**c-Myc and Caspase-2 Are Involved in Activating Bax during Cytotoxic Drug-induced Apoptosis**

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Activation of Bax following diverse cytotoxic stress has been shown to be an essential gateway to mitochondrial dysfunction and activation of the intrinsic apoptotic pathway characterized by cytochrome c release with caspase-9/-3 activation. Interestingly, c-Myc has been reported to promote apoptosis by destabilizing mitochondrial integrity in a Bax-dependent manner. Stress-induced activation of caspase-2 may also induce permeabilization of mitochondria with activation of the intrinsic death pathway. To test whether c-Myc and caspase-2 cooperate to activate Bax and thereby mediate intrinsic apoptosis, small interfering RNA was used to efficiently knock down the expression of c-Myc, caspase-2, and Apaf-1, an activating component in the apoptosome, in two human cancer cell lines, lung adenocarcinoma A-549 and osteosarcoma U2-OS cells. Under conditions when the expression of endogenous c-Myc, caspase-2, or Apaf-1 is reduced 80–90%, cisplatin (or etoposide)-induced apoptosis is significantly decreased. Biochemical studies reveal that the expression of c-Myc and caspase-2 is crucial for cytochrome c release from mitochondria during cytotoxic stress and that Apaf-1 is only required following cytochrome c release to activate caspases-9/-3. Although knockdown of c-Myc or caspase-2 does not affect Bax expression, caspase-2 is important for cytosolic Bax to integrate into the outer mitochondrial membrane, and c-Myc is critical for oligomerization of Bax once integrated into the membrane.

It is well documented that cytotoxic stress and DNA damage induce apoptosis by affecting the permeability of mitochondria to facilitate activation of the intrinsic apoptotic pathway (1, 2). Activation is characterized by mitochondrial dysfunction with release of caspase activators including cytochrome c that activate procaspases-9 and -3 that liquefy the cell from within and effect the morphologic signs of apoptosis including membrane blebbing, cell shrinkage, and DNA fragmentation (1, 2). It has been reported that activation of the proapoptotic Bcl-2 family members, Bax (or Bak), is an essential gateway to mitochondrial dysfunction required for cell death induced by diverse cytotoxic stress (3). Overexpression of Bax or the addition of purified recombinant Bax to isolated intact mitochondria can trigger the release of cytochrome c, indicating a direct role for Bax in disrupting mitochondrial integrity (4–7). The three-dimensional solution structure of Bax demonstrates a high degree of similarity to the conformation of Bcl-XL (8), and Bax, Bcl-2, and Bcl-X, have all been shown to possess channel-forming activity in artificial membranes (9–12). Bax is normally localized in the cytoplasm or peripherally associated with the outer mitochondrial membrane (OMM) in its inactive monomeric form (8). In response to apoptotic stimuli, Bax translocates to, and/or integrates into, the OMMs, where it undergoes dimerization and oligomerization to potentially form a channel that somehow facilitates release of apoptogenic factors including cytochrome c from the dysfunctional mitochondrion (4–7, 13–15). For example, UV irradiation of cultured cells or treatment with staurosporine induces Bax oligomerization in the OMMs that facilitates cytochrome c release with subsequent caspase-9/-3 activation (14). However, what regulates Bax translocation to and insertion into the OMMs and its oligomerization remains unclear.

Recently, it has been reported that cytotoxic stress also causes activation of caspase-2 upstream of mitochondria and that caspase-2 is required for the permeabilization of mitochondria to promote cytochrome c release (16, 17). Furthermore, it has been shown that caspase-2 may be required for the translocation of Bax to the OMMs (17). Interestingly, the c-Myc oncoprotein has been reported to be involved in the induction of apoptosis in a Bax-dependent mechanism (18–20). However, the mechanism by which Bax is activated and inserted into the OMMs and the role of c-Myc, if any, in this process remains unclear. Whether c-Myc simply drives apoptosis through its transcriptional modulation of target genes (21), including Bax (22), or whether c-Myc promotes apoptosis by destabilizing mitochondrial integrity is also not clear (23, 24). Therefore, it remains to be clarified whether c-Myc stimulates Bax activity at the mitochondria (23) or whether c-Myc can functionally cooperate with Bax to induce apoptosis (24).

