Several years ago, it was discovered that apoptosis is a central step in the development of cancer (Hanahan and Weinberg 2000). The apoptotic defect can, as in follicular lymphoma, be due to overexpression of Bcl-2, but more commonly results from mutations that ablate function of the p53 tumor suppressor, which acts upstream of Bcl-2. Particularly pertinent to this paper, it is now recognized that the resulting defects in apoptotic signaling may prove valuable for therapy. Indeed, the recently described ABT-737 is a promising “BH3 mimetic” of Bad. We find that, like Bad, ABT-737 kills cells efficiently only if Mcl-1 is absent or down-regulated. Thus, manipulation of apoptosis by targeting the Bcl-2 family has exciting potential for cancer treatment.

In 1988, the bcl-2 gene, which had been identified earlier as a novel gene translocated in follicular lymphoma to an immunoglobulin locus, was shown to act by preventing cell death rather than by driving cell proliferation (Vaupel et al. 1988). This discovery engendered the now widely accepted concept that impairment of apoptosis is a central step in the development of cancer (Hanahan and Weinberg 2000). The apoptotic defect can, as in follicular lymphoma, be due to overexpression of Bcl-2, but more commonly results from mutations that ablate function of the p53 tumor suppressor, which acts upstream of Bcl-2. Particularly pertinent to this paper, it is now recognized that the resulting defects in apoptotic signaling may prove valuable for therapy. Indeed, the recently described ABT-737 is a promising “BH3 mimetic” of Bad. We find that, like Bad, ABT-737 kills cells efficiently only if Mcl-1 is absent or down-regulated. Thus, manipulation of apoptosis by targeting the Bcl-2 family has exciting potential for cancer treatment.

The Bcl-2 protein family, which largely determines commitment to apoptosis, has central roles in tumorigenesis and chemoresistance. Its three factions of interacting proteins include the BH3-only proteins (e.g., Bim, Puma, Bad, Nova), which transduce diverse cytotoxic signals to the mammalian pro-survival proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1), whereas Bak and Bax, when freed from pro-survival constraint, provoke the mitochondrial permeabilization that triggers apoptosis. We have discovered unexpected specificity in their interactions. Only Bim and Puma, which mediate multiple cytotoxic signals, engage all the pro-survival proteins. Nova and Bad instead bind subsets and cooperate in killing, indicating that apoptosis requires neutralization of different pro-survival subsets. Furthermore, Mcl-1 and Bcl-xL, but not Bcl-2, directly sequester Bak in healthy cells, and Bak is fixed only when BH3-only proteins neutralize both its guards. BH3-only proteins such as Bim are tumor suppressors and mediate many of the cytotoxic signals from anticancer agents. Hence, compounds mimicking them may prove valuable for therapy. Indeed, the recently described ABT-737 is a promising “BH3 mimetic” of Bad. We find that, like Bad, ABT-737 kills cells efficiently only if Mcl-1 is absent or down-regulated. Thus, manipulation of apoptosis by targeting the Bcl-2 family has exciting potential for cancer treatment.
somehow allows Bax and Bak to oligomerize in the mitochondrial outer membrane and thereby provoke its permeabilization.

We are exploring how the interaction of different Bcl-2 family members triggers the cell death switch, how this protein family regulates tissue homeostasis, how disruption of that regulation promotes tumorigenesis, and how impaired apoptosis affects cancer therapy. We briefly review in this symposium our recent findings on the roles of BH3-only proteins in transmission of specific death signals, evidence for specificity in interaction of family members, and our new model for the regulation of Bak. Finally, we discuss recent findings that highlight the therapeutic prospects for targeting the Bcl-2 family with small molecules.

