Caspase-2 Can Trigger Cytochrome c Release and Apoptosis from the Nucleus

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The cysteine proteases specific for aspartic residues, known as caspases, are localized in various subcellular compartments and play specific roles during the regulative and the executive phase of the cell death process. Here we investigated the subcellular localization of caspase-2 in healthy cells and during the execution of the apoptotic program. We have found that caspase-2 is a nuclear resident protein and that its import into the nucleus is regulated by two different nuclear localization signals. We have shown that in an early phase of apoptosis caspase-2 can trigger mitochondrial dysfunction from the nucleus without relocalizing into the cytoplasm. Release of cytochrome c occurs in the absence of overt alteration of the nuclear pores and changes of the nuclear/cytoplasmic barrier. Addition of leptomycin B, an inhibitor of nuclear export, did not interfere with the ability of caspase-2 to trigger cytochrome c release. Only during the late phase of the apoptotic process can caspase-2 relocalize in the cytoplasm, as consequence of an increase in the diffusion limits of the nuclear pores. Taken together these data indicate the existence of a nuclear/mitochondrial apoptotic pathway elicited by caspase-2.

Members of the aspartate-specific cysteine family of proteases known as caspases play a critical role in apoptosis (1). Caspases can be divided into initiator caspases and effector caspases based on the presence of a large prodomain at their amino-terminal region (2). Initiator caspases generally act at the apex of a proteolytic cascade, whereas effector caspases act downstream and are involved in the cleavage of specific cellular proteins (death substrates) (3). Once processed, death substrates modulate the morphological changes characterizing the apoptotic process (4, 5). The long prodomains of the initiator caspases trigger/facilitate the activation of the proenzymes through the interaction with specific adaptor molecules (6). Caspase-2, caspase-8, caspase-9, and caspase-10 are the long prodomain caspases involved in the apoptotic process.

A plethora of studies have demonstrated that caspase-2 has structures mostly in the nucleus (22, 26), similar to the filamentous structures formed by death effector domains of Fas-associated death domain and caspase-8 or by other proteins (27–30). In the case of caspase-2 these structures are due to CARD-mediated oligomerization of the protein (22). Overall these data, sometimes controversial, still do not clarify the

1 The abbreviations used are: GFP, green fluorescent protein; CARD, caspase recruitment domain; LMB, leptomycin B; NLS, nuclear localization signal; NPC, nuclear pore complex; DTT, dithiothreitol; TRITC, tetramethylrhodamine B isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; GST, glutathione S-transferase; aa, amino acid; DN, dominant negative; HA, hemagglutinin.
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subcellular localization of caspase-2 and the relative implication for its death activity.

The goals of this work were to clarify the subcellular localization of caspase-2 in healthy cells and to identify the regions responsible for its subcellular targeting. Furthermore, we have investigated whether during apoptosis caspase-2 could translocate in different subcellular compartments and the relationship between its subcellular localization and its ability to trigger mitochondrial dysfunction.

EXPERIMENTAL PROCEDURES

Culture Conditions—IMR90-E1A, IMR90-E1A caspase-9 DN (31), and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 ng/ml) and incubated at 37 °C in 5% CO2 atmosphere. Leptomycin B (Sigma) was used to expose the final concentration of 5 ng/ml.

Transfection, Microinjection, and Time Lapse Analysis—Transfections were performed using the calcium phosphate precipitation method. Cells were seeded 24 h before transfection at 1.2 x 10^6 cells/ml and analyzed 12 h after removal of the precipitates. For caspase-2 killer was transfected with pCAGGS-caspase-2C and pCAGGS-KK135136AA mutant. The GST-caspase-2 prodomain was obtained by subcloning the N-terminal fragment containing full-length human RAIDD into the cloning sites: primer rC2N, 5'-CATCATCTCGAGTCATGTGGGAGGGTGTCCTGG-3'; primer sC2N, 5'-CATCATATGCTTTCTGGGCTTCAGCAT-3'; primer r27N, 5'-CATCATAGCTCATGCTGCCATGACGGAAGGACA-3'; and primer s27N, 5'-CATCATCTCTGCGCCATGAGAAGGACA-3'.

In vitro mutagenesis to generate caspase-2NLS-(131-143) was performed as described previously (32). The following sets of primer were used: primer C2N, 5'-CATCATAGCTCATGCTGCCATGACGGAAGGACA-3'; primer sC2N, 5'-CATCATATGCTTTCTGGGCTTCAGCAT-3'; primer r27N, 5'-CATCATCTCTGCGCCATGAGAAGGACA-3'; and primer s27N, 5'-CATCATCTCTGCGCCATGAGAAGGACA-3'.

The GST-caspase-2 promodan was obtained by subcloning the caspase-2 promodan from pCAGGS3HA-caspase-2 promodan into the EcoRI/XhoI sites of pGEX4T1 (Amersham Biosciences).

