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Signaling Pathway for Apoptosis: A Racetrack for Life or Death

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Abstract Apoptosis, or programmed cell death, is a gene-directed mechanism activated as a suicidal event to get rid of excess, damaged, or infected cells. The recent astounding pace of research in this area has expanded our horizon of understanding that this mechanism is regulated largely by pro- and anti-apoptosis factors acting for or against the final death event. The driving force behind these factors, either pro-apoptosis or pro-survival, is largely determined by signal transduction pathways, starting with the initiation of a death signal at the plasma membrane, and following through a complex cytoplasmic network before reaching the end point of cell demise. Enmeshed in this intricate cytoplasmic network are many checkpoints, where complexes of pro- and anti-apoptosis factors function to facilitate or deter the death signals. The culmination of the balancing act between these two camps of factors at these signal transduction checkpoints may then result in the final decision to die or to live. Thus, the eventual death of a cell may require successful passage through all the checkpoints, a mechanism Nature has provided as a safeguard to prevent erroneous triggering of death. With the advent of a new biotechnology revolution at the dawn of the new millenium, we look forward to an exciting era when we can gain fuller understanding of the operation of all these checkpoints. Ultimately, this gain will pave the way to control the apoptosis event at the checkpoints, and to support the organism's functionality as long as possible. J. Cell. Biochem. Suppls. 32/33:95–102, 1999.

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Apoptosis, or programmed cell death (PCD), is essential to normal mammalian development. Features including cytoplasmic shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation generally distinguish cells committed to activation of programmed cell death from other types of death invoked by necrotic processes. Genes that modulate the mechanisms leading to apoptotic cell death are being discovered at an extraordinary pace. Characterization of these genes shows that this gene-directed cell suicidal program is regulated by many parallel and converging signal transduction pathways, depending on different cell types, different differentiation stages, and different extracellular cues giving rise to a multitude of intertwining signals. The most exquisitely controlled apoptotic signals occur during normal mammalian development, particularly in the central nervous system, where the correct number of postmitotic cells and synaptic connections needs to be established with precise spatial and temporal sequences, dictating the exact cell mass to form different compartments in the central nervous system. Similar regulations are also in effect for the cardiomyocytes in the heart to create a defined tissue mass for the functional dynamics of the cardiac chambers. In other tissues, the activity of apoptosis gene-directed suicidal programs are also observed throughout the organismic life span, when cells are no longer wanted functionally, such as those which have migrated to the tips of the intestinal villi, or when cells are damaged through infection, toxin exposure, or ultraviolet (UV) or oxidative damage. In this last case, apoptotic signals induced by injury or stress produce deleterious damage to irreplaceable

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postmitotic cells, such as neurons. Hence, cells have evolved very strict mechanisms to control conflicting and converging pathways, deciding whether to die or to live, with such detrimental potential. For example, one of the most efficient pathways to induce apoptosis, the tumor necrosis factor (TNF) receptor, resembles a race between caspase activation and NF-κB activation, having as the outcome cellular death or survival, respectively. The decision to die or live is related to how the winners of opposing forces get through traps set up by the diametrically opposed pathways. Although details remain obscure, insights are rapidly emerging.

The purpose of this review is to describe some of the key pathways that regulate pro- and anti-apoptotic signals, and the ongoing race between antagonistic pathways. The ultimate decision as to the functional operation in any given tissue results from the balance between the two genetic forces. For example, the strategy for protection from neurodegeneration is not to have the apoptotic event occur, whereas the strategy for the eradication of cancer plaques is to use apoptotic signals to get rid of the neoplastic cells. In all, evolution provides a longstanding molecular strategy not only to maintain the proper critical mass for each tissue's operational function, but also to maintain such defined mass with the regulation to insure the continuous working throughout the life span of an organism as long as possible, even up to a hundred years in the case of centenarians.

