Distinct roles for extracellular-signal-regulated protein kinase (ERK) mitogen-activated protein kinases and phosphatidylinositol 3-kinase in the regulation of Mcl-1 synthesis

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Alterations in the expression of various Bcl-2 family members may act as one means by which a cell's survival may be regulated. The mechanism by which cytokines regulate expression of Bcl-2 family members was examined in the haemopoietic cell line TF-1. Cytokine-induced Mcl-1 protein expression was shown to be controlled through a pathway dependent upon phosphatidylinositol 3-kinase (PI 3-kinase). The cytokine-induced increase in mRNA transcription was not dependent upon PI 3-kinase, thus dissociating the immediate-early transcription factors responsible for Mcl-1 transcription from the PI 3-kinase signalling pathway. In contrast, Mcl-1 mRNA levels were dependent upon MEK [mitogen-activated protein kinase (MAPK)/extracellular-signal-

regulated protein kinase] activation, suggesting a role for the Ras/MEK/MAPK pathway in Mcl-1 transcription. Activation of PI 3-kinase was shown to be necessary to stimulate Mcl-1 protein translation. This was not due to any effect on prolonging the half-life of the protein. Finally, the lipid second messenger ceramide was shown to cause a reduction in Mcl-1 protein translation, probably via its ability to inhibit protein kinase B activation, providing further clues regarding the death-inducing effect of this lipid.

Key words: apoptosis, cytokine, protein translation, signal transduction.

INTRODUCTION

The signalling pathways activated by cytokines that are specifically involved in inhibition of apoptosis have been an area of active research for several years. In 1995, Yao and Cooper [1] and our group [2] provided the first suggestions that the activation of phosphatidylinositol 3-kinase (PI 3-kinase) was a key step in blocking apoptosis of neuronal and haemopoietic cells, respectively. Significantly, both of these initial studies suggested that the signalling pathway initiated by activation of Ras, and culminating in the activation of the mitogen-activated protein kinases (MAPKs) p44 erk1 and p42 erk2, was thought to play a lesser role in prevention of apoptosis. Since then, numerous studies have implicated PI 3-kinase as an anti-apoptosis signal, and a major focus has been on the role of the serine/threonine kinase, protein kinase B (PKB) or Akt, in mediating this effect (reviewed by Datta et al. [3]). PKB is thought to promote survival through phosphorylation of various cellular proteins, including the Forkhead family of transcription factors FKHR, FKHL1 and AFX [4–7], the apoptosis-initiating protease caspase-9 [8], glycogen synthase kinase-3 [9] and Bad [10,11], although the exact role of each of these downstream events in the context of cell survival is still not well defined and, in some cases, conflicting results have been reported [12,13].

The Bcl-2 family of genes and proteins have now been well described as mediators of either cell survival or cell death (reviewed in [14,15]). The pro-survival members include Bcl-2, Bcl-X_L, Bcl-w, A1 and Mcl-1, whereas members of the family that promote apoptosis include Bax, Bad, Bak and Bid. The major factor determining whether or not the proteins promote survival is the presence of several homologous domains, known as Bcl-2 homology (or BH) domains. These are thought to

mediate protein–protein interactions and it appears that a BH3 domain in the absence of others is one criterion that distinguishes the pro-apoptosis proteins. Whereas overexpression of these gene products has been useful in delineating their intracellular functions, the means by which the genes are regulated by cytokines or inducers of apoptosis has not been completely defined. Furthermore, regulation at the level of protein synthesis could also play an important role in determining the intracellular concentration of the Bcl-2 family members.

The emphasis of the current work has been on changes in expression of the Bcl-2 family member Mcl-1, which was the only detectable Bcl-2 family member whose level of protein expression was altered significantly by cytokine treatment of TF-1 cells. Mcl-1 was originally identified as a result of its up-regulation in a human myeloblastic leukaemia cell line that was induced to differentiate along the monocyte lineage [16]. It can act as a survival factor in multiple haemopoietic cell types, both in tissue culture [17] and when expressed in transgenic mice [18]. The role of Mcl-1 as a survival mediator in haemopoietic cells in response to vascular endothelial growth factor has also been reported [19]. Part of the effect of Mcl-1 may result from its ability to associate with Bax [17], although this has not been studied in great detail. The main effect of Mcl-1 seems to be to prolong survival, as opposed to inhibition of apoptosis, especially when compared with Bcl-2 or Bcl-X_L. In fact, a very recent study has shown that the *mcl-1* gene knockout is embryonic lethal, but without any obvious effects on apoptosis during embryogenesis [20]. Further genetic analysis is required to delineate Mcl-1 function in the developed animal. *mcl-1* has recently been described as an early response gene when cells are stimulated with granulocyte/macrophage colony-stimulating factor (GM-CSF), suggesting that it may play a role in cytokine-mediated survival of haemo-

