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The role of caspase-2 in stress-induced apoptosis

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Abstract

Caspase-2 is the most evolutionarily conserved of all the caspases, yet it has a poorly defined role in apoptotic pathways. This is mainly due to a dearth of techniques to determine the activation status of caspase-2 and the lack of an abnormal phenotype in caspase-2 deficient mice. Nevertheless, emerging evidence suggests that caspase-2 may have important functions in a number of stress-induced cell death pathways, in cell cycle maintenance and regulation of tumour progression. This review discusses recent advances that have been made to help elucidate the true role of this elusive caspase and the potential contribution of caspase-2 to the pathology of human diseases including cancer.

Keywords: caspase-2 • apoptosis • PIDD • RAIDD • tumour suppressor

Caspase function

Apoptosis allows for the removal of defective, redundant, infected, transformed or senescent cells from a population. The genetically controlled pathways that regulate apoptosis are prone to dysfunction; therefore, defects in these pathways contribute to diverse pathological conditions including cancer, diabetes, AIDS, ischemia-reperfusion injury and neurodegenerative disorders. The contribution of apoptosis to pathology of disease is often based on molecular data from patients or mice with defects in known regulators of apoptosis. Among these key regulators of apoptosis is the caspase family of proteases that mediates the proteolytic disassembly of the dying cell *via* cleavage of specific substrates [1].

Caspases are normally present in cells as inactive precursors that are activated at the onset of and during the progression of apoptosis. However, their functions are not restricted to cell death regulation. For example, caspase-1 cleaves the pro-inflammatory cytokines, pro-intereukin-1β and pro-interleukin-18 to their mature forms and has fundamental roles on variety of aspects of innate immune function [2]. Caspases therefore have diverse roles in protecting the body from disease [3].

The caspases can be divided into two groups: initiator caspases, including caspase-1, -8, -9 and -10, and executioner caspases, such as caspase-3 and -7 [4]. Executioner caspases are responsible for the cleavage of numerous structural and regulatory proteins, required to dismantle the cell [1]. Initiator caspases are so-called because they are the first to be activated in a pathway. For example, many genotoxic stresses trigger the activation of caspase-9 by engaging the mitochondrial pathway, inducing mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release [5]. Caspase-8, in contrast, is activated by death receptors, such as Fas and TNFR1 [6]. These initiator caspases function primarily to cleave and activate executioner caspases, but they can cleave additional proteins to promote apoptosis. For

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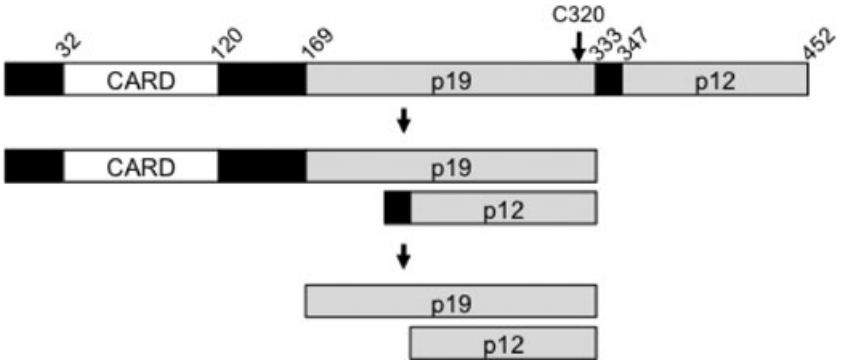

Fig. 1 Schematic of the caspase-2 protein. The proposed mechanism of caspase-2 processing that occurs upon dimerization is shown. Note only one member of the dimer is depicted. C320 represents the active site cysteine.

example, in some cell types caspase-8 cleaves the BH3-only protein Bid to promote MOMP [7].

Initiator caspases are activated by induced proximity of inactive monomers to form an active dimer [8, 9], which is facilitated by the recruitment of initiator caspases to large molecular weight protein complexes that can be considered 'activation platforms'. Examples of known activation platforms are the death-inducing signalling complex (DISC) for caspase-8 and the Apaf-1 apoptosome for caspase-9 [10].

Caspase-2: initiator or executioner caspase?

Although caspase-2 is the most evolutionarily conserved of all the caspases [11], it has a poorly defined role in apoptosis. It has been difficult to formally characterize caspase-2 as an initiator or an executioner caspase because it possesses features of both groups. The substrate specificity of caspase-2 has more in common with executioner caspases than initiator caspases. All caspases cleave after an Aspartic acid residue (termed the P1 position) but have different preferences for the 3–4 amino acids immediately preceding the Aspartic acid (P2-P5). Caspase-2, like caspase-3 and -7, shows a strong preference for an Aspartic acid residue in the P4 position of its substrates while other caspases can tolerate different amino acids in this position [12, 13]. On the other hand, caspase-2 shares sequence similarities with initiator caspases. The initiator caspases all possess long prodomains that contain a protein:protein interaction domain such as a caspase recruitment domain (CARD) or a death effector domain [14, 15]. Homotypic interactions between adaptor proteins and caspases containing CARDs and death effector domains govern the recruitment of initiator caspases to their activation platforms to result in caspase activation. Caspase-2 contains a CARD in its prodomain suggesting that it is activated similar to initiator caspases [15].

