Myeloid Cell Leukemia 1 Is Phosphorylated through Two Distinct Pathways, One Associated with Extracellular Signal-regulated Kinase Activation and the Other with G₂/M Accumulation or Protein Phosphatase 1/2A Inhibition*

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Protein kinase C activators and microtubule-damaging drugs stimulate BCL2 phosphorylation, which has been associated with either enhancement or inhibition of cell viability. In a Burkitt lymphoma cell line, both types of agents likewise stimulated phosphorylation of myeloid cell leukemia 1 (MCL1), another viability-promoting BCL2 family member. However, while MCL1 phosphorylation induced by the protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), did not affect its electrophoretic mobility, microtubule-damaging agents, such as taxol, induced MCL1 phosphorylation associated with a band shift to decreased mobility. Inhibitors of extracellular signal-regulated kinase (ERK) activation blocked TPA-induced MCL1 phosphorylation but not the taxol-induced band shift. TPA-induced MCL1 phosphorylation occurred rapidly and was not associated with decreased viability, while the taxol-induced band shift occurred upon extended exposure as cells accumulated in G₂/M followed by cell death. Protein phosphatase 1/2A inhibitors also induced the MCL1 band shift/phosphorylation. Thus, MCL1 undergoes two distinct types of phosphorylation: (i) TPA-induced, ERK-associated phosphorylation, which does not alter the electrophoretic mobility of MCL1, and (ii) ERK-independent phosphorylation, which results in an MCL1 band shift and is induced by events in G₂/M or protein phosphatase 1/2A inhibitors.

Both anti- and proapoptotic BCL2 family members undergo phosphorylation, as is seen with BCL2, BCLX, and BAD. The role of this post-translational modification has been well defined for the proapoptotic family member, BAD, where phosphorylation promotes association with a 14-3-3 protein instead of BCLX, thereby freeing BCLX to exert its antiapoptotic activity (1–6). However, the role of phosphorylation in the case of BCL2 and other antiapoptotic family members is not yet completely understood. BCL2 phosphorylation is induced by several types of agents. These include growth factors and protein kinase C activators, such as erythropoietin, interleukin-3, and bryostatin-1 (7–9). They also include microtubule-directed agents, such as taxol and nocodazole, as well as the protein phosphatase 1/2A inhibitor, okadaic acid (10–18). BCL2 phosphorylation has been associated with viability promotion in some cases and with cell death in others (8, 18). These varying results could relate to the fact that BCL2 phosphorylation has been studied by several investigators using different agents in varied cell lines.

Our studies focus on myeloid cell leukemia 1 (MCL1), an antiapoptotic BCL2 family member identified by its rapid up-regulation during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of myeloid ML-1 cells (19). Previous studies on MCL1 regulation indicated that TPA increases MCL1 expression within 3 h through an extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent signal transduction pathway, which activates a serum response factor-Erk-1 transcription factor complex (20, 21). Microtubule-disrupting agents such as nocodazole and vinblastine similarly increase ERK activation and MCL1 expression within 3 h, as does okadaic acid (20, 22). MCL1 may also be regulated at the level of mRNA and protein turnover, since both can be rapidly degraded (t½ = 1–3 h) (22, 23).

In the work described here, we investigated whether MCL1 is phosphorylated, as are BCL2 and BCLX. We approached this question by using various agents that induce BCL2 phosphorylation and testing for a similar effect on MCL1. We first tested for MCL1 phosphorylation in the presence of TPA as an activator of protein kinase C and ERK (20). We then examined the effects of microtubule damaging agents and protein phosphatase 1/2A inhibitors. Our results show that, along with rapid ERK activation, TPA stimulates MCL1 phosphorylation without changing its electrophoretic mobility. In contrast, microtubule-damaging agents that produce a G₂/M cell cycle arrest (e.g. taxol) and protein phosphatase 1/2A inhibitors (e.g. okadaic acid) induce ERK-independent MCL1 phosphorylation associated with a band shift to reduced electrophoretic mobility. Thus, the viability-promoting BCL2 family member, MCL1, can undergo two distinct types of phosphorylation; one is associated with ERK activation and does not change its electrophoretic mobility, while the other results in an ERK-independent MCL1 band shift and is induced by G₂/M events or protein phosphatase 1/2A inhibitors.