To construct pEGFP-N1-caspase-2 and pEGFP-C3-caspase-2, the entire coding region of caspase-2 was amplified from pCAGGS-caspase-2 by PCR using rC2N and sC2N primers. The amplification products were inserted in pEGFP-N1 or pEGFP-C3 vectors.

RESULTS

Subcellular Localization of Caspase-2—Caspase-2 localization was investigated by subcellular fractionation of IMR90-E1A cells. Nuclear and cytosolic extracts were prepared by detergent lysins and were used to visualize caspase-2, the different fractions by Western analysis. As shown in Fig. 1a, caspase-2 was revealed both in the nuclear and in the cytosolic fractions. The quality of the fractionation was verified by probing the lysates for well defined nuclear and cytoplasmic markers as illustrated in Fig. 1a. To confirm the observed caspase-2 subcellular distribution, fractionation was performed by a different...
that should represent microsomal and mitochondrial fractions, respectively (Fig. 1b). Here again fractionation quality was verified by controlling the distribution of specific subcellular markers. The subcellular localization of caspase-2 was next investigated by confocal microscopy using the affinity-purified anti-caspase-2 antibody (18). In IMR90-E1A endogenous caspase-2 was prevalently revealed as a nuclear staining, with the exclusion of the nucleoli (Fig. 1c). The nuclear staining was undetectable after pre-absorption of the antibody with recombinant caspase-2 (Fig. 1c, PA).

Confocal analysis of the overexpressed caspase-2 by using the specific antibody revealed the same diffuse nuclear staining as obtained for the endogenous caspase-2 (Fig. 1d). Nuclei were visualized by propidium iodide staining (Fig. 1d, PI). When caspase-2wt was co-expressed with its previous identified adaptor RAIDD/CRADD (21, 34) again, it was prevalently observed as a diffuse nuclear staining, whereas RAIDD/CRADD showed both a nuclear and a cytoplasmic distribution (data not shown).

To clarify the apparent discrepancy between the nuclear-cytoplasmic localization of caspase-2 observed after biochemical fractionation and its exclusively nuclear localization detected by immunofluorescence analysis, we decided to analyze the subcellular localization of the overexpressed caspase-2 by subcellular fractionation. We used GFP-tagged caspase-2 to exclude epitope masking in the immunofluorescence assay. To reduce the killer activity of caspase-2, low amounts of the expression plasmid were transfected, and the analysis was performed soon after transfection (within 10 h). Under these conditions few cells entered apoptosis (data not shown), and caspase-2-GFP was mainly revealed as unprocessed procaspase (see Fig. 1f).

Similarly to the untagged protein, caspase-2-GFP was mainly detected as a diffuse nuclear staining with the exclusion of the nucleoli by confocal microscopy (Fig. 1e). On the other hand ectopically expressed caspase-2-GFP was detected both in the nucleus and the cytoplasm, by means of subcellular fractionation and Western blotting. Similarly endogenous caspase-2 was detected both in the nucleus and in the cytoplasm.

In conclusion these results suggest that the accumulation of caspase-2 in the cytosolic compartment observed after subcellular fractionation might be ascribed to a dissociation of caspase-2 from the nuclei occurring during the experimental procedure.

**Dissection of the Caspase-2 Prodomain, Implications for Nuclear Localization and Killer Activity—**In order to map the regions of caspase-2 required for its targeting to the nuclear compartment, a number of different deleted constructs were generated, as outlined in Fig. 2a. We created a caspase-2 lacking the prodomain-(Δ1–152), because it has been demonstrated that murine caspase-2 lacking this region is impaired in nuclear localization (22). We also created a caspase-2 lacking the first 27 aa (Δ1–27), because a nuclear localization sequence is present in this region, and it has been shown previously (22, 26) that this region is required for the nuclear localization of the caspase-2 prodomain. Finally, a caspase-2 version lacking both the amino-terminal region and the CARD domain-(Δ1–104) and a caspase-2 consisting only of the prodomain-(Δ153–435) were also generated. All the deleted derivatives were cloned in pcDNA3 expression vector containing an HA epitope tag at the amino terminus.

