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Role of protein kinase activity in apoptosis

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Abstract. The transmission of signals from the plasma membrane to the nucleus involves a number of different pathways all of which have in common protein modification. The modification is primarily in the form of phosphorylation which leads to the activation of a series of protein kinases. It is now evident that these pathways are common to stimuli that lead to mitogenic and apoptotic responses. Even the same stimuli under different physiological conditions can cause either cell proliferation or apoptosis. Activation of specific protein kinases can in some circumstances protect against cell death, while in others it protects the cell against apoptosis. Some of the pathways involved lead to activation of transcription factors and the subsequent induction of genes involved in the process of cell death or proliferation. In other cases, such as for the tumour suppressor gene product p53, activation may be initiated both at the level of gene expression or through pre-existing proteins. Yet in others, while the initial steps in the pathway are ill-defined, it is clear that downstream activation of a series of cysteine proteases is instrumental in pushing the cell towards apoptosis. In this report we review the involvement of protein kinases at several different levels in the control of cell behaviour.
Key words. Apoptosis; protein tyrosine kinase; protein kinase C; cell cycle; PI3-kinase; DNA-PK.

Introduction

It is now well recognized that apoptosis is an active regulated process relying on both gene induction and protein activation [1–3]. A multitude of factors, both extracellular and intracellular, have been described that initiate and/or control the process [4–7]. Many of these factors also participate in cell proliferation and cell differentiation as members of signal transduction pathways [8–11]. Since the early 1980s when it was demonstrated that chromatin was fragmented into oligonucleosomal-sized fragments [12], there has been a preoccupation with nuclear events. It is now evident from cell-free systems capable of inducing apoptosis and from enucleated cells that factors in the cytoplasm play a predominant role in the process. While the search for the elusive endonuclease capable of degrading DNA into the characteristic pattern of fragments, continues, it is now clear that families of cysteine and serine proteases also participate by recognizing and cleaving specific sites in a limited number of protein substrates [13–21]. These proteases are not synthesized de novo in response to the apoptotic stimulus but already pre-exist in the cytoplasm, where they are activated by cleavage [22–25]. In some cases this cleavage is initiated by other family members, as in the case of the interleukin-1β (IL-1β)-converting enzyme (ICE) family [6, 7, 26]. In other cases, as in cytotoxic T-cell killing, serine proteases such as granzyme B (fragmentin-2) are responsible for activation of ICE-like proteases [27, 28]. It is still not clear how these cysteine and serine proteases are in turn receptive to upstream signals that initiate the series of events involved. The take-home message from both receptor- and non-receptor-mediated signalling is that a cascade of events are initiated involving the activation of protein tyrosine kinases, phospholipases, phosphatidylinositol 3-kinases and consequent downstream changes that involve the mobilization of Ca^{2+}, activation of protein kinase C (PKC), activation of the MAP kinase and other pathways and in turn the activation of transcription factors [29–34]. There is evidence also in the case of apoptosis that either the inhibition of protein tyrosine kinases [25, 35, 36] or, on the other hand, the activation of these enzymes leads to the induction of apoptosis [37, 38]. As is the case of signal transduction pathways that mediate proliferative events, changes in the phosphorylation state of proteins also play an important role in apoptosis. In this review we will address the importance of phosphorylation/dephosphorylation events in the process of apoptosis at various levels in signal transduction pathways.

Importance of pre-existing proteins

Evidence accumulated from cells in culture, cell-free extracts and enucleated cells demonstrates that the factors required for apoptosis are already present in the cell and that no new RNA or protein synthesis is required [39–43]. A greater knowledge of this process has helped to resolve the apparent differences that existed between murine thymocytes and a variety of hu-

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man cell lines. While dependence of murine thymocytes on de novo protein synthesis for apoptosis as evidenced by the use of inhibitors such as cycloheximide has been reported [3, 43–45], not only did this compound fail to prevent apoptosis in human cells [46, 47], but it was shown in some cases to exacerbate the process [48–51]. Inhibitors have been useful in delineating a variety of metabolic steps but have the disadvantage that they are differentially cytotoxic in different cell types. The evidence that emerges in several systems undergoing apoptosis is that pre-existing proteins are of key importance [46, 52–54]. In this respect comparison can be made with signal transduction pathways involving receptor-ligand interaction which leads to a cascade of events that are largely controlled by phosphorylation and activation of proteins [55, 56]. We have shown previously that apoptosis induced by either heat treatment or ionizing radiation exposure of human cell lines is accompanied by dephosphorylation of a limited number of specific proteins [48]. In addition, inhibitors of phosphatases 1 and 2A, okadaic acid and calyculin A delayed the process of apoptosis in response to a variety of damaging agents [48]. At longer times after treatment okadaic acid, because of its toxicity, brought on apoptosis. Ohoka et al. [57] have also demonstrated, using okadaic acid, that protein dephosphorylation is an essential step for glucocorticoid-induced apoptosis in murine T-cell hybridomas. Activation of serine/threonine phosphatases is correlated with the temperature-dependent induction of death in *Nicotiana tabacum* by tobacco mosaic virus, and okadaic acid inhibited the onset and the extent of the hypersensitive response in vitro [58]. Paradoxically, okadaic acid is also capable of inducing apoptosis in myeloid cells [59–62]. Ishida et al. [63] demonstrated the late onset of apoptosis in myeloid leukemia cells exposed to okadaic acid. Here again caution must be exercised in interpreting the findings on inhibitior studies. It is evident that the window of inhibition for okadaic acid depends very much on the cell type, presumably reflecting the responses of the cell to the specific apoptotic stimulus and the stage at which phosphatase activity is critical. Other evidence for a role for protein phosphorylation/dephosphorylation in apoptosis will be discussed below in the section on protein tyrosine kinases.

