Published in final edited form as:
Biochem Pharmacol. 2012 January 15; 83(2): 199–206. doi:10.1016/j.bcp.2011.10.008.

Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis by phosphorylation of Mcl-1, promoting its degradation and freeing Bak from sequestration

Rong Chu, **David T. Terrano**, and **Timothy C. Chambers***
Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205

Abstract

Mcl-1 is one of the major anti-apoptotic members of the Bcl-2 family of apoptotic regulatory proteins. In this study we investigated the role of Mcl-1 in mitotic arrest-induced apoptosis. Vinblastine treatment of KB-3 cells initially resulted in a phosphatase-sensitive mobility shift in Mcl-1 and then subsequent loss of Mcl-1 protein expression which was prevented by MG132, suggesting that phosphorylation triggered proteosome-mediated degradation. Mcl-1 phosphorylation/degradation was a specific response to microtubule inhibition and did not occur in response to lethal concentrations of DNA damaging agents. Vinblastine treatment caused degradation of Mcl-1 in cells in which apoptosis was blocked by Bcl-xL overexpression, indicating that Mcl-1 degradation was not a consequence of apoptosis. A partial reversible phosphorylation of Mcl-1 was observed in synchronized cells traversing mitosis, whereas more extensive phosphorylation and subsequent degradation of Mcl-1 was observed if synchronized cells were treated with vinblastine. Mcl-1 phosphorylation closely paralleled cyclin B expression, and specific cyclin-dependent kinase (Cdk) inhibitors blocked vinblastine-induced Mcl-1 phosphorylation, its subsequent degradation, and improved cell viability after mitotic arrest. Co-immunoprecipitation studies indicated that Mcl-1 was complexed with Bak, but not Bax or Noxa, in untreated cells, and that Bak became activated in concert with loss of Mcl-1 expression. These results suggest that Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis via Mcl-1 phosphorylation, promoting its degradation and subsequently releasing Bak from sequestration.

Keywords

Mcl-1; Cdk1; microtubule inhibitors; apoptosis; Bcl-2 proteins

1. Introduction

Together with Bcl-xL and Bcl-2, Mcl-1 is one of the major anti-apoptotic members of the Bcl-2 family (Reviewed in 1, 2). It differs from Bcl-xL and Bcl-2 in that it does not contain a well-defined BH4 domain, and also in contrast to other anti-apoptotic Bcl-2 proteins, its

*To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Mail Slot 516, 4301 W. Markham St., Little Rock, AR 72205, USA. Tel: 501 686 5755; Fax: 501 686 8169; chamberstimothyc@uams.edu.

expression is subject to rapid alterations in response to external stimuli. Mcl-1 expression is typically induced in response to proliferation, differentiating, or survival signals, and the key role of Mcl-1 in supporting cell survival is highlighted in experiments with hematopoietic stem cells, which fail to differentiate and do not survive if Mcl-1 is conditionally knocked-out (3). Conversely, down-regulation of Mcl-1 is often an early event in cells subjected to apoptotic stimuli, and high levels of expression of Mcl-1 are observed in multiple myeloma and other cancers, and this correlates with enhanced survival, drug resistance, and poor prognosis (1, 2). Mcl-1 is primarily localized to the outer mitochondrial membrane where it suppresses the release of cytochrome c from the mitochondria by binding and neutralizing pro-apoptotic Bcl-2 family members including Bak, tBid, Puma and Bim (4-8).

Post-translational modifications of Mcl-1 are largely responsible for the rapid turnover occurring in response to specific stimuli, and Mcl-1 is subject to fairly complex regulation involving different kinases including JNK, ERK, p38 and GSK-3β (2, 3). For example, phosphorylation of Mcl-1 at T92 and T163 mediated by ERK prolongs the half-life of Mcl-1 protein (9, 10), while phosphorylation of Mcl-1 at S159 mediated by GSK-3β promotes Mcl-1 ubiquitination and degradation (11). Mcl-1 is also phosphorylated during mitosis at Ser64 and this enhances Mcl-1 stability and its anti-apoptotic function (12).

Because Mcl-1 expression is a key regulator of cell survival and cell death, it was of interest to examine Mcl-1 in the context of mitotic arrest-induced apoptosis. We show that in synchronized KB-3 cells traversing mitosis, Mcl-1 is partially and transiently phosphorylated, whereas when treated with microtubule inhibitors, Mcl-1 phosphorylation is more robust and sustained and results in Mcl-1 degradation. Selective inhibition of Cdk1 during mitotic arrest inhibits Mcl-1 phosphorylation and in turn inhibits Mcl-1 degradation. Furthermore, we show that Cdk1-mediated phosphorylation and subsequent degradation of Mcl-1 leads to the release of bound Bak and subsequent Bak activation. These results provide novel insight into the signaling mechanisms that regulate mitotic arrest-induced cell death. A preliminary report of these findings has been published (13).

2. Materials and methods

2.1 Materials

Antibodies to Mcl-1 (catalog # sc-12756) and cyclin B1 (catalog # sc-245) and Protein A/G PLUS-Agarose beads were purchased from Santa Cruz (Santa Cruz, CA); antibodies to GAPDH (catalog # 2118), Bcl-xL (catalog # 2762), phospho-H3 histone (Ser10) (catalog # 97015), and Bax (catalog # 2772) were obtained from Cell Signaling Technology (Beverly, MA); antibody to poly(ADP-ribose) polymerase (PARP) (catalog # 556362) was purchased from Pharmingen (Franklin Lakes, NJ); phospho-H1 histone antibody (catalog # 06-597) was from Upstate Biotechnology (Waltham, MA); antibody to Noxa (catalog # IMG-349A) was from Imgenex (San Diego, CA); antibody to Bak (catalog # AM04) was from Calbiochem (Gibbstown, NJ); and antibody to active Bak (NT) (catalog # 06-536) was from Millipore (Billerica, MA). Thymidine and MG132 were purchased from EMD Biosciences (Gibbstown, NJ). Aminopurvalanol A and RO3306 were purchased from Axxora (San Diego, CA) and ZM447439 was from Tocris Bioscience (Ellisville, MO). Lambda protein phosphatase was obtained from New England BioLabs (Ipswich, MA). Vinblastine, vincristine, Taxol™, doxorubicin, and VP-16, and all other chemicals not specifically indicated, were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and synchronization

The KB-3 human carcinoma cell line was maintained in monolayer culture at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2

mM L-glutamine, 50 U/ml penicillin, and 50 g/ml streptomycin. KB-3 cells were synchronized by a double thymidine block method as described previously (14). Briefly, cells (10^6) in a 100-mm dish were incubated in medium containing 2 mM thymidine for 16 h, released into normal medium for 9 h, and then incubated for 16 h in medium containing 2 mM thymidine. KB-3 cells stably overexpressing HA-tagged Bcl-xL were described previously (15).