In cell lysates caspase-2 is spontaneously recruited to a large molecular weight protein complex in a CARD-dependent manner suggesting that caspase-2 is activated by dimerization [16]. Upon dimerization, the caspase-2 precursor promotes its own cleavage to generate the fully processed enzyme (Fig. 1). First, caspase-2 is auto-processed at D333 separating it into a large (p37) and a small (p14) subunit that remain associated. Further processing at D169 and D347 removes the prodomain and interdomain linker respectively, generating the p19/p12 heterodimer [17, 18]. Kumar and colleagues demonstrated that although cleavage of caspase-2 is required for full activity of caspase-2, a non-cleavable mutant of caspase-2 retains 20% activity of the wild-type protein. In contrast, only dimeric species of caspase-2 are active, in the sense that they have the ability to cleave substrate. Therefore cleavage of caspase-2 is not required for the initial activation of the caspase. Rather it appears that autocatalytic processing of caspase-2 stabilizes the active enzyme [19]. This manner of activation by dimerization supports caspase-2 being a member of the initiator caspase group.

However, unlike other initiator caspases, caspase-2 does not directly activate executioner caspases. Caspase-2 has been shown to cleave caspase-7, but only at lower than cellular pH and therefore it may not be a physiologically relevant cleavage event [20]. Instead, caspase-2 activates the executioner caspases indirectly by inducing MOMP. Caspase-2 appears to induce MOMP through cleavage and activation of the pro-apoptotic Bcl-2 family protein Bid [21, 22]. Bid, in turn induces release of cytochrome c from mitochondria and subsequent apoptosis (Fig. 2). Thus caspase-2 appears to be activated upstream of the mitochondrial pathway. Supporting these observations a number of independent studies have shown that caspase-2-induced apoptosis is blocked by the anti-apoptotic protein Bcl-xL that prevents MOMP [21, 23, 24].

Caspase-2 is also processed downstream of the mitochondria. Caspase-2 cleavage was observed in cytochrome c-activated cell lysates and this was shown to be a result of caspase-3-mediated cleavage of caspase-2 [25]. Furthermore, caspase-2 cleavage induced by etoposide and γ-irradiation was not detected in Apaf-1 or caspase-9 deficient cells indicating that this incidence of caspase-2 processing is dependent on apoptosome activation [26, 27]. As discussed, most evidence suggests that caspase-2 activation occurs prior to MOMP but

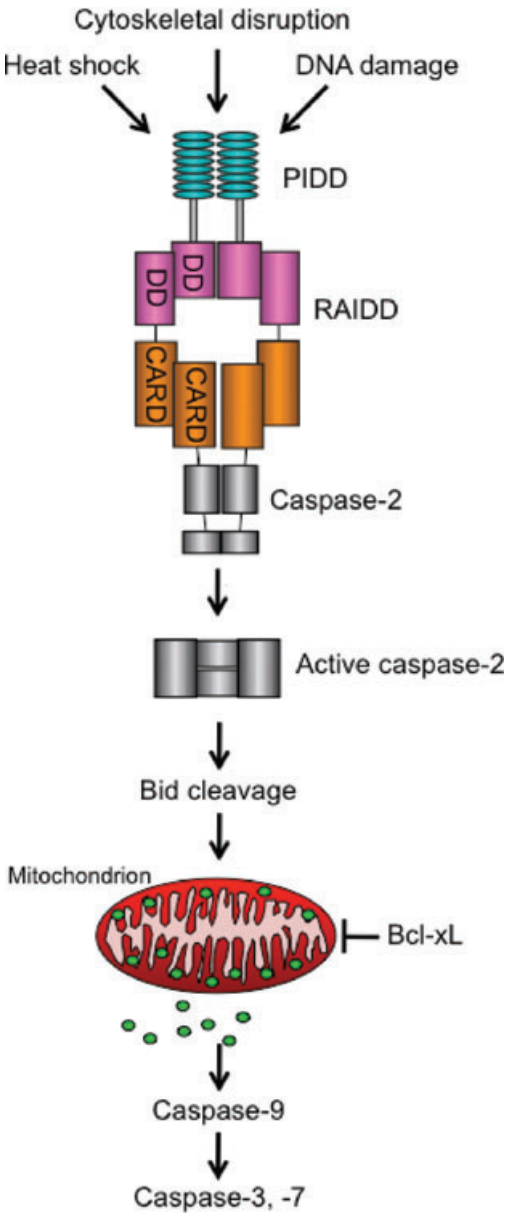

Fig. 2 The caspase-2 activation pathway. A schematic representation of the proposed mechanism for capase-2 activation by the PIDDosome. Inactive caspase-2 monomers are recruited to the PIDDosome in response to certain cellular stresses. This results in dimerization and activation of caspase-2. Caspase-2 cleaves and activates Bid to induce MOMP eventually resulting in activation of executioner caspases by caspase-9-mediated cleavage.

there may be context or cell type-specific events where caspase-2 is activated downstream of the mitochondria. However, currently there is no conclusive evidence that the cleavage of caspase-2 downstream of MOMP is sufficient to activate the caspase and this is often considered to be a bystander cleavage event during apoptosis.