EXPERIMENTAL PROCEDURES

Cell Culture, Drug Treatment, and Assessment of Cell Viability—The BL41 Burkitt lymphoma cell line was obtained from Dr. Surrerendra Sharma (Brown University, Providence, RI). The BL41-3 subline that endogenously expresses high levels of MCL1 was derived in our laboratory as described previously (53) and a clone of this subline (BL41-3 clone 10) was derived by picking individual colonies from methocellu—

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‡ The abbreviations used are: MCL1, myeloid cell leukemia 1; TPA, 12-O-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PBS, phosphate-buffered saline.
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RESULTS

TPA Activates the ERK Pathway and Induces MCL1 Phosphorylation without a Band Shift—We first examined the effect of TPA on $^{32}P$ incorporation into MCL1 while monitoring total MCL1 protein in parallel by Western blotting (Fig. 1). These studies were primarily carried out in BL41-3 cells because preliminary experiments had shown that TPA does not significantly alter the level of MCL1 expression, allowing effects on MCL1 phosphorylation to be monitored in the absence of potentially confounding changes in MCL1 protein levels. MCL1 consists principally of a doublet of two closely spaced bands (~40–42 kDa) (20). The doublet exhibited low levels of $^{32}P$ incorporation in untreated, control BL41-3 cells and increased $^{32}P$ labeling following exposure to TPA (Fig. 1). The TPA-induced increase in MCL1 phosphorylation occurred rapidly (within 5 min) and in conjunction with the appearance of activated ERK, primarily ERK2, since only a low level of ERK1 was detected (Fig. 1). ERK activation and increased MCL1 phosphorylation were seen at a TPA concentration of 10 nM and were not markedly further stimulated at higher concentrations. The effect of TPA on MCL1 phosphorylation was not associated with a change in the electrophoretic mobility of the MCL1 protein (band shift) or with a loss of BL41-3 cell viability.

FIG. 1. TPA activates ERK and induces MCL1 phosphorylation without changing its electrophoretic mobility. Radiolabeled BL41-3 cells were exposed to TPA (or Me$_2$SO as a vehicle control) at the indicated times and concentrations. They were then assayed for $^{32}P$ incorporation into immunoprecipitated MCL1 ($^{32}P$ MCL1) and for total MCL1 protein on the same blot. Unlabeled cells were also treated with TPA and analyzed for phosphorylated or total ERK protein by Western analysis. Cells were greater than 96% viable following treatment with 10 nM TPA for 24 h.

Cells were incubated with ECL (Amersham Pharma Biotech) followed by exposure to film.

Cell Cycle Analysis and Cell Sorting—After treatment, cells were rinsed with PBS, stored as necessary at 4 °C in 75% ethanol (in PBS), and then resuspended in PBS containing 0.1 mg/ml propidium iodide and 1 mg/ml RNase A. After incubation at 37 °C for 30 min, cell cycle distribution was assessed using a FACScan (Becton Dickinson). The Modfit for Mac version 2.0 program (Verity Software House) was used to estimate the percentage of cells in the various phases of the cell cycle.

TPA Treatment—To determine whether the TPA band shift was associated with phosphorylation, cell lysates were exposed to $\lambda$-phosphatase as follows. Cells (2 × 10$^6$) were washed with PBS and lysed as above in immunoprecipitation lysis buffer that contained protease inhibitors but not phosphatase inhibitors (except sodium phosphate where indicated). After centrifugation (13,200 rpm for 10 min), lysates were incubated in the presence or absence of $\lambda$-phosphatase for 15–30 min at 30 °C, using the buffer specified in the supplier's protocol (New England BioLabs). For the taxol-treated cells, okadaic acid (1 μM) was added to the cell lysates as discussed under “Results.” Samples were then boiled for 5 min in SDS sample buffer, and MCL1 was assayed by Western blotting.