2.3. Preparation of cell extracts and immunoblotting

Whole cell extracts were prepared by suspending cells in 0.25 ml of lysis buffer (25 mM HEPES [pH 7.4], 0.3 M NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM EDTA, EDTA-free complete protease inhibitor tablets [Roche], 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μM okadaic acid). The suspension was incubated for 45 min on ice with occasional mixing; insoluble material was removed by centrifugation (15 min at 12,000 × *g*), and protein concentration in the supernatant was determined using the BioRad protein assay. For lambda phosphatase treatment, cell extracts containing 0.1 mg protein were incubated with 400 U lambda phosphatase for 30 min at 30°C. Immunoblotting was performed using 60 μg protein/lane and apparent molecular weights were as follows: Mcl-1, 40 kDa; GAPDH, 38 kDa; intact PARP, 116 kDa; cleaved PARP, 85 kDa; Bcl-xL, 30 kDa; cyclin B, 50 kDa; phospho-H1, 32 kDa; phospho-H3, 17 kDa; Bak, 30 kDa; Bax, 23 kDa; and Noxa, 11 kDa.

2.4. Immunoprecipitation

KB-3 cells were lysed in 0.5 ml of lysis buffer (40 mM HEPES [pH 7.4], 120 mM NaCl, 1% CHAPS, 1 mM EDTA, supplemented with protease and phosphatase inhibitors (EDTA-free complete protease inhibitor tablets [Roche], 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μM okadaic acid]) by incubating on ice for 45 min and centrifugation at 12,000 × g for 15 min. The extract (1 mg) was precleared with 20 μl agarose beads, according to the manufacturers' directions (Santa Cruz), and to the supernatant was added 5 μg of rabbit polyclonal antibody to Mcl-1 or Bak. After mixing for 3 h at 4°C, the lysates were then incubated with 50 μl of protein A/G PLUS-Agarose beads for 1 h at 4°C. The beads were pelleted by centrifugation at 1,000 × g for 5 min and washed three times with 0.2 ml of lysis buffer. The beads were resuspended in 100 μl of 2 × SDS loading buffer and incubated for 1 h at 37°C. The immunoprecipitated samples were resolved by 12.5% acrylamide SDS-PAGE gels (Bio-Rad) and analyzed by immunoblotting.

2.5 Other methods

Bak activation was assessed as described previously (16). Briefly, cells were lysed in buffer containing 1% CHAPS as described above, extracts subjected to immunoprecipitation using an antibody (NT) which recognizes the conformationally active form of Bak, and immunoprecipitates analyzed for the presence of Bak by immunoblotting. Apoptosis assays were conducted as described previously (15) using a kit from Roche Applied Science which quantitatively measures soluble oligonucleosomes generated via DNA fragmentation during the apoptotic process.

3. Results

3.1 Microtubule inhibitors induce Mcl-1 phosphorylation and degradation

Immunoblotting of KB-3 cell extracts with sc-12756 antibody against Mcl-1, as described in Materials and methods, revealed the presence of a major band at 40 kDa (see Figs. 1-6),

corresponding to the full-length anti-apoptotic form, and the shortened form was not detected (data not shown). KB-3 cells were treated with 30 nM vinblastine for periods up to 48 h, and Mcl-1 expression monitored by immunoblotting. Mcl-1 underwent several changes, including an initial increase in expression, then a pronounced mobility shift, then loss of expression (Fig. 1A). The mobility shifted form of Mcl-1 reverted to the unshifted form if cell lysates were treated with lambda phosphatase, showing that the decreased mobility induced by vinblastine was due to phosphorylation (Fig. 1B). When cells were co-treated with vinblastine and the proteosome inhibitor MG132, Mcl-1 expression was maintained and increased (Fig. 1C), suggesting that loss was due to proteosome-mediated degradation. Mcl-1 expression was also increased in cells treated with MG132 only (Fig. 1C), consistent with reports showing that it is normally subject to rapid turnover via proteosome-mediated degradation in unstimulated cells (3). Microtubule inhibitors including vinblastine, vincristine and paclitaxel all induced phosphorylation and degradation of Mcl-1 with a similar time course, whereas the DNA damaging agents doxorubicin and VP-16 did not (Fig. 2). Note that all five drugs induced apoptosis under these conditions, as indicated by PARP cleavage (Fig. 2).

3.2 Mcl-1 degradation is not a consequence of apoptosis

Our previous studies have shown that when KB-3 cells are treated with 30 nM vinblastine, apoptosis begins at around 28 h post-treatment, as indicated by PARP cleavage and cytochrome c release (17). Mcl-1 degradation occurs by 24 h post-treatment (Fig. 1, 2) and is therefore likely a pre-apoptotic event. To confirm this, we used KB-3 cells stably overexpressing Bcl-xL (KB3-Bcl-xL) (Fig. 3A). Whereas 48 h vinblastine treatment of KB-3 cells strongly induced apoptosis, KB3-Bcl-xL cells were markedly vinblastine resistant, exhibiting a much lower level of apoptosis (Fig. 3B). However, under these same conditions, Mcl-1 phosphorylation and degradation occurred to a similar extent and with similar kinetics in both KB-3 and KB3-Bcl-xL cell lines (Fig. 3C). These data demonstrate that Mcl-1 degradation is not a consequence of apoptosis.

