Two kinds of BIR-containing protein - inhibitors of apoptosis, or required for mitosis

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Journal of Cell Science 114, 1821-1827 © The Company of Biologists Ltd

Summary

The baculoviral IAP repeat (BIR) is a zinc-binding fold. Some BIR-containing proteins (BIRCs), including several from insect viruses, insects and vertebrates, are inhibitors of cell death and act by binding to active caspases. Their ability to do so can be antagonized by pro-apoptotic insect proteins such as Grim, HID and Reaper, or the mammalian protein Diablo/Smac. Members of one structurally distinct subgroup of BIR-containing proteins, which are present in yeasts and *Caenorhabditis elegans* as well as insects and vertebrates, do not act as caspase inhibitors; instead, they are required for chromosome segregation and cytokinesis, and act in concert with inner centromere protein (INCENP) homologues and aurora kinase homologues.

Key words: Survivin, XIAP, IAP, BIRC, Cytokinesis, Apoptosis, Mitosis, INCENP, Aurora kinase

Introduction

Members of the IAP (inhibitor of apoptosis) family of proteins were first identified as viral products used by baculoviruses to inhibit defensive apoptosis of host cells and thereby allow the virus more time to replicate (Crook et al., 1993). Since that time, many cellular IAP homologues have been found, some of which can also inhibit apoptosis. However, a role in physiological cell death has not been established for all IAPs, and members of a structurally distinct subfamily play essential roles in cell division, rather than having a direct role in the regulation of apoptosis (Uren et al., 1999; Uren et al., 2000). Because all IAPs bear one or more characteristic zinc-finger motifs termed baculoviral IAP repeats (BIRs), the whole family of proteins (see Fig. 1) is more precisely termed BIR-containing proteins (BIRps or BIRC proteins) rather than IAPs.

Identification of cellular BIR-containing proteins

Most baculoviral BIRps contain two BIRs and a RING-finger domain, another zinc-binding motif. Several baculoviruses encode more than one BIRp, and some also carry a gene for the unrelated caspase inhibitor p35 (Clem et al., 1991). An anti-apoptotic role for all of these IAPs, in particular those from viruses that also carry p35 genes, has not yet been demonstrated.

The first non-viral IAP found was NAIP (BIRC1; Roy et al., 1995), a candidate for the gene mutated in the inherited disease spinal muscular atrophy (SMA). It is now clear that SMA is caused by mutations in the closely linked *SMN* gene, which encodes a protein involved in splicing, rather than by abnormalities in NAIP (Lefebvre et al., 1995; Liu et al., 1997; Jablonka et al., 2000). Furthermore, there are several, closely linked, *NAIP* genes (Yaraghi et al., 1998), but none of them corresponds to the published *NAIP* cDNA (Liston et al., 1996; Xu et al., 1997) that was used to demonstrate the NAIP anti-apoptotic activity (Yamamoto et al., 1999). Therefore it is currently unclear which if any of the NAIP gene products are inhibitors of apoptosis. Nevertheless, although deletion of one of the *NAIP* genes, *NAIP1*, in the mouse did not affect development or behavior, it appeared to reduce the survival of certain neuronal subsets following experimentally induced seizures (Holcik et al., 2000).

Other non-viral BIR-containing proteins have been identified by database searches, by sequencing of proteins from complexes that associate with the cytoplasmic domain of tumour necrosis factor receptor 2 (TNFR2) or by genetic screens in *Drosophila*. Thus BIRC2 (also known as cIAP1, MIHB or hIAP2) and BIRC3 (also known as cIAP2, MIHC or hIAP1) were found bound to TNF-receptor-associated factors TRAF1 and TRAF2 in association with TNFR2, and BIRC4 (also known as XIAP, MIHA or hILP) and ML-IAP/Livin were found as ESTs in database searches (Rothe et al., 1995; Liston et al., 1996; Uren et al., 1996; Duckett et al., 1996; Vucic et al., 2000; Kasof and Gomes, 2001). *Drosophila* BIRps DIAP1 (Thread) and DIAP2 (DIHA) were found in screens of flies expressing the cell death inducer *reaper* and in database searches (Hay et al., 1995; Uren et al., 1996). Chicken BIRC2 (ITA) was identified in a search for IL-2-induced genes in chicken thymocytes (Digby et al., 1996). All of these proteins bear 1-3 BIR domains and, like the baculoviral BIRps but unlike BIRC1, BIRC5 or BIRC6, have a single RING-finger domain. BIRC2 and BIRC3 also have a caspase-recruitment domain (CARD).

