Loss of MYC Confers Resistance to Doxorubicin-induced Apoptosis by Preventing the Activation of Multiple Serine Protease- and Caspase-mediated Pathways*

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The nuclear proto-oncogene c-MYC (hereafter called MYC) encodes a sequence-specific transcription factor, which plays an essential role in important cellular processes as different as proliferation, differentiation, apoptosis, and tumorigenesis (1). A role for MYC in regulating apoptosis was first demonstrated by studies in which overexpression of MYC under growth-limiting conditions resulted in cell death (2, 3). The dual capacity of MYC to stimulate cell proliferation in the presence of the appropriate survival factor(s) and to trigger apoptosis in their absence ensures that cell growth is strictly controlled by the microenvironment (3). Subsequent results have shown that MYC-induced apoptosis requires interaction at the cell surface between Fas and its ligand (FasL) and that MYC acts down-stream of the Fas receptor by sensitizing cells to the Fas death signal (4). Moreover, it has been shown that this sensitization step is represented by cytochrome c release (5). MYC has also been reported to sensitize cells to TNFα-induced apoptosis (6). Finally, deregulation or overexpression of MYC enhances the apoptotic response to different inducers, such as cytokine withdrawal, glucose deprivation, hypoxia, DNA damage, and cancer therapeutics (7).

Most data presented so far have dealt with the role of over-expressed MYC in inducing apoptosis. However, MYC also appears to have a role in regulating apoptosis when it is expressed at physiological levels. For instance, antisense oligonucleotides specifically blocking MYC expression resulted in a reduction of T-cell receptor activation-induced death (8). In addition, Sedivy and co-workers (9) recently reported that cells lacking MYC are resistant to cell death induced by etoposide. Moreover, they showed that the defect in DNA damage-initiated apoptosis in the G2 phase of the cell cycle was rescued by ectopic expression of Cyclin A, thus linking DNA damage-induced apoptosis and cell cycle progression (9). Somewhat contrasting results using the same cell line were reported by Soucie et al. (10). In fact, these investigators showed that in absence of MYC, cells could undergo cytochrome c-independent cell death when treated with etoposide and suggested that MYC regulates a mitochondrial amplification step (10). To clarify these contrasting results we decided to use the same cellular model to further investigate the role of MYC in drug-induced apoptosis.

In this study we have confirmed that the absence of MYC impairs the apoptotic response to etoposide and to another topoisomerase II inhibitor, doxorubicin. Moreover, we have shown that the absence of MYC does not affect camptothecin-induced apoptosis and that it has intermediary effects of the ability of taxol and staurosporine to induce apoptosis. Furthermore, we have investigated the molecular mechanisms involved in doxorubicin-induced apoptosis. Here, we report that together with the classical mitochondrial pathway, doxorubicin triggers two other pathways involved in executing cell death. Interestingly, we show that all these apoptotic pathways are impaired in MYC-deficient cells. Finally, we demonstrate that one of the pathways contributing to the cell death process induced by doxorubicin is represented by the activation of serine proteases, which occurs in parallel and upstream of caspase activation.

EXPERIMENTAL PROCEDURES

Reagents—Etoposide, doxorubicin, taxol, camptothecin, staurosporine, tosyl lysine chloromethyl ketone, pepstatin A, CA-074 Me,
MDL28170, and E64d were purchased from Calbiochem Pharmaceuticals. The Fas ligand kit was from Upstate Biotechnology, MFL4 was from Pharmingen.

Cell Culture and Stable Cell Lines—TGR-1 and HO15.19 Rat1 fibroblasts were a kind gift of J. Sedivy (Brown University, Providence, RI) and have been described previously (11). 7M3 is a representative clone of HO15.19 re-expressing Myc. TGR-1-BcXL is a representative clone of fluorescence-activated cell sorter (CHAPS, 3-[3-cholamidopropyl]dimethy lammonium)-1-propanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; wt, wild type; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone.

