Caspase-3 and caspase-7 but not caspase-6 cleave Gas2 in vitro: implications for microfilament reorganization during apoptosis

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SUMMARY

Apoptosis is characterized by proteolysis of specific cellular proteins by a family of cystein proteases known as caspases. Gas2, a component of the microfilament system, is cleaved during apoptosis and the cleaved form specifically regulates microfilaments and cell shape changes. We now demonstrate that Gas2 is a substrate of caspase-3 but not of caspase-6. Proteolytic processing both in vitro and in vivo is dependent on aspartic residue 279. Gas2 cleavage was only partially impaired in apoptotic MCF-7 cells which lack caspase-3, thus indicating that different caspases can process Gas2 in vivo. In vitro Gas2 was processed, albeit with low affinity, by caspase-7 thus suggesting that this caspase could be responsible for the incomplete Gas2 processing observed in UV treated MCF-7 cells. In vivo proteolysis of Gas2 was detected at an early stage of the apoptotic process when the cells are still adherent on the substrate and it was coupled to the specific rearrangement of the microfilament characterizing cell death. Finally we also demonstrated that Gas2 in vitro binds to F-actin, but this interaction was unaffected by the caspase-3 dependent proteolytic processing.

Key words: Actin, PARP, MCF-7, Death substrate

INTRODUCTION

Cell death by apoptosis is characterized by cellular and nuclear shrinkage, membrane blebbing, condensation of nuclear chromatin and DNA fragmentation (Wyllie et al., 1980). A family of cystein proteases called caspases play a critical role in the execution of the apoptotic program from nematodes to mammals (Cohen, 1997; Crynes, and Yuan, 1998). Caspases contain a QACXG pentapeptide in which the cysteine participates in catalysis and are characterized by the absolute requirement of an aspartic residue in the substrate P1 position (Nicholson et al., 1995; Thornberry et al., 1997). Like many other cellular proteases, caspases are synthesised as inactive proenzymes that can be activated upon an apoptotic signal. These enzymes can be broadly subdivided by the nature of their pro-domain. Some caspases have long prodomains while others contain smaller pro-domains. The long pro-domain is important for apoptotic stimuli-dependent recruitment of the caspases into aggregates, which favour pro-enzyme activation (Kumar and Colussi, 1999). Long pro-domain caspases seem to lie at the apex of a hierarchically ordered proteolytic cascade, while caspases containing a short pro-domain seem to act downstream, and for this reason they are also known as executioner caspases (Salvesen and Dixit, 1997).

Executioner caspases, by specifically cleaving selected cellular proteins, or death substrates, govern the morphological changes characterizing the apoptotic phenotype (Porter et al., 1997; Tan and Wang, 1998). Death substrates therefore play an important role in determining the final apoptotic phenotype. Caspase-dependent cleavage can inactivate death substrates such as in the case of PARP, nuclear lamin, Bcl-2 and β-catenin (Lazebnik et al., 1994; Takahashi et al., 1996; Brancolini et al., 1997). Alternatively cleavage can activate the substrate, examples of such substrates, include MEKK1, p21-activated kinase, protein kinase Cδ and Gas2 (Emoto et al., 1995; Brancolini et al., 1995; Cardone et al., 1997; Rudel and Bokoch, 1997).

In some circumstances a relationship between the caspase-dependent processing of a death substrate and a particular aspect of the apoptotic phenotype has been established. For example the cleavage of the inhibitor of caspase-activated DNAse is responsible for the nuclear morphological changes and degradation of nuclear DNA (Liu et al., 1997; Enari et al., 1998). However, for a large number of death substrates, how their processing relates to specific alterations of the different cellular compartments, and how it is finely orchestrated are still open questions.

The microfilament system plays an important role in regulating the apoptotic phenotype (Cotter et al., 1992). Some death substrates, involved in regulating actin architecture have been identified. Gelsolin is cleaved by caspase-3 and the cleaved fragment can disrupt actin filaments in the absence of calcium (Kothakota et al., 1997). α-fodrin is cleaved during apoptosis by caspase-3 and possibly also by calpain; this cleavage could contribute to the remodelling of the cell cortex (Janicke et al., 1998b; Waterhouse et al., 1998). Gas2 is another
component of the microfilament system which is proteolytically processed during apoptosis (Lee et al., 1999). Overexpression of the apoptotic form of Gas2 triggers microfilament reorganization and cell condensation. Gas2 represents a putative caspase substrate since its cleavage during cell death is dependent on aspartic residue at position 279 (Brancolini et al., 1995).