Abbreviations used: PI 3-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; GM-CSF, granulocyte/macrophage colony-stimulating factor; ERK, extracellular-signal-regulated protein kinase; FBS, fetal bovine serum; MEK, MAPK/ERK kinase; IL, interleukin; 4E-BP1, eukaryotic initiation factor 4E binding protein-1.

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poietic cells [21,22]. Our current studies investigating several signalling events involved in regulation of Mcl-1 have found some unique differences from those previously reported [21,22]. In contrast to the findings of Wang et al. [22], which suggested that PI 3-kinase regulates transcription of Mcl-1, a novel aspect of our study has been the demonstration that while the Ras/extracellular-signal-regulated protein kinase (ERK) pathway is important for regulation of *mcl-1* gene expression, the PI 3-kinase pathway is not involved in the regulation of its mRNA expression, but rather is essential for production of Mcl-1 protein. These findings suggest that an additional effect of the PI 3-kinase pathway in promoting cell survival, and thereby inhibiting apoptosis, may be mediated specifically by the increased translation of pro-survival proteins such as Mcl-1.

EXPERIMENTAL

Antibodies and reagents

Anti-Mcl-1 antibody (sc-819) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Bak (06-536), anti-Bax (06-499) and anti-PKB (06-558) specific antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-phospho-Ser-473 PKB antibody (9270), and the anti-phospho-MAPK (9106) were purchased from New England Biolabs (Beverly, MA, U.S.A.). The RiboQuant Multi-Probe RNase Protection Assay System, including the multi-probe template sets hAPO-2 (45121P) and hAPO-2c (45609P), were purchased from Pharmingen (San Diego, CA, U.S.A.). C₂-, C₂-dihydro- and C₆-ceramides were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.). LY-294002 was from Calbiochem (La Jolla, CA, U.S.A.). U0126 was from Promega (Madison, WI, U.S.A.). [³⁵S]Methionine and [α -³²P]UTP were purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Recombinant human GM-CSF was from R&D Systems (Minneapolis, MN, U.S.A.). RPMI 1640, fetal bovine serum (FBS), Protein G-Sepharose beads and Trizol reagent were purchased from Canadian Life Technologies (Burlington, ON, Canada).

Cell-culture conditions and stimulations

RPMI 1640 was supplemented with 10% FBS, 10 μ M 2-mercaptoethanol, 100 units/ml penicillin G and 100 μ g/ml streptomycin prior to using for cell culture. The factor-dependent haemopoietic cell line TF-1 (American Type Culture Collection) was passaged in RPMI 1640 supplemented with 1% CGMI (human GM-CSF)-conditioned medium. Prior to stimulation, cells were starved of cytokine and cultured overnight in low-serum medium (0.5% FBS, v/v). Cells were then washed once with PBS and incubated at 10⁶ cells/ml in low-serum medium with and without recombinant human GM-CSF.

RNase-protection assays

Total RNA was isolated from cultured cells using Trizol reagent and subjected to the RiboQuant Multi-Probe RNase Protection Assay System according to the manufacturer's specifications. Briefly, a 2 μ g portion of total RNA was incubated overnight at 56 °C with a ³²P-labelled antisense RNA probe set generated by T7 polymerase-directed *in vitro* transcription. Irrelevant mouse, human and yeast RNAs were also incubated overnight with probe sets to control for non-specific hybridization. Following hybridization, excess probe and other single-stranded RNA were subjected to RNase digestion and 'RNase-protected' probes were purified and separated on denaturing polyacrylamide gels. Protected mRNA species in the original RNA sample were

quantified based on the intensity of the corresponding protected probe fragment, as determined by direct quantification with a Molecular Imager (Bio-Rad). To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping-gene transcript, L32.

