THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 24, Issue of June 15, pp. 21907–21915, 2001
Printed in U.S.A.

Caspase-2-induced Apoptosis Is Dependent on Caspase-9, but Its Processing during UV- or Tumor Necrosis Factor-dependent Cell Death Requires Caspase-3*

Received for publication, December 21, 2000, and in revised form, March 14, 2001
Published, JBC Papers in Press, March 16, 2001, DOI 10.1074/jbc.M011565200

Gabriela Paroni‡§, Clare Henderson‡, Claudio Schneider‡¶, and Claudio Brancolini‡‖

From the ‡Dipartimento di Scienze e Tecnologie Biomediche, Sezione di Biologia, Universita' di Udine, P. le Kolbe 4, Udine 33100, Italy and ¶Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie AREA Science Park, Padriciano 99, Trieste 34142, Italy

Mammalian caspases are a family of cysteine proteases that plays a critical role in apoptosis. We have analyzed caspase-2 processing in human cell lines containing defined mutations in caspase-3 and caspase-9. Here we demonstrate that caspase-2 processing, during cell death induced by UV irradiation, depends both on caspase-9 and caspase-3 activity, while, during TNF-α-dependent apoptosis, capase-2 processing is independent of caspase-9 but still requires caspase-3. *In vitro* procaspase-2 is the preferred caspase cleaved by caspase-3, while caspase-7 cleaves procaspase-2 with reduced efficiency. We have also demonstrated that caspase-2-mediated apoptosis requires caspase-9 and that cells co-expressing caspase-2 and a dominant negative form of caspase-9 are impaired in activating a normal apoptotic response and release cytochrome *c* into the cytoplasm. Our findings suggest a role played by caspase-2 as a regulator of the mitochondrial integrity and open questions on the mechanisms responsible for its activation during cell death.

Caspases belong to a conserved family of cysteine proteases playing a critical role in apoptosis and proinflammatory cytokine maturation (1). In normal cells, caspases are present as zymogens that are cleaved at aspartic sites during cell death. Enzyme processing generates the active form, which is constituted by a heterodimer consisting of two small and a two large subunits (2).

Caspases involved in the apoptotic process can be divided into initiator caspases and effector caspases based on the presence of a large prodomain at their amino terminus region. In general, effector caspases possess short prodomains and are cleaved by initiator caspases, which possess large prodomains. Effector caspases are involved in the cleavage of the death substrates, thus modulating the morphological changes characterizing the apoptotic process (3).

The long prodomains of the initiator caspases function in inducing dimerization and activation of the proenzymes through interaction with specific adaptor molecules. Caspase-2, caspase-8, caspase-9, and caspase-10 are the long prodomain caspases involved in the apoptotic process (4).

The generation of caspase-deficient mice for initiator caspases has indicated that these enzymes regulate cell death in a tissue- and stimulus-specific fashion (4). In particular, caspase-9 is the critical player of the apoptotic stimuli acting through mitochondrial dysfunction (5, 6), while caspase-8 is critical for the apoptotic pathways generated by death receptors (7).

Caspase-2-deficient mice showed an apoptotic deficit in the oocytes, following exposure to chemotherapeutic drugs, and in B lymphoblasts following incubation with perforin and granzyme B (8). Additional studies have shown that this initiator caspase is essential for specific apoptotic pathways such as β-amyloid-induced neuron death and salmonella-induced macrophage death (9, 10).

The identification of RAIDD/CRADD as an adaptor protein for caspase-2 has suggested that caspase-2 could be involved also in apoptosis triggered by TNF-α. Recruitment of caspase-2 to the TNF-α receptor is regulated by the interaction between the CARD domain present in the prodomain of caspase-2 and a similar CARD domain present in RAIDD/CRADD (11, 12).

Different apoptotic insults can cause caspase-2 processing, which generally occurs by two proteolytic steps. A first cleavage at aspartic 316 generates two fragments: one of 32–33 kDa containing the prodomain and the large subunit and a second fragment of 14 kDa containing the small subunit. Subsequent cleavages at Asp^{152} and Asp^{330} lead to the formation of the large and the small subunits of 18 and 12 kDa, respectively. The appearance of the 32–33-kDa fragment has been generally used as marker of caspase-2 activation (13–15).

Despite significant evidence for an involvement of caspase-2 in different apoptotic pathways, its specific role is not completely understood. Only recently, a caspase-2 substrate has been identified in golgin-160, thus suggesting that caspase-2 could act as an executioner caspase involved in modulating Golgi integrity (16).

In the present study, we have attempted to further analyze caspase-2 activity and its hierarchy with respect to the common apoptotic pathways. We have used human cell lines containing defined mutations in caspase-9 and caspase-3 to unveil the relationships between the mitochondrial pathway and caspase-2 proteolytic processing in response to different apoptotic triggers. In addition, we have analyzed the ability of caspase-2 to induce cell death in cells defective in caspase-9 activity.

EXPERIMENTAL PROCEDURES

Antibody Production—Rabbits were immunized with His-tagged caspase-2 fragment (residues 15–436) purified from *Escherichia coli* transformed with the construct pQE32-caspase-2. Briefly, after induc-

* This work was supported by a grant from the Ministero della Sanità (to C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a fellowship from the Fondazione Italiana per la Ricerca sul Cancro.

‖ To whom correspondence should be addressed. Tel.: 0432-494382; Fax: 0432-494301; E-mail: cbrancolini@makek.dstb.uniud.it

tion with isopropyl-1-thio-β-D-galactopyranoside, bacteria where collected by centrifugation at 3500 rpm for 5 min and lysed in 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme, 0.5 mM phenylmethylsulfonyl fluoride. After sonication, the insoluble fraction was resuspended in sample buffer (2% SDS, 10% glycerol, 120 mM Tris-HCl, pH 6.8, 0.005% bromphenol blue, 1% β-mercaptoethanol) and then run on an SDS-10% polyacrylamide gel. The gel was stained with Coomassie Blue R-250 to identify the caspase-2 band, which was then electroeluted. The protein was dialyzed overnight in PBS and purified by nickel chromatography using a His-trap column (Qiagen), eluted with an imidazole buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 250 mM imidazole), and then used to immunize rabbits.

Purified recombinant caspase-2 was cross-linked to an Affi-Prep 10 column (Bio-Rad) and used to affinity-purify antibody to caspase-2 from rabbit antiserum. The serum was loaded onto the column at a slow flow rate, washed with 10 mM Tris-HCl, pH 7.4, and then washed with a high salt buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.4). Caspase-2 antibodies were eluted using 10 mM glycine, pH 2.5, and neutralized with pH 7.5 with Tris-HCl.

Plasmids—To generate $pcDNA_3$HA caspase-2 C303G, the entire coding region of human caspase-2 was amplified from $pGDSV7_S$ caspase-2 by polymerase chain reaction.

In vitro mutagenesis to substitute Cys^{303} with Gly was performed as previously described (17). The following set of primers was used: primer A (CATGAATTCATGGCCGCTGACAGGGGACGC), primer B (ATCCAGGCCGGCCGTGGAGAT), primer C (ATCTCCACGGCCGGCCTGGAT), and primer D (CATCTCGAGTCATGTGGGAGGGTGTCCTGG).

Caspase-2 C303G cDNA was subcloned in pcDNA3HA as *Eco*RI/*Eco*RI and *Eco*RI/*Xho*I fragments.

Caspase-2 C303G was subcloned from $pcDNA_3$HA caspase-2 C303G as *Sph*I/*Xho*I fragment in pQE32 to generate a His-tagged caspase-2 construct.

To generate pEGFPN1Bid and pBSKBid, the entire coding region of human Bid was amplified by polymerase chain reaction from a human fetal cDNA library. The following set of primers was used: primer E (CATGAATTCTGATGGACTGTGAGGTCAACAAC) and primer F (CATGGATCCCGGTCCATCCCATTTCTGGCTAA).

Cell Lines and Induction of Apoptosis—Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Cells at 70–80% confluence were treated for 36 h with 1 μg/ml cycloheximide plus 20 ng/ml TNF-α or 10 ng/ml TNF-α (MCF-7pBC3 and MCF-7pBC3mut) or with 1 μg/ml cycloheximide (Sigma) alone as a control.