Protein tyrosine kinases

Cross-linking of the immunoglobulin (Ig) receptor by anti-Ig antibodies or binding to antigen initiates a series of well-defined steps that include activation of protein tyrosine kinase, hydrolysis of phosphatidylinositol and the mobilization of Ca^{2+} [64–67]. Activation is achieved by phosphorylation of specific sequence motifs [antigen receptor activation motif (ARAM)] within Igα and Igβ which constitute the B-cell receptor (BCR) [68]. These phosphorylated sites act as binding sites for protein tyrosine kinases, which subsequently become activated to phosphorylate different substrates [68]. One of these substrates, phospholipase C-γ2, is activated by phosphorylation to catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) [69]. These products act as second messengers for the mobilization of Ca^{2+} ($InsP_3$) and PKC activation (DAG). A second substrate is phosphatidylinositol 3-kinase (PI3-kinase), which phosphorylates PIP_2 to produce phosphatidylinositol 3,4,5-triphosphate (PIP_3), also a second messenger, the exact role of which remains undefined [56, 68].

Engagement of the BCR leads not only to cell proliferation and differentiation but also to apoptosis. For example, B cells expressing self-reactive Ig are eliminated by the binding of self-antigens to surface Ig on immature B cells [70, 71], and cross-linking of surface Ig on immature B-cell lines induces apoptosis [72–74]. As pointed out above, in the case of cell proliferation in response to surface Ig cross-linking, activation of one or more protein tyrosine kinases (PTKs) is an early event [75, 76]. It might be expected that the transmission of an inhibitory response leading to anergy or cell death would also involve PTK activation. Yao and Scott [38] described a close association between level of activatable Blk, a nonreceptor protein tyrosine kinase, and the inhibitory phenotype in immature B-lymphoma cells. They also demonstrated that exposure of CH31 lymphoma cells to antisense oligonucleotide for the nonreceptor PTK, Blk, prevented anti-μ-chain-mediated growth inhibition and subsequent apoptosis, emphasizing the importance of tyrosine phosphorylation in this process [77]. Activation of PTK during apoptosis has also been reported for acute lymphoblastic leukemia cells treated with interferon α (IFNα) which was accompanied by tyrosine phosphorylation of a 135-kDa protein [37]. In addition, exposure of human B-lymphocyte precursors to ionizing radiation caused enhanced tyrosine phosphorylation of multiple substrates under conditions where DNA fragmentation was occurring and the morphological features characteristic of apoptosis were present [33]. The PTK inhibitors herbimycin and genistein prevented the radiation-induced phosphorylation and inhibited apoptosis. Immunoprecipitation followed by protein kinase assay revealed that the enzymes responsible for phosphorylation were not the Src family tyrosine kinases. Evidence for a role for PTK activation in apoptosis has also been provided in staphylococcal enterotoxin B-activated peripheral T cells [78]. At the molecular level it has been demonstrated that the ARAM sequence in the cytoplasmic tails of the Igα/Igβ heterodimeric component of BCR is critical for transmitting growth arrest and apoptotic signals [79]. As in the case of signalling for prolifera-

tion, these functions of ARAM are positively regulated by tyrosine phosphorylation.

The mouse B-lymphoma cell line WEHI231 is known to undergo apoptosis upon cross-linking of surface IgM by anti-IgM antibodies. Variant lines which are resistant to apoptosis induced in this way have been shown to express dramatically reduced levels of HS1 protein, which associates with Lyn, Blk and Fyn when it is tyrosine-phosphorylated shortly after cross-linking of the surface IgM [80]. Expression of the human HS1 protein from an expression vector into one of the variant lines restored the sensitivity of the cells to anti-IgM-induced apoptosis, implying a crucial role for the HS1 protein in B-cell antigen receptor-mediated signalling leading to apoptosis [81]. B cells from HS1-null mutant mice are also resistant to apoptosis induced by multivalent cross-linking of surface IgM [82].