The BIR domain is one of ~20, structurally distinct, zinc-finger folds in which zinc ions are coordinated by cysteine and histidine residues (Hinds et al., 1999). The BIR fold can be separated into two halves. The N-terminal half contains two α-helices and a universally conserved arginine residue. Near the start of the C-terminal half, which contains the zinc ion coordinated by three cysteine residues and a histidine, is a universally conserved glycine residue.

A subfamily of BIR proteins that bear structurally distinct BIRs have been identified in yeasts, invertebrates and vertebrates. This includes mammalian BIRC5 (also known as

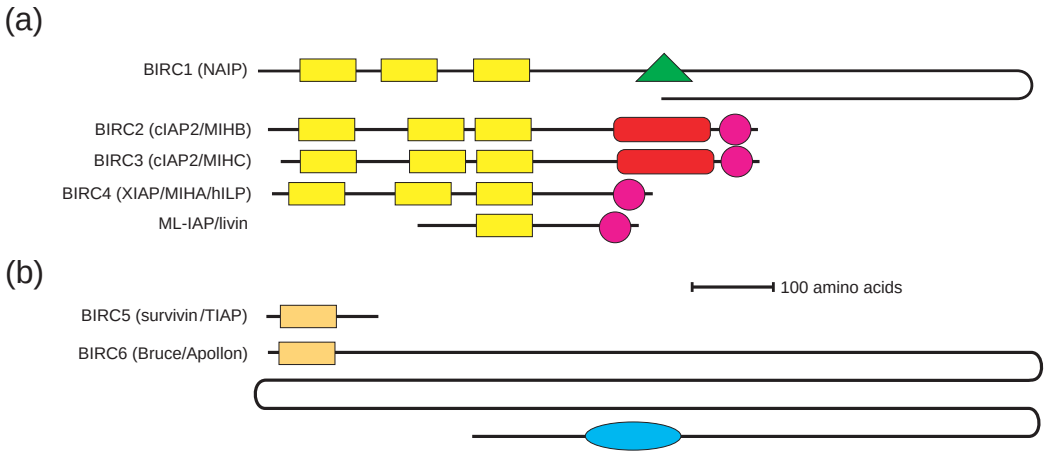

Fig. 1. Human BIR-containing proteins. Human BIR containing proteins are grouped according to their structure and known or presumed function. Those implicated in the regulation of apoptosis are shown above (a), and those that appear to have cell cycle functions are below (b). All bear BIR domains (yellow boxes). Most of those implicated in cell death control also bear RING-finger domains (purple circles). BIRC2 and BIRC3 also have CARDs (red ovals). BIRC1 (of which there are several, closely linked copies) has a nucleotide-binding loop (green triangle). The two proteins that bear a structurally distinct BIR, BIRC5 and BIRC6, are the smallest and largest BIR-containing proteins. BIRC6 bears a ubiquitin-conjugating domain (blue oval). The BIRs in BIRC proteins from yeasts and *C. elegans*, and the *Drosophila* protein deterin, have structures like those of BIRC5 and BIRC6. Terminology from HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature).

survivin or TIAP) and BIR6 (also known as BRUCE or Appolon), *Drosophila* Deterin, *Caenorhabditis elegans* BIR-1 and BIR-2, *Schizosaccharomyces pombe* BIR1 and *Saccharomyces cerevisiae* Bir1p (Uren et al., 1998; Jones et al., 2000; Hauser et al., 1998). BIRs from these survivin-like proteins usually contain three additional residues, often including a proline, between the universally conserved glycine residue in the middle of the fold and the first zinc-binding cysteine residue. In addition, unlike the other BIRp genes, most of these genes have introns just before the universally conserved glycine residue.