3.3 Mcl-1 undergoes partial and transient phosphorylation during traverse of mitosis

Because Mcl-1 was phosphorylated in response to vinblastine treatment which induces mitotic arrest (17), it was important to determine if Mcl-1 was also phosphorylated during normal mitosis. For this purpose, KB-3 cells were synchronized at the G1/S boundary using double thymidine block. Previous studies using propidium iodide staining and flow cytometry, as well as immunoblotting for several cyclins, indicated that following release from the block, cells progress through S, G2, M, and back into G1 phase, in a highly synchronized fashion (14). Different flasks of KB-3 cells were released from the block into medium containing either vehicle or vinblastine, and harvested at defined time points thereafter. During the 9-12 h period post-release, which comprised late G2 phase into mitosis and the beginning of the next G1 phase (14), in the control set, Mcl-1 underwent partial and transient phosphorylation (Fig. 4). This occurred in close parallel with cyclin B expression which increased and decreased in the predicted cyclic pattern (Fig. 4). When released in the presence of vinblastine, Mcl-1 phosphorylation was initiated as cells entered late G2 phase and mitosis at 9-10 h post-release, and, at later time points in the presence of drug, phosphorylation was sustained and resulted in loss of Mcl-1 expression (Fig. 4), as observed in asynchronous cells (Fig. 1A). This occurred in concert with sustained high levels of cyclin B expression (Fig. 4).

3.4 Cdk inhibitors inhibit Mcl-1 phosphorylation and degradation and improve cell viability during mitotic arrest

The profile of Mcl-1 phosphorylation described above, with partial and transient phosphorylation during mitosis, versus complete and sustained phosphorylation in response

to mitotic arrest, strongly correlated with cyclin B expression, suggested a possible role for Cdk1/cyclin B. In order to test this, Cdk inhibitors were used. However, there is a critical issue that must be considered in designing experiments of this type, because Cdk inhibitors prevent cell cycle progression, and adding them to cell cultures prior to or with vinblastine could indirectly inhibit Mcl-1 phosphorylation by blocking the ability of cells to reach M phase. To preclude this possibility, KB-3 cells were synchronized by double thymidine block, treated with vinblastine one hour after release into S phase, and then as cells approached M phase, were treated with Cdk inhibitors. This strategy, depicted in Fig. 5A, allowed timely use of Cdk inhibitors at M phase while permitting the cells to progress to M phase in their absence, and is a critical and necessary feature of the experimental design. We chose two specific small molecule Cdk inhibitors, purvalanol A and RO3306 (18, 19). While both compounds are highly selective for Cdks versus other kinases (18, 19), they inhibit Cdks in general. However, Cdk1/cyclin B is the only Cdk active during M phase, a prediction that we have demonstrated experimentally in KB-3 cells (20), and therefore under the conditions of this experiment (Fig. 5A), purvalanol A and RO3306 can be considered highly selective for Cdk1/cyclin B. Comparison of lanes 1 and 5 in Fig. 5B shows that vinblastine induced Mcl-1 phosphorylation during the interval from 11 h to 13 h post-release as expected. In the presence of Cdk inhibitors during this interval, Mcl-1 phosphorylation was strongly inhibited, with Mcl-1 failing to undergo the distinct phosphorylation-dependent mobility shift seen with vehicle. Furthermore, purvalanol A and RO3306 strongly inhibited phosphorylation of the known Cdk1 substrate H1 histone under the same conditions (Fig. 5B). Note that during this interval, H3 histone phosphorylation levels were maintained (Fig. 5B), indicating that the reduced phosphorylation of Mcl-1 in the presence of Cdk inhibitors was not a result of mitotic slippage which is accompanied by phosphatase activation and H3 dephosphorylation (20). As a further test for specificity, we employed ZM477439, an inhibitor of Aurora kinases, which are also active during mitosis and mitotic arrest. As shown in Fig. 5C, vinblastine-induced Mcl-1 phosphorylation occurred normally in the presence of ZM477439, whereas phosphorylation of histone H3 at Ser10, a known target site of Aurora kinases, was strongly inhibited, demonstrating the effectiveness of the inhibitor under these conditions. The results presented in Fig. 5A-C provide strong and properly controlled experimental evidence implicating Cdk1/cyclin B in vinblastine-induced Mcl-1 phosphorylation.

If phosphorylation of Mcl-1 triggers its degradation, Cdk inhibitors would be expected to not only inhibit phosphorylation but also protect Mcl-1 from degradation. To test this, KB-3 cells were synchronized by double thymidine block, treated with vinblastine one hour after release, and then at 11 h post-release, when Mcl-1 phosphorylation was initiated, cells were treated with either DMSO or the Cdk inhibitors, and then harvested at 17 h post-release. As shown in Fig. 5D, in synchronized cells treated with vinblastine and then treated with vehicle for the 6 h period between 11 and 17 h post-release, Mcl-1 was largely degraded, as observed earlier (Fig. 4). However, when treated with either of two Cdk inhibitors, purvalanol A or RO3066, during the same 6 h period, Mcl-1 was largely protected from degradation and, consistent with Fig. 5B, migrated in the unshifted, unphosphorylated form (Fig. 5D). Based on these results, it would be predicted that inhibition of Cdk1 in this context would promote cell survival via maintenance of Mcl-1 expression. To test this hypothesis, synchronized KB-3 cells were treated with vinblastine, and then treated at 11 h post-release with vehicle or the Cdk inhibitors. Cells were followed microscopically, and it was quite apparent that cells exposed to the inhibitors were protected. Thus, a greater proportion was adherent and morphologically normal, versus those exposed to vehicle which were predominantly rounded and detached. Representative results, from triplicate plates of cells quantifying adherent versus non-adherent cells for each condition, are shown in Fig. 5E. Consistent with these findings, viability was significantly increased in cells co-treated with the Cdk inhibitors, as determined by trypan blue exclusion (Fig. 5F).

