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**Review**

# Caspase-mediated activation of PAK2 during apoptosis: proteolytic kinase activation as a general mechanism of apoptotic signal transduction?

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## Abstract

**p21-activated kinase 2 (PAK2) is proteolytically cleaved during apoptosis through the action of DEVD-sensitive caspase(s). This cleavage event causes PAK2 activation, and PAK2 activity is implicated in regulation of the biochemistry and morphology of the apoptotic cell. PAK2 is just one example of a number of identified caspase targets that are protein kinases involved in regulating various aspects of cell function. We hypothesize that this may reflect their important role in regulating the controlled and orderly demise of the dying cell.**

**Keywords:** p21-activated kinase; caspases; cell death; signal transduction

**Abbreviations:** PAK2, p21-activated kinase 2; JNK, c-Jun amino terminal kinase; FADD, Fas associated death domain; DAP-kinase, death-associated protein kinase; ERK, extracellular signal regulated kinase; MAP kinase, mitogen-activated protein kinase; MEKK1, mitogen/extracellular signal regulated kinase kinase kinase 1; FAK, focal adhesion kinase; DNA-PK, DNA-dependent protein kinase; CAM KII, calmodulin dependent protein kinase II

It is now generally understood that the process of regulated cell death plays a critical homeostatic role in a wide variety of cellular processes, ranging from basic organismal development to formation of the mature immune and nervous systems. All cells contain the machinery to undergo the regulated death response known as apoptosis (Jacobson *et al*, 1997; Ellis *et al*, 1991; Majno and Joris, 1995; King and Cidlowski, 1995). Apoptosis enables an organism to eliminate unwanted or defective cells through an orderly process of cellular disintegration that has the advantage of not inducing an undesirable inflammatory response. Apoptotic elimination of cells occurs during normal tissue development and turnover, as well as in a variety of pathological conditions. Indeed, growing evidence indicates that improper regulation of apoptosis contributes to disorders such as cancer, viral infection, autoimmune disease, neurodegenerative disorders, stroke, anemia, and AIDS (Wyllie, 1997). Understanding the underlying control mechanisms for apoptosis may therefore be of considerable therapeutic benefit.

The ability of an organism to maintain cellular homeostasis is critically dependent upon a balance between cell proliferation, differentiation and death. It is clear from our knowledge of cancer that uncontrolled cell proliferation can be harmful to the organism. It makes sense then that the processes involved in cell proliferation be tightly linked to the mechanisms involved with cell death, a concept supported by observations that genes involved in proliferation are also often capable of modulating or inducing cell death (Jacobson *et al*, 1997; Ellis *et al*, 1991; Majno and Joris, 1995; King and Cidlowski, 1995). In addition, recent evidence suggests that the critical role of the cytoskeleton in regulating cell proliferation signals may extend to the process of cell death as well (Meredith *et al*, 1993; Chen *et al*, 1997).

Apoptosis can be triggered by a wide variety of extrinsic signals (Fas ligand, tumor necrosis factor, growth factor withdrawal, kinase inhibitors, viral or bacterial infection, oncogenes, cytotoxic T lymphocytes, ceramide, UV irradiation, glutamate, heat shock, chemotherapeutic drugs, etc.) (Wyllie, 1997). Some apoptotic signals act in many types of cells (e.g., UV irradiation), while others are cell-type specific (e.g., glutamate). Some of these signals can induce either apoptosis or necrosis (e.g., TNF), while others may trigger apoptosis or mitogenesis (e.g., ceramide). While the reasons for these differences are unclear at present, a common cell death program or pathway exists; that is, a cascade of cellular events that is common from cell to cell, regardless of the initial death stimulus. There is substantial evidence for this, including the common and orderly morphological changes observed during all cell death and the existence of conserved genetic elements found in widely divergent organisms (e.g., the CED-3 proteases and CED-9: BCL-2 proteins in *C. elegans* and their equivalents in mammalian organisms) (Ellis *et al*, 1991; Martin and Green, 1995; Chinnaiyan and Dixit, 1996). Thus, even though the agents which initiate apoptosis vary from cell to cell, there appears to be a basic biochemical machinery underlying the process of regulated cell death.

The ICE/CED-3 caspases are ubiquitous and critical components of the cell death machinery. The caspases are a family of cysteine proteases with the distinctive property of cleaving immediately after aspartate residues (Chinnaiyan and Dixit, 1996; Whyte, 1996; Nicholson and Thornberry, 1997). Relatively few apoptotic substrates for

caspases have been identified, and their exact roles in the execution of the apoptotic program remain unclear (Rosen and Casciola-Rosen, 1997). An emerging theme appears to be the identification of kinases as caspase targets. Many of these kinases are known to be involved in normal cellular signaling cascades that are important components of cellular growth andlor stress responses (Hunter, 1997).

## Kinases as caspase targets

The activation of both tyrosine and serine/threonine kinases during apoptosis induced by a variety of stimuli has been reported (e.g., Eischen *et al*, 1994). Inhibitors of tyrosine kinases can block subsequent apoptotic events, while some kinase inhibitors (e.g., staurosporine) can be effective inducers of apoptotic cell death. In support of the critical role of protein phosphorylation events in apoptotic signaling, protein phosphatases also appear to be important modulators of apoptotic responsiveness (Morana *et al*, 1996; Sato *et al*, 1995).

