Growth-factor-dependent phosphorylation of Bim in mitosis

Mário GRÃOS, Alexandra D. ALMEIDA and Sukalyan CHATTERJEE¹

Centro Biologia Desenvolvimento, Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal

The regulation of survival and cell death is a key determinant of cell fate. Recent evidence shows that survival and death machineries are regulated along the cell cycle. In the present paper, we show that BimEL [a BH3 (Bcl-2 homology 3)-only member of the Bcl-2 family of proteins; Bim is Bcl-2-interacting mediator of cell death; EL is the extra-long form] is phosphorylated in mitosis. This post-translational modification is dependent on MEK (mitogen-activated protein kinase/extracellular-signal-regulated kinase) and growth factor signalling. Interestingly, FGF (fibroblast growth factor) signalling seems to play an essential role in this process, since, in the presence of serum, inhibition of FGF receptors abrogated phosphorylation of Bim in mitosis. Moreover,

we have shown bFGF (basic FGF) to be sufficient to induce phosphorylation of Bim in serum-free conditions in any phase of the cell cycle, and also to significantly rescue cells from serum-deprivation-induced apoptosis. Our results show that, in mitosis, Bim is phosphorylated downstream of growth factor signalling in a MEK-dependent manner, with FGF signalling playing an important role. We suggest that phosphorylation of Bim is a decisive step for the survival of proliferating cells.

Key words: apoptosis, Bim (Bcl-2-interacting mediator of cell death), cell cycle, fibroblast growth factor (FGF), mitosis, signal transduction.

INTRODUCTION

The regulated co-ordination of cell division and cell death is a key determinant for normal physiological functions and cell number homeostasis [1,2]. Apoptosis (programmed cell death) is a mechanism by which organisms eliminate unwanted or damaged cells [3–5]. Cells have developed surveillance mechanisms that control the progression through the cell cycle by ensuring that the initiation of one event occurs only after successful completion of the previous one [6]. The evidence pointing to the cross-talk between the cell cycle and apoptosis is sparse, but not lacking, although the details are still largely unknown [7–9]. Caspases and the pro-apoptotic Bcl-2 family of proteins have been shown to be up-regulated in S phase after G₀ arrest [10,11]. In addition, Bcl-2 and Mcl-1 have been reported to influence and be influenced by the cell cycle (for a review see [12]). Moreover, activation of several cell-cycle-related proteins has been implicated in apoptosis in post-mitotic neurons [13]. This communication between cell-cycle regulators and the apoptotic machinery led us to the hypothesis that competence for self-destruction of cells might change along the cycle.

The mitochondria play a central role in apoptosis by releasing death-promoting molecules, under apoptotic stimuli such as cytochrome *c*, thus engaging downstream executioner pathways for subsequent cell death [4,5]. The mitochondrial ‘gates’ are crucial in initiating or restraining the downstream cascades that lead to apoptosis. The Bcl-2 family proteins, consisting of both pro- and anti-apoptotic members, share from one to four homology domains (BH1–BH4), and act as gatekeepers by regulating the translocation of death-promoting molecules from the mitochondrial intermembrane space [12,14]. The members of the Bcl-2 family of proteins are regulated at various levels, each of them being specifically downstream of different, but partially overlapping, apoptotic and survival signals, but all of them in unison

sense a wide range of stimuli [15]. Moreover, the ability of these proteins to interact among themselves through the BH domains provides cells with a mechanism for integration of information from within and around the cell (for a review see [12]). A subset of Bcl-2-related proteins share only the BH3 domain, and are referred to as ‘BH3-only proteins’ [16]. All members of this subgroup are pro-apoptotic, and are thought to exert their function by activating or enhancing other pro-apoptotic Bcl-2-related proteins, or by preventing the action of anti-apoptotic proteins, hence tilting the balance between anti- and pro-apoptotic Bcl-2 molecules [12].

Bim (Bcl-2-interacting mediator of cell death) is a BH3-only protein that exists mostly in three isoforms, BimS (short), BimL (long) and BimEL (extra-long), generated by alternative splicing [17]. BimL and BimEL have been reported to be modulated at the level of expression [18–21], post-translational modifications and localization. In healthy cells, BimL and BimEL are sequestered to the microtubules (MTs). In response to several apoptotic stimuli, BimL and BimEL are released and re-localized to the intracellular organelles, and trigger apoptosis [22,23]. Release of Bim from the MTs and subsequent apoptosis has also been described to occur as a consequence of phosphorylation downstream of the JNK (c-Jun N-terminal kinase) pathway [24]. Interestingly, other reports have shown that Bim may be attenuated through phosphorylation downstream of survival pathways in a MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase]/ERK-dependent manner [25–28], resulting in degradation [29–31] and possibly loss of ability to interact with anti-apoptotic Bcl-2 and Bcl-X_L.

In the present paper, we report that in NIH 3T3 cells, Bim is phosphorylated in mitosis downstream of growth factor signalling in a MEK-dependent manner, suggesting that this pro-apoptotic molecule is attenuated in this phase of the cell cycle. Moreover, we show that FGF (fibroblast growth factor) signalling, shown

Abbreviations used: bFGF, basic fibroblast growth factor; BH, Bcl-2 homology; Bim, Bcl-2-interacting mediator of cell death; P-Bim, phosphorylated Bim; BimEL, extra-long Bim; BimL, long Bim; BimS, short Bim; Cdk, cyclin-dependent kinase; CIP, calf intestine phosphatase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; FGF, fibroblast growth factor; HA, haemagglutinin; HEK, human embryonic kidney; IP, immunoprecipitation; MEK, mitogen-activated protein kinase/ERK kinase; MT, microtubule; PI3K, phosphoinositide 3-kinase; TBST, Tris-buffered saline with Tween 20; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

¹ To whom correspondence should be addressed (email sukalyan@igc.gulbenkian.pt).

previously to be involved in survival and proliferation [32,33], plays a major role in phosphorylation of Bim in mitosis. This report provides evidence for the cross-talk between survival and apoptotic machineries in mitosis, indicating intricate quality-control mechanisms in surveillance of cell division.

EXPERIMENTAL

Materials

DMEM (Dulbecco's modified Eagle's medium) with GlutaMAX™-I and 1000 mg/ml glucose, heat-inactivated FBS (foetal bovine serum), non-essential amino acids, PBS, Lipofectamine™, Plus™ reagent and human recombinant bFGF (basic FGF) were obtained from Invitrogen. Nocodazole, taxol, thymidine, poly(L-lysine) and anti-(active ERK p42/p44) antibodies were from Sigma. SU5402, roscovitine, U0126 and LY294002 were from Calbiochem. Purvalanol A was from Tocris (Bristol, U.K.). Anti-Bim antibody was from Stressgen. Anti-pSer⁶⁵-Bim antibody was from Upstate Biotechnology. Anti-HA (haemagglutinin) monoclonal antibody was from Santa Cruz Biotechnology. Anti-(total ERK p42/p44), anti-Akt and anti-pAkt antibodies were from Cell Signaling Technology. Protein A-Sepharose beads were from Amersham Biosciences. NheI, XhoI and CIP (calf intestine phosphatase) enzymes were from New England Biolabs.