3.5 Functional role for Mcl-1 in sequestration of Bak

In order to determine the mechanism whereby phosphorylation and degradation of Mcl-1 promotes cell death induced by microtubule inhibitors, we sought to identify key binding partners of Mcl-1 by co-immunoprecipitation. Mcl-1 was immunoprecipitated from untreated or vinblastine-treated KB-3 cells and samples probed by immunoblotting for Mcl-1 and several pro-apoptotic Bcl-2 proteins implicated in binding Mcl-1, including Bak, Bax and Noxa (Fig. 6A). In untreated cells (lane 3), Bak but not Bax or Noxa co-precipitated with Mcl-1, suggesting that Mcl-1 selectively interacts with Bak. Mcl-1 immunoprecipitates from vinblastine treated cells (lane 4), in which Mcl-1 was present in the phosphorylated form and at a reduced level as expected, showed a correspondingly reduced level of Bak. Since total Bak expression was unchanged (Fig. 6A, lanes 1 and 2), these results suggest that Mcl-1 phosphorylation/degradation frees Bak from Mcl-1 imposed sequestration. These findings were confirmed by reciprocal immunoprecipitations: Mcl-1 was present in Bak immunoprecipitates from control cells but undetectable in Bak immunoprecipitates from vinblastine treated cells (Fig. 6B). In addition, Mcl-1 was absent from immunoprecipitates of Bax or Noxa (data not shown), consistent with the results in Fig. 6A. To determine if loss of Mcl-1 binding promotes Bak activation, extracts were subjected to immunoprecipitation with an antibody which recognized the conformationally active form of Bak. As shown in Fig. 6C, active Bak was readily detected in extracts from vinblastine-treated cells but was not detected in extracts from untreated cells. To determine whether Mcl-1 phosphorylation was sufficient, in of itself, to disrupt the interaction with Bak, we prepared extracts from cells co-treated with vinblastine and MG132, where the phosphorylated form of Mcl-1 was protected from degradation and thus present in abundance (see Fig. 1C). Bak was present in relative abundance in immunoprecipitates of highly phosphorylated Mcl-1 (Fig. 6D), indicating that it is degradation of Mcl-1 triggered by phosphorylation, not phosphorylation itself, that releases Bak from Mcl-1 sequestration.

4. Discussion

Microtubule-inhibiting, or anti-mitotic, drugs have been in widespread use as antitumor agents for many decades, yet the mechanisms underlying their lethal effects are still far from clear. Such drugs typically cause mitotic arrest and subsequent apoptotic cell death, but the signaling pathways that link these processes remain obscure. Understanding how these agents work at the molecular level downstream of mitotic arrest is critical for elucidating the factors that dictate sensitivity and resistance to these agents and thus for appropriate patient selection.

The results presented here show that a key response to microtubule inhibition is the phosphorylation and degradation of Mcl-1, one of the major anti-apoptotic members of the Bcl-2 family of apoptotic regulatory proteins. DNA damaging agents under conditions that induced apoptosis did not induce these events, suggesting that Mcl-1 phosphorylation/degradation may be more specifically related to apoptosis occurring in response to mitotic arrest. Studies with synchronized cells and selective Cdk inhibitors, used in a carefully controlled fashion to ensure timely and specific Cdk inhibition during mitotic arrest but not at earlier times, strongly implicated Cdk1/cyclin B in Mcl-1 phosphorylation in this context. The fact that two structurally dissimilar Cdk inhibitors gave essentially identical results in several different experiments (Figs. 5B, 5D, 5E, 5F) further strengthens this conclusion. The Cdk inhibitors not only inhibited Mcl-1 phosphorylation, but also inhibited vinblastine-induced Mcl-1 degradation, and significantly improved cell survival after mitotic arrest. These results are consistent with a key role for Mcl-1 in maintenance of cell viability, and highlight the importance of the signaling mechanisms required for its elimination during mitotic arrest. Endogenous levels of Mcl-1 appear to be functionally sufficient, because

KB-3 cells stably overexpressing Mcl-1 exhibit the same sensitivity to vinblastine as parental cells (data not shown).

The finding that degradation of Mcl-1 occurred in a Cdk1-dependent fashion provides a clear and definitive example of pro-apoptotic signaling by overactive Cdk1. Recent work from our laboratory has shown that Bcl-xL and Bcl-2 are also phosphorylated and inactivated by Cdk1-mediated phosphorylation (20). Thus a major mechanism for pro-apoptotic signaling by Cdk1 is the functional elimination (Bcl-xL, Bcl-2) or actual elimination (Mcl-1) of anti-apoptotic proteins Bcl-2 proteins. Using synchronous cell cultures, we also showed that Mcl-1 is partially phosphorylated during normal mitosis, but that this does not lead to Mcl-1 degradation, whereas the extensive phosphorylation occurring in response to overactive Cdk1 during mitotic arrest does lead to Mcl-1 degradation. These results suggest that Mcl-1, like Bcl-2 and Bcl-xL (20), may act as a sensor for Cdk1 signal duration, and interpretation of its degree of phosphorylation may enable a cell to readily distinguish a normal mitosis from an abnormal mitotic arrest and respond accordingly.

Since our original and novel observation that microtubule inhibition leads to Mcl-1 phosphorylation and degradation in KB-3 cells (13), we have made similar observations in a number of other cell lines including colon carcinoma[1]. In addition, recent and independent studies in other laboratories have similarly shown that Mcl-1 is subject to degradation in response to microtubule inhibitors and other anti-mitotic drugs. For example, in three of four multiple myeloma cell lines, mitotic death after treatment with the kinesin spindle protein inhibitor ARRY-520 was associated with loss of Mcl-1 expression, whereas escape from mitotic death in the other cell line was associated with retention of Mcl-1 expression (21). Whether phosphorylation was the mechanism triggering Mcl-1 degradation in the myeloma cell lines was not examined. In U2OS cells, paclitaxel induced Mcl-1 degradation which was triggered by Cdk1-mediated phosphorylation (22), consistent with our results. Another recent report further strengthened the generality of this signaling pathway by showing in several primary and cancer cell lines Mcl-1 phosphorylation and degradation in response to anti-mitotic agents (23).