Tyrosine phosphorylation is not always associated with the onset of apoptosis. On the contrary, inhibition of tyrosine kinase causes apoptosis in HL60 and MO7e cells [35]. Granulocyte/macrophage colony-stimulating factor (GM-CSF) increases tyrosine phosphorylation in both neutrophils [83] and eosinophils [84] and inhibits cell death in vitro. Pretreatment with the tyrosine kinase inhibitor genistein prevented the increase in phosphorylation and blocked the protective effect of GM-CSF, suggesting that tyrosine kinase activity plays a key role in preventing apoptosis. Selective targeting of the membrane-associated CD19-lyn kinase complex in vitro with B43-Gen, a CD19 receptor-specific immunoconjugate containing the protein tyrosine kinase inhibitor genistein, triggered rapid apoptotic cell death in radiation-resistant lymphoma cells [85]. Additional compelling evidence in support of a role of PTK in protecting against apoptosis comes from experiments with enucleated cells. Deprivation of these cytoplasts of growth factor or treatment with the protein kinase inhibitor staurosporine leads to cytoplasmic changes characteristic of apoptosis [86]. Deregulation of tyrosine kinase activity in chronic myelogenous leukemia as a consequence of the Philadelphia chromosomal rearrangement and the formation of a chimeric *bcr-abl* gene is anti-apoptotic [87, 88]. Antisense oligonucleotides to *bcr-abl* mRNA selectively inhibit leukemia cell proliferation [89] and induce apoptosis in BV173 cells [88]. Cortez et al. [90] have demonstrated that the antiapoptotic and transforming activation of *bcr-abl* results from overlapping but distinct signals which include Ras activation and induction of c-*myc* mRNA. Activation of v-*abl* suppresses apoptosis induced both by growth factor withdrawal and by exposure of these cells to hydroxyurea [91]. Translocation and activation of PKCβII appears to play a role in this suppression of apoptosis [92]. The emphasis in this section has been on the catalytic activity of kinases, putting phosphate groups onto proteins. However, protein phosphorylation is also controlled by phosphatases that dephosphorylate these molecules. Use of the tyrosine phosphatase inhibitor phenylarsene oxide (PAO) causes an increase in protein tyrosine phosphorylation and an inhibition of apoptosis in human eosinophils and neutrophils, and inhibition of tyrosine kinase activity with herbimycin or genistein reversed the effects of PAO on tyrosine phosphorylation and granulocyte apoptosis [84]. Clearly, alteration in the extent of tyrosine phosphorylation of specific proteins determines the fate of these cells, and this is controlled by the activation of both kinases and phosphatases. In the example cited above, inhibition of protein tyrosine phosphatase leads to inhibition of apoptosis. On the other hand, in B-cell lymphoma and myeloid leukemia cell lines, the tyrosine phosphatase inhibitor bis(maltolato)oxovanadium (iv) (BMLOV) activates cell signal pathways in a lineage-specific manner, resulting in desensitization of receptor-mediated signalling and induction of apoptosis [93]. Thus, protein tyrosine phosphatase both limits the onset or extent of apoptosis by preventing protein phosphorylation (eosinophil and neutrophil cell death) and, in lymphoid cells, induces apoptosis by limiting protein tyrosine phosphorylation. CD45, a transmembrane tyrosine-specific phosphatase, has been shown to be associated with both induction and suppression of apoptosis in lymphoid cells. Induction of apoptosis in rat thymocytes by dexamethasone or heat treatment is accompanied by an early increase in cells expressing a high molecular weight isoform (190–220 kDa) of CD45 (CD45RC) [94]. Another isoform of CD45, 160 kDa in size (CD45RO), mediates cell adhesion and suppresses apoptosis in CS-21 mouse lymphoma cells [95]. The data presented in this section further emphasize the importance of phosphorylation/dephosporylation not only in the control of cell proliferation but also in the process of apoptosis.

Protein kinase C involvement in apoptosis

In this review the words 'contradiction' or 'paradox' are used with reference to the role of a specific molecule in apoptosis. PKC is no exception to this. Several reports demonstrate that activators of PKC such as the tumour-promotor phorbol 12-myristate 13-acetate (PMA) inhibit apoptosis [96–100], whereas others have shown that inhibition of PKC activity inhibits apoptosis [101–104]. As with other molecules, the role of PKC depends on the cell type, the state of activation of the cell, phase of the cell cycle and the nature of the agent being used. As in the case of control of cell proliferation, it is very likely that PKC is an intermediate in signal transduction as part of one or more pathways in apoptosis. The word 'pathways' is used advisedly, since there are multiple isoforms of PKC, and they appear to function in different ways in apoptosis [105]. PKC represents a family of serine/threonine kinases regulated by lipid and

classified according to Ca^{2+} dependence and phorbol ester binding activity. To date 12 isoforms have been described that differ in tissue distribution, location within the cell, dependence on Ca^{2+} and activation by different agents [106–111]. Conventional PKCs (α, $\beta 1$, $\beta 2$, γ) are Ca^{2+}-dependent and bind phorbol ester. Novel PKCs (δ, ε, η, θ and μ) bind phorbol ester but lack the second constant region C2 and are thus independent of calcium. Atypical PKCs (ζ, λ and ι) also lack the C2 region and possess only one of the two zinc fingers found in the other PKCs; hence they are calcium-independent and do not bind phorbol esters or diacyglycerol. It is now evident that specific isoforms respond to specific stimuli, but the responses are cell-type specific; for example PKCβ and PKCε have been shown to be activated in response to IFNα in Daudi cells [112, 113]. Null mutant mice lacking the γ isoform of PKC, which is concentrated in the brain, show altered function of the γ-aminobutyric acid (GABA) receptor in response to ethanol but not to diazepines or barbiturates [114]. Macrophage colony stimulating factor (M-CSF) specifically activates PKCα and promotes differentiation of granulocyte macrophage colony-forming cells into neutrophils [115]. The atypical PKCζ has been implicated in the response of U937 cells to TNFα (116), and is required for NF-kB activation as well as for mitogenic signalling in Xenopus oocytes and mammalian cells [117]. It also has a role in NGF-induced differentiation of PC12 cells [118].