FIG. 1. Myc sensitizes cells to apoptosis induced by several different chemotherapeutic agents. Dose-response curves of TGR-1 (MYC wt) and HO15.19 (MYC null) cells treated with different chemotherapeutic drugs. Cells were incubated with increasing doses of the chemotherapeutic drugs for 72 h, and the level of apoptosis was assessed by sub-G1 content using FACS analysis. Values represent the mean ± SD of three experiments.

Lexington, KY); and anti-cleaved caspase-3 (Asp-175) (Cell Signaling Technology, Beverly, MA).

Immunofluorescence—Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. Permeabilization and staining with monoclonal anti-cytochrome c (clone 6H2.B4, Pharmingen) was performed according to Goldstein et al. (14). Cells were counterstained with DAPI.

Fluorogenic Assay for Caspase-3/7 Activity—Cell lysates were obtained as described for immunoblotting. 30 mg/ml proteins were used for fluorogenic assays as described (15). Briefly, lysates were incubated with the synthetic substrate ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-AMC, Alexis) (40 μM) in an assay buffer containing 100 mM Hepes-NaOH, pH 7.4, containing 10% sucrose, 0.1% Nonidet P-40, 1 mM EDTA, 5 mM dithiothreitol, supplemented with the protease inhibitors aprotonin and leupeptin (20 mg/ml each), pepstatin (1 mg/ml), and phenylmethylsulfonyl fluoride (1 mM). Cleavage of the fluorogenic substrate was monitored for 15 min by amido-4-methylcoumarin release in a fluorescence spectrophotometer (excitation/emission wavelengths 380/460 nm). Caspase activity was expressed as arbitrary units of fluorescence/min/mg of protein, and the values obtained at each time point were divided by the value of the relative untreated sample to obtain the fold increase.

RESULTS

Myc-deficient Cells Are Resistant to Topoisomerase II Inhibitors and Display Different Levels of Sensitivity toward Other Cytotoxic Drugs—To confirm previous reports in which Myc was shown to play a physiological role in chemotherapy-induced apoptosis (9, 10), Myc wild type (wt) (TGR-1) and MYC null (HO15.19) cells were incubated with increasing doses of a selection of chemotherapeutic drugs, and the level of apoptosis was assessed by FACS analysis at different time points between 24 and 72 h (Fig. 1 and data not shown). The drugs tested are currently employed in cancer therapy and are representative of different classes of compounds known to affect different targets.

The abbreviations used are: FADD, Fas-associated death domain; DN, dominant negative; PARP, poly(ADP-ribose)polymerase; crmA, cytotoxic response modifier A; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; CHAPS, 3-[3-cholamidopropyl]dimethy lammonium)-1-propanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; wt, wild type; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone.

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As evident from the data presented in Fig. 1 MYC null cells, despite showing a higher rate of spontaneous cell death than the parental cells in untreated cultures, were resistant to topoisomerase II inhibitors up to relative high concentrations. In fact, a 20-fold higher concentration of etoposide was needed to induce apoptosis to a similar extent in MYC null fibroblasts as compared with wt fibroblasts (Fig. 1A). The difference was even more striking in the case of doxorubicin where a 30-fold higher concentration of the drug killed 25% less MYC null cells as compared with wt cells (Fig. 1B). Loss of Myc expression also partially impaired the apoptotic response toward taxol and staurosporine (Fig. 1, C and D), though to a different extent. As reported previously (9), MYC null cells are not resistant to all apoptotic stimuli, because a normal apoptotic response was triggered when HO15.19 cells were treated with camptothecin (Fig. 1E).

To study in more detail the mechanism by which MYC null cells are resistant to drug-induced apoptosis, we decided to use doxorubicin as a model drug, because even at very low doses it is very active toward wt cells but completely ineffective toward MYC null cells.