A key question is whether Cdk1 acts alone or in concert with other kinases to phosphorylate Mcl-1 during mitotic arrest. In U2OS cells, mitotic phosphorylation of Mcl-1 was reported to be largely Cdk1-dependent with two major phosphorylation sites, Ser64 and Thr92 (22). In HCT116 cells, major sites of Mcl-1 phosphorylation during mitotic arrest were found to be Ser64, Thr92, Ser121, Ser159, and Thr163 (23). Preliminary evidence was presented that Cdk1 mediated phosphorylation of Mcl-1 may cause loss of binding of PP2A phosphatase, allowing several other kinases, including p38, JNK, and CKII, to complete Mcl-1 phosphorylation leading to its degradation (23). The fact that Mcl-1 phosphorylation can be effectively inhibited by Cdk inhibitors (Fig. 5B) is consistent with these other reports and also consistent with the idea that Cdk1 may be an initiating kinase that primes Mcl-1 for action by other kinases. In preliminary studies, we have found that a Thr92Ala mutant of Mcl-1 expressed in KB-3 cells becomes phosphorylated and degraded in response to vinblastine treatment with the same kinetics as wild-type Mcl-1 (data not shown), suggesting that another site(s) can substitute.

Our work makes an additional important contribution by defining a rational mechanism whereby loss of Mcl-1 leads to apoptosis. We showed that in untreated cells Bak is bound to Mcl-1, and this appeared to be a selective interaction as other candidate pro-apoptotic proteins, including Bax and Noxa, were not detected in Mcl-1 immunoprecipitates. In

[1]Unpublished observations

addition, Bak underwent conformational changes recognized by an active Bak antibody in response to vinblastine treatment and in concert with loss of Mcl-1 expression. Thus phosphorylation dependent Mcl-1 degradation during mitotic arrest leads to loss of sequestration of Bak permitting Bak activation and apoptosis. Importantly, highly phosphorylated Mcl-1, derived from cells co-treated with vinblastine and MG132, retained the ability to physically interact with Bak, indicating that Mcl-1 degradation is the key step in Bak release. Intriguingly, we have shown previously that Cdk1 phosphorylates Bcl-xL during mitotic arrest in KB-3 cells and that this results in loss of Bax binding and subsequent translocation of Bax from the cytosol to the mitochondria (24). Collectively, these results emphasize the profound manner in which overactivated Cdk1 contributes to mitotic arrest-induced apoptosis, by facilitating the freeing of two key pro-apoptotic effector proteins, Bak and Bax, from sequestration by two key anti-apoptotic Bcl-2 proteins, Mcl-1 and Bcl-xL, respectively.

Acknowledgments

This work was supported by National Institutes of Health Grant CA-109821 from the National Cancer Institute and by funds from the Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences.

Abbreviations

Cdk	cyclin-dependent kinase
JNK	c-Jun N-terminal kinase
ERK	extracellular response kinase
GSK	glycogen synthase kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PARP	poly(ADP-ribose) polymerase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid

References

[1]. Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. Cell Mol Life Sci. 2009; 66:1326–1336. [PubMed: 19099185]

[2]. Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. FEBS Lett. 2010; 584(14):2981–2989. [PubMed: 20540941]

[3]. Opferman JT. Unraveling Mcl-1 degradation. Cell Death and Differ. 2006; 13:1260–1262.

[4]. Shimazu T, Degenhardt K, Nur-E-Kamal A, Zhang J, Yoshida T, Zhang Y, Mathew R, White E, Inouye M. NBK/BIK antagonizes Mcl-1 and Bcl-xL and activates Bak-mediated apoptosis in response to protein synthesis inhibition. Genes Dev. 2007; 21:929–941. [PubMed: 17403773]

[5]. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007; 26:1324–1327. [PubMed: 17322918]

[6]. Han J, Goldstein LA, Hou W, Rabinowich H. Functional linkage between Noxa and Bim in mitochondrial apoptotic events. J Biol Chem. 2007; 282:16223–16231. [PubMed: 17374615]

[7]. Clohessy JG, Zhuang J, de Boer J, Gil-Gomez G, Brady HJ. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Bio. Chem. 2006; 281:5750–5759. [PubMed: 16380381]

[8]. Kim R. Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem Biophys Res Commun. 2005; 333:336–343. [PubMed: 15922292]

[9]. Ding WX, Ni HM, Chen XY, Yu J, Zhang L, Yin XM. A coordinated action of Bax, PUMA, and p53 promotes MG132-induced mitochondrial activation and apoptosis in colon cancer cells. Mol Cancer Ther. 2007; 6:1062–1069. [PubMed: 17363499]

[10]. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. Mcl-1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene. 2004; 23:5301–5315. [PubMed: 15241487]

[11]. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of Mcl-1. Mol Cell. 2006; 21:749–760. [PubMed: 16543145]

[12]. Kobayashi S, Lee SH, Meng XW, Mott JL, Bronk SF, Werneburg NW, Craig RW, Kaufmann SH, Gores GJ. Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1. J Biol Chem. 2007; 282:18407–18417. [PubMed: 17463001]

[13]. Chu R, Terrano DT, Chambers TC. Phosphorylation and degradation of mcl-1 in vinblastine-induced apoptosis. Proc Am Assoc Cancer Res. 2009 Abstract#4601.

