ANTIAPOPTOTIC PROTEINS

The Bcl-2 and Inhibitor of Apoptosis Protein Families

Quinn L. Deveraux, PhD, Sharon L. Schendel, PhD, and John C. Reed, MD, PhD

Apoptosis, or programmed cell death, ensures that the genesis of new cells by way of cell division is controlled appropriately and offset by cell loss.[103] Cell death is a natural accompaniment of the physiology of self-renewing tissues in which terminally differentiated cells turn over to be replaced by new cells, such as in the skin, intestine, immune system, mammary gland, and uterus. Developmental organization requires removal of some cells for achieving the final desired structures, such as digits and canals, and ensuring proper cytoarchitectures of most organs, such as the kidney, heart, and brain. Moreover, elimination of cells compromised by viral infection, oxidation, hypoxia, or DNA damage is important for maintaining healthy tissues.[32]

Apoptotic defects are the primary lesion in some types of cancer and leukemia, allowing malignant cells to survive longer than their intended life span and endowing these cells with a selective survival advantage relative to their normal counterparts. Therefore, cell death can contribute to neoplastic expansion in the absence of increased cellular division.[84] Excessive apoptosis, however, has been implicated in spinal muscular atrophy (SMA), autoimmune disease, acquired immunodeficiency syndrome (AIDS), Alzheimer's disease, myocardial infarction, and stroke.[32] Thus, appropriate regulation of apoptosis is critical to development and maintenance of normal tissues.

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CELL DEATH PATHWAYS

The biochemical basis for most of the morphologic changes associated with apoptosis, such as membrane blebbing, chromatin condensation, and DNA fragmentation, can be traced to the actions of a family of cysteine proteases called the *caspases*. Once activated, the caspases can cleave cytoskeletal and nuclear matrix-associated proteins that are required for cellular integrity, such as lamins, inhibitors of DNA degradation enzymes, such as inhibitor of caspase-activated deoxyribonuclease (ICAD), and DNA repair enzymes,

From the The Burnham Institute Program on Apoptosis and Cell Death Research, La Jolla, California

such as DNA-protein kinase (PK) and poly (adenoside diphosphate [ADP]-ribose) polymerase (PARP), to name only a few of the known caspase substrates. Caspase-mediated cleavage of these and other cellular proteins facilitates the ordered dismantling of the cell and the irreversible destruction of its genome.

Most caspases are synthesized initially as inactive precursors (zymogens) that undergo proteolytic processing to produce subunits that form the active heterotetrameric protease. In mammalian cells, activation of the caspase zymogens has been reported to occur through at least three independent mechanisms: (1) cleavage by upstream active caspases; (2) cleavage by granzyme B, an aspartate-specific serine protease found in the granules of cytolytic T-cells; and (3) autoprocessing of zymogens with assistance from other caspase-interacting proteins, which can occur in either a cis- or transmanner (reviewed in references 90 and 105).

Cellular and genotoxic stresses, such as those inflicted by chemotherapeutic drugs and radiation, can induce the expression of proapoptotic members of the Bcl-2 family. At least one of these proteins, Bax, has been shown to promote the release of cytochrome c from mitochondria (reviewed in references 37 and 86). Once released, cytochrome c catalyzes the formation of the *apoptosome* a deoxyadenosine (dATP)– or adenosine triphosphate (ATP)-dependent complex consisting of the apoptosis protease activating factor 1 (Apaf-1) protein and procaspase-9.[57, 59, 86, 136] Apoptosome formation results in activation of bound procaspase-9, which then can directly cleave and activate procaspase-3, resulting in additional caspase activation and apoptosis (Fig. 1). In addition to fostering procaspase-3 activation, caspase-9 may have other functions, because in some cells, it can translocate to the nucleus following apoptotic stimulus.[53]

Another prototypical mechanism for triggering autoprocessing and activation of caspases requires the recruitment of procaspase-8 to plasma membrane receptor complexes, such as Fas, which is a member of the tumor necrosis factor (TNF) family of cell death receptors. Procaspase-8 possesses approximately 1% the activity of the processed fully active protease. When brought into close apposition by oligimerization around Fas receptor complexes, these zymogens transprocess each other, yielding autonomous, active caspase-8.[47, 67, 104] Once activated, caspase-8 then can directly activate procaspase-3 and other downstream caspases (see Fig. 1).

Bcl-2 FAMILY PROTEINS

In certain apoptotic pathways, Bcl-2 family proteins govern a cell's decision to heed or ignore death signals. The progenitor of this family is the Bcl-2 protein, first identified at a chromosomal breakpoint in human B-cell lymphomas.[117] The family subsequently has expanded and now includes at least 18 members with representatives from mammalian species, viruses, and *Caenorhabditis elegans*.

The family can be divided into two groups: (1) antiapoptotic, which includes Bcl-2 and Bcl-x_L; and (2) proapoptotic, which includes Bax and Bid (Fig. 2). As in all families, some members are of close relation, whereas other members can claim only distant relation. In this respect, all family members share pockets of sequence similarity, denoted BH1, -2, -3, and -4.[16] The BH3 domain is common to almost all family members, and some family members, including Bid, Bad, and Hrk, have the BH3 domain as their only link to the family. All of these so-called "BH3-only" proteins are proapoptotic. The BH4 domain is unique to antiapoptotic proteins and is found at the extreme amino terminus of these proteins.

Deletion mutagenesis has suggested that these regions of sequence similarity are important in regulating protein-protein interactions between the family members to form either homo- or heterodimers.[99, 129, 133] The fate of cell seems to lie with the relative amounts of the pro- and antiapoptotic proteins and the identity of the predominating protein complexes.[52]

FORM FOLLOWING FUNCTION?