It has been established for some time that immature thymocytes are susceptible to glucocorticoid – and Ca^{2+} ionophore – stimulated cell death [1, 3, 12]. Agents that stimulate PKC, including 12-*O*-tetradecanoylphorbol-13 acetate (TPA) and phorbol 12,13-dibutyrate (PDBu), block DNA fragmentation in thymocytes [97, 119]. This was interpreted to be due to PKC activation blocking apoptosis by preventing Ca^{2+}-stimulated endonuclease activity [97]. Given the role of PKC in signal transduction and the complexity of this family of enzymes, it is unlikely that this is the underlying explanation for the effects of PKC. Other studies suggest that glucocorticoid-induced apoptosis is dependent on PKC since it is inhibited by PKC inhibitors [119]. While phorbol esters are protective in the studies cited above, they have also been demonstrated to cause DNA cleavage in mouse thymocytes [103] and potentiate glucocorticoid-induced cell death in human CEM-C7 cells [120]. Activation of PKC by phorbol esters in a variety of different cell types has been shown either to prevent apoptosis induced by different agents or to enhance the process of apoptosis (as discussed above). It remains unclear why activation of PKC in one cell type is protective and in another capable of exacerbating the process of apoptosis. However, the description of 12 different isoforms of PKC provides some insight into the varied responses. Phorbol esters bind to other proteins as well as PKC [121, 122] and are not uniform in their effects on individual isoforms of PKC; in some cases activation occurs, while in others downregulation is evident [123]. In a study of PKC isozyme distribution in relation to Bcl-2 expression in stratified squamous epithelium, Knox et al. [124] have shown that cytoplasmic PKCδ and ε together with nuclear PKCβ and δ are associated with cell survival. The expression of these isozymes is coincident with the presence of Bcl-2 protein, while their loss accompanies apoptosis. Iwata et al. [125] have shown that glucocorticoid selectively induces an increase in Ca^{2+}-independent PKC activity in the particulate fraction of immature thymocytes but not in mature T cells. This increase in activity was prevented by inhibitors of RNA and protein synthesis and involved the selective translocation of PKCε from the cytosol to the particulate fraction. Thus, this process involved de novo protein synthesis but also a change in the phosphorylation state of PKCε on translocation. In the breast cancer cell line MCF-7, phorbol esters caused growth inhibition associated with retention of cell viability, but overexpression of PKC led to cell death when cells were treated with phorbol ester [126]. These authors were unable to determine whether the overexpression of this isoform was responsible for the initiation of the death signal or whether concurrent changes in expression in other isoforms played a role. Selective activation of PKC$\beta 1$ during myeloid differentiation in the cell lines U937 and HL60 is associated with onset of apoptosis [105, 127]. Overexpression of PKCξ in U937 cells, induced to undergo differentiation by phorbol esters, switches the molecular programme to death rather than differentiation [128]. On the other hand, when spontaneously apoptotic U937 cells were isolated from exponentially growing cells, they were shown to exhibit increased expression of PKCβ and reduced expression of PKCζ [105]. As described above, ionizing radiation is capable of inducing apoptosis in different cell types [33]. We have referred to evidence that the activation of PTK is an early step in this process leading to enhanced phosphorylation of several substrates [33]. The same group has shown that the events downstream of PTK activation include stimulation of phosphatidylinositol turnover; covalent modification of serine/threonine-specific protein kinases including PKC; and activation of the transcription factor NF-kB [129]. Activation of PKC was prevented by the PTK inhibitors herbimycin A and genistein. A role for PKC in the radiation response has also been provided in other studies [130, 131]. Recent results demonstrate that the response of U937 cells to ionizing radiation includes activation of PKCδ [132]. Both activation of PKCδ and endonucleolytic cleavage of DNA were blocked by Bcl-2 and Bcl-xL, supporting an association with apoptosis. Furthermore, activation of PKCδ occurred by cleavage at a site similar to that recognized by interleukin-1β converting enzyme (ICE), and the

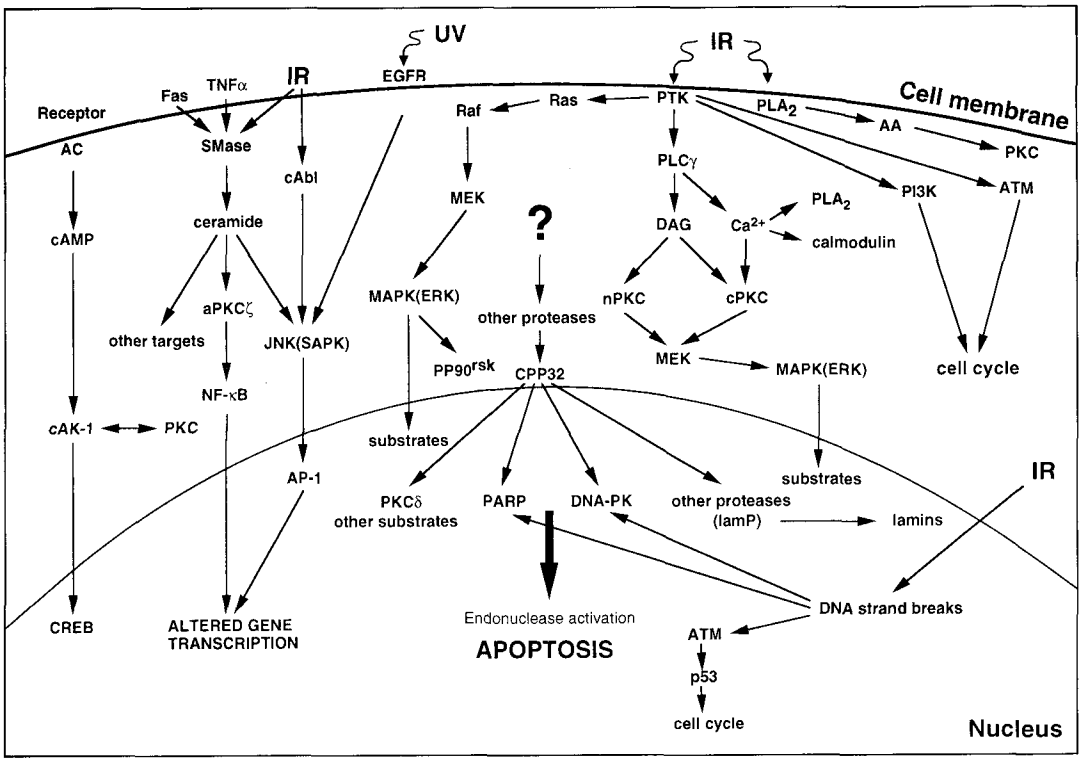

Figure 1. Signal transduction pathways implicated in apoptosis.

ICE-like protease inhibitor YVAD-CMK blocked the proteolytic activation of PKCδ and DNA fragmentation in irradiated cells [132]. The apparent contradictions related to the role of PKC in protecting against apoptosis or inducing the process can be explained to some extent by the cell type and the stimulus in question. It is apparent from the limited amount of data on the different isoforms of PKC that activation or down-regulation of individual species may be critical in determining the final outcome in a specific cell type.