RECEPTOR SIGNALING

Signaling through the tumor necrosis factor receptor (TNF-R) gene family plays a significant role in mediating pro- and anti-apoptotic cellular events. TNF-R1 mediates opposing mechanisms established at the early stages of activation through the "death domain" (DD) [Ashkenazi and Dixit, 1998]. Upon TNF binding, trimerization of TNF-R1 receptors results in aggregation of DDs, allowing for the recruitment of TRADD. TRADD mediates recruitment of TRAF1/2, which leads to activation of both JNK/SAPK and, to a lesser extent, NF-κB pathways; recruitment of FADD leads to apoptosis by caspase-8 activation, and RIP leads to anti-apoptotic mechanisms by NF-κB activation. Moreover, RIP can recruit RAIDD, which in turn can recruit caspase-2 and induce apoptosis. Insight from RIP knockout mice, where thymocytes and embryonic fibroblasts tend to undergo extensive apoptosis under various stimuli, suggests that NF-κB and its pro-survival response may be the dominant force [Kelliher et al., 1998]. Hence the TNF-R1 signaling complex is formed by interaction of TRADD, TRAF, FADD, and RIP, allowing multiple signals to be implemented and positively or negatively regulated for both pro- and anti-apoptotic events (Fig. 1). There is now evidence to suggest that the initial event of TNF signaling is mediated through a negative regulator of the TNF-R1 signaling complex formation, termed silencer of death domains (SODD) [Jiang et al., 1999]. Before TRADD association, SODD is bound to the intracellular domain of TNF-R1, and is dissociated upon ligation with TNF. SODD may also be involved in modulating the duration of TNF signaling, since the TNF-R1 signaling complex is dissociated and reassociated within minutes of TNF exposure [Jiang et

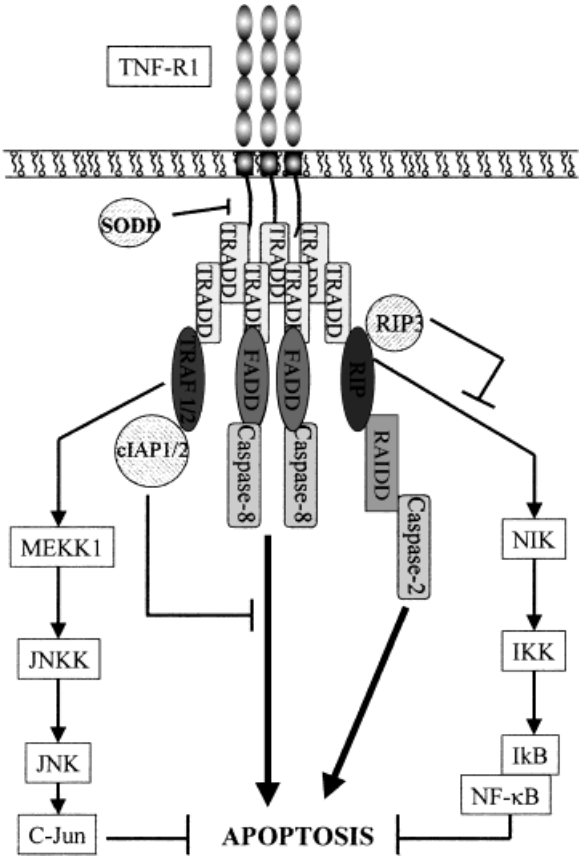

Fig. 1. Major proteins involve in the pro-apoptotic and pro-survival pathways induce by oligomerization of tumor necrosis factor receptor (TNF-R1). TRADD, TNF-receptor-associated death domain protein; TRAF, TNR receptor-associated factor; RIP, receptor interacting protein; FADD, Fas-associated death domain protein; IAP, inhibitors of apoptosis.

al., 1999]. Therefore, SODD may act as a negative regulator, essentially by inhibiting downstream effector molecules including NF-κB, by modulating how long the TNF signaling complex remains associated. However, SODD function is not limited to TNF-R1 signaling, as it can associate with the DR3 receptor to mediate similar downstream cascades [Jiang et al., 1999]. Since other roles of SODD have not yet been established, it is possible that SODD may be involved in transducing other signals. It is likely that other SODD-like molecules will be discovered, and it would be particularly interesting to see how such negative regulators may play a role in transducing signals in other cell types and differentiation paradigms.