BH3-ONLY MEDIATORS OF CYTOTOXIC SIGNALS

As reviewed elsewhere (Huang and Strasser 2000; Bouillet and Strasser 2002; Puthalakath and Strasser 2002; Strasser 2005), the BH3-only proteins are sensors and transducers of multiple cytotoxic insults. Presumably, their multiplicity has evolved to allow exquisite control over cell death. For example, Bim and Bmf are regulated in part by sequestration to the cytoskeleton. Thus, Bim can be sequestered by dynein motor complexes to the microtubules (Puthalakath et al. 1999), and Bmf by the myosin V complex to the actin cytoskeleton (Puthalakath et al. 2001). Interestingly, Bim is unleashed in response to paclitaxel (Taxol), which affects microtubules, whereas Bmf can be activated by loss of cell attachment to the matrix (anoikis) (Puthalakath et al. 2001) and by treatment with HDAC inhibitors (Zhang et al. 2005). Thus, certain BH3-only proteins seem to represent sentinels positioned to regulate stress to specific subcellular compartments. To enable responses to other cytotoxic cues, however, these proteins are controlled in multiple ways. Bim, for example, can be regulated at the transcriptional level, by phosphorylation and by protein turnover (Bouillet and Strasser 2002; Puthalakath and Strasser 2002; Akiyama et al. 2003).

Disruption of the genes encoding BH3-only proteins has provided important insights into their physiological functions. Mice deficient in Bim have been particularly informative. Their defects have revealed that Bim is critical for removing superfluous hematopoietic cells, for apoptotic responses to other cytotoxic cues, however, these proteins are controlled in multiple ways. Bim, for example, can be regulated at the transcriptional level, by phosphorylation and by protein turnover (Bouillet and Strasser 2002; Puthalakath and Strasser 2002; Akiyama et al. 2003).

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Disruption of the genes encoding BH3-only proteins has provided important insights into their physiological functions. Mice deficient in Bim have been particularly informative.
The BH3-only proteins have been generally assumed to interact promiscuously with all the pro-survival proteins, but recently we have unexpectedly found that most exhibit notable selectivity (Chen et al. 2005). Quantitative analysis using an optical biosensor to determine the affinity of various BH3 peptides for the five Bcl-2-like proteins revealed that the interactions varied more than 10,000-fold in affinity. Bim and Puma avidly engaged all five pro-survival proteins, but some of the BH3-only proteins showed preferences. Most strikingly, Bim bound tightly to Bcl-2, Bcl-xL, and Bcl-w but not to Mcl-1 or A1, whereas Noxa instead bound only Mcl-1 and A1 (Fig. 2A).

Importantly, the binding data correlated with pro-apoptotic activity. Bim and Puma, which bind all the pro-survival proteins, potently induced apoptosis, whereas ones

that bind only a subset of them (e.g., Bad, Noxa) were considerably weaker killers (Chen et al. 2005). This finding probably explains why cells that lack Bim or Puma exhibit much more marked defects in apoptosis than cells lacking one of the other BH3-only proteins (Fig. 1). Notably, however, Bad and Noxa, which bind complementary targets, cooperated to induce potent killing (Fig. 2B). We therefore think that apoptosis usually requires neutralizing more than one type of pro-survival protein.

The basis for binding specificity of specific BH3 domains is becoming clearer as more structures of these proteins emerge, such as a high-resolution view of Bim bound to Bcl-xL (Liu et al. 2003) and the structures we have determined for Bcl-w (Hinds et al. 2003) and Mcl-1 (Day et al. 2005). Consideration of these structures allowed us to identify two mutations to the Noxa BH3 peptide that greatly increased its binding to Bcl-xL (Chen et al. 2005). Such findings have significant implications for the search for BH3 mimetic drugs (see below).

**SEQUESTRATION MODEL FOR Bak**

Activation of Bax or Bak is essential for apoptosis in most if not all cells (Lindsten et al. 2000), and both clearly act downstream of the BH3-only proteins (Cheng et al. 2001; Zong et al. 2001), but how the pro-survival proteins prevent activation of Bak and Bax has remained controversial (Adams 2003). Although Bax and Bak have largely redundant function, their regulation must differ significantly, because Bak resides in the mitochondrial membrane of healthy cells, whereas Bax is cytosolic prior to a death signal, probably because in healthy cells its hydrophobic carboxy-terminal membrane anchor is buried within a surface groove (Suzuki et al. 2000).