Functions of caspase-2

Role in development

The requirement of caspase-2 in regulating cellular processes and its contribution to pathology of disease has been difficult to determine, primarily because caspase-2 deficient mice survive normally and do not exhibit any prominent developmental defects [28]. This is in contrast to the lethal phenotypes associated with deficiencies in caspase-8 and -9 [29].

The only developmental abnormality associated with caspase-2 deficiency is that the female mice have excess oocytes [28]. These mice retain a strong neomycin resistance cassette in their genome that may produce defects in expression of neighbouring genes and therefore account for or enhance the observed phenotype. It has not been published if caspase-2 deficient mice that have been independently generated also possess excess oocytes [26]. Nevertheless, a role for caspase-2 in oocyte development is supported by an observation that oocytes from caspase-2 deficient mice were resistant to DNA damage-induced apoptosis [28] and studies in Xenopus oocyte extracts [30]. Oocytes have low levels of glycolysis and glucose metabolism proceeds primarily through the pentose phosphate pathway. Inhibition of the pentose phosphate pathway in Xenopus oocyte extracts was shown to induce caspase-2 activation and apoptosis, suggesting caspase-2 is metabolically regulated [30].

Caspase-2 in aging

Despite the subtle phenotype of caspase-2 deficient mice at birth, they appear to have certain aging-related defects. The maximum lifespan of caspase-2 null mice was reported to be approximately 10% shorter than wild-type mice. Older caspase-2 deficient mice displayed aging-dependent bone loss that was partially due to enhanced bone resorption. Additional aging-related traits were reported to be associated with loss of caspase-2 including reduced hair regrowth, lower body fat content and increased protein oxidation [31]. These defects may be a result of compromised apoptosis due to the loss of caspase-2 but this has not been shown directly. Nonetheless the observed defects likely contribute to the shorter lifespan of caspase-2 deficient animals, implicating a role for caspase-2 in aging.

Caspase-2 is a tumour suppressor

Recent evidence suggests that caspase-2 may have a role in the suppression of tumorigenesis. Loss of caspase-2 in a lymphoma

model (induced by transgenic c-Myc on the μ-enhancer (Eμ-Myc)) greatly accelerated tumour growth [32]. Furthermore, caspase-2 deficient mouse embryonic fibroblasts (MEF) showed evidence of enhanced transforming potential *in vitro* and have increased genomic instability [32]. Thus caspase-2 may be an important safeguard against tumour generation. One of the hallmarks of tumorigenesis is that cancer cells avoid apoptosis, thus caspase-2 may suppress tumours by inducing apoptosis of potential tumour cells. Alternatively, caspase-2 may suppress tumour growth by inducing cell-cycle arrest. Consistent with a potential cell-cycle function, caspase-2 null MEF have been reported to proliferate at higher rates [32]. Furthermore, caspase-2 has been observed to induce cell-cycle arrest after DNA damage [32, 33]. It is not clear if the observed tumour suppression is a result of the induction of apoptosis or cell-cycle arrest by caspase-2. In addition, the downstream targets of caspase-2 in tumour suppression have yet to be fully determined.

Caspase-2 substrates

As discussed, caspase-2 appears to induce apoptosis by cleaving Bid. A number of other proteins have been identified as targets of caspase-2 but in many cases their impact on caspase-2-mediated apoptosis or on additional functions of caspase-2 (*e.g.* cell cycle arrest) is unclear.

Golgin-160 is a Golgi membrane protein that has been described as a substrate for caspase-2. Cleavage of golgin-160 contributes to the fragmentation of the Golgi apparatus during apoptosis. Golgin-160 is also cleaved by caspase-3 and -7; however, caspase-2 has been detected at the Golgi apparatus and therefore may have more access to golgin-160 than other caspases [34]. The role of golgin-160 in normal (non-apoptotic) conditions is unclear but expression of a version of this protein that is resistant to caspase cleavage has been shown to protect cells from stress-induced apoptosis [35]. This suggests that golgin-160 cleavage may induce apoptosis directly rather than merely contributing to the execution phase of apoptosis by mediating organelle destruction.

A recent study suggests that RIP1 is a proteolytic target of caspase-2 and that cleavage of RIP1 by caspase-2 contributes to its tumour suppressive function [36]. RIP1 functions to induce NFκB activation in response to tumour necrosis factor (TNF)-α and DNA damage [37, 38]. Among the many NFκB target genes is survivin, which is a member of the IAP protein family. Survivin is an essential mitotic gene that is indispensible for several steps in cell division and is expressed at high levels in the majority of human tumours [39]. Thus caspase-2-mediated cleavage of RIP1 has been proposed to inhibit NFκB activation resulting in silencing of survivin gene expression and suppression of tumour growth [36].

A number of additional proteins have been proposed to be substrates for caspase-2 including ICAD, PARP, BAD, the transcription factor CUX1 and protein kinase Cδ [40–44] (for a full list see [45]). Many of these observations are based on *in vitro* cleavage assays using recombinant caspase-2 and need to be verified in cells. Furthermore all of these proteins can also be cleaved by caspase-3 and/or caspase-7, thus the physiological relevance of caspase-2 mediated cleavage of these substrates is unclear.