One of the most interesting members of the TNF receptor family is the p75 neurotrophin receptor [reviewed by Barker, 1998]. Unlike the TNF receptors, however, the signaling pathways that are mediated by p75 after binding by nerve growth factor and other neurotrophins (brain-derived neurotrophic factor, neurotrophin-3) are less understood. Based on its structural homology with the TNF receptor family, p75 is expected to mediate signals that promote cell death and survival [Barker, 1998]. This has been demonstrated by transgenic expression of only the intracellular domain of p75, which is able to induce death in certain neuronal populations, suggesting that apoptotic signals may be mediated through p75 independent of its extracellular function [Majdan et al., 1997]. Also, in p75-deficient mice, a higher number of cholinergic neurons are present in the basal forebrain, suggesting an absence of apoptosis during development [Van der Zee et al., 1996; Yeo et al., 1997]. In accordance with the TNF-receptor model of signaling, p75 may be involved in the recruitment of other adapter molecules through interactions with its death domain. p75 can also mediate neuronal survival through collaborative signaling with the TrkA neurotrophin receptor, to mediate survival signal transduction cascades [Barker and Shooter, 1994; Verdi et al., 1994]. These observations have led to the suggestion that parameters such as the ratio of p75 to TrkA receptor may regulate the extent of neuronal death, presenting a different means by which the TNF receptor family may regulate a cell's fate [reviewed in Casaccia-Bonnefil et al., 1998].

In dying neuronal cells that are dependent on neurotrophins such as NGF for survival, the apoptotic cascades are still unclear. Interestingly, sympathetic neurons are resistant to cytochrome *c*-mediated cell death for a limited time after NGF withdrawal [Deshmukh and Johnson, 1998]. Such a model suggests that sympathetic neurons may have intrinsic survival mechanisms allowing them to remain viable in the absence of trophic signaling. Indeed, this would be a useful mechanism for neurons, allowing them to survive as long as possible in unfavorable conditions, such as the absence of NGF, or in vulnerable situations caused by injury or stress. Deshmukh and Johnson [1998] suggest that sympathetic neurons adapt a "competence-to-die," independent of Bax function and protein synthesis, before cytochrome-c-mediated cell death can occur. Such a multi-step mechanism toward cell death, in addition to ongoing parallel signaling pathways, is consistent with a model of cell death that resembles a "race" between opposing signals to decide cell fate.

DUAL ROLE OF THE BCL-2 PROTEIN FAMILY

As described above, cells' responses to any external stimuli are regulated by many cross-talking signal transduction pathways, due to their exposure to a continuous bombardment of a variety of different signals at the cell surface. In the situation where cells have to integrate two conflicting signals, with an apoptotic signal represented for example by TNF, and a survival signal represented for example by a growth factor such as the epidermal growth factor (EGF), or insulin growth factor, IGF-1, the outcome may be decided by which of the two signals can win the control of a major commitment point: a race to die or live. In mammalian cells, this commitment point is located at the mitochondria, and is predominantly determined by the function of members of the Bcl-2 protein family.

The Bcl-2 protein family is composed so far of 15 members, divided into two functional classes: the pro-survival members, Bcl-2, Bcl-x_L, A1/Bfl-1, Bcl-w, NR-13, MCL-1, Boo; and the pro-apoptotic members, Bax, BAD, Bid, Bak, Bok/Mtd, Bik, Bim, Hrk, and Diva. The means by which these proteins exert their pro-survival or pro-apoptotic functions are extremely varied, as if different proteins followed different evolutionary paths to achieve their protective or killing modalities. All these proteins share regions of homology, designated BH1–4. A subset of pro-apoptotic Bcl-2 family members, named