Western Blotting—Western blotting for MCL1 or ERK was carried out as described previously with minor modifications (20). Lysates from equal amounts of total cells were electrophoresed on a 10 or 12.5% polyacrylamide gel followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). $^{32}P$-labeled MCL1 was detected by autoradiography using X-Omat Blue XB-1 film (Eastman Kodak Co., Rochester, NY). The membrane was then subjected to Western blotting for total MCL1 protein as described below.

Cell viability was assessed in cells incubated with Hoechst 33342 dye (2 μg/ml) for 15 min and visualized using a UV microscope. Cells exhibiting normal nuclear morphology (unaltered chromatin) were scored as viable, and those containing condensed, fragmented nuclei were scored as dead (34–36).

$^{32}P$ Incorporation into MCL1—For radiolabeling, cells were washed twice in phosphate-free RPMI 1640 (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum (Life Technologies) and were resuspended (1 × 10$^7$ cells/ml) in this medium in the presence of $^{32}P$-orthophosphate (120 μCi/ml; NEN Life Science Products). Cells were radiolabeled for 1 h prior to the addition of drugs, except in the case of taxol, when cells were radiolabeled during the final 3 h of treatment. After drug treatment, cells were collected and rinsed once with PBS and then incubated for a 30-min incubation at 4 °C with 600 μl of immunoprecipitation lysis buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 0.2% (v/v) Nonidet P-40, 2 mM EDTA, 2 mM DTA), to which the following protease and phosphatase inhibitors were added: Complete Inhibitors™ (Roche Molecular Biochemicals), 100 μg/ml aprotinin, 1 μg/ml pepstatin A, 80 mM sodium fluoride, 40 mM sodium phosphate, and 1 mM sodium orthovanadate. Lysates were centrifuged at 15,200 rpm for 10 min, and the supernatant was incubated with a rabbit polyclonal anti-MCL1 antibody (23) for 30 min at 4 °C with gentle agitation. This was followed by a 1-h incubation with 5 μg of protein A-Sepharose beads (Sigma). The beads were rinsed with the immunoprecipitation lysis buffer, collected by centrifugation at 6000 rpm, resuspended in SDS sample buffer, and boiled for 5 min. Electrophoresis was carried out at 130 V for 16 h on a 12.5% SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), $^{32}P$-labeled MCL1 was detected by autoradiography using X-Omat Blue XB-1 film (Eastman Kodak Co., Rochester, NY). The membrane was then subjected to Western blotting for total MCL1 protein as described below.

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RESULTS

TPA Activates the ERK Pathway and Induces MCL1 Phosphorylation without a Band Shift—We first examined the effect of TPA on $^{32}P$ incorporation into MCL1 while monitoring total MCL1 protein in parallel by Western blotting (Fig. 1). These studies were primarily carried out in BL41-3 cells because preliminary experiments had shown that TPA does not significantly alter the level of MCL1 expression, allowing effects on MCL1 phosphorylation to be monitored in the absence of potentially confounding changes in MCL1 protein levels. MCL1 consists principally of a doublet of two closely spaced bands (~40–42 kDa) (20). The doublet exhibited low levels of $^{32}P$ incorporation in untreated, control BL41-3 cells and increased $^{32}P$ labeling following exposure to TPA (Fig. 1). The TPA-induced increase in MCL1 phosphorylation occurred rapidly (within 5 min) and in conjunction with the appearance of activated ERK, primarily ERK2, since only a low level of ERK1 was detected (Fig. 1). ERK activation and increased MCL1 phosphorylation were seen at a TPA concentration of 10 nM and were not markedly further stimulated at higher concentrations. The effect of TPA on MCL1 phosphorylation was not associated with a change in the electrophoretic mobility of the MCL1 protein (band shift) or with a loss of BL41-3 cell viability.

