Role of Prodomain in Importin-mediated Nuclear Localization and Activation of Caspase-2*

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Caspase-2 is unique among mammalian caspases because it localizes to the nucleus in a prodomain-dependent manner. The caspase-2 prodomain also regulates caspase-2 activity via a caspase recruitment domain that mediates oligomerization of procaspase-2 molecules and their subsequent autoactivation. In this study we sought to map specific functional regions in the caspase-2 prodomain that regulate its nuclear transport and also its activation. Our data indicate that caspase-2 contains a classical nuclear localization signal (NLS) at the C terminus of the prodomain which is recognized by the importin αβ heterodimer. The mutation of a conserved Lys residue in the NLS abolishes nuclear localization of caspase-2 and binding to the importin αβ heterodimer. Although caspase-2 is imported into the nucleus, mutants lacking the NLS were still capable of inducing apoptosis upon overexpression in transfected cells. We define a region in the prodomain that regulates the ability of caspase-2 to form dot- and filament-like structures when ectopically expressed, which in turn promotes cell killing. Our data provides a mechanism for caspase-2 nuclear import and demonstrate that association of procaspase-2 into higher order structures, rather than its nuclear localization, is required for caspase-2 activation and its ability to induce apoptosis.

Caspase activation is a key event in the execution of apoptosis (reviewed in Refs. 1 and 2). Based on structure and function, caspases are grouped into two main classes (reviewed in Refs. 3 and 4). The initiator caspases, including caspase-2, have a long prodomain that mediates their autoactivation during apoptosis. The effector caspases, including caspase-3, -6, and -7, have short prodomains and require cleavage by initiator caspases for their activation. Effector caspases are primarily responsible for the cleavage of a large number of cellular substrates which leads to the morphological changes that characterize apoptotic cell death (1–4). The long prodomain of caspase-2 contains a caspase recruitment domain (CARD), a six α-helical motif shared by other initiator caspases including CED-3 in the worm, DRONC in Drosophila, and caspase-9 in mammals (3–6). A structurally and functionally related motif, the death effector domain (DED), is found in the prodomains of some other initiator caspases including caspase-8 and -10 (3, 4). The proximal activation of initiator caspases in vivo is regulated by adaptor proteins that, like the caspases, contain CARDs or DEDs (3, 4). In response to an apoptotic stimulus, adaptor proteins bind to initiator caspases via their CARDs or DEDs and recruit them to specific death complexes. High local concentrations of procaspase molecules are generated, allowing their activation and autoprocessing. When expressed ectopically, however, activation of initiator caspases usually occurs spontaneously because of high intracellular concentrations of caspases (3, 4). Upon overexpression in transfected cells, via its CARD-containing prodomain, caspase-2 associates into distinctive dot-like and filamentous nuclear structures that may represent sites of caspase-2 homodimerization and activation (7–9). In a similar fashion, the DEDs of caspase-8 and -10 mediate their formation into structures in the cytoplasm called death effector filaments (10). Because of their propensity to homodimerize at high concentrations, overexpression of initiator caspases, including caspase-2, is sufficient to trigger apoptosis in transiently transfected cells (11–13). In fact, enforced dimerization of effector caspases is sufficient to trigger their activation (14–16).

Caspase-2 has been shown recently (17–20) to be the most proximal caspase in the caspase cascade activated during stress signaling. It appears to be required for mitochondrial permeabilization and the release of apoptogenic factors such as cytochrome c and Diablo (17–20). Although adaptor proteins have been described for most initiator caspases, an in vivo adaptor for caspase-2 has yet to be identified. Via its CARD, caspase-2 can interact with the CARD-containing proteins RAIDD (21, 22) and ARC (23), although the physiological significance of these interactions remains to be confirmed. Following gel filtration, endogenous caspase-2 elutes in high molecular weight fractions of cell lysates incubated at 37 °C, suggesting that caspase-2 has the ability to be recruited to and/or form a large protein complex in vivo, in a similar manner to the Apaf-1-mediated caspase-9 apoptosome (24).