Inhibition of apoptosis

Our understanding of how BIRps can inhibit apoptosis has come mainly from analysis of baculoviral IAPs, the *Drosophila* proteins DIAP1 and DIAP2, and the mammalian proteins BIRC2-BRC4 (see Fig. 2). These experiments have shown that

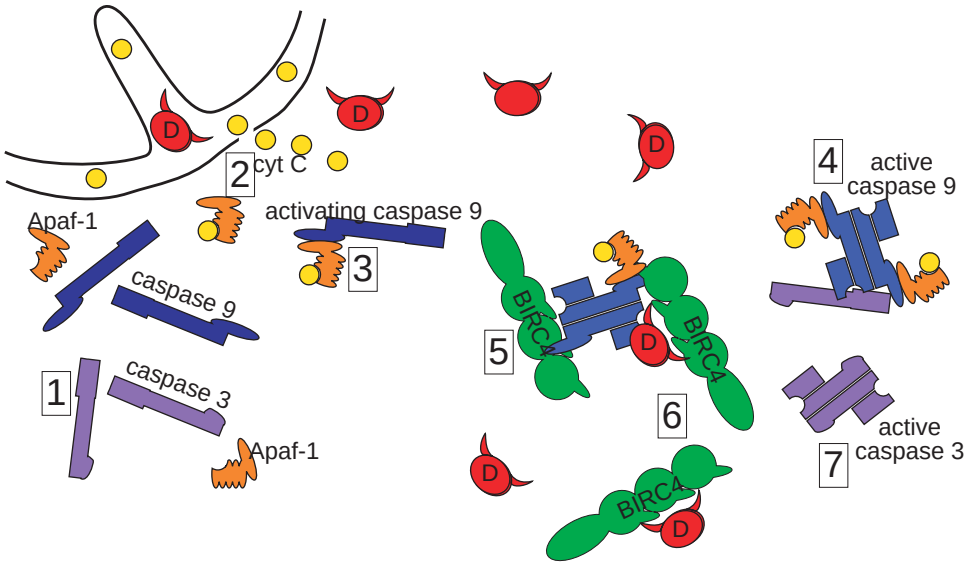

Fig. 2. Mechanism of action of BIRC4. In healthy cells, caspases are in their inactive, uncleaved, zymogen form (1). Release of cytochrome C (cyt C) from the mitochondria allows it, and dATP, to bind to Apaf-1, which activates it (2). Through the N-terminal CARDs, Apaf-1 can interact with caspase 9, causing it to activate (3). Active caspase 9 can cleave and activate caspase 3 (4), (7). If sufficient free BIRC4 is present in the cytosol, it can bind to processed caspase 9 (5) and thereby prevent it from activating caspase 3. However, if enough Diablo/Smac dimers (D) are also released from the mitochondria (1), they can bind to BIRC4 and displace caspase 9 (6), freeing it to activate caspase 3 (7). In this simplified model, many proteins or protein complexes are shown as monomers, even though it is possible that some of these proteins (such as caspase zymogens and BIRCs) form dimers or higher-order complexes.

these BIRps can inhibit cell death by binding to and inhibiting caspases, the key effector proteins of apoptosis (Deveraux et al., 1997; Roy et al., 1997; Hawkins et al., 1999; Meier et al., 2000).

BIRC4 can inhibit caspase 3 and caspase 9 with an IC_{50} of ~10 nM. A fragment including its second BIR (BIR2) and the flanking region N-terminal to it can bind to and inhibit active, processed, caspase 3, and BIR3 can bind to processed caspase 9 (Takahashi et al., 1998; Sun et al., 2000; Sun et al., 1999). Comparison of point mutant BIRC4 proteins that cannot bind to caspase 3 with mutants that cannot bind to caspase 9 suggests that inhibition of caspase 9 is more important for its ability to inhibit apoptosis (J. Silke, unpublished observations).