In this study we have characterized the relationships between Gas2 processing, caspases activation and changes of the microfilament system. We demonstrate that Gas2 can bind F-actin in vitro and it is a substrate of caspase-3 and caspase-7, but not of caspase-6. While caspase-3 cleaves PARP and Gas2 with similar affinity, caspase-7 was 1000-fold more efficient in cleaving PARP respect to Gas2. Proteolytic processing of Gas2 in vivo during apoptosis is coupled to changes of the microfilament system but this processing dose not interfere with its ability to bind F-actin.

MATERIALS AND METHODS

Cells lines and culture conditions

NIH-3T3, MCF-7 and COS-7 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

In each experiment 2.5x10^5 cells/ml were seeded in 35 mm Petri dishes. Cells were transfected as previously described (Brancolini et al., 1995).

For density-dependent inhibition, cells were plated at 10^4/cm^2 in 10% FCS. 24 hours after plating the medium was changed every 2 days. For induction of apoptosis in MCF-7 cells culture medium was removed, dishes were washed once with PBS, UVC irradiated (180 J/m² in PBS) and fresh medium, containing 10% FCS was added to the cells. 24 hours later non adherent and adherent cells were harvested, washed in PBS, and solubilized in SDS-PAGE buffer.

Purification of recombinant caspase-3 and caspase-6 and in vitro proteolytic assay

Caspase-3 was expressed in bacteria using the pQE-12 expression system (Qiagen). Cells were grown to an OD of 0.2. Cloning of sequences 276-314 was performed in E. coli (Brancolini et al., 1992). The fusion protein GST-Gas2wt and GST-Gas2 (276-314) was purified as previously described (Brancolini et al., 1992).

Microinjection

Microinjection was performed using the Automated Injection System (Zeiss Oberkochen, Germany). Cells were injected with 50 ng/ml of each expression vector (Brancolini et al., 1995). Each cell was injected for 0.5 seconds at a constant pressure of 150 hPA. Under these conditions, approximately 0.05 pl of sample was injected, which corresponded to about 500 plasmid copies.

Immunofluorescence microscopy

NIH 3T3 cells were grown under the described conditions and then fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. Fixed cells were washed with PBS/0.1 M glycine, pH 7.5, and then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. The coverslips were treated with the first antibodies the anti-h-TR OKT9, the pan anti-gas2 or the anti-Gas2 carboxy terminus, respectively, for 1 hour in a moist chamber at 37°C. For triple immunofluorescence, to detect actin filaments hTR and carboxy-terminal domain of Gas2, coverslips were incubated with biotinylated anti-mouse antibodies (Southern) streptavidin AMCA-conjugated (Jackson), TRITC anti-Rabbit (Dako) and phalloidin FITC (Sigma) for 1 hour at 37°C.

Actin filaments were detected using FITC-phallloidin or TRITC-phallloidin (Sigma) and nuclei were labeled with propidium iodide. Cells were examined by epifluorescence with a Zeiss Axiovert 35 microscope or a Zeiss laser scan microscope (LSM 410) equipped with a 488 λ argon laser and a 543 λ helium neon laser. The following sets of filters were used: rhodamine (BP546, FT580, LP 590), fluoresceine (450-490, FT 510, LP520).

Binding of Gas2 to actin

For the construction of the GST-Gas2Δ276-314 fusion protein, oligonucleotides oligo: 5’-ATGGATCCCGAATGCATGGCAGCTGCCCT-GAGC-3’, and oldw: 5’-AGAGCTTTCAGATCTGCAGCA-3’, containing BamHI and HindIII sites were used to generate a polymerase chain reaction fragment of Gas2Δ276-314 which was cloned in pGEX3 vector. The fusion proteins GST-Gas2wt and GST-Gas2Δ276-314 were expressed in E. coli and purified as previously described (Brancolini et al., 1992).