The subcellular localization of the overexpressed proteins was analyzed using both the anti-caspase-2 and the anti-HA antibodies with similar results. Representative cells are shown in Fig. 2b. Overexpressed caspase-2wt was detected as a diff-
FIG. 2. Deletion constructs of caspase-2. 

a, schematic representation of caspase-2 and deleted derivatives. Prodomain, large subunit (LS), and small subunit (SS).

b, immunofluorescence analysis of IMR90-E1A cells overexpressing caspase-2 wt and its deleted derivatives. IMR90-E1A
The Prodomain of Caspase-2 Contains a Second Nuclear Localization Signal—Previous studies have suggested that caspase-2 can also be targeted to the nuclear compartment independently from the previous identified bipartite NLS (22). We have demonstrated that caspase-2-(Δ1–104), which lacks the bipartite NLS and the CARD, still showed a prevalent nuclear localization. This observation prompted us to investigate whether other potential NLS were present in the prodomain of caspase-2. Other types of NLS described include those found in the proto-oncogene c-myc (PAAKRVKLD) where proline and aspartic acid residues flank a central basic cluster (36). Sequence analysis revealed that this type of NLS is present in the prodomain of caspase-2 between aa 131 and 143 (see Fig. 3a). To investigate the role of this second NLS (NLS-II) in the nuclear localization of caspase-2, the two lysines at position 135 and 136 were replaced by two alanines (K135A/K136A).

Caspase-2 K135A/K136A showed a predominant diffuse cytosolic staining when overexpressed in IMR90-E1A cells, even though some nuclear staining, as above described for Δ1–27, was still detectable (Fig. 3b). In contrast, caspase-2 wt was exclusively nuclear under the same experimental conditions. This residual nuclear localization could be both ascribed to a simple diffusion in the nuclear compartment of the overexpressed protein, which size is behind the exclusion limits of the nuclear pore, or to a residual nuclear import activity of the remnant NLS.

To confirm that the nuclear localization of caspase-2-(Δ1–104) lacking NLS-I was dependent on NLS-II, we used a caspase-2-(Δ1–104) fragment, where the lysines 134–135 were substituted with alanines. As shown in Fig. 3b, whereas caspase-2-(Δ1–104) was mainly nuclear, caspase-2-(Δ1–104) K135A/K136A showed a prevalent cytosolic localization.

To determine whether the newly identified NLS-II could target a cytosolic protein to the nucleus, a peptide containing NLS-II was fused to the reporter protein β-galactosidase. The immunofluorescence analysis demonstrated that β-galactosidase containing the caspase-2 NLS-II was localized prevalently in the nucleus, and on the contrary β-galactosidase lacking the caspase-2 NLS-II was localized prevalently in the cytoplasm.

To characterize further the caspase-2 K135A/K136A mutant, we evaluated its ability to dimerize in an in vitro pull-down assay using GST-caspase-2 prodomain. Caspase-2 K135A/K136A was able to interact with caspase-2 similarly to the wt form (Fig. 3c).

By having demonstrated that caspase-2 K135A/K136A was still able to dimerize with caspase-2 and that it was impaired in the nuclear localization, the next question was to study its killer activity.

A cell death assay was performed as described above, and the results obtained are summarized in Fig. 3d. Caspase-2 K135A/K136A was able to induce cell death similarly to caspase-2 wt in IMR90-E1A cells, and moreover, caspase-2 wt and caspase-2 K135A/K136A showed similar capacity to induce cytoplasmic relocalization of cytochrome c in IMR90-E1A caspase-9 DN cells (Fig. 3c).
Subcellular Localization of Caspase-2 Fused to GFP in Vivo—The demonstration that the caspase-2 mutants (K135A/K136A and Δ1–152), which showed a predominant cytoplasmic localization, were equally able to induce cell death and cytochrome c release raises the question of a possible cytoplasmic function of caspase-2 during apoptosis. To address this point, we decided to study if ectopically expressed caspase-2 can re-localize from the nucleus into the cytoplasm during cell death. 

FIG. 3. Caspase-2 contains a second nuclear localization sequence (NLS-II). a, schematic representation of caspase-2 where the putative nuclear import sequences (NLSs) are underlined. Prodomain, large subunit (LS), small subunit (SS). b, immunofluorescence analysis of IMR90-E1A cells overexpressing caspase-2 wt and its deleted or point-mutated derivatives. IMR90-E1A cells after transfection with the relative constructs were fixed and processed for the immunofluorescence analysis to visualize caspase-2. Bar, 7 μm. c, immunofluorescence analysis of IMR90-E1A cells overexpressing β-galactosidase or β-galactosidase containing caspase-2 NLS-II. IMR90-E1A cells after transfection with the relative constructs were fixed and processed for the immunofluorescence analysis to visualize β-galactosidase. Bar, 7 μm. d, in vitro caspase-2 binding. A GST-caspase-2-prodomain (GST-caspase-2) and GST as control, immobilized on glutathione-Sepharose beads, were incubated with the in vitro translated products of the indicated constructs. After washing, proteins bound to the beads were evaluated by SDS-PAGE. e, IMR90-E1A cells were co-transfected with the indicated constructs and pEGFPN1 as a reporter. The appearance of the apoptotic cells was scored after 20 h from transfection. Cells showing a collapsed morphology and presenting extensive membrane blebbing were scored as apoptotic. Data represent arithmetic means ± S.D. of four independent experiments. f, IMR90-E1A caspase-9 DN cells were co-transfected with the indicated constructs and pEGFPN1 as a reporter. After 20 h immunofluorescence assay was performed using anti-cytochrome c antibody. Cells co-expressing GFP and caspase-2 or caspase-2 K135A/K136A were scored for cytochrome c release from mitochondria. Data represent arithmetic means ± S.D. of three independent experiments.
Because this relocalization cannot be unambiguously analyzed by biochemical fractionation, we decided to monitor the subcellular localization of active caspase-2 during apoptosis by confocal microscopy. We used a fusion of caspase-2 with GFP at the carboxyl terminus (caspase-2-GFP) to visualize the proenzyme and, after its processing, the active tetramer and a fusion of caspase-2 with GFP at the amino terminus (GFP-caspase-2) to visualize the proenzyme and, after its processing, the prodomain.