Despite the important role the Bcl-2 protein family plays in cell death pathways, the exact biochemical mechanism by which the Bcl-2

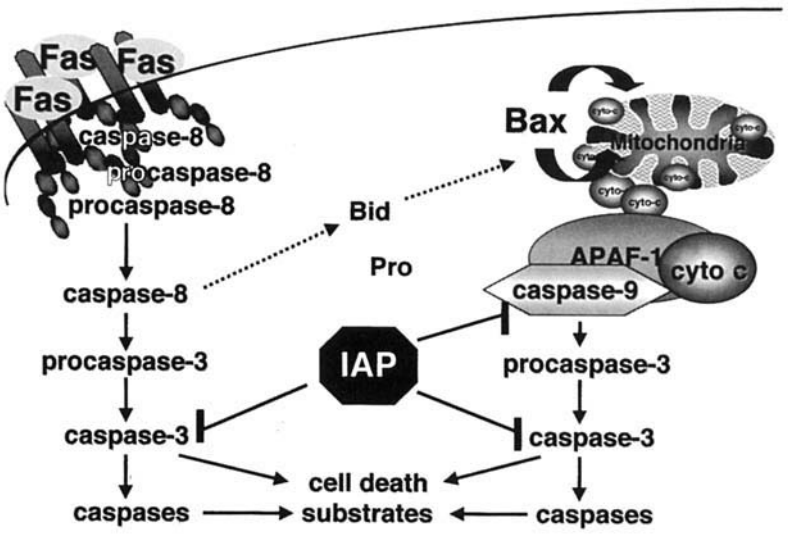

Figure 1. Apoptotic pathways. Several members of the tumor necrosis factor (TNF) family of death receptors (Fas) recruit caspase-8 to their cytosolic domains on ligand binding, resulting in proteolytic activation of this proximal caspase. Once activated, caspase-8 can induce directly or indirectly the activation of caspase-3. A separate pathway for caspase activation is dependent on mitochondria and involves release of cytochrome c (cyto c) from this organelle into the cytosol—an event often mediated by proapoptotic Bcl-2 family members, such as Bax. On entering the cytosol, cyto c catalyzes the formation an Apaf-1/caspase-9 complex (apoptosome). The apoptosome activates caspase-3, initiating a caspase cascade that executes the apoptotic program. In some cells, Fas-induced cell death depends on mitochondria. In these cells, activated caspase-8 cleaves Bid, which then targets the mitochondria and promotes cyto-c release. Inhibitor of apoptosis proteins (IAPs) can block each of these apoptotic pathways by directly inhibiting distinct caspases.

family of proteins modulates apoptosis remains unclear. A possibility for the biochemical function of the Bcl-2 protein family was suggested following the determination of the 3-dimensional structure of Bcl-x_L.[74]

Bcl-x_L is a bundle of 7 helices arranged in three layers. The outer two layers of amphipathic helices enclose between them two central helices. These two helices are long (each approximately 20 residues) and have a pronounced hydrophobicity.

The Bcl-x_L structure bears a strong resemblance to the previously determined structures of the membrane translocation domains of the bacterial toxins diphtheria and colicins A and E1.[74] The diphtheria toxin membrane translocation domain forms a channel in the endosomal membrane, through which the ADP-ribosylating subunit passes,[60] while the colicins kill sensitive *Escherichia coli* strains by way of the formation of a highly conductive ion channel that depolarizes the target cell's plasma membrane, resulting in cell death.

Although the colicin and diphtheria toxins attack very different organisms, the structures of these toxins use a similar *cloak-and-dagger* strategy in which the hydrophobic *dagger* is hidden within a *cloak* of amphipathic helices that allows these proteins to exist in a soluble state, but under certain conditions the hydrophobic dagger is *unsheathed* allowing the protein to insert into membranes.

The coordinates of the three-dimensional Bcl-x_L structure can be used as a scaffold on which to build models for the other Bcl-2 family members. Despite their opposing functions and their isolated regions of sequence similarity, models for Bcl-2 and Bax could be built using the Bcl-x_L structure for a guide.[93] Both proteins share a similar silhouette, in that they are α-helical bundles having at their core two long central hydrophobic helices. The three-dimensional structure for Bid has also been determined, and it also shows the same characteristics.[16, 70] The structural similarity between these Bcl-2 family members

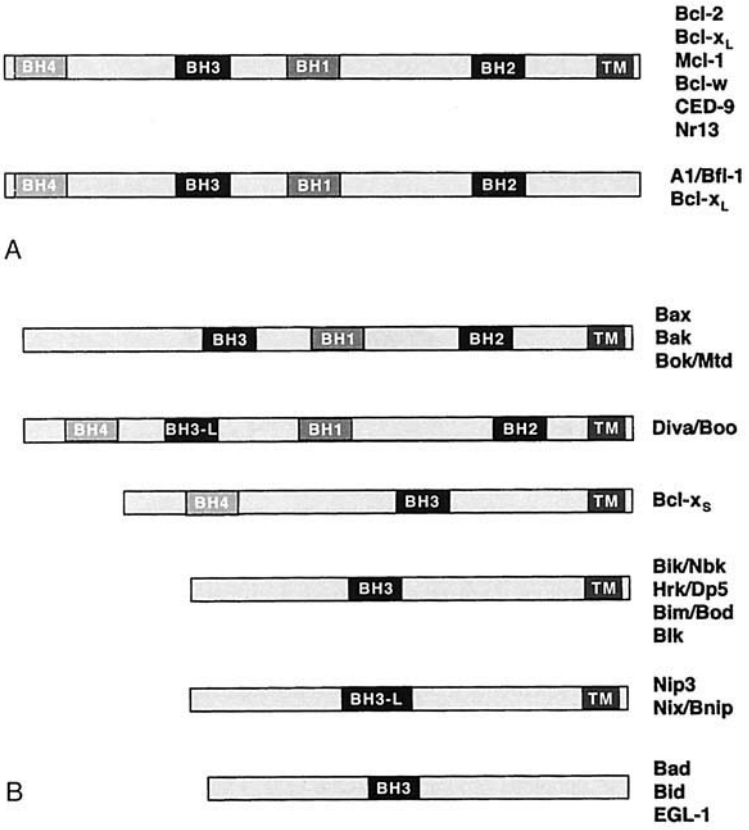

Figure 2. Bcl-2 protein family homology domain organization. Two subgroups are indicated: antiapoptotic (*A*). which discourages cell death, and proapoptotic (*B*) which promotes cell death. The relative locations of the homology domains (BH1, -III 2, -III 3, and -III 4) and the C-terminal transmembrane domain (TM) are indicated. For the pore-forming proteins, the putative pore-forming helices fall between the BH1 and BH2 domains, with the exception of Bid.

and the pore-forming domains of bacterial toxins suggests that the Bcl-2 protein family may possess pore-forming potential.