For UV treatment, culture medium was removed, dishes were washed once with PBS[1] and UVC (180 J/m^2)-irradiated, and fresh medium, containing 10% fetal calf serum, was added to the cells (17). Cells were harvested by scraping with a rubber policeman 15 or 18 h later. When required floating cells were collected separately from the adherent cells. After washes in PBS, cells were resuspended in SDS sample buffer sonicated boiled for 3 min and analyzed by Western blot.

Transfections were performed by the calcium phosphate precipitation method. Cell were seeded 24 h before transfection and analyzed 20 h after removal of the precipitates. Cells were transfected with 2 μg of each expression vector together with 200 ng of pEGFPN1 (Invitrogen) to identify transfected cells.

In Vitro Proteolytic Assay—Caspase-3 was expressed in bacteria and purified as previously described (18) using the pQE-12 expression system (Qiagen). Density scanning of the ~20-kDa fragments of the autoprocessed caspases, as evidenced after electrophoretic separation and Coomassie Blue staining, was used to estimate the amount of active enzyme. Purified caspase-7 was obtained from Alexis, and caspase-2 was from Chemicom.

The different caspases, poly(ADP-ribose) polymerase (PARP) and Bid, were *in vitro* translated with ^{35}S using the TNT-coupled reticulocyte lysate system (Promega). 1 μl of each *in vitro* translated protein was incubated with increasing amounts of caspase-3 or caspase-7 in 15 μl of the appropriate buffer (final volume) for 1 h at 37 °C. Reactions were terminated by adding one volume of SDS gel loading buffer and boiling for 3 min.

Immunoblotting—For Western blotting, proteins were transferred to 0.2-μm pore-sized nitrocellulose (Schleicher & Schuell) using a semidry blotting apparatus (Amersham Pharmacia Biotech) (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 1 h in Blotto-Tween 20 (17) (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% nonfat dry milk, and 0.1% Tween 20) and incubated overnight at room temperature with the specific antibody: anti-caspase-2, anti-actin, and anti-p85 PARP fragment (Promega). Blots were then rinsed three times with Blotto-Tween 20 and incubated with peroxidase-conjugated goat anti-rabbit (Sigma) or goat anti-mouse (Sigma) for 1 h at room temperature. The blots were then washed four times in Blotto-Tween 20, rinsed in phosphate buffer saline, and developed with Super Signal West Pico, as recommended by the vendor (Pierce).

Indirect Immunofluorescence Assay—For indirect immunofluorescence assays, transfected cells were fixed with 3% paraformaldehyde in PBS for 1 h at room temperature. Fixed cells were washed with PBS and 0.1 M glycine, pH 7.5, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. The coverslips were treated with anti-cytochrome *c* (Promega) or with anti-cytochrome *c* oxidase, diluted in PBS for 1 h in a moist chamber at 37 °C. They were then washed with PBS twice, followed by incubation with tetramethylrhodamine isothiocyanate-conjugated anti-mouse (Sigma) for 30 min at 37 °C. Nuclei were evidenced by Hoechst staining.

Cells were examined by epifluorescence with a Zeiss Axiovert 35 microscope or with a LEICA TCS laser scan microscope equipped with a 488 λ argon laser and a 543 λ helium neon laser.

RESULTS

Production of a Caspase-2-specific Antibody—We produced an antibody against *E. coli* expressed caspase-2 fragment in order to study caspase-2 activation during cell death. The antiserum was purified on a caspase-2 affinity column and tested for caspase-2 detection by Western blot. 293 cells were transfected with HA-tagged catalytic inactive caspase-2 as positive control. Anti-caspase-2 antibody recognizes a single band migrating at around 48 kDa, showing a similar electrophoretic mobility to the band detected with the anti-HA antibody in caspase-2-transfected cells. The intensity of the band detected by the anti-caspase-2 antibody was dramatically increased in caspase-2-transfected cells (Fig. 1*a*).

Caspase-2 processing during apoptosis can be followed by the appearance of different proteolytic fragments. A first cleavage at aspartic 316 generates two fragments: a first fragment of 32–33 kDa containing the prodomain and the large subunit and a second fragment of 14 kDa containing the small subunit. Subsequent cleavages at Asp^{152} and Asp^{330} generate the large and the small subunits of 18 and 12 kDa, respectively (13). To confirm the specificity of the anti-caspase-2 antibody, we investigated caspase-2 processing by Western blot during apoptosis.

MDA cells were UV-irradiated, and cellular lysates were prepared 24 h later. In UV-irradiated cells, a band migrating at ~33 kDa was detected, the appearance of which parallels the rate of cell death in the culture as confirmed by β-catenin processing (19). This band is similar in size to the previously identified intermediate processed form of caspase-2, p33. The small and large subunits of caspase-2 were detected only after long exposure of the blot (Fig. 1*b*). In conclusion, this evidence indicates that the produced antibody specifically recognizes caspase-2 both in normal and apoptotic cells.

We next investigated the appearance of the 33-kDa form of caspase-2 in different cell lines when apoptosis was induced by UV irradiation. As shown in Fig. 1*c*, the processed form of caspase-2 was detected upon apoptosis induction in most of the cell lines tested, with the exception of the MCF-7 cells. The anti-caspase-2 antibody also showed cross-species reactivity, since it detected a band of 50 kDa in the cellular lysates of murine fibroblasts NIH 3T3 that is converted in a p33 form upon induction of apoptosis (data not shown).

IMR90-E1A Containing a Dominant Negative Form of Caspase-9 and MCF-7 Containing a Catalytically Inactive Caspase-3 Show a Defective Apoptotic Response following UV

[1] The abbreviations used are: PBS, phosphate-buffered saline; CHX, cycloheximide; CI, catalytically inactive; DN, dominant negative; PARP, poly(ADP-ribose) polymerase; PLAP, placental alkaline phosphatase; TNF, tumor necrosis factor; FACS, fluorescence-activated cell sorting.

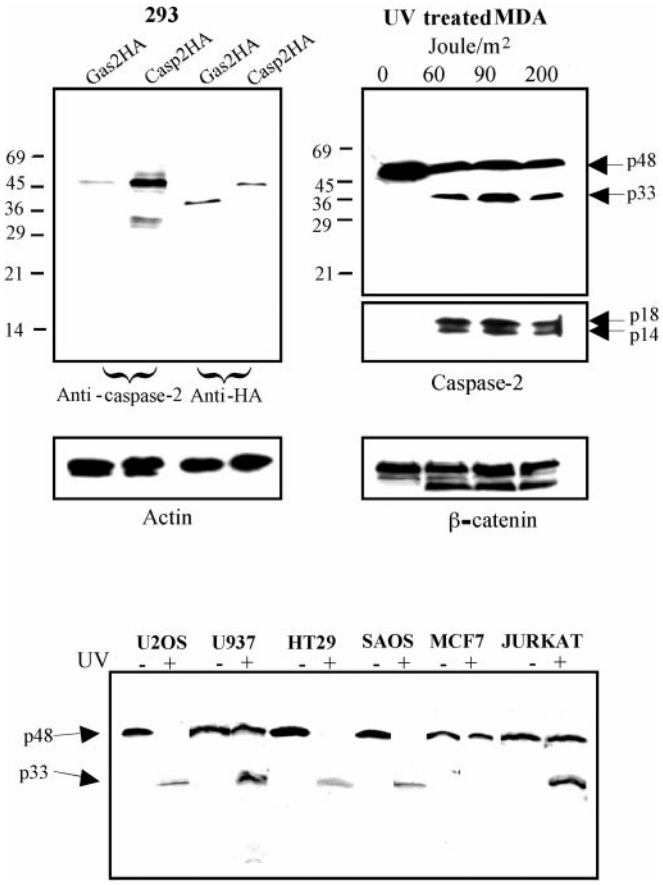

FIG. 1. **Characterization of anti-caspase-2 antibody.** *a*, equal amounts of 293 total cell lysates transfected with pcDNA3HAGas-2 or pCDNA3HA caspase-2 C303G were subjected to 15% SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed using anti-caspase-2, anti-HA, or anti-actin antibodies. *b*, 293 cells were UV-irradiated as indicated. After 24 h, lysates from both adherent viable cells and nonadherent apoptotic cells were combined and were Western blotted with anti-caspase-2 antibody. *c*, different cell lines were UV-irradiated (120 J/m^2) or untreated. 20 h later, untreated (−) and apoptotic cells (+) were harvested. Western analysis was performed using anti-caspase-2 antibody.