Measuring caspase-2 activation during apoptosis

There is a large literature describing stimuli that potentially activate caspase-2 during apoptosis but the ability of these to actually engage caspase-2 is in many cases still debated [46, 47]. A number of these agents are described in Table 1 and include stimuli that induce DNA damage, cytoskeletal disruptors, ER stress, death receptor ligation and certain forms of neuronal stress including trophic factor withdrawal and β-amyloid treatment [48–53].

Much of the controversy regarding the stimuli and pathways that activate caspase-2 can be attributed to different methods used to assess caspase-2 activation (Table 1). As discussed, cleavage does not initiate activation of caspase-2 and it is likely that cleavage alone is not sufficient to activate caspase-2. Therefore it is difficult to interpret conclusions that are based solely on measuring caspase-2 cleavage by Western blot. Similarly, assays using fluorogenic substrates or inhibitors based on the preferred cleavage site for caspase-2, VDVAD, to indicate specific activation of caspase-2 are problematic since this site can also be efficiently cleaved by caspase-3 [54]. A protein-based inhibitor termed AR-F8 has been developed that inhibits caspase-2 activity with a high degree of specificity and may be a superior alternative to the classical VDVAD-based inhibitor. AR-F8 is an ankyrin repeat based protein modified to bind the surface of caspase-2 such that it stabilizes a conformation that renders the active site inaccessible to substrates [55]. The disadvantage of AR-F8 is that it is protein-based and hence not readily taken up into cells and is therefore not ideal for *in vivo* use. However, this inhibitor may provide a basis for the development of novel small molecule cell-permeable inhibitors with specificity for caspase-2.

Specific knockdown of caspase-2 by siRNA is another tool that is widely used to study the caspase-2 pathway. Lazebnik and colleagues observed that knockdown of caspase-2 using siRNA resulted in resistance to etoposide-induced death [48]. However, it was subsequently shown that additional siRNA sequences targeting caspase-2 did not have the same effect suggesting that off-target effects of the siRNA may have contributed to resistance of the cells to etoposide-induced apoptosis. Moreover, studies using caspase-2 deficient cells indicate that caspase-2 is not essential for DNA damage-induced apoptosis by etoposide, dexamethasone or γ-irradiation in thymocytes or by etoposide or UV in MEF [26, 56].

RNAi of caspase-2 has been similarly employed to conclude that caspase-2 is required for apoptosis induced by ER stress by Brefeldin A [50] but caspase-2 deficient cells are susceptible to a number of stimuli that engage ER stress pathways [56]. Aside from

Table 1 Examples of stimuli that have been reported to activate caspase-2

Drug type	Examples	Relationship to MOMP	Cell type	Assay used to measure caspase-2
DNA- damage inducers	Etoposide	post-MOMP (Apaf1-dependent) [27] or pre-MOMP [48, 106]	Jurkat [27, 106], IMR90E1A [48], Hela [23]	Cleavage [27, 106], siRNA [48], VDVAD*, VDVADase† [106], BiFC [23]
	Cisplatin	Pre-MOMP [107, 108]	LLC-PK1 [107], Caov4 (express p53) [108]	VDVAD [107], VDVADase, cleavage [108], siRNA [107, 108]
	5-FU	Pre-MOMP	HCT116	VDVAD, VDVADase, siRNA [109]
	Doxorubicin	ND	Mouse oocytes	Resistance of Caspase-2-/- cells [28]
	γ-irradiation	Bcl2 insensitive death in absence of CHK1 [91], Post-MOMP [26]	HEK293 [33], zebrafish embryos with silenced CHK1 [91], thymocytes [26]	bVAD pulldown [33], cleavage [26, 33], c2 morpholino [91]
	Daunorubicin	Pre-MOMP (VDVAD blocks cyt c release)	H1299 (expressing p53)	VDVAD, siRNA [110]
	UV	Post-MOMP (requires Bax)	HCT116	Cleavage [111]
Cytoskeletal disruptors	Zoledronic acid	Pre-MOMP	MEF [49]	Resistance of caspase-2-/- cells [49]
	Cytochalasin D,	Pre-MOMP	MEF [49], hela [23]	Resistance of caspase-2-/- cells [49], BiFC [23]
	Vincristine	Pre-MOMP	MEF [49], hela [23]	Resistance of caspase-2-/- cells [49], BiFC [23]
	Paclitaxel	Pre -MOMP	MEF [49]	Resistance of caspase-2-/- cells [49]
Neuronal stress	β-amyloid	ND	Hippocampal neurons, sympathetic neurons, PC12	Cleavage, antisense, resistance of caspase-2-/- cells [53]
	Transient global ischemia	Pre-MOMP (PIDD-dependent Bid cleavage)	hippocampal CA1 neurons.	Cleavage [112]
	Hypoxia	ND	SK-N-MC	Cleavage [113]
	NGF withdrawal	Post-MOMP (inhibited by Bcl2) [114]	Neuronal PC12 cells, sympathetic neurons	Cleavage [59], antisense [52, 114]
	NGF	ND	Mature oligodendrocytes	Cleavage [115]
ER stress	Brefeldin A	Pre-MOMP	Bax/Bak deficient MEF, Hela	Cleavage and siRNA [50, 116]
	Tunicamycin	Pre-MOMP (cleavage not blocked by Bcl2)	U937, Hela	Cleavage, VDVADase [117] siRNA [116]
	Bortezomib	Pre-MOMP	Human myeloma (H929, 8226/S)	siRNA, VDVAD [118]
	Sorafenib	ND	U937	siRNA [119]