Western Blotting—Western blotting for MCL1 or ERK was carried out as described previously with minor modifications (20). Lysates from equal amounts of total cells were electrophoresed on a 10 or 12.5% polyacrylamide gel followed by transfer to a polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat dry milk in PBS and incubated with the primary antibody, either for 1 h with a monoclonal anti-MCL1 antibody (21) (diluted 1:2000 in PBS) or overnight with the polyclonal ERK antibodies that recognize phosphorylated (activated) ERK (26, 27, 29–31). U0126 and PD98059 have previously been used to implicate the ERK pathway in a number of signaling cascades such as the induction of T cell proliferation and in the nerve growth factor-...
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Fig. 2. The TPA-induced increase in MCL1 phosphorylation is blocked by inhibitors of ERK activation. A, BL41-3 cells were preincubated for 15 min in the presence of U0126, its inactive analog U0124, or Me2SO. Cells were then either treated with TPA or Me2SO for 15 min. 32P incorporation into MCL1 and ERK activation were assayed as in Fig. 1. In addition, cells exposed for up to 100 min with 25 μM U0126 alone did not demonstrate a decrease in basal 32P incorporation into MCL1 (not shown). B, an experiment similar to that shown in A was performed using PD98059.

Fig. 3. Microtubule-damaging agents and protein phosphatase 1/2A inhibitors decrease the electrophoretic mobility of MCL1. BL41-3 cells (A–C) or ML-1 cells (D) were exposed to microtubule-damaging agents, protein phosphatase 1/2A inhibitors, or TPA using the indicated concentrations and exposure times. The MCL1 protein was then assayed by Western blotting.

(Figs. 1 and 3A). In ML-1 cells, MCL1 was also phosphorylated without undergoing a band shift (data not shown). In summary, TPA stimulated both ERK activation and phosphorylation of MCL1 without producing a band shift.

To better understand the role of the ERK pathway in stimulating MCL1 phosphorylation, BL41-3 cells were incubated with the selective MEK inhibitor U0126 or PD98059 prior to TPA application. U0126, used at the lowest concentration found in preliminary experiments to block TPA-induced ERK activation, also blocked the TPA-induced increase in MCL1 phosphorylation (Fig. 2A). PD98059 likewise blocked the TPA-induced MCL1 phosphorylation, while U0124, an inactive analog of U0126, did not (Fig. 2, A and B). In contrast, these inhibitors did not affect basal MCL1 phosphorylation (Fig. 2; see figure legend), which agreed with the fact that basal phosphorylation occurred in the absence of substantial activated ERK (Figs. 1 and 2). Overall, MEK inhibitors blocked TPA-induced ERK activation and the associated increase in MCL1 phosphorylation, while having little effect on basal phosphorylation levels. Thus, TPA-induced MCL1 phosphorylation without a band shift appeared to relate, either directly or indirectly, to MEK-induced ERK activation.

Microtubule-damaging Agents and Protein Phosphatase 1/2A Inhibitors Produce an MCL1 Band Shift That Reflects Phosphorylation—We next tested microtubule-damaging agents and protein phosphatase 1/2A inhibitors for their ability to induce a band shift of the MCL1 protein as was seen with BCL2 (10, 11). Exposure of BL41-3 cells to okadaic acid or taxol decreased the electrophoretic mobility of MCL1, which was readily detectable as a band shift on Western analysis (Fig. 3A). This effect was not seen with TPA (Fig. 3A and Figs. 1 and 2) but was seen with another phosphatase inhibitor, calyculin A, and with other microtubule-damaging agents including nocodazole, vinblastine, and colchicine (Fig. 3, B and C, and data not shown). Microtubule-disrupting agents, such as vinblastine, also induced an MCL1 band shift in ML-1 cells (at 10 h), which occurred following the increase in MCL1 expression seen within 2.5–5 h (Fig. 3D). We note that, in BL41-3 cells, two rather diffuse shifted bands were seen with okadaic acid, while primarily one shifted band was seen with taxol. This difference could relate to the time course of effects of these agents, since the shift occurred more rapidly with okadaic acid than with taxol as shown further below (Figs. 5B and 7A). For further studies of the MCL1 band shift, we used BL41-3 cells exposed to 1 μM okadaic acid or to 0.1 μM taxol, where a 10-fold lower concentration of either agent produced little or no shift (Fig. 3, B and C). Overall, the present findings demonstrate that an MCL1 protein band shift occurs in the presence of protein phosphatase 1/2A inhibitors and microtubule-damaging agents, including agents that cause both microtubule disruption (e.g. nocodazole and vinblastine) and stabilization (e.g. taxol).