**Myc Status Determines the Resistance to Apoptosis of HO15.19 Cells**—The apoptotic defect in HO15.19 cells was revealed using drugs whose apoptotic effect is known to be dependent, completely or in part, on the presence of wild type p53 (reviewed in Ref. 16). Previous data have suggested (9) that TGR-1 and HO15.19 cells contain wild type p53 and that the induction of p53 in HO15.19 is partly impaired upon treatment with etoposide and cisplatin. To understand whether the treatment of TGR-1 and HO15.19 cells with doxorubicin results in stabilization of p53, we treated the cells with 0.01 µg/ml doxorubicin for 24 h and determined the level of p53 by Western blotting. As shown in Fig. 2A, the treatment of the cells resulted in a robust accumulation of p53 with no differences observed between the MYC null and wt cell lines. Moreover, the accumulation of Bax, a classical p53 target involved in apoptosis (17), following doxorubicin treatment strongly suggests that functional p53 is induced in the two cell lines (Fig. 2B). Our data therefore suggest that, at least for some kinds of drugs, p53 induction alone is not sufficient but that a concomitant presence of Myc is necessary to induce apoptosis after DNA damage. Moreover, the apoptotic defect was specifically due to the loss of Myc and not to secondary mutations accumulated as a consequence of Myc absence, because re-expression of Myc in HO15.19 cells rescued doxorubicin-induced apoptosis both in short term (Fig. 2C) and in long term (Fig. 2D) assays.

**Lack of Effector-Caspase Activation in myc Null Cells**—The signaling cascades triggered once the apoptotic stimulus is recognized as such (induction phase) can be largely modulated, mainly by the Bcl2 family of apoptotic regulators (decision phase), before the apoptotic program is executed via activation of the caspases (execution phase). Because we observed that the Bcl2 family member Bax is induced in both TGR-1 and HO15.19 cells, we concluded that the induction phase and at least part of the decision phase are not impaired even in absence of functional Myc. To assess whether the apoptotic process is blocked at the level of the execution phase in MYC null cells, we monitored the integrity of poly(ADP-ribose)polymerase (PARP) as a marker of caspase activation (Fig. 2E). The cleavage product of PARP is evident upon doxorubicin treatment of TGR-1 and 7M3 cells but is absent in HO15.19 cells, indicating that Myc is required to activate the execution program in response to doxorubicin treatment. Defective activation of caspases however, is not a generic defect of MYC null cells, because PARP is normally cleaved when HO15.19 cells are exposed to camptothecin.

**FIG. 2.** Myc status determines the resistance to apoptosis of HO15.19 cells and correlates with a lack of activation of effector caspases. A, induction of p53 in MYC wt and null cells upon treatment with doxorubicin. Cell lysates were prepared at the indicated times after treatment with doxorubicin, and Western blots were probed with antibodies to the indicated proteins. Vinculin served as a loading control. B, Bax is induced in both MYC wt as well in MYC null cells 24 h after doxorubicin addition. Western blots were probed with the indicated antibodies. Actin served a loading control. C, Myc re-expression rescues apoptosis in HO15.19 cells. Pictures of TGR-1, HO15.19, and MYC-infected HO15.19 (7M3) cells treated (Doxo) or untreated (Cnt) with 0.01 µg/ml doxorubicin for 72 h. Colony assay of TGR-1, HO15.19, and 7M3 cells untreated (Cnt) or treated (Doxo) with 0.01 µg/ml doxorubicin. The experiment was performed as described under “Experimental Procedures.” D, lack of PARP cleavage in Myc-deficient cells following doxorubicin treatment. Anti-PARP immunoblot of TGR-1, HO15.19, and 7M3 cell lysates obtained at different time points after treatment with doxorubicin (Doxo, top part) and camptothecin (Cpt, bottom part). An arrow indicates cleaved PARP. Vinculin served as a loading control.