We have recently explored the activation of Bak and have proposed that Bak is normally sequestered by two specific pro-survival Bcl-2 family members (Willis et al. 2005). Importantly, in healthy cells we found that Bak associates directly with both Mcl-1 and Bcl-xL, but not with Bcl-2, Bcl-w, or A1 (Willis et al. 2005). The interaction requires the BH3 of Bak (Fig. 3), so that domain presum-
ably fits into the groove on Mcl-1 and Bcl-xL. Indeed, a Bak BH3 peptide binds avidly to both of these pro-survival proteins but not to the others (Willis et al. 2005). When cytotoxic signals activate BH3-only proteins that can engage both Mcl-1 and Bcl-xL, the BH3-only proteins displace Bak (Fig. 4). Accordingly, Noxa can bind to Mcl-1 and displace Bak, but Bak-mediated cell death also requires neutralization of Bcl-xL by other BH3-only proteins, such as Bad. Hence, Noxa can kill mouse embryonic fibroblasts lacking Bcl-xL but not those lacking Bcl-2 (Willis et al. 2005).

We were intrigued to find that Noxa not only displaces Mcl-1 from Bak, but also triggers Mcl-1 degradation in a proteasome-dependent manner (Willis et al. 2005). Interestingly, Mcl-1 degradation in response to genotypic damage apparently also involves a newly described ubiquitin E3 ligase (denoted Mule for Mcl-1 ubiquitin ligase E3) (Zhong et al. 2005). Notably, Mule possesses a BH3 domain similar to Bak, allowing its specific association with Mcl-1 and not other Bcl-2 family members. At present, it is unclear whether Mule acts downstream of Noxa, perhaps displacing Mcl-1 from Noxa, or whether Noxa and Mule instead lie on two independent pathways to Mcl-1 destruction by the proteasome.

Our observations suggest that Bak is held in check solely by Mcl-1 and Bcl-xL and induces apoptosis if, and only if, released from both its guards (Fig. 4). Once freed from them, Bak may spontaneously associate with itself to induce the damage to intracellular membranes that elicits cell death. The mechanism by which Bak (or Bax) aggregates in the membrane is not known, but we found that it required the Bak BH3 domain (Willis et al. 2005). That observation may mean that a key step in the oligomerization of Bak (and perhaps also of Bax) is formation of Bak-Bak homodimers in which a Bak molecule with an exposed BH3 domain has inserted it into the groove on another molecule of Bak that retains a “receptor” conformation (Fig. 4).

The Bcl-2 family in tumorigenesis

As reviewed elsewhere (Cory and Adams 2002; Cory et al. 2003), pro-survival family members such as Bcl-2 and Bcl-xL are overexpressed in many human tumors, and transgenic mouse models have provided compelling direct evidence that their overexpression contributes to neoplasia. Overexpression of Bcl-2 in the B-lymphocyte compartment, for example, was shown over a decade ago to provoke a marked expansion of nonproliferating B cells (McDonnell et al. 1989; Strasser et al. 1991), and tumors developed rapidly when a cooperating oncogene such as c-myc was coexpressed (Strasser et al. 1990). The tumors provoked by Bcl-2 alone arose only after a protracted period and comprised pre-B lymphomas and plasmacytomas.

Figure 4. Model for the regulation of Bak. The essence of the model (Willis et al. 2005) is that, in healthy cells, at least a portion of Bak exists in the mitochondrial outer membrane in a form bound to Mcl-1 and Bcl-xL, via the BH3 (red beak) of Bak. Bak can be released for death duty only by BH3-only proteins that engage both Mcl-1 (e.g., Noxa) and Bcl-xL (e.g., Bad). Bak may then aggregate into a form (as yet unknown) that permeabilizes the mitochondrial outer membrane, provoking release of apoptogenic proteins such as cytochrome c that induce caspase activation. We speculate that some of the Bak molecules may normally remain in a “receptor” conformation to account for the observation that Bak oligomerization also requires the Bak BH3 domain (Willis et al. 2005). (Reprinted, with permission, from Willis et al. 2005.)
Bcl-2 SWITCH IN TUMORGENESIS AND THERAPY

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tomas, many of which exhibited c-myc translocations (McDonnell and Korsmeyer 1991; Strasser et al. 1993).