"BH3–only" and including Bid, BAD, Bik, and Hrk, heterodimerizes with anti-apoptotic members such as Bcl-2 and Bcl-x_L, and somehow prevents the members of the latter category from supporting survival [reviewed in Adams and Cory, 1998]. The best-described example of such heterodimerization is the BAD-Bcl-x_L association. In apoptotic cells, BAD is found associated with Bcl-x_L, probably preventing its association with Apaf-1 and the subsequent formation of the "apoptosome" required for cellular death (Fig. 2). In growth-stimulated cells, the activation of PI3K signal transduction pathway leads to Akt/PKB activation and subsequent BAD phosphorylation. Alternatively, BAD phosphorylation can be accomplished by interleukin-3 (IL-3), which can activate a second kinase, protein kinase A (PKA), located at the mitochondrial membrane [Harada et al., 1999]. Phosphorylation of BAD inactivates its pro-apoptotic function by preventing its association with Bcl-x_L, because phosphorylated BAD is sequestered by the 14–3-3 protein in the cytoplasm away from the functional site, the mitochondria. This cytoplasmic sequestration of BAD therefore leaves the mitochondrial Bcl-x_L alone and free to perform its pro-survival operation, probably by binding to Apaf-1 and preventing the formation of a functional "apoptosome" (see below). This example provides an obvious illustration of how preventing the heterodimerization with BAD can hinder the apoptotic pathway from reaching the finish line, leading to the final cellular demise. Another example is the fact that Akt/PBK can also phosphorylate caspase-9, a member of the "apoptosome." This phosphorylation annuls this caspase's typical pro-apoptotic function [Cardone et al., 1998]. These examples show that a single action such as protein modification via phosphorylation is able to inhibit an apoptotic process. Obviously, there are many multiple factors such as these examples poised to intercept the many signaling steps and rescue cells from ever reaching the final stage to die.

It appears that Akt/PKB protein possesses more anti-apoptotic functions, independent from the two examples described above. These functions lie upstream of caspase-9 processing, since the release of cytochrome *c* (see below) is inhibited by Akt/PKB activation [Kennedy et al., 1999]. This exclusive cellular feature was recently demonstrated otherwise by the identification of another protein, FKHRL1, as a third substrate for Akt/PKB phosphorylating action, in addition to BAD and caspase-9 [Brunet et al., 1999]. Upon phosphorylation by Akt, this protein associates with 14–3-3 protein. Under serum withdrawal conditions, it translocates to the nucleus and activates Fas ligand and other potentially apoptotic gene expressions.

Apoptotic function by heterodimerization is not always an operational rule of thumb for all members of the Bcl-2 family. Moreover, conflicting results can be found with the same protein as to whether its function requires the dimerized biochemical form to occur. For example, Boo/Diva, a recently identified Bcl-2 homologue, is bound to Apaf-1 to regulate cell death. This protein was identified independently by

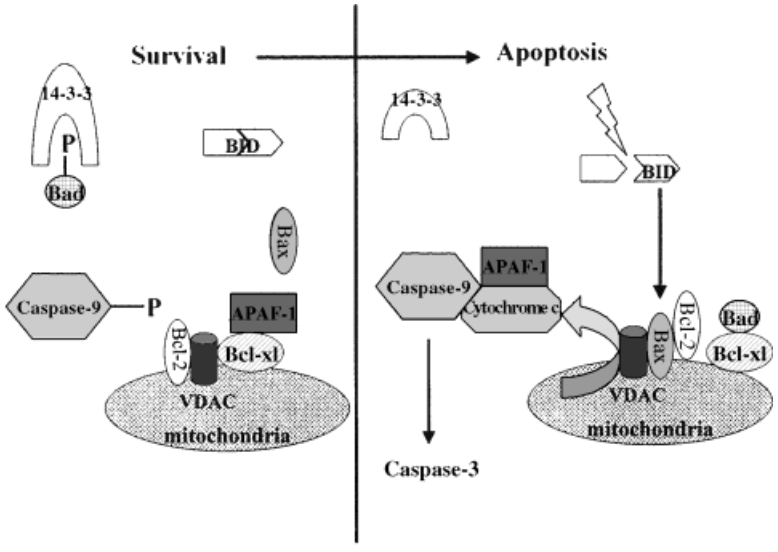

Fig. 2. Integration of pro-apoptotic and pro-survival signals at the mitochondria.

two groups who, however, describe it variously as anti- or pro-apoptotic in action [Inohara et al., 1998; Song et al., 1999]. This difference is largely due to variations in their observation, specifically on the feature of heterodimerization potential. While the group that termed the protein Diva shows that it does not dimerize with any known members of the Bcl-2 family, the group that termed it Boo demonstrates that it dimerizes with Bok and Bak, preventing the latter two proteins from exerting their pro-apoptotic function.