Continued

Table 1 Continued

Drug type	Examples	Relationship to MOMP	Cell type	Assay used to measure caspase-2
Oxidative Stress (ROS)	HDAC inhibitors LAQ-824 [120], SAHA, oxamflatin, depsipeptide [121]	Pre-MOMP (blocked by Bcl2) [121]	U937 [120], CEM [121]	Cleavage [121], siRNA [120]
	Helicobacter pylori	ND	AGS epithelial cells	FAM-VDVAD-FMK [122]
	2,3-dimethoxy-1,4-naph-thoquinone (DMNQ)	Pre-MOMP	Neural stem cells C17.2	VDVADase, VDVAD [123]
	Dopamine		Rat N27 cells	VDVADase [124]
Death receptors	TRAIL	Pre-MOMP (bid cleavage) [125]	Jurkat (J16,Fas sensitive clone), HCT116	siRNA [125, 126]
	Anti-Fas	Pre-MOMP [51]	SKW6.4, U937, Jurkat	Cleavage, VDVADase [76] antisense [51]
	TNF	Independent? (cleaved by caspase-3) [127]	Human fibroblasts [127], Hela [23]	Cleavage [127], BiFC [23]
Miscellaneous	Heat shock	Pre-MOMP	mouse splenocytes, Jurkat [24], Hela [23]	bVAD pulldown [24], BiFC [23]
	Brucella abortus	ND	Macrophage	VDVAD, shRNA [128]
	Resveratrol	Pre-MOMP	HCT116	Cleavage, VDVADase, anti-sense [129]
	PRIMA-1met (reactivates mutant p53)	Pre-MOMP	H1299	VDVADase, VDVAD, siRNA [130]
	Granzyme B		lymphoblasts	Resistance of Caspase-2-/- cells [28]
	BCR ligation	ND	B104 (B cell lymphoma)	Cleavage, VDVAD [131]

*VDVAD refers to inhibition using VDVAD.fmk.
†VDVADase refers to activity based on cleavage of fluorogenic conjugated VDVAD.

potential off-target effects of siRNA targeting sequences, there are a number of additional reasons that may explain the differences in results between the two approaches. Caspase-2-dependency of a specific pathway or stimulus may vary according to cell type due to differential expression of caspase-2 regulatory factors (see below). Similarly, there may be different requirements for caspase-2 in mouse cells compared to human cell lines. Studies of cells from caspase-3-deficient mice show that the apoptotic responses of the deficient cells vary greatly depending on the genetic background of the mice [57, 58]. Furthermore, compensatory mechanisms may exist in caspase-2 deficient animals. For example, silencing of caspase-2 in PC12 cells and primary sympathetic neurons using antisense oligonucleotides led to resistance to trophic factor withdrawal-induced apoptosis suggesting an important role for caspase-2 in neuronal death [53, 59]. However, sympathetic neurons from caspase-2 deficient animals showed no resistance to nerve growth factor withdrawal [28]. This was subsequently demonstrated to be due to the ability of caspase-9 to compensate for deficiency of caspase-2. Caspase-9 appears to be expressed at higher levels in brain and sympathetic neurons from caspase-2 null animals. Consequently, down-regulation of caspase-9 rescued caspase-2 deficient sympathetic neurons from nerve growth factor withdrawal induced apoptosis, while caspase-9 knockdown in wild-type cells had no effect [60]. Thus silencing of caspase-2 using RNAi or antisense techniques can reveal functions of caspase-2 that can be masked by the development of parallel compensatory pathways in caspase-2 deficient animals.

Activation of caspase-2 in certain scenarios may not be sufficient to commit a cell to apoptosis but rather has additional consequences for the cell such as cell-cycle arrest. For example, Shi *et al.* demonstrated that γ-irradiation enhanced caspase-2 activation at doses that did not induce apoptosis [33]. The method used to measure caspase-2 activity in this study was an *in situ* trapping and labelling technique known as bVAD pulldown.

The bVAD pulldown approach uses a biotinylated irreversible pan-caspase inhibitor VAD (bVAD) that covalently binds to the first caspase activated in response to a stimulus. Thus the initiator caspase can be pulled down and identified using streptavadin.

This method was also used to identify caspase-2 as the initiator caspase activated in response to heat shock [24]. In cells exposed to heat shock, bVAD pulled down full-length caspase-2, indicating that the active form of caspase-2 is not processed. Although informative, this approach requires a large number of cells and thus is not suitable for many cell types. In addition, zVAD.fmk, the inhibitor that bVAD is derived from, does not inhibit caspase-2 as efficiently as other caspases [61]. Consequently, in cases of incomplete inhibition of caspase-2 by bVAD, downstream caspases would be identified by bVAD, precluding an accurate measurement of caspase-2 activity.