To test whether c-Myc and caspase-2 are involved in activating Bax at the mitochondria that initiates activation of the intrinsic apoptotic pathway, a small interfering RNA (siRNA) strategy was used to efficiently knock down the expression of c-Myc, caspase-2, or Apaf-1 in human lung adenocarcinoma A-549 and osteosarcoma U2-OS cells. Cytotoxic drugs were then applied to induce apoptosis. Results indicate that the

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2 The abbreviations used are: OMM, outer mitochondrial membrane; siRNA, small interfering RNA; MEF, mouse embryonic fibroblast.
expression of endogenous c-Myc, caspase-2, and Apaf-1 is essential for drug-induced apoptosis to occur in this experimental system. Furthermore, biochemical analysis reveals that caspase-2 is important for integration of Bax into the OMMs, whereas c-Myc is critical for Bax oligomerization after integration.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human lung adenocarcinoma A-549 cells and osteosarcoma U2-OS cells (ATCC) were grown and maintained respectively in F-12K and McCoy’s 5A medium (ATCC) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Cellgro) in 5% CO₂ and 95% humidified air at 37°C.

**Transfection with siRNAs**—Apaf-1 siRNA duplex with the target sequence 5’-AAT TGG TGC ACT TTT ACG TGA-3’ (nucleotides 978–998) and caspase-2 siRNA duplex with the target sequence 5’-AAA CAG CTG TTG ATG CAA-3’ (nucleotides 94–114) were purchased from Dharmacon. Double-stranded c-Myc siRNA and negative control siRNA were purchased from Ambion (silencer™ c-Myc siRNA control, catalog number 4604). A-549 cells were cultured in 100-mm dishes (Falcon) in 12 ml of F-12K medium complemented with 10% fetal bovine serum and transfected at 30% of confluency by adding 90 μl of siPORT™ amine transfection agent (Ambion) and 60 μl of 20 μM Apaf-1 or caspase-2 siRNA or 16 μl of 75 μM c-Myc or negative control siRNA (final concentration 100 nM). Control cells were treated with 90 μl of siPORT™ amine transfection agent alone. Cells were rinsed with the medium after 20 h of incubation and then maintained in culture for an additional 28 h. U2-OS cells were cultured and transfected similarly as A-549 cells except that 60 μl of siPORT™ amine transfection agent was added to each 100-mm dish.

**Analysis of Protein Expression**—Following siRNA transfection for 48 h, cells were harvested after trypanotizing for 10 min and lysed, and proteins were extracted and quantitated as described (25, 26). Equal amounts of lystate protein (50 μg) were subjected to SDS-PAGE followed by immunoblotting with an anti-cytochrome c antibody (Cell Signaling).

**Drug-induced Apoptosis**—Following siRNA transfection for 48 h, A549 cells and U2-OS cells were treated with 40 or 30 μM cisplatin, respectively, or 50 μM etoposide (Sigma) for 24 h. Cell viability was measured using the trypan blue exclusion method as described (27, 28).

**Caspase-3 Activity Assay**—Following siRNA transfection for 48 h, cells were treated with 40 μM cisplatin or 50 μM etoposide for 20 h. Apo-ONE™ homogeneous caspase-3 assay was performed following the manufacturer’s manual (Promega) as described (29).

**Cytochrome c Release**—Following siRNA transfection for 48 h, cells were treated with cisplatin for 18 h and then harvested after trypanotizing for 10 min. To assess cytochrome c release, the cytosolic fraction was obtained using a digitonin-based subcellular fractionation technique as described (30–32). Equal amounts of cytosolic fractions (80 μg) were subjected to SDS-PAGE followed by immunoblotting with an anti-cytochrome c antibody (Pharmingen).

**RESULTS**

**Transfection of siRNA Specifically Knocks Down the Expression of c-Myc, Caspase-2, and Apaf-1 in A549 and U2-OS Cells**—Cytotoxic stress, such as DNA damage, induces apoptosis through the mitochondrial pathway, and proapoptotic Bax (or Bak) is required to disrupt mitochondrial membrane integrity to initiate stress-induced apoptosis (1–3). Recent reports show that caspase-2 and c-Myc may be involved in destabilizing mitochondrial integrity (16, 17, 23, 24). To test whether c-Myc and caspase-2 are involved in activating Bax during stress-induced apoptosis, an siRNA strategy was utilized to knock down the expression of c-Myc, caspase-2, and Apaf-1 in human lung carcinoma A-549 and osteosarcoma U2-OS cells. After 48 h of transient transfection, endogenous c-Myc, caspase-2, and Apaf-1 levels were reduced 80–90% (Fig. 1). Demonstrating specificity of this system, a negative control siRNA (pRNA), with no sequence homology to any known gene, had no effect on the expression of the proteins measured, and none of the siRNAs used affected the expression of caspase-9. Although it was reported that Bax may be a transcriptional target of c-Myc and mediator of c-Myc-induced apoptosis (22), Bax expression is not affected by knockdown of c-Myc, caspase-2, or Apaf-1 in this experimental system (Fig. 1). These data indicate that c-Myc does not affect steady-state levels of Bax under these experimental conditions.