One of the most distinguishing features of caspase-2 is its ability to localize to the nucleus constitutively (7, 25–28), although its role in this subcellular compartment is not known. A recent study (27) reports that caspase-2 is retained in the nucleus until the late stages of apoptosis, suggesting that the nucleus is the site of procaspase-2 activation. Supporting this, another study has shown that in the absence of cytosolic fac-

death effector domain; WT, wild type; PDM, caspase-2 prodomain; HA, hemagglutinin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
We also define a region in the prodomain at the N terminus of nucleus via the well characterized classical pathway erodimer, whereas a single point mutation abolishes binding, the GFP N-terminal fusion vector pEGFP-N1 (Clontech). To generate restriction sites, respectively. Amplified products were subcloned into the catalytically inactive mutant (C320G) caspase-2 (11, 12, 30) as a amplification using caspase domain. Caspase-2 mutants containing a K152A substitution – Asp169), CARD was ampli - 

This study focuses on dissecting the functional regions of the caspase-2 prodomain that modulate nuclear transport and enzime activation. By using various caspase-2 mutants, we define a classical NLS in the prodomain of mouse caspase-2 that contains a conserved Lys residue critical for its function. This newly characterized NLS is shown to be necessary and sufficient for nuclear transport of caspase-2. We provide evidence that this NLS strongly interacts with the importin α/β heterodimer, whereas a single point mutation abolishes binding, suggesting that caspase-2 is likely to be imported into the nucleus via the well characterized classical pathway in vivo. We also define a region in the prodomain at the N terminus of the CARD that regulates the formation of dots/filaments that correlate with the cell killing ability of caspase-2.

EXPERIMENTAL PROCEDURES

Expression Constructs for Localization Studies—WT and catalytically inactive C320G mutant mouse caspase-2 GFP and C2PD-GFP constructs have been described previously (7, 15, 26). Caspase-2 GFP fusions containing various prodomain deletions were generated by PCR amplification using Hire polymerase, with either the wild-type (WT) or the catalytically inactive mutant (C320G) caspase-2 (11, 12, 30) as a template and forward and reverse primers containing XhoI and BamHI restriction sites, respectively. Amplified products were subcloned into the GFP N-terminal fusion vector pEGFP-N1 (Clontech). To generate prodomain deletion mutants PDM125, PDM44, and PDMACARD, coding regions were amplified using appropriate primers. An initiation codon was inserted in the forward primers immediately 5’ of the codons encoding Ile64, Val65, and Leu122, respectively. To construct PDMLink, which contains only the CARD region (Met1–Thr121) and lacks the prodomain linker region (Leu122–Asp169), CARD was amplified and cloned into the XhoI and BamHI sites of pEGFP-N1. Next, the region encoding the caspase-2 p18 and p14 subunits (the caspase do-

Immunoprecipitation—For immunoprecipitation, cells were lysed on ice in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, and Complete protease inhibitors (Roche Molecular Biochemicals). Cellul-

The components of the yeast two-hybrid system were purchased from Clontech. Caspase-2 lacking the first 44 amino acids (PDM44) was amplified and subcloned into the pAS2.1 plasmid vector, and the Gal4 activation domain vector, pACT2. The pAS2.1-PDM44 and pACT2-PDM44 constructs were co-transformed into Saccharomyces cerevisiae strain Y190 as was pAS2.1-PDM44 and empty pACT2 vector and pACT2-PDM44 with empty pACT2 vector. Cells containing both vectors were selected on SD medium lacking Leu and Trp as described previously (13). Interacting fusion proteins were screened for β-galactosidase activity in a colony-lift filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) according to the instructions provided by Clontech. Positive and negative controls for two-hybrid assays were as described earlier (13).
The Caspase-2 Linker Region of the Prodomain Mediates Nuclear Localization—In previous studies (7) we have shown that nuclear transport of caspase-2 is mediated by the prodomain region of the molecule. When ectopically expressed, caspase-2 concentrates in the nucleus of transfected cells in distinct structures resembling dots or filaments. A potential bipartite NLS identified in the N terminus of the caspase-2 prodomain was shown by us to be necessary but not sufficient for nuclear transport of caspase-2 (7), suggesting the presence of an additional element(s) in the caspase-2 prodomain that is required for nuclear transport of caspase-2.

The caspase-2 prodomain is 169 amino acids in length and consists of a 34-residue N-terminal region (Met1–Pro34), a CARD (Asp35–Thr121), and a 48-amino acid region (Leu122–Asp169) linking the CARD to the enzymatic domain encompassing the p18 and p14 subunits (Fig. 1A). Because the first 34 amino acids in the prodomain do not appear to be involved in any specific function (7, 12, 13), we have divided the caspase-2 prodomain into two regions consisting of the CARD (Met1–Thr121) at the N terminus and the linker region at the C terminus (Leu122–Asp169). For localization studies, we generated a series of prodomain mutants containing a GFP tag at the C terminus (Fig. 1B).