In addition to similarity to pore-forming proteins, the structure of Bcl-x_L reveals other details about how this protein is regulated. For example, a long loop lacking defined secondary structure intervenes between the first and second helices of Bcl-x_L. This loop sequence is a feature of only the antiapoptotic family members, and, although it is dispensable to their protective action, this region may represent a regulatory domain, because it is vulnerable to protein digestion[106] and possesses several phosphorylation sites.[14] Thus, posttranslational modifications or conformational changes occurring in this domain may act as a means for modulating the protective effects of Bcl-2 and Bcl-x_L.[71]

The BH1, -2, and -3 domains cluster together on one side of the molecule, forming a hydrophobic cleft. This structural feature, along with results from site-directed mutagenesis studies, suggests that this patch may participate in hydrophobic protein-protein interactions between Bcl-2 family members. A peptide corresponding to the BH3 domain of the proapoptotic family member, Bak, was able to nestle into the cleft, which is just wide enough to accommodate the α-helix of dimerizing partners.[91]

IN VITRO CHANNEL FORMATION

In order for the Bcl-2 family proteins to form pores they must have α-helices that are of sufficient length to completely span a

membrane bilayer, and these helices must be largely lacking in charged residues. Each residue of an α-helix donates 1.5 Å to the overall helix length. If a typical lipid bilayer has a hydrophobic cross-section of approximately 30 Å,[73] then it follows that the helix must contain at least 20 residues. Bcl-x_L has two α-helices that satisfy this requirement: the two central helices. Although two α-helices are insufficient to enclose a channel lumen, the tendency of the Bcl-2 protein family to form dimers suggests that two or more molecules could coalesce to form a channel (reviewed in reference 93).

In vitro channel-forming ability has been demonstrated by several family members: Bcl-2, Bcl-x_L, Bax, and, discussed later, Bid.[4, 72, 94–95, 97] Bcl-2, Bcl-x_L, and Bax each form channels in large unilamellar liposomes and in planar bilayer systems, from which information about channel characteristics, such as conductance and ion selectivity, can be gained. Each protein displayed a population of channels with varied conductance states, ranging from 20 pS to nearly 2000 pS.[93] The colicin E1 channel also produces 20 pS channels in planar lipid bilayers,[12] and this channel is predicted to be composed of four transmembrane α-helices, two hydrophobic and two amphipathic.[20] By analogy, the 20 pS channel formed by the Bcl-2 protein family members also could consist of a four-helix bundle, but in contrast to colicin, which has a monomeric channel, it is likely that two molecules must donate the their central fifth and sixth α-helices to form a conductive channel. Indeed, the channel-forming activity appears to lie within these helices, because their removal abolishes the channel activity for Bcl-2 and Bax.[68, 94] In the case of Bax, oligomerization appears to be a crucial event for channel formation as the insertion-competent state of the protein elutes in gel filtration experiments as an oligomer of 160 kDa suggesting that at least six subunits of Bax may participate in channel formation.[5] This oligomerization state may also account for the larger conductances observed for Bax channels of up to 2000 pS.[4]

A PORE ALTERNATIVE

That several Bcl-2 family members form channels in vitro while claiming allegiance to opposing family branches, that is, anti- and proapoptotic, at first appears to be a paradox. Their similar structures may confer on most family members the ability to induce ion conductance in vitro, but this general trait may acquire a different purpose in vivo. It is possible that ion conductance, while detectable in vitro, may not be relevant to the Bcl-2 protein family function. The ability of these proteins to exist either in a soluble, globular form, or a membrane-inserted form may represent a means by which these proteins take on alternative conformations and in doing so, expose parts of the proteins that normally may be tucked out of reach. Such a situation could apply to regulation of protein-protein interactions. Bcl-2 and Bcl-x_L have displayed an affinity for a variety of proteins, including the protein kinase Raf-1, the protein phosphatase calcineurin, the *C. elegans* protein CED-4, and the Hsp70 modulating protein BAG-1 (Reviewed in reference 85). These protein-protein interactions are governed by the N-terminal BH4 domain[133] as mutations in this region abolish these interactions. The BH4 domain also appears important to formation of Bcl-2–Bax heterodimers as site-directed mutations in the Bcl-2 BH4 domain abolished Bcl-2–Bax interactions and the protective effect afforded by Bcl-2 against Bax and staurosporine-induced cell death.[41] It is possible that in the uninserted soluble state, the BH4 domain is tucked against the protein and becomes accessible on insertion of the central hydrophobic helices.[94]

A MATTER OF PLACEMENT: Bcl-2 FAMILY PROTEINS AND THE MITOCHONDRIA

The mitochondria, play significant roles in apoptosis regulation (Reviewed in references 37–38 and 119). Most Bcl-2 family proteins have at their C-terminus a stretch of approximately 20 hydrophobic residues, which seems to be critical to localize these proteins to mitochondria and to other cellular membranes, including the nuclear envelope and the endoplasmic reticulum (ER). Bcl-2 and Bcl-x_L appear to be permanently localized to the mitochondrial membrane,[18, 54] while other Bcl-2 family proteins, largely the proapoptotic

members, such as Bax, are transient mitochondrial residents that change their cellular address from cytosolic to mitochondrial in response to several death signals.[39, 125] The predicted hydrophobic central fifth and sixth α-helices of Bax seem to play a role in this change of address, because stripping these helices of charged residues (purported to line the aqueous lumen of a Bax channel) and substituting alanines resulted in a protein that was constitutively localized to mitochondria and hyperactive in its proapoptotic activity or "gain of function."[77]

Although the Bcl-2 family proteins commonly are thought to inhabit only the outer mitochondrial membrane,[119] immunoelectron microscopy revealed a nonuniform distribution of Bcl-2 in mitochondrial membranes, suggesting that this protein may be located preferentially at zones of adhesion, which join the outer and inner membranes,[22, 54] a fact that could have importance in how these proteins might regulate the mitochondria's role in apoptosis.

As outlined previously, in some cell death pathways, escape of cytochrome c from the intermembrane space of mitochondria represents a key event in initiating the caspase activation cascade. Indeed, tissues from patients with end-stage human cardiomyopathy showed accumulation of cytosolic cytochrome c accompanied by caspase-3 activation.[76] Once liberated from the mitochondria, cytochrome c is free to participate in formation of the apoptosome.[57] In certain cells, other proteins that redistribute from the intermembrane space to the mitochondria include caspase-9 and caspase-3[53, 109] and apoptosis inducing factor (AIF), which results in nuclear morphology changes.[51] The mechanism by which these proteins pass into the cytoplasm remains unclear, although the Bcl-2 family proteins clearly regulate their escape.