Irradiation—To study the hierarchy of caspase-2 processing in respect to different caspases during apoptosis *in vivo*, we decided to use cell lines containing defined mutations in specific caspases.

We took advantage of the IMR90 cells transformed with E1A oncogene and containing a dominant negative form of caspase-9 (caspase-9 DN) (20). As a control IMR90 fibroblasts expressing E1A alone were used. It has been previously demonstrated that IMR90-E1A cells expressing the caspase-9 DN do not show all of the "classical" apoptotic features when challenged by etoposide treatment, whereas in these cells cytochrome *c* release from mitochondria was reported as normal (20).

We also used the human MCF-7 breast carcinoma cell line, which is devoid of caspase-3 due to the functional deletion of the *CASP-3* gene (21). This cell line was used to reintroduce wild type caspase-3 or its catalytic inactive point-mutated derivative caspase-3 (CI) as a control (22).

We used FACS analysis to confirm that the different cell lines selected showed different susceptibility to enter cell death by apoptosis following UV irradiation.

UV-irradiated IMR90-E1A cells underwent apoptosis as evidenced by cell morphology, chromatin condensation, and detachment from the adhesion substrate. Levels of apoptosis were assessed by FACS analysis using propidium iodide staining (Fig. 2*a*). Analysis of DNA content (Fig. 2) revealed a significant increase of cells with sub-G_1 DNA content, indicative of apoptosis (19), when IMR90-E1A cells were UV-irradiated. On the contrary, IMR90-E1A cells containing caspase-9 DN were impaired in entering efficiently apoptosis following UV irradiation, as evidenced by FACS analysis. Indeed, in these cell lines, floating cell fragments could be detected upon UV irradiation (data not shown).

Reintroduction of wild type caspase-3 in MCF-7 cells renders these cells susceptible to apoptosis following UV irradiation, whereas cells expressing a catalytic inactive form of caspase-3 were partially resistant to UV-dependent cell death (Fig. 2*b*). Moreover, when MCF-7 caspase-3 CI cells were UV-irradiated, some cell debris was detected floating in the medium (data not shown).

Caspase-2 Processing in Human Fibroblasts Expressing a Catalytic Inactive Form of Caspase-9—Having selected two human cell lines containing defined mutations in caspase-3 and caspase-9, we analyzed the relationships between caspase-2 processing and the above mentioned caspases by inducing cell death with two different stimuli: UV irradiation and TNF-α.

As mentioned before, some dead cells floating in the medium were observed also in the case of UV-irradiated IMR90-E1A-caspase-9 DN that were resistant to apoptosis as judged by FACS analysis. These dead cells might be indicative of a necrotic or "frustrated apoptotic" response that occurs following extensive DNA damage and/or mitochondrial dysfunction.

In our studies, we have focused our attention on the status of caspase-2 in the population of dead cells that can be isolated as nonadherent cells (Fig. 3, *D*) from the population of still viable cells (Fig. 3, *V*). It is important to note that the population of nonadherent cells was consistently reduced in cells expressing the catalytic inactive caspases (data not shown), but in our Western analysis the total amount of protein lysates loaded for each sample was normalized.

In apoptotic IMR90-E1A cells, almost all caspase-2 was processed to the p33 form, as shown in Fig. 3*a*, while in floating cells from IMR90-E1A-caspase-9 DN (*D*), caspase-2 processing was largely impaired. The same lysates were also analyzed for PARP processing by using an antibody specific for the cleaved form. Processing of PARP was observed in both cell lines although the presence of a dominant negative form of caspase-9 clearly reduced the amount of cleaved PARP detectable. Actin was used as loading control.

We next analyzed the dependence of caspase-2 processing on caspase-9 in response to the activation of a different apoptotic pathway. IMR90-E1A and IMR90-E1A-caspase-9 DN were treated with TNF-α in the presence of cycloheximide, and Western blot analysis was performed using lysates prepared from TNF-α/CHX- and CHX-treated or untreated cells. Nonadherent dead cells (Fig. 3, *D*) and adherent viable cells (*V*) were harvested separately.

In TNF-α-triggered cell death, despite of the presence of a dominant negative form of caspase-9, caspase-2 was normally processed, thus generating the p33 fragment (Fig. 3*b*). PARP processing during apoptosis induced by TNF-α was independent from caspase-9 activity. Here again, detection of actin was used as a loading control.

Caspase-2 Processing during Apoptosis in MCF-7 Cells—The previous experiment clearly shows that caspase-2 processing following UV irradiation is dependent on caspase-9. Since caspase-9 activates caspase-3 in response to DNA damage (20), we next analyzed if caspase-3 is critical for caspase-2 processing *in vivo*.

Human MCF-7 cells were UV-irradiated, and 18 h later nonadherent dead cells (Fig. 3, *D*) and adherent viable (*V*) cells

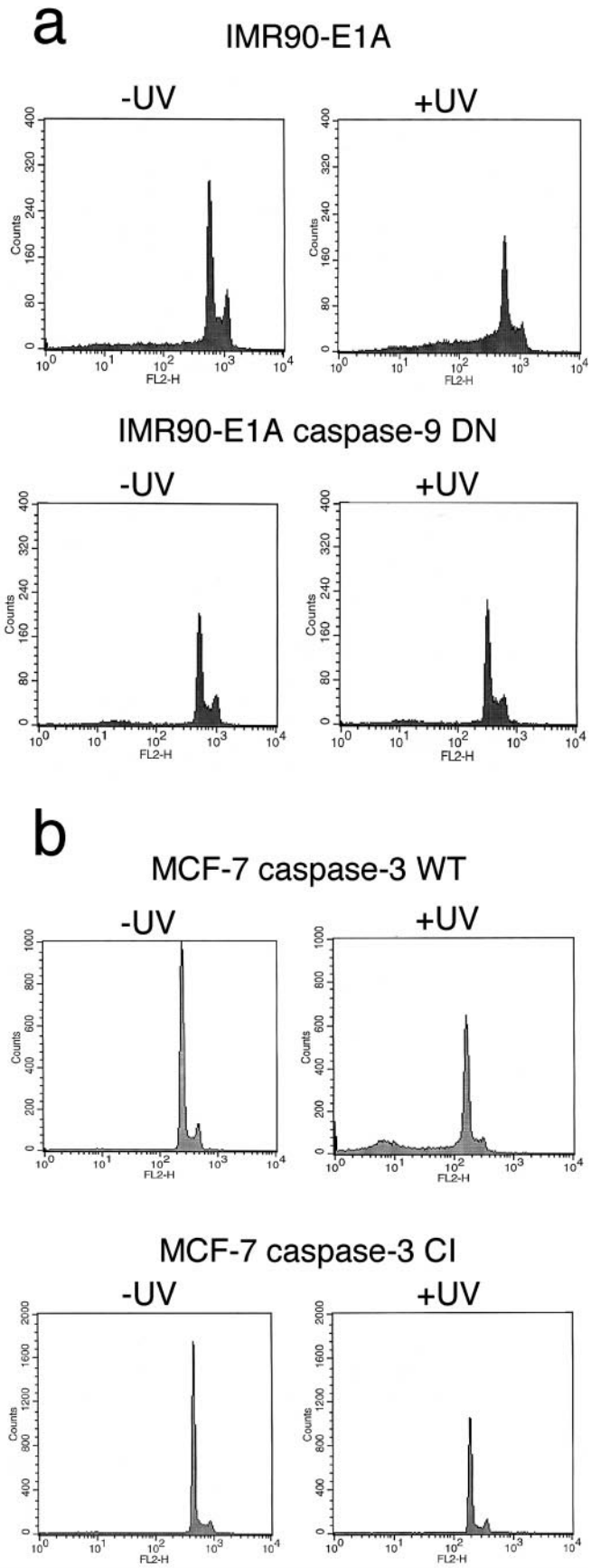

FIG. 2. **Cell cycle analysis after UV treatment.** Flow cytometric analysis of apoptosis after propidium iodide staining. IMR90-E1A, IMR90-E1A caspase-9 DN, MCF-7 caspase-3, and MCF-7 caspase-3 CI were UV-irradiated (180–200 J/m^2) and 15–18 h later processed for the analysis.