An F-actin cosedimentation assay was used to determine if Gas2 bound actin filaments. Actin polymerisation was induced by adding 40 μl of F-actin buffer (10 mM phosphate buffer, pH 7.4, 160 mM NaCl, 1 mM MgCl₂, 0.2 mM DTT, 0.2 mM ATP 1 mM PMSF) to 10 μl of 2 mg/ml actin (Sigma) in G-buffer (2 mM Tris-HCl, pH 7.5, 0.2 (transfer buffer: 20% methanol, 48 mM Tris, 39 mM glycine and 0.0375% SDS). After staining with Ponceau S, the nitrocellulose sheets were saturated for 2 hours in Blotto-Tween 20 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl 5% non-fat dry milk and 0.1% Tween-20) and incubated overnight at room temperature with the specific antibody: anti-Gas2 or anti-β-catenin (Transduction Laboratories). Immunodecorations were performed as previously described (Brancolini et al., 1992). For [35S]methionine labeling, BALB/c grown for 7 days in 10% FCS, were labeled for 12 hours in 1 ml of methionine-free DMEM, containing 100 μCi/ml [35S]methionine (Amersham). After washing with cold PBS, cells were lysed on the dish by addition of 0.5 ml lysis buffer (150 mM NaCl, 20 mM TEA, pH 7.5, 0.8% SDS). After boiling, 0.5 ml of quench buffer (100 mM NaCl, 20 mM TEA, pH 7.5, 4% Triton X-100) was added containing (final concentrations) 1 mM PMSF and 10 mg/ml each of aprotinin, leupeptin, antipain, and pepstatin. The lysates were cleared by centrifugation in an Eppendorf centrifuge for 2 minutes and used for immunoprecipitation as previously described (Brancolini et al., 1992). Protein A-Sepharose was recovered by centrifugation, washed 3 times in wash buffer (20 mM TEA, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF) and finally resuspended in caspase-3 digestion buffer. Immune complexes were released by boiling for 5 minutes in sample buffer.
RESULTS

Caspase-3, but not caspase-6 cleaves Gas2 in vitro

Gas2, a component of the microfilament system, is proteolytically processed during apoptosis induced by different stimuli. This processing removes the carboxy-terminal region of Gas2, thus unmasking a potent microfilament and cell shape reorganizing activity (Brancolini et al., 1995).

Even though it has been demonstrated that Gas2 cleavage during apoptosis is dependent on the aspartic residue 279, whether caspases are directly responsible for such processing is still unknown. Executioner caspases are generally involved in the processing of different death substrates; among them caspase-3 and caspase-6 show different substrate specificities (Takahashi et al., 1996), therefore, as a first step towards the identification of the caspase involved in Gas2 processing, we analyzed whether the executioner caspase-3 and caspase-6 were able to cleave Gas2 in vitro.

Recombinant caspases were made in bacteria and purified as described in Materials and Methods. Full-length Gas2 cDNA was in vitro translated and then incubated with purified caspase-3 (Fig. 1a) or purified caspase-6 (Fig. 1b). Treatment with increasing amounts of caspase-3 for 30 minutes at 37°C specifically cleaved Gas2, thus producing a band showing similar electrophoretic mobility (≈31 kDa) with respect to Gas2 as detected in extracts of apoptotic cells.

PARP (poly-ADP-ribose polymerase), a well-defined substrate of caspase-3 (Nicholson et al., 1995) was similarly incubated with increasing amounts of the recombinant caspase-3 over the same time course. Even though partial PARP processing was observed after incubation with 0.01 ng of caspase-3, 10 ng of caspase-3 were required for the full processing of both Gas2 and PARP.

In vitro translated Gas2 was also incubated with increasing amounts of caspase-6 for 2 hours at 37°C. Caspase-6 was unable to cleave the in vitro translated Gas2 when used at up to 200 ng. Lamin A, a previously characterized substrate of caspase-6 (Takahashi et al., 1996) and β-catenin, which is cleaved at multiple sites during apoptosis (Brancolini et al., 1997, 1998) were both fully processed under the same experimental conditions (Fig. 1b).

