Biochemical Pharmacology 83 (2012) 199–206

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

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ARTICLE INFO

Article history:
Received 15 September 2011
Accepted 11 October 2011
Available online 18 October 2011

Keywords:
Mcl-1
Cdk1
Microtubule inhibitors
Apoptosis
Bcl-2 proteins

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.

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 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

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.

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doi:10.1016/j.bcp.2011.10.008

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, Paclitaxel, 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 1000 × *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 Section 2, 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).