While it is hard to reconcile the results shown above with the Diva/Boo protein, this conflicting picture provides us with a cautionary reminder on using non-physiological conditions to assess the true function of a protein involved in apoptosis. This caution should be especially borne in mind when the popular transfection method is used to assess the functional role of a given gene. For example, transfection with TNF-R1-associated genes such as TRADD, FADD, or RIP can lead to flooding the cell milieu with an excess of partners for heterodimerized biochemical action. The final success of apoptosis may be solely due to the fact that this allows close proximity of caspase-8 by forming a large partner complex, leading to autoprocessing of caspase-8. In fact, the overabundance of transgene expression leads to the caspase-8 activation in an artificial scenario, which could never occur in real physiological conditions.

Emerging from the complexities of biochemical heterodimerization is the picture of a focal point in the fight to survive or to die, the "apoptosome," with Apaf-1 the key member. This focal point is therefore a reasonable target for the balance of action, once different Bcl-2 members are activated in response to various stress stimuli. The action to precipitate the final death may be a "tug-of-war" of the biochemical forces between the many pro-apoptotic and pro-survival members of the large Bcl-2 family. In all, it points toward Apaf-1, the base unit to form the "apoptosome," as an important focus in effecting successful cellular death.

Another important component of the "apoptosome" is cytochrome *c*. Upon release from the mitochondria, it associates with Apaf-1, caspase-9, and dATP, which leads to autoprocessing of caspase-9 and further activation of effector caspases such as caspase-3, caspase-6, and caspase-7. Here, Bcl-2 members again show apoptotic involvement, playing a gatekeeper role in regulating the mitochondrial release of cytochrome *c*. For example, Bid, a member of the Bcl-2 family, when cleaved by caspase-8, translocates to the mitochondria; this translocation induces the release of cytochrome *c*. This action of Bid is potentially due to its ability to form channels in liposomes and planar bilayers, like its sister proteins, Bcl-x_L and Bax [Schendel et al., 1999]. How these presumed channels promote the release of cytochrome *c* is still being debated. The fact that members such as Bax, Bak, Bcl-x_L, and Bcl-2 can associate with two components of the permeability transition (PT) pore, the adenine nucleotide translocator (ANT) [Marzo et al., 1998] and the recently described voltage-dependent anion channel (VDAC) [Shimizu et al., 1999; Narita et al., 1998], argues that, in part, the regulation of cytochrome *c* release is due to the control of PT openings. A recent paper suggests that this regulation may be at the level of the mitochondrial ATP/ADP exchange [Van der Heiden et al., 1999]. Bcl-x_L, thought to be pro-survival in its function, promotes efficient exchange of ADP for ATP under serum-withdrawal conditions, preventing mitochondria from undergoing swelling, cytochrome *c* release, and PT opening. Thus, members of the Bcl-2 family can function in either promoting, such as Bid protein, or preventing, such as Bcl-x_L, the release of cytochrome *c*, and perform these functions in both pro-survival and pro-death roles. In all, these results point to the channel-forming activity as a means for Bcl-2 proteins to participate in the regulation of cells' suicidal path.