*The Activation of the Fas (CD95) Receptor Is Not Required for Doxorubicin-induced Apoptosis*—Chemotherapeutic drugs have been reported to activate effector caspases either via death receptor-mediated activation of caspase-8 (extrinsic pathway) or via mitochondria-mediated activation of caspase-9
To understand whether the resistance to doxorubicin-induced apoptosis in MYC null cells is because of a defective activation of Fas/FasL, we incubated TGR-1 and HO15.19 cells with 25 ng/ml of purified FasL. Activation of the Fas receptor is not required for doxorubicin-induced apoptosis. The percentage of cell death of TGR-1 cells exposed to 0.01 μg/ml doxorubicin (Doxo) in the presence or absence of 30 μg/ml MFL4, an antagonistic antibody able to block the activation of Fas. As a control, TGR-1 cells treated with 25 ng/ml purified FasL in the presence or absence of 30 μg/ml MFL4 were analyzed. Values represent the mean ± S.D. of three independent experiments.

Anticancer drugs may trigger apoptosis also by acting directly on different components of the Fas signaling pathway, e.g. by triggering a Fas ligand-independent, FADD-mediated activation of the Fas death pathway (19). Moreover, anticancer drugs can directly activate caspase-8, the upstream caspase in the Fas pathway, in a Fas-independent manner (20). To assess the involvement of FADD or caspase-8 in doxorubicin-induced cell death, TGR-1 cells were infected with retroviruses expressing dominant negative (DN) versions of human caspase-8 or human FADD. The “empty” retrovirus (Pinco) was used as control. Forty-eight hours after infection the cells were treated with 0.01 μg/ml doxorubicin or 25 ng/ml recombinant FasL. Seventy-two hours after drug treatment floating cells were pooled with those harvested by trypsinization, and DNA content was evaluated by FACS analysis. Apoptosis was evaluated only in GFP-positive gated cells. Values represent the mean ± S.D. of three independent experiments.
Subsequently, we sought to determine whether the mitochondrial pathway mediates doxorubicin-induced apoptosis in TGR-1 cells (Fig. 3D). Cytochrome c release was studied by immunofluorescence in cells treated with doxorubicin or, as a positive control, with camptothecin. In healthy cells cytochrome c is mitochondrial as indicated by the typical punctuate distribution of the staining in all cell lines (Fig. 3D, a, c, and e). Following doxorubicin treatment of TGR-1 and 7M3 cells, the staining becomes intense and diffuse, which is consistent with a cytoplasmic distribution of cytochrome c released from mitochondria, (Fig. 3D, g and k). This staining pattern was not observed in HO15.19 cells (Fig. 3D, i) indicating that the block in the apoptotic machinery resides upstream of the mitochondria. Once again, the defect is specifically related to the inability of MYC null cells to die in response to doxorubicin, because cytochrome c is released as result of camptothecin treatment (Fig. 3D, o).

**Overexpression of BclXL Is Not Sufficient to Inhibit Doxorubicin-induced Apoptosis**—To understand whether the inability to activate the mitochondrial pathway could account for the lack of drug-induced apoptosis in MYC null cells, we tested whether an exogenously applied mitochondrial block would be sufficient to abolish doxorubicin-induced apoptosis. To this end, we infected TGR-1 cells with a retrovirus overexpressing human BclXL, a member of the Bcl2 family, which blocks the release of cytochrome c from the mitochondria (21). Several clones were obtained that overexpressed BclXL, and when we tested BclXL expression we discovered that compared with TGR-1 HO15.19 cells expressed very high levels of endogenous BclXL, similar to those of the overexpressing clones. We found that the high levels of BclXL are a consequence of the absence of Myc, because re-expression of Myc in 7M3 cells restored BclXL expression to normal levels (Fig. 4A). The finding that HO15.19 cells express high levels of BclXL could account for the inability of these cells to release cytochrome c upon doxorubicin stimulation.