Stimulation and immunoblotting

Cells were deprived of cytokine and plated in low-serum medium (0.5% FBS, v/v) for 8 h followed by re-stimulation with 10 ng/ml GM-CSF for the indicated times in the presence or absence of the PI 3-kinase inhibitor LY-294002 (up to 50 μ M), in the presence or absence of the MEK (MAPK/ERK kinase) inhibitor U0126 (up to 25 μ M) or in the presence or absence of ceramide analogues (up to 25 μ M). Pre-incubation with inhibitors was generally for 15 min followed by cytokine stimulation. Cells were lysed with ice-cold solubilization buffer (10 mM Tris/HCl, pH 7.4/150 mM NaCl/5 mM EDTA/1% Nonidet P40/1% deoxycholic acid/0.25 mM PMSF/1 μ M pepstatin/0.5 μ g/ml leupeptin/10 μ g/ml soya bean trypsin inhibitor) and nuclei were pelleted by centrifugation (20000 g, 1 min). Supernatants were transferred to new tubes and normalized for total protein using a Pierce protein assay. Equal volume of 2 $\times$ reducing sample buffer was added to each sample followed by boiling for 5 min. Cell extracts (50 μ g) were resolved on SDS/PAGE (12% gel) and proteins transferred to nitrocellulose by semi-dry blotting. Membranes were blocked with 5% (w/v) skimmed milk containing 0.05% sodium azide overnight at room temperature and probed with a 1:500 dilution of antibodies specific for Mcl-1, Bak, Bax and Bcl-X_L at room temperature. Primary antibody was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham). In cases where blots are shown, they are representative of at least three (usually more) independent experiments that gave similar results.

Determination of Mcl-1 half-life

To determine the effect of PI 3-kinase inhibition on the half-life of Mcl-1 in TF-1 cells, exponentially growing cells were incubated for 30 min in methionine-free medium supplemented with 0.5% dialysed FBS and 10 ng/ml GM-CSF. Cells were pulse-labelled with 1 mCi/ml [³⁵S]methionine (ICN; 10 mCi/ml stock solution, > 1000 Ci/mmol) for 15 min at 37 °C and chased in growth medium containing 0.5% FBS, 10 ng/ml GM-CSF and 10 mM methionine in the presence or absence of LY-294002. At various times after the chase, 1 $\times$ 10⁷ cells/sample (1 mg of solubilized protein) were lysed in 500 μ l of solubilization buffer immediately followed by removal of nuclei by centrifugation (20000 g, 1 min). Whole-cell lysates were normalized for total protein using the Pierce assay. The supernatant was transferred to new chilled tubes and pre-cleared twice, for 45 min each time, with 20 μ l of packed volume of Protein G-Sepharose. After pre-clearing, the beads were pelleted and lysates transferred to new chilled tubes followed by the addition of 4 μ g of anti-Mcl-1 antibody. Immunoprecipitations were performed overnight at 4 °C with continuous mixing. The samples then received 20 μ l of a 1:1 suspension of Protein G-Sepharose beads in solubilization buffer with mixing at 4 °C for an additional 1 h. The beads were pelleted and washed five times with fresh solubilization buffer. In between washes, the beads and bound Mcl-1 protein were transferred to new chilled tubes to minimize non-specific binding of [³⁵S]methionine. The beads were then resuspended in 20 μ l of 2 $\times$ SDS sample buffer followed by boiling for 5 min. Mcl-1 immune complexes were resolved by SDS/PAGE (12% gels) and visualized by autoradiography. The Mcl-1 specific bands were quantified with a PhosphorImager.

Translational studies

TF-1 cells were starved of cytokine and cultured overnight in low-serum medium (0.5% FBS, v/v). Cells were then washed three times with PBS and seeded in methionine-free medium supplemented with 0.5% dialysed FBS. After 30 min at 37 °C, cells were pretreated with LY-294002, U0126, C₆- or C₂-ceramide, or vehicle alone for an additional 15 min. Labelling was initiated by adding 1 mCi/ml [³⁵S]methionine in growth medium containing 0.5% dialysed FBS and 10 ng/ml GM-CSF. At various times after labelling, 1 × 10⁷ cells were lysed in 500 µl of solubilization buffer immediately followed by removal of nuclei by centrifugation (20000 g, 1 min). Whole-cell lysates were normalized for total protein using the Pierce assay. Cell extracts were pre-cleared and subjected to immunoprecipitation with anti-Mcl-1 antibody and analysed as described above.