Certainly, many of the kinases whose activity is stimulated by pro-apoptotic stimuli are acting as upstream signals which regulate the initiation of apoptotic death. Such upstream kinases include cJun amino terminal kinase (JNK), p38 MAP kinase, the ERK MAP kinases, and perhaps ceramide activated kinase (Chuang *et al*, 1997; Cosulich and Clarke, 1996; Mathias *et al*, 1991 and references therein). Activation of the ERK MAP kinase pathway correlates strongly with inhibition of cell death responses to growth factor withdrawal in neuronal cell lines (Xia *et al*, 1995) and to Fas receptor stimulation in Jurkat cells (Holmström *et al*, 1998). JNK has been shown to be stimulated through the action of a protein termed Daxx in 293 and Hela cells (Yang *et al*, 1997). Daxx is recruited and apparently activated through the death domain of the Fas receptor, and through its ability to stimulate JNK it acts cooperatively with the FADD pathway to induce apoptosis. Activation of the JNK pathway by small GTPases is also sufficient to induce apoptosis in Jurkat T cells (Chuang *et al*, 1997). In both cases, JNK activity can be shown to lie upstream of caspase activation. JNK activity antagonizes the anti-apoptotic effects of BCL-2 and may do so by phosphorylating BCL-2 directly (Park *et al*, 1997; Maundrell *et al*, 1997). JNK signaling may also enhance expression of Fas ligand, thereby initiating further death signaling through the Fas receptor (Hueber *et al*, 1997).

The ability of PI-3 kinase inhibitors to enhance cell death responses has suggested that the products of this lipid kinase regulate an anti-apoptotic mechanism. This has recently been shown to be mediated through the ability of $PIP_3$ to regulate the serine/threonine protein kinase Akt. Activated Akt phosphorylates BAD and thereby prevents it from complexing: with and blocking the anti-apoptotic activity of BCL-$x_L$ (Gajewski and Thompson, 1996; del Peso *et al*, 1997; Datta *et al*, 1997).

Death-associated protein kinase (DAP-kinase) is a $Ca^{2+}$/calmodulin-dependent serine/threonine kinase whose ectopic expression induced apoptotic death in a number of cell types (Cohen *et al*, 1997; Jin *et al*, 1997). Originally identified as a mediator of $\gamma$ interferon-induced cell death (Deiss *et al*, 1995), it has been established that the catalytic activity of this 160 kDa kinase is required for its death-inducing properties. DAP kinase is associated with the actin cytoskeleton, and it has been proposed that DAP kinase provides a mechanism to link suppression of apoptosis to metastasis in turmor cells (Inbal *et al*, 1997). There is no indication that DAP kinase is a substrate for caspase-mediated proteolysis, and DAP kinase therefore may be a direct mediator of cell death responses to certain stimuli.

In contrast to the kinases that are acting upstream of the caspase cascades to initiate and/or regulate the apoptotic response, recent studies have established that the activation of a number of kinases occurs subsequent to the activation of the caspase cascades. Some of these kinases are activated indirectly through caspase action on other substrates, but an increasing number have been found to be directly cleaved by caspases, leading to modulation of their catalytic activity. Indeed, while the number of identified caspase substrates remains relatively small, a significant portion of these substrates turn out to be signaling kinases with an impressive array of cellular regulatory activities (Table 1).

## Caspase-mediated cleavage of p21-activated kinase 2 (PAK2)

The p21 activated kinases (PAKs 1, 2, 3 or $\alpha$, $\gamma$, $\beta$, respectively) are a closely structurally related family of serine/threonine kinases whose activity can be regulated by the binding of the small GTPases Rac and Cdc42 (Manser *et al*, 1994; Knaus *et al*, 1995; Martin *et al*, 1995; Sells and Chernoff, 1997). PAKs are also members of a much larger family of signaling kinases related to the Ste20 kinase involved in pheromone responsiveness and polarized morphogenesis in budding yeast (reviewed in Sells and Chernoff, 1997). Mammalian PAKs have been implicated in the regulation of a number of cellular activities. These include regulation of MAP kinase signaling pathways (including JNK, p38 and ERK), the cell cycle, oxidant generation in phagocytic leukocytes, and cytoskeletal dynamics. Interestingly, a PAK-like kinase has also been shown to physically associate with the Nef protein of HIV, which is in turn capable of inducing apoptosis when overexpressed in Jurkat T cells (Cullen, 1996).