[14]. Fan M, Du L, Stone AA, Gilbert KM, Chambers TC. Modulation of mitogen-activated protein kinases and phosphorylation of Bcl-2 by vinblastine represent persistent forms of normal fluctuations at G2-M. Cancer Res. 2000; 60(22):6403–6407. [PubMed: 11103805]

[15]. Upreti M, Lyle CS, Skaug B, Du L, Chambers TC. Vinblastine-induced apoptosis is mediated by discrete alterations in subcellular location, oligomeric structure, and activation status of specific Bcl-2 family members. J Biol Chem. 2006; 281:15941–15950. [PubMed: 16574665]

[16]. Upreti M, Chu R, Galitovskaya EN, Smart SK, Chambers TC. Key role for Bak activation and Bak-Bax interaction in the apoptotic response to vinblastine. Mol Cancer Ther. 2008; 7(7):2224–2232. [PubMed: 18645031]

[17]. Brantley-Finley C, Lyle CS, Du L, Goodwin ME, Hall T, Szwedo D, Kaushal GP, Chambers TC. The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide. Biochem Pharmacol. 2003; 66:459–469. [PubMed: 12907245]

[18]. Bain J, Plater L, Elliott M, Shpiro N, Hastie J, Mclauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. Biochem. J. 2007; 408:297–315. [PubMed: 17850214]

[19]. Vassilev LT, Tovar C, Chen S, Knezevic D, Zhao X, Sun H, Heimbrook DC, Chen L. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:10660–10665. [PubMed: 16818887]

[20]. Terrano DT, Upreti M, Chambers TC. Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation act as a functional link coupling mitotic arrest and apoptosis. Mol Cell Biol. 2010; 30:640–656. [PubMed: 19917720]

[21]. Tunquist BJ, Woessner RD, Walker DH. Mcl-1 Stability Determines Mitotic Cell Fate of Human Multiple Myeloma Tumor Cells Treated with the Kinesin Spindle Protein Inhibitor ARRY-520. Mol Cancer Ther. 2010; 9:2046–2056. [PubMed: 20571074]

[22]. Harley ME, Allan LA, Sanderson HS, Clarke PR. Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. EMBO J. 2010; 29:2407–2420. [PubMed: 20526282]

[23]. Wertz IE, Kusam S, Lam C, Okamoto T, Sandoval W, Anderson DJ, Helgason E, Ernst JA, Eby M, Liu J, Belmont LD, Kaminker JS, O'Rourke KM, Pujara K, Kohli PB, Johnson AR, Chiu ML, Lill JR, Jackson PK, Fairbrother WJ, Seshagiri S, Ludlam MJ, Leong KG, Dueber EC, Maecker H, Huang DC, Dixit VM. Sensitivity to antitubulin chemotherapeutics is regulated by Mcl-1 and FBW7. Nature. 2011; 475(7354):122.

[24]. Upreti M, Galitovskaya EN, Chu R, Tackett AJ, Terrano DT, Granell S, Chambers TC. Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance. J Biol Chem. 2008; 283(51):35517–35525. [PubMed: 18974096]

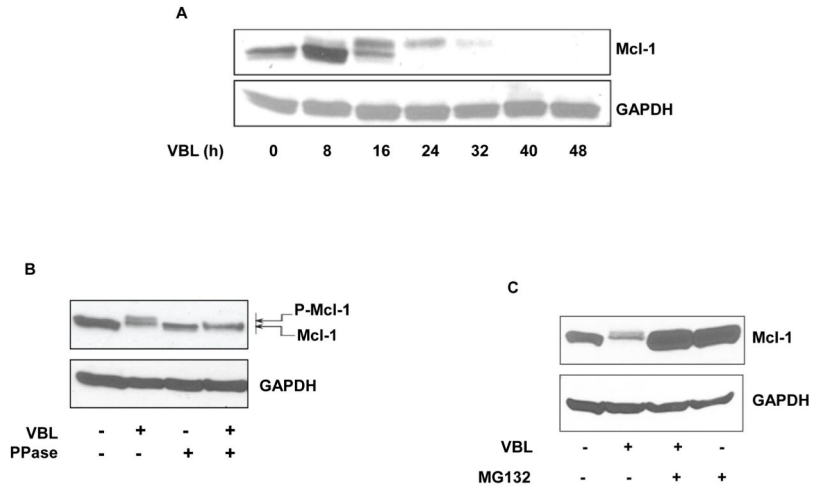

Fig. 1. Kinetics of vinblastine-induced Mcl-1 phosphorylation and degradation

A. KB-3 cells were treated with 30 nM vinblastine (VBL) for the times indicated and cell extracts subjected to immunoblotting for Mcl-1 or GAPDH as a loading control. **B.** Lysates of KB-3 cells that were either untreated or treated with vinblastine (VBL) (30 nM, 24 h) were incubated with or without lambda phosphatase (PPase, 400 U) for 30 min at 30°C. Immunoblotting was performed for Mcl-1 or GAPDH as a loading control. Phosphorylated Mcl-1 (P-Mcl-1) and unphosphorylated Mcl-1 (Mcl-1) are indicated by arrows. **C**. KB-3 cells with or without 20 μM MG132 pretreatment (30 min) were treated with or without 30 nM vinblastine (VBL) for 24 h, and cell extracts were immunoblotted for Mcl-1 or GAPDH as a loading control.

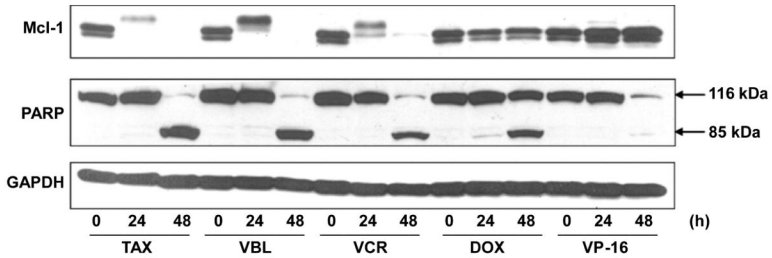

Fig. 2. Mcl-1 is phosphorylated and degraded in response to microtubule inhibition but not DNA damage

KB-3 cells were untreated or treated for 24 or 48 h with 30 nM paclitaxel (TAX), 30 nM vinblastine (VBL), 30 nM vincristine (VCR), 1 μM doxorubicin (DOX), or 15 μM VP-16, and cell extracts subjected to immunoblotting for Mcl-1 or poly(ADP)-ribose polymerase (PARP), with uncleaved (116 kDa) and cleaved (85 kDa) species indicated. GAPDH was used as a loading control.