Other protein kinases

As is the case for signalling pathways activated in response to receptor engagement, it would be expected that pathways involved in apoptosis will be composed of many intermediate factors (fig. 1). We have already discussed the involvement of PTKs and PKC isoforms in previous sections. As outlined in figure 1, there are a number of other protein kinases that might be important in apoptosis. Previous studies from this laboratory failed to find evidence for protection against radiation or heat-induced apoptosis in BM13674 cells treated with TPA [48]. Failure to exacerbate the extent of radiation-induced apoptosis by inhibitors of PKC with the same cell line was supportive of the TPA data [133]. But the specific protein kinase A (PKA) inhibitor, isoquinoline sulfonamide (H89), gave rise to a significantly increased level of apoptosis post irradiation compared with that observed in cells only exposed to radiation. The same concentration of H89 which was effective in increasing apoptosis markedly reduced PKA activity. Inhibitors of other protein kinases did not lead to an additional increase in apoptosis over and above that observed with radiation alone [133]. Contrary to these results, McConkey et al. [134] have reported that elevated cAMP stimulates DNA fragmentation and cell death in rat thymocytes. cAMP has also been shown to induce cell death in the myelocytic rat cell line IPC-81 [135]. The use of cAMP analogues demonstrated that the cAMP-dependent protein kinase isoenzyme 1 (cAK1) was most efficient in inducing cytolysis, indicating that selective activation of this isoenzyme was instrumental in inducing apoptosis. Additional evidence for the importance of cAK1 in apoptosis was derived from a mutant rat leukemia cell line which was resistant to cAMP-induced apoptosis [136]. The mutant cell line was characterized by a mutation in one of the genes coding for the regulatory subunits of cAK1 that reduced its binding affinity. Apoptosis correlated with the extent of binding of cAMP to the subresponsive regulatory subunit of cAK1. Microinjection of the catalytic subunit of cAK1 also induced apoptosis in IPC-81 cells [137]. Using PMA as an activator of PKC and the nondegradable PKA activator Sp-5,6-DCI-cBIMPS, Ojeda et al. [138] demonstrated a strong synergism in induction of apoptosis in murine thymocytes; thus, the cross-talk between PKA and PKC signal transduction

pathways yields a potentiation rather than an antagonism. It remains to be clarified which isoforms of PKC and PKA are involved in the cross-talking; cAK1, however, is a likely candidate.

The cytokine tumour necrosis factor (TNF) induces a variety of biological responses that include generation of cAMP [139], activation of phospholipase C [74, 140], enhanced phosphorylation of cellular proteins [141, 142] and activation of NF-kB [143]. The observed activation of protein kinase activity [144, 145] and inhibition of phosphatase activity [146, 147] could account for the phosphorylation changes observed in response to incubation of cells with TNF. There are two receptors for TNF, p75 and p55, which mediate its biological activity [148, 149], but the precise roles of these receptors are not yet determined [150]. Darnay et al. [151] have shown that the p75 TNF receptor is phosphorylated in vivo, and that selective triggering of this receptor in the rat/mouse T-cell hydridoma PC60 is capable of inducing apoptosis in these cells [152]. Immunoprecipitation of p75 has identified an associated protein kinase, casein kinase-1 (CK-1), and an inhibitor of CK-1 prevented p75 kinase activity from co-immunoprecipitating with p75 [152]. In addition, CK-1-specific peptide substrates are phosphorylated by p75 kinase activity in vitro, and a partially purified CK-1 phosphorylates p75 in vitro. CK-1 phosphorylation of the p75 TNF receptor negatively regulates TNF-induced signalling to apoptosis.

In recent years, the importance of ceramide as a regulator of apoptosis and growth suppression has emerged [153, 154]. Ceramide is generated from sphingomyelin by sphingomyelinase, which is activated in response to ligation of Fas, TNFα, IL-1, IFNγ and nerve growth factor (NGF) receptors. Ceramide has also been reported to be produced in response to ionizing radiation [155], and ceramide-induced apoptosis is inhibited by phorbol ester. Several targets for ceramide action have been proposed: Kolesnick's group has reported on a ceramide-activated protein kinase (CAPK) which has been shown to phosphorylate Raf [156], and Hannun's group has described a ceramide-activated protein phosphatase (CAPP) which belongs to the phosphatase 2A family of serine/threonine protein phosphatases and is inhibited by okadaic acid. A third target is PKCζ, which can be activated by ceramide in vitro [157]. The proto-oncogene product Vav is a guanine nucleotide exchange protein for Ras which contains a cysteine-rich motif similar to that in PKC and has been shown to bind phorbol ester. Gulbins et al. [158] have recently demonstrated that ceramide enhanced Vav exchange activity. Ras activation has also been shown to be important in Fas-mediated apoptosis, and this appears to occur via ceramide action [159]. Ras-neutralizing antibody and a dominant negative *ras* mutant interfered with Fas-induced apoptosis. Treatment of HL-60 cells with either TNFα or ceramide results in the prolonged activation of JNK (c-Jun amino terminal protein kinase) activity with little or no effect on ERK1 (extracellular signal-regulated kinases) or ERK 2 kinases [160]. However, only TNFα treatment results in activation of NF-kB. These results contrast with those of Gulbins et al. [159], who showed mitogen-activated protein kinase (MAPK) activation (ERK1 and ERK2) in response to ceramide. Whether the different targets of ceramide action mediate distinct responses to this regulator has yet to be determined, and there is still much to be learned about the mechanisms that lead to apoptosis resulting from generation of ceramide.