The ability of caspase-2-GFP and GFP-caspase-2 to undergo autocatalytic activation was investigated by transient transfection in 293 cells. We used a fusion of caspase-2 with GFP at the carboxyl terminus (caspase-2-GFP) to visualize the proenzyme and, after its processing, the active tetramer. We used an antibody specific for the small subunit of caspase-2 in immunoblot analysis to evaluate caspase-2 processing (Fig. 4a). Caspase-2-GFP and GFP-caspase-2 both were detected as processed forms. Moreover, in the case of caspase-2-GFP the molecular size of the small subunits was increased as a consequence of the GFP. This indicates that the GFP, when fused to the carboxyl terminus of caspase-2, assembles into the active tetramer.

We next performed a time-lapse analysis to evaluate the ability of GFP-caspase-2 and caspase-2-GFP to induce apoptosis. The time-lapse analysis was performed in IMR90-E1A cells 30–60 min after microinjection, and frames were collected every 2 min for 12 h. Selected frames of a representative experiment, at the indicated times, are shown in Fig. 4, b–d. The localization of GFP-caspase-2 in nuclear dots precedes cell death. A time-lapse sequence of cells undergoing apoptosis following caspase-2 overexpression is shown in Fig. 4d. Each filled square represents a single cell at the time of the death as established by nuclear collapse.
overexpressed caspase-2 as described above for fixed cells. Within 4 h after microinjection almost 90% of the overexpressing cells had died by apoptosis as judged by nuclear fragmentation and membrane blebbing (data not shown).

A different scenario was observed when GFP-caspase-2 was overexpressed. Initially, GFP caspase-2 was detected as a dif-

![Fig. 5. Caspase-2-dependent cytochrome c release from mitochondria. IMR90-E1A cells were injected with pEGFP-N1-caspase-2 (caspase-2-GFP) or with pEGFP-C3-caspase-2 (GFP-caspase-2) (20 ng/µl). After 3 h cells were fixed and processed for immunofluorescence to visualize caspase-2-GFP, cytochrome c (Cyt-c), and GFP-caspase-2. Nuclei were stained with propidium iodide (PI). Images were obtained using a Leica TCS confocal microscopy and are displayed in pseudocolors. a, non-apoptotic cells; b, cells in an early apoptotic phase; c, cells in a late apoptotic phase. Bar, 8 µm.](image-url)
fuse nuclear staining, but at later time points (see Fig. 4c, 1.51) small dots were observed in the nucleus containing GFP-caspase-2, which increased in number and size over time (Fig. 4c, 2.41). These dots resemble previously described (22) nuclear structures enriched in caspase-2 prodomain observed in cultured cells after overexpression of catalytic inactive caspase-2.
or of the prodomain alone in fusion with GFP. We have also noticed that these dots partially co-localize with the promyelocytic leukemia oncogenic domain (26) and that their appearance anticipates cell death. The time dependence of apoptosis induced by caspase-2 in relation to the appearance of the nuclear dots containing caspase-2 is summarized in Fig. 4d. About 98% of the cells die by apoptosis 100 min after the appearance of caspase-2 in the nuclear dots. The occurrence of cell death shortly after the appearance of caspase-2 in dot-like structures could explain the failure to clearly observe these structures in transient transfection experiment with untagged caspase-2. Because localization of caspase-2-GFP in the nuclear dots was less evident, it is possible that such dots are enriched in a processed form of caspase-2 consisting of the prodomain but lacking the small subunit.

In summary, these experiments indicate that the fusion to GFP did not affect the subcellular localization of caspase-2, its catalytic activity, and its ability to induce cell death.