The Bcl-2 protein family member Bax may provide a direct route for cytochrome c out of the mitochondria. Treatment of isolated mitochondria with recombinant Bax resulted in release of more than 30% of the total cytochrome c, suggesting that the Bax protein itself may be capable of forming a pore large enough to allow cytochrome c release.[48]

Alternatively, mitochondrial swelling, which eventually compromises outer membrane integrity, may result in cytochrome c leaking out into the cytosol. This swelling and subsequent rupture of the outer mitochondrial membrane could be induced directly through the channel activity of Bcl-2 family proteins,[120] or the Bcl-2 family could indirectly control mitochondrial volume by affecting the activity of the mitochondrial permeability transition pore (PTP).[135] The PTP pore allows passage of solutes with a molecular mass not exceeding 1500 Da. Although all of the components of PTP are not yet defined, the core participants appear to be the adenine nucleotide translocator (ANT) the voltage-dependent anion channel (VDAC).[135] ANT and VDAC are localized to the inner and outer mitochondrial membranes, respectively. A variety of parameters, including membrane potential, matrix pH, and oxidation state,[8] affect the conductance state of the PTP. Opening of the PTP results in a rapid membrane depolarization.

Bcl-2 family proteins could regulate the cytochrome c release through interactions with proteins involved in the PTP. VDAC was reconstituted in liposomes and in the presence of recombinant proapoptotic proteins Bax and Bak the opening of VDAC was promoted, while Bcl-x_L appears to close the channel through direct binding. In cytochrome c–loaded VDAC vesicles, Bax and Bak induced a loss or potential and cytochrome c release that could be inhibited by Bcl-x_L.[100] Although obtained from in vitro experiments, these results suggest that Bcl-2 family proteins may directly bind to VDAC and alter its activity, which should affect the activity of the PTP pore in mitochondria.

Another interaction that has been described is between Bax and ANT.[11] Again, ANT was reconstituted into lipid bilayers and its channel activity measured. On addition of Bax to these lipid bilayers, a composite channel is formed with an electrophysiological profile that differs from the channels formed by either Bax or ANT alone. This channel appears even under conditions where Bax has no detectable channel activity. In contrast, when reconstituted into lipid bilayers in the presence of Bcl-2, there is inhibition of channel formation.

The fact that ANT is inner membrane and that Bax is traditionally thought to have an outer mitochondrial localization poses some difficulty for thinking about this model. This can be remedied by the fact that the Bcl-2 family proteins do not appear to have a uniform mitochondrial distribution, but rather appear to cluster at adhesion sites where the outer and inner membrane are in contact.[54] An analogy can be drawn to the method of colicin action. In the case of colicins, many molecules may bind to the outer wall of the target *E. coil* cell, but very few access the inner membrane space, and only one colicin molecule seems to be necessary to deliver the lethal channel. Only those colicin molecules that bind to an outer membrane receptor, that is, associated with inner membrane-bound proteins and found at adhesion zones, seem to be capable of inserting to form their channel.[96] The same scenario also could exist for Bcl-2 family proteins. Most of the population may exist at the outer membrane surface, however, those molecules that are at contact sites, which themselves appear to be transient,[98] may be the active population in that they are in proper position to interact with PTP pore components.

CASPASE-8 Bid CLEAVAGE: A MITOCHONDRIAL LINK TO THE "Fas" TRACK

In response to Fas receptor ligation, procaspase-8 is recruited to the death receptor complex where local aggregation allows the processing of caspase-8 from the zymogen to active form within the death induced signaling complex (DISC), which includes in addition to procaspase-8 and Fas, Fas-associated death domain (FADD).[75] After activation at the DISC, caspase-8 is released and is available to activate downstream caspases, such as caspase-3 (see Fig. 1). There are two *tracks* a cell can follow with regards to DISC formation. Type 1 cells respond to Fas engagement by the activation of large amounts of caspase-8 by the DISC, whereas Type II cells have reduced DISC formation and consequently lower amounts of activated caspase-8. Examples of Type I and type II cells are lymphocytes and hepatocytes, respectively.[92] The presence of cytosolic cytochrome c in compromised cardiac tissue and the expression of Bcl-2 in these cells suggests that cardiomyocytes might fall into the type II category. Type I cells cannot be rescued from cell death by Bcl-2 or Bcl-x_L overexpression, whereas type II cells can. This fact, along with a reduced $\Delta\psi_m$, suggests that type II cells may take a mitochondrial detour along their cell death pathway.

The amplification of Fas-mediated death signals by way of the mitochondria in type II cells suggested that there must be an intermediary substrate that caspase-8 cleaves with the cleavage product assisting in promoting cytochrome c release. This substrate was revealed by several groups to be the proapoptotic Bcl-2 protein family member, Bid.[56, 64]

Bid is a 195 residue, 22 kDa protein that lacks the hydrophobic COOH-terminal domain, which confers a largely cytosolic localization.[121] Bid interacts with Bcl-2, Bcl-x_L, and Bax by way of its BH3 domain and can annul the cytoprotective effects of Bcl-2 and Bcl-x_L.[64] The Bid amino acid sequence contains a putative caspase-8 cleavage site (57-L-Q-T-G-61) within its NH_2 terminus and Bid is indeed cleaved between residues 59 and 60 by caspase-8 *in vivo* and *in vitro*.[56, 64] Following cleavage, the truncated Bid translocates to the mitochondria where it is a potent inducer of cytochrome c release, suggesting that the truncated Bid may play a role in increasing the permeability of the mitochondria membrane, allowing cytochrome c escape.

The three-dimensional structure of Bid shows a strong similarity to Bcl-x_L despite its modest sequence similarity to Bcl-x_L and other Bcl-2 family members. This structural similarity again implied that Bid might possess pore-forming capacity, and indeed BID does, but with a twist: Only the cleaved form of BID is able to form conductive channels in vitro.[95] The cleavage of Bid removes the amino-terminus, which results in an increased exposure of hydrophobic surface area, most notably of the central helix pair (α4 and α5) that are the putative pore-forming regions for Bid. This increase in exposed hydrophobic surface area may promote membrane insertion. Also, the cleaved form has

an increased accessibility of the BH3 domain that is involved in dimerization with other Bcl-2 family proteins,[91] suggesting that the cleavage may promote protein-protein interactions that may modulate activity of other Bcl-2 family members involved in cytochrome c release, most notably, Bax.