We then tested several clones for their biological response to doxorubicin, and to our surprise none of the tested clones was protected from apoptosis, despite expressing high levels of BclXL (Fig. 4B and data not shown). A possible explanation for the lack of protection could be that the overexpressed BclXL is mislocalized and/or non-functional. However, immunofluorescence studies showed that BclXL was correctly localized at the mitochondria, because in untreated cells the signal from BclXL colocalized with that from cytochrome c (Fig. 4C, f). Moreover, we observed that overexpressed BclXL is functional, because it blocks cytochrome c release after doxorubicin exposure (Fig. 4D, compare d with h). Despite that, BclXL is not able to prevent apoptosis as evident from the typical morphological features such as condensation and fragmentation of the nuclei (Fig. 4D, c and g). However, a modest but reproducible delay in the onset of the apoptotic process was observed in all of the experiments performed with BclXL overexpressing clones (data not shown).
Inhibition of Caspase-3 Activation Does Not Protect TGR-1 Cells from Doxorubicin-induced Apoptosis—As shown in Fig. 5A, we observed cleaved, and therefore active, caspase-3 in the presence of a mitochondrial block obtained by BclXL overexpression. We therefore sought to determine whether caspase-3 is the pivotal activator/effector of the alternative pathway triggered by doxorubicin. To this end we infected both TGR-1 and TGR-1-BclXL with a XIAP point mutant (W310A) that does not interact with caspase-9 and only inhibits caspase-3/7 (22). As shown in Fig. 5B, the W310A mutant suppressed most of the caspase-3/7 activity elicited by doxorubicin treatment in both TGR-1 and in TGR-1-BclXL. Unexpectedly, W310A overexpression, although being able to slightly delay the onset of cell death in TGR-1-BclXL, did not prevent the cumulative number of cell deaths (Fig. 5C). A later Smac- and/or HtrA2/Omi-mediated relief of caspase inhibition, following the release of these proteins from activated mitochondria, might explain the finding that W310A overexpression is not able to suppress cell death in TGR-1 cells. In agreement with such a notion, we detected a residual caspase-3/7 activity in cells expressing W310A (Fig. 5A). A later Smac- and/or HtrA2/Omi release should be blocked as a consequence of mitochondrial protection afforded by BclXL overexpression. These observations led us to search for additional and non-conventional mechanisms of caspase-3 activation triggered by doxorubicin.

Serine Protease Activation Occurs prior to Early Caspase Activation in Doxorubicin-induced Apoptosis—Recent studies (reviewed in Refs. 23 and 24) have indicated that proteases other than caspases (including cathepsins, calpains, and serine proteases) also have roles in mediating and promoting apoptosis. In fact, their inhibition completely, or in some cases partially, prevents cell death induced by different stimuli. Moreover, depending on the cell type and on the inducer non-caspase proteases can be activated either upstream or downstream of caspases. To investigate whether non-caspase proteases could play a role in activating caspase-3 and/or executing cell death upon doxorubicin treatment, we induced apoptosis in presence of a mitochondrial block obtained by BclXL overexpression. These observations led us to search for additional and non-conventional mechanisms of caspase-3 activation triggered by doxorubicin.

These findings suggest that the mitochondrial pathway is not the only executor of doxorubicin-induced apoptosis in TGR-1 cells and that an alternative pathway is activated upstream or concomitantly when cells are exposed to the drug. A corollary emerging directly from these observations is that this second pathway is also under the control of Myc, because the mitochondrial block described in MYC null cells cannot account solely for the resistant phenotype.
completely blocked, when pre-incubating TGR-1 cells with 100 μM tosyl lysine chloromethyl ketone (trypsin-like serine protease inhibitor) for 1 h before the addition of 0.01 μg/ml doxorubicin (Doxo) for the indicated amount of time, and caspase-3 activation was measured by Western blotting. Anti-cytochrome c staining of TGR-1-GFP (a) and TGR-1-crmA (c) cells treated with doxorubicin for 30 h. DAPI counterstaining is also shown (b, d). C, a combination of serine protease inhibitors, the blocking of caspase-3/7 and mitochondrial activation is required to protect against doxorubicin-induced apoptosis. Pictures of TGR-1 cells expressing the indicated proteins treated with 0.01 μg/ml doxorubicin for 72 h. D, colony assay of TGR-1 cells expressing the indicated proteins and HO15.19 cells. Cells were seeded at low density after a 24-h treatment with 0.01 μg/ml doxorubicin, as indicated under “Experimental Procedures.”