The end-point of the many pathways activated by external stimuli is the activation of transcription factors such as NF-kB, c-Jun, c-Fos, AP-1 and many others [161]. Several reports have described the activation of c-jun after growth factor withdrawal from sympathetic neurons [162, 163] and exposure of CEM-C7 cells to radiation or dexamethasone [164]. The role of c-Jun in apoptosis remains unclear; however, it has been shown using antisense oligonucleotides for c-jun and an inhibitor of AP-1 activation that AP-1 is critical for apoptosis in HL60 cells [165]. The transcription activity of c-Jun is enhanced by signalling through several pathways, including the MAPK pathway. The MAPK family members include ERK, JNK and p38 protein kinase [19, 166, 167]. While activation of JNK and p38 occurs in response to a variety of environmental stimuli; JNK and p38 induce apoptosis under defined conditions [42, 168, 169]. Rat pheochromocytoma PC-12 cells differentiate into cells resembling sympathetic neurons in the presence of NGF, and removal of NGF causes morphological changes characteristic of apoptosis and DNA fragmentation [34]. Under these conditions there was a significant increase in the activity of JNK and p38 kinase, and this increase preceded the induction of apoptosis, suggesting that the activation of these kinases might contribute to its onset. Staurosporine, an inducer of apoptosis, also increased the activities of these kinases, and several agents that prevented apoptosis inhibited kinase activation [34]. The conclusion drawn from this work is that a dynamic balance exists between growth factor-activated ERK and stress-activated JNK, p38 pathways which determines the ultimate fate of the cell.

Apoptosis and the cell cycle

Over the past 20–30 years the emphasis on the effects of DNA-damaging agents on cells has concentrated on the nature of the damage and the capacity of the cell to repair that damage [170]. Recently, more emphasis has been placed on other aspects of the damage response [171–176]. It is now evident that members of signal transduction pathways for mitogen and other receptor-

mediated responses also participate in the transmission of signals initiated by agents such as ultraviolet (UV) light and ionizing radiation [33, 130, 131, 171, 172, 177]. As we have pointed out above, the end-point of these pathways is frequently the activation of transcription factors. Another major end-point is the control of cell cycle progression, which in turn regulates the rate of cell proliferation. Cell cycle control in response to damaging agents such as UV and ionizing radiation involves both protein activation/stabilization and increased expression of some genes [172, 178, 179]. Exposure of eukaryotic cells to ionizing radiation leads to the activation of several checkpoints that control the progression of cells between different phases of the cell cycle [180]. Progression of cells from G1 phase to S phase is normally controlled by cyclin-dependent kinases (cyclin E-cdk2 and cyclin DI-cdk4), the phosphorylation status of these kinases, the substrates of these kinases, such as the retinoblastoma protein, and cyclin-kinase inhibitors [179, 181]. Signals arising due to DNA damage activate the product of the tumour suppressor gene p53 to prevent the progression of cells from G1 to S phase [180, 181]. This is achieved by p53-activated induction of the cdk inhibitor p21/WAF1, which binds to and inhibits cyclin-kinase complexes, preventing the phosphorylation of specific substrates and in turn the passage of cells from G1 to S phase [177, 183, 184]. Wild-type p53 blocks progression of cells from G1 to S phase in response to DNA damage [180, 182] or when it is overexpressed in cells [185]. After exposure of cells to DNA-damaging agents, p53 is translocated from the cytoplasm to the nucleus, where it can bind to specific DNA sequences to positively or negatively regulate transcription [183]. After treatment with radiation and other agents, the half-life of p53 increases from 10–30 min to several hours [186]. Since p53 has multiple phosphorylation sites, it has been suggested that phosphorylation is the means of stabilization [187, 188]. When rat embryo fibroblasts transfected with activated *ras* are exposed to phorbol ester, co-operation with wild-type p53, to bring about growth arrest, is observed [189]. Under these conditions a specific enhancement of wild-type p53 phosphorylation occurs, and there is an enhancement of binding of p53 to DNA. The PKC mode of phosphorylation in vitro also stimulates p53 DNA binding activity. Delphin and Baudier [189] propose that PKC and p53 participate in a negative feedback control of phosphoinositide signals common to mitogenic stimulation. Wild-type p53 leads to either G1 arrest or apoptosis in different cell types, but no G1 arrest is observed with mutant p53, and apoptosis can be prevented [180, 190].

There is now evidence that the signals that control proliferation as a consequence of cell cycle progression are also involved in mediating apoptosis. The potential role of activation of cyclin-dependent kinases in the initiation and/or progression through apoptosis has recently been investigated in different cell types. While studies have implicated the activation of cyclin A-dependent kinases in chemically induced apoptosis of S-phase Hela cells [191] and in myc-overexpressing fibroblasts [192], others have pointed to a role for selective activations of $p34^{cdc2}$-associated kinases in protease- induced apoptosis in lymphoma cells [193], cyclin B1/cdc2 kinase complexes in DNA damage-induced apoptosis [194] and cyclin E-, but not cyclin A-, containing complexes in cytosine arabinoside-induced apoptosis of HL60 cells [195]. Meikrantz et al. [191] demonstrated that apoptosis can be induced in HeLa cells by the same agents that induce premature mitosis in hamster cells. Under these conditions cyclin A-dependent kinase activity increases. When L929 fibroblasts are arrested early in G1 phase, prior to cyclin-A synthesis, they are protected from TNFα-induced apoptosis [196]. On the other hand when positive regulators of cyclin-A transcription c-*myc* and adenovirus E1A are ectopically expressed, they lead to apoptosis [197, 198]. Shi et al. [193] have suggested that the initiation of abortive mitosis through premature $p34^{cdc2}$ activation may be a general mechanism for induction of apoptosis, acting as a convergence point for different apoptotic signals. More recent data employing FT-210 cells exposed to a diverse array of apoptotic-inducing stimuli, where $p34^{cdc2}$ was degraded, failed to change susceptibility to undergo apoptosis, indicating that $p34^{cdc2}$ activation was not generally obligatory for apoptosis [199]. In addition, Norbury et al. [200] have shown that activation of cdc2 is not involved in the induction of apoptosis in quiescent thymocytes. However, it is possible that other cyclin-dependent kinases are active in promoting apoptosis in nonproliferating cells.