Plasmid constructs

HA-Bim fusion was constructed by subcloning a HA tag in-frame with the N-terminus of BimEL into the NheI and XhoI sites of pCMS-EGFP + BimEL as described in [25]. The frame was confirmed by local automatic sequencing facilities.

Cell culture and cell-cycle synchronization

NIH 3T3, Swiss 3T3 and HEK-293T (human embryonic kidney) cells were cultured in DMEM plus GlutaMAX™-I and 10% (v/v) FBS at 37°C, under 5% CO₂, except that the medium for HEK-293T cells was supplemented with 0.1 mM minimum essential medium non-essential amino acids. Mitotic NIH 3T3 cells obtained by shake-off were arrested previously by treating with nocodazole (100 ng/ml) or taxol (400 nM) for 12 h. The detached (enriched in mitosis) and adherent cells (non-mitotic) were collected separately. G₁/S-phase NIH 3T3 cells were obtained by double-thymidine block: cells at 40–50% confluence were incubated with 2.5 mM thymidine for 12 h, washed with PBS, and cultured with fresh medium for 8 h, followed by a second treatment with 2.5 mM thymidine for 12 h. Mitotic arrest was obtained by thymidine–nocodazole block: NIH 3T3 cells at 70–80% confluence were arrested in G₁/S phase using 2.5 mM thymidine for 12 h, washed with PBS, and cultured in the presence of 100 ng/ml nocodazole for 12 h. G₂-phase cells were obtained as described for mitotic cells, except that Cdk (cyclin-dependent kinase) inhibitor roscovitine (35 µM) or purvalanol A (15 µM) was added 5 h post-G₁/S release. G₀ quiescent cells were obtained by culturing Swiss 3T3 cells in DMEM supplemented with 0.5% (v/v) FBS for 32 h. Transient serum-deprivation experiments were performed by washing NIH 3T3 cells three times with serum-free medium, followed by incubation with fresh serum-free DMEM for 2 h.

DNA content analysis by flow cytometry (FACS)

Cells were collected after scraping, washed with cold PBS and centrifuged at 3300 g for 5 min. Cells were resuspended in 300 µl

of cold PBS and fixed by adding 700 µl of 100% ethanol (70% final), incubated for 20 min at 4°C, centrifuged at 3300 g for 5 min and resuspended in PBS and 0.1% (v/v) Triton X-100 (Sigma) containing 5 µg/ml RNase (Roche) for 20 min at room temperature (21°C). Cells were then stained with propidium iodide (Sigma) by incubating with 25 µg/ml propidium iodide in PBS. After 10 min, cells were washed twice with PBS, centrifuged at 3300 g for 5 min and resuspended in PBS. Cells were analysed on a FACScalibur flow cytometer, and results were analysed using CellQuest (Becton Dickinson).

Protein extraction, protein determination, SDS/PAGE and Western blotting

Cells were lysed using cold lysis buffer [25 mM Hepes, 5 mM MgCl₂, 1 mM EGTA and 0.5% (v/v) Triton X-100, pH 7.5, supplemented with 2 mM NaF, 1 mM DTT (dithiothreitol), 2 mM PMSF, 20 µg/ml aprotinin, 1.5 µg/ml benzamidine, 10 µg/ml leupeptin and 1 µg/ml pepstatin A] and centrifuged at 20000 g for 15 min at 4°C. Supernatants were collected, and 6 × sample buffer [350 mM Tris/HCl, pH 6.8, 10.3% (w/v) SDS, 300 µl/ml glycerol, 93 µg/ml DTT, 0.12 mg/ml Bromophenol Blue] was added to a final concentration of 1× and heated at 99°C for 5 min. Protein determination was achieved using Bio-Rad Protein Assay reagent before the addition of sample buffer. Samples were loaded and separated on SDS/discontinuous 4–12% (w/v) acrylamide–bisacrylamide (Bio-Rad) gels. Blotting was done using nitrocellulose membranes (Scheicher & Schuell). Membranes were blocked with PBS and 5% (w/v) non-fat dried milk for 1 h, incubated with antibodies diluted 1:1000 in blocking solution (1 h at room temperature), washed with PBS and 0.1% (v/v) Tween 20, incubated with the respective secondary antibodies diluted 1:1000 in blocking solution (1 h at room temperature) and washed with PBS. For anti-pSer⁶⁵-Bim antibody staining, blocking was carried out overnight at 4°C in TBST [Tris-buffered saline with 0.1% (v/v) Tween 20] and 5% (w/v) non-fat dried milk. Primary antibody dilution was 1:2000 in TBST with 5% (w/v) non-fat dried milk (1 h at room temperature), and washes were performed using TBST and 0.5% (w/v) BSA. An ECL[®] (enhanced chemiluminescence) kit (Amersham Biosciences) was used for detection, according to the manufacturer's instructions.

CIP assay

Protein extracts of mitotic cells were obtained as described above. Before the addition of sample buffer, extracts were quantified and incubated with CIP in 1× Buffer 3 (New England Biolabs) at 50 units of enzyme per 100 µg of total protein at 30°C for 30 min.

Transient transfection and IP (immunoprecipitation)

Transient transfections were performed in 2.5 × 10⁵ HEK-293T cells by using 0.5 µg of cDNA, pre-complexed with 0.3 mg/ml Plus™ Reagent and 0.12 mg/ml Lipofectamine™, according to the manufacturer's instructions. After 90 min of incubation at 37°C under 5% CO₂, the medium containing the DNA complexes was replaced by fresh medium. After a further 18 h, cells were treated or not with 20 ng/ml bFGF for 15 min and then collected. Cells were harvested, washed with cold PBS and homogenized in IP buffer [40 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2% (v/v) Nonidet P40, 20% (v/v) glycerol, 50 mM NaF, 1 mM β-glycerophosphate, 1 mM PMSF, 20 µg/ml aprotinin, 1.5 µg/ml benzamidine, 10 µg/ml leupeptin and 1 µg/ml pepstatin A). Homogenates were centrifuged at 20000 g for 10 min at 4°C, and the supernatants were incubated with solid Protein A-Sepharose

beads pre-treated with 0.2 mg/ml anti-HA antibody or anti-pSer⁶⁵-Bim antibody. After 3 h of incubation on a rocking platform at 4°C, the samples were washed three times with IP buffer for 10 min, heat-denatured and analysed by SDS/PAGE.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay

Cells were plated on poly(L-lysine)-coated coverslips. Cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde for 25 min at room temperature, then washed again with PBS and permeabilized using PBS and 0.1% (v/v) Triton X-100 for 15 min at room temperature. After washing with PBS, FITC-conjugated TUNEL staining was carried out using the *In Situ* Cell Death Detection Kit (Roche), according to the manufacturer's instructions. Cells were washed three times with PBS and incubated with 100 ng/ml DAPI (4,6-diamidino-2-phenylindole) (Sigma) for 5 min at room temperature for nuclei staining. Cells were washed with PBS, and coverslips were mounted on glass slides using Vectashield mounting medium (Vector). Cells were visualized under a Leica DM LB2 microscope. Image processing and cell counting were carried out using Image J software.

RESULTS

BimL and BimEL, but not BimS, are phosphorylated in mitosis, but not in G₁/S or G₂ phases of the cell cycle

Using established cell-synchronization methods [34], NIH 3T3 cells were arrested in G₁/S phase or in mitosis (metaphase arrest). The metaphase-arrested cells exhibited the typical round and loosely attached mitotic phenotype, in contrast with the untreated cycling cells or cells arrested in G₁/S phase (Figure 1A). DNA-content analysis revealed that nocodazole- or taxol-treated cells detached by shake-off were predominantly in mitosis, displaying mostly 4n (tetraploid) DNA content, in contrast with untreated cycling mixed population of cells, which were highly enriched in G₁ phase (Figure 1B). The remaining adherent population of nocodazole- or taxol-treated cells was mostly enriched in G₂ phase, consisting of cells that had not yet entered mitosis, as assessed by DNA-content analysis (enrichment in 4n DNA content, Figure 1B, panel 3) and morphology (flat adherent cells, not shown). When re-plated in fresh medium, metaphase-arrested cells were alive and competent to resume the cell cycle. At 12 h after re-plating, morphological features (results not shown) and flow-cytometry analysis revealed that they were viable, since we could not detect a significant sub-G₁ population of cells. Moreover, the cells were able to proliferate, exhibiting more cells with 2n (diploid) DNA, indicating exit from mitosis and entry into G₁ phase (Figure 1B, panel 5). Western-blot analysis of whole-cell protein extracts using anti-Bim polyclonal antibody detected the three most abundant alternative splice isoforms of the protein (BimS, L and EL) (Figure 1C). Extracts obtained from shaken-off metaphase-arrested cells exhibited slow-migrating bands for BimL and BimEL (but not for BimS), in addition to the bands observed migrating in the same position as protein obtained from control or G₁/G₂-phase adherent cells (Figure 1C). All further experiments will focus only on the BimEL isoform.

In order to rule out that the slow-migrating band of Bim could be due to the MT-perturbing agents used to arrest cells in metaphase (see the Experimental section), we obtained an enriched population of mitotic cells without using nocodazole or taxol. Cells were arrested in G₁/S phase (monitored by flow cytometry) and then released, by washing and adding fresh medium, resulting in cell-cycle progression. At 8 h post-release, a considerable

amount of cells had already reached mitosis, endorsed by morphological features (results not shown) and FACS analysis (Figure 2A, upper panel). The cells were shaken off, and Western-blot analysis of protein extracts obtained from the detached cells revealed that the post-translational modification of BimEL took place in normal mitotic cells independently of pharmacological intervention (Figure 2A). To test whether this slow-migrating band was due to phosphorylation, a regulatory mechanism widely used by cells during mitosis [35], extracts prepared from the detached cells were treated with CIP or left untreated. The slow-migrating band was lost on phosphatase treatment, indicating it was indeed a phosphorylated form of Bim (Figure 2A, lower panel).

To assess if nocodazole could induce phosphorylation of Bim, cycling Swiss 3T3 cells were arrested in G₀ phase and were then maintained for 20 h in the presence of the drug, in order to allow the activation of any putative signalling cascades that might induce phosphorylation of Bim. As a positive control for phosphorylated Bim (P-Bim), G₀-phase cells were cultured in the presence of serum and nocodazole, resulting in cell-cycle re-entry and mitotic arrest 28 h later (Figure 2B, upper panel). Western-blot analysis of extracts from nocodazole-treated G₀-phase cells demonstrated clearly that it failed to induce phosphorylation of Bim, as observed by the protein migrating at a level similar to untreated cycling cells. In contrast, phosphorylation was clearly evident in mitotic cells that were allowed to re-enter the cycle after G₀ arrest (Figure 2B, lower panel). These results confirmed that phosphorylation of Bim takes place in mitosis and is independent of nocodazole treatment. Hence, for all further experiments, nocodazole was used as the mitosis-arresting agent.

To investigate the phosphorylation status of BimEL in G₂ phase, the Cdk inhibitor roscovitine was used to prevent Cdk1 activation and consequent entry into mitosis [36]. G₂-arrested cells were tetraploid to the same extent as mitotic cells, in contrast with G₁/S-arrested cells (Figure 3A). Morphological features allowed us to ensure that these cells were indeed in G₂ phase and not in mitosis, since they were flat in shape and strongly adherent, whereas the latter displayed a rounded-up shape and were loosely attached to the tissue culture dish (Figures 3B and 3C). Western-blot analysis confirmed that, similarly to G₁/S, G₂-arrested cells did not possess significant amounts of P-Bim, in contrast with mitotic cells (Figure 3D). These results show that if cells are held in G₂ phase and restrained from entering mitosis by inhibiting Cdks (most likely due to inhibition of Cdk1, the primary engine of mitosis), Bim is not phosphorylated. These results support the conclusion that, under cell culture conditions, Bim is specifically phosphorylated in mitosis and not in G₁/S or G₂ phases of the cell cycle.

Inhibition of Cdks or MEK, but not PI3K (phosphoinositide 3-kinase) induces loss of phosphorylation of Bim in mitosis

Cdk1 in association with cyclin B are the most significant and decisive players for entry and maintenance of mitosis [35]. Taking into account that Bim is specifically phosphorylated in this phase of the cell cycle, we assessed the importance of mitosis-related signalling for the phosphorylation event to occur by perturbing mitosis through pharmacological inhibition of Cdk1 in metaphase-arrested cells. Roscovitine or purvalanol A treatment on mitotic cells greatly reduced phosphorylation of Bim, in contrast with cells held in mitosis for the same amount of time without inhibiting Cdks (Figures 3E and 4E).