PRO-APOPTOTIC VERSUS ANTI-APOPTOTIC SIGNALING

A general model by which receptor oligomerization induces apoptosis is depicted in Figure 2. Caspase-8 is processed at the TNF receptor. This autoproteolytic processing produces the active form of caspase-8, which in turn cleaves Bid and allows the latter to translocate along with its sister, Bax, to the mitochondria. There, Bid, with its pore-forming activity, associates with Bax, and induces a conformational change in Bax, promoting the release of cytochrome *c* release by the PT [Desagher et al., 1999]. The released cytochrome *c* is then associated with Apaf-1 and caspase-9, and in the presence of dATP, causes the caspase-9 to be processed, possibly by autoproteolysis. The processed caspase-9, the active form, causes further acti-

vation of other caspases such as caspase-3, caspase-8, caspase-7, and caspase-6 [reviewed in Cryns and Yuan, 1998]. Therefore, TNF receptor oligomerization starts all these cascades of caspase activation via three essential steps: the initial caspase-8 autoprocessing, leading to Bid translocation and cytochrome *c* release, and formation of the Apaf-1 complex, setting the stage for further caspase activation.

By contrast, TNF receptor oligomerization can induces an alternate signal, starting with TRADD recruiting RIP, initiating a cascade of phosphorylation, and culminating in IκB degradation and NF-κB activation [reviewed in Ashkenazi and Dixit, 1998]. So far, no evidence has been described for a preferential activation, either pro- or anti-apoptotic, via the action of either caspase-8 or RIP. If both cascades are activated simultaneously, we do not know which cascade will be the dominant force at the finish line, nor which will execute the final commitment to die or to live.

Nevertheless, it is certain that the ability to construct an efficient apoptosome is the key step in the race for a cell to establish a successful survival or apoptotic signal.

A new family of inhibitors of apoptosis has recently been identified, called IAPs, composed of XIAP, cIAP1, cIAP2, NAIP, BRUCE, and survivin [reviewed in Deveraux and Reed, 1999]. Overexpression of XIAP, cIAP1, cIAP2, NAIP, or survivin protects cells from TNF- or serum deprivation-induced apoptosis. This cytoprotective effect seems to be mediated by a few selected mechanisms at several levels of the apoptotic cascade. For example, one level of action is caspase inhibition, by direct binding between cIAP1/2 or XIAP and caspase-3, -7, or –9. Also, cIAP1 and cIAP2, along with TRAF1 and TRAF2, can be transcriptionally regulated by NF-κB, and together inhibit caspase-8-mediated apoptosis. This scenario gives cIAP the potential to have two locations to hinder apoptotic death: 1. at the plasma membrane level, and 2. at the mitochondrial level, both of which prevent the formation of an efficient "apoptosome."

The involvement of the NF-κB pathway in regulating apoptosis is further evidenced by the fact that the Bcl-2 homologue bfl-1/A1, a pro-survival member, is found to be up-regulated by active Rel/NF-κB [Zong et al., 1999]. Similarly, two other pro-survival Bcl-2 proteins, Bcl-2 and Bcl-x_L, are found to be transcriptionally up-regulated by NF-κB in primary hippocampal neurons in response to TNFα [Tamatani et al., 1999]. Moreover, Bcl-2 can promote IκBα degradation, leading to NF-κB up-regulation [de Moissac et al., 1998]. This suggests an amplification loop where Bcl-2 gets up-regulated by growth factors, in turn activating NF-κB, and further up-regulation of other pro-survival Bcl-2 members. In any case, the fact that in many instances TNF can induce apoptosis only in the presence of transcription and translation inhibitors hints that the downstream action of Akt/PKB can somehow delay the apoptotic pathway long enough to allow new proteins such as cIAP1, cIAP2, Bcl-2, Bcl-x_L and A1/bfl-1 to be synthesized. These newly produced proteins can form the added fortified means leading to a sustained survival state.