**Expression of c-Myc, Caspase-2, and Apaf-1 Is Essential for Drug-induced Apoptosis**—Transient transfection with siRNAs alone has no effect on the viability of A-549 and U2-OS cells (data not shown). Cisplatin and etoposide, DNA-damaging drugs that are used clinically in cancer chemotherapy, were
used to treat these cells. When treated with cisplatin for 24 h, about 50% of the cells died, as measured by trypan blue exclusion (Fig. 2A). However, when c-Myc expression is knocked down, cisplatin-induced cell death is significantly inhibited since only 10–15% cells die. Similarly, when Apaf-1 or caspase-2 is knocked down, cisplatin-induced cell death is reduced to about 20%. Importantly, the negative control siRNA (nRNA) has no effect on cisplatin-induced apoptosis (Fig. 2A). Therefore, we can conclude that optimal cell death requires c-Myc, caspase-2, and Apaf-1. Activation of procaspases-9 and -3 is a key event in activating the execution phase of intrinsic apoptosis. To test whether these cells underwent apoptosis by this route, caspase-3 activity was measured following siRNA transfection and cisplatin treatment. Results reveal that caspase-3 activation is significantly inhibited when c-Myc, caspase-2, or Apaf-1 is knocked down (Fig. 2B), suggesting that c-Myc, caspase-2, and Apaf-1 are three important mediators of drug-induced caspase-3 activation and apoptosis. Similar results are observed for single agent treatment with etoposide (data not shown).

**c-Myc and Caspase-2, but Not Apaf-1, Are Important for Cytochrome c Release**—During stress-induced activation of the intrinsic apoptotic pathway, caspase-9/-3 activation is initiated by cytochrome c release from the mitochondria into the cytosol (7, 34). Cytochrome c facilitates the formation of the apoptosome that contains Apaf-1 that recruits and activates procaspase-9, which in turn cleaves and activates its downstream executioner, procaspase-3 (35, 36). Using a digitonin-based subcellular fractionation technique (Fig. 3), results indicate that cisplatin-induced cytochrome c release is significantly decreased when either c-Myc or caspase-2 is knocked down, whereas, as expected, knockdown of Apaf-1 has no effect on cytochrome c release (Fig. 3). These results demonstrate that both c-Myc and caspase-2 are essential for permeabilization and dysregulation of mitochondria in this experimental system.

Recent reports proposed cytoplasmic proteins 14-3-3/H9258 and Ku-70 as Bax inhibitors that normally sequester Bax in the cytosol, thereby preventing Bax-induced cytochrome c release,

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**FIGURE 1.** siRNAs specifically knock down the expression of c-Myc, Apaf-1 (A-1), and caspase-2 (C-2). A-549 cells and U2-OS cells were cultured in 100-mm dishes in 12 ml of medium complemented with 10% fetal bovine serum and transfected with c-Myc, Apaf-1, or caspase-2 siRNA or negative control siRNA (nRNA) for 48 h as described under “Experimental Procedures.” Control cells were treated with the amine transfection agent alone for the corresponding time. Cells were then harvested and lysed, and proteins were extracted and quantitated. Equal amounts of protein lysates (50 μg) were subjected to SDS-PAGE followed by immunoblotting with c-Myc, Apaf-1, caspase-2 (Casp-2), caspase-9 (Casp-9), Bax, and actin antibodies. The representatives of three independent experiments were shown.

**FIGURE 2.** c-Myc, caspase-2 (C-2), and Apaf-1 (A-1) are important for cisplatin-induced apoptosis. A, following siRNA transfection for 48 h, A-549 cells and U2-OS cells were treated with 40 and 30 μM cisplatin, respectively, for 24 h. Cell viability was measured using the trypan blue exclusion method. nRNA, negative control siRNA. B, following siRNA transfection for 48 h, cells were treated with 40 μM cisplatin for 20 h, and Apo-ONE™ homogeneous caspase-3 activity assay was performed as described under “Experimental Procedures.” Caspase-3 activity is quantitated by relative fluorescence units. The two-tailed t test was used to determine significant differences (*, p < 0.05).