Bid-null mice were generated and found to be resistant to treatment with anti-Fas antibody, which is normally lethal to wild-type mice.[130] In keeping with the two types of cell death for Fas-induced apoptosis, the Fas ligation in the Bid-null mice was reduced in hepatocytes, but the response of thymocytes and fibroblasts was largely unaffected.[130]

Another study suggests that Bid works independently of caspase cleavage and in concert with Bax to promote cytochrome release. In this case, the BH3 domain of Bid would bind to the hydrophobic cleft of Bax, which would promote its translocation to the mitochondria and perhaps foster a membrane-inserted *active* state.[24] In this capacity, Bid acts more as a chaperone than a direct effector of cytochrome c release.

Thus, a role has emerged for Bid in at least some apoptotic pathways, in which it seems to function as a messenger for relaying signals induced by Fas or TNF at the cytoplasmic membrane, to the mitochondria. In other contexts, Bid may function more as a chaperone to promote Bax activation.

INHIBITOR OF APOPTOSIS PROTEIN FAMILY PROTEINS

Possibly one of the most ancient evolutionary pressures for a cell suicide program can be attributed to viruses. Death of the infected host cell stymies viral propagation—thereby protecting uninfected neighboring cells; however, viruses have co-evolved strategies for promoting cell survival by targeting conserved steps in the host cell-death program.[13, 51, 134] Based on their antiapoptotic function during viral infection, inhibitor of apoptosis proteins (IAPs) were first discovered in baculovirus.[9, 21] Intriguingly, ectopic expression of some Baculoviral IAPs in cultured mammalian cells suppressed cell death induced by several apoptotic stimulus, suggesting that IAPs must block a conserved step in the cell-death program.

A novel 70 amino acid domain termed the *Baculoviral inhibitory repeat* (BIR) characterizes the IAP-protein family.[9, 21] Based on sequence homology to the BIR domain, yeast, worms, flies, birds, mice, pigs, and humans all appear to encode IAP relatives, although many of these proteins have yet to be tested for antiapoptotic function. Six human IAP relatives have been identified including; NAIP, cIAP1/HIAP-2, cIAP2/HIAP-1, XIAP/hILP, Survivin, and BRUCE.[2, 30, 40, 58, 87] NAIP, XIAP, cIAP1, cIAP2, and Survivin all exhibit antiapoptotic function in various experimental systems, thus confirming their membership in the IAP family of proteins.

Up to three tandem copies of the BIR domain can occur within the known human IAP-family proteins (Fig. 3). The repeated BIRs are highly conserved within a particular IAP and between distinct family members—ranging from approximately 30% to 90% identity. The conserved presence and spacing of cysteine and histidine residues present within the BIR domain coordinate zinc-binding.[108] Some IAPs also contain a zinc-binding ringfinger motif at their C-terminus. Although the role of the ringfinger motif with respect to IAP antiapoptotic function is unknown, in all cases at least one BIR is required for IAP-mediated suppression of cell death (reviewed in reference 25).

HOW DO INHIBITOR OF APOPTOSIS PROTEINS SUPPRESS APOPTOTIC PATHWAYS?

Recent studies have demonstrated that several of the human IAPs (XIAP, cIAP1, and cIAP2) directly inhibit caspases.[26, 89] XIAP, cIAP1, and cIAP2 bind and potently inhibit caspase-3, to 7 and -9 but not caspase-1, 6, 8, or 10 or CED-3.[79–81] Survivin also can be coimmunoprecipitated with caspase-3, -7, and -9, and it suppresses apoptosis induced by overexpression of these caspases, implying that Survivin also is a caspase inhibitor.[113] More recently, IAPs from other species, including, *Drosophilia, Lepidopteran*, and *Baculovirus*, have been shown to function by inhib-

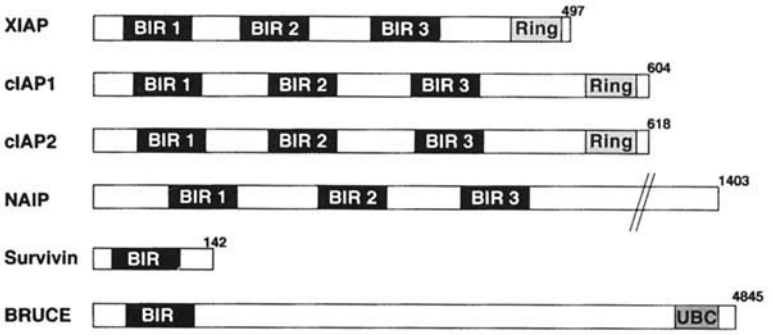

Figure 3. Human IAP proteins. Highly conserved BIR domains that can be present at 1 to 3 copies (BIR 1–3) characterize IAPs. XIAP, cIAP1 and cIAP2, also contain a C-terminal ring domain (Ring). Survivin and BRUCE have a single BIR domain. In addition to a single N-terminal BIR domain, BRUCE also has ubiquitin conjugating (E2) domain (UBC) at its C-terminus; however, the functional significance of the BIR or UBC domain in BRUCE has yet to be reported.

iting specific caspases.* Thus, caspase inhibition appears to be a conserved mechanism by which IAP family proteins suppress cell death.

Caspase-8 and -9 represent the pinnacle caspases in the Fas/TNF-family death receptor and cytochrome c/Apaf-1 pathways, respectively (see Fig. 1). Although human IAPs (XIAP, cIAP1, and cIAP2) do not bind or inhibit caspase-8, they do bind to and inhibit its substrate, caspase-3, thereby arresting the proteolysis cascade and providing protection from Fas/caspase-8–induced apoptosis.[26–27, 89] In contrast, in mitochondria-dependent pathways for caspase activation, XIAP, cIAP1, and cIAP2 directly bind to the apical caspase, procaspase-9, and prevent its processing and activation induced by cytochrome c, and Apaf-1.[27] Presumably, IAP interaction with procaspase-9 occurs on recruitment to the apoptosome complex, but this has yet to be determined.