Aspartic 279 is required for caspase-3 dependent Gas2 processing in vitro

It has been demonstrated that the proteolytic processing of Gas2 during apoptosis is dependent on an aspartic residue at position 279 (Brancolini et al., 1995). Therefore, we next analyzed whether proteolytic processing of Gas2, as mediated by caspase-3 in vitro, was also dependent on aspartic 279.

Gas2wt and Gas2D279A cDNAs were in vitro translated and treated with increasing amounts of purified caspase-3 for 30 minutes at 37°C as shown in Fig. 2. Substitution of the aspartic acid at position 279 of Gas2 completely abolishes the caspase-3 dependent cleavage of Gas2. Gas2wt, under the same experimental conditions, was proteolytically processed as above reported. Furthermore, addition of the specific caspase-3 inhibitor DEVD-CHO efficiently suppressed Gas2wt in vitro processing.

Immunopurified Gas2 is cleaved by caspase-3

The use of reticulocyte lysates in our in vitro proteolytic assays cannot exclude the possibility that Gas2 is an indirect target of caspase-3. In fact different procaspases, which are activated after the addition of the purified caspase-3 might be present in the lysates. In order to clarify if caspase-3 indeed directly cleaves Gas2, we decided to isolate Gas2 from the cells, by immunoprecipitation. Density arrested BALB/c cells were labeled for 12 hours with [35S]methionine and after cell lysis immunoprecipitations were performed using antibodies against Gas2, as described in Materials and Methods. When the immunopurified Gas2 was incubated with purified caspase-3 proteolytic processing was observed, as above reported, and this cleavage was inhibited by addition of DEVD-CHO (Fig. 3). In summary we can conclude that Gas2 is a direct substrate of caspase-3 and that the aspartic residue 279 is critical both for its in vitro and in vivo proteolytic processing.

Gas2 proteolytic processing during apoptosis was partially impaired in UV irradiated MCF-7 cells

Human MCF-7 breast carcinoma cell line is devoid of caspase-
3 due to the functional deletion of the CASP-3 gene (Janicke et al., 1998a). Despite the lack of caspase-3, MCF-7 cells are still sensitive to different apoptotic stimuli, thus representing an ideal system to test if this caspase is critical for cleaving a specific death substrate during apoptosis (Janicke et al., 1998b; Tang and Kidd, 1998).

Gas2 is expressed at low levels, almost undetectable in MCF-7 cells, therefore a Gas2 DNA expression construct was transiently transfected in these cells. After transfection cells were UV irradiated and 16 hours later apoptotic and non-apoptotic cells were harvested separately. Western blotting analysis revealed that Gas2 was only partially processed to a 31 kDa form exclusively in the non-adherent, apoptotic cell population (Fig. 4). Approximately 50% of Gas2 was cleaved in the apoptotic MCF-7 cells. As control COS-7 cells were transfected with Gas2 cDNA, UV irradiated and apoptotic and non-apoptotic cells were harvested separately. In this case western analysis revealed that Gas2 was fully processed to a 31 kDa form in the apoptotic population.

The same lysates were also analyzed for β-catenin processing, a death substrate cleaved by caspase-3 in vitro. In vivo β-catenin is cleaved at different sites giving rise to three major forms of around 65-70 kDa (Brancolini et al., 1997, 1998). When analyzed in apoptotic MCF-7 cells β-catenin processing was impaired. In the case of β-catenin only the unprocessed form at ~92 kDa, albeit at a lower level, was evident. The reduced amount of β-catenin could be from a proteolytic degradation unrelated to caspase processing (Willert and Nusse, 1998). β-Catenin processing was observed in apoptotic COS-7 cells as previously reported (Brancolini et al., 1997).

To confirm that the partial cleavage of Gas2 in apoptotic MCF-7 cells was dependent on caspases we analyzed if the general caspase inhibitor zVAD.fmk was able to inhibit its processing in UV treated MCF-7 and if this processing was dependent on aspartic 279.