**Subcellular Localization of Caspase-2 during Different Phases of the Apoptotic Response**—At least two different phases can be identified during apoptosis induced by caspase-2. An initial apoptotic phase during which cytochrome c is released from the mitochondria, but no significant alterations of the nuclear morphology can be observed, and a late phase when drastic alterations of the nuclear morphology are evident. Therefore, in order to evaluate caspase-2 localization during early and late apoptosis, we performed a triple immunofluorescence analysis in IMR90-E1A cells to visualize caspase-2-GFP, cytochrome c, and nuclei. More than 300 cells were analyzed in triplicate experiments, and the results obtained are exemplified in Fig. 5. As expected in non-apoptotic cells both caspase-2-GFP and GFP-caspase-2 were revealed as a diffused nuclear staining (Fig. 5a). Again in cells at an early apoptotic stage, as judged by cytochrome c release, both caspase-2-GFP and GFP-caspase-2 were localized in the nucleus (Fig. 5b). However, whereas caspase-2-GFP was prevalently detected as a diffused nuclear staining, GFP-caspase-2 was observed prevalently in nuclear dots, thus suggesting a different subnuclear localization of the active enzyme with respect to the prodomain.

Caspase-2-GFP can be observed both in the nucleus and in the cytoplasm (Fig. 5c, caspase-2-GFP, arrowheads) in cells at late apoptotic phase, as judged by nuclear morphology (Fig. 5c, PI), whereas GFP-caspase-2 was prevalently localized to the nuclear compartment (Fig. 5c, GFP-caspase-2).

Even in IMR90-E1A caspase-9 DN cells, release of cytochrome c following caspase-2 activation occurs in the absence of relocation of the enzyme in the cytoplasm (data not shown).

**The Subcellular Localization of Ran but Not Nuclear Pores Were Altered by Caspase-2**—The above-described studies suggest that caspase-2 can trigger mitochondrial dysfunction from the nucleus and that, only during the final events of the apoptotic process, active caspase-2 can translocate, at some extent, into the cytoplasm. How can caspase-2 trigger cytochrome c release from the nucleus?

In eukaryotic cells macromolecular traffic between the nucleus and the cytoplasm can occur by simple diffusion, for particles of less than 50/60 kDa, or mediated by soluble transport receptors that shuttle through the nuclear pore complex (NPC) for larger molecules (38). Cargo molecules are recog-

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**Fig. 7. Caspase-2 can induce cytochrome c release without altering the nuclear/cytoplasmic barrier.** a, IMR90-E1A caspase-9 DN cells were injected with pcDNA HA-caspase-2 (50 ng/μl), pEGFP-N1-Bid (5 ng/μl), and 66-kDa dextran (1 mg/ml). After 3 h cells were fixed and processed for immunofluorescence to visualize Bid-GFP and dextran. Images were obtained using a Leica TCS confocal microscopy. Bar, 1 μm. b, IMR90-E1A caspase-9 DN cells were injected with pEGFP-N1-caspase-2 (20 ng/μl) and 66-kDa dextran (1 mg/ml). After 3 h cells were fixed and processed for immunofluorescence to visualize caspase-2-GFP, dextran and cytochrome c. Images were obtained using a Leica TCS confocal microscopy. Bar, 1 μm.

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nized via import or export targeting signals by the transport receptors, which are able to associate with components of the NPC (39). The small GTPase Ran is highly enriched in the nucleus in its GTP-bound form, and it regulates nuclear-cytoplasmic transport (40). Ran-GTP binds to importins, inducing the release of imported cargo, on the other side exportins interact with their substrates only in the nucleus in the presence of Ran-GTP, and they release the substrates in the cytoplasm after GTP hydrolysis (41).

The translocation of the apoptotic signal triggered by caspase-2 from the nucleus to the mitochondria could occur (i) by simple diffusion, (ii) by regulated nuclear export, and (iii) by increased diffusion (i.e. by increasing the diffusion limits of the nuclear pores).

Interestingly in apoptotic cells the diffusion limit of the nuclear pores is increased, thus allowing the redistribution throughout the cell of soluble proteins normally restricted to the nucleus or to the cytoplasm (42). In addition in apoptotic cells Ran subcellular localization is altered, thus suggesting a dysfunction of nuclear transport (42, 43).

Therefore, in order to investigate how nuclear localized caspase-2 could trigger cytochrome c release, we first evaluated if caspase-2 could regulate Ran localization and nuclear pores status. We used Bid translocation to the mitochondria as
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Marker of caspase-2 activation (18).

pcDNA3-caspase-2 and pGFPN1Bid were co-expressed by nuclear microinjection in IMR90-E1A. Cells were fixed 3 h later and processed for immunofluorescence to visualize Bid-GFP, nuclei, and p62, a component of the plug of the nuclear pores and a marker for nuclear pore integrity (42) (Fig. 6a) or Bid-GFP, nuclei, and Ran (Fig. 6b).

The antibody against p62 similarly detected the nuclear rim in cells presenting Bid-GFP as a diffuse staining or translocated to mitochondria, thus excluding overt alterations of the nuclear pores. As expected a large part of Ran was concentrated in the nucleus in cells presenting a diffuse localization of Bid-GFP. On the contrary Ran was distributed throughout the cell and therefore released from the nucleus in cells presenting Bid translocated to the mitochondria (Fig. 6b).