In further studies, we first confirmed that the MCL1 protein present in cells treated with okadaic acid or taxol undergoes phosphorylation. 32P incorporation into MCL1 was markedly increased with 1 μM okadaic acid but was unaltered with a 10-fold lower concentration or with its inactive analog, 1-norokadone (1 μM; Fig. 4A and data not shown). The shifted MCL1 band seen in cells treated with 0.1 μM taxol also exhibited 32P incorporation, although the increase was not as pronounced in this case (Fig. 4A). This experiment was carried out at a relatively early time point to minimize taxol-induced toxicity (see
Fig. 4. The MCL1 band shift induced by okadaic acid or taxol is associated with phosphorylation. A, BL41-3 cells were either treated or not treated with okadaic acid or taxol as indicated and then assayed for 32P incorporation into MCL1 as in Fig. 1. B, lysates from BL41-3 cells that had been treated or not treated with okadaic acid or taxol were incubated at 30 °C in the absence or presence of λ-phosphatase. Sodium phosphate was added to the indicated samples. In the experiment with taxol, okadaic acid was added to the indicated lysates as described under “Experimental Procedures.” MCL1 was analyzed by Western blotting.

Fig. 5B), and a considerable amount of unshifted MCL1 remained present. Nevertheless, these experiments demonstrate that the shifted MCL1 band is phosphorylated in cells treated with okadaic acid or taxol.

To clarify the relationship between the MCL1 band shift and phosphorylation, lysates from cells treated with the above agents were incubated with λ-phosphatase in an in vitro reaction. If phosphorylation contributed to the MCL1 band shift, incubation with the phosphatase would be expected to revert the shifted bands to a nonshifted position. Using cells treated with okadaic acid, where the band shift occurred rapidly, we found that λ-phosphatase indeed caused a reversion of the MCL1 band shift (Fig. 4B). The reversion was inhibited by adding excess sodium phosphate as a means of inhibiting the phosphatase (39). This experiment also yielded information concerning the two bands of the MCL1 doublet, since the incubation with λ-phosphatase did not collapse these two to a single band. Instead, two bands remained after phosphatase treatment of MCL1 from either untreated or okadaic acid-treated cells, under conditions where the band shift was reverted in the latter case (Fig. 4B). Therefore, the MCL1 band shift seen in the presence of okadaic acid appeared to result from phosphorylation, while the MCL1 doublet did not.

In the above experiment, samples that were not exposed to λ-phosphatase were incubated at 30 °C in parallel with those that were exposed to the enzyme. This experimental design ensured that any difference seen in the presence versus the absence of the phosphatase related to the activity of the enzyme itself, as opposed to events that might simply occur as a result of the 30 °C incubation. When a similar experiment was carried out using lysates from taxol-treated cells, the shifted MCL1 protein reverted to a nonshifted position during the 30 °C incubation in the absence as well as in the presence of λ-phosphatase (data not shown). This suggested that an endogenous phosphatase might be dephosphorylating MCL1 upon incubation at 30 °C. Since reversion of shifted MCL1 was seen with lysates from taxol-treated but not okadaic acid-treated cells, we hypothesized that it might be inhibited by okadaic acid. Indeed, the addition of okadaic acid to the cell lysates in vitro prevented reversion of the taxol-induced MCL1 band shift, while having no detectable effect on the MCL1 protein from untreated control cells. Therefore, lysates from taxol-treated cells were incubated at 30 °C in the presence of okadaic acid. Under these conditions, λ-phosphatase (which is not sensitive to okadaic acid) (39) caused the taxol-induced shifted MCL1 band to revert to a nonshifted position (Fig. 4B). This effect was again prevented by the addition of excess sodium phosphate. In summary, the MCL1 band shift as induced by either taxol or okadaic acid related to phosphorylation, since incubation with λ-phosphatase resulted in reversion to a nonshifted position.