To test whether the caspase-2 CARD alone is capable of mediating nuclear transport, we fused the mouse caspase-2 CARD directly to GFP and assessed the localization of the fusion protein in transfected COS cells. Unlike the caspase-2 prodomain-GFP fusion that forms elaborate filaments in the nucleus (Fig. 2A), the CARD-GFP construct is predominantly localized as perinuclear filamentous structures (Fig. 2B). Thus the CARD alone cannot localize efficiently to the nucleus in the absence of the linker region, despite the fact that it contains the bipartite NLS. Indeed, when the CARD is fused directly to the caspase domains, deleting the linker region (PDMΔLink), the GFP fusion protein forms aggregates in the cytoplasm (Fig. 2C), indicating that the CARD in conjunction with the caspase domains cannot mediate nuclear transport. On the other hand, a GFP fusion lacking the CARD but containing the linker region connected to the caspase domains (PDMΔCARD) localizes diffusely in the nucleus, in the absence of the previously characterized NLS (Fig. 2D).

To test further the ability of the linker region to direct nuclear transport, we fused the mouse caspase-2 linker region to caspase-3. Addition of the caspase-2 linker region to caspase-3 was sufficient to drive transport of the usually cytoplasmic caspase-3-GFP fusion protein wholly to the nucleus (Fig. 2, E and F), confirming that this region directs nuclear localization. Collectively, our localization data point to the linker region of mouse caspase-2 as containing an element(s) that is required for nuclear transport of caspase-2.

The Caspase-2 Prodomain Contains a Functional NLS in the Linker Region—Our deletion mutant analysis implicated a role for the linker region of the caspase-2 prodomain in mediating nuclear transport. An examination of the sequence of the caspase-2 prodomain linker region identified a putative NLS spanning residues 149–156 that is similar to the NLS of the c-Myc protein (29). This 8-amino acid region contains conserved Pro, Lys, and Leu residues (PPHKQLRL) that correspond to identical residues in c-Myc and that are conserved in human, rat, and mouse caspase-2 (Fig. 3A).

In order to determine whether this putative NLS is functional, we carried out site-directed mutagenesis studies. By analogy with the well characterized c-Myc NLS, we predicted that Lys152 would be a critical residue. Mouse caspase-2 mutants were generated in which this Lys152 residue was substituted with an Ala (K152A). In all cases, catalytically inactive forms of each fusion protein were used for localization studies to prevent cell killing. As shown in Fig. 3, K152A mutation resulted in a strikingly altered caspase-2 localization. Whereas caspase-2-GFP characteristically accumulates in the nucleus forming dots or filaments (Fig. 3B), the mutant caspase-2-GFP (K152A) was mostly localized outside and exclusive of the nucleus, forming dot-like aggregates in the cytoplasm (Fig. 3C). Interestingly, the K152A mutant retains the ability to form aggregates despite being restricted to the cytoplasm, suggesting that nuclear localization is not necessary for caspase-2 to form aggregates when overexpressed.

To determine whether mutation of Lys152 also affects nuclear localization...
localization of the caspase-2 prodomain, we generated a mutant caspase-2 prodomain-GFP (K152A) construct and transfected it into COS cells. As mentioned before, caspase-2 prodomain-GFP forms filament-like structures in the nucleus of transfected COS cells (Fig. 3D). Similar to the data obtained for the full-length molecule, K152A mutation resulted in caspase-2 prodomain-GFP localizing outside of the nucleus (Fig. 3E). The pattern of localization of the prodomain (K152A) mutant resembled that of CARD-GFP (compare Fig. 3E to Fig. 2B) with many cells exhibiting filamentous structures that lie outside and often around the nucleus. Thus, as for the full-length mutant, caspase-2 prodomain-GFP (K152A) is still able to form aggregates, in this case filament-like structures, despite its impaired nuclear localization. We also constructed a K152A mutant of the PDM CARD-GFP construct that normally localizes diffusely in the nucleus (Fig. 3F). Once again, mutation of Lys\(^{152}\) resulted in a localization pattern exclusive of the nucleus (Fig. 3G). Collectively, these mutagenesis studies clearly define another nuclear localization signal in the mouse caspase-2 prodomain in which Lys\(^{152}\) is essential for the nuclear localization of full-length caspase-2, the caspase-2 prodomain, and a mutant of caspase-2 lacking the CARD.