Cytoskeletal regulation by PAKs appears to be complex, requiring both the C terminal kinase domain and independent regulatory activities of the N terminus mediated through protein-protein interactions. PAK1 has been localized to areas of active membrane ruffling in fibroblasts, and the introduction of PAK1 mutants modified in the N terminal regulatory domain into cells induces membrane ruffling and polarized cell morphologies reminiscent of motile cells (Dharmawardhane *et al*, 1997; Sells *et al*, 1997; Manser *et al*, 1997). Indeed, PAK1 is abundant in the leading edge of oriented- or motile cells. These effects are largely independent of

**Table 1** Characteristics of kinases known to be proteolytically cleaved by mammalian caspases

| Kinase | Caspase site | Consensus phosphorylation | Substrates | Regulatory activities |
|---|---|---|---|---|
| PAK2 | SHVD[212] | [KRES/T][d] | p47[phox d], MEK1[f] myosin light chain[g] | Cytoskeleton, MAPK pathways, NADPH oxidase |
| MEKKI | GVED[871] DTVD[874] | ND | MKK4[h], MEK1[?] | MAPK pathways |
| FAK | ND | [EDXXY] | Paxillin[j], p130cas, shc | Adhesion; cytoskeleton, nuclear signaling |
| DNA-PK | DEVD[2712] WVGD[2983] | [EPPLSQEAFA-DLWKK][a] | p53[a,b] | DNA repair, cell cycle |
| PKCδ | DMQD[330] | S/TXK/R[c] K/RXS/T | Many | Pleiotropic signaling, cytoskeleton |
| PKCθ | DEVD[354] | S/TXK/R[c] K/RXS/T | Many | Pleiotropic signaling, cytoskeleton |
| PITSLRE | YVPD[393] | ND | (Cyclins)? | Cell cycle, chromosome segregation, growth control |
| PRK2 | ND | ND | ? | Cytoskeleton |
| CaMKII | ND | XRXXS/T[j] XRXXS/TV | Many | Pleiotropic signaling |
| Akt | ND | ND | BAD, others | Anti-apoptotic; mitogenic |
| Raf-1 | ND | ND | MEK1, MEK2 | Mitogenic; antiapoptotic |

[a]Lees-Miller S *et al.* (1992) Mol. Cell. Biol. 12:5041–5049. [b]Shieh SY *et al.* (1997) Cell 91:325–334. [c]Pearson R and Kemp BE. (1991) Meth. Enzy. 200:62–76. [d]Tuazon PT *et al.* (1997) Biochem. 36:16059–64. [e]Knaus UG *et al.* (1995) Science 269:221–23. [f]Frost JA *et al.* (1997) EMBO J. 21:6426–38. [g]Ramos E *et al.* (1997) Receptors and Signal Transduction 7:99–110. [h]Yan M *et al.* (1994) Nature 372:798–800. [j]Burridge K and Chrznowsky-Wodnicka M. (1996) Annu. Rev. Cell. Dev. Biol. 12:463–519

PAK kinase activity, but do require intact proline-rich SH3 binding domains in the PAK N terminus. The ability of PAK to interact with components of mammalian and yeast signaling pathways through these SH3-binding domains has been demonstrated (Leeuw *et al*, 1995; Galisteo *et al*, 1996; Bokoch *et al*, 1996). Also relevant to their cytoskeletal regulatory function is the ability of PAKs to phosphorylate and modulate the activity of myosin. PAK and PAK-related enzymes phosphorylate the heavy chain of myosin from Acanthamoeba, thereby stimulating its actin-dependent $Mg^{2+}$-ATPase activity (Wu *et al*, 1996; Brzeska *et al*, 1997). This effect has not yet been shown to occur with any mammalian myosins. PAKs, particularly PAK2, have also been found to phosphorylate the light chain of mammalian myosins *in vitro*, with a resulting increase in actin-dependent ATPase activity (Tuazon and Traugh, 1984; Ramos *et al*, 1997). The possibility that PAKs regulate the actinomyosin system of cells may explain much of the reported biological activity of PAKs.

Recently, several laboratories have shown that PAK2 is a substrate for proteolytic cleavage by DEVD-sensitive caspases during apoptosis induced by a variety of stimuli in different types of cells (Rudel and Bokoch, 1997; Lee *et al*, 1997). The 62 kDa PAK2 is cleaved into a 34 kDa C terminal fragment and a 28 kDa N terminal fragment with a time course that parallels apoptotic death in Jurkat cells. In intact cells the cleavage is inhibitable by both Ac-YVAD-cmk and Ac-DEVD-CHO, but in apoptotic cell lysates the cleavage is at least 100-fold more sensitive to DEVD inhibitors, indicating the involvement of a DEVD-sensitive caspase. This is supported by the ability of recombinant CPP32/caspase-3 to cleave PAK2 *in vitro*. The closely related PAK1 and PAK3 proteins are not proteolytically cleaved under the same conditions.

PAK2 cleavage takes place adjacent to aspartate residue #212, which lies in the region between the regulatory N terminus and the catalytic C terminus. Since removal of inhibitory constraints imposed by the N terminus is thought to be involved in normal PAK activation by GTPases, the kinase activity of the PAK2 C terminal fragment was evaluated in cells stimulated by Fas receptor crosslinking and was found to be constitutively activated. These data suggest that PAK2 may become a highly active kinase as a consequence of caspase-mediated cleavage (Figure 1). Moreover, the released N terminal fragment may itself be able to exert regulatory effects towards the cytoskeleton. However, it should be pointed out here that actual physical separation of the PAK2 fragments resulting from cleavage at Asp212 during apoptosis has not yet been demonstrated. It is possible that additional signals, e.g., binding of active Rac or Cdc42, are still necessary for PAK2 activation to occur *in vivo*.