The Taxol-induced MCL1 Band Shift Is Associated with Events in the G2/M Phase of the Cell Cycle but Not ERK Activation—Since taxol is a microtubule-stabilizing agent and has a mechanism of action different from that of TPA, we further explored how taxol brings about its effects on MCL1 phosphorylation and how its effects differ from those of TPA. The 0.1 μm concentration of taxol that induced the MCL1 band shift did not induce ERK activation as assayed upon exposure of BL41-3 cells for 3, 6, 12, and 24 h (data not shown), a result similar to observations in ML-1 cells (20). The microtubule-disrupting agent nocodazole likewise did not activate ERK in BL41-3 cells, which differed from the observations in ML-1 cells. Furthermore, inhibitors of ERK activation, U0126 and PD98059, did not block the taxol- or nocodazole-induced MCL1 band shift (Fig. 5A and data not shown). Thus, unlike TPA, the effects of the microtubule-dam-
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The fact that microtubule-stabilizing and -disrupting agents had similar effects on MCL1 suggested that the band shift may relate to the accumulation of cells in the G2/M phase of the cell cycle, which occurs with both types of agents. To investigate this possibility, we monitored the time course of the effects of taxol on cell cycle distribution and the MCL1 band shift. Cells began to accumulate in G2/M within 3 h after the application of taxol, with increasing accumulation occurring at 6 and 12 h (Fig. 5B). In parallel, a shifted MCL1 band was faintly detected at 3 h, with increasing amounts being apparent at 6 and 12 h. A substantial proportion of MCL1 remained unshifted at 12 h, when a majority of cells were in G2/M. This has also been seen with BCL2 (40, 41) and may indicate that only cells in a particular fraction of G2/M demonstrate the band shift. Cell death began to appear when a majority of cells had accumulated in G2/M (at 12 h and beyond; Fig. 5B). In short, the taxol-induced MCL1 band shift paralleled G2/M accumulation and was followed by cell death.

To further test for an association of the MCL1 band shift with events in G2/M, we carried out cell sorting and assayed for the shifted MCL1 band in populations enriched for cells in G2/M versus G0/G1/S. With taxol-treated cells, we found that the shifted MCL1 band was present in the population of cells sorted for G2/M DNA content but not those with a lower (large) G2/G1/S phase DNA content (Fig. 5C). When untreated control cells were sorted, a shifted band was also faintly detectable in the G2/M-enriched population. Thus, a small proportion of cells may normally demonstrate an MCL1 band shift during cell cycle transit through G2/M, and the prominent band shift seen with taxol may reflect the accumulation of cells in this phase.

Based on the above association with G2/M, we hypothesized that prevention of cell entry into G2/M might prevent the taxol-induced MCL1 band shift. We examined this using aphidicolin, which prevents passage through S phase by inhibiting DNA polymerase α (42). Taxol-induced G2/M accumulation and the MCL1 band shift were reduced with 0.025 μg/ml aphidicolin and were blocked with higher, nontoxic concentrations (∼0.25 μg/ml; Fig. 6A). The taxol-induced MCL1 band shift thus appeared to depend upon cell passage into G2/M.