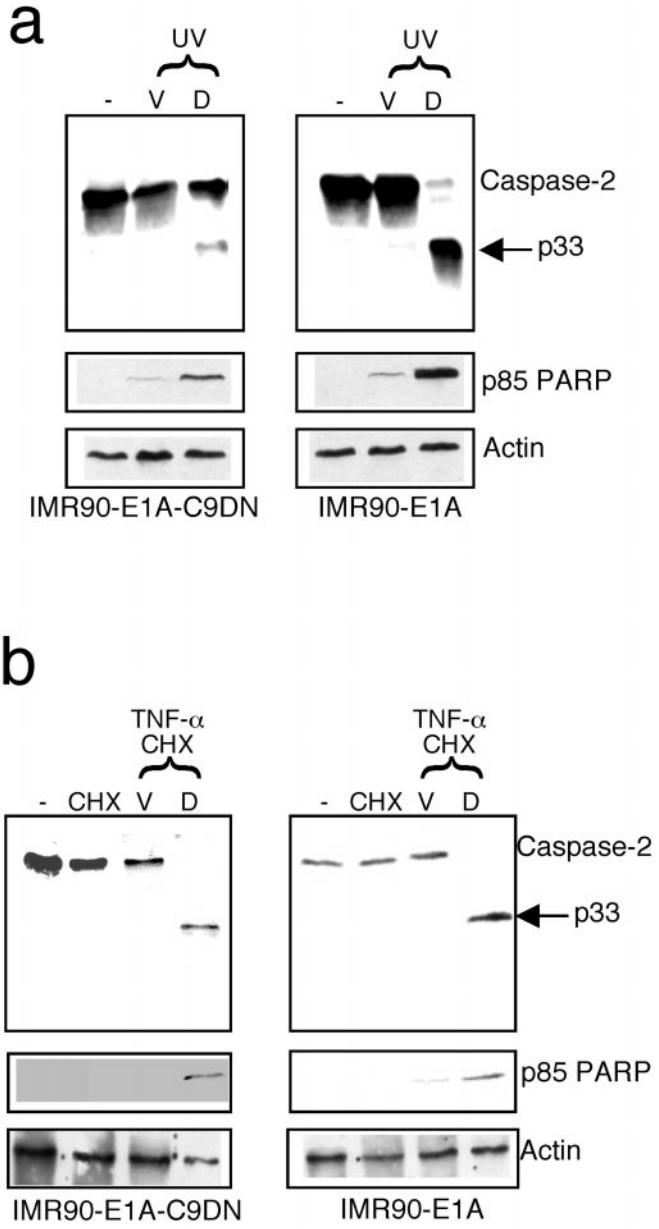

FIG. 3. **Caspase-2 processing in human fibroblasts expressing a catalytic inactive form of caspase-9.** *a*, IMR90-E1A and IMR90-E1A caspase-9 DN were UV-irradiated or untreated. After 15 h, lysates from both adherent viable cells (*V*) and nonadherent dead cells (*D*) were harvested separately. Western analysis was performed using anti-caspase-2, anti-PARP, or anti-actin antibody as described. *b*, IMR90-E1A and IMR90-E1A caspase-9 DN were treated with TNF-α (20 ng/ml) and cycloheximide (1 μg/ml), treated with cycloheximide (1 μg/ml) alone, or left untreated. 36 h later, adherent and nonadherent treated cells were collected separately, while in untreated cells nonadherent apoptotic cells were almost undetectable. Western analysis was performed using anti-caspase-2, anti-PARP, or anti-actin antibody as a loading control.

were harvested and combined, and lysates were prepared for Western analysis.

As shown in Fig. 4*a*, in UV-irradiated MCF-7 wild type caspase-3, a consistent fraction of caspase-2 could be detected as the p33-processed form, while in MCF-7 caspase-3 CI cells, caspase-2 processing was undetectable. Processing of PARP was reduced but still detectable in MCF-7 caspase-3 CI as described above in IMR90-E1A caspase-9 DN.

We next analyzed the dependence of caspase-2 processing on caspase-3 under TNF-α-induced apoptosis. MCF-7 wild type caspase-3 and caspase-3 CI cells were treated with TNF-α in

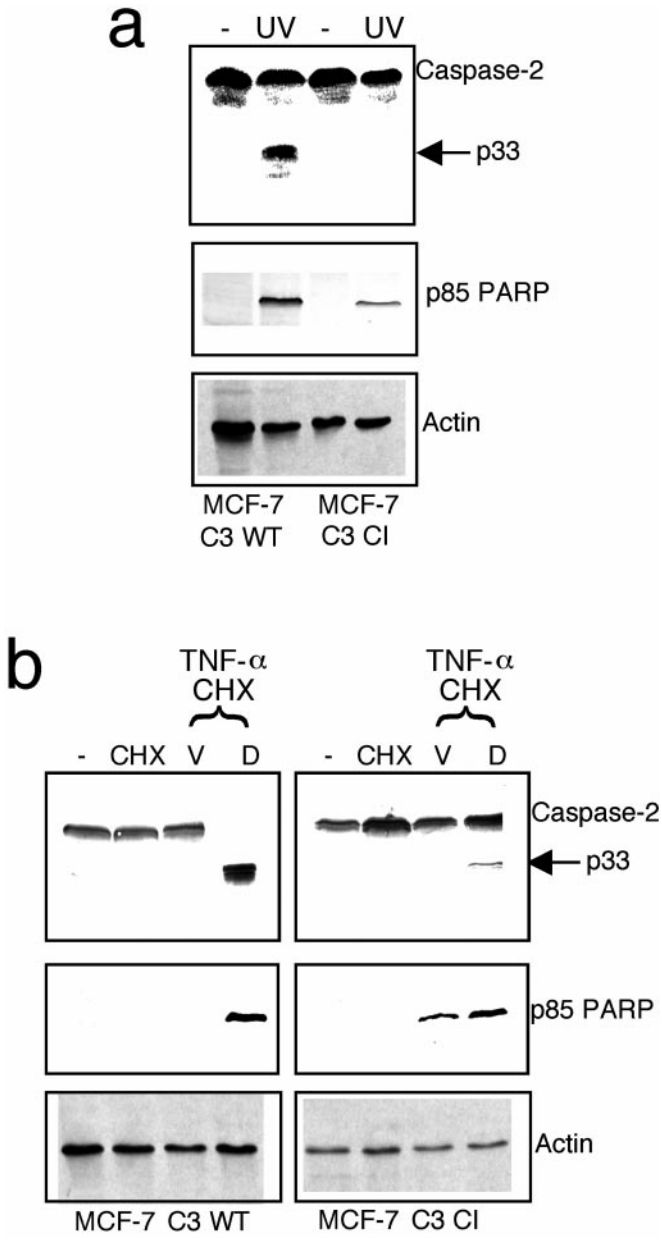

FIG. 4. **Caspase-2 processing during apoptosis in MCF-7 cells.** *a*, MCF-7 caspase-3 and MCF-7 caspase-3 CI were UV-irradiated or untreated. 18 h later, lysates from both adherent viable cells and nonadherent apoptotic cells were combined. Western analysis was performed using anti-caspase-2, anti-PARP, or anti-actin antibody as a loading control. *b*, MCF-7 caspase-3 and mutated MCF-7 caspase-3 were treated with TNF-α (10 ng/ml) and cycloheximide (1 μg/ml), treated with cycloheximide (1 μg/ml) alone, or left untreated. 36 h later, adherent and nonadherent treated cells were collected separately, while in untreated cells nonadherent apoptotic cells were almost undetectable. Western analysis was performed using anti-caspase-2, anti-PARP, or anti-actin antibody as a loading control.

the presence of cycloheximide for 36 h, and Western blot analysis was performed. Nonadherent dead (Fig. 4, *D*) and adherent viable (*V*) cells were harvested separately.