### Table 1. Caspase Activity in MCF-7 and COS-7 Cells

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**Fig. 2.** In vitro protease assays. (a) [35S]Methionine-labeled in vitro translated Gas2wt and Gas2D279A were incubated for 30 minutes at 37°C with 0.1-100 ng of purified caspase-3, with 0.1-100 ng of purified caspase-3 in the presence of 0.2 μM Ac-DEVD-CHO, or buffer alone.

**Fig. 3.** In vitro protease assays. BALB/c fibroblasts were labeled with [35S]methionine and after cell lysis immunoprecipitations were performed as described in Materials and Methods. Immunocomplexes were resuspended in caspase-3 buffer and incubated for 30 minutes with 50 ng of purified caspase-3, or with 50 ng of purified caspase-3 in the presence of 0.2 μM Ac-DEVD-CHO. In vitro translated Gas2 is also shown.

**Fig. 4.** Gas2 processing during apoptosis in MCF-7 cells. COS-7 and MCF-7 cells were transfected with Gas2wt, after 3 days in 10% FCS cells were UV irradiated (120 J/m²). 20 hours later non-apoptotic (N) and apoptotic floating cells (A) were harvested separately. (B) MCF-7 cells were transfected with Gas2wt and Gas2D279A. After 3 days in 10% FCS cells were UV irradiated (120 J/m²) and 20 hours later both apoptotic and non-apoptotic cells were combined for western analysis. zVAD-fmk was used at 100 μM final concentration. Western analysis was performed using the indicated antibodies.
zVAD.fmk was able to efficiently counteract apoptosis in UV treated MCF-7 cells (data not shown). To compare Gas2 processing in UV treated MCF-7 cell in the presence or not of zVAD.fmk, adherent and non-adherent cells were combined for this analysis. As shown in Fig. 4, zVAD.fmk completely abolished Gas2 processing in UV treated MCF-7 cells. In addition in apoptotic cells expressing the Gas2D279A point mutants proteolytic processing was undetectable thus confirming the involvement of a caspase.

**Caspase-7 dependent processing of Gas2 in vitro**

The limited Gas2 proteolytic processing observed in apoptotic cells suggests that it could also be a substrate for another executioner caspase. Therefore we analyzed if in vitro the executioner caspase-7 was also able to process Gas2.

In vitro translated Gas2 was incubated with increasing amounts of purified caspase-7 for 90 minutes at 37°C. As shown in Fig. 5, 200 ng of caspase-7 were required for the full processing of Gas2 while partial Gas2 processing was observed after incubation with 20 ng of caspase-7. Under the same experimental conditions, full processing of PARP was observed after incubation with 2 ng of purified caspase-7. This analysis therefore suggests that caspase-7 can cleave Gas2 in vitro even though with a lower affinity when compared to PARP.

**Caspase-dependent processing of Gas2 during apoptosis is coupled to actin reorganization**

We next analyzed if Gas2 processing at its carboxy-terminal domain was coupled to the previously described changes in actin organization occurring during the execution phase of the apoptotic process (Brancolini et al., 1997). During apoptosis cells sever contacts with neighboring cells, retract from the adhesion substrate, dismantle stress fibers and extensively accumulate actin in the perinuclear region.

gas2 was co-expressed with transferrin receptor (hTR) in growing NIH3T3 cells by nuclear microinjection, and apoptosis was induced 6 hours later by removing serum from the culture medium. After 12 hours cells were fixed and analyzed for triple immunofluorescence using antibodies against the hTR, the carboxy-terminal of Gas2 (anti-Gas2-CT) and FITC-phalloidin. The anti-Gas2-CT antibody was able to detect the protein in morphologically normal cells, failing to recognize the Gas2 form present in apoptotic cells (Brancolini et al., 1995).

As shown in Fig. 6A cells displaying a non-apoptotic

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**Fig. 5. Caspase-7 in vitro processing.** [35S]Methionine labeled in vitro translated Gas2wt and PARP were incubated for 90 minutes at 37°C with 0.2-200 ng of purified caspase-7.