Finally, since the effect of taxol related to G2/M accumulation and since a shifted MCL1 band was faintly detectable in untreated G2/M-sorted cells, we hypothesized that synchronized cells passing as a cohort through G2/M might demonstrate an MCL1 band shift. To test this, cells were arrested in S phase by incubation with aphidicolin, followed by transfer to fresh drug-free medium to allow reinitiation of the cell cycle. A cohort of cells passed through G2/M 6–8 h after release, and a shifted MCL1 band was detected at ∼8–10 h (Fig. 6B). The fact that G2/M accumulation was marked at 6 h but little shifted MCL1 was present until 8 h suggested that the band shift may occur in late, rather than early, G2/M. The shifted band represented only a small proportion of the total MCL1 protein, suggesting that it was present at low levels or in a small proportion of cells within G2/M. For example, the shift may be a transient event that normally occurs within a specific window in late G2/M. Therefore, during cell passage through G2/M, the MCL1 protein is subjected to phosphorylation associated with a band shift.

The Okadaic Acid-induced MCL1 Band Shift Is Not Dependent on G2/M Accumulation or ERK Activation—In a final set of experiments, we monitored the time course of the okadaic acid-induced MCL1 band shift and tested for the involvement of ERK or G2/M accumulation. The MCL1 band shift was seen within 1.5 h after application of 1 μM okadaic acid and could not be meaningfully monitored for longer than ∼6 h due to loss of MCL1 protein expression (Fig. 7A). While this concentration of okadaic acid activated ERK, as in other cells (20, 43), complete inhibition of the ERK activation with U0126 did not prevent the MCL1 band shift (Fig. 7B). In addition, okadaic acid (1 μM) did not cause a substantial increase in the percentage of cells in G2/M (Fig. 7C), and pretreatment of cells with aphidicolin did not prevent the okadaic acid-induced MCL1 band shift (Fig. 7D), indicating that unlike taxol, the okadaic acid-induced band shift was not associated with G2/M accumulation.

DISCUSSION

Studies of the BCL2 family member BAD have uniformly demonstrated inhibition of proapoptotic activity upon phosphorylation (1–6). However, studies of BCL2 and BCLX have not yielded unequivocal results upon examination of different phosphorylation-stimulating agents in different systems. The studies reported here examined the phosphorylation of MCL1, a viability-promoting member of the BCL2 family that is similar to BCL2 and BCLX (but not BCLW or A1) in that it contains an upstream loop region in addition to the BCL2 homology domains (44, 45). The loop region undergoes phosphorylation and is a regulatory region of BCL2 and BCLX (8, 18, 46–50). In our experiments, we used a cell line that endogenously expresses abundant MCL1 and tested the effects of a variety of agents known to influence BCL2 phosphorylation. Our results revealed differences in MCL1 phosphorylation as induced by various types of agents. TPA stimulated MCL1 phosphorylation without changing its electrophoretic mobility, while microtubule-damaging agents, such as taxol, produced an MCL1 band shift that represented phosphorylation. Various signaling pathways appeared to be involved in the differing manifestations of MCL1 phosphorylation, since the TPA-in-
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The MCL1 phosphorylation/band shift induced by microtubule-damaging agents occurred with an extended time course in association with $G_2/M$ arrest and was blocked when cells were prevented from entering $G_2/M$ with aphidicolin. ERK was not involved, since taxol and nocodazole did not activate this pathway in BL41-3 cells (although nocodazole caused rapid ERK activation in ML-1 cells) (20), and the band shift induced by these agents was not blocked by U0126 or PD98059, similar to a recent report on the BCL2 band shift (52). An MCL1 band shift was also seen in populations enriched for cells in $G_2/M$ by cell sorting or cell synchronization. Taken together, our results suggest that the MCL1 phosphorylation/band shift occurs in cycling cells at a point in late $G_2/M$ and that increasing the number of cells at this point results in an increase in the shifted MCL1 band. In other words, events in late $G_2/M$, rather than ERK activation, appear to underlie the effects of the microtubule-damaging agents on the MCL1 phosphorylation/band shift.