We have recently shown that inhibition of activity of members of the cyclin-dependent kinase family, specifically $p58^{cyclin\ A}$ kinases, followed by restoration to normal levels is associated with radiation-induced apoptosis in a susceptible Burkitt's lymphoma cell line (Williams et al., unpublished). On the other hand, cyclin kinase activity decreased and remained low in an isogenic Burkitt's cell line characterized by resistance to radiation-induced apoptosis. In both cases, inhibition of cyclin kinase activity was accompanied by an increase in p21/WAF1. In the apoptosis-susceptible cell line WAF1 was subsequently degraded but remained intact in the resistant line, implicating WAF1 in the regulation of cyclin-dependent kinases in radiation-induced apoptosis. In other cell lines activation of cdk activity is observed during apoptosis [191–195], but in the susceptible Burkitt's lymphoma cell line an initial decrease in activity followed by a return to normal values occurred. It is not certain whether this recovery is typical of the population of cells as a whole or whether it represents an apoptotic subpopulation showing cdk activation.

Ectopic expression of a 58-kDa protein kinase (PITSLREβ1), a member of the *cdc2* gene family, causes abnormal chromosome segregation and decreased growth [201]. Lahti et al. [202] have recently shown that the decreased growth rate is due to apoptosis and is accompanied by accumulation of PITSLRE kinase. It is suggested that this kinase may lie within an apoptotic signalling pathway and may be a target for serine proteases.

In addition to activating a checkpoint at the G1/S phase transition, DNA-damaging agents also lead to arrest in G2 phase and ultimately cell death [203, 204]. Treatment of Chinese hamster ovary cells with etoposide causes inhibition of $p34^{cdc2}$ kinase and a transient G2 delay [205]. Upon recovery of kinase activity, cytotoxicity results due to gross chromosomal fragmentation presumably as a consequence of abnormal $p34^{cdc2}$ activity. Cells undergoing granzyme B-mediated apoptosis also activate $p34^{cdc2}$, giving rise to chromatin condensation and DNA fragmentation typical of apoptosis [193]. In this respect it resembles mitotic catastrophe where cdc2 is active out of mitosis [193]. Cells transfected with Wee1 kinase, an enzyme that negatively regulates $p34^{cdc2}$ by phosphorylation, become resistant to apoptosis induced by granzyme B or perforin [206]. Wee1 inhibits apoptosis by preventing the dephosphorylation and activation of $p34^{cdc2}$.

As at other levels of signal transduction, phosphorylation and dephosporylation events are associated with control of cell cycle progression. Apoptosis has been demonstrated in all phase of the cell cycle, and in some cases, such as for p53 involvement, phosphorylation is required for apoptosis to occur. In other cases, such as the premature activation of $p34^{cdc2}$, dephosphorylation is required. Clearly, as in normal cell cycle control, kinases and phosphatases also participate in the changes required for apoptosis at various stages of the cell cycle.

Phosphatidylinositol 3-kinase

The transmission of signals from the cell surface to the cytoplasm and ultimately the nucleus is initiated by the phosphorylation of specific sites in the cytoplasmic tails of receptors and receptor-associated molecules. This is achieved by autophosphorylation in some cases, while in other cases a soluble protein tyrosine kinase is responsible for the phosphorylation [207]. This phosphorylation leads to the recruitment and activation of enzymes involved in signal transduction, such as PLCγ and phosphatidylinositol 3-kinase (PI3-kinase) [56, 208]. PI3-kinase is a heterodimer composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit [209]. The 85-kDa subunit binds to other proteins and associates with and is phosphorylated by the catalytic subunit, giving rise to an activated complex [56]. There is evidence that PI3-kinase is a dual specificity kinase, acting as a lipid kinase and a serine/threonine protein kinase [210]. This enzyme is activated by growth factors and other agents and plays a role in growth, differentiation, transformation and apoptosis, presumably being mediated by 3-phosphoinositides [56, 211, 212].

During the normal development of the nervous system, a large proportion of developing neurons is eliminated by apoptosis [42]. Survival of individual neurons is determined by the secretion of a series of neurotrophins from their target cells [213, 214]. One of these neurotrophins, NGF, is responsible for both cell survival and differentiation by activating a protein tyrosine kinase [215, 216]. NGF is also capable of preventing apoptosis in rat pheochromocytoma PC-12 cells [217, 218].

Specific inhibitors of PI3-kinase, wortmannin and LY294002, abrogated the ability of NGF to prevent apoptosis in PC-12 cells, suggesting that PI3-kinase played a role in this process [76]. Furthermore, cells expressing platelet-derived growth factor (PDFG) receptor were protected against apoptosis by PDGF, but cells expressing a mutant form of the receptor that failed to activate PI3-kinase were not protected [76]. These results suggest that one important role of PI3-kinase is to ensure cell survival by preventing apoptosis. Since this enzyme has dual specificity for lipid and proteins, it is possible that protection is mediated by the products of PIP_2 hydrolysis and/or by the phosphorylation of protein substrates.