Caspase-2 can induce Bid translocation and cytochrome c release independently from caspase-9; therefore the relocalization of Ran was assayed in IMR90-E1A caspase-9 DN. As shown in Fig. 6c and summarized in Fig. 6d, caspase-2 can induce redistribution of Ran from the nucleus to the cytoplasm independently from caspase-9, and this event correlates with the translocation of Bid to the mitochondria.

**Nuclear Caspase-2 Can Modulate Mitochondrial Integrity without Altering the Nuclear/Cytoplasmic Barrier**—The above-described studies do not exclude a possible effect of caspase-2 on the diffusion limits of the nuclear pore that could not be evidenced by p62 staining. To test this hypothesis we investigated the ability of caspase-2 to alter the diffusion limits of the nuclear pores. Fluorescent 66-kDa dextran, which does not diffuse passively through the nuclear pores, can be used as marker for nuclear breakdown in living cells (44). Here again, because caspase-9 is described to directly or indirectly disrupt the nuclear-cytoplasmic barrier, we decided to investigate if caspase-2 can specifically alter the permeability of the nuclear barrier in cells lacking caspase-9 activity. The nuclei of IMR90-E1A caspase-9 DN cells were injected with pcDNA3-caspase-2, dextran-TRITC 66-kDa, and pGFP-Bid. 4 h later cells were fixed to visualize Bid-GFP and dextran. As expected, in cells showing Bid-GFP in its unprocessed form (diffuse nuclear and cytosolic distribution), the 66-kDa dextran showed a nuclear localization (Fig. 7a). The same nuclear localization of the 66-kDa dextran was preserved in cells showing Bid-GFP translocated to the mitochondria. Similar results were obtained when caspase-2-GFP was co-expressed with dextran-TRITC 66-kDa, and caspase-2 activation was evaluated by cytochrome c distribution (Fig. 7b). Hence nuclear caspase-2 can trigger mitochondrial dysfunction without altering the nuclear/cytoplasmic barrier.

**Caspase-2 Can Induce Cytochrome c Release and Apoptosis in the Presence of Leptomycin B**—Nuclear pore complex protein CRM1 is able to recognize specific nuclear export sequences and mediate nuclear export from the nucleus (45). CRM1 is the target of the cytotoxic LMB. By direct binding to CRM1, LMB disrupts nuclear export sequence-dependent nuclear export. To understand if active nuclear export mediated by CRM1 could be implicated in triggering cytochrome c release once caspase-2 is activated in the nucleus, we investigated the effect of LMB on cytochrome c release in cells overexpressing caspase-2. IMR90-E1A caspase-9 DN cells were pretreated with LMB (5 ng/ml) 2 h before microinjection with plasmid encoding GFP-caspase-2. After further incubation for 3 h in the presence of LMB cells were fixed and processed for immunofluorescence. As shown in Fig. 8a, release of cytochrome c after activation of caspase-2 was independent from LMB treatment. Fig. 8b represents the quantitative analysis. Under the same experimental conditions LMB was able to inhibit the nuclear export of IxB-α (Fig. 8c) (45).

**Disruption of the Nuclear/Cytoplasmic Barrier during the Late Phase of the Apoptotic Process Could Permit Caspase-2 Translocation into the Cytoplasm**—To clarify the mechanism by which active caspase-2 can exit from the nucleus during the late apoptotic phase (see Fig. 5c), we analyzed the diffusion limits of the nuclear/cytoplasmic barrier in vivo throughout the apoptotic process triggered by caspase-2.

pGFPN1-caspase-2 and 66-kDa TRITC dextran were microinjected in the nucleus of IMR90-E1A cells, and soon afterward the cells were subjected to a time-lapse analysis. Frames were collected every 2 min during an 8-h period. Selected frames of a representative experiment, at the indicated times, are shown in Fig. 9a.

Caspase-2-GFP and dextran were well confined in the nucleus even though alterations of the nuclear morphology, indicative of the apoptotic process, were evident (starting from 2.30 h after microinjection). Localization of the 66-kDa dextran into the cytoplasm was first detected after 2.36 h and was more evident 2.42 h after microinjection. During the same time points caspase-2-GFP was more confined in the altered nucleus relative to the 66-kDa dextran. This observation might suggest that a fraction of caspase-2-GFP was not soluble at the indicated time points.

The time-lapse studies indicate that apoptosis triggered by caspase-2 in IMR90-E1A could induce alterations of the nuclear permeability, possibly as a consequence of caspase-9 activation (42). We next wanted to study if caspase-2 could alter nuclear permeability also independently from caspase-9. To this end pGFPN1-caspase-2 and 66-kDa TRITC dextran were microinjected in the nucleus of IMR90-E1A caspase-9 DN, and soon afterward cells were subjected to the time-lapse analysis. Frames were collected every 2 min during an 8-h period. Selected frames of a representative experiment at the indicated times are shown in Fig. 9b.