Okadaic acid, like the microtubule-damaging agents, induced an MCL1 band shift. However, the band shift induced by okadaic acid occurred more rapidly than that induced by microtubule-damaging agents and was not dependent on cells accumulating in $G_2/M$. We speculate that okadaic acid might induce the MCL1 band shift by activating the pathway that produces the band shift without requiring $G_2/M$ accumulation, by activating other pathways, or by inhibiting the dephosphorylation of MCL1. This may relate to the observation that, with okadaic acid, both bands of the MCL1 doublet shift, while with taxol, primarily one shifted band was detected. The rapid action of okadaic acid may have stimulated a shift of both MCL1 bands, while the gradual accumulation of cells in $G_2/M$ with taxol allowed the occurrence of other events that differentially affected the two bands of the doublet (e.g. differential formation or degradation of the bands).

The present results on MCL1 phosphorylation invite a comparison with previous findings on the various agents that increase MCL1 expression in cell lines where the gene is inducible. As discussed above, stimulation of the ERK pathway appears to be linked to both MCL1 expression and the phosphorylation without band shift. Some microtubule-damaging agents can also affect both MCL1 expression and the phosphorylation with band shift, although these occur through different mechanisms; MCL1 expression is rapidly increased by microtubule-disrupting but not microtubule-stabilizing agents, while the phosphorylation/band shift is produced by both types of agents. The increase in MCL1 expression is associated with ERK activation and occurs rapidly (20), while the phosphorylation/band shift is instead associated with the eventual accumulation of cells in $G_2/M$. This difference in timing is readily apparent upon application of microtubule-disrupting agents to ML-1 cells, where the increase in MCL1 protein levels occurs well before the band shift. In summary, ERK activation is associated with the stimulation of MCL1 expression and phosphorylation without a band shift, while events in $G_2/M$ underlie...
the ERK-independent MCL1 phosphorylation/band shift. The parallels between agents that induce MCL1 phosphorylation and those previously found to increase MCL1 levels suggest a coordination between MCL1 protein expression and its phosphorylation.

It remains to be determined how these findings with MCL1 relate to previous observations with BCL2. Interestingly, MCL1 phosphorylation induced by TPA is reminiscent of that seen with these agents in the case of BCL2. BCL2 undergoes phosphorylation at Ser^70 with both growth factors and microtubule-damaging agents, and recent findings show that microtubule-damaging agents induce the phosphorylation of additional residues (e.g., BCL2 Ser^57 and Thr^69) (8, 18) (10–13, 18). We do not know whether the two types of MCL1 phosphorylation observed here differ in their effects, although the phosphorylation without band shift induced by TPA was associated with the maintenance of cell viability, while the phosphorylation/band shift induced by taxol was followed by cell death. Our future work is aimed at addressing this question by further characterizing MCL1 phosphorylation, starting with the identification of the sites involved with the different types of agents. Thus, just as the proapoptotic family member, BAD, is phosphorylated by various kinases, the same may be true for antiapoptotic family members such as MCL1, where the present studies have shown that MCL1 is phosphorylated through two distinct pathways.

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REFERENCES

1. Gajewski, T. F., and Thompson, C. B. (1996) Cell 87, 589–592
2. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. (1996) Mol. Cell 2, 413–422
3. Scheid, M. P., and Duronio, V. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 7439–7444
4. Wang, H. G., Pathan, N., Ethell, I. M., Krajewska, S., Yamaguchi, Y., Shibaaski, P. McKeon, F., Bole, T., Franke, T. F., and Reed, J. C. (1999) Science 284, 339–343
5. Zha, J., Harada, H., Yang, E., Jocket, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
6. Schuurman, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C., and Bokoch, G. M. (2000) Mol. Cell. Biol. 20, 453–461
7. May, W. S., Tyler, P. G., It0, T., Armstrong, D. K., Qatsha, K. A., and Davidson, N. E. (1994) J. Biol. Chem. 269, 26865–26870
8. Ito, T., Deng, X., Carr, B., and May, W. S. (1997) J. Biol. Chem. 272, 11671–11673
9. Ruivo, P. P., Deng, X., Carr, B. K., and May, W. S. (1998) J. Biol. Chem. 273, 25436–25442
10. Haldar, S., Jena, N., and Croce, C. M. (1994) Biochem. Cell Biol. 72, 455–462
11. Haldar, S., Jena, N., and Croce, C. M. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 4597–4611
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