TIAM1. We next sought to identify the precise cleavage site that yields the M_r 75,000 TIAM1 product. Cleavage at either amino acid 945 or 993 (Fig. 1) could potentially produce a peptide of the observed size. To determine which of these sites was used, TIAM1 mutants deleting either the NH_2 -terminal 945 or 993 amino acids were generated. HA-tagged forms of these proteins were expressed in COS cells, and their electrophoretic mobilities were compared with the M_r 75,000 product generated in Fas-ligated Jurkat cells. As seen in Fig. 4A, the $\Delta 993$ but not the $\Delta 945$ mutant comigrated with the peptide from Fas-treated Jurkat cells (its slightly reduced mobility is likely due to the HA tag). Similarly, *in vitro* translated HA- $\Delta 993$ comigrated with the cleavage product of C1199 in protease reactions (Fig. 4B).

Amino acid 993 of TIAM1 occurs within the sequence DETD₉₉₃. Thus, cleavage at this site is consistent with the fact that generation of the M_r 75,000 fragment is inhibited by the DEVD peptide (Fig. 3). To unequivocally demonstrate that this motif represents the correct processing site in TIAM1,

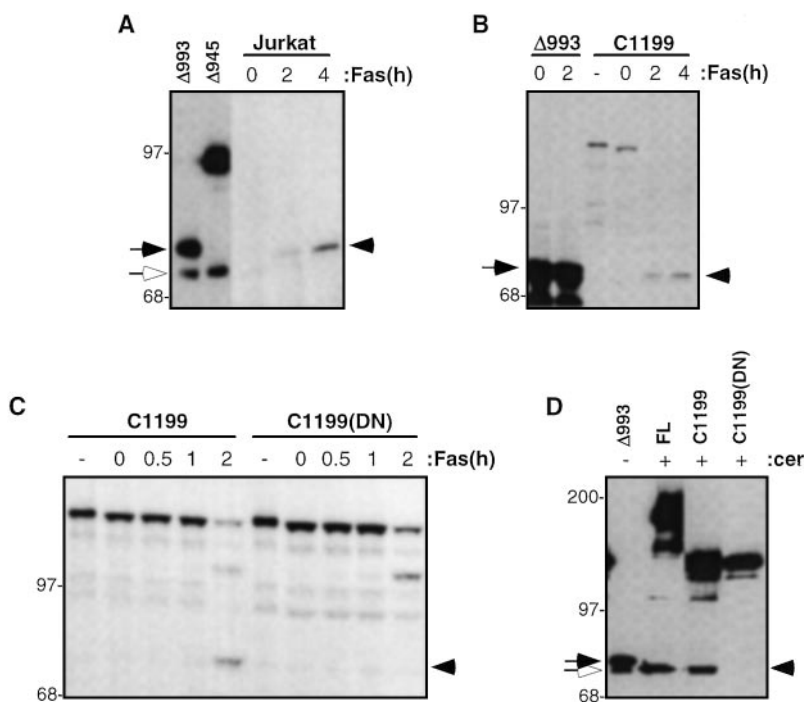


Fig. 4. Mapping of the TIAM1 caspase site *in vitro* and *in vivo*. **A**, HA-tagged forms of $\Delta 945$ and $\Delta 993$ (corresponding to the peptides generated by use of sites C and D, respectively, in Fig. 1) were expressed in COS cells, and their electrophoretic mobilities were compared with that of the cleavage product produced in Jurkat cells by Fas treatment. Blots were probed with anti-TIAM antibody. **B**, TIAM1 C1199 or $\Delta 993$ was *in vitro* translated and subjected to protease reactions as described in the Fig. 3 legend. *In vitro* translated products were left untreated (-) or incubated with extracts prepared from Jurkat cells treated with anti-Fas for the indicated times. ^{35}S -labeled peptides were detected by autoradiography. **C**, *in vitro* protease reactions were performed as described in the Fig. 3 legend using either C1199 or C1199(DN), in which the DETD₉₉₃ motif was mutated, as substrate and analyzed by autoradiography. **D**, HMN1 cells were transfected with the indicated TIAM1 constructs and then treated with C2-ceramide for 2 h as indicated. Whole cell lysates were immunoblotted with anti-TIAM antibody. Arrowhead, caspase cleavage product produced *in vivo* or *in vitro*; closed arrow, HA-tagged $\Delta 993$; open arrow, degradation product of transfected TIAM1 mutants that was not observed in untransfected cells.