**FIGURE 3.** c-Myc and caspase-2 (C-2), but not Apaf-1 (A-1), are crucial for cytochrome c release. Following siRNA transfection for 48 h, cells were treated with 40 μM cisplatin (Sigma) for 18 h and then harvested. The cytosolic fraction was obtained using a digitonin-based subcellular fractionation technique. Equal amounts of cytosolic fractions (50 μg) were subjected to SDS-PAGE followed by immunoblotting with an anti-cytochrome c (Cyto C), anti-14-3-3θ, and anti-Ku-70 antibodies. The representatives of three independent experiments were shown. nRNA, negative control siRNA.
and that 14-3-3 and Ku-70 may be directly cleaved by some caspases to release Bax upon apoptotic stimulation (37, 38). However, we have not observed any significant change of 14-3-3 and Ku-70 expression levels that may suggest specific cleavage or degradation (Fig. 3).

_Caspase-2, but Neither c-Myc nor Apaf-1, Is Important for Bax Integration into the Outer Mitochondrial Membranes—_In non-stressed cells, Bax is located in the cytosol, where it may be peripherally but not integrally associated with the OMM (8). Following a death stimulus, Bax is translocated and integrated into the OMM through conformational exposure of its C-terminal transmembrane domain (8, 39). This process has been shown to precede mitochondrial dysfunction resulting in cytochrome c release into the cytosol (33, 40). It has been shown that alkali extraction of mitochondrial membranes will denature any peripherally associated (i.e. non-integrated) proteins that may otherwise pellet with the isolated membranes and thereby appear to be integral to the OMM. Importantly, this alkali extraction strategy can distinguish whether Bax is peripherally associated with or integrally inserted into the OMM (33). Studies were performed using this technique to determine whether caspase-2 or c-Myc affects the membrane integration of Bax during drug treatment. Results reveal that when caspase-2 is knocked down, the membrane-integrated Bax is decreased by 60–70% (Fig. 4), whereas knockdown of either c-Myc or Apaf-1 has no effect on Bax integration into the OMM. As a control, prohibitin is normally located in the inner mitochondrial membrane and is not affected in this experimental system (29).

_c-Myc Is Critical for Bax Oligomerization after Integration into the Outer Mitochondrial Membranes—_It has been reported that c-Myc can promote apoptosis by destabilizing mitochondrial integrity with cytochrome c release (23, 24). However, the mechanism is not clear. In particular, it remains controversial whether c-Myc potentiates apoptosis by stimulating Bax activity at the mitochondria or whether c-Myc functionally cooperates with Bax but works independently of Bax activation to induce apoptosis (23, 24). It is also controversial whether c-Myc activation can stimulate a detectable conformational change in Bax in mitochondria (23, 24). Interestingly, it has been consistently demonstrated that Bax is present as a dimer or oligomer following insertion into the OMM and that Bax oligomerization may be required for any channel-forming activity in artificial liposomes (13–15). Thus, the Bax oligomers are likely the functionally active form of Bax that facilitates dysfunction of the OMM with cytochrome c release. To test whether c-Myc or caspase-2 affects Bax oligomerization, mitochondria were isolated and subjected to cross-linking. Following cisplatin treatment for 18 h, Bax dimers, trimers, and tetramers are clearly detected in isolated mitochondrial membranes (Fig. 5). When c-Myc is knocked down, Bax oligomerization is significantly reduced. In contrast, knockdown of Apaf-1 has no effect on Bax oligomerization, although apoptosis is inhibited. These results suggest that c-Myc, either directly or through an as yet unidentified protein or cofactor, may indirectly facilitate Bax oligomerization in the OMM. These results are consistent with the report that c-Myc is required for Bax activation at the mitochondria, as detected using the conformation-specific Bax 6A7 antibody (23). Fur-
that caspase-2 is necessary for efficient cytochrome c release (42, 43). In this study, we examined the role of caspase-2 in drug-induced apoptosis in two human cancer cell lines. Consistent with the reports mentioned above, our results indicate that caspase-2 is necessary for efficient Bax activation in the mitochondrial membrane (Fig. 4), findings consistent with those reported for caspase-2 being required to translocate Bax to mitochondria (17). Taken together, these data suggest that caspase-2 activation may be the critical linkage point between caspase-2 and mitochondrial dysfunction. However, how caspase-2 mediates Bax translocation and/or integration into the mitochondrial membrane is not yet clear. It was recently reported that cytoplasmic protein 14-3-3 may act to inhibit Bax by directly binding and sequestering Bax in the cytosol and that 14-3-3 or Ku-70 may be directly cleaved by some protease/caspase to release Bax upon apoptotic stimulation, now allowing it to become integral to the OMM and disrupt mitochondrial integrity (37, 38). Therefore, we tested whether caspase-2 can activate Bax by degrading 14-3-3 or Ku-70 but did not observe any significant degradation of 14-3-3 or Ku-70 to indicate their role in Bax activation, regardless of whether or not caspase-2 is knocked down (Fig. 3).