RESULTS

RNase-protection analysis of Bcl-2 family expression

In TF-1, a human erythroleukaemia cell line, the presence of GM-CSF or interleukin (IL)-3 is necessary for growth and survival [23,24]. Following a period of cytokine deprivation, and re-addition of GM-CSF, RNase protection assays using the RiboQuant hAPO-2 template set were used to measure the level of expression of Bcl-2 family members to assess their involvement in the pro-survival effect of this cytokine. GM-CSF caused an increase in the level of Mcl-1 message that was both time- (Figure 1) and dose-dependent (results not shown). This increase appeared to be transient, in that it was maximal by 2 h and declining by 4 h (Figure 1). These data are generally consistent with *mcl-1* being an early response gene [21]. The other Bcl-2 family members detected included Bik, Bax, Bak and Bcl-X_L. When message levels were quantified and normalized with respect to

Figure 1 GM-CSF treatment increases Mcl-1 and Bcl-X_L mRNA levels

TF-1 cells were deprived of cytokine and plated in low-serum medium for 8 h followed by re-stimulation with GM-CSF for 0, 2 and 4 h. Total RNA was isolated and analysed by RNase-protection assays using the hAPO-2 multi-probe template set. Radioactivity was quantified by a Molecular Imager. To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping-gene transcript, L32. Data represent means ± S.D. of triplicate determinations from three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 2 MEK activity is required for GM-CSF-induced up-regulation of Mcl-1 mRNA

TF-1 cells were starved of cytokine and cultured overnight in low-serum medium. Cells were then re-stimulated with GM-CSF alone for 2 h (lane 1) or with GM-CSF in the presence of LY-294002 (50 µM; lane 2), C₆-ceramide (50 µM; lane 3) or the MEK inhibitor U0126 (25 µM; lane 4). Total RNA was isolated and analysed by RNase-protection assays using the hAPO-2 multi-probe template set. To control for equal loading between samples, the level of each mRNA species was normalized against that of the housekeeping-gene transcript, L32. Data for the unstimulated, GM-CSF-alone and LY-294002-treated samples are the means ± S.D. from triplicate determinations. The C₆-ceramide- and U0126-treated samples are the means ± range from duplicate determinations. Results are representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the L32 transcript, Bcl-X_L message was also found to increase with GM-CSF treatment. The other Bcl-2 family members did not change significantly in response to GM-CSF stimulation (Figure 1).

The signalling pathways activated by the GM-CSF receptor responsible for the effects on Mcl-1 and Bcl-X_L were investigated, at the levels of both mRNA and protein expression. The PI 3-kinase pathway was blocked selectively with wortmannin or LY-294002, at concentrations of 100 nM or 50 µM, respectively, which we and many others have shown previously to be effective in blocking PI 3-kinase activity [25]. We also used the MEK inhibitor U0126 [26] to assess the requirement for enzymes downstream of MEK activation. Finally, we were interested in comparing the effect of a cell-permeable ceramide, C₆-ceramide, which is known to induce apoptosis in these cells, at least partially through the inhibition of PKB/Akt [27,28].

TF-1 cells were deprived of cytokine and then stimulated with GM-CSF for 2 h, in the presence or absence of the various compounds. When the level of Mcl-1 message was normalized against the level of L32 message, the increase observed following a 2 h incubation with GM-CSF was not affected by pre-incubation of the TF-1 cells with LY-294002 (Figure 2).

Figure 3 Inhibition of PKB and MAPK by various inhibitors

TF-1 cells were starved of cytokine for 8 h followed by a 15 min pre-incubation with LY-294002 (50 μ M), C₆-ceramide (25 μ M) or U0126 (25 μ M) where indicated, followed by a 10 min stimulation with GM-CSF. Cells were harvested and equal amounts of protein lysates analysed by immunoblotting for phospho-MAPK (top panel) or phospho-PKB (middle and lower panels).

Treatment with wortmannin also had no effect (results not shown). C₆-ceramide treatment, which we have shown previously inhibits PKB/Akt activation by promoting dephosphorylation of Ser-473 [28], had no effect on Mcl-1 message up-regulation (Figure 2). Thus neither PI 3-kinase nor any of its downstream effectors are necessary for the up-regulation of Mcl-1 message induced by GM-CSF. However, blocking the ERK pathway by the MEK inhibitor U0126 completely blocked the increase in Mcl-1 message (Figure 2). Another inhibitor of MEK, PD98059, gave similar results (not shown). Generally, we found that U0126 was a more potent and reproducible inhibitor of MEK, possibly because of solubility problems with PD98059 at concentrations required to fully inhibit MEK. Because of this, we performed most experiments with U0126.