It has been shown that the MEK/ERK pathway is critical for survival and required for G₂/M progression [37]. We addressed the role of the MEK/ERK pathway in phosphorylation of Bim in mitosis by inhibiting MEK1 and 2, the upstream kinases and

Figure 1 Metaphase-arrested cells display phosphorylation of BimL and BimEL protein, but not BimS

(A) Morphology of NIH 3T3 cells in culture. Light-microscopy images of: proliferating mixed population (for conditions see the Experimental section) (panel 1), cells enriched in G₁/S-phase by double-thymidine block (panel 2), and cells enriched in mitosis by nocodazole block (panel 3). (B) DNA-content profile (propidium iodide staining) of NIH 3T3 cells enriched in different phases of the cell cycle. DNA content was assessed by scoring cells as a function of propidium iodide intensity in the FL2-A channel (2n and 4n DNA content indicated as a percentage of total cells respectively). Cycling cells (control) consisted mainly of G₁-phase cells (2n) (panel 1). Cells treated with nocodazole (Noc., 100 ng/ml) or taxol (Tax., 400 nM) for 12 h, as indicated (panels 2–4): mitotic cells were obtained by shake-off (s.o.), and the detached cells were collected (panels 2 and 4); the remaining adherent cells were collected by scraping, consisting mainly of G₂-phase (4n) cells (panel 3). Shaken-off mitotic cells were competent to re-enter the cell cycle by re-plating in fresh nocodazole-free medium (panel 5). (C) Whole-cell protein extracts were separated by SDS/PAGE, and Western-blot analysis was performed by using polyclonal anti-Bim antibody. NIH 3T3 cells were obtained at different phases of the cell cycle (as indicated). Extracts from mitotic-arrested cells displayed slow-migrating bands (arrows) for BimL and BimEL, but not for BimS (lanes 2 and 4), in comparison with cycling cells (lane 1) and G₂-enriched populations (lanes 3 and 5). Noc., nocodazole; Tax., taxol; s.o., shake-off.

activators of ERK1 and 2. By using its specific inhibitor U0126 [38], phosphorylation of Bim was fully reversed, suggesting that the MEK/ERK pathway plays an important role for phosphorylation of Bim in mitosis (Figure 3F). Results from other investigators using other cell types and stimuli also implicate the MEK/ERK pathway in phosphorylating Bim [25,29–31]. Inhibition of PI3K (another well known pro-survival kinase) by using its specific inhibitor LY294002 [39] had no effect on phosphorylation of Bim, since the migration pattern of Bim was equal to that of untreated mitotic cells (Figure 3F). The specificity of MEK and PI3K inhibitors was assessed by using anti-phospho antibodies specific for their respective targets, ERK1/2 and Akt. Levels of phosphorylated ERK and Akt decreased dramatically in the presence of their respective kinase inhibitors (U0126 for

MEK and LY294002 for PI3K), whereas no change was observed in presence of inhibitors for their reciprocal kinases. Protein levels of total ERK and total Akt remained unchanged in all conditions (results not shown). The involvement of the MEK/ERK pathway ushered in the 'usual suspects' for the survival cascades and growth factor signalling, suggesting a cross-talk between the cell cycle and growth factor signal reception by the cell.

bFGF causes phosphorylation of Bim independently of the cell cycle in a MEK/ERK-dependent manner

FGF signalling has been shown to regulate mitogenic and survival cascades via MEK and ERK [40]. Hence, we set out to investigate whether bFGF, a growth factor known to activate the MEK/ERK

Figure 2 *Bona fide phosphorylation of Bim in mitosis*

(A) Cells were arrested in G₁/S phase, as observed by FACS analysis (upper panel, left), and mitotic cells were obtained by shake-off (s.o.) after 8 h post-G₁/S release (upper panel, right; indicated by arrow). Protein extracts were prepared from the mitosis-enriched population of cells, and an aliquot of the extracts was treated with CIP. Extracts were separated by SDS/PAGE, followed by Western blot with anti-Bim antibody (lower panel). Mitotic cells display P-Bim (lower panel, lane 1), which could be abrogated by treatment with the phosphatase (lower panel, lane 2). (B) DNA-content analysis (upper panel) and anti-Bim Western blot (lower panel) of cycling cells (Control; left and lane 1), cells arrested in mitosis after G₀ release by addition of 10 % FBS and 100 ng/ml nocodazole (Noc) for 28 h (G₀ → NocSer28h; centre and lane 2), and G₀-arrested cells treated with 100 ng/ml nocodazole for 20 h (G₀ + Noc20h; right and lane 3).

pathway in NIH 3T3 fibroblasts [41], was competent to induce phosphorylation of Bim and override the requirement for cells to be in mitosis for Bim to be phosphorylated. As shown in Figure 4(A), incubating a heterogeneous population of cells with 1, 5 or 10 ng/ml bFGF resulted in increasingly higher levels of P-Bim. At 10 ng/ml, the phosphorylation was even higher than that of mitotic cells, in contrast with protein obtained from cells in G₁/S (Figure 4A). To confirm that the slow-migrating band of Bim was indeed a phosphorylated form of the protein, HEK-293T cells were transfected with empty vector or HA-Bim, and extracts were immunoprecipitated using anti-HA or anti-pSer⁶⁵-

Bim antibodies. As shown in Figure 4(B), the anti-HA antibody was competent to immunoprecipitate both the normal (control) and the slow-migrating species of HA-Bim (upon bFGF treatment of the cells) equally well (left-hand panel). In contrast, the anti-pSer⁶⁵-Bim antibody recognized the slow-migrating HA-Bim, but could only marginally immunoprecipitate the normal migrating band, indicating that P-Bim accounts for the slow-migrating band (Figure 4B, right-hand panel). To understand further if bFGF was acting through the MEK/ERK pathway, G₁/S and mitotic cells were treated with or without the MEK inhibitor U0126, and then stimulated with or without bFGF. In Figure 4(C), we present Western blots for the conditions indicated for phosphorylated active ERK (P-ERK), total ERK (as control) and Bim. As expected, there was a robust activation of ERK and phosphorylation of Bim by treating cells with bFGF alone (Figure 4C, lanes 3 and 7), indicating that Bim is downstream of the MEK/ERK pathway, which in turn is downstream of bFGF signalling. Lanes 2, 4, 6 and 8 of Figure 4(C) show that U0126 greatly reduced basal phosphorylation and activation of ERK in all phases of the cell cycle, with no significant amounts of P-Bim detectable even in the presence of bFGF (lanes 4 and 8). Our results presented in Figure 4(C) regarding phosphorylation of Bim downstream of bFGF and in mitosis were confirmed further by Western blot, using the anti-pSer⁶⁵-Bim antibody (Figure 4D, middle panel), showing that the intensity of signal obtained in each experimental condition using the anti-pSer⁶⁵-Bim antibody was consistent with the slow-migrating pattern of Bim using the anti-(total Bim) antibody (Figure 4D, upper panel).