To circumvent some of these problems, we recently described a technique to directly measure caspase-2 activation in single cells [23]. Using bimolecular fluorescence complementation (BiFC) we measured the first step in the caspase-2 activation pathway; recruitment to its activation platform. BiFC uses 'split' fluorescent proteins such as Venus, which is a brighter more photostable version of YFP [62]. Venus can be split into fragments that alone are not fluorescent, but maintain the ability to refold into the intact fluorescent protein when in sufficient proximity. The refolding of split Venus fragments is rapid; in one study it was detected in less than five minutes. Thus it is highly suitable for single-cell imaging [63, 64]. Refolding of split YFP (an earlier incarnation of this technique), in contrast, requires over 30 min. to reach completion [63]. When caspase-2 was fused to each half of split Venus, recruitment of caspase-2 to its activation platform and subsequent induced proximity resulted in enforced association of the two halves of Venus that was directly measured as an increase in fluorescence. Using this approach, caspase-2 recruitment to its activation platform was observed in response to heat shock, DNA damage by etoposide, cytoskeletal disruption by vincristine and inhibition of the pentose phosphate pathway by DHEA [23]. The advantage of using the BiFC approach over other methods used to measure caspase-2 activation is that it allows direct visualization of the caspase-2 activation platform in cells. Therefore it enabled accurate measurement of *when* caspase-2 was activated and *where* in the cell it was activated. For example, when Hela cells expressing the caspase-2 BiFC components were exposed to a lethal heat shock temperature, we observed induced proximity of caspase-2 in the cytosol as a series of fluorescent spots that began to appear approximately 5 hrs after stimulation and prior to MOMP. Although caspase-2 BiFC is a powerful technique to measure caspase-2 activation in cells it needs to be carefully controlled to verify specificity of interaction. Thus we determined that disrupting the binding between caspase-2 and its adaptor protein RAIDD dramatically decreased heat shock-induced caspase-2 BiFC.

The experimental systems involving bVAD pulldown and caspase-2 BiFC both suggest an apical role for caspase-2 in heat shock-induced cell death. However, a number of studies, primarily using caspase-2 deficient cells, fail to see a requirement for caspase-2 in heat shock-induced apoptosis [56, 65, 66]. Heat shock is a quite a broad stimulus of cell death and likely engages apoptosis pathways in a number of ways. For example, heat has been shown to directly activate Bax and Bak, which would bypass the requirement for caspase-2 [67]. In addition, heat has been shown to induce up-regulation of FasL in activated T lymphocytes through activation of the transcription factor heat shock factor 1 (HSF-1), to induce death in target cells [68]. Caspase-2-deficient lymphocytes treated with FasFc to block the Fas pathway were shown to be profoundly resistant to heat shock-induced apoptosis compared to wild-type cells [24]. Together these results suggest that a caspase-8-dependent component may contribute to heat shock-induced apoptosis in certain cell types. Overall, the precise role of caspase-2 in heat shock and the physiological relevance of caspase-2 in heat shock pathways have yet to be clarified. However, as has been observed in the case of DNA-damage-induced caspase-2 activation, the primary function of caspase-2 activation in response to heat shock may not be in the engagement of apoptosis but to regulate additional, as yet unidentified, cellular events.

Caspase-2 activation platforms

Caspase-2, like other initiator caspases is activated by dimerization upon recruitment to its activation platform [19]. The activation platform for caspase-2 appears to be a complex of proteins termed the PIDDosome. The PIDDosome consists of the proteins PIDD, RAIDD and caspase-2. When activated, PIDD binds and induces oligomerization of the adaptor protein RAIDD [69]. The binding is mediated through a protein-protein interaction motif present in both proteins known as a death domain (DD). RAIDD, in turn, binds to caspase-2 *via* a heterotypic CARD:CARD interaction [70]. This results in induced proximity of caspase-2 molecules facilitating dimerization (Fig. 2). The crystal structure of the PIDD DD and RAIDD DD core complex of the PIDDosome suggests that five DDs of PIDD bind seven DDs of RAIDD [71]. This structure predicts that seven caspase-2 molecules are recruited to the PIDDosome in total.

A second caspase-2 activation platform has been identified to contain PIDD, caspase-2 and the nuclear serine/threonine kinase DNA-PKcs and does not require RAIDD. This so-called nuclear PIDDosome seems to function primarily in the maintenance of a cell cycle checkpoint rather than apoptosis [33]. The existence of a nuclear PIDDosome is consistent with numerous reports that have shown caspase-2 localized to the nucleus and suggest that it functions from the nucleus [72–74]. The caspase-2 sequence contains a classical nuclear localization sequence and mutation of this region prevents caspase-2 entry into the nucleus [72]. Surprisingly, using caspase-2 BiFC we found was that active caspase-2 was located in the cytoplasm in response to etoposide and other stimuli regardless of the presence or absence of the nuclear localization sequence, suggesting that caspase-2 is not activated in the nucleus [23]. However, activation of capase-2 in the nucleus may be relatively low compared to RAIDD-dependent activation of caspase-2 in the cytosol and thus the caspase-2 BiFC approach may not be sensitive enough to detect active caspase-2 in the nucleus. Indeed, Shi *et al.* reported that there is a constitutively active pool of caspase-2 in the nucleus of untreated cells [33] and it is not clear if this pool could be detected using the caspase-2 BiFC methodology.