In the same experiment, the effect of MEK or PI 3-kinase inhibitors, or ceramide treatment, on the GM-CSF-induced increase in Bcl-X_L message was also assessed. Similar to the effects seen with Mcl-1, the increase in Bcl-X_L message was inhibited by U0126, suggesting the involvement of MEK/ERK (Figure 2). However, PI 3-kinase inhibition also blocked the increase in Bcl-X_L message (Figure 2). Together, these results suggest that a MEK-controlled and a PI 3-kinase-controlled pathway together regulate Bcl-X_L transcription. C₆-ceramide had a relatively small effect on Bcl-X_L message. LY-294002 treatment had a much more profound effect than that of

Figure 4 PI 3-kinase and MEK activities are required for GM-CSF-induced Mcl-1 up-regulation

TF-1 cells were deprived of cytokine, plated in low-serum medium for 8 h and re-stimulated with GM-CSF in the presence or absence of LY-294002 (50 μ M) or the MEK inhibitor U0126 (25 μ M). Cells were harvested after 0, 2 and 4 h and equal amounts of protein lysates were analysed by immunoblotting for the expression of Mcl-1. The same membrane was subsequently re-probed with antibodies specific to Bax, and Bcl-x_L. Unstim, unstimulated.

ceramide. This result is consistent with the ability of ceramide to partially inhibit PKB activation as a result of Ser-473 dephosphorylation, whereas LY-294002 completely inhibits PKB [28]. Thus PKB is likely to be involved in PI 3-kinase-regulated Bcl-X_L, but not Mcl-1, mRNA levels.

Phospho-specific antibodies for PKB and ERK1/2 were used to confirm that each of these pathways was blocked selectively by the respective inhibitors. LY-294002 and C₆-ceramide had their expected effects on PKB Ser-473 phosphorylation (Figure 3), whereas these drugs had no effect on p44 erk1 or p42 erk2 phosphorylation. The lack of any effect of PI 3-kinase inhibitors on ERK activation in TF-1 cells is consistent with an earlier study in which we reported the same phenomenon in MC/9 cells [25]. As expected, U0126 blocked ERK1/2 phosphorylation without any effect on PKB phosphorylation (Figure 3).

Analysis of Bcl-2 family protein levels

Incubation of TF-1 cells in the presence of either PI 3-kinase or MEK inhibitors during overnight incubations with GM-CSF caused a significant reduction in the level of Mcl-1 protein without affecting the levels of other Bcl-2 family proteins (results not shown). To mimic conditions used when examining mRNA levels, cytokine was removed and the serum was reduced for 8 h to lower the basal level of Mcl-1 protein, followed by re-stimulation with GM-CSF for short periods of time (2 and 4 h) in the presence or absence of PI 3-kinase or MEK inhibitors. This allowed us to determine whether LY-294002 or U0126 were specifically blocking a GM-CSF-stimulated signal required for the up-regulation of Mcl-1 protein synthesis. Figure 4 shows that 2 and 4 h re-stimulation with GM-CSF caused a large increase in Mcl-1 protein, which was largely blocked by either LY-294002 or U0126. The effects seen with U0126 might be expected, based on the observation that this compound blocked the increase in Mcl-1 message. However, inhibition of Mcl-1 protein expression by PI 3-kinase inhibition occurred despite there being no effect of LY-294002 on Mcl-1 mRNA levels.

Figure 5 C₆-ceramide inhibits GM-CSF-induced increase in Mcl-1 protein levels

(A) TF-1 cells were deprived of GM-CSF, placed in low-serum medium and re-stimulated with GM-CSF in the presence or absence of C₆-ceramide (25 μ M) or LY-294002 (50 μ M). After re-stimulation (4 h), TF-1 cells were lysed in solubilization buffer and equal amounts of their protein lysates were analysed by immunoblotting for the expression of Mcl-1 and Bak. (B) Similar to (A), except that LY-294002 and C₆-ceramide were used at the indicated concentrations and Mcl-1, Bax and Bcl-X_L were immunoblotted.

Analysis of Bcl-X_L protein expression proved to be very different. Despite the large increase in the level of Bcl-X_L mRNA following treatment with GM-CSF, which was blocked by the various inhibitors, all of the treatments tested resulted in little or no consistent change in the level of Bcl-X_L protein in several experiments (Figure 4). The same was true following 16 h of treatment with the various inhibitors (results not shown). This suggests that the half-life of Bcl-X_L protein is much longer than that of Mcl-1, but more detailed comparisons will be required to verify this.