Experiments shown in Figures 4(C) and 4(D) showed that total Bim protein levels seem to be significantly higher in G₁/S-phase conditions than in mitosis. Although detailed analysis of this observation was beyond the scope of the present work, it is in agreement with a report showing that several BH3-only proteins (including Bim) are up-regulated in G₁/S-phase, downstream of E2F1 [11]. P-Bim may also undergo degradation in mitosis, thus contributing to the relative lower amount of protein in this phase of the cell cycle. These issues are currently being addressed in our laboratory.

Cdk inhibitor purvalanol A [42] (or roscovitine, as shown earlier in Figure 3E) significantly reduced phosphorylation of Bim in mitosis. However, bFGF was able to override this effect (Figure 4E, lanes 4–6). Similar results were obtained when using a cycling population of cells (Figure 4E, lanes 1–3). These results not only showed that the Cdk inhibitors used did not interfere with MEK pathway, but also suggest that bFGF- and mitosis-induced phosphorylation of Bim are outcomes of independent pathways. Alternatively, it is also likely that phosphorylation of Bim in mitosis might occur due to increased sensitivity of cells to growth factors signalling through MEK during this phase of the cell cycle. This hypothesis would explain why Bim undergoes phosphorylation in mitosis without additional growth factors, and why this effect could be reverted by inhibiting Cdks, keeping in mind that Cdk1 in association with CyclinB is the major driving force for mitosis. This would also explain why we were able to block phosphorylation of Bim in mitosis by inhibiting MEK, and to induce phosphorylation of Bim in non-mitotic cells by treating them with exogenous bFGF. Furthermore, phosphorylation of Bim through activation of growth factor signalling pathways and consequent activation of MEK/ERK corroborates results from other researchers [25–29,31].

Phosphorylation of Bim in mitosis is dependent on growth factors

To test the significance of growth factors in phosphorylating Bim in mitosis, we transiently deprived mitotic cells of growth factors

Figure 3 Bim is phosphorylated in mitosis, but not in G₁/S or G₂ phase, and inhibition of Cdks and MEK, but not PI3K, correlates with loss of phosphorylation of Bim in mitosis

Cells were arrested in G₁/S or G₂ phase, or mitosis (Mit), as indicated. **(A)** DNA-content analysis was performed by FACS, and the percentage of tetraploid cells was scored. G₂-phase and mitotic cells exhibited equivalent levels of tetraploidy (representative of at least three independent experiments). G₂- and metaphase-arrested cells could be distinguished morphologically by light microscopy: G₂ cells were flat and adherent **(B)**, in contrast with rounded-up and loosely attached mitotic cells **(C)**. **(D)** Anti-Bim Western blot performed with extracts obtained from G₁/S-phase (lane 1), G₂-phase (lane 2) and mitotic (Mit; lane 3) cells. **(E)** Anti-Bim Western blot performed with extracts obtained from mitotic cells (Mit; lane 3) or cells arrested in mitosis for 4 h in the absence (Mit4h; lane 4) or in presence of Cdk inhibitors 15 μ M purvalanol A (Mit Purv4h; lane 1) or 35 μ M roscovitine (Mit Rosc4h; lane 2). **(F)** Anti-Bim Western blot of extracts prepared from mitotic cells treated for 3 h with inhibitors for MEK (U0126) (M + U0 35; lane 2) or PI3K (LY294002) (M + LY 35; lane 3) at the indicated concentrations (micromolar range), or left untreated during the same period of time (M; lane 1).

by serum starvation and assayed for phosphorylation of the protein. In Figure 5(A) we show, using Western blot, that phosphorylation of Bim and ERK in mitosis in the presence of serum could be reduced significantly by transiently serum-starving the cells, which could be reverted by adding exogenous bFGF. This result clearly illustrates that growth factors are indeed necessary for phosphorylation of Bim in mitosis, in agreement with earlier results regarding bFGF (Figure 4).

We next sought to clarify the contribution of FGF signalling by using SU5402 [43] to pharmacologically inhibit FGF receptors and assess the status of Bim in mitosis. However, we first titrated the inhibitor by treating cells with or without SU5402 at different concentrations (Figure 5B). Western-blot analysis showed that bFGF was sufficient to induce phosphorylation of BimEL in the absence of serum. Phosphorylation was prevented in the presence of increasing concentrations of SU5402, being completely abrogated by inhibiting the receptors for 2 h at 100 μ M (Figure 5B). Under the same conditions, we were also able to fully revert the phosphorylation phenotype of Bim and reduce phosphorylation

of ERK in mitosis in the presence of serum (Figure 5C). This result suggests strongly that FGF signalling plays a critical role in inducing phosphorylation of Bim in mitosis in NIH 3T3 fibroblasts.

Phosphorylation of Bim correlates with bFGF rescuing of serum-deprivation-induced cell death

Based on our results showing that serum deprivation caused a loss of phosphorylation of Bim in mitosis, and that bFGF was sufficient to rescue this phenotype (Figure 5A), we investigated whether bFGF alone was competent to rescue cells from serum-deprivation-induced apoptosis. Mitotic cells were transiently deprived of serum to extinguish growth factor signalling and abrogate phosphorylation of Bim. Cells were then released from metaphase arrest, resulting in progression through cytokinesis to G₁-phase (results not shown). Prolonged serum starvation resulted in high levels of cell death, in contrast with cells cultured in the presence of serum or bFGF (Figure 6A). As assessed by

Figure 4 Addition of exogenous bFGF causes phosphorylation of Bim in a MEK/ERK-dependent manner, independently of the cell cycle