Once caspases are activated, however, the pro-apoptotic pathway gains a serious advantage over the pro-survival pathway, because many of the players involved in the anti-apoptotic pathway are substrates for caspases. Both Bcl-2 and Bcl-x_L are cleaved by caspases during apoptosis [Cheng et al., 1997; Clem et al., 1998]. Once cleaved, they are converted to pro-apoptotic proteins; results showing that mutant protein in the cleavage site protects cells from undergoing apoptosis suggest that this cleavage is necessary for a cell to die. Moreover, caspases can cleave signal transduction proteins such as Akt and raf-1, turning off major effectors of the pro-survival pathway [Widmann et al., 1998]. Furthermore, caspase-3 cleaves the regulatory A subunit of phosphatase 2A (PP2A), increasing its activity by 4.5-fold [Santoro et al., 1998]. This increase in PP2A activity may act in concert with the cleavage of signaling protein to efficiently shut off a growth-factor survival pathway, dependent on phosphorylation, leaving no other choice but for the cell to die. Another category of substrate for caspases, which therefore has an effect on cell fate, is the cleavage of proteins involved in translation, such as eIF4G and eIF-2alpha, or involved in segregation of new protein into the endoplasmic reticulum (ER), such as the signal recognition particle (SRP) [Marissen and Lloyd, 1998; Satoh et al., 1999; Utz et al., 1998]. These events completely turn off translation and translocation of new protein into the ER. These results imply that translation of new proteins is not required for efficient apoptosis, but that these cleavages may further inhibit the NF-κB sur-

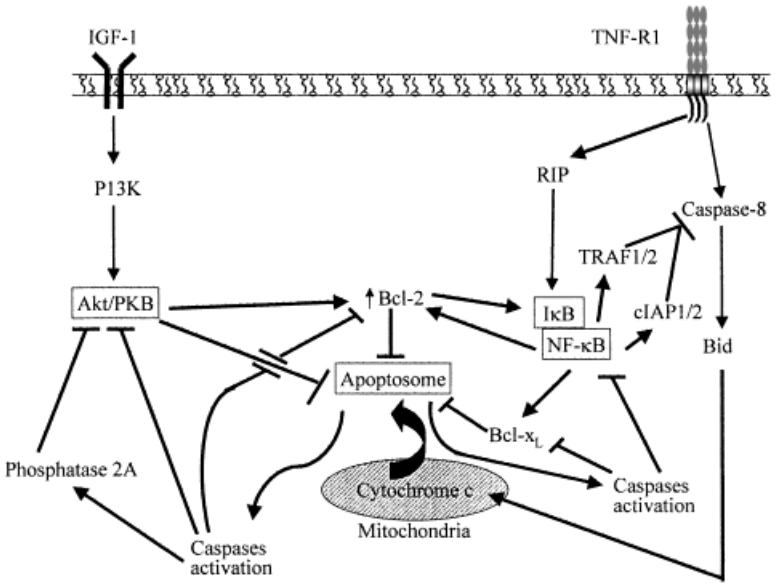

Fig. 3. Major checkpoints where pro-survival or pro-apoptotic pathways take a major advantage on the racetrack for cell fate.

vival pathway, which, often, is dependent on translation of new proteins such as cIAP1/2, A1, Bcl-2, and Bcl-x_L to efficiently block apoptosis.

CONCLUSION

In a molecular race in which cell fate is the final outcome, the decision to live or to die must be a well-calculated one, and the process leading to the eventual death must have many checkpoints (summarized in Fig. 3). Until recently, we viewed many complex apoptotic pathways from a reductionist point of view, as if they were determined by a few genes and a few checkpoints. While the discovery of these few apoptosis-related genes set the exciting pace of research in this area, it nevertheless reveals the fact that Mother Nature is clever in her evolutionary design to make certain that the operational mode for key cellular events such as apoptosis follows the principle of Darwinian selection. In operation, cells must be designed to permit flexibility and plasticity for the primary mode of action of decision to die or to live. There may be many redundant, compensatory, and symbiotic signaling pathways, and they may be highways where networking traffic is not governed by a single system of operation. In the end, we may find that there are hundreds of genes' action involved in a single checkpoint, and the final determination to die or to live is orchestrated by these hundreds of genes via several dozens of checkpoints. With the advent of high-throughput biochip technology, we are poised at the revolutionary step to discover the identities of the hundreds of members of the molecular tug of war on the racetrack to live or to die. This discovery will pave the step leading to our ultimate goal, which is to have cells die on genetic cue when they are not needed, and to rescue cells from death when they are wanted.

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