The effects on protein expression of Mcl-1 and other Bcl-2 family proteins were investigated in greater detail. As shown in Figure 5, the presence of GM-CSF maintained elevated levels of Mcl-1 protein, which were decreased in cells starved of cytokine for 16 h. When the cells were incubated with LY-294002 to block PI 3-kinase activity, or C₆-ceramide, which may have multiple effects but can also inhibit PKB activation (see Figure 3 and [28]), the level of Mcl-1 protein was greatly decreased in both starved cells and cells incubated with GM-CSF. Also, the effects of both LY-294002 and C₆-ceramide were dose-dependent (Figure 5B), although only a few concentrations were tested. As shown in the lower panels of Figures 5(A) and 5(B), the levels of Bcl-X_L, Bak and Bax proteins were not changed significantly by the various treatments.

The potential role of p70 S6 kinase in the synthesis of Mcl-1 was also investigated by inhibition using rapamycin. At

Figure 6 Inhibition of p70 S6 kinase activity attenuates Mcl-1 up-regulation

TF-1 cells were deprived of cytokine (C), plated in low-serum medium for 8 h and re-stimulated with GM-CSF in the presence or absence of the PI 3-kinase inhibitor LY-294002 (10–50 μ M) or the p70 S6 kinase inhibitor rapamycin (25–100 ng/ml). Cells were harvested 6 h after re-stimulation and equal amounts of protein lysates were analysed by immunoblotting for the expression of Mcl-1. Two representative experiments are shown. Samples in the lower panel were on the same gel as some of the samples shown in Figure 5(B) and thus the first five lanes are repeated to allow direct comparison.

Figure 7 PI 3-kinase activity has no effect on the half-life of Mcl-1

TF-1 cells were pulse-labelled with [³⁵S]Met for 15 min and chased in low-serum growth medium containing methionine (10 mM) and GM-CSF in the presence or absence of LY-294002 (50 μ M). At the indicated times after chase (min), whole-cell lysates were prepared and Mcl-1 was immunoprecipitated. Mcl-1 was resolved on an SDS/PAGE gel and visualized and quantified by a Molecular Imager. The level of Mcl-1 at each time point was converted to the percentage of Mcl-1 remaining with respect to the zero time point and plotted to determine half-life values. This experiment was performed in duplicate with similar results; one set of samples is shown.

100 ng/ml, rapamycin completely inhibits cytokine activation of p70 S6 kinase [29], and as shown in Figure 6 there was partial inhibition of Mcl-1 protein synthesis. The inhibition by rapamycin was not as complete as blocking PI 3-kinase with LY-294002, nor was it as effective as ceramide. This suggests that p70

Figure 8 PI 3-kinase inhibition blocks Mcl-1 protein translation

TF-1 cells were placed in medium containing [35 S]Met and then stimulated with GM-CSF alone or with LY-294002, C₆-ceramide or U0126. After 1, 3 or 4 h Mcl-1 was immunoprecipitated, fractionated by SDS/PAGE and quantified by a Molecular Imager. The autoradiogram used to generate the data is shown in the lower panel. This is representative of three independent experiments that showed similar results. Unstim, unstimulated.

S6 kinase may play a partial role in regulation of Mcl-1 protein synthesis.

Effects on degradation or translation of Mcl-1 protein

Pulse-chase experiments were carried out to analyse the rate of degradation of Mcl-1. Labelling of TF-1 cells for a 15 min pulse with [35 S]methionine, followed by quenching with unlabelled methionine in the presence or absence of LY-294002 or C₆-ceramide, showed that there was no difference in the rate of degradation of Mcl-1 (Figure 7). Results of this analysis revealed that the half-life of Mcl-1 protein was approx. 20–30 min, but blocking PI 3-kinase with LY-294002 did not result in an enhanced degradation of the protein. The band indicated as Mcl-1 was identified by immunoblotting, and was the only band detected with the anti-Mcl-1 antibody. Other 35 S-labelled bands are likely to represent non-specifically precipitated proteins.

When cells were labelled with [35 S]methionine and the synthesis of Mcl-1 protein was examined for up to 4 h, there was an enhancement of Mcl-1 protein synthesis in response to incubation with GM-CSF. Likewise, there was clearly a reduction in Mcl-1 protein translation caused by incubation with LY-294002, ceramide or U0126 (Figure 8). Together, these results support the conclusion that synthesis of Mcl-1 protein is dependent on PI 3-kinase activity, even though PI 3-kinase is not involved in regulating expression of Mcl-1 mRNA.