In this paper we confirm and extend previous observations that some classes of anticancer drugs induce an apoptotic response that is dependent on Myc. Moreover, through a detailed analysis of doxorubicin-induced apoptosis we demonstrate that together with the classical mitochondrial pathway two other pathways are concomitantly triggered to execute cell death, all of which are impaired in Myc deficient cells. Finally, we demonstrate that the activation of serine proteases occurs in parallel with and upstream of caspase activation and significantly contributes to doxorubicin-induced apoptosis.

The first significant finding is that the apoptotic response to
some classes of anticancer drugs is dependent on Myc. Previous results (9) have shown that Myc is required for etoposide-induced apoptosis. We tested another inhibitor of the same class, namely doxorubicin, because etoposide and doxorubicin are structurally unrelated molecules that in addition to topoisomerase II also have other targets (26). We also tested a broad range of other anticancer drugs and found that the absence of Myc greatly reduces apoptosis induced by taxol, partially impairs staurosporine-induced death, but is irrelevant for camptothecin-induced apoptosis demonstrating a differential requirement for Myc in the induction of apoptosis. Our data are in apparent contrast to results reported by another group (10), who did not observe the resistance of MYC null cells to etoposide, doxorubicin, and taxol at any tested concentration. A main difference in the experimental approach may account for this as we, for instance, used lower concentrations of the tested drugs. Remarkably, the highest differences in apoptotic response between MYC wt and null cells are evident for the lowest concentrations of each drug. Accordingly, at the highest concentration tested a significant amount of cell death occurs even in absence of Myc.

The most significant finding presented in this paper is the identification of the diverse Myc-dependent apoptotic pathways triggered by doxorubicin. It is well accepted that chemotherapeutic drugs induce cell death by triggering either the extrinsic or intrinsic pathway of caspase activation (18). The extrinsic pathway is initiated by death receptor-mediated activation of caspase-8, which subsequently activates caspase-3, the main effector caspase. Mitochondrial activation is not required for the extrinsic pathway, and when it occurs it represents a positive feedback loop that amplifies the extent of caspase activation (27). Therefore Bcl2 or BclXL overexpression usually does not protect from apoptosis triggered through the extrinsic pathway. In several models, signaling through Fas/FasL has been shown to be involved in doxorubicin-induced apoptosis (28), for example via up-regulation at the transcriptional level of the FasL (27). Moreover, expression of FasL in activated T-cells is regulated by Myc (29) through a “non-canonical” DNA binding element (30), and Myc null B cells derived from conditional knock-out mice express low levels of Fas and FasL following activation (31). Despite the fact that these results suggest a role for Myc in the regulation of drug-induced apoptosis mediated by Fas/FasL we could not demonstrate a role for these proteins in doxorubicin-induced apoptosis (Fig. 3). Indeed, neither the block of the receptor nor the interruption of the effectors downstream of the receptor were effective in reducing doxorubicin-induced apoptosis.