Another dimension has been added to the role of PI3-kinase in apoptosis with the cloning and identification of the gene mutated in the human genetic disorder ataxia-telangiectasia (A-T) [219]. This gene, called ATM (A-T Mutated), is related to PI3-kinase and a series of proteins from yeast, *Drosophila* and mammalian cells through an N-terminal PI3-kinase domain [219–222]. The products of these related genes are involved in DNA repair [223], cell cycle control [224] and the shaping of chromosomes [225, 226]. A-T is a human genetic disorder characterized by immunodeficiency, neurological abnormalities, radiosensitivity and a defective radiation response, cell cycle abnormalities and a predisposition to cancer [227–229]. The defect in a PI3 kinase-like protein in A-T is compatible with the range of abnormalities observed in this syndrome. The chromosome instability and cancer predisposition that characterizes cells from A-T patients appears to be due to failure to respond to DNA damage by the activation of cell cycle checkpoints [182, 230–234]. However, the increased sensitivity to radiation does not appear to be due to a defective p53 response which permits A-T cells to progress from GI to S phase without delay after exposure to radiation [230–232]. On the contrary, Meyn et al. [235] have shown that A-T fibroblasts and lymphoblastoid cells undergo apoptotic cell death in response to radiation or streptonigrin exposure, under

conditions where no appreciable apoptosis was present in control cells. Even though the p53 response is defective in A-T, apoptosis appears to be p53-mediated. Suppression of apoptosis occurred in A-T fibroblasts when p53 was functionally inactivated by a dominant negative p53 gene or by the E6 gene of human papilloma virus [227, 235]. Loss of p53 function in transfected A-T cells was accompanied by the acquisition of a near-normal resistance to ionizing radiation, which led the authors to suggest that p53-mediated apoptosis is the major cause of radiosensitivity in A-T cells, in culture. Results with lymphoblastoid cells also demonstrate more apoptosis in irradiated A-T cells, but the extent of apoptosis in these cultures only accounts for approximately 30% of the total cell death at 72 h post irradiation (Khanna et al., unpublished). Since it has been demonstrated that different regions of the p53 molecule control cell cycle arrest and apoptosis, and since there does not appear to be a correlation between these two functions, it seems likely that p53 mediates apoptosis by a mechanism other than through G1 delay.

Proteolytic cleavage of DNA-dependent protein kinase

In a recent review, Martin and Green [236] have invoked 'Death by a thousand cuts' to account for the series of proteolytic events that contribute to the process of apoptosis. While this may eventually involve a thousand cuts or more, it is likely that a much smaller number of strategic proteolytic cleavages, aimed at a set of critical targets, will represent the initial stage in one or more pathways to apoptosis. It is now clear that degradation of macromolecules is not confined to DNA, but a series of functionally and structurally important cellular proteins are also degraded during the process of apoptosis [20, 52, 236]. These proteins include poly(ADP-ribose) polymerase (PARP) (15, 20), nuclear lamins [21], U1-70 kDa [14], fodrin [236], hnRNPC [17] and topoisomerase 1 [52].

A gene essential for developmentally programmed cell death in *Caenorhabditis elegans*, *Ced-3*, has been shown to be homologous to the mammalian ICE-like protease required for processing pro-ILIβ [7, 25]. A family of these ICE-like proteases has now been identified [15, 18, 20, 26, 237–239], and there is good evidence that proteases play an essential role in apoptosis [22, 236, 240]. It is notable that the substrates described for these enzymes are involved either in nuclear, cytoskeletal or membrane structures, or they have an important functional role. PARP and topoisomerase are implicated in DNA repair and chromosomal segregation, and their degradation during apoptosis would favour DNA fragmentation.

We have recently added another important substrate to that group, DNA-PK. This enzyme comprises a 460-kDa catalytic subunit (DNA- PKcs) and a DNA-binding component, Ku, made up of two subunits 70 and 80 kDa in size [241, 242]. The cloning of a cDNA for DNA-PKcs has been described recently [223]. It is related to several other proteins through a PI3-kinase domain but has only been shown to phosphorylate proteins. This enzyme is involved in DNA double-strand break repair, V(D)J recombination and transcription [243–245]. We and others have demonstrated that DNA-PKcs is selectively degraded during apoptosis induced by different agents in different cell types [54, 246]. There was a parallel loss of DNA-PK activity, but the DNA-binding component of the enzyme remained intact under these conditions. The cleavage pattern of DNA-PKcs during apoptosis was the same as that observed when purified DNA-PKcs was incubated with the ICE-like protease CPP32. Microsequence analysis revealed that the cleavage sites were the same. These results show that an important protein kinase is degraded in apoptosis, and as with PARP, this degradation would tip the balance in favour of DNA fragmentation. The cleavage of DNA-PKcs and PARP would also help to conserve ATP, which is required for the two activities, and allow apoptosis, an energy-requiring process, to proceed efficiently.

Conclusions

Control of development, differentiation, cell proliferation and cell death involves a variety of changes at the level of gene expression as well as modifications to existing proteins. These modifications frequently involve changes to the phosphorylation state of proteins which may either activate or suppress the activity of a specific protein. At a variety of levels in signal transduction pathways, protein kinases and/or phosphatases are activated in order to ensure the transmission of specific signals from the plasma membrane to the nucleus. While de novo protein synthesis is required for apoptosis to proceed in murine cells, no such requirement has been demonstrated in several other cell types undergoing apoptosis. Similar to events documentated during cell proliferation, protein kinases participate at different stages in cells undergoing apoptosis. The decision to proliferate or die depends on the stage of ontogeny (as in lymphoid cells), the nature of the stimulus received, the cell type and the stage of the cell cycle. Experiments with cell-free extracts and enucleated cells have revealed that pre-existing proteins in the cytoplasm are capable of initiating apoptois. The challenge that remains in the field of apoptosis is a description of the upstream or triggering events that are responsible for initiating the process, although it is conceivable that there will be no single trigger since so many agents working in so many different ways are capable of causing apoptosis. It is more likely that there will be common intermediates

and perhaps cross-talk between pathways. A description of the role of specific protein kinases/phosphatases and their substrates will assist in delineating these pathways. A greater understanding of the individual steps involved will offer further enlightenment for the design of strategies designed to enhance or suppress specific steps as therapeutic approaches to the control of cancer, virus infection and other important biological processes.

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