PAK activity is implicated in regulation of at least two important processes which may contribute to the apoptotic death pathway. These conclusions were based on studies of stable Jurkat cell lines expressing an inducible kinase dead PAK1 (H83L, H86L, K299R) construct. Because of the high degree of sequence identity in the catalytic domains of PAK1 *vs* PAK2, this construct serves as a dominant inhibitor of both forms of PAK. Jurkat cells expressing dominant negative PAK1 (H83L, H86L, K299R) still underwent nuclear fragmentation and cell

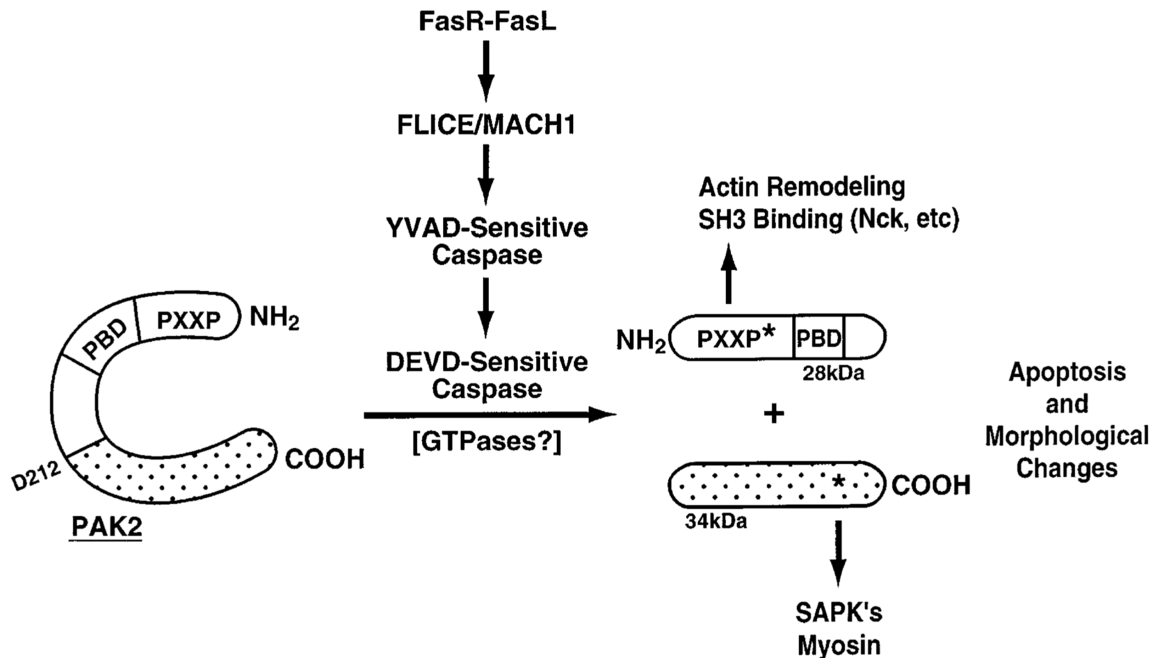


**Figure 1** Schematic diagram indicating the likely activities of PAK2 activated due to caspase-mediated cleavage during apoptosis. The N- and C-terminal fragments formed may have distinct regulatory activities, as suggested by previous studies on PAK (see references). The possibility that activation post cleavage may require additional signals from Rac or Cdc42 GTPases requires investigation. PBD, p21-binding domain or GTPase-binding domain; PXXP, proline-rich SH3 binding domains in N terminus; D212 indicates the Caspase3 cleavage site on PAK2; SAPKs, stress activated protein kinases

death in response to Fas ligation; however the cells were inhibited in their ability to fragment into apoptotic bodies (Rudel and Bokoch, 1997). Phosphatidylserine exposure was actually enhanced in the same experiments, perhaps as a consequence of the blockade of cytoskeletal and morphological changes in these cells.

We observed that PAK function is required for the stimulation of the c-Jun amino terminal kinase (JNK) pathway by Fas in Jurkat cells (Rudel *et al*, 1998). JNK activation by Fas receptor is partially blocked by caspase inhibitors, as has been demonstrated with a number of other apoptotic stimuli as well. Expression of dominant negative PAK blocked Fas-induced activation of JNK, but did not affect activation stimulated by phorbol esters. Inhibition of JNK activity was not sufficient to block cell death induced by Fas receptor crosslinking. However, activation of the JNK pathway by Cdc42 (Chuang *et al*, 1997) or expression of the constitutively active PAK C terminus was sufficient to induce an apoptotic response, albeit with a time course that was much slower than that of Fas.