Caspase-2 was fully processed when apoptosis was induced in wild type caspase-3 MCF-7 cells treated with TNF-α and CHX. In apoptotic MCF-7 caspase-3 CI cells, caspase-2 processing was largely impaired.

PARP was similarly processed in both cell lines, thus confirming the induction of cell death.

In Vitro Caspase-3-mediated Processing of Caspase-1, -2, -3, -6, -7, -8, and -9—Having demonstrated that caspase-3 is the

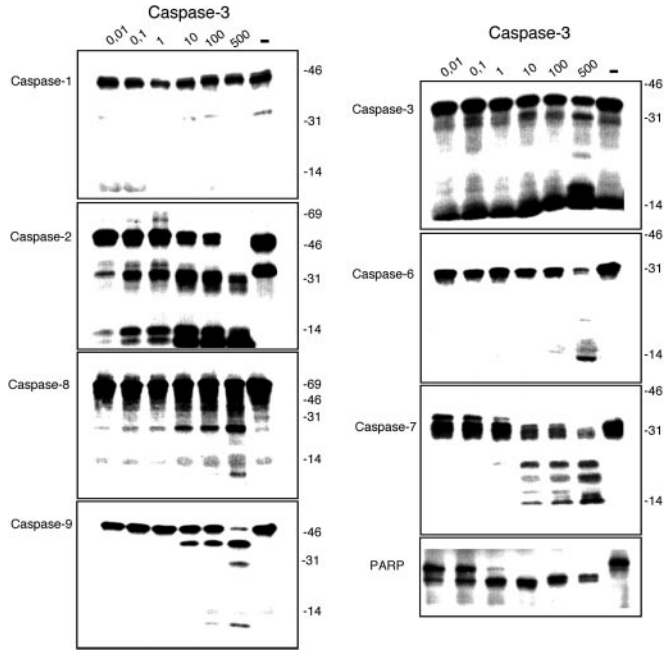

FIG. 5. **Caspase-2 is the preferred caspase cleaved by caspase-3 *in vitro*.** [^{35}S]Methionine-labeled *in vitro* translated products of the indicated caspases and PARP were incubated for 1 h at 37 °C with increasing amounts of recombinant caspase-3 or with caspase-3 buffer alone.

critical enzyme for an efficient processing of caspase-2 *in vivo*, we wanted to analyze the ability of caspase-3 to cleave caspase-2 *in vitro* with respect to other long and short prodomain caspases involved in the apoptotic response.

In vitro proteolytic assays using recombinant caspases-3 were performed. Full-length caspase-1, caspase-2, caspase-3, caspase-6, caspase-7 caspase-8, and caspase-9 cDNA were i*n vitro* translated and then incubated with purified caspase-3 (Fig. 5).

Treatment with an increasing amount of purified caspase-3 for 30 min at 37 °C leads to the processing of all of the tested caspases with the exception of caspase-1. Among the different long prodomain caspases, some processing of caspase-2 to generate the p18/p12 active form was detectable after incubation with only 0.01 ng of caspase-3, while the full processing of the p48 form was detectable when 500 ng of caspase-3 were used. In contrast, caspase-9 and caspase-8 were weak substrates of caspase-3 and only partially processed after incubation with 10 ng of the recombinant caspase-3. Among the different short prodomain caspases analyzed, caspase-7 was the most efficiently cleaved by caspase-3, and partial processing was detectable after incubation with 10 ng of recombinant caspase-3.

PARP, a well defined substrate of caspase-3 (4, 23), was used as a control under the same conditions. From this analysis, we can suggest that caspase-2, among the different caspases analyzed, is the best substrate for caspase-3.

In Vitro Processing of Caspase-2 Can Be Mediated by Caspase-7—Residual caspase-2 processing was observed when apoptosis was induced by TNF-α in MCF-7 cells defective for caspase-3 activity. Since caspase-3 and caspase-7 show overlapping cleavage consensus sequences and share many death substrates *in vivo* (4), we asked whether caspase-7 was able to cleave caspase-2 in an *in vitro* assay.

In vitro translated caspase-2 was incubated with increasing amounts of purified caspase-7 for 60 min at 37 °C. As shown in Fig. 6*a*, 1 μg of caspase-7 was required for the full processing of caspase-2, while partial caspase-2 processing was observed after incubation with 10 ng of caspase-7. Under the same

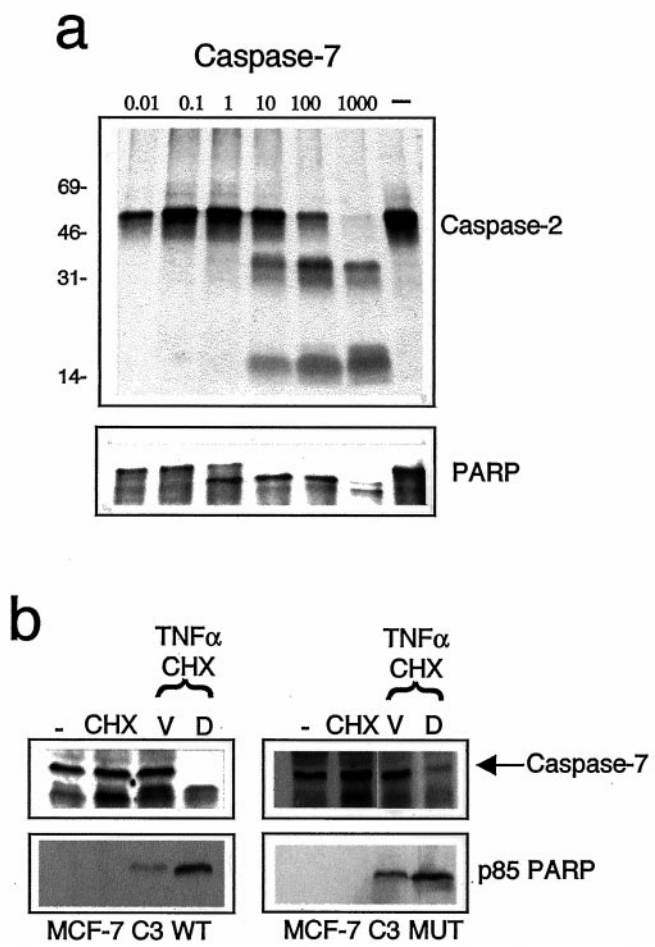

FIG. 6. **Processing of caspase-2 can be mediated by caspase-7.** *a*, [^{35}S]methionine-labeled *in vitro* translated caspase-2 and PARP were incubated for 1 h at 37 °C with increasing amounts of recombinant caspase-7 or with caspase-7 buffer alone. *b*, MCF-7 caspase-3 and MCF-7 caspase-3 CI were treated with TNF-α (10 ng/ml) and cycloheximide (1 μg/ml), treated with cycloheximide (1 μg/ml) alone, or left untreated. 36 h later, adherent and nonadherent treated cells were collected separately, while in untreated cells nonadherent apoptotic cells were almost undetectable. Western analysis was performed using anti-caspase-7 antibodies.