the two aspartate residues in this sequence were mutated to asparagine in the context of the C1199 protein [denoted as C1199(DN)]. Analogous substitutions in other caspase substrates have been shown to abolish cleavage (12, 13). Mutation of these residues completely blocked formation of the M_r 75,000 product in *in vitro* protease reactions (Fig. 4C), indicating that this is indeed the relevant site. The intermediate M_r 115,000 product persisted when the C1199(DN) mutant was exposed to Fas-ligated Jurkat cell extracts, confirming the specificity of the mutation. Based on the electrophoretic mobility of $\Delta 945$ (which migrates at M_r 95,000), it is likely that the M_r 115,000 band is generated by cleavage at amino acid 737 (Fig. 1, site B).

To determine whether cleavage occurs at residue 993 *in vivo*, various TIAM1 mutants were transfected into HMN1 cells (Fig. 4D). After treatment with ceramide, cell extracts were immunoblotted with anti-TIAM1 antibody. In cells transfected with the FL or C1199 proteins, a band that comigrated with $\Delta 993$ was produced on ceramide treatment. In contrast, C1199(DN) was resistant to proteolysis, demonstrating unequivocally that aspartate 993 is the caspase site used *in vivo*.

The TIAM1 Cleavage Product Fails to Localize to the Plasma Membrane and Is Defective in Activation of Rac *in Vivo*. TIAM1 functions as a Rac-specific GEF (26, 30). Previous work has suggested that recruitment of TIAM1 to the plasma membrane is essential for its ability to activate Rac and requires the NH₂-terminal PH domain of TIAM1 (17, 18, 22). Because cleavage of TIAM1 at amino acid 993 removes this domain, we examined the subcellular localization of the $\Delta 993$ peptide. HMN1 cells were transfected with cDNA encoding either the FL or $\Delta 993$ TIAM1 and then analyzed by immunofluorescence microscopy. As seen in Fig. 5A, strong

plasma membrane staining of FL TIAM1 was observed. In contrast, $\Delta 993$ was found exclusively in the cytoplasm. Thus, caspase-mediated cleavage of TIAM1 affects its intracellular distribution.

Because $\Delta 993$ is unable to localize to the plasma membrane, we predicted that it would be incompetent in activating Rac. To test this, we compared the ability of FL and $\Delta 993$ TIAM1 peptides to activate Rac *in vivo* by monitoring its binding to the p21-binding domain of PAK fused to glutathione-S-transferase (GST-PBD) (31, 32). GST-PBD binds to Rac specifically in its active, GTP-bound form. COS cells were transfected with FL or $\Delta 993$ peptide, starved to quiescence mitogenic signals, and then lysed in the presence of GST-PBD. GST-PBD was precipitated using glutathione-agarose beads, and associated, active Rac was detected by immunoblotting. As seen in Fig. 5B, FL TIAM1 potently activated endogenous Rac over levels seen in vector-transfected cells. In contrast, the $\Delta 993$ peptide, when expressed at levels comparable to FL TIAM1, failed to significantly stimulate GTP loading of Rac. These results suggest that caspase-mediated cleavage of TIAM1 leads to its functional inactivation.

FL but not $\Delta 993$ TIAM1 Activates JNK and SRF. The results above indicate that caspase-mediated processing of TIAM1 abrogates its ability to activate Rac. To determine whether it also blocked signaling downstream of Rac, we examined the activation of two Rac effectors, JNK1 and the transcriptional activator, SRF. TIAM1 constructs were co-transfected with GST-tagged JNK1 into COS cells. GST-JNK was isolated using glutathione-agarose beads and then subjected to *in vitro* kinase assays using ATF2 as substrate. Whereas constitutively active Rac (Rac61L) and FL TIAM1 potently activated JNK1, $\Delta 993$ had no effect, even when expressed at much higher levels (Fig. 6A).