Given the original link of translocated human BCL2 with follicular lymphoma, it was surprising that the Ig enhancer-driven BCL2 transgene did not yield that tumor type. Recently, however, mice bearing a transgene that imposed overexpression of Bcl-2 throughout the hematopoietic compartment (Ogilvie et al. 1999) have yielded the first animal model of follicular lymphoma (Egle et al. 2004b). Intriguingly, these B-lymphoid tumors were preceded by grossly enlarged germinal centers, development of which required help from the expanded pool of CD4 T cells produced by this transgene. As these mice are also subject to autoimmune disease, it seems likely that antigenic or autoimmune stimulation through the B-cell antigen receptor plays a critical role in development of the tumors. It is therefore tempting to think that antigenic stimulation also has a role in human follicular lymphoma, as some other evidence has previously suggested (Zelenetz et al. 1992).

Since Bcl-2 pro-survival family members are oncoproteins, we wanted to determine whether their BH3-only antagonists can act as tumor suppressors. Bim was of particular interest because it is a major antagonist of Bcl-2 in the lymphoid compartment (Bouillet et al. 2001). Since overexpression of Bcl-2 markedly increases the rate of tumor development in our well-studied Eμ-mykl transgenic mice (Strasser et al. 1990), we tested whether loss of Bim would act similarly. Indeed, loss of even a single allele of Bim accelerated tumorigenesis (Egle et al. 2004a). Because the acceleration primarily reflected leukemia of mature B cells, the tumor suppressor role of Bim may be confined to particular stages of development. Pertinently, homozygous deletions of the human BIM locus have now been found in a significant fraction of human mantle cell lymphomas (Tagawa et al. 2005).

Significantly, Bim appears to act as a tumor suppressor by mediating the apoptosis induced by Myc under suboptimal growth conditions. In lymphocytes, overexpressed Myc appears to lower the threshold for apoptosis by reducing levels of Bcl-xL and Bcl-2 while elevating those of Bim (Egle et al. 2004a). Thus, Bim deficiency presumably contributes to oncogenesis by allowing more B-cell lineage cells to survive in the face of overexpressed Myc, just as does loss of p53 in cells that retain Bim. Accordingly, most of the tumors from the Bim-deficient cells retained p53 function, whereas most of those arising in Bim-proficient cells have lost p53 function.

To better gauge how oncogene activation affects cell survival and tumorigenesis in different cell lineages, we have characterized transgenic mouse strains with Myc expressed in most hematopoietic cells (Smith et al. 2005). Surprisingly, the types of tumors that arise depends on the level of Myc (D.P. Smith et al., in prep.).

IMPLICATIONS OF APOPTOTIC REGULATION FOR CYTOTOXIC THERAPY

Cytotoxic therapy for cancer will undoubtedly benefit from a deeper understanding of how the Bcl-2 family is regulated, in particular from insights into the critical roles of specific BH3-only proteins in the responses to different chemotherapeutic agents (Fig. 1). For example, recent evidence indicates that the apoptosis induced in chronic myeloid leukemia cells by Gleevec (imatinib) relies in part on Bim (Kuribara et al. 2004). Indeed, we find that both Bim and Bad have critical roles in this response (J. Kuroda and A. Strasser, unpubl.).

A striking illustration of the way in which understanding of apoptotic mechanisms could affect future treatment strategies emerged from a recent collaborative study with Eileen White and colleagues. Just as we had reported with lymphocytes (Bouillet et al. 1999), the sensitivity of epithelial carcinoma cells to paclitaxel (Taxol) proved to require Bim (Tan et al. 2005). If the tumor cells contained an activated Ras oncogene, however, the cells were rendered refractory to paclitaxel, because excess Ras activity caused Bim to be targeted for proteasomal degradation. Nevertheless, Bim levels and sensitivity to paclitaxel could both be restored by addition of the clinically relevant prosaptoinhibitor bortezomib (PS-341). These results provide a rational basis for combining paclitaxel and bortezomib in therapy, particularly in cells where the Ras pathway is active (Tan et al. 2005).

In the killing of some human cancer cell lines, bortezomib also exhibits synergy with TRAIL, a ligand for certain death receptors. A study with Andrew Kraft and colleagues revealed the likely basis for the synergy: Bortezomib provokes higher levels of Bk and/or Bim in the various lines, apparently by inhibiting their degradation (Nikrad et al. 2005). These findings indicate that combination chemotherapy may soon have a more rational foundation.