The Caspase-2 NLS Is Recognized by Importin \(\alpha/\beta\)-Dependent on Lys\(^{152}\)—NLSs normally mediate nuclear entry by conferring interaction with the cellular nuclear import machinery and, in particular, recognition by members of the NLS-recognizing importin superfamily (29). The best understood nuclear import pathways represent those mediated by either the importin \(\alpha/\beta\) heterodimer, where importin \(\alpha\) interacts with the NLS directly or importin \(\beta\) alone (29). To assess the ability of caspase-2 to be recognized by importins, GFP fusion protein expressing constructs were derived for the caspase-2 prodomain (residues 19–167) and the caspase-2 linker (residues 122–167) with or without the K152A mutation. Proteins were purified by affinity chromatography and then tested for their ability to bind to importin \(\alpha\), importin \(\beta\), or the importin \(\alpha/\beta\) dimer using native gel electrophoresis and fluorimaging. Both wild-type caspase-2 constructs were recognized by both importin \(\alpha\) alone and the importin \(\alpha/\beta\) heterodimer, as indicated by reduced mobility of the complexes of GFP fusion proteins with importins in the gel (Fig. 4). Binding of the importin \(\alpha/\beta\) heterodimer resulted in a greater shift in mobility than that of importin \(\alpha\) alone due to the larger molecular weight of the complex. Importin \(\beta\), in contrast, did not show any binding, consistent with the idea that caspase-2 contained a conventional NLS recognized by importin \(\alpha\), the importin \(\alpha/\beta\) heterodimer, and thus is completely comparable with the “classical” nuclear import substrate SV40 large T antigen. Results for the NLS of the latter fused to GFP in this respect are indicated in Fig. 4 (rightmost lanes and not shown); GFP alone did not show any shift in mobility due to the addition of importins (not shown). Experiments were also performed for caspase-2-GFP fusion protein constructs containing the single point mutation K152A (Fig. 4), results indicating a complete lack of binding of either importin \(\alpha\) or the importin \(\alpha/\beta\) heterodimer to the mutant proteins. Clearly, Lys\(^{152}\) is required for importin recognition of caspase-2, consistent with the idea that Lys\(^{152}\) is a critical residue within the second caspase-2 NLS. These results suggest that in \(\text{in vivo}\) caspase-2 nuclear localization is likely to be mediated by the classical importin \(\alpha/\beta\)-mediated nuclear import pathway.

CARD Is Necessary for Caspase-2 Localization to Dot-like and Filamentous Structures—As mentioned above, nuclear localization does not appear to modulate the ability of full-length caspase-2, caspase-2 prodomain, or the caspase-2 CARD to aggregate in dot- or filament-like structures. In previous work defining the first NLS of caspase-2, we observed that deletion of the first 44 amino acids of the caspase-2 prodomain (a deletion that removes most of helix 1 of the caspase-2 CARD and also deletes the putative bipartite NLS) not only impaired nuclear localization of the prodomain but also resulted in the loss of dot- and filament-like structures. We hypothesized that the first helix of the CARD is critical in mediating the ability of caspase-2 to localize in dot- or filament-like structures. To investigate this region further, we constructed deletion mutants of full-length caspase-2, where either the first 25 amino acids or the first 44 amino acids were deleted from caspase-2 and fused to GFP (Fig. 5A). As shown before, caspase-2-GFP forms dot-like structures in the nucleus (Fig. 5A). Removal of the first 25 amino acids had little effect on both nuclear localization of caspase-2-GFP and the presence of dots or filaments in the nucleus (Fig. 5B). However, deletion of the first 44 amino acids resulted in loss of dot-like or filamentous structures (Fig. 5C), defining residues 25–44 as important for promoting the assembly of caspase-2-GFP into dots and filaments. Because residues 35–44 comprise most of the first helix of CARD including several basic residues likely to participate in electrostatic interactions, we propose that the first helix of the caspase-2 CARD is required for the ability of caspase-2 to form dot- or filament-like structures.