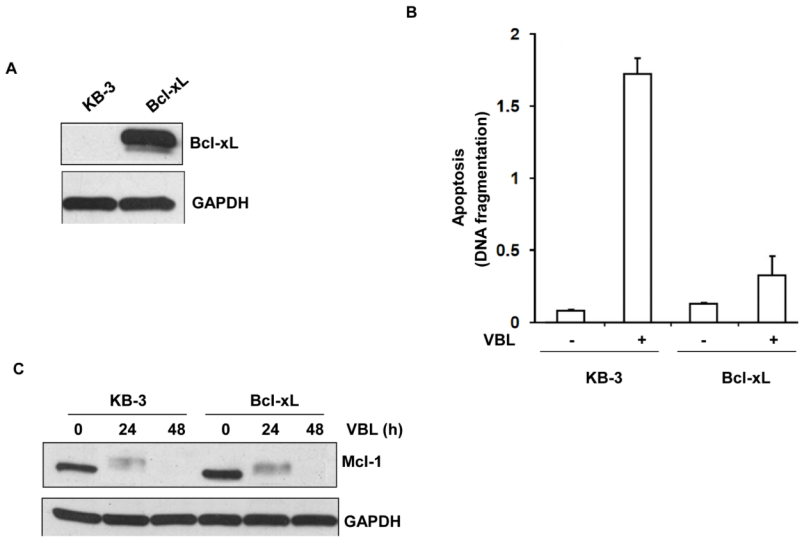

Fig. 3. Mcl-1 degradation is not a consequence of apoptosis

A. Bcl-xL expression in parental KB-3 cells and in cells stably overexpressing HA-Bcl-xL. Immunoblots of Bcl-xL and GAPDH as a loading control are shown. Note that endogenous Bcl-xL is below the level of detection under these exposure conditions. **B.** Parental KB-3 cells or cells overexpressing HA-Bcl-xL were either untreated or treated with 30 nM vinblastine (VBL) as indicated for 48 h and subjected to apoptosis assay, as described in Materials and Methods. Results show mean ± S.D. (n = 6). **C.** Parental KB-3 cells or cells overexpressing HA-Bcl-xL were treated with 30 nM vinblastine (VBL) for the times indicated and extracts subjected to immunoblotting for Mcl-1 or GAPDH.

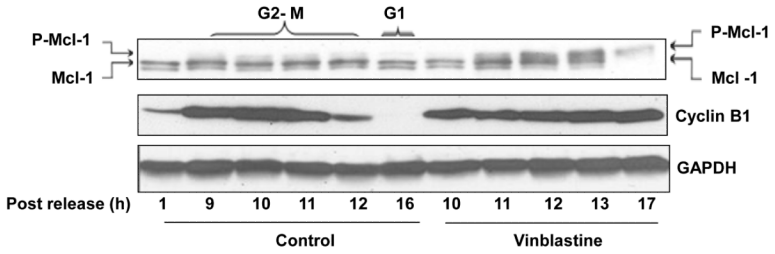

Fig. 4. Kinetics of vinblastine-induced Mcl-1 phosphorylation in synchronized KB-3 cells with or without vinblastine treatment

KB-3 cells were synchronized at the G1/S boundary by double thymidine block and either left untreated (control) or treated with 30 nM vinblastine 1 h after release, and then harvested at the indicated times after release. In the untreated control group, time points corresponding to late G2-M phases and G1 phase are indicated. Extracts were subjected to immunoblotting for Mcl-1, cyclin B1, or GAPDH. Phosphorylated Mcl-1 (P-Mcl-1) is indicated.