It has been reported that c-Myc oncoprotein promotes apoptosis by destabilizing mitochondrial integrity (18). Interestingly, Bax-deficient MEF cells have been found to be markedly resistant to c-Myc-induced apoptosis, and c-Myc is unable to induce cytochrome c release in Bax-deficient MEFs (24). In contrast, MEFs deficient in either Bak or Bid remain sensitive to c-Myc-induced apoptosis (24), excluding their role in the c-Myc apoptosis mechanism. Importantly for the mechanism, ectopic expression of Bax in Bax-deficient MEFs restores sensitivity to c-Myc-induced apoptosis, indicating a requisite role for Bax (24). However, Bax does not appear to be a transcriptional target of c-Myc, at least in this system, because c-Myc knockdown has no effect on steady-state Bax expression levels in the cells used or in cells tested by others including Rat-1 fibroblasts and MEFs (23, 24, 44, 45). Thus, we propose that c-Myc may somehow indirectly trigger activation of existing Bax. Several findings support this notion. First, we find that neither Bax expression nor the integration of Bax into the mitochondrial membranes is influenced by c-Myc knockdown (Figs. 1 and 4). Second, c-Myc activation does not affect Bax expression or its subcellular localization (23, 24, 44). Third, when endogenous c-Myc is knocked down by 80–90%, Bax oligomerization is significantly reduced, and cytochrome c is not released (Figs. 3 and 5). Importantly, in this experimental circumstance, cytotoxic drug-induced apoptosis is blocked (Fig. 2).

It has been reported that etoposide or taxol-induced Bax activation, as detected by the conformation-specific 6A7 antibody, does not occur in c-Myc null Rat-1 fibroblasts, whereas reintroduction of c-Myc into the c-Myc null cells restores Bax...
activation, cytochrome c release, and apoptosis (23). Consistent with this report, our data support a role for c-Myc in activating Bax because knockdown of c-Myc significantly reduces Bax oligomerization following its integration into the mitochondrial membranes (Fig. 5). This novel experimental finding supports a model whereby Bax is an effector molecule cooperating with c-Myc during cytotoxic drug-induced apoptosis. However, since our co-immunoprecipitation studies did not detect any direct interaction between c-Myc and Bax, or between c-Myc and other Bcl-2 family members tested, including Bcl-XL, Bak, Bid, and Bad (data not shown), we suspect that it is unlikely that c-Myc directly activates Bax. Alternatively, although it is formally possible that c-Myc may indirectly regulate Bax activity through transcriptional modulation of a Bax activator that functions at the mitochondria to facilitate Bax oligomerization (Fig. 6), it is also possible that another as yet unidentified protein may be involved in such a process. Thus, further studies are required to identify such a potential c-Myc regulated activator of Bax.

In sum, this study has examined several key steps required for mitochondrial dysfunction and activation of the intrinsically apoptotic pathway. Our findings provide novel biochemical evidence that c-Myc and caspase-2 can functionally cooperate to promote drug-induced apoptosis by activating Bax at the level of the mitochondria. That is, caspase-2 is important for Bax integration into the outer mitochondrial membrane, and c-Myc is critical, but apparently acts indirectly, to facilitate Bax oligomerization. Furthermore, neither caspase-2 nor c-Myc is sufficient alone to fully activate Bax to initiate apoptosis. Thus, the blockade of these two rate-limiting steps, either by RNA interference or by small molecule inhibitor, may efficiently inhibit the apoptotic process and thus might have merit to suggest a novel therapeutic approach in diseases where apoptosis plays a pathological role.

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