**Fig. 6. Gas2 proteolytic cleavage in vivo: in situ analysis.** 24 hours after seeding NIH3T3 cells were microinjected with pGDSV7gas2wt and pGDSV3h-TR. After 6 hours, serum was removed to induce apoptosis and 16 hours later cells were fixed and processed for immunofluorescence analysis to visualize actin filaments, (phalloidin-FITC) (A,D), h-TR, (OKT9 and anti-mouseAMCA) (B,E) and Gas2 using anti-carboxy-terminal antibodies (C) or anti-amino-terminal antibodies (F) with anti-rabbit TRITC as second antibody. Bar, 5 μm.
phenotype were positive for staining with both anti-hTR (Fig. 6B) and anti-Gas2-CT (Fig. 6C) antibodies. On the contrary an apoptotic cell showing the previously characterized reorganization of the microfilament system (Fig. 6A, arrow) presented positive staining for hTR (Fig. 6B), failing to be detected with the anti-Gas2-CT antibody (Fig. 6C). Similar results were obtained in different experiments. Using an antibody specific for the amino-terminal domain of Gas2 both apoptotic (Fig. 6D, arrow) and normal cells scored positive for hTR expression (Fig. 6E) and Gas2 staining (Fig. 6F).

**Gas2 and its apoptotic deleted version can similarly bind to F-actin in vitro**

Gas2 can co-localize with the actin filaments, enriched at the cell periphery and in the membrane ruffles, but it is unknown if Gas2 binds directly to F-actin. Therefore, a co-sedimentation assay was used to determine if Gas2 could bind to F-actin in vitro. Gas2 was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein and purified by affinity chromatography on glutathione-agarose beads. The fusion protein was incubated alone or with purified porcine muscle F-actin, as described in Materials and Methods and centrifuged to pellet F-actin. The supernatants and pellets were separated on SDS-polyacrylamide gels and stained with Coomassie blue.

A large part of GST-Gas2 was detected in the pellet fraction when centrifuged together with F-actin. On the contrary GST protein was mainly detected in the supernatant if centrifuged in the presence of F-actin. Since GST-Gas2 was undetectable in the pellet when centrifuged in the absence of F-actin, we can conclude that Gas2 co-sedimented with F-actin.

We next analyzed if the apoptotic processed form of Gas2 was also able to co-sediment with F-actin. A carboxy-deleted version of Gas2 (Δ276-314) was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein. Gas2wt-GST and Gas2Δ276-314-GST were incubated with F-actin and centrifuged after 30 minutes at 37°C to pellet the F-actin.

SDS-PAGE was performed on the supernatants and pellets, and gels were Coomassie blue stained: approximately the same amount of Gas2wt-GST fusion protein, and its apoptotic form, Gas2Δ276-314-GST fusion protein co-sedimented with F-actin. Here again GST alone did not co-sediment with F-actin.

**DISCUSSION**

An important step in the understanding of the proteolytic cascade regulating cell death by apoptosis is to establish which death substrate is cleaved by a specific caspase. In this report we have shown that Gas2 is a substrate of caspase-3. Purified caspase-3 can cleave immunopurified Gas2, thus indicating that the processing is direct. On the contrary caspase-6 was unable to cleave Gas2 in vitro even though its specific substrates lamin A (Takahashi et al., 1996) and β-catenin were efficiently cleaved.

Aspartic residue 279 at the carboxy-terminal region of Gas2 is required for its processing both in vivo during apoptosis (Brancolini et al., 1995) and in vitro by caspase-3, thus strengthening the hypothesis that caspase-3 could be responsible for cleaving Gas2 also in vivo. Caspase-3, in addition to the requirement for a P1 Asp, shows preference for an anionic aspartic residue in the P4 residue (DXXD) (Nicholson et al., 1995; Thornberry et al., 1997). Aspartic residue 279 in Gas2 does not show a canonical caspase-3 consensus sequence, but instead has an SRVD motive. A serine residue in position P4 is also present in the caspase-3 death substrate sterol-regulatory element binding protein SREBP-1 and the p21-protein activated kinase 2 PAK-2 (Wang et al., 1996; Rudel and Bokoch, 1997; Tan and Wang, 1998). It is interesting to note that a dominant negative PAK construct inhibited the formation of the apoptotic bodies during Fas-induced apoptosis (Rudel and Bokoch, 1997). A similar altered apoptotic phenotype was also reported in the case of disruption of the microfilament system following cytochalasin treatment (Cotter et al., 1992). Gas2 is also involved in regulating microfilament changes during apoptosis (Brancolini et al., 1995), thus two caspase-3 substrates with the same unusual P4 residue seem to be both involved in regulating cell shape changes.