THERAPEUTIC POTENTIAL OF “BH3 MIMETICS”

As discussed elsewhere (Baell and Huang 2002; Cory et al. 2003; Cory and Adams 2005), because abnormalities in cancer cells, such as p53 mutations, disturb the transduction of cytotoxic signals to the Bcl-2 family (i.e., prevent Puma/Noxa induction), an anticancer drug that mimicked a BH3-only protein should be efficacious (Fig. 5). Two of our findings are pertinent to the design of such BH3 mimetics. First, our demonstration (Chen et al. 2005) of preferential binding by certain BH3 domains (Fig. 2A) suggests that it should eventually be feasible to target a specific Bcl-2-like protein, such as one needed to maintain a particular tumor type, or at least a subset of the pro-survival proteins. The specificity should increase the therapeutic index of a BH3 mimetic by reducing deleterious effects on normal tissues. Second, our evidence (Willis et al. 2005) that only Mcl-1 and Bcl-xL, guard Bak (Fig. 4) suggests that targeting these pro-survival proteins may be particularly effective, even in tumors that greatly overexpress Bcl-2. In view of the frequent overexpression of Bcl-2 in cancer cells, that could be a significant advantage.

Development of a small-molecule inhibitor of a protein–protein interaction is very challenging. As reviewed elsewhere (Baell and Huang 2002; Cory et al. 2003), several putative BH3 mimetics have been reported, but most
of these compounds bind to their target with only micromolar affinity, whereas BH3 peptides bind with nanomolar affinity (Chen et al. 2005). That raises significant questions about their specificity. Since BH3-only proteins require either Bax or Bak to kill cells (Cheng et al. 2001; Zong et al. 2001), a true BH3 mimetic should not kill cells lacking both Bax and Bak. We find, however, that several compounds inferred to be BH3 mimetics but having low affinity for their putative targets kill bax−/−bak−/− fibroblasts as readily as wild-type fibroblasts (A. Wei et al., in prep.). Hence, these compounds kill cells in a nonspecific manner.

As discussed further elsewhere (Cory and Adams 2005), strong proof of principle that BH3 mimetics have promise for cancer therapy has come very recently from Abbott Laboratories (Oltersdorf et al. 2005). By structure-based design with Bcl-xL as a target, Oltersdorf et al. have developed a compound, denoted ABT-737, with high (low nM) affinity for Bcl-xL, Bcl-2, and Bcl-w, albeit very poor affinity for Mcl-1 and A1. With many tumor cell lines, they report that ABT-737 displays strong synergy in inducing apoptosis with conventional chemotherapeutic agents, as well as γ-radiation. Moreover, as a single agent, ABT-737 kills tumor cells of certain types; intriguingly, the susceptible cells include not only follicular lymphoma and chronic lymphocytic leukemia samples, but also small-cell lung carcinoma (SCLC). Most strikingly, with xenografts of two different SCLC lines, ABT-737 at the highest dose tested induced stable regression of the tumors in 77% of the mice (Oltersdorf et al. 2005). Remarkably, the drug was tolerated well, the only adverse effects noted being some drop in lymphocytes and platelets.

In view of the considerable promise of ABT-737, we have been studying its mode of action (A. Wei et al., in prep.). As expected for an authentic BH3 mimetic, its ability to kill fibroblasts requires Bax or Bak. Its pro-apoptotic activity on wild-type fibroblasts, however, was relatively weak. Since the binding of ABT-737 to pro-survival proteins closely resembles that of Bad (Fig. 2A), we tested whether Noxa would augment killing by ABT-737, as it does with Bad (Fig. 2B), by engaging Mcl-1 and promoting its degradation (Willis et al. 2005). Indeed, fibroblasts overexpressing Noxa became far more sensitive to ABT-737. Furthermore, the enhanced activity was clearly mediated by the inactivation of Mcl-1, because RNAi against Mcl-1 as well as Noxa overexpression rendered two human tumor cell lines far more sensitive to ABT-737, as shown in Figure 6 for the breast cancer cell line MCF-7.