The proteolytic cleavage and activation of an endogenous PAK in Xenopus oocytes by caspases has also been recently reported (Saure *et al*, 1997). Interestingly, in this system PAK activity appeared to be anti-apoptotic, as the expression of a catalytically inactive PAK C terminal fragment induced cell death. This effect was suppressed by addition of BCL-2 or a DEVD caspase inhibitor peptide, and rescued by competing the dominant negative mutant with constitutively active PAK. The effects of PAK on cell death in the Xenopus system appear to be linked to effects of PAK to modulate cell cycle arrest.

Overall, these data suggest that PAK(s) play important roles in controlling membrane, morphological and signaling responses during apoptosis. This can be a result of an activation process distinct from the normal means of PAK regulation (i.e., the binding of activated Rac or Cdc42) that involves caspase-mediated proteolysis, although similar regulation through GTPase-regulated signals remains a possibility. While PAK2 appears to be one of the important downstream targets of caspase action, it now appears that it is not just an isolated example of a kinase regulated by caspase-mediated proteolysis. An increasing number of other kinases appear to be targets of apoptotic regulation via caspases as well.

## Other protein kinases proteolytically cleaved and regulated during apoptosis

### MEKK1

As mentioned above, the ability of certain apoptotic stimuli to induce signaling via the JNK and p38 MAPK pathways is dependent upon caspase-mediated events. This is also true during the process of anoikis, or apoptosis due to loss of integrin-initiated adherence signals (Frisch *et al*, 1996). One potential mechanism for activation of the JNK and p38 kinase cascades is through the recently described proteolytic activation of MEKK1 by caspases (Cardone *et al*, 1997).

MEKK1 serves to couple multiple hormonal signals preferentially to activation of the JNK pathway in a variety of cell types. Full length MEKK1 has been recently cloned and shown to be stimulated by the small GTPases Ras and Rac/Cdc42 (Fanger *et al*, 1997). During anoikis of MDCK cells induced by cell-matrix detachment, MEKK1, which migrates as a series of 160–200 kDa bands representing multiple phosphorylated states of the full length protein, was shown to be cleaved to fragments of ~78 kDa and ~100 kDa. This cleavage was inhibitable by CrmA, and was shown to occur with purified recombinant caspases-3, -7, and -8 *in vitro*. Cleavage in apoptotic extracts was extremely sensitive to inhibition by Ac-DEVD-CHO, but less sensitive to Ac-YVAD-CHO. The proteolytic cleavage site was localized to aspartate residues within the sequence aa868–871 (GVED) and aa871–874 (DTVD). Since truncation at these sites would remove the regulatory N terminus from the full length 1493 aa. MEKK1, activation of MEKK1 was predicted and shown to occur. Transient expression of the constitutively active C-terminal MEKK1 cleavage product induced apoptosis as assessed by the criterion of abnormal nuclear morphology, consistent with a prior report that MEKK1 was able to induce apoptosis, and the observation that dominant negative forms of MEKK1 inhibited apoptosis and anoikis (Johnson *et al*, 1996). Caspase-mediated MEKK1 cleavage and activation may thus contribute to nuclear changes during apoptosis and may enhance signals through the JNK pathway which promote cell death. MEKK1, as well as other stimuli which activate JNK (e.g., activated Cdc42), also increase caspase activity, suggesting positive feedback loops may exist which promote an apoptotic cascade.

## Focal adhesion kinase (FAK)

It is a well established phenomenon that attachment of cells via integrins to extracellular matrix components such as fibronectin, vitronectin, and collagen has protective effects against apoptotis induced by other stimuli (Meredith *et al*, 1993). At least part of this protective effect appears to be due to the activation by adhesion of the tyrosine kinase known as focal adhesion kinase or FAK (Frisch and Francis, 1994; Hungerford *et al*, 1996). FAK localizes to focal adhesions and may regulate the formation of focal adhesion assemblies in spreading or migrating cells, as well as signaling pathways to the nucleus which suppress apoptosis and enhance proliferation.

FAK has been shown to be proteolytically cleaved during apoptosis induced by Apo-2L and/or Fas receptor crosslinking in both adherent and suspended Jurkat T cells (Wen *et al*, 1997). Cleavage correlated with the time course of caspase-mediated PARP proteolysis, as well as with cell death. The intact 120–125 kDa FAK is sequentially cleaved to an 85 kDa product, followed by a 77 kDa fragment. While both fragments were also observed in MCF-7 breast cancer cells, only the 85 kDa fragment was detected in H460 non-small cell lung cancer cell line. Earlier reports had also demonstrated FAK cleavage during c-*myc*-induced apoptosis, and both FAK cleavage and apoptosis were inhibited by adhesion to fibronectin or collagen matrices (Crouch *et al*, 1996). Apo-2L- and Fas-induced FAK proteolysis in intact cells was inhibited by 40 $\mu$M ZVAD-FMK but not by YVAD-CHO at up to 200 $\mu$M. DEVD-CHO preferentially blocked formation of the 77 kDa product, but also blocked formation of the 85 kDa fragment at higher concentrations. *In vitro* studies indicated that cleavage to the 85 kDa product was catalyzed by caspase-3 and caspase-7, while 77 kDa product was formed in response to caspase-6, either from the intact FAK or from the 85 kDa fragment. The initial cleavage generates a 33 kDa C terminal piece containing an intact kinase domain and the 85 kDa fragment which consists of the remaining N terminus. The second cleavage event removes an additional 8 kDa from the N terminal piece, leaving the 33 kDa kinase domain intact. While activity of the individual pieces was not determined directly in this study, analysis of FAK activity during apoptosis induced in Jurkat cells indicates that FAK activity decreases (Wen *et al*, 1997). It is not clear if this is due to loss of activity resulting from the observed cleavage events, or whether the decreased activity is a consequence of competitive/dominant negative effects of the generated fragments. The suggestion is, however, that FAK activity is compromised due to caspase-mediated proteolysis during certain forms of apoptosis. The loss of FAK activity may contribute to detachment of the dying cell from the substratum and to the morphological changes occurring during apoptosis, as well as to the loss of FAK-mediated anti-apoptotic signals.