The presence of a serine in position P4 of Gas2 could also explain the only partial impairment of its processing in UV treated apoptotic MCF-7 cells, which lack functional caspase-3. Approximately 50% of Gas2 was proteolytically processed in this cell line, while processing of the caspase-3 substrate β-
catenin was completely impaired. Processing of gelsolin and α-fodrin was also impaired in TNF or staurosporin treated apoptotic MCF-7 cells, while more data are necessary to understand the rate of PAK-2 processing (Janicke et al., 1998b; Tang and Kidd, 1998). The partial Gas2 processing was dependent on a caspase activity, since treatment with the broad caspase inhibitor zVAD.fmk completely abolished such processing and the Gas2D279A point mutants was unprocessed in apoptotic MCF-7.

These results indicate that Gas2 in UV treated MCF-7 cells is also the target of a caspase different from caspase-3. In vitro Gas2 can be cleaved, although with low affinity by caspase-7. In fact, while caspase-3 cleaves PARP and Gas2 with similar efficiency, caspase-7 was approximately 1000-fold more active on PARP than on Gas2. Caspase-7 could be responsible for the observed partial proteolytic processing of Gas2 in UV treated MCF-7 cells and the low affinity, demonstrated in vitro, might explain the incompleteness of this proteolytic event. However, it should be clarified whether apoptosis in UV treated MCF-7 is characterized by caspase-7 activation (Janicke et al., 1998b; Lim et al., 1999; Slee et al., 1999).

Overexpression of caspase-2, -3, -6, -7, -8, and -9 leads to apoptosis that is indistinguishable, in terms of microfilament changes, from apoptosis induced by serum deprivation (C. Brancolini, unpublished results). Different death substrates are regulators of the actin architecture, thus supporting the hypothesis that caspases can directly modulate microfilament reorganization during cell death. In this context it will be important to understand how the processing of the gelsolin, α-fodrin Gas2 and PAK-2 is coordinately regulated. It is possible that during the different steps of the apoptotic process specific requirements in terms of actin dynamics are necessary and that the caspase dependent modulation of the different microfilament regulating proteins is temporally and spatially modulated.

Our studies demonstrate that Gas2 was already proteolytically processed and therefore fully active, for triggering microfilament changes, when cells were still adherent to the extracellular matrix and the actin cytoskeleton underwent the specific apoptotic reorganization. Even though the execution phase of apoptosis is difficult to order sequentially, because its onset is asynchronous across a cell population, this evidence suggests that Gas2 processing might be an early event.

We have also demonstrated that Gas2 in vitro can bind F-actin and that this interaction does not seem to be modulated after caspase-3 processing since the Gas2 deleted version Δ276-314, resembling the apoptotic form, co-precipitated with F-actin in a co-sedimentation assay. Gas2 binding to actin filaments should therefore be required to exert its effect on the actin cytoskeleton. In supporting this hypothesis we reported that Gas2-deleted versions losing larger fragments of the carboxy-terminal region of up to aa 171 showed reduced ability to co-localize with actin filaments in vivo and reduced ability to induce morphological changes (Brancolini et al., 1995). But how to explain the reorganization of the actin filaments induced by overexpression of Gas2Δ276-314?

In the case of gelsolin, which shows both actin monomer-binding and F-actin severing activities, caspase-3 cleavage generates a fragment which may preferentially sever actin filaments rather than bind monomeric actin (Kothakota et al., 1997). Therefore it could be possible that caspase-3 regulates the ability of Gas2 to modulate actin dynamics instead of its ability to bind F-actin. Alternatively Gas2 could act as an anchor for some factors modulating actin dynamics and this function could be being regulated by caspase-3 processing. Additional studies will therefore be needed to distinguish between these possibilities.

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