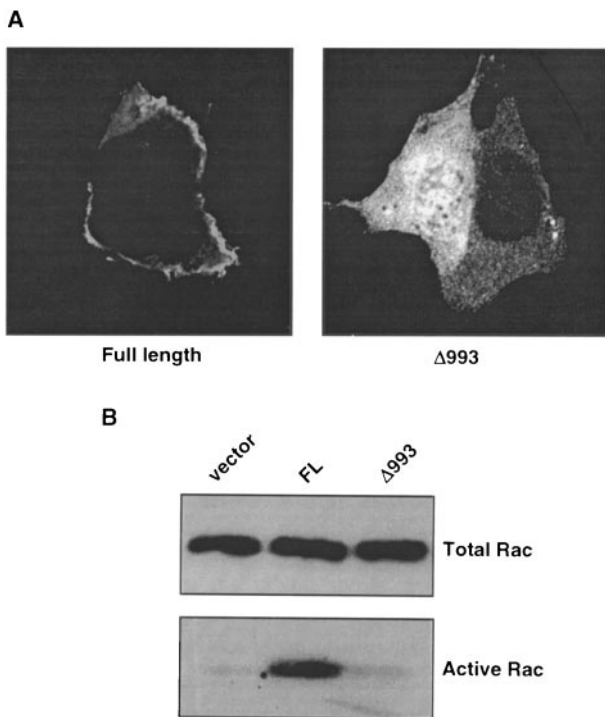


Fig. 5. TIAM1 $\Delta 993$ cannot localize to the plasma membrane TIAM1 or activate Rac *in vivo*. **A**, HMN1 cells were transfected with the indicated TIAM1 constructs. TIAM1 peptides were visualized by indirect immunofluorescence. **B**, COS cells were transfected with the indicated TIAM1 construct and then starved for 24 h. Cells were lysed in the presence of GST-PBD and precipitated using glutathione-agarose beads. Whole cell lysates (*Total Rac*) and GST-PBD pull-downs (*Active Rac*) were subjected to immunoblotting with anti-Rac antibody; whole cell lysates were also immunoblotted with anti-TIAM1 to confirm comparable expression of TIAM1 constructs (data not shown).

To further examine the ability of the $\Delta 993$ peptide to signal to Rac effectors, we tested its activation of SRF. Rac61L potently stimulates the transcriptional activity of SRF, as measured by a luciferase reporter gene driven by a SRE. To assess the potency of TIAM1 constructs in activating SRF, NIH3T3 cells were cotransfected with the SRE-luciferase reporter gene and TIAM1 constructs. As seen with the JNK kinase assays, FL TIAM1 strongly activated SRF, whereas $\Delta 993$ was inactive (Fig. 6B). Previous studies have shown that Rac activates JNK and SRF by independent signaling pathways (33). Thus, processing of TIAM1 by caspases abrogates its ability to signal to Rac and multiple effector pathways.

Rac Activity Is Reduced on Ceramide Treatment of HMN1 Cells. To investigate whether TIAM1 cleavage results in inactivation of Rac in a physiological setting, endogenous Rac activity was monitored at various time points during ceramide-induced apoptosis in HMN1 cells. As seen in Fig. 7A, untreated HMN1 cells contain high levels of active Rac. On addition of ceramide, a progressive decrease in Rac-GTP levels was observed. Levels of active Rac began to decline as early as 15 min after ceramide addition and continued to fall for at least 2 h, in parallel with the gradual processing of FL TIAM1 (Fig. 7B). These results are consistent with a role