## DNA-dependent protein kinase (DNA-PK)

DNA-PK is a predominantly nuclear serine/threonine kinase that is structurally related phosphatidylinositol 3-kinase and the ataxia telangiectasis gene product (Hungerford *et al*, 1996). DNA-PK is activated by double stranded DNA breaks, and is intimately involved in the repair of such breaks (see Han *et al*, 1996 for referenced reviews on this topic). DNA-PK is thought to be the product of the severe combined immunodeficiency (scid) gene, and animals mutated at this locus are deficient in repair of double stranded DNA breaks (Biedermann *et al*, 1991). Cells defective in this enzyme are sensitized to killing by ionizing radiation, while a cell line resistant to etoposide-induced apoptosis did not activate DNA-PK. Auto-reactive antibodies identified in human sera have been found to react with fragments of DNA-PK formed during apoptosis, and this may be an important component of autoimmune disease (Casciola-Rosen *et al*, 1995).

DNA-PK consists of a 460 kDa catalytic subunit and the heterodimeric Ku regulatory subunit, made up of 70- and 86-kDa proteins. The catalytic subunit of DNA-PK has been shown to be proteolytically cleaved in response to inducers of apoptosis, including staurosporine and Fas (Casciola-Rosen *et al*, 1995, 1996; Song *et al*, 1996; Han *et al*, 1996; McConnell *et al*, 1997). Cleavage of the 460 kDa catalytic subunit results in the formation of a pair of 165/168 kDa cleavage products (also described as 150 kDa in some reports) and a single 240–250 kDa fragment. Cleavage correlates with the onset of cell death and with activation of caspase-3 (CPP32). Proteolysis was inhibited in the

presence of 1 $\mu$M of the caspase-3 inhibitory peptide, Ac-DEVD-CHO, but not by up to 50 $\mu$M Ac-YVAD-CHO. Proteolysis to generate the same fragments was observed *in vitro* in the presence of recombinant caspase-3, but not caspase-1 (ICE), caspase-4; or caspase-6. DNA-PK contains two potential caspase cleavage sites (aa 2709–2713 (DEVDN), and aa 2979–2983 (DWVGD)) which would generate fragments of the appropriate size as observed in these studies, and caspase action at these sites has been confirmed by N-terminal sequence analysis of cleavage fragments (Song *et al*, 1996). It is of interest that the cleavage of DNA-PK during apoptosis correlates with the loss of its catalytic activity (Song *et al*, 1996; McConnell *et al*, 1997), suggesting that this may contribute to the DNA fragmentation that occurs during regulated cell death. In addition, DNA-PK normally phosphorylates and regulates the activity of the p53 tumor suppressor gene product. The loss of this activity is likely to contribute to dysfunctional regulation of cell cycle progression (Lees-Miller *et al*, 1992; Shieh *et al*, 1997).

## Protein kinase C family members (PKC$\delta$:PKC$\theta$)

The protein kinase Cs have long been known to be important components of intracellular signaling. This family of enzymes can be divided into the classical calcium-dependent forms, and into calcium-independent novel and atypical groups. Two structurally related members of the novel group, PKC$\delta$ and PKC$\theta$, have been shown to be proteolytically cleaved during ionizing radiation-, Fas-, and TNF-induced apoptosis and ionizing radiation-, Ara-C-, etoposide-, cisplatinum-induced cell death, respectively (Emoto *et al*, 1995, 1996; Ghayur *et al*, 1996; Datta *et al*, 1997). Cleavage of the 78 kDa PKC$\delta$ to a 40 kDa fragment was inhibited by BCL-2/BCL-$X_L$ expression and by treatment of cells with 10 $\mu$M YVAD-CMK (DEVD peptides were not tested). Similarly, the 78 kDa PKC$\theta$ cleavage to a 40 kDa fragment was blocked by BCL-$X_L$, CrmA, or p35 expression. *In vitro* analysis demonstrated that PKC$\theta$ was cleaved specifically by caspase 3, but not caspases 2, 4, 6, and 7 (Datta *et al*, 1997); PKC$\delta$ was cleaved by caspase 3, but not caspase 1 (Ghayur *et al*, 1996).