Formation of Aggregates Is Not a Consequence of Caspase-2 Homodimerization—Because the PDM\(^{44}\) mutant lacks most of helix 1 of the CARD, we hypothesized that the loss of aggregate formation may reflect an inability of the PDM\(^{44}\) mutant to homodimerize via its CARD. We have demonstrated in yeast that caspase-2 can homodimerize in a prodomain-dependent manner (13). Co-immunoprecipitation experiments were carried out to evaluate whether the PDM\(^{44}\) mutant could ho-
modimerize in mammalian cells. GFP-tagged caspase-2 constructs were co-transfected with equal amounts of HA-tagged constructs and GFP fusions precipitated with an anti-GFP monoclonal antibody. As expected, both wild-type caspase-2 and the PDMΔ25 mutants co-precipitated their HA-tagged counterparts (Fig. 5D), demonstrating that caspase-2 can homodimerize in mammalian cells. Surprisingly, however, the PDMΔ44 mutant was also able to homodimerize, despite lacking part of the first helix of CARD. The ability of PDMΔ44 to homodimerize in mammalian cells suggests that homodimerization between two caspase-2 molecules does not require helix 1 of the CARD and that caspase-2 homodimerization does not correlate with aggregate formation.

To confirm this result, we performed yeast two-hybrid analysis on cells harboring pAS2.1 and pACT2 PDMΔ44 fusion constructs. Fig. 5E shows that co-expression of pAS2.1 and pACT2 fusion constructs results in a positive β-galactosidase assay (bottom panel), whereas cells harboring either fusion construct with the corresponding empty vector did not contain β-galactosidase activity (top and middle panels). Thus whereas PDMΔ44 is unable to form aggregates it is still capable of homodimerization. This indicates that homodimerization between two caspase-2 molecules is not necessarily sufficient in itself to mediate aggregate formation.

An Intact CARD but Not Nuclear Localization Is Necessary for Apoptosis-induced by Caspase-2 Overexpression—Our data suggest that aggregate formation that occurs upon caspase-2 overexpression requires helix 1 of the CARD, although dimerization is not dependent on this region. To determine whether the ability of caspase-2 to aggregate is important for cell killing, NIH3T3 cells were transfected with wild-type caspase-2 or the prodomain mutants, PDMΔ25 or PDMΔ44, and assessed for the presence of apoptotic morphology 24 h later. As shown previously, overexpression of caspase-2 kills NIH3T3 cells very efficiently (>95% death within 24 h) (Fig. 6). The PDMΔ25 mutant is slightly less effective than wild-type caspase-2, inducing cell death of ~70% of transfected cells. Interestingly, the PDMΔ44 mutant kills transfected cells very inefficiently with less than 20% of transfected cells displaying apoptotic morphology at this time point. Thus an intact CARD is essential for the formation of higher order caspase-2 structures as well as its ability to kill efficiently transfected cells, suggesting that formation of dots or filaments correlates with cell killing potential, at least in the context of the precursor molecule.

It could also be argued that the PDMΔ44 mutant cannot kill due to its impaired nuclear localization. However, the PDMΔLink caspase-2 mutant that lacks the newly identified NLS and does not localize to the nucleus was still able to induce...
significant levels of cell death (Fig. 6). In addition, the K152A mutants with impaired nuclear localization can also kill transfected cells at comparable levels to the wild-type versions (data not shown). Thus it is unlikely that impaired nuclear localization per se of the PDM44 mutant can explain its inability to kill transfected cells. This is consistent with the observation that caspase-2 aggregate formation still occurs in the absence of nuclear localization.

**DISCUSSION**

By immunofluorescence, endogenous caspase-2 is primarily localized to the nucleus and also the Golgi complex, showing a diffuse localization pattern (26, 28). In contrast, ectopically expressed caspase-2 is mostly nuclear, where it aggregates into structures resembling dots and filaments (7, 9, 27). By using various deletion mutants of the caspase-2 prodomain, we have been able to assign specific regions of the caspase-2 prodomain to the functions of apoptosis, nuclear localization, and the formation of nuclear and cytoplasmic aggregates (summarized in Table 1).

Our previous studies had identified a putative bipartite NLS that resides at the N terminus of caspase-2 and overlaps the first helix of the CARD (7). Deletion of this region results in the loss of nuclear localization as well as a loss of higher order structures, but it is not sufficient to direct nuclear transport of GFP (7). In this study we identify and characterize a second NLS present in the linker region of the caspase-2 prodomain. Our data show that in contrast to the previously proposed NLS, this signal is sufficient to drive nuclear import of another protein that normally resides in the cytoplasm. We identify a critical Lys residue within this NLS that is absolutely required for nuclear transport of caspase-2, suggesting that this sequence represents a genuine, functional NLS. Mutation of this Lys residue abolishes interaction with importin α and an importin α/β heterodimer in vitro, suggesting a mechanism for NLS-mediated caspase-2 nuclear import. Given that this second NLS is sufficient to mediate nuclear transport, the role of previously identified N-terminal bipartite NLS is currently unclear.