(A) Anti-Bim Western blot of whole-cell extracts obtained from G₁/S- (lane 1), mitotic (M; lane 2) and cycling NIH 3T3 (lanes 3–5) cells. Cycling NIH 3T3 cells were treated for 10 min with bFGF at 1 ng/ml (lane 3), 5 ng/ml (lane 4) and 10 ng/ml (lane 5). (B) Western blots (WB) of IP of extracts obtained from HEK-293T cells transfected with empty vector (EV; lanes 1 and 4) or HA-Bim fusion (lanes 2, 3, 5 and 6). Cells were left untreated (lanes 1, 2, 4 and 5) or treated for 15 min with 20 ng/ml bFGF before extraction (lanes 3 and 6). IPs were performed using anti-HA antibody (left-hand panel) or anti-pSer⁶⁵Bim antibody (right-hand panel). Samples were separated by SDS/PAGE, blotted on to a nitrocellulose membrane and stained using an anti-HA antibody. (C) G₁/S- or metaphase-arrested cells were treated with or without 35 μ M MEK inhibitor U0126 (UO) for 3 h, and then treated with or without bFGF (10 ng/ml for 10 min), as indicated. bFGF caused activation of ERK and phosphorylation of Bim (lanes 3 and 7), which was prevented in presence of MEK inhibitor (lanes 4 and 8). Mitosis-induced phosphorylation of Bim was reduced by inhibition of MEK (lanes 5 and 6). (D) Western blot (WB) of NIH 3T3 whole-cell extracts obtained from G₁/S-phase cells treated with 10 ng/ml bFGF for 10 min (lane 2) or left untreated (lane 1). Mitotic cells were left untreated (lane 3) or treated with 35 μ M MEK inhibitor U0126 for 3 h (UO; lane 4) or 10 ng/ml bFGF for 15 min (lane 5). Membranes were stained using total anti-Bim antibody (upper panel), anti-pSer⁶⁵-Bim antibody (middle panel) or anti-ERK antibody for loading control. (E) bFGF-induced phosphorylation of Bim was not affected by Cdk inhibitors. Cycling (lanes 1–3) or mitotic-arrested cells (lanes 4–6) were treated with or without 15 μ M purvalanol A (Purv) for 3 h and then treated or not with 10 ng/ml bFGF for 10 min, as indicated. bFGF-treated cells displayed phosphorylation of Bim in the presence of purvalanol A (lanes 3 and 6). Treatment with the Cdk inhibitor purvalanol A significantly reduced phosphorylation of Bim in mitosis (lanes 4 and 5), which could be rescued by subsequent treatment with 10 ng/ml bFGF for 10 min (lane 6).

Figure 5 Growth-factor-dependent phosphorylation of Bim in mitosis

(A) NIH 3T3 cells were arrested in mitosis. Cells were transiently serum-deprived (–Ser; see the Experimental section) in the presence of nocodazole (100 ng/ml) and in the absence (lane 2) or in the presence of 100 ng/ml bFGF (lane 3). As a control, cells were washed with serum-free medium and cultured for 2 h in the presence of 10% (v/v) FBS and nocodazole (100 ng/ml) (+Ser, lane 1). Cell lysates were resolved by SDS/PAGE and immunoblotted with anti-Bim, anti-P-ERK and anti-(total ERK) antibodies. (B) Cycling NIH 3T3 cells were transiently serum-deprived (–Ser) in the absence (lane 1) or in the presence of 100 ng/ml bFGF (lanes 2–4) and in the presence of 50 μ M (lane 3) or 100 μ M (lane 4) of FGF receptor inhibitor SU5402 (SU). Cell lysates were resolved by SDS/PAGE and immunoblotted with anti-Bim antibody. (C) NIH 3T3 cells were arrested in G₁/S (lane 1) or in mitosis (lanes 2–3). Mitotic-arrested cells were treated further (lane 3) or not (lane 2) with 100 μ M FGF receptor inhibitor SU5402 (SU) for 2 h. Cell lysates were resolved by SDS/PAGE and immunoblotted with anti-Bim, anti-P-ERK and anti-(total ERK) antibodies.

DNA-content analysis, 38% of the serum-starved cells were apoptotic, displaying sub-G₁ DNA content. Treating cells with bFGF alone significantly reversed this effect (Figure 6B). We do not exclude a requirement for other growth factors, as reflected by the lack of complete rescue of cell death. Moreover, we observed significant levels of apoptosis to occur in conditions of serum-starvation in early G₁ and even before cytokinesis. Serum-deprived cells displayed apoptotic features at 3 and 6 h post-mitotic release, as observed by light microscopy and TUNEL assay (results not shown, and Figures 6C and 6D), which could be reversed by treatment with bFGF. These data suggest that decision-making events for the initiation of apoptosis may occur even during mitosis. Taken together, our results strongly indicate that FGF signalling, leading to activation of survival pathways

Figure 6 EFGF rescues cells from serum-deprivation-induced cell death

(A) Light-microscopy images of NIH 3T3 cells. Cells were arrested in mitosis, washed with serum-free medium, shaken-off and re-plated on poly(L-lysine)-coated six-well plates. Cells were transiently serum-deprived in the presence of nocodazole (100 ng/ml), washed and cultured in fresh medium with 10% serum (+Ser; panel 1) or without serum (–Ser) in the absence (panel 2) or in the presence (panel 3) of 200 ng/ml bFGF for 24 h in the presence of 2.5 mM thymidine. (B) Apoptosis of cells under the same conditions as in (A) was quantified by scoring the percentage of sub-G₁ population of cells by FACS DNA-content analysis using propidium iodide. Treatment with bFGF (200 ng/ml) significantly rescued cells from serum-deprivation-induced apoptosis (** $P < 0.01$ using Student's *t* test). (C) Cells were treated as in (A), except that after shake-off, cells were re-plated on coverslips and times of incubation were 3 h or 6 h (as indicated) instead of 24 h. Cells were visualized under a fluorescence microscope after staining nuclei with FITC-conjugated TUNEL and DAPI (4,6-diamidino-2-phenylindole). TUNEL-positive cells were considered to be apoptotic. DAPI was used to stain all cells (nuclear staining). (D) Images obtained from conditions described in (C) were processed using Image J software. The cells positive for TUNEL- (apoptotic cells) and DAPI-stained cells (total cells) were counted separately using the same software. At least 850 cells were counted in each condition, and apoptotic cells were scored as a percentage of total cells (left-hand panel) and fold increase of apoptosis (right-hand panel) after 3 h treatments (open bars) and 6 h treatments (closed bars). These results are representative of three independent experiments.

(MEK/ERK) and phosphorylation of Bim, attenuates the pro-apoptotic consequences of serum deprivation.

DISCUSSION

The harmonious orchestration of cell-fate decisions primarily requires regulation of cell division and cell death through intra- and inter-cellular signalling. These processes are intricately connected, and each one of them may modulate the other, influencing physiology and pathology [1,2]. The cross-talk between cell cycle and apoptosis is still poorly understood [10,11]. In the present study, we have focused on the regulation of cell survival/death signalling in mitosis, specifically the pathways that regulate the pro-apoptotic Bcl-2 family BH3-only protein Bim.