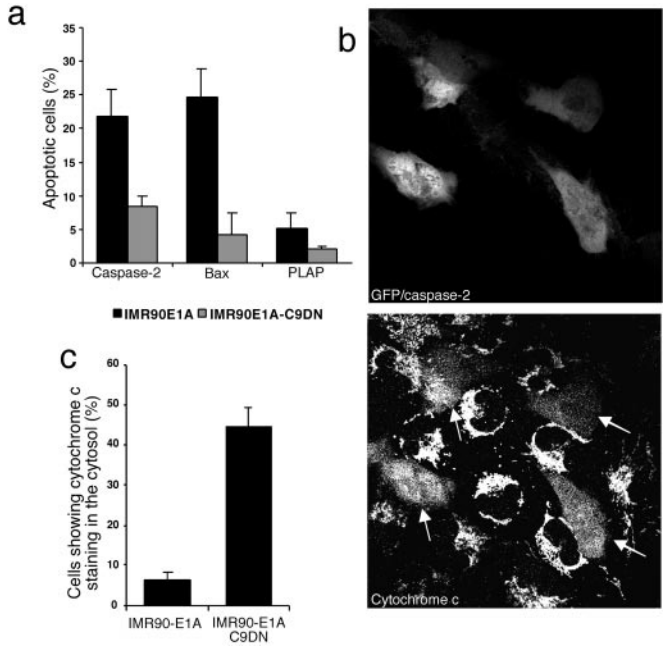

FIG. 7. **Apoptosis induced by caspase-2 triggers cytochrome *c* release and requires caspase-9 activity.** a, in IMR90-E1A caspase-9 DN and IMR90-E1A cells, pGDSV7S-caspase-2, pGDSV7S-bax, or pGDSV7S-hPLAP was co-transfected with pEGFPN1 as a reporter. The appearance of apoptotic cells was scored after 20 h from transfection. *b*, IMR90-E1A caspase-9 DN cells were co-transfected with pGDSV7S-caspase-2 and pEGFPN1 as a reporter. After 20 h, an immunofluorescence assay was performed using anti-cytochrome *c* antibody. *c*, IMR90-E1A caspase-9 DN and IMR90-E1A cells co-expressing caspase-2 and GFP were scored for cytochrome *c* release from mitochondria.

experimental conditions, full processing of PARP was observed after incubation with 10 ng of purified caspase-7. This analysis suggests that caspase-7 can cleave caspase-2 *in vitro,* although with a lower efficiency when compared with PARP.

It is possible that caspase-7 is responsible for the limited proteolytic processing of caspase-2 during apoptosis in MCF-7 caspase-3 CI cells (25). We therefore analyzed if caspase-7 was activated when these cells were treated with TNF-α/CHX.

We have analyzed by Western blot caspase-7 expression, as shown in Fig. 6*b*, with an antibody that recognizes the caspase-7 proenzyme migrating at around 34 kDa. Disappearance of the 34-kDa band was considered as evidence for caspase-7 processing. Nonadherent apoptotic and adherent nonapoptotic cells were harvested separately.

Caspase-7 was processed in MCF-7 wild type caspase-3 cells during apoptosis, and its processing was reduced but still detectable in apoptotic MCF-7 containing catalytic inactive caspase-3.

Apoptosis Induced by Caspase-2 Overexpression Triggers Cytochrome c Release and Requires Caspase-9 Activity—The dependence of caspase-2 processing on caspase-3 argues that caspase-2 acts as an effector caspase and that its activation is a downstream event in the proteolytic cascade triggering cell death.

On the other hand, caspase-2 possesses a prodomain, which is a marker for caspases acting at the apex of the proteolytic cascade, and its overexpression can trigger cell death by apoptosis, possibly as a consequence of caspase-2 activation following prodomain-mediated oligomerization (14, 15, 26, 27).

If caspase-2 induces apoptosis simply by acting as an effector caspase, its ability to induce cell death should be independent from mutations in the regulative caspases such as caspase-9.

Therefore, we have investigated the ability of caspase-2 to efficiently induce cell death when overexpressed in cells impaired in caspase-9 activity.

IMR90-E1A caspase-9 DN and IMR90-E1A were co-transfected with caspase-2 and GFP used as a reporter, and the appearance of apoptotic cells was scored 24 h after transfection.

As shown in Fig. 7*a*, the apoptotic response triggered by caspase-2 overexpression in IMR90-E1A caspase-9 DN was impaired, thus demonstrating the dependence on caspase-9. Under the same conditions, PLAP (placental alkaline phosphatase), used as a control, was unable to induce cell death.

The dependence of caspase-2 from caspase-9 to efficiently induce apoptosis was similarly observed in the case of Bax, which is a well known modulator of the mitochondrial integrity (28).

Caspase-9 is part of the apoptosome that triggers cell death in response to mitochondrial stress and subsequent release of mitochondrial components such as cytochrome *c* (29–32).

Caspase-2 in turn requires caspase-9 to mediate apoptosis, therefore suggesting that caspase-2 acts upstream of the mitochondrial pathway. Therefore, we next analyzed by immunofluorescence whether overexpression of caspase-2 leads to cytochrome *c* release from mitochondria.

As exemplified in Fig. 7*b*, IMR90-E1A caspase-9 DN cells overexpressing caspase-2 showed relocalization of cytochrome

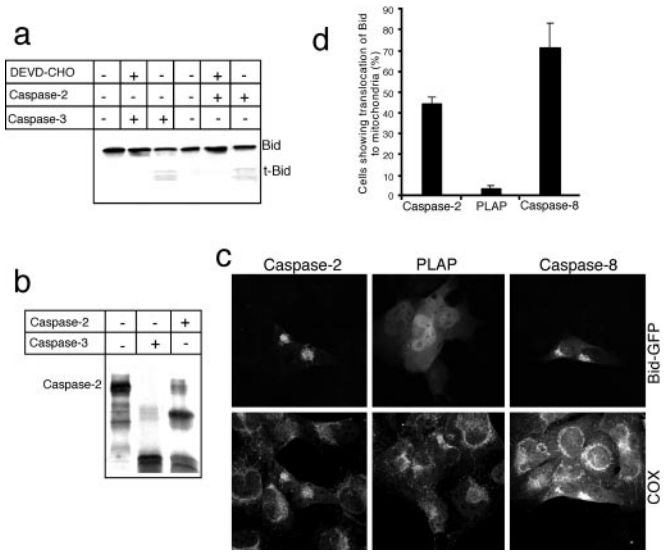

FIG. 8. **Caspase-2 overexpression triggers the translocation of Bid to mitochondria.** *a*, [^{35}S]methionine-labeled *in vitro* translated Bid was incubated for 1 h at 37 °C with recombinant caspase-2 or caspase-3. *N*-Acetyl-Asp-Glu-Val-Asp-aldehyde was used at a concentration of 1 μM. *b*, [^{35}S]methionine-labeled *in vitro* translated caspase-2 was incubated for 1 h at 37 °C with recombinant caspase-2, caspase-3, or buffer alone. *c*, IMR90-E1A caspase-9 DN cells were co-transfected with pGDSV7S-caspase-2 and pEGFPN1-Bid, with pGDSV7S-PLAP and pEGFP-Bid, or with pGDSV7S-caspase-8 and pEGFPN1-Bid. After 20 h, immunofluorescence assays were performed using anti-cytochrome *c* oxidase (*COX*) antibody. *d*, IMR90-E1A caspase-9 DN cells co-expressing caspase-2 and Bid-GFP, PLAP and Bid-GFP, or caspase-8 and Bid-GFP were scored for translocation of Bid-GFP to mitochondria.

c, as a diffused staining in the cytoplasm (see *arrows*). In nonoverexpressing cells, cytochrome *c* showed a filamentous, perinuclear distribution, an indication for mitochondrial localization. Fig. 7*c* shows a quantitative analysis of cytochrome *c* localization in IMR90-E1A and IMR90-E1A caspase-9 DN cells overexpressing caspase-2. In almost 50% of IMR90-E1A caspase-9 DN cells overexpressing caspase-2, a diffuse cytosolic staining for cytochrome *c* can be observed.

Caspase-2 Overexpression Triggers the Translocation of Bid to Mitochondria—Bid is a proapoptotic member of the Bcl-2 family of proteins that contains only a BH3 domain (33). Bid is a substrate of caspase-8 that, once processed, translocates to mitochondria and potently induces cytochrome *c* release (34, 35). We therefore investigated if Bid was translocated to mitochondria in IMR90-E1A-caspase-9 DN cells, overexpressing caspase-2.

Bid is specifically cleaved at Asp^{59} by caspase-8 in the Fas apoptotic signaling pathway (34, 35). *In vitro*, caspase-2 and caspase-3 can partially cleave Bid at the same aspartic residue (34). As shown in Fig. 8*a*, we confirmed that both caspase-2 and caspase-3 could cleave Bid. However, it is important to note that the same amount of recombinant caspase-2 and caspase-3 that was able to cleave Bid only partially was sufficient to efficiently process caspase-2 (Fig. 8*b*).

Recently, a Bid bearing a C-terminal GFP tag has been used to monitor its subcellular localization during apoptosis. Bid processing and its subsequent myristoylation at glycine 60 promote its targeting to mitochondria (36).