Our findings of the second NLS are consistent with a recent study (27) that reported a homologous sequence in the human caspase-2 prodomain. Two Lys residues were mutated in this sequence to demonstrate a functional role in nuclear transport of caspase-2. Comparison of the human and mouse caspase-2 NLS sequences reveals the presence of only a single Lys in the mouse sequence that is also conserved in the rat. This Lys corresponds to the same position of the critical Lys in the c-Myc NLS. Thus by first establishing the conserved nature of the caspase-2 NLS, we have been able to distinguish exactly what Lys residue is the most critical component of this signal. More importantly, we have been able to propose a mechanism for caspase-2 nuclear import in vivo by demonstrating interaction of the caspase-2 NLS with the importin-α moiety of the importin α/β heterodimer. Together our results suggest that caspase-2 is imported into the nucleus via this NLS by the same mechanism used by well characterized nuclear import substrates such as the SV40 T antigen, c-Myc oncoprotein, and the retinoblastoma tumor suppressor protein (29).

What is the role of caspase-2 in the nucleus? Surprisingly, the caspase-2 mutants defective in nuclear localization are still able to form aggregates and induce cell death upon overexpression, suggesting that nuclear localization may be dispensable for caspase-2 function. As these data were obtained using ectopically expressed protein, it remains formally possible that endogenous nuclear caspase-2 serves a specific function in apoptosis. One possibility is that caspase-2 may be sequestered in the nucleus to prevent its unfettered activation, given its propensity to oligomerize and autoactivate. As we demonstrate in this study, however, caspase-2 forms dots and filaments in the nucleus that correlate with cell killing ability, suggesting that this is the site for procaspase-2 activation. Supporting this, a recent study shows that caspase-2 is retained in the nucleus until the late stages of cell death and that apoptosis can be triggered by caspase-2 overexpression in the presence of leptomycin B, an inhibitor of nuclear export (27).

With these studies in mind, caspase-2 may serve as a sensor in the nucleus for receiving signals that are transmitted via this subcellular compartment. If true, we would predict that caspase-2 must cleave at least one nuclear substrate to trigger cell death. It is therefore puzzling that caspase-2 overexpression still kills cells when nuclear localization of caspase-2 is abrogated, suggesting that there must be a similar caspase-2 substrate in the cytoplasm. In one study, Bid translocation to mitochondria and cytochrome c release occurred while caspase-2 remained in the nucleus suggesting that nuclear caspase-2 causes mitochondrial permeabilization indirectly (27). In contrast, other studies (18, 19) have shown that either nuclear or recombinant caspase-2 is sufficient to cause cytochrome c release from isolated mitochondria directly. Thus, caspase-2 seems to be able to induce mitochondrial permeabilization both indirectly from the nucleus as well as directly when supplied exogenously.

Deletion of the first 44 amino acids of caspase-2 resulted in caspase-2-GFP localizing diffusely in the cytoplasm without forming dots and filaments. Although disrupted in its nuclear localization, caspase-2 aggregation still occurs in the absence of nuclear localization.
localization, we have shown that nuclear localization is not required for caspase-2 to form higher order structures. Loss of higher order structure formation correlated with a loss of cell killing ability, suggesting that formation of dots/ fila ments is a visual indicator of caspase-2 activity and consequent cell death. Surprisingly, despite its inability to aggregate into higher order structures and its inability to kill cells, this mutant was still capable of homodimerization. These observations suggest that although caspase-2 aggregation into a large complex is required for caspase-2 to form higher order structures. Loss of aggregation are distinct events.

One possibility is that in overexpressing cells, formation of aggregates represents oligomers of dimerized procaspase-2 molecules. Supporting this idea, gel filtration analysis shows that purified recombinant caspase-2 elutes as an oligomeric complex, the formation of which is dependent on the prodomain. This suggests that caspase-2 may not require an adaptor molecule for its oligomerization and activation as high localized concentrations in vivo would be sufficient to form a large complex, and formation of a large complex may be sufficient for caspase-2 autoactivation (24). Thus CARD-dependent aggregation is a likely mechanism for procaspase-2 activation and apoptosis induced by caspase-2 overexpression. As caspase-2 activation occurs rapidly in response to a variety of cell death signals (34), oligomerization may be an efficient mechanism to mediate activation in vivo. However, the mechanisms that facilitate and control caspase-2 oligomerization in vivo remain to be discovered.

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