Our results showed that BimEL was phosphorylated specifically in mitosis. We have also shown that this post-translational modification occurred in metaphase-arrested and also in normal mitotic cells obtained from a cycling population, suggesting a biological role for phosphorylation of Bim. Our data indicate that certain signalling pathways activated during mitosis might trigger this post-translational modification. This idea was reinforced by the fact that perturbation of mitosis through pharmacological inhibition of Cdk1 (an essential kinase during mitosis)

during metaphase arrest was sufficient to significantly reduce phosphorylation of Bim. Furthermore, inhibition of MEK/ERK pathway in this phase of the cell cycle resulted in complete loss of phosphorylation of Bim, unveiling a link between cell cycle and survival/apoptosis players MEK, ERK and Bim.

In agreement with other reports [25,28–31], we found that exogenous activation of MEK/ERK pathway was sufficient to phosphorylate Bim in interphase (G₁/S-arrested or cycling cells). Namely, bFGF, a known activator of the MEK/ERK pathway through FGF receptors, was competent to induce phosphorylation of Bim in a MEK/ERK-dependent manner. We have also shown this pathway to be competent to induce phosphorylation of Bim during metaphase arrest, even in the presence of mitosis-perturbing agents, such as Cdk inhibitors (as discussed above). This result suggested that either growth-factor-dependent phosphorylation of Bim and phosphorylation of Bim in mitosis were independent events or growth factor signalling was being activated as a consequence of cells being in mitosis. The second hypothesis appeared to be more plausible, since phosphorylation of Bim in mitosis was dependent on growth factors, as shown in the serum-deprivation and inhibition of MEK experiments. Concomitantly, inhibition of FGF receptors using a specific inhibitor also prevented phosphorylation of Bim in mitosis in the presence of serum, indicating that FGF signalling plays a pivotal role in

this biochemical event. Overall, our data were consistent with reports describing phosphorylation of Bim in cycling cells to occur downstream of growth factor signalling in a MEK/ERK-dependent manner, such as nerve growth factor, epidermal growth factor, serum, or interleukin-2 or -3, depending on the cell types used [25–29,31].

Growth-factor-dependent MEK/ERK-mediated phosphorylation of Bim has been shown to restrict the apoptotic activity of the protein [25–28,31]. Although the mechanisms by which P-Bim is restrained from causing apoptosis are still not understood, P-Bim has been described to be targeted for proteasome degradation [29–31]. However, other mechanisms responsible for loss of pro-apoptotic properties of Bim, such as relocation or revised interaction with interacting pro- or anti-apoptotic partners cannot be excluded. Moreover, it has been reported that Bim is kept constrained from initiating apoptosis by sequestration to the MTs, being mobilized off them on stimulation by pro-apoptotic agents [22,24]. It is well documented that, in mitosis, MTs undergo profound rearrangements during the spindle formation. Innumerable MAPs (MT-associated proteins) are displaced from MTs, and MT-stabilizing proteins are loaded on to them [44,45]. We speculate that Bim may be displaced from MTs during the spindle formation, and pose a danger of turning on apoptosis due to release and relocation, hence the need to phosphorylate to incapacitate the molecule. These issues are currently being addressed in our laboratory.

We have observed that mitotic-arrested cells transiently deprived of serum were able to progress to G₁ phase when released (by washing away nocodazole), but showing a significant number undergoing apoptosis, as compared with cells treated with 10 % FBS or bFGF at the time of release. bFGF alone was able largely to rescue these dying cells, although not completely, suggesting that FGF signalling is relevant, but probably not the only player involved. Our results, together with a number of reports from different research groups showing that MEK/ERK-dependent phosphorylation of Bim attenuates the pro-apoptotic features of the protein, strongly suggest that phosphorylation of Bim is an important step during mitosis, establishing a link between cell cycle and survival signals. Based on our data, we suggest that loss of phosphorylation of Bim in mitosis may trigger apoptosis at the M to G₁ transition. Regulation of apoptosis through post-translational modification mechanisms may be specially important in mitosis, since transcription is essentially shut down due to chromatin condensation, hence *de novo* RNA and protein synthesis become highly constrained.

The Bim-knockout mice show no striking generalized phenotype. The strongest effects observed mainly concern the immune system, consisting of a 2–4-fold increase in B- and T-cell numbers, and being prone to autoimmunity. Nevertheless, the mice are largely uncompromised [46]. The possibility of compensation by other redundant BH3-only proteins in the Bim-knockout mouse during development cannot be excluded. Numerous evidence published recently has established that phosphorylation of Bim influences the function of the protein towards pro- [24,47] or anti-apoptosis [25,26,29–31]. Our results, taken together with these findings, encourage us to speculate whether a knock-in mouse carrying the phosphorylation mutants of Bim will exhibit a more drastic phenotype with respect to viability than Bim-knockout. This approach might uncover new functional implications for regulation of Bim in development.

We thank Dr E. Lam for help with cell-synchronization techniques and for providing cell lines; Dr L. A. Greene for the pCMS-EGFP + BimEL plasmid; Professor M. Raff and Dr M. Mallo for discussion; Dr J. Leon, D. Calado, S. Godinho and M. Rebelo for discussion and comments on the manuscript; Chatterjee laboratory members (Dr T. Pais,

C. Figueiredo, A. Mena, A. Veloso, R. Peixoto and M. Matos) for help provided, discussion and comments on the manuscript; N. Moreno for assistance with imaging techniques. This work was funded by research grant POCTI/BCI/42249/2001 from Fundação para a Ciência e a Tecnologia (FCT), Portugal, and Instituto Gulbenkian de Ciência/Fundação Calouste Gulbenkian to S. C. M. G. and A. D. A. are recipients of FCT fellowships SFRH/BD/2729/2000 and fellowship associated to grant POCTI/CBO/47565/2002 respectively. M. G. is on the Programa Gulbenkian de Doutoramento em Biologia e Medicina (Gulbenkian Ph.D. Programme in Biology and Medicine).