Overexpression of IAP-family proteins has been shown to suppress apoptosis induced by Bax and other proapoptotic Bcl-2 family proteins, which are known for their ability to target mitochondria and induce cytochrome c release.[10, 26, 48, 66, 125] The IAPs, however, do not interfere with Bax-mediated release of cytochrome c,[48, 34] an observation that is consistent with data indicating that the human IAPs (at least XIAP, cIAP1, cIAP2, and Survivin) block caspase activation and apoptosis downstream of Bax, Bik, Bak and cytochrome c.[26–27, 31, 82, 89, 113]

At least for XIAP, the ability to inhibit caspase-3 and -7 has been attributed to its BIR2 domain and sequence just N-terminal to the BIR2 domain,[108, 111] whereas the ability to inhibit caspase-9 localizes to the BIR3-ring region of XIAP.[28] Therefore, at least some IAPs have evolved distinct caspase inhibitory domains that may, in part, explain their versatility and effectiveness as antiapoptotic proteins.

IAPs and more specifically BIR domains, however, may have other functions. BIR-containing proteins have recently been identified in the yeast strains *Schizosaccharomyces pombe* and *Saccharomyces cerevesiae*. Because yeast do not appear to contain caspaselike proteases, yeast BIR proteins presumably have functions other than caspase inhibition. Consistent with this idea, yeast BIR proteins are reported to facilitate cell division.[118, 131] Similarly, recent genetic analysis of a *C. elegans* BIR-containing gene demonstrated its essential role in cytokinesis, rather than apoptosis.[35]

Interestingly, the single BIR-domain of the IAP family member Survivin, seems most closely related to the BIR domains found in yeast and worms, which as reviewed previously are reported to function in cell division and not in cell death. The scenario for

*References 26–28, 44, 49, 89, 108, 111, 113, 122

human Survivin, however, may not be as straight forward. Indeed, Survivin is expressed in the G_2/M phase of the cell cycle in a cycle-regulated manner.[55] At the beginning of mitosis, Survivin associates with microtubules and disruption of Survivin-microtubule interaction results in loss of Survivin's antiapoptotic function and increased caspase-3 activity. These and other results suggest that Survivin may countact a default induction of apoptosis at the G_2/M "checkpoint" of the cell cycle.[55] Thus, the human IAP Survivin appears to bridge the evolutionary gap between the nematode and yeast BIR proteins—which are regulators of cell division, and other viral, fly and human IAPs—that are antiapoptotic proteins.

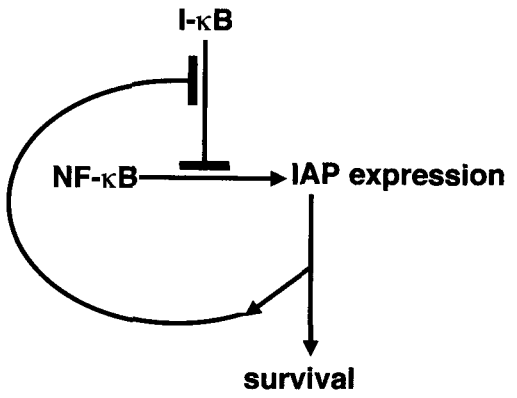

Figure 4. Antiapoptotic activity of NF-κB. At least one proposed mechanism by which NF-κB suppresses cell death is through its ability to induce the expression of IAPs, which in turn block apoptosis. IAPs also may facilitate the destruction of the NF-κB inhibitor Iκ-B, thereby potentiating a positive feedback loop that promotes cell survival.

INHIBITOR OF APOPTOSIS PROTEINS, SIGNAL TRANSDUCTION, AND APOPTOSIS

cIAP2 has been functionally implicated in TNF-induction of nuclear factor (NF-κB) and protection from apoptosis.[17] First, TNF-α has been shown to induce expression of cIAP2 though stimulation of NF-κB. Second, overexpression of cIAP2, reportedly can also lead to NF-κB activation. Third, cIAP2 expression suppresses cell death induced by TNF-α through the receptor TNFR-1. A dominant form of the NF-κB inhibitor I-κB (resistant to degradation), blocks these cIAP2 activities, implying that cIAP2 participates in a positive feedback mechanism regulating NF-κB activation by targeting I-κB for degradation (Fig. 4). Moreover, a mutant of cIAP2 lacking the C-terminal ring domain inhibited NF-κB induction by TNF and enhanced TNF killing. Based on these findings, the authors[17] suggested that cIAP2 is critically involved in TNF signaling events that induce NF-κB, which are required for suppression of TNF-induced apoptosis.

Is the induction of IAP-family genes, however, critical for the antiapoptotic effect of NF-κB? Studies of the effects of TNF-α on IAP-family gene expression in endothelial cells suggests the answer to this question may be difficult to obtain because of redundancy in IAP family genes. Transcription of cIAP1, cIAP2, and XIAP genes was found to be strongly up-regulated on treatment of endothelial cells with the TNF-α, interleukin (IL)-1β, and LPS—reagents that lead to NF-κB activation.[102] In these studies, overexpression of I-κB suppressed NF-κB activation and prevented the induction of all these IAP-family genes. I-κB overexpression also sensitized endothelial cells to TNF-α–induced apoptosis. Ectopic expression of at least one of the IAPs, XIAP, suppressed the I-κB effect, thereby protecting endothelial cells from TNF-α–induced apoptosis, suggesting that XIAP represents one of the NF-κB–regulated genes that can counteract the apoptotic signals caused by TNF-α–induced activation of caspase-8.[102] Thus, although we do not know whether IAP expression is necessary for NF-κB-mediated protection against TNF-α, it is sufficient. Based on these and similar reports, it may be worth considering whether dysfunctional regulation of the IAPs occurs in sepsis and some inflammatory conditions, where cytokine-induced endothelial cell death occurs.

INHIBITOR OF APOPTOSIS PROTEIN AND Bcl-2 FAMILY PROTEINS IN DISEASE

Misregulation of the balance between life and death at the cellular level, can contribute

to acute and chronic disease. Resistance to cell-death stimuli can result in an expanded population of diseased cells, as in the case of some carcinomas, and may play a role in angiogenesis and cardiovascular-related diseases. Excessive cell death, however, can contribute to autoimmune and neurodegenerative diseases and acute conditions, such as ischemia and excessive tissue damage following trauma. Therefore, it is perhaps not surprising that dysregulation of Bcl-2 and IAP family proteins is increasingly implicated in the pathology of human diseases.