Similar to caspase-2, when PIDD is knocked out in mice no dramatic phenotype is observed. Moreover, PIDD null thymocytes are susceptible to a number of stimuli that induce apoptosis, including heat shock, DNA damaging agents such as etoposide, cisplatin and γ-irradiation or others including taxol, sorbitol, cytokine withdrawal or TNF-α [56, 75]. These studies suggest that PIDD is not essential for caspase-2 activation-induced apoptosis indicating that additional caspase-2 activation platforms may exist.

For example, when RAIDD was discovered it was initially considered to be a component of the TNF-receptor complex, thus proposing a potential platform to activate caspase-2 [70]. However, there is little conclusive evidence that caspase-2 is robustly activated directly by the TNF DISC. Furthermore deficiency of RAIDD had no effect on TNF-induced death of MEF [76].

Recruitment of caspase-2 to the Fas DISC was observed in T and B cells, but was not able to initiate FasL-induced apoptosis in the absence of caspase-8 [77]. A recent report suggests that caspase-2 is recruited to the Fas DISC during DNA damage-induced apoptosis. Treatment of HCT116 cells with 5-fluorouracil (5-FU) reportedly induced p53 up-regulation of Fas and cleavage of caspase-2 by caspase-8 at the DISC [78]. However, it was not determined that caspase-2 recruitment to the DISC was required for 5-FU-induced death in these cells, thus the significance of caspase-2 as a component of the Fas DISC is unclear.

Other reports suggest that caspase-2 is recruited to promyelocytic leukaemia protein nuclear bodies (PML-NBs) in the nucleus [79, 80]. PML-NBs are nuclear macromolecular complexes that potentially regulate multiple cellular processes including apoptosis [81]. PML-NBs act as scaffolds for numerous proteins and immunofluorescence studies show co-localization of GFP tagged caspase-2 with PML-NBs suggesting a potential activation platform for caspase-2 [80]. Furthermore, a protein called SP100 appears to reside at these nuclear structures and contains a putative CARD domain [79]. However, a potential interaction between caspase-2 and SP100 has not been confirmed experimentally. Therefore it is unknown what physiological role, if any, PML-NBs have in the activation of caspase-2.

Regulation of caspase-2 activation

The difficulty in assessing caspase-2 activation may indicate that this caspase is subject to tight regulatory control. Indeed, in the absence of cellular stressors, caspase-2 activation appears to be suppressed by a number of mechanisms.

Inhibition of caspase-2 by Hsp90α

Heat stress induces apoptosis, but also activates the protective factor HSF-1 [82]. HSF-1 is a transcription factor that induces the expression of a number of heat shock proteins (Hsps) including Hsp90α [83]. We observed that HSF-1 potently inhibits caspase-2 activation in response to heat shock and this is mainly due to the activity of the chaperone Hsp90α [23]. Thus Hsp90α appears to raise the threshold for caspase-2 activation during heat shock-induced apoptosis. Heat shock proteins have been similarly implicated as regulators of other initiator caspases. For example, Hsp70 and Hsp90 have been shown to inhibit apoptosome assembly by binding to Apaf-1 [84–86]. Hsp90α may inhibit caspase-2 activation in a similar fashion by binding one of the proteins of the caspase-2 activation platform such as PIDD or RAIDD, but this has yet to be determined.

Caspase-2 activity is suppressed by phosphorylation at distinct sites

Caspase-2 activation also appears to be suppressed by phosphorylation at three distinct sites. The first is in the prodomain of caspase-2 and is subject to metabolic regulation. In Xenopus oocytes, phosphorylation of caspase-2 at S135 suppresses its activation [30]. This appears to be the result of NADPH production by the pentose phosphate pathway. High levels of NADPH activate calcium/calmodulin-dependent protein kinaseII that phosphorylates caspase-2. Caspase-2 phosphorylated at S135 appears to be bound to 14–3-3ζ in the presence of nutrients. Nutrient depletion induces the release of caspase-2 from 14–3-3ζ allowing dephosphorylation of caspase-2 by the protein phosphatase 1 (PP1) [87].

The second site of phosphorylation of caspase-2 is in the linker domain between the large and small subunit (S340 in human beings) [88]. Phosphorylation at this site by cdk1–cyclin B1 appears to suppress caspase-2 activation during cell division. Dephosphorylation of caspase-2 by PP1 appears to induce cells to undergo mitotic cell death.

Finally, caspase-2 activation appears to be suppressed in human cells by phosphorylation at S157. Phosphorylation at this residue is induced by protein kinase CK2, a constitutively active growth factor-independent serine threonine kinase. Inhibition of PKCK2 sensitized cells to TRAIL-induced apoptosis and promoted caspase-2 dimerization in a PIDD-independent fashion. PKCK2 is elevated in a number of tumour lines that are also resistant to TRAIL treatment and it has been proposed that suppression of caspase-2 activation in these cells is responsible for the resistance to TRAIL [89].