Because the level of Mcl-1 in hematopoietic cells is tightly regulated by cytokines, we tested whether cytokine deprivation would also augment killing by ABT-737. The test employed a cytokine-dependent myeloid cell line, FDC-P1, engineered to overexpress Bcl-2. Indeed, the starved cells were far more sensitive to the drug, despite the presence of abundant Bcl-2, which protects the untreated cells from death on cytokine deprivation. A likely explanation for the increased sensitivity is that the
cytokine deprivation both lowered the level of Mcl-1 and elevated that of Bim.

Since killing by ABT-737, like that by Bad, is greatly augmented if Mcl-1 is neutralized by Noxa or eliminated by RNAi, ABT-737 may prove most effective as a single agent in tumors with low or negligible levels of Mcl-1. Furthermore, its synergy with various chemotherapeutic agents and radiation probably reflects down-regulation of Mcl-1 by these agents (Oltersdorf et al. 2003; Njiahvan et al. 2003). In view of the results discussed above, the cytotoxic action of ABT-737 may be mediated preferentially through release of Bak (Fig. 4).

CONCLUSIONS

Impaired apoptosis is a central step in tumorigenesis. Although loss of p53 function is the most frequent cause, the Bcl-2 family probably contributes directly or indirectly in a significant proportion of human tumors. Over-expression of the pro-survival proteins, particularly Bcl-2, is frequent, and in some cell types, loss of a BH3-only antagonist such as Bim can have a similar effect. Gain of Bcl-2 or loss of Bim is tumorigenic in part because both counter the cell death impetus resulting from oncogene activation, such as constitutive Myc expression.

The eight or more BH3-only proteins appear to be the essential transducers of most cell death signals, including those elicited by most conventional cytotoxic agents (Fig. 1). Hence, better understanding of their specific roles and the complex ways they are regulated is likely to provide a more rational foundation for therapy, particularly combination chemotherapy, which remains highly empirical.

The interaction of BH3-only proteins with the pro-survival proteins exhibits more specificity than has generally been expected (Chen et al. 2005). Bim and Puma bind promiscuously to all the pro-survival proteins, and this may well account for their greater pro-apoptotic activity and the more marked apoptotic defects observed upon knockout of their genes (Fig. 1). The other BH3-only proteins, however, bind preferentially to different subsets, with Bad and Noxa engaging complementary groups (Fig. 2A). Since they also exhibit synergy in cell killing (Fig. 2B), we have suggested that commitment to apoptosis requires neutralization of more than one subset of the pro-survival proteins (Chen et al. 2005).

Insight into the issue of how the pro-survival family members regulate commitment to apoptosis has come from our studies on Bak killing (Willis et al. 2005). To our surprise, Bak appears to be kept under control by only two of its pro-survival counterparts, Bcl-xL and Mcl-1, and Bak-2 and Bcl-xL seem to have no role in guarding it (Fig. 4). In healthy cells, Bak molecules associate with these guardians via the Bak BH3 (Fig. 3). In our model, if both Bcl-xL and Mcl-1 are inactivated, Bak may spontaneously aggregate and trigger damage to the outer mitochondrial membrane and probably other membranes (Fig. 4). As yet, we do not know whether Bak is regulated somewhat similarly.

The recently developed BH3 mimetic ABT-737 (Oltersdorf et al. 2005), along with findings with certain constrained BH3 peptides (Walencky et al. 2004), has provided strong proof of principle that triggering apoptosis of tumor cells by directly engaging the pro-survival proteins (Fig. 5) represents a promising new approach to cancer therapy (Cory and Adams 2005). The binding profile of ABT-737 mimics that of Bad (Fig. 2A). Accordingly, we found that the drug mediated Bax/Bak-dependent apoptosis, its potency was limited unless Mcl-1 was down-regulated (Fig. 6). Hence, we surmise that ABT-737 will prove most efficacious as a single agent in tumors in which Mcl-1 is low or absent. Its killing action is markedly enhanced, however, by treatment that provoke Mcl-1 down-regulation, such as exposure to conventional cytotoxic agents or cytokine deprivation. Thus, it may well prove efficacious on a wider range of tumors in combination with agents that down-regulate Mcl-1. The success of ABT-737 in preclinical mouse models (Oltersdorf et al. 2005) suggests that for many tumors the Bcl-2-guarded gateway to cell death is the Achilles’ heel. Hence, BH3 mimetics seem destined to be valuable new weapons in the war on cancer.

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