Caspase-2 and the 66-kDa dextran were well confined in the nucleus up to 4.00 h after microinjection. Some dextran staining can be observed in the cytoplasm 4.50 h after microinjection. This becomes more evident at later time points and parallels a decreased signal in the nucleus (see Fig. 9b, 5.20 h after microinjection). Here again, although some caspase-2-GFP can relocalize into the cytoplasm, it is more confined in the nucleus with respect to the 66-kDa dextran during the same time course. Again, this observation might suggest that a fraction of nuclear caspase-2-GFP was not soluble at the indicated time points. It is important to note that the described time schedule can differ among cells. These differences probably reflect a differential responsiveness of IMR90-E1A cells to caspase-2 overexpression (data not shown).

To establish clearly the timing of caspase-2 activation in relation to the increased permeability of the nuclear barrier, we used Bid-GFP as a reporter for caspase-2 activation. pcDNA3-caspase-2, pGFPN1Bid, and dextran-TRITC were co-expressed by nuclear microinjection in IMR90-E1A caspase-9 DN cells, and soon after microinjection cells were subjected to time-lapse analysis. Frames were collected every 2 min during the period of 8 h. Selected frames of a representative experiment, at the indicated times, are shown in Fig. 9c.

We observed translocation of Bid-GFP to the mitochondria as early as 4 h after microinjection (see Fig. 9c, arrowhead 4.01–4.15). During the same time course the first evidence of an effect of caspase-2 on the nuclear diffusion limits can be detected 2 h later (see Fig. 9c, arrowhead 5.47 and 5.51) with the appearance of the dextran staining in the cytoplasm. Taken together these results demonstrate that the alteration of the
nuclear/cytoplasmic barrier is a late event triggered by caspase-2.

Here again it is important to note that the schedule of Bid-GFP translocation and of dextran release from the nucleus can differ among cells (data not shown).

**DISCUSSION**

The proteolytic machinery controlling apoptosis includes numerous caspases that are localized in different subcellular compartments and play specific roles during the regulative and the executive phases of the cell death process (3–5).

The subcellular localization of caspase-2 is still debated. By means of immunofluorescence analysis endogenous caspase-2 was observed in the cytosol, in the nucleus, and in the Golgi (20, 25), whereas ectopically expressed endogenous caspase-2 accumulated mainly in the nucleus (22, 26). A different study, using subcellular fractionations, has indicated a prevalent cytosolic localization of endogenous caspase-2 (24). Our immunofluorescence studies support a nuclear localization for both endogenous and ectopically expressed caspase-2. In contrast, upon subcellular fractionation a large amount of caspase-2 was detected in the cytoplasm. We analyzed the subcellular localization of caspase-2-GFP both by fractionation and by confocal microscopy and present data strongly suggesting that the cytosolic localization of caspase-2, observed after fractionation, might be ascribed to a dissociation of caspase-2 from the nuclei during the experimental procedure. However, we cannot exclude that the nuclear import of caspase-2 could be regulated thus making possible its accumulation in the cytoplasm in certain cell types (20). Further studies are necessary to unambiguously answer this point.

The bipartite NLS present within the amino-terminal 44-amino acid residues is necessary for the nuclear localization of caspase-2 (22, 26). In the current study we have confirmed that this sequence is a critical NLS of caspase-2, and in addition we have identified a second, previously unrecognized NLS within the prodomain of caspase-2. The existence of a second NLS was suggested by Colussi et al. (22) to explain the failure of the amino-terminal 44-amino acid sequence to drive the nuclear localization of a reporter protein. This second NLS (NLS-II) is placed between aa 131 and 143 (PLYKKLRLSTD) and resembles the NLS of the proto-oncogene c-myc, where proline and aspartic acid residues define a central basic cluster (36). Our present study shows that NLS-II can drive the nuclear import of a cytoplasmic protein. The two separate NLSs present in the prodomain of caspase-2 could functionally collaborate with each other, and thus provide the cells with greater versatility in regulating caspase-2 nuclear import.