DISCUSSION

Over the last few years, there has been an increased understanding of the role of Bcl-2 family members in regulating a cell's decision to undergo apoptosis, or to survive. In many instances, the

overexpression of various Bcl-2 family proteins has been correlated with a function of the particular family member as either a pro-survival or pro-apoptotic agent. Therefore, the implication has been that alterations in the expression of Bcl-2 family members could play a part in a cell's fate, and as a consequence, the possible cellular signal-transduction pathways that regulate the Bcl-2 family of proteins can determine whether a cell survives or undergoes apoptosis. We have been interested in regulation of the Bcl-2 family proteins by phosphorylation [12,30] or, in the present study, by regulation of protein expression. Our aim is to determine how these events may be involved in the pro-survival signal provided by cytokines such as GM-CSF and IL-3.

GM-CSF-induced signalling events trigger activation of both the Ras/MAPK pathway and the PI 3-kinase cell-survival pathways. The Ras/MEK/ERK pathway is involved in the transduction of growth signals in virtually all types of cell and couples a growth and survival stimulus initiating at the cell surface to transcription factors controlling gene expression [31,32]. Our group [12,30] and others [33–35] have demonstrated that phosphorylation of the pro-apoptotic protein Bad at Ser-112 is mediated by the MEK/ERK pathway and that this MEK-dependent phosphorylation of Bad may be a contributing factor in the ability of cytokines to promote cell survival. The present study suggests that the Ras/MEK/ERK pathway also serves a role in the transcriptional activation of the pro-survival factor Mcl-1, as has been reported previously [36,37]. Unlike inhibition of MEK, blocking PI 3-kinase and PKB did not alter the up-regulation of Mcl-1 message following stimulation with GM-CSF. However, inactivating PI 3-kinase did block the increase in Mcl-1 protein synthesis induced by GM-CSF and this effect was not due to an increase in Mcl-1 degradation or alterations in Mcl-1 protein stability. Thus GM-CSF-induced Mcl-1 up-regulation is controlled at the translational level by a PI 3-kinase-controlled pathway. The inhibitory effects of ceramide treatment support a role for PKB in mediating this PI 3-kinase effect, based on reports showing that ceramide can inhibit PKB activation, but there are also other possible effects of this lipid. However, p70 S6 kinase, which can be regulated partially by PI 3-kinase and is involved in regulation of protein translation [38], also appears to be playing a role and this will require further investigation.

Our findings are contradictory to the work of Wang and colleagues [22], which suggests that PI 3-kinase regulates *mcl-1* gene expression through the activation of an immediate-early transcription factor. In their study, Wang and co-workers demonstrated that PI 3-kinase inhibitors or dominant negative PI 3-kinase mutants block the transcriptional up-regulation of Mcl-1 mRNA. The major factor that may contribute to the discrepancies observed between the two studies is the extent of cross-talk between the PI 3-kinase and MAPK pathways. First, the Wang group performed some of their work in the IL-3-dependent pro-B cell line BAF/3. In these cells, blocking with a dominant negative p85 has been shown to inhibit activation of MAPK [39]. In another study, overexpression of dominant negative p85 was shown to block IL3-induced ERK activation in FDC-P1 cells [40], but we have shown that inhibition of PI 3-kinase has no effect on IL-3-induced MAPK activation in MC/9 cells [25] as well as in FDC-P1 cells (D. Chiu and V. Duronio, unpublished work). Therefore, we suggest that the effects of the dominant negative p85 are not necessarily due to inhibition of PI 3-kinase and thus one cannot attribute inhibitory effects of dominant negative p85 to be due to effects solely on the PI 3-kinase pathway. Since the studies by Wang and co-workers did not address the effects of blocking PI 3-kinase on

MAPK activation, it is possible that the effects seen on Mcl-1 mRNA transcription were manifested through a reduction in MAPK signalling. In TF-1 cells, we have shown clearly that blocking PI 3-kinase had no effect on MAPK activation (Figure 3). Again, this is perfectly consistent with our previous studies on another cell line [25]. Blocking PI 3-kinase had no effect on Mcl-1 mRNA levels, but we could abolish increases in Mcl-1 mRNA by inhibiting MEK. Our results suggest that the PI 3-kinase pathway is not regulating transcription of Mcl-1, and thus the method of inhibiting PI 3-kinase, and the corresponding effects on MAPK activity, must be considered prior to drawing conclusions about the specific role of PI 3-kinase. We cannot conclude, however, that PI 3-kinase pathways do not regulate transcription of Bcl-2 family proteins in general, since we have shown that cytokine-induced increases in Bcl-X_L mRNA were blocked by PI 3-kinase inhibitors in the same experiments.