Structural studies have shown that IAPs inhibit effector caspases 3 and 7 in a fundamentally different way from the other caspase inhibitory proteins p35 and CrmA (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). CrmA and p35 act as pseudosubstrates and bear sequences designated P4-P1, in which P1 is the aspartic acid residue following which cleavage occurs. In the case of p35, these residues are P4DQMDP1GF, and, in the case of CrmA, they are P4LVADP1CA. Although residues 148DISD151 of BIRC4 were also proposed to act as a pseudosubstrate for caspases, this seemed unlikely, because, firstly, BIRC4 is not cleaved by caspases at this site, and, secondly, mutation of a putative P1 aspartate residue in BIR2 (D151A) only modestly reduced inhibitory activity, whereas mutation of the putative P4 (D148A) caused complete loss of activity (Sun et al., 1999). Solving of the structures of BIR2 of BIRC4 bound to caspases explained the paradox: the linker residues N-terminal to BIR2 (148DISD151) do bind to the active site of the caspases, but in the reverse orientation compared with peptide substrates CrmA and p35 - i.e. with 148D closest to the catalytic cysteine residue of the caspase.

Biochemical and NMR studies have shown that BIR3 of BIRC4 can bind to and inhibit active caspase 9 (Sun et al., 2000). In this case, the flanking region N-terminal to the core BIR fold does not appear to be important for this interaction; this indicates that these two regions of BIRC4 inhibit caspase 3 and caspase 9 in different ways. BIRC4 only binds to processed caspase 9 (Ekert et al., 2001), and processing produces an N-terminal peptide in caspase 9 that resembles that of Diablo, Grim, HID and Reaper. It therefore appears that processed caspase 9 binds to BIR3 of BIRC4 in the same way as the IAP antagonists do (Srinivasula et al., 2001).

The *Drosophila* IAPs DIAP1 and DIAP2 also function by binding to and inhibiting caspases (Kaiser et al., 1998; Hawkins et al., 1999; Meier et al., 2000). In addition, they can bind to the pro-apoptotic signalling proteins Reaper, Grim and HID (Wang et al., 1999; Vucic et al., 1998). These proteins bind to the IAPs and thereby prevent them from inhibiting caspase activity. Screens in *Drosophila* for enhancers or suppressors of *reaper*-induced apoptosis yielded *diap1* genes that have mutations in regions encoding BIR1 and BIR2. Furthermore mutations that suppress Reaper/Grim induced apoptosis (Goyal et al., 2000; Lisi et al., 2000) are in the analogous residues of BIR3 of BIRC4 that would disrupt interaction of BIR3 with Diablo/Smac (Wu et al., 2000; Liu et al., 2000). These results indicate that, like the caspases, Diablo/Smac, Grim, HID and Reaper bind to BIR domains or the regions flanking them.

In common with the insect pro-apoptotic proteins Grim, HID and Reaper, the mammalian IAP-binding protein Diablo/Smac also binds to BIRps through its N-terminal residues (Chai and Shi, 2000), and indeed there is a small but significant similarity between the processed N-termini of these molecules (Silke et al., 2000). Because increasing amounts of Diablo/Smac promote formation of a BIRC4-Diablo complex, while simultaneously decreasing the amount of BIRC4 bound to caspase 9, Diablo (and presumably Grim, HID and Reaper) appears to work by binding to the BIRps and displacing the caspases (Ekert et al., 2001; Srinivasula et al., 2001). In the case of BIRC4, the structural basis for this displacement is competition between Diablo and caspase 9 for binding to the same residues of BIR3 (Liu et al., 2000; Wu et al., 2000). Therefore IAPs are inhibitors of caspase activity that are themselves under inhibitory control by proteins such as Grim, HID and Reaper, or Diablo/Smac.