Proteolytic cleavage of both forms of PKC resulted in increased activity of the 40 kDa kinase fragment, as assessed by phosphorylation of exogenous substrates by the partially purified kinase and by in-gel kinase assays, as well as by assay of the recombinant cleavage construct. This is likely a result of cleavage at aa327–331 (DMQDN) in PKC$\delta$ (Ghayur *et al*, 1996) and aa351–355 (DEVDK) in PKC$\theta$ (Datta *et al*, 1997) at the V3 region to form a constitutively active catalytic domain fragment. Overexpression of the constitutively active PKC$\theta$ catalytic 40 kDa fragment, but not the full length or a kinase-dead fragment, induced nuclear and DNA fragmentation concomitant with cell death. In contrast to the pro-apoptotic effects resulting from proteolytic cleavage of PKC$\delta$ and PKC$\theta$, other work has shown that PKC$\zeta$ and PKC$\lambda/\iota$ interact with Par-4 and abrogate the ability of Par-4 to induce apoptosis (Diaz-Meco *et al*, 1996), while the atypical PKC$\iota$ protects K562 leukemia cells against drug-induced apoptosis (Murray and Fields, 1997), as does PKC$\beta$II in HL60 cells (Whitman *et al*, 1997). It is also of interest that PKC$\theta$ has recently been shown to be required for cell cycle progression and to modulate the formation of particular types of cytoskeletal structures (Tang *et al*, 1997).

A recent report has shown that the protein kinase C-related kinase 2 (PRK2 kinase) is proteolyzed by caspase 3 at two sites *in vitro*, as well as during apoptosis induced by Fas or staurosporine (Cryns *et al*, 1997). Both cleavage sites *in vivo* were within the regulatory domain of this serine/threonine kinase, suggesting its activity may be upregulated during apoptosis. It is of particular significance that PRK2 kinase has been shown to bind and be stimulated by the small GTPases Rac and Rho (Vincent and Settleman, 1997). PRK2 appears to be involved in cytoskeletal regulation by these GTPases, and it is interesting to speculate that activation of both PRK2 and PAK2 by caspases enables them to exert important regulatory influences on the cytoskeletal dynamics of the apoptotic cell.

## PITSLRE Kinase

PITSLRE$\beta$I is a 58 kDa member of a family of p34cdc2-related serine/threonine kinases which are expressed in both tissue specific- and ubiquitous fashion (Lahti *et al*, 1995 and references therein). Expression of PITSLRE$\beta$I, induces teleophase delay, abnormal segregation of chromosomes and decreased growth rates due to apoptosis in Chinese hamster ovary cells. Apoptosis can be induced by ectopic expression of an amino-terminal (aa 1–74) deleted mutant containing an active catalytic domain. The levels of PITSLRE message and activity were observed to increase in Fas-stimulated Jurkat T cells. This was associated with the formation of 43–50 kDa C terminal PITSLRE catalytic fragments. Generation of these cleavage products was inhibited, along with Fas-induced cell death, by treatment with TPCK Curiously, cysteine protease inhibitors were reported to have no effect on either Fas-induced PITSLRE cleavage or cell death in these studies. However, recent work has shown that PITSLRE is cleaved during TNF-induced apoptosis, and that cleavage is sensitive to CrmA and BCL-2 (Beyaert *et al*, 1997). Both ICE/caspase-1 and CPP32/caspase-3 were able to produce cleavage of PITSLRE kinase *in vitro* at Asp 393 within the sequence YVPDS to generate the 43 kDa C terminal fragment. *In vivo* analysis in caspase-1 deficient cells indicated that cleavage of PITSLRE was normal, suggesting that caspase-3 may be the physiologically relevant protease. Removal of portions of the PITSLRE regulatory N terminus are known to increase kinase activity and increased activity is observed during apoptotic responses in several cell lines. Furthermore, the N terminus contains two nuclear localization signals and a region capable of binding to SH2 domains, suggesting that cleavage during apoptosis may be a mechanism to modulate localization and/or interactions with other proteins, as well as activity.

## Calmodulin-dependent protein kinase II (CaM KII)

Both tumor necrosis factor and UV light rapidly stimulate calcium independent CaM KII activity in U937 and several other cell lines (Wright *et al*, 1997). Cell lines resistant to TNF- or UV-induced apoptosis, such as K562 cells, do not activate CaM KII. Calcium independent CaM KII activation was blocked by VAD-fmk and TPCK, suggesting that proteolysis was required for the activation process. Proteolytic cleavage to an active catalytic fragment by caspases was implied, but not demonstrated by these data. Activation of this enzyme may be of interest, as two mechanistically distinct inhibitors of CaM KII block DNA fragmentation and cell death in U937 cells. CaM KII has also been implicated in regulation of processes as diverse as ion channel function, calcium homeostasis and cytoskeletal regulation.