REFERENCES

- Jacobson, M. D., Weil, M. and Raff, M. C. (1997) Programmed cell death in animal development. *Cell* **88**, 347–354
- Vaux, D. L. and Korsmeyer, S. J. (1999) Cell death in development. *Cell* **96**, 245–254
- Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257
- Marsden, V. S. and Strasser, A. (2003) Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. *Annu. Rev. Immunol.* **21**, 71–105
- Jiang, X. and Wang, X. (2004) Cytochrome c-mediated apoptosis. *Annu. Rev. Biochem.* **73**, 87–106
- Elledge, S. J. (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* **274**, 1664–1672
- Castedo, M., Perfettini, J. L., Roumier, T. and Kroemer, G. (2002) Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death Differ.* **9**, 1287–1293
- Guo, M. and Hay, B. A. (1999) Cell proliferation and apoptosis. *Curr. Opin. Cell Biol.* **11**, 745–752
- O'Connor, L., Huang, D. C., O'Reilly, L. A. and Strasser, A. (2000) Apoptosis and cell division. *Curr. Opin. Cell Biol.* **12**, 257–263
- Nahle, Z., Polakoff, J., Davuluri, R. V., McCurrach, M. E., Jacobson, M. D., Narita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D. and Lowe, S. W. (2002) Direct coupling of the cell cycle and cell death machinery by E2F. *Nat. Cell Biol.* **4**, 859–864
- Hershko, T. and Ginsberg, D. (2004) Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. *J. Biol. Chem.* **279**, 8627–8634
- Cory, S., Huang, D. C. and Adams, J. M. (2003) The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* **22**, 8590–8607
- Greene, L. A., Biswas, S. C. and Liu, D. X. (2004) Cell cycle molecules and vertebrate neuron death: E2F at the hub. *Cell Death Differ.* **11**, 49–60
- Ferri, K. F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3**, E255–E263
- Puthalakath, H. and Strasser, A. (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ.* **9**, 505–512
- Huang, D. C. and Strasser, A. (2000) BH3-only proteins – essential initiators of apoptotic cell death. *Cell* **103**, 839–842
- Bouillet, P., Zhang, L. C., Huang, D. C., Webb, G. C., Bottema, C. D., Shore, P., Eyre, H. J., Sutherland, G. R. and Adams, J. M. (2001) Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. *Mamm. Genome* **12**, 163–168
- Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L. and Coffey, P. J. (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr. Biol.* **10**, 1201–1204
- Stahl, M., Dijkers, P. F., Kops, G. J., Lens, S. M., Coffey, P. J., Burgering, B. M. and Medema, R. H. (2002) The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J. Immunol.* **168**, 5024–5031
- Chalmers, C. J., Balmanno, K., Hadfield, K., Ley, R. and Cook, S. J. (2003) Thrombin inhibits Bim (Bcl-2-interacting mediator of cell death) expression and prevents serum-withdrawal-induced apoptosis via protease-activated receptor 1. *Biochem. J.* **375**, 99–109
- Wang, Z., Malone, M. H., He, H., McColl, K. S. and Distelhorst, C. W. (2003) Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. *J. Biol. Chem.* **278**, 23861–23867
- Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M. and Strasser, A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell* **3**, 287–296
- Day, C. L., Puthalakath, H., Skea, G., Strasser, A., Barsukov, I., Lian, L. Y., Huang, D. C. and Hinds, M. G. (2004) Localization of dynein light chains 1 and 2 and their pro-apoptotic ligands. *Biochem. J.* **377**, 597–605

- 24 Lei, K. and Davis, R. J. (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2432–2437
- 25 Biswas, S. C. and Greene, L. A. (2002) Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. *J. Biol. Chem.* **277**, 49511–49516
- 26 Seward, R. J., von Haller, P. D., Aebersold, R. and Huber, B. T. (2003) Phosphorylation of the pro-apoptotic protein Bim in lymphocytes is associated with protection from apoptosis. *Mol. Immunol.* **39**, 983–993
- 27 Shiniyo, T., Kuribara, R., Inukai, T., Hosoi, H., Kinoshita, T., Miyajima, A., Houghton, P. J., Look, A. T., Ozawa, K. and Inaba, T. (2001) Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. *Mol. Cell. Biol.* **21**, 854–864
- 28 Wang, P., Gilmore, A. P. and Streuli, C. H. (2004) Bim is an apoptosis sensor that responds to loss of survival signals delivered by EGF but not those provided by integrins. *J. Biol. Chem.* **279**, 41280–41285
- 29 Ley, R., Balmano, K., Hadfield, K., Weston, C. and Cook, S. J. (2003) Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* **278**, 18811–18816
- 30 Luciano, F., Jacquelin, A., Colosetti, P., Herrant, M., Cagnol, S., Pages, G. and Auberger, P. (2003) Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* **22**, 6785–6793
- 31 Ley, R., Ewings, K. E., Hadfield, K., Howes, E., Balmano, K. and Cook, S. J. (2004) Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim_{EL} kinases" that bind to the BH3-only protein Bim_{EL} causing its phosphorylation and turnover. *J. Biol. Chem.* **279**, 8837–8847
- 32 Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211–225
- 33 Ornitz, D. M. and Itoh, N. (2001) Fibroblast growth factors. *Genome Biol.* **2**, REVIEWS3005
- 34 Whitfield, M. L., Sherlock, G., Saldanha, A. J., Murray, J. I., Ball, C. A., Alexander, K. E., Matese, J. C., Perou, C. M., Hurt, M. M., Brown, P. O. and Botstein, D. (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Mol. Biol. Cell* **13**, 1977–2000
- 35 Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature (London)* **344**, 503–508
- 36 Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G. and Moulino, J. P. (1997) Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243**, 527–536
- 37 Liu, X., Yan, S., Zhou, T., Terada, Y. and Erikson, R. L. (2004) The MAP kinase pathway is required for entry into mitosis and cell survival. *Oncogene* **23**, 763–776
- 38 Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feese, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273**, 18623–18632
- 39 Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**, 5241–5248
- 40 Yamada, S., Taketomi, T. and Yoshimura, A. (2004) Model analysis of difference between EGF pathway and FGF pathway. *Biochem. Biophys. Res. Commun.* **314**, 1113–1120
- 41 Kovalenko, D., Yang, X., Nadeau, R. J., Harkins, L. K. and Friesel, R. (2003) Sef inhibits fibroblast growth factor signaling by inhibiting FGFR1 tyrosine phosphorylation and subsequent ERK activation. *J. Biol. Chem.* **278**, 14087–14091
- 42 Gray, N. S., Wodicka, L., Thunnissen, A. M., Norman, T. C., Kwon, S., Espinoza, F. H., Morgan, D. O., Barnes, G., LeClerc, S., Meijer, L. et al. (1998) Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. *Science* **281**, 533–538
- 43 Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955–960
- 44 Cassimeris, L. (1999) Accessory protein regulation of microtubule dynamics throughout the cell cycle. *Curr. Opin. Cell Biol.* **11**, 134–141
- 45 Walczak, C. E. (2000) Microtubule dynamics and tubulin interacting proteins. *Curr. Opin. Cell Biol.* **12**, 52–56
- 46 Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Kontgen, F., Adams, J. M. and Strasser, A. (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286**, 1735–1738
- 47 Putcha, G. V., Le, S., Frank, S., Besirli, C. G., Clark, K., Chu, B., Alix, S., Youle, R. J., LaMarche, A., Maroney, A. C. and Johnson, Jr, E. M. (2003) JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* **38**, 899–914

Received 16 