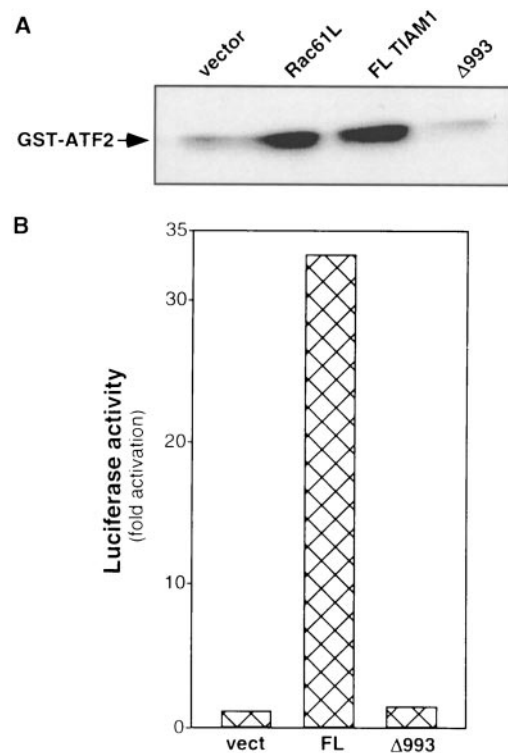


Fig. 6. FL but not $\Delta 993$ TIAM1 activates signaling downstream of Rac. **A**, COS cells were transfected with JNK/pEBG encoding GST-JNK and the indicated TIAM1 constructs or constitutively active Rac (Rac61L). GST-JNK was purified using glutathione-agarose beads and subjected to *in vitro* kinase assays using GST-ATF2 as substrate. Phosphorylated GST-ATF2 was visualized by autoradiography. Data are representative of three independent experiments. **B**, TIAM1 constructs were cotransfected with a SRE-luciferase reporter construct into NIH3T3 cells. Cells were starved for 24 h and then assayed for luciferase activity. Data are presented as fold activation over cotransfection with empty vector and represent the average of three independent experiments performed in duplicate. For both **A** and **B**, TIAM1 immunoblots were performed to ensure comparable expression of the TIAM1 mutants (data not shown).

for caspase-mediated cleavage of TIAM1 in the down-regulation of Rac during apoptosis. Significant levels of apoptosis were observed at 2 h of ceramide treatment (Fig. 7B); thus, the cleavage of TIAM1 and activation of Rac preceded cell death.

Discussion

Our results suggest that cleavage and inactivation of TIAM1 by caspases may contribute to the down-regulation of Rac activity during apoptosis. This represents a novel mechanism for modulating GEF activity and GTPase signaling. In contrast to reversible regulatory modifications, such as phosphorylation, the proteolytic processing of TIAM1 irreversibly blocks signaling to Rac. The cleavage of TIAM1 in three different cell types in response to diverse apoptotic stimuli suggests that it may be a conserved early event in multiple forms of cell death.

Previous studies have demonstrated that TIAM1 must be localized to the plasma membrane to signal to Rac and its downstream effectors (17, 18, 22). Two different mutants that

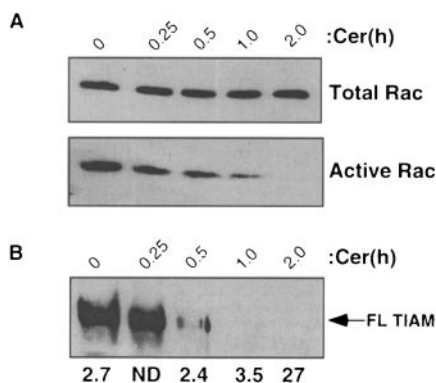


Fig. 7. Rac is down-regulated on ceramide treatment in HMN1 cells. HMN1 cells were starved overnight and then stimulated with C2-ceramide (50 μ M) for the indicated times. *A*, cells were lysed in the presence of GST-PBD and precipitated with glutathione-agarose beads. The associated, active Rac was detected by immunoblotting. *Top panel*, total Rac present in whole cell lysates; *bottom panel*, active Rac present in GST-PBD pull-downs. *B*, in parallel, lysates were immunoprecipitated with anti-TIAM antibody, followed by immunoblotting with the same antibody. The percentage of dead cells at each time point, as determined by trypan blue uptake, is indicated below each lane; ND, not determined. At 3 h of ceramide treatment, 43% of cells were dead.

deleted either the NH₂-terminal 909 or 1011 amino acids of TIAM1 failed to translocate to the membrane (17, 18, 22). Here, we shown that deletion of the NH₂-terminal 993 amino acids also causes cytoplasmic retention. In all three peptides, the NH₂-terminal PH domain, which mediates binding to phosphatidylinositol-3,4,5-trisphosphate at the plasma membrane, is removed (19). Accordingly, these peptides fail to activate Rac at this locale. Notably, at very high levels of expression, Δ 993 was able to induce activation of Rac,⁴ presumably in the cytoplasm. However, this pool of Rac was still unable to signal to downstream effectors efficiently (Fig. 6).⁴ These results are consistent with the work of others, which demonstrated that Rac must be activated at the plasma membrane to signal to JNK and PAK (18, 34). We further tested whether expression of the cleavage-resistant mutant of TIAM could prevent Rac inactivation in response to ceramide. Although we found that this was the case, interpretation of this result was complicated by the fact that the cleavage-sensitive form could also do so.⁴ This was due to the fact that on overexpression, a significant fraction of the transfected TIAM remained uncleaved (see Fig. 4D) and therefore competent to activate Rac. Even on titrating down the dose of transfected DNA, a substantial amount of FL TIAM remained intact, suggesting that caspase levels in the cell are limiting.