## Raf-1, Akt

Survival signals initiated by both Raf-1, through the ERK MAP kinase pathway, and Akt, through phosphorylation of BAD, have protective effects against apoptosis initiated by a variety of agents (see Discussion above). It has been recently reported that both Raf-1 and Akt are proteolytically degraded in a caspase-dependent (i.e., Ac-YVAD-CMK- and $Bclx_L$-dependent) manner (Widmann *et al*, 1998). The caspase responsible for these cleavages and the site(s) of cleavage are unknown. Degradation of both enzymes occurred relatively late during the apoptotic response, but correlated with loss of both Raf-1 and Akt activity. Thus, caspase action may specifically turn off these survival pathways that could otherwise interfere with the ongoing apoptotic response.

# Conclusions

It is remarkable that an increasing number of kinases capable of regulating intracellular signaling are targets for caspases during the cell death program. Certainly these kinases are at least capable of modulating the apoptotic responsiveness of the cell, and it may be hypothesized that they may additionally serve as critical control elements for regulating and coordinating the complex biochemical and morphological changes accompanying apoptotic death. It is interesting to speculate that, just as activation of kinase signaling pathways in response to hormones and other physiological stimuli regulate cell growth and proliferation through their action on specific regulatory targets, the activation (or inhibition) of specific kinases through their irreversible proteolytic cleavage by caspases might control a specific sequence of events that produces the well-known apoptotic program (Figure 2). Thus, the concept of a 'death by a thousand cuts' resulting from the promiscuous action of caspases (Martin and Green, 1995), may be misleading; rather, 'death via a specific series of caspase-initiated kinase signaling events' may be more fundamental to the controlled apoptotic responses which lead to the orderly demise of the cell. Indeed, the sensitivity of apoptotic responses to

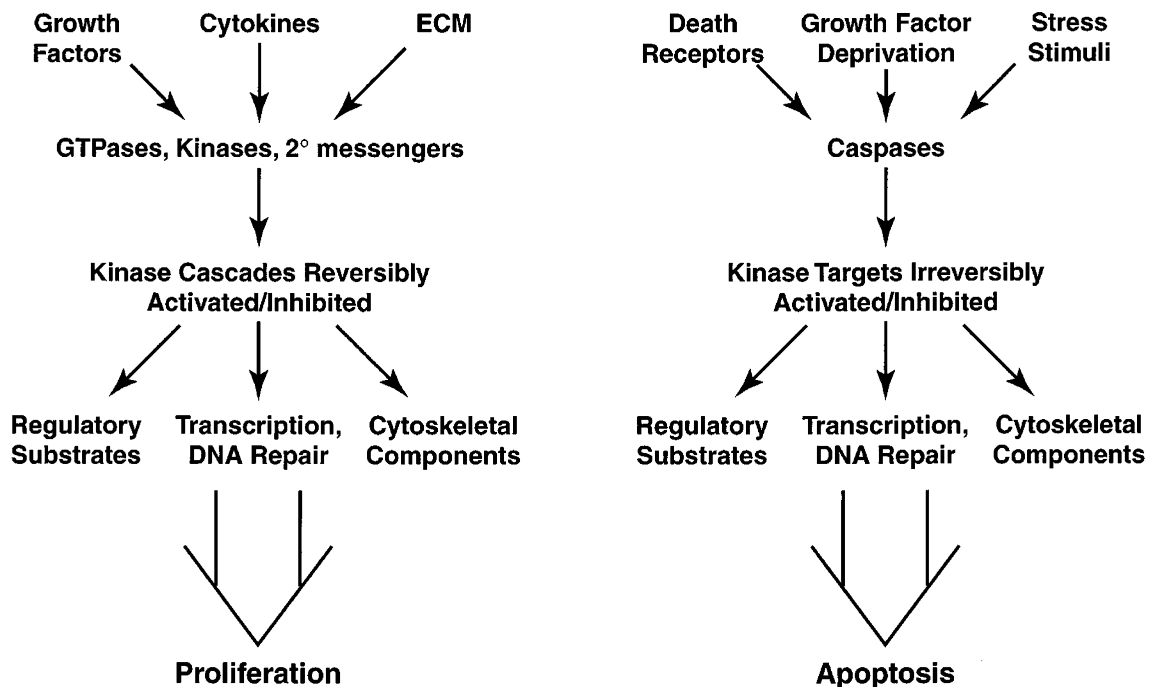


**Figure 2** Parallels in the kinase signaling pathways that regulate cell proliferation and those that may regulate the cell death program. Just as kinase activation by extracellular signals controls cell growth, kinase activation by caspases may initiate and regulate a specific series of cellular events that lead to the characteristic responses of the apoptotic cell. ECM, extracellular matrix

protein kinase inhibitors and protein phosphatases suggests that there may be some truth to this hypothesis. Certainly, additional studies to identify kinase targets for caspases and to determine their specific contributions to the apoptotic program are warranted. An understanding of the mechanisms through which the kinases serving as caspase targets are acting to regulate the apoptotic program should lead to new insights into the underlying biochemical mechanisms involved in controlling normal and pathological cell death.

## Note added in proof

A recent paper describes the PAK-related kinase MST1 as an additional caspase target during staurosporine- and Fas-induced apoptotic death in BJAB human B lymphoma cell lines

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