The prodomain of caspase-2 also contains the CARD, which is composed by six antiparallel amphipathic α-helices, and mediates homophilic interactions, thus triggering proenzyme activation (35, 46). Caspase-2, containing deletions or alterations of the CARD, was impaired in inducing cell death. Surprisingly, a caspase-2 without the prodomain (Δ1–152) was able to induce cell death and cytochrome c release similarly to the wt form, whereas a shorter deletion of the prodomain (Δ1–121), was also unable to induce cell death. Therefore, it is possible that the prodomain contains an inhibitory domain, which should be located between aa 104 and 151 likely between aa 121 and 151. An inhibitory effect of the prodomain was previously suggested by the observation that active recombinant caspase-2 could be obtained by expressing in E. coli a fragment lacking the prodomain (Δ1–152). Expression of full-length caspase-2 did not show proteolytic activity even though it was processed into

**FIG. 9.** An increase of the diffusion limits of the nuclear pore is a late event during the apoptotic response elicited by caspase-2. a, time-lapse images of a representative IMR90-E1A cell double-stained for caspase-2-GFP and dextran. Frames at selected times after microinjection (as indicated) of a representative cell injected with pEGFP-N1-caspase-2 (20 ng/μl) and 66-kDa dextran (1 mg/ml) are shown. Bar, 2 μm. b, time-lapse images of a representative IMR90-E1A caspase-9 DN cell double-stained for caspase-2-GFP and dextran. Frames at selected times after microinjection (as indicated) of a representative cell injected with pEGFP-N1-caspase-2 (20 ng/μl) and 66-kDa dextran (1 mg/ml) are shown. Bar, 2 μm. c, time-lapse images of a representative IMR90-E1A caspase-9 DN cell double-stained for Bid-GFP and dextran. Frames at selected times after microinjection (as indicated) of a representative cell injected with pEGFP-HA-caspase-2 (50 ng/μl), pEGFP-N1-Bid (5 ng/μl), and 66-kDa dextran (1 mg/ml) are shown. Bar, 2 μm.
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a prodomain large subunit and a small subunit (37). It is interesting to note that an autoinhibitory domain has been recently discovered in procaspase-3. Caspase-3 is under strict regulatory self-control by a "safety catch" Asp-Asp-Asp tripeptide, contained within the proenzyme itself (48).

The identity of apoptotic signals specifically activating caspase-2 is unknown. However, upon ectopic expression, one can artificially activate caspase-2, possibly as consequence of the forced oligomerization. This phenomenon has allowed us to study the apoptotic signals originated by caspase-2 in the nucleus.

We have observed that activated caspase-2 can trigger the release of cytochrome c from the nucleus, and only at a later phase can the active enzyme partially translocate into the cytoplasm.

Caspase-2 triggered the release of cytochrome c in the cytoplasm when (i) active caspase-2 was still confined in the nucleus, (ii) no changes of the diffusion limits of the nuclear pores were evident, (iii) Ran accumulated in the cytoplasm, and (iv) the CRM1-dependent nuclear export was inhibited by LMB treatment.

These observations can be explained with two hypothetical mechanisms as follows: a nuclear factor, regulated by caspase-2, may exit the nucleus by a CRM1-independent pathway, or alternatively, the nuclear factor may translocate to the cytoplasm by simple diffusion.

In a simple model system the nuclear factor should be cleaved directly or indirectly by caspase-2, and once cleaved it should be able to exit the nucleus and to propagate the signal to the mitochondria. Unfortunately, the number of known death substrates, cleaved by caspase-2, is limited. Besides caspase-2 autocatalysis, it has been reported that caspase-2 can cleave Golgin-160 and Bid (18, 25, 49). Golgin-160 is a peripheral Golgi membrane-associated protein, and therefore its involvement in transferring the apoptotic signal from the nucleus to the cytoplasm is unlikely. Bid has been localized in the cytosol by subcellular fractionation (50). Nevertheless, when overexpressed as GFP fusion or epitope-tagged (18, 49, 64) kDa in its uncomplexed form (37, 49). This implies that processed caspase-2, even though not complexed to other proteins, cannot exit the nucleus by diffusion.

Caspase-9 directly or indirectly is responsible for increasing nuclear permeability during apoptosis, which is due to alterations of nuclear pores (42). Caspases can cleave some nuclear pore elements, thus suggesting a possible mechanism for the increase of the nuclear diffusion limit (42, 43, 54), but alternative mechanisms could also be evoked (55). We found that caspase-2 can increase the nuclear diffusion limits. This effect was shown to be independent from caspase-9, indicating that caspase-2 can activate alternative pathways to increase nuclear permeability.

One possibility is that caspase-2 might cleave directly some of the more than 50 proteins of the vertebrate nuclear pore complex (38, 56). However the observed delay, ~2.0 h, between caspase-2 activation, as indicated by Bid-GFP processing, and the diffusion of dextran out of the nucleus, argues against a direct processing of the nuclear pore components by caspase-2. Hence we suggest an indirect effect of caspase-2 on the nuclear-cytoplasmic barrier.

In conclusion our data clarify the subcellular localization of caspase-2 and demonstrate the existence of two NLSs regulating its nuclear import. Active caspase-2 can mediate cytochrome c release from the nucleus thus indicating the existence of a nuclear/mitochondrial apoptotic apoptotic pathway.

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