Rac and Cdc42 have been reported to have both positive and negative roles in apoptosis. Both proteins can promote apoptosis in multiple hematopoietic lineages and neuronal cells (4–10). The majority of these studies demonstrated that ectopic expression of constitutively active mutants of the G proteins could induce apoptosis. One study further analyzed regulation of endogenous Rac, showing that it was rapidly

activated (activation occurred within 1 min and was sustained for at least 40 min) on C6-ceramide or Fas treatment of Jurkat cells (7, 8). We have measured Rac activation in our Jurkat cells at the time points described in their work, but we did not observe similar activation.⁴ Although the basis for this difference remains unclear, clonal variation is the most likely reason. Consistent with this possibility, various studies have described distinct regulation of the Rac effector, JNK, in different Jurkat clones on Fas treatment. For example, in the study cited above, JNK activation was seen 5 min after Fas stimulation, peaked at 20 min, and then declined rapidly by 30 min (7). In Jurkat clones used by us and others, activation did not occur until 1 h after Fas stimulation and was sustained for at least 3–4 h (23, 35). This difference strongly suggests that signaling pathways may be distinctly regulated in different Jurkat clones.

TIAM1 has also been shown to promote apoptosis in certain cell types. Treatment of promyelocytic HL-60 or myeloid U937 cells with the apoptotic agent bufalin induced a modest increase in endogenous TIAM1 mRNA and protein levels (36). Furthermore, overexpression of TIAM1 enhanced bufalin-induced apoptosis, whereas expression of antisense TIAM1 RNA inhibited apoptosis.

In contrast to the work described above, numerous studies also reveal a role for Rac pathways in protection against apoptosis. For example, constitutively active Rac blocks Ras-induced apoptosis in fibroblasts in part by activating the transcription factor nuclear factor κ B (1). Rac also functions in insulin-mediated survival of fibroblasts (3) and in interleukin 3-mediated survival of BaF3 cells (2). Similarly, the Rac effector PAK1 can also promote cell survival. PAK1 plays a key role in interleukin 3-mediated survival of lymphoid progenitor cells (37). An important substrate of PAK1 in this response is Bad, a proapoptotic member of the Bcl-2 family of proteins (37). Phosphorylation by PAK1 inhibits the apoptotic activity of Bad.

Thus, Rac can have opposite effects on apoptosis. One factor that likely contributes to these differences is the use of distinct cell types and apoptotic stimuli. Rac may be activated to different levels and/or with altered kinetics, depending on the cellular context. This may in turn determine which downstream effector pathways become activated. This is superimposed on the activation of cell- and stimulus-specific parallel signaling pathways, all of which together dictate the biological outcome. Such cell type-specific effects have been amply demonstrated for Ras (1, 38–44) and for JNK2 (45). Targeted disruption of JNK2 in mice revealed distinct roles for JNK2 in apoptosis of lymphocytes: JNK2 was required for anti-CD3- but not Fas-induced apoptosis of immature thymocytes and was not required for activation-induced apoptosis of mature T cells (45). Thus, a single protein can regulate apoptosis in a stage- and stimulus-dependent manner.

Another possible contributing factor for the disparate effects of Rac is the levels of expression achieved in the different studies. Indeed, the various groups used transient transfections, stable transfections, and microinjection techniques, methods that typically yield very different expression levels. Supporting this possibility, recent work has demon-

⁴ Unpublished observations.

strated that within a single cell type, Ras can induce either proliferation or apoptosis, depending on its level of expression (1).