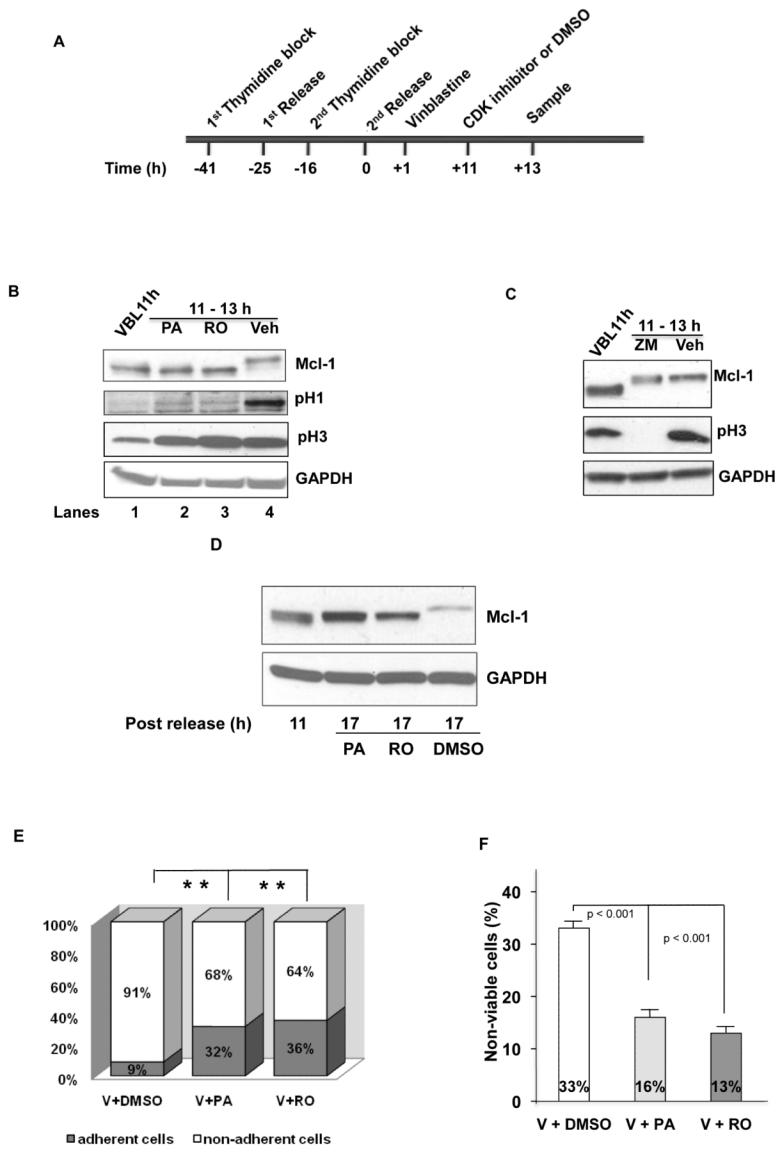

Fig. 5. Cdk inhibitors inhibit vinblastine-induced Mcl-1 phosphorylation and degradation and improve cell survival after mitotic arrest

A. Experimental design. The timing of events for cell synchrony by double thymidine block and M phase-specific timing of Cdk1 inhibition following vinblastine treatment are shown. **B.** KB-3 cells were synchronized at the G1/S boundary by double thymidine block and were treated with 30 nM vinblastine (VBL) 1 h after release. At 11 h post-release, cells were either harvested *(lane 1)* or treated with 1 μM purvalanol A (PA), 1 μM RO3306 (RO), or 0.1% DMSO vehicle (Veh) for 2 h and harvested at 13 h post-release. Whole-cell extracts were prepared and immunoblotted for Mcl-1, phospho-H1 histone (pH1), phospho-H3 histone (pH3) or GAPDH. **C.** Conditions as in B, except that the Aurora kinase inhibitor, ZM477439 (ZM) at 1 μM, was used instead of Cdk inhibitors. **D.** KB-3 cells were synchronized at the G1/S boundary by double thymidine block and treated with 30 nM vinblastine (VBL) 1 h after release. At 11 h post-release (PR), cells were either harvested or treated for 6 h with 0.1% DMSO vehicle, 1 μM purvalanol A (PA), or 1 μM RO3306 (RO), and harvested at 17 h post-release. Whole-cell extracts were prepared and immunoblotted for Mcl-1 or GAPDH. **E.** KB-3 cells were synchronized at the G1/S boundary by double

thymidine block and treated with 30 nM vinblastine (V) 1 h after release. At 11 h post-release, 0.1 % DMSO, 1 μM purvalanol A (PA), or 1 μM RO3306 (RO) was added. At 24 h post-release, adherent and non-adherent cells were collected and counted. The results show the percentage of adherent versus non-adherent cells for each condition averaged from n = 3. ** $p < 0.001$. **F.** Conditions as in E, except that the total population of cells was collected and subjected to trypan blue exclusion assay. The percent trypan blue positive, non-viable cells for each condition (mean ± S.D., n = 3) are shown with p values.

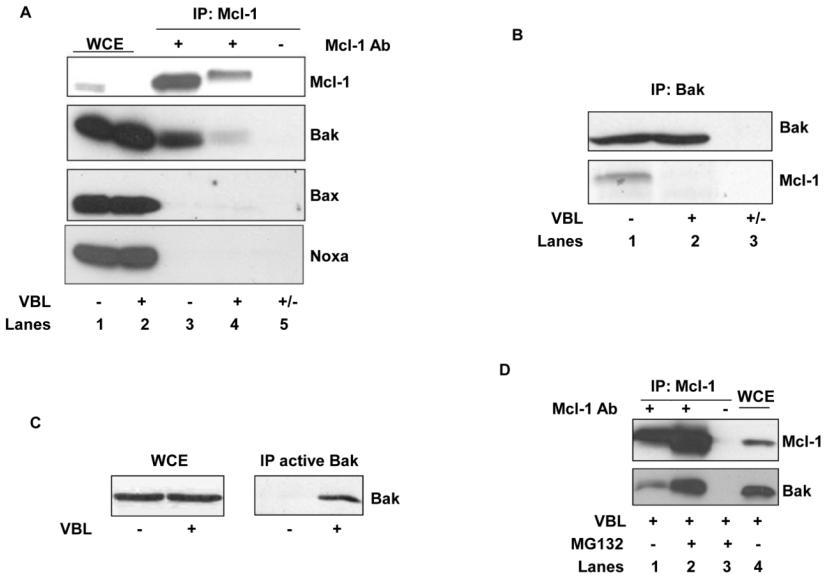

Fig. 6. Mcl-1 interacts with Bak but not Bax or Noxa and loss of Mcl-1 leads to Bak activation

A. KB-3 cells were untreated or treated for 16 h with 30 nM vinblastine (VBL) as indicated and cell extracts subjected to immunoprecipitation (IP) with Mcl-1 antibody, as described in Materials and methods. Immunoprecipitates (lanes 3 and 4) and whole cell extracts (WCE) (lanes 1 and 2) were immunoblotted for Mcl-1, Bak, Bax, or Noxa, as indicated. Lane 5 represents a mock immunoprecipitation, conducted in the absence of antibody, using a 1:1 mixture of samples. **B.** Samples and conditions as in A, except that immunoprecipitations carried out using Bak antibody. **C.** KB-3 cells were untreated or treated for 36 h with 30 nM vinblastine and extracts subjected to immunoprecipitation (IP) with antibody to active Bak. Whole cell extracts (WCE) or immunoprecipitates were probed for Bak expression by immunoblotting. **D.** KB-3 cells were treated with 30 nM vinblastine for 16 h in the absence or presence of 20 μM MG132 as indicated and cell extracts subjected to immunoprecipitation (IP) with Mcl-1 antibody and probed for Mcl-1 and Bak. A whole cell extract (WCE, from the sample treated with VBL), as well as a mock immunoprecipitation conducted in the absence of Mcl-1 antibody, using the co-treated sample, were also immunoblotted for Mcl-1 or Bak.