A wide variety of physiological signals, such as those generated by cytokines and growth factors, can activate the sphingomyelin pathway resulting in the production of the lipid second messenger ceramide, which is a well-known inducer of apoptosis [41]. The serine/threonine kinase PKB is a well-characterized downstream effector of PI 3-kinase, and reports by several groups have identified PKB as a downstream target of ceramide-mediated apoptosis [27,28]. Recent work from our laboratory has shown that PKB phosphorylation at an activating site, Ser-473, may be a target of ceramide-activated protein phosphatases [28]. Thus our current study suggests that the mechanism by which ceramide may function to inhibit a GM-CSF-induced PI 3-kinase-dependent increase in Mcl-1 protein is through the inactivation of PKB.

An involvement of PKB in the PI 3-kinase-dependent regulation of Mcl-1 protein appears probable, since PKB was shown recently to phosphorylate components of the translational machinery. For example, eukaryotic-initiation-factor-4E-binding protein-1 (4E-BP1) phosphorylation by the PKB pathway inactivates the function of 4E-BP1 to repress mRNA translation [42,43]. In addition, PI 3-kinase, via phosphatidylinositol-dependent kinase-1 and PKB, may have multiple inputs on p70 S6 kinase, a known regulator of protein synthesis [38], although we have shown here that the effects of rapamycin (and thus complete p70 S6 kinase inhibition) on Mcl-1 protein up-regulation were not as dramatic as those of blocking PI 3-kinase (Figure 6). Thus there are several possible ways in which PI 3-kinase-dependent pathways affect translation of Mcl-1 protein.

Another important observation in this study is the contrast in regulation of mRNA levels of Bcl-2 family members, compared with expression of the corresponding proteins. The greatest contrast is observed in comparing Bcl-X_L and Mcl-1. Whereas the change in Bcl-X_L mRNA expression was even more dramatic than that of Mcl-1, there was no significant change in the level of Bcl-X_L protein. This demonstrates the importance of analysing the protein levels before drawing conclusions based solely on changes in mRNA levels. The more dramatic changes in the level of Mcl-1 protein compared with the other proteins can probably be attributed to the presence of PEST sequences (protein domains containing a high proportion of proline, glutamic acid, serine and threonine residues) in the N-terminal region of Mcl-1, which is not conserved in other Bcl-2 family proteins [44]. It is interesting that a recent study suggested that deletion of the PEST domain from Mcl-1 did not change its half-life [45], but the half-life of the protein was determined by blocking protein synthesis with cycloheximide, which may complicate such measurements.

The implication from this study and others [21,22] investigating changes in Mcl-1 expression is that increased Mcl-1 protein

contributes to the anti-apoptotic effect of GM-CSF. Huang et al. [46] have also suggested that stem-cell factor and IL-5 may promote survival via increased expression of Mcl-1. However, there is no strong evidence that this is the primary role of Mcl-1. In fact, the loss of the *mcl-1* gene in mice results in the failure of embryos to implant [20] and thus it plays a key role in mammalian development. Changes in Mcl-1 have also been observed in haemopoietic cell differentiation [16] and its precise role in differentiation remains to be determined. One could easily speculate that changes observed in Mcl-1 expression may also play a role in cytokine effects on cell differentiation.

In summary, through binding to its receptor, GM-CSF can induce anti-apoptotic signalling via stimulation of the Ras/ERK and PI 3-kinase signal-transduction cascades. Several research findings suggest that the cytoprotective actions of these signalling pathways may converge on members of the Bcl-2 family. Our data here demonstrate that ERK- and PI 3-kinase-dependent pathways are required for GM-CSF-induced up-regulation of the Mcl-1 pro-survival factor. Specifically, we show that the PI 3-kinase pathway is most important for translation of Mcl-1 protein, rather than for increased gene transcription. In addition, we provide evidence that treatment of cells with ceramide, which is known to cause apoptosis and inhibit activation of PKB, also correlates with partial inhibition of the translation of Mcl-1 protein, but not transcriptional activation of Mcl-1.

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