Although we have shown that Rac activity is down-regulated on ceramide treatment, its precise role in apoptosis of HMN1 cells remains unclear. Whereas our results are consistent with a role for Rac and TIAM in promoting survival (because they are inactivated during apoptosis), transient overexpression of FL TIAM1, C1199(DN), or Δ 993 in HMN1 cells did not inhibit ceramide-induced apoptosis.⁴ This is not surprising because numerous other parallel, proapoptotic pathways are still activated normally. Indeed, Rac61L itself did not prevent ceramide-induced apoptosis of HMN1 cells,⁴ which suggests that other pathways are sufficient to override any antiapoptotic signal that Rac and presumably TIAM1 might convey. Furthermore, it is unlikely that blocking cleavage of TIAM1 would have dramatic effects on apoptosis because certain Rac effectors such as MEKK and PAK can be cleaved and activated directly by caspases (12, 13) and can therefore be regulated independently of TIAM1/Rac. In turn, downstream targets such as JNK could be activated even if TIAM1 processing were inhibited. Thus, it remains to be determined whether TIAM1 cleavage plays an accessory role in the execution of apoptosis or whether it occurs as a secondary response to caspase activation. Future studies will be aimed at addressing this question and identifying Rac effectors that might be sensitive to TIAM1 processing.

Materials and Methods

Tissue Culture and Transfections. Jurkat T lymphocytes were cultured in RPMI 1640 containing 10% FBS (10% FBS/RPMI). COS and HMN1 neuroblastoma motor neuron cells (46) were maintained in DMEM containing 10% FBS and pen/strep (10% FBS/DME). Rat pheochromocytoma PC12 cells were maintained in RPMI 1640 supplemented with 10% horse serum, 5% FBS, and pen/strep. NIH3T3 cells were cultured in DMEM containing 5% calf serum and pen/strep. All cells were maintained in 5% CO₂.

Cells were induced to undergo apoptosis by the following protocols: Jurkat cells were seeded at 8×10^5 cells/ml in 10% FBS/RPMI 18–24 h before an experiment. Cells were then resuspended at a concentration of 2×10^6 cells/ml and treated with anti-Fas antibody (100 ng/ml; clone CH11; Kamiya Biomedical Co.) for the indicated times. HMN1 cells were seeded at 50% confluence 24 h before an experiment and then starved in SF DMEM for 16–18 h. C2-ceramide (50 μ M; Calbiochem) diluted in SF DMEM was added to cells for the indicated times (47). To induce apoptosis in PC12 cells, exponentially growing cells were resuspended by trypsinization and then washed three times in SF medium. Cells were seeded in SF medium (3.4×10^5 cells/60-mm dish) in the absence or presence of zVAD-fmk as described previously (24). For Jurkat and HMN1 cells, zVAD-fmk (50–100 μ M; Kamiya Biomedical Co. or Enzyme Systems) was added 30 min before apoptotic induction.

COS, NIH3T3, and HMN1 transfections were performed using LipofectAMINE. Briefly, cells were seeded in 35-mm dishes 16–18 h before transfection. Each well was transfected with a total of 2 μ g of DNA for 4–5 h. Cells were

washed and allowed to recover overnight in growth medium and then starved or treated as indicated.

Constructs and Plasmids. FL TIAM1/pCANmyc was kindly provided by Dr. Anne Crompton; C1199/pUTSV (encoding the COOH-terminal 1199 amino acids of murine TIAM1) was obtained from Dr. John Collard. C1199 was subcloned into HA-pcDNA (a derivative of pcDNA3 containing a HA tag) as follows: C1199 was excised from pUTSV by digestion with *Mlu*I, followed by blunting with T4 DNA polymerase, and then digestion with *Spe*I. This fragment was subcloned into HA-pcDNA, which was digested with *Ap*aI, blunted, and then digested with *Xba*I. HA- Δ 945/pcDNA was generated by digesting C1199/pUTSV with *Dra*III and *Mlu*I, followed by blunting, liberating a fragment that encodes the COOH-terminal 646 amino acids of TIAM. This fragment was subcloned into HA-pcDNA that had been digested with *Eco*RI and then blunted with T4 DNA polymerase. HA- Δ 993/pcDNA was generated by digesting HA- Δ 945/pcDNA with *Bam*HI, which removed amino acids 946–1130. The deleted fragment was replaced by a PCR product encoding amino acids 994–1130. The sequence of this construct was confirmed by automated sequencing. HA-C1199(DN)/pcDNA was generated by PCR-mediated ligation; primers were designed to convert amino acids 990 and 993 from aspartate to asparagine. Further details are available on request. The sequence of this mutant was confirmed by automated sequencing.