We have generated a similar GFP-fused Bid to analyze its subcellular localization in IMR90-E1A-caspase-9 DN cells overexpressing caspase-2. Caspase-8 and PLAP were also overexpressed as a control. A confocal microscopy analysis is shown in Fig. 8*c*. GFP showed a spotted localization, which was coincident with mitochondria, as evidenced by staining with an antibody against cytochrome *c* oxidase (*COX*), in cells co-expressing caspase-2 and Bid-GFP. On the contrary, GFP showed a diffuse nuclear/cytosolic distribution in cells co-expressing Bid-GFP and PLAP. As expected, Bid-GFP was observed co-localizing with mitochondria in caspase-8-overexpressing cells. Fig. 8*d* shows a quantitative analysis of Bid-GFP localization in IMR90-E1A caspase-9 DN cells overexpressing caspase-2, PLAP, and caspase-8. In almost 50% of IMR90-E1A caspase-9 DN cells overexpressing caspase-2, Bid-GFP was translocated to mitochondria.

DISCUSSION

In this report, we have analyzed the dependence of caspase-2 processing on caspase-9 and caspase-3 during apoptosis *in vivo*.

We have used human fibroblasts overexpressing catalytically inactive caspase-9 (20) and MCF-7 cells in which wild type caspase-3 or its catalytic inactive mutant has been reintroduced (22). Apoptosis was induced by stimuli that activate different apoptotic pathways, namely UV irradiation, which provokes DNA damage and mitochondrial dysfunction (30, 31), and activation of the death receptor for TNF-α (37).

By FACS analysis, we noticed that both cells lines were resistant to UV-dependent apoptosis, in agreement with the previous results obtained using MEF from mice knock-out for caspase-9 or ES cells −/− for caspase-3 (6, 38). However, some debris floating in the medium were observed when cells containing catalytic inactive caspase-9 or caspase-3 were challenged with UV. These cell fragments probably represent the result of a "frustrated apoptosis" that is activated in these cell lines following extensive DNA damage and mitochondrial dysfunction. The ability to enter some sort of frustrated apoptosis was also confirmed by the detection of PARP. Aberrant apoptosis and PARP cleavage has been reported also in the case of MEF from caspase-3 −/− (38), thus confirming our evidence. In our cellular systems, it is possible that UV and TNF-α trigger PARP processing through the activation of caspase-7 (25).

To observe caspase-2 activation during apoptosis, we have produced a rabbit antiserum that recognizes the p48-unprocessed form and the p33-cleaved form (13). In the current study, we have followed the appearance of the p33 and the disappearance of the p48 form as signs of caspase-2 processing and activation.

Our data suggest that during apoptosis induced by UV irradiation, efficient processing of caspase-2 is dependent on caspase-9 and caspase-3.

Different results indicate that *in vitro* caspase-3 can cleave caspase-2 (13, 39, 40, 41). Caspase-2 processing can be triggered by caspase-2 itself and by caspase-3 *in vitro*, and it is dependent from Asp residues at position 316 (13). Caspase-2 and caspase-3 share a strong requirement for Asp in P4 and are generally classified in the group II caspases (42). However, some distinctions in terms of recognition of cleaved target sequences are suggested by the requirement of caspase-2 for a P5 residue (43).

Caspase-3-dependent processing of caspase-2 during apoptosis *in vivo* has been suggested by using the inhibitor peptide *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (13). The use of this inhibitor cannot exclude an effect on different caspases such as caspase-2 itself or caspase-7. Our results, obtained by using cell lines containing defined mutations in caspase-9 and caspase-3, unambiguously demonstrate a critical role of caspase-3 in the processing of caspase-2 *in vivo* in human cells.

The *in vitro* proteolytic assay suggests that among the different caspases tested, caspase-2 was the best substrate for caspase-3, thus reinforcing the idea that caspase-3 is the caspase involved in caspase-2 processing during UV. In this respect, a more detailed analysis will be necessary to specifically determine the affinity of caspase-3 for processing the

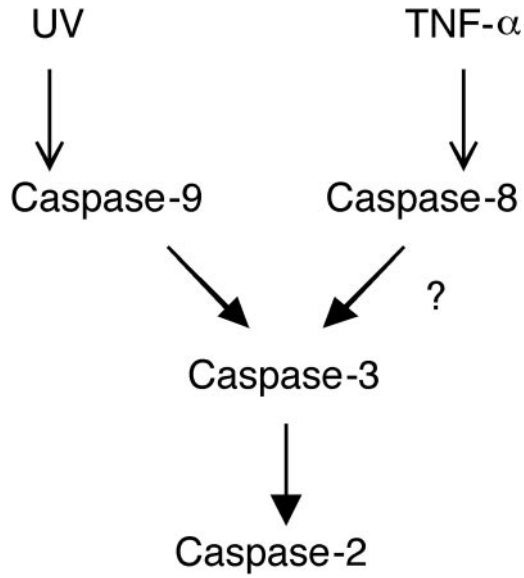

FIG. 9. **A model for caspase-2 processing during UV- or TNF-α-induced apoptosis.**

different caspases. Caspase-2 processing in response to TNF-α was normal in the presence of mutated caspase-9, whereas in cells expressing mutated caspase-3 cleavage it was extremely reduced.

Therefore, caspase-9 seems dispensable for caspase-2 processing in response to TNF-α; on the contrary, caspase-3 is required. A different apoptotic pathway not involving the cytochrome *c* and the "apoptosome," but depending on caspase-8, might be evoked as schematized in Fig. 9.

In vitro and *in vivo* studies suggest that the limited processing of caspase-2 still detectable in TNF-α-treated MCF-7 caspase-3 CI could be dependent on caspase-7. Caspase-7 can process at some extent caspase-2 *in vitro*, and it is activated, although at reduced levels, in TNF-α-treated MCF-7 cells catalytically inactive for caspase-3.

Caspase-2 has been shown to associate through its prodomain with RAIDD/CRADD (death adaptor molecule), involved in recruiting caspase-2 to the TNF receptor-1 signaling complex (11, 12). In principle, RAIDD/CRADD should be responsible for inducing caspase-2 dimerization and activation following TNF-α treatment.

In our cells deprived of functional caspase-3, we were unable to observe efficient processing of caspase-2 when apoptosis was induced by TNF-α treatment, but processing was observed in the same cellular background following reintroduction of a functional caspase-3. There are different explanations for this contradictory result. It is possible that caspase-2 in MCF-7 cells is not involved in TNF-α apoptosis; alternatively, caspase-2 processing is not required for switching on its proteolytic activity following TNF receptor-1 activation, as demonstrated for caspase-9 (44).

In this respect, it will be important to understand if unprocessed caspase-2 in complex or not with CRADD can show some proteolytic activity. In this context, it is important to note that a link has been reported between caspase-2 processing and its apoptotic activity in different experimental models (45).

As summarized in Fig. 9, if caspase-2 processing is dependent on caspase-3 it is possible that caspase-2 acts as an effector caspase and that its activation is a late event during apoptosis. The limited number of caspase-2-specific substrates so far identified counters a role of this enzyme as an effector caspase. Indeed, apart from caspase-2 itself, only recently golgin-160 has been reported to be cleaved at a specific site by caspase-2. Golgin-160 can be cleaved also, at different sites by caspase-3 and caspase-7 (16). Our limited knowledge about caspase-2 substrates makes it difficult to hypothesize a role for this caspase as an effector caspase.

Alternatively, the caspase-3-dependent processing of caspase-2 could be the result of an amplification loop, which is a common event during the apoptotic response (37, 46).

The requirement of caspase-9 during apoptosis induced by caspase-2 overexpression reinforces a role of caspase-2 as a regulative caspase. We are conscious that, although caspase-2 expression can be modulated *in vivo* (47), its overexpression is an artificial way to induce apoptosis. However, it represents the only possible way to selectively activate a caspase-2-specific apoptotic pathway.

We observed that in cells containing catalytic inactive caspase-9 and overexpressing caspase-2, cytochrome *c* was released from the mitochondria and accumulated in the cytosol. A similar behavior was observed when apoptosis was induced by Bax overexpression. This result suggests that caspase-2 can act as a regulative caspase upstream of caspase-9 by regulating mitochondrial integrity, thus triggering the release of mitochondrial components including cytochrome *c*, which regulate caspase-9 activation (32). A role of caspase-2 in regulating mitochondrial integrity is also supported by the ability of Bcl-2 to inhibit effectively caspase-2-mediated cell death (27).