Alternatively spliced isoforms of caspase-2

Alternative splicing of the caspase-2 gene results in two distinct isoforms: caspase-2L that is pro-apoptotic and caspase-2S, a truncated version of caspase-2 that is anti-apoptotic. Caspase-2L and caspase-2S appear to be differentially expressed in different tissues. Caspase-2L is expressed in a range of tissues while caspase-2S is predominantly expressed in heart, skeletal muscle and brain [90]. Caspase-2 splicing appears to be regulated by a candidate tumour suppressor RBM5, which binds caspase-2 pre-mRNA and promotes formation of the caspase-2L species [91]. Therefore the differential expression patterns of the two isoforms of caspase-2 may be associated with levels of expression of RBM5 in various tissues.

Regulation of caspase-2 during cell-cycle

Caspase-2 has also been reported to be inhibited by the cell-cycle regulating protein Chk1. When Chk1 is inhibited, caspase-2 induces apoptosis in response to γ-irradiation-induced DNA damage. This pathway appears to be downstream of ataxia telagiectasia mutated (ATM) and ATM and RAd3 related (ATR) and is independent of p53 [92]. This observation suggests a role for caspase-2 in the regulation of cell-cycle. Consistent with this idea, caspase-2 deficient cells have higher rates of proliferation [32]. Furthermore, activation of caspase-2 has been reported to delay G2 to M progression suggesting a cell cycle checkpoint function for caspase-2 [32, 33]. This appears to correlate with a third phosphorylation event induced by DNA-PKcs at S122 [33]. In contrast to the other phosphorylation events, this phosphorylation activates caspase-2 rather than suppressing it.

Caspase-2 in disease

Cancer

A role for caspase-2 as a tumour suppressor suggests that caspase-2 may be absent or defective in certain cancers. Caspase-2 is located on human chromosome 7 and maps to the q34–35 segment [93]. The 7q region is frequently deleted or affected in haematological neoplasms [94]. Studies analyzing caspase-2 levels in cancer have so far shown conflicting results. Decreased levels of caspase-2 correlated with drug-resistance in childhood forms of acute lymphoblastic leukaemia (ALL) [95] and decreased caspase-2 and RAIDD expression was observed in mantle cell lymphoma tumour samples [96]. In contrast, increased levels of caspase-2 in acute myelogenous leukaemia and adult ALL have been associated with lower patient survival [97, 98]. Many of these studies were carried out on small sample sizes, which may contribute to the contradictory results. In light on the recent observations that caspase-2 is a potential tumour suppressor, it seems timely to expand on these early investigations.

Neurodegenerative disorders

Caspase-2 has also been implicated in pathologies associated with aging. Caspase-2 activation has been observed in response to a number of forms of neuronal stress including β-amyloid, which is the main constituent of amyloid plaques in Alzheimer's disease [53, 59, 60]. Furthermore, increased caspase-2 has been observed in the brains of Alzheimer's disease patients [99]. Huntington's disease is a neurodegenerative disorder that is caused by the expansion of CAG repeats of the huntingtin gene. Expression of a form of the huntingtin gene that codes for 40 glutamines was shown to be associated with increased caspase-2 expression, in addition to increased levels of caspase-1, -3 and -7 [100]. Furthermore, increased caspase-2 expression has been reported in neuritic profiles of the striatum from cases of Huntington's disease [101]. Caspase-2 has been shown to interact with huntingtin in a polyglutamine repeat-length dependent manner since it binds longer forms more efficiently. Caspase-2 and huntingtin co-localize in neuronal processes and axonal terminals in primary striatal cultures and this has been postulated to promote neuritic degeneration *via* cleavage of huntingtin by caspase-2 to yield cytotoxic products [101].

Ischemia-reperfusion injury

In a chick cardiomyocyte model of ischemia-reperfusion inhibition of caspase-2 using an inhibitor dramatically decreased cell death [102]. In this type of injury the majority of cellular damage is a result of oxidative stress and a number of conditions that promote oxidative stress have been suggested to activate caspase-2 (Table 1). Caspase-2 binds a CARD-containing protein called ARC that is expressed almost exclusively in heart and skeletal muscle [103]. ARC was shown to protect cardiomyocytes from oxidative stress induced apoptosis and may do so through inhibition of caspase-2 [104]. Although indirect, these studies may suggest that targeting caspase-2 could protect cells from oxidative stress resulting from ischemia-reperfusion-associated injury. Of course, much more research is required to determine a direct role for caspase-2 in these pathways.

Concluding remarks

Caspase-2 has in some reviews been termed 'the Cinderella caspase' [105] since it has long been considered to have minor or redundant functions in apoptosis pathways. Recently it has moved to the forefront of caspase research, but there are still a number of controversies surrounding caspase-2 such as the exact components of its activation platform or what stimuli lead to its activation. It remains to be seen what the true role for caspase-2 is. Is it mandatory for apoptosis in certain contexts or does it primarily function in the maintenance of cell-cycle? With new tools at our disposal to dissect these pathways, including genetic models and probes to specifically assess activity, we will be able to more fully investigate role of this elusive caspase in cancer and other human diseases.

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