Immunoprecipitations and Immunoblotting. After apoptotic induction, cells were washed twice in ice-cold PBS and then lysed in radioimmunoprecipitation assay buffer [20 mM Tris (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.7 μ g/ml pepstatin, 0.5 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT]. Cells were solubilized for 10 min on ice and then pelleted in a microcentrifuge at 4°C for 10 min. Supernatants were immunoprecipitated with anti-TIAM1 antibody (sc-872; Santa Cruz Biotechnology, Inc.) and protein A-agarose (Sigma Chemical Co.) for 4 h. Immunoprecipitates were washed six times in radioimmunoprecipitation assay buffer, boiled in sample buffer, and then fractionated by SDS-PAGE. Proteins were transferred to nitrocellulose and then immunoblotted with anti-TIAM antibody (1:1000 dilution). Detection was performed using enhanced chemiluminescence. In all cases, whole cell lysates, as well as the supernatants from the TIAM1 immunoprecipitations, were also probed to ensure equal amounts of total protein in each sample.

In Vitro Protease Reactions. *In vitro* protease reactions were performed as described previously (27). Briefly, lysates were prepared from Jurkat cells [wild-type or clone I2.1 (27, 28)] that had been treated with anti-Fas for various times or left untreated. TIAM1/pcDNA constructs were transcribed and translated *in vitro* in the presence of [³⁵S]methionine using the TNT system (Promega). [³⁵S]Methionine-labeled products were incubated with Jurkat lysates for 1 h at 37°C, fractionated on Laemmli gels, and then visualized by autoradiography. Where indicated, DEVD-CHO (Enzyme Systems) was added to the Jurkat lysate for 30 min on ice before addition of the *in vitro* translated product.

Immunofluorescence. HMN1 cells were seeded on acid-washed coverslips and then transfected with FL or Δ 993 TIAM constructs using LipofectAMINE. Cells were fixed with formaldehyde using standard methods and then detected with anti-HA (for Δ 993) or anti-myc (for FL) antibodies, followed by Cy3-labeled antimouse IgG.

Rac Activation Experiments. COS or HMN1 cells were transfected or induced to undergo apoptosis as indicated. Cells were lysed in G protein lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.5% NP40, 5 mM β -glycerophosphate, 1 mM DTT, and protease inhibitors] containing GST-PBD. Cells were solubilized for 5–10 min on ice and then pelleted in a microfuge for 10 min. Supernatants were precipitated with glutathione-Sepharose beads (Pharmacia) for 30 min and then washed three times in G protein lysis buffer. Samples were fractionated on 12% SDS-PAGE gels; the bottom portion was immunoblotted with anti-Rac antibody (Upstate Biotechnologies, Inc.), and the upper portion was stained with Coomassie Blue to ensure that equal amounts of GST-PBD were present. GST-PBD, encoding amino acids 70–117 of human PAK1 fused to GST, was kindly provided by Dr. Jonathan Chernoff (Fox Chase Cancer Center); the protein was purified as described previously (48).

Luciferase and Kinase Assays. NIH3T3 cells were transiently cotransfected with TIAM1 mutant constructs and a luciferase reporter gene driven by a variant SRE that monitors TCF-independent SRF activity (33). After transfection, cells were allowed to recover overnight in 5% CS/DMEM. The next morning, cells were starved in 0.5% FBS/DMEM. Cells were harvested 24 h later, and luciferase activity was measured according to the manufacturer's instructions (Promega Corp.). In parallel, a fraction of the lysate was subjected to SDS-PAGE and immunoblotted with anti-TIAM1 antibody.

For JNK assays, COS cells were cotransfected with JNK/pEBG encoding GST-JNK and the various TIAM1 constructs. After transfection, cells were starved in 0.5% FBS/DMEM for 24 h. Cells were harvested and subjected to kinase assays using GST-ATF2 as substrate as described previously (23, 48). In parallel, a fraction of the lysate was immunoblotted with anti-GST or anti-TIAM1 antibodies to confirm equal expression in all samples.

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