The intrinsic or mitochondrial pathway is triggered through the action of pro-apoptotic members of the Bcl2 family, which regulate the mitochondrial release of cytochrome c. This release allows apoptosome formation and caspase-9 activation resulting in caspase-3 activation and apoptosis. Overexpression of anti-apoptotic members of the Bcl2 family blocks the mitochondrial pathway at the level of cytochrome c release and therefore protects cells from apoptosis (18). We found that in absence of Myc the mitochondrial pathway is not activated upon doxorubicin treatment as measured by lack of cytochrome c release. Interestingly, the lack of mitochondrial activation may be because of an increase in the endogenous levels of BclXL in MYC null cells. This increase appears to be a direct consequence of Myc loss, because its re-expression re-establishes the normal levels of BclXL in the fibroblasts. This finding is in agreement with a previously reported role of Myc in repressing BclXL levels (32). Myc-mediated suppression of BclXL is also consistent with the concept that Myc sensitizes cells to apoptosis through the regulation of cytochrome c release (5). However, the lack of Myc-mediated mitochondrial activation is not sufficient to account for the doxorubicin-resistance of MYC null cells, because BclXL overexpression does not prevent doxorubicin-induced apoptosis.

To identify other mechanisms involved in doxorubicin-induced apoptosis we searched for “atypical” pathways of caspase activation. Reviewing the literature we noticed several examples in which caspase-3 is activated independently of the mitochondrial pathway (33, 34). Moreover, caspase-3 can be directly activated by non-caspase enzymes like the calcium-dependent enzyme calpain, lysosomal enzymes (35), or enzymes contained in the granules of cytotoxic cells like granzymes and perforins (36, 37). Often caspase-3 activation represents the crucial effector in which inhibition blocks apoptosis (38). Interestingly we found that caspase-3 was in part activated independently of the mitochondria suggesting that besides the classical intrinsic pathway, doxorubicin can also activate caspases independently of the mitochondria. Moreover, this pathway is dependent on Myc also, because caspase-3 (Fig. 6A) is not activated in doxorubicin-treated MYC null cells. Finally, the finding that doxorubicin-induced cell death was not blocked by caspase-3 inhibition in BclXL-overexpressing MYC wt cells (Fig. 5C) suggests that a third caspase-3-independent, but Myc-dependent, pathway is activated by doxorubicin. Although such a mechanism is not commonly used, several examples have been reported in which apoptosis can occur in absence of caspase-3 activation (reviewed by Ref. 38). Caspase-3-independent apoptosis may involve other caspases. Alternatively, or in addition, another class of proteases, namely serine proteases, shown to be involved in some forms of apoptosis (24) may contribute to caspase-3 activation. Significant to this study, it has been shown previously (39) that apoptosis triggered in rat fibroblasts by Myc overexpression can be inhibited by the use of a serine protease inhibitor (39). In our study, the inhibition of serine proteases allowed a complete suppression of doxorubicin-induced apoptosis only if it was concomitant with mitochondrial and caspase-3 inhibition (Fig. 6, C and D) suggesting that activation of serine proteases represents the third Myc-dependent pathway triggered by doxorubicin. In agreement with this suggestion are recent data demonstrating that the levels of two serine protease inhibitors are increased in MYC null cells (40).
three different but linked Myc-dependent protease pathways (Fig. 7). Together with the classical mitochondrial pathway a serine protease-mediated pathway participates in early caspase-3 activation. However, activation of the mitochondrial pathway is independent of serine protease activity, because cytochrome c release occurs in the presence of serine protease inhibition. Moreover, another pathway is likely to converge on caspase-3 activation, because a XIAP mutant specific for caspase-3 is required together with the inhibition of serine proteases and BclXL to block apoptosis. According to recent data (33) a possible candidate for this additional pathway could be caspase-12. Our results also show that the serine proteases have targets other than caspase-3, because their inhibition is required together with caspase-3 inhibition to protect against cell death. In support of other targets of serine proteases are recent mutational analysis data of the serine protease HtrA2/Omi showing that both XIAP binding and the catalytic activity of HtrA2/Omi contribute to its pro-apoptotic activity (41). Interestingly, HtrA2/Omi was recently reported (42) to be a p53-sensitive protein showing that both XIAP binding and the catalytic activity of HtrA2/Omi to test this hypothesis. Because MYC-dependent Activation of Serine Proteases and Caspases

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