Although Du et al. (Du et al., 2000) found that the BIR-containing protein BIRC5 (survivin) in cell lysates was able to bind to Diablo/Smac-coupled beads, this BIRC-protein–antagonist interaction is unlikely to occur in vivo, because Diablo/Smac is in the mitochondria and, unlike other BIRC proteins, BIRC5 is present only in cells undergoing mitosis (see below).

Observations that the RING-finger domains of several other types of protein are involved in protein ubiquitination and proteosomal degradation led two groups to test for this function in the RING-finger domains of the IAPs (Yang et al., 2000; Huang et al., 2000). Although strong evidence that the RING fingers of BIRps can also mediate auto- or trans-ubiquitination has accumulated, this activity does not appear to be essential for their anti-apoptotic activity. RING-dependent ubiquitination might, however, be important for regulating the levels of IAPs or their target caspases.

Other IAP-binding proteins

Apart from binding to antagonists (i.e. Diablo/Smac, Grim, HID and Reaper) and caspases, IAPs have also been reported to bind to a number of signalling proteins. BIRC2 and BIRC3 were initially found binding to TRAF1 and TRAF2 in complexes associating with the cytoplasmic domain of TNFR2 (Rothe et al., 1995). Although these associations were confirmed in yeast two-hybrid experiments (Uren et al., 1996), the significance of these findings is not known. IAPs from mammals and insects have also been implicated in BMP signalling, but in opposite ways. Yeast two-hybrid experiments using BIRC4 as bait pulled out TAB1, a protein that associates with TAK1 and transforming growth factor receptors. In this way, BIRC4 is proposed to enhance BMP signalling (Yamaguchi et al., 1999). Yeast two-hybrid experiments using *Drosophlia* DIAP1 as bait pulled out Thick veins, a protein that interacts with a Dpp type I receptor in the fly, in an interaction that is proposed to decrease BMP signalling (Oeda et al., 1998).

Another yeast two-hybrid experiment using BIRC4 as bait pulled out the TRAF-like protein Xaf-1/Xap-1 (Liston et al., 2001). Because this protein is exclusively nuclear, whereas BIRC4 is cytoplasmic, and because its ability to bind BIRC4 is much lower than that of Diablo/Smac (Verhargen et al., 2000), the significance of this association is unclear.

In vivo roles of anti-apoptotic BIRps

Knockout and overexpression studies of *diap1* show that it has an essential role in control of apoptosis during development. Flies lacking *diap1* die during embryogenesis, exhibiting massive apoptosis. Because this phenotype is not affected by additional deletion of *HID*, *grim* and *reaper*, the pro-apoptotic activity of these proteins appears to be achieved by antagonism of DIAP1, at least during embryogenesis. One partial loss-of-function mutation of *diap1* (*Th4*) gives rise to thread-like antennae, in which cells that make up the aristae die (Hay et al., 1995).

Knockout and overexpression studies of mammalian IAPs suggest that they are less important in the control of developmental apoptosis in mammals than in the fly. In mammals, members of the BCL-2 family might well be the main regulators of apoptosis. Nevertheless, mammalian IAPs could be important in disease, because the BIRC3 gene is often translocated in mucosa-associated lymphoid tissue (MALT) lymphomas (Dierlamm et al., 1999), and ML-IAP/Livin is often abnormally expressed in melanoma cell lines (Vucic et al., 2000; Kasof and Gomes, 2001).

BIRC proteins with roles in mitosis

The first member of a subfamily of BIRps that have roles in mitosis to be identified was survivin, a mammalian protein that bears a single BIR (Ambrosini et al., 1997). The sequences encoding survivin first appeared in the databases in antisense orientation as EPR-1, apparently resulting from insertion of the cDNA into the cloning vector in reverse orientation (Altieri, 1994; Zaman and Conway, 2000). Reports that survivin can bind to and inhibit caspase 3 (Tamm et al., 1998; Conway et al., 2000; Shin et al., 2001) may be incorrect, because studies using purified survivin show it cannot bind to or inhibit purified caspase 3 at physiological concentrations (Verdecia et al., 2000; Banks et al., 2000), and survivin does not possess residues analogous to those of BIRC4 that bind to caspase 3 (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001).

Survivin exists as a dimer, probably as a ‘bow tie’ like structure in which the long C-terminal α-helix extends out from the dimerization interface (Verdecia et al., 2000; Chantalat et al., 2000), rather than forming dimers around an extra zinc ion (Muchmore et al., 2000). Analysis of *survivin* expression showed that it is cell cycle regulated and is expressed in all dividing cells, including cancer cells (Kobayashi et al., 1999; Li and Altieri, 1999). Altieri and co-workers have speculated that it is phosphorylated by p34cdc2 kinase (O’Connor et al., 2000), but, because the target threonine residue is not present in survivin homologues from fish or invertebrates, this is not likely to be of key importance for its function.

BIRps identified in yeasts and *C. elegans* bear BIRs that most closely resemble those of survivin, and, like the *survivin* gene, several of these genes contain introns within the BIR-encoding sequences. The expression and function of these genes, like *survivin*, are linked to the cell cycle (Uren et al., 1998; Speliotes et al., 2000; Uren et al., 2000; Uren et al., 1999; Zhao et al., 2000).

Budding yeast cells lacking Bir1 form spores inefficiently and have a phenotype similar to that of *Chl1* mutants, which lack a component of the kinetochore (Uren et al., 1999; Gerring et al., 1990). Bir1 was picked up in yeast two-hybrid experiments using the key kinetochore protein, NDC10, as bait (Yoon and Carbon, 1999). These results showed that *S. cerevisiae* Bir1 acts with kinetochore proteins and has a role in chromosome segregation.

Fission yeast *BIR1* mutants display a ‘cut’ phenotype, in which the septum forms in dividing yeast before the chromatin has segregated into the daughter cells. In these yeast, the spindle forms but does not elongate (Uren et al., 1999; Rajagopalan and Balasubramanian, 1999). Once again, a survivin-like protein is involved in cytokinesis and or chromosome segregation.

In *C. elegans* embryos in which BIR-1 production was inhibited by RNA interference, the DNA replicated but cytokinesis failed to be completed (Fraser et al., 1999). Furthermore, AIR-2, a *C. elegans* member of the aurora/AIR/Ipl1p family of serine threonine kinases, failed to localize correctly (Speliotes et al., 2000). Because transgenic expression of human *survivin* partially suppresses this phenotype, survivin and the *C. elegans* BIR proteins must act in a similar way. Furthermore, because genetic data indicate that BIR1 is not involved in cell death pathways in *C. elegans*, these experiments suggest survivin does not have a direct role in apoptosis in mammals.

Mouse embryos lacking *survivin* closely resemble *C. elegans* in which BIR-1 production is inhibited. In both cases, the chromatin replicates, but cytokinesis is abnormal because cleavage furrows that begin to form are not completed. The phenotypes in both the mouse and *C. elegans* resemble those of embryos lacking INCENP (inner centromere protein) homologues (Uren et al., 2000; Kaitna et al., 2000).

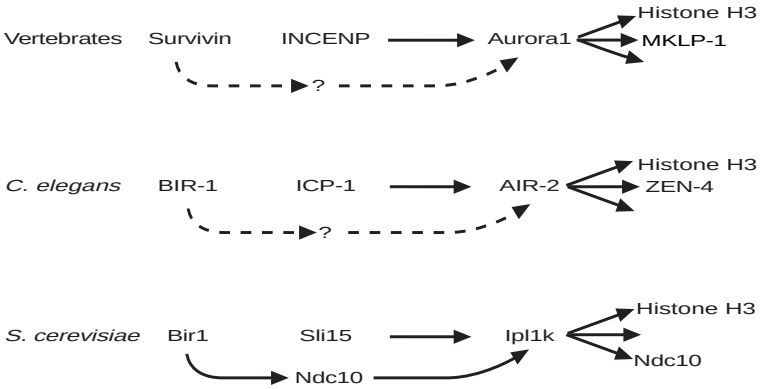

Fig. 3. Conservation of biochemical pathways in which BIRC5 and its homologues act. In mammals, BIRC5, INCENP and aurora 1 kinase are all chromosome passenger proteins. The same is true for the *C. elegans* proteins BIR-1 and AIR-2. Furthermore, BIR-1 is needed for correct localization of AIR-2, but whether this involves direct interactions is not known. In the yeast *S. cerevisiae*, Bir1p binds to Ndc10p, which is a substrate of the aurora kinase homologue Ipl1 kinase. Ipl1 kinase binds directly to the INCENP homologue, Sli15p, just as INCENP binds directly to aurora 1, and ICP-1 binds to AIR-2. Once activated, the kinases can phosphorylate substrates, including histone H3 and kinesins. Absence of, or inhibition of, BIRC5, INCENP or aurora 1 in mammalian cells, or homologues in other organisms, leads to failure of cytokinesis and/or imprecise chromosome segregation.

Furthermore, the localization of survivin homologues and INCENP homologues in the worm and in vertebrates is similar, and characteristic of so-called 'chromosome passenger' proteins. These proteins localize to the centromeres until the metaphase-anaphase transition but then remain in the equatorial zone as the chromosomes separate, eventually localizing to the mid-body at telophase, after which they are degraded (Speliotes et al., 2000; Uren et al., 2000, Skoufias et al., 2000).

In yeasts, invertebrates and vertebrates, INCENP homologues bind directly to and appear to localize aurora kinase homologues. These serine-threonine kinases are responsible for phosphorylating histones and kinesins during cytokinesis (Kim et al., 1999; Adams et al., 2000; Kaitna et al., 2000). Thus, it appears that, in a pathway conserved from yeast to vertebrates, survivin-like proteins directly or indirectly control INCENP and Aurora kinases, which themselves directly interact (see Fig. 3). Together these proteins are required to coordinate chromosome segregation with cytokinesis (Uren et al., 2000).

The tight correlation of survivin message and protein abundance with mitosis (Zhao et al., 2000) explains why survivin is much easier to detect in cancer cells (which are cycling) than in cells from normal adult tissue (which are mostly in G_0). To date, there has been no evidence to indicate that survivin expression is abnormal in cancer cells or that cancer cells bear mutations to DNA sequences that encode survivin or regulate its expression.

Conclusions and perspectives

Cells commonly activate their apoptotic mechanisms in response to stresses, including disturbances to their cell cycle machinery, such as when apoptosis is induced by agents that affect microtubules, including taxol and colchicine (Tang et al., 1994; Martin and Cotter, 1990). However, it would be wrong to conclude from this that microtubules are inhibitors of apoptosis. Similarly, because survivin is essential for mitosis, it should not be surprising that inhibiting it provokes an apoptotic response, but this does not mean survivin is an inhibitor of cell death.

Although plants have many different zinc-finger proteins, no plant gene that encodes a BIR fold has been identified. BIR-containing proteins are found in yeasts and animals, but anti-apoptotic BIRps are found only in insects and vertebrates. These observations suggest that the BIR fold evolved in the common ancestor of yeasts and animals, and was involved in cell division. Cytokinesis is very different in plants, compared with yeasts and animals, and they appear not to use either a survivin-like BIR-containing protein or an INCENP homologue. The anti-apoptotic BIR proteins may have arisen following duplication of the gene that encoded the ancestral mitotic BIR protein, an event that presumably occurred after the evolution of nematodes, in an ancestor shared by insects and vertebrates. The addition of the RING-finger domain must have come about soon after this event. These new BIRps were used by these organisms to inhibit caspases, but they retained the ancient, survivin-like BIRp for cytokinesis. Insects and vertebrates consequently bear both types of BIR protein; some of these have roles in cell division, whereas others prevent apoptosis by inhibiting caspase activity.

DLV is a scholar of the Leukemia and Lymphoma Society. This work was supported in part by the Anti-Cancer Council of Victoria.

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