Caspase-2 has been localized in different subcellular compartments as well as in mitochondria (16, 24, 48). However, we think that additional studies are required to establish if caspase-2 is responsible for the release of cytochrome *c* when localized in the mitochondria.

Bid is a proapoptotic member of the Bcl-2 family proteins that contains only a BH3 domain and can be cleaved by caspase-8 following Fas/TNF receptor-1 activation (34, 35). Caspase-8-dependent processing of Bid is required for its translocation to mitochondria, where it induces cytochrome *c* release. Cleaved Bid undergoes *N*-myristoylation on the exposed glycine 60, and this post-translation modification is required for its targeting to mitochondria (36).

We have observed that overexpression of caspase-2 can induce the translocation of Bid from cytosol to mitochondria. Therefore, it is possible that in cells overexpressing caspase-2, Bid is cleaved and myristoylated and can mediate the release of cytochrome *c*. *In vitro* caspase-2 can process, albeit weakly, Bid, thus indicating that *in vivo* caspase-2 might directly mediate Bid translocation to mitochondria. However, we cannot exclude the possibility that Bid translocation in response to caspase-2 overexpression is not directly regulated by caspase-2.

In conclusion, our findings focus attention on caspase-2 as a regulator of the mitochondrial integrity and raise questions concerning the mechanisms responsible for its activation during cell death.

Acknowledgments—We are grateful to Y. Lazebnik, L. Faleiro, and J. Rodriguez for IMR90-E1A, IMR90-E1A caspase-9 DN, MCF-7 wild type caspase-3, and MCF-7 caspase-3 catalytic inactive cell lines and anti-caspase-7 antiserum. We also thank F. Demarchi and C. Kuhne for carefully reading the manuscript and for helpful suggestions, A. Risso for helping with the FACS analysis, and M. Stebel for support in the generation of the anti-caspase-2 antiserum.

REFERENCES

1. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
2. Cohen, G. M. (1997) *Biochem. J.* **326,** 1–16
3. Salvesen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446
4. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) *Annu. Rev. Biochem.* **68,** 383–424
5. Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998) *Cell* **94,** 325–337
6. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. (1998) *Cell* **94,** 339–352

7. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P., and Wallach, D. (1998) *Immunity* **9,** 267–276
8. Bergeron, L., Perez, G. I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K. E., Flaws, J. A., Salter, J. C., Hara, H., Moskowitz, M. A., Li, E., Greenberg, A., Tilly, J. L., and Yuan, J. (1998) *Genes Dev.* **12,** 1304–1314
9. Troy, C. M., Rabacchi, S. A., Friedman, W. J., Frappier, T. F., Brown, K., and Shelanski, M. L. (2000) *J. Neurosci.* **4,** 1386–1392
10. Jesenberger, V., Procyk, K. J., Yuan, J., Reipert, S., and Baccarini, M. (2000) *J. Exp. Med.* **192,** 1035–1046
11. Ahmad, M., Srinivasula, S. M., Wang, L., Talanian, R. V., Litwack, G., Fernandes-Alnemri, T., and Alnemri, E. S. (1997) *Cancer Res.* **4,** 615–619
12. Duan, H., and Dixit, V. M. (1997) *Nature* **385,** 86–89
13. Li, H., Bergeron, L., Cryns, V., Pasternack, M. S., Zhu, H., Shi, L., Greenberg, A., and Yuan, J. (1997) *J. Biol. Chem.* **34,** 21010–21017
14. Colussi, P. A., Harvey, N. L., and Kumar, S. (1998) *J. Biol. Chem.* **273,** 24535–24542
15. Colussi, P. A., Harvey, N. L., Shearwin-Whyatt, L. M., and Kumar, S. (1998) *J. Biol. Chem.* **273,** 26566–26570
16. Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A., and Rosen, A. (2000) *J. Cell Biol.* **149,** 603–612
17. Brancolini, C., Benedetti, M., and Schneider, C. (1995) *EMBO J.* **14,** 5179–5190
18. Sgorbissa, A., Benetti, R., Marzinotto, S., Schneider, C., and Brancolini, C. (1999) *J. Cell Sci.* **112,** 4475–4482
19. Brancolini, C., Sgorbissa, A., and Schneider, C. (1998) *Cell Death Differ.* **5,** 1042–1050
20. Fearnhead, H. O., Rodriguez, J., Givek, E-E., Guo, W., Kobayashi, R., Hannon, G., and Lazebnik, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13664–13669
21. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J. Biol. Chem.* **273,** 9357–9360
22. Faleiro, L., and Lazebnik, Y. (2000) *J. Cell Biol.* **151,** 951–960
23. Nicholson, D. W., Ambereen, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376,** 37–38
24. Zhivotovsky, B., Samali, A., Gahm A., and Orrenius, S. (1999) *Cell Death Differ.* **6,** 644–651
25. Germain, M., Affar, E. B., D'Amours, D., Dixit, V. M., Salvesen, G. S., and Poirier, G. G. (1999) *J. Biol. Chem.* **274,** 28379–28384
26. Butt, A. J., Harvey, N. L., Parasivam, G., and Kumar, S. (1998) *J. Biol. Chem.* **273,** 6763–6768
27. Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78,** 739–750
28. Pastorino, J. G., Tafani, M., Rothman, R. J., Marcineviciute, A., Hoek, J. B., and Farber, J. L. (1999) *J. Biol. Chem.* **274,** 31734–31739
29. Zhou, H., Li, Y., and Wang, X. (1999) *J. Biol. Chem.* **274,** 11549–11556
30. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) *Science* **288,** 1053–1058
31. Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X., and Williams, R. S. (2000) *Cell* **101,** 389–399
32. Green, D. R. (2000) *Cell* **102,** 1–4
33. Huang, D. C., and Strasser, A. (2000) *Cell* **103,** 839–842
34. Li, H., Zhu. H., Xu, C. J., and Yuan, J. (1998) *Cell* **94,** 491–501
35. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998*) Cell* **94,** 481–490
36. Zha, J., Weiler, S., Oh, K. J., Wei, M. C., and Korsmeyer, S. J. (2000) *Science* **290,** 1761–1765
37. Ashkenazi, A., and Dixit, V. M. (1999) *Curr. Opin. Cell Biol.* **11,** 255–260
38. Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W., and Mak, T. W. (1998) *Genes Dev.* **12,** 806–819
39. Van de Craen, M., Declercq, W., Van den Brande, I., Fiers, W., and Vandenabeele, P. (1999) *Cell Death Differ.* **11,** 1117–1124
40. Xue, D., Shaham. S, and Horvitz, H. R. (1996) *Genes Dev.* **10,** 1073–1083
41. Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) *J. Cell Biol.* **144,** 281–292
42. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) *J. Biol. Chem.* **272,** 17907–17911
43. Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D., and Wong, W. W. (1997) *J. Biol. Chem.* **272,** 9677–9682
44. Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., and Salvesen, G. S. (1999) *J. Biol. Chem.* **274,** 8359–8362
45. Allet, B., Hochmann, A., Martinou, I., Berger, A., Missotten, M., Antonsson, B., Sadaul, R., Martinou, J., and Bernasconi, L. (1996) *J. Cell Biol.* **132,** 479–486
46. Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) *Nature* **400,** 886–891
47. Kumar, S., Kinoshita, M., Noda, M., Copeland, N. G., and Jenkins, N. A. (1994) *Genes Dev.* **8,** 1613–1626
48. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M. C., Alzari, P. M., and Kroemer, G. (1999) *J. Exp. Med.* **18,** 381–394

Caspase-2-induced Apoptosis Is Dependent on Caspase-9, but Its Processing during UV- or Tumor Necrosis Factor-dependent Cell Death Requires Caspase-3

Gabriela Paroni, Clare Henderson, Claudio Schneider and Claudio Brancolini

J. Biol. Chem. 2001, 276:21907-21915.
doi: 10.1074/jbc.M011565200 originally published online March 16, 2001