HEART AND VASCULAR RELATED DISEASES

Nuclear factor κB seems to play an important role in controlling the induction of apoptosis in human and rat vascular smooth muscle cells (SMCs).[33] Reportedly, SMCs in high-density culture are resistant to apoptosis, which correlated with the expression of cIAP1 and high NF-κB activity. Transfection of Iκ-B, inhibitor of NF-κB, reduced human cIAP1 mRNA levels. These data suggest that NF-κB activity increases expression of cIAP1, which confers protection from cell death. Consistent with this idea, antisense inhibition of IAP-1 sensitized high-density SMCs to cell-death induction.[33] Based on their data, the author[33] suggested that cIAP1 is transcriptionally regulated by NF-κB and that SMCs at high density are protected by an antiapoptotic mechanism that involves increased expression of NF-κB and cIAP1.

Using differential display, cIAP2 was reportedly one of the cytokine-responsive genes from endothelial cells that can be regulated by monocyte conditioned medium or TNF-α. Furthermore, in vivo expression of cIAP2 was detected in endothelial cells overlying lesions heavily infiltrated by monocytes and foam cells. These results suggest that cIAP2 may play an important role in the molecular processes involved in vascular diseases, such as atherosclerosis.[43]

Several studies have detected the presence of Bcl-2 protein family members in cardiac myocytes. In rat heart, antiapoptotic Bcl-2 and Bcl-x_L were expressed to high levels in neonatal cardiac tissue and their presence was maintained throughout development. The proapoptotic proteins Bad and Bax, while present at high levels in neonatal hearts, were absent in adult hearts.[19] Although the functional significance of these observations remains to be investigated, the presence of these proteins may suggest that they play roles developing, modeling and maintaining the adult heart by regulating apoptosis.

In this regard, reperfusion of ischemic myocardium causes cardiomyocyte apoptosis that reportedly occurs in concert with down-regulation of Bcl-2 gene expression.[69] In these studies, ischemic preconditioning (PC) mediated by cyclic episodes of short-term ischemia and reperfusion, reportedly reduced apoptotic cell death. PC was shown to initiate a signaling pathway by potentiating tyrosine kinase phosphorylation, which lead to the activation of p38 MAP kinase and MAPKAP kinase 2. Based on observations that NF-κB plays a crucial role in this signaling pathway and can be a target of oxygen free radicals and that Bcl-2 is reported to be an antioxidant gene, the authors[69] hypothesized that reactive oxygen species might play a role in this signaling process. Alternatively, NF-κB may influence the expression of other antiapoptotic proteins, such as the IAPs, thereby conferring protection against ischemic insult in cardiomyocytes.

Expression of p53 in ventricular myocytes was shown to result in a significant increase in Bax and was sufficient to trigger apoptosis.[50] In these studies, expression of Bcl-2 was sufficient to prevent p53-mediated apoptosis and p53-dependent transcription of Bax in ventricular myocytes.[50] These studies suggest that pro- and antiapoptotic proteins can influence ventricular remodeling after injury. This may have clinical significance since inappropriate loss of myocardial cells has been suggested to contribute to conduction defects and heart defects.

NEURONAL AND NEURODEGENERATIVE DISEASES

The *NAIP* gene was first identified because of its apparent deletion in patients with spinal

muscular atrophy (SMA), a hereditary motorneuron degenerative disease.[58, 88] Though the primary genetic defect in SMA has been ascribed to an adjacent gene,[63] *SMN*, rather than *NAIP*, patients with the severest forms of this disease appear to harbor deletions at 5q13.1 that encompass the *SMN* and *NAIP* genes. Intriguingly, the survival motor neuron gene (SMN) protein has been reported to bind Bcl-2 and enhance Bcl-2-mediated protection from apoptosis,[45] raising the possibility that two survival genes may be lost in more severely affected individuals.

Consistent with the primary defect in SMA being attributed to the SMN gene, it recently was reported that *NAIP*-deleted mice develop normally. The survival of pyramidal neurons in the hippocampus after kainic acid-induced limbic seizures is, nevertheless, greatly reduced in the *NAIP* knock-out animals. The authors[42] concluded that although *NAIP* is not necessary for normal development of the murine central nervous system; it is required for neuronal survival in pathological conditions.

NAIP also may be involved in adaptive responses to ischemia. Transient forebrain ischemia selectively elevates levels of *NAIP* in rat neurons that are resistant to ischemia-reperfusion.[127] Up-regulation of endogenous *NAIP* expression or intracerebral injection of *NAIP*-encoding adenoviruses reportedly reduces ischemic damage in the rat hippocampus, suggesting that *NAIP* may play a role in conferring resistance to ischemia-induced cell death.[127] In cell culture experiments, however, transfection of primary cerebellar granule cell neurons with adenoviruses encoding NAIP, XIAP, cIAP1, or cIAP2 delayed but did not prevent apoptosis induced by K^+ depolarization and serum deprivation. Nonapoptotic cell death induced by L-glutamate was unaffected by these IAP-family proteins.[101] Thus, IAPs are apparently insufficient to protect some types of neurons from insults often associated with ischemia.

Nevertheless, it was reported that adenovirus-mediated overexpression of XIAP prevented the production of catalytically active caspase-3 and degeneration of CA1 neurons after transient forebrain ischemia.[126] CA1 neurons protected in this manner appeared to function normally, as assessed by immunohistochemical detection of the neuronal activity marker nerve growth factor inducible-A and by spatial learning performance in the Morris water maze. The authors[126] concluded that XIAP overexpression permits CA1 neurons to survive and operate properly after an ischemic insult.

CANCER

As described here and reviewed previously, the gene encoding Bcl-2 protein was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in B-cell lymphomas.[117] Bcl-2 contributes to neoplastic cell expansion by preventing cell turnover because of programmed cell death. In this regard, overexpression of antiapoptotic Bcl-2 and Bcl-x_L also has been documented in many other types of human tumors, including cancers of the prostate, colon, breast, and lung. Moreover, antiapoptotic Bcl-2 family proteins have been associated with chemoresistance and radioresistance in some types of malignancy (reviewed in references 46, 83, 85, 107).

The IAPs also are implicated increasingly in the oncogenic process. For example, the oncoprotein v-Rel, a member of the Rel/NF-κB family of transcription factors, induces malignant transformation and inhibits apoptosis. The chicken homolog of cIAP1 (ch-IAP1) was found to be up-regulated following expression of v-Rel in fibroblasts, a B-cell line, and in spleen cells.[132] Expression of exogenous ch-IAP1 in temperature-sensitive v-Rel–transformed spleen cells inhibited apoptosis in these cells at the nonpermissive temperature Based on these results, it appears that ch-IAP1 is induced and functions as a suppressor of apoptosis in the v-Rel–mediated transformation process.[132]

cIAP2 and a novel gene, named *MLT*, are recurrently rearranged in marginal zone cell lymphomas of mucosa-associated lymphoid tissue (MALT).[29] This t(11;18)(q21;q21) rearrangement appears to be the key genetic lesion and is found in approximately 50% of cytogenetically abnormal low-grade MALT lymphomas. Based on these data, it was sug-

gested that the resulting cIAP2-MLT fusion may have enhanced anti-apoptotic function, thereby contributing to the oncogenesis of MALT lymphoma.[29]

The IAP member Survivin is expressed in a high proportion of the commonest human cancers but not in normal terminally differentiated adult tissues.[2] The assessment of Survivin expression in human tumor specimens included in situ RNA hybridization and immunohistochemical analysis, confirming expression in tumor cells but not admixed stromal cells or adjacent normal tissues.[3] Thus, altered Survivin expression seems to define a common event associated with the pathogenesis of most human cancers. Moreover, reductions in Survivin expression achieved using antisense strategies cause apoptosis and sensitization to anticancer drugs, at least in some tumor cell lines, implying that Survivin expression can be important for cell survival or chemoresistance in carcinomas.[3]

Not all tumors, however, express Survivin and even within a given type of cancer, heterogeneity in Survivin expression may be observed. Immunohistochemical assessments of Survivin expression in tumors in which either immunointensity, percentage immunopositivity, or have been measured to segregate Survivin negative from positive (Survivin low from high) tumors suggest that Survivin expression (or higher levels of Survivin expression) is associated a poor prognosis in neuroblastomas, colon, and gastric cancers.[1–3, 62] Though preliminary, assessments of Survivin expression may be of prognostic significance for patients with some types of cancer.

In this regard, a recent study revealed that Survivin expression was positive in 118 of 167 breast carcinoma cases (70.7%) having histological stages I to IH.[114] In contrast, no Survivin expression was detected in adjacent normal tissue. Survivin-positive samples strongly correlate with Bcl-2 expression and exhibited reduced apoptosis (low apoptotic index). Patients with a low apoptotic index had lower survival rates than the group having a high apoptotic index. The authors[114] suggest that apoptosis inhibition by Survivin alone, or in cooperation with Bcl-2, is a significant prognostic parameter of worse outcome in breast carcinoma.

Endothelial cell activation and dysfunction can play a prominent role in physiological processes, such as angiogenesis, and in the pathophysiology of atherosclerosis.[116] Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that can act as an endothelial cell mitogen and seems to be a major survival agent for endothelial cells during angiogenesis and vasculogenesis. VEGF has been shown to mediate this latter function, in part through the induction of Bcl-2 expression and the activation of the P13 kinase–Akt/PKB signaling pathway.[116] Additionally, VEGF was reported to increase XIAP and Survivin protein levels 2.9- and 19.1-fold, respectively, in human umbilical vein endothelial cells, suggesting that VEGF mediated survival may be, in part, mediated by inducing expression of these IAPs. The authors[116] suggest that these results raise the possibility of therapeutically targeting XIAP or Survivin in antiangiogenic therapy as a means of suppressing tumor growth, in addition to directly targeting tumor cells that express these survival proteins.

Consistent with the above observations, a separate study reported that stimulation of quiescent endothelial cells with mitogens, including VEGF and basic fibroblast growth factor (bFGF), increased Survivin expression approximately 16-fold.[78] Survivin protein concentration was minimal in the endothelium of nonproliferating capillaries of normal skin, whereas it became massively up-regulated in newly formed blood vessels of granulation tissue in vivo. Ectopic expression of Survivin reduced caspase-3 activity and counteracted apoptosis induced by TNF-α/cycloheximide in endothelial cells[78] suggesting that antiapoptotic proteins may play an important role in the angiogenic process.

IMMUNE DISEASE

As outlined above, increased activity or expression of antiapoptotic proteins can adversely influence the maintenance of healthy cells by suppressing apoptosis. In contrast, lack of antiapoptotic protein function can

result in excessive apoptosis. A recent example of this concept was described for cartilage-hair hypoplasia (CHH) syndrome—a rare autosomal recessive disease characterized by increased T-cell apoptosis and cell-mediated or combined immunodeficiency.[128] This study reported that CHH was associated with altered expression of Fas, Fas ligand (FasL), IAP, Bax, and Bcl-2. Increased apoptosis in CHH correlated with increased expression of Fas, FasL, and Bax and decreased expression of Bcl-2 and IAPs compared with the control. These data suggest that increased apoptosis of T cells contributes to lymphopenia and immunodeficiency in CHH, and that increased T-cell death, in this case, is mediated by altered expression of pro- and antiapoptotic proteins.[128]

Changes in Fas, FasL, and Bcl-2 expression have also been reported in circulating T cells in patients with HIV infection,[6, 23, 26] further suggesting a problem with regulation of apoptosis genes in immunodeficiency states. Conversely, autoimmune disorders are commonly characterized by a failure to remove autoreactive lymphocytes. In this context, studies of transgenic and knock-out mice have provided examples of autoimmunity that is caused by changes in the expression of Bcl-2, Bcl-x_L, and Fas.[61, 65, 79, 112, 123] Alterations in the expression or function of apoptosis-regulating genes, such as Bcl-2 and Fas, also have been described in humans with lupus or other autoimmune disorders.[7, 15, 80–81, 115] Also, the HIV protease reportedly cleaves Bcl-2.[106] Further, the HIV tat protein can sensitize T cells to Fas-dependent apoptosis.[124] Thus, defects in apoptosis regulation are intricately associated with immune system diseases.

SUMMARY

The balance between pro- and antiapoptotic proteins can determine cellular fate. In this regard, the Bcl-2 and IAP protein families have evolved as highly conserved regulators of cell death. A further testament to their critical roles in maintaining balance between cell life and death may be the increasing implication of Bcl-2 and IAP proteins in the pathologies of human diseases. Although much has been learned about these families of proteins, future studies of the Bcl-2 and IAP families are sure to hold more exciting discoveries and will continue to reveal new strategies for combating human diseases.

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Address reprint requests to

John C. Reed, MD, PhD
The Burnham Institute
10901 N Torrey Pines Road
La Jolla, CA 92037

e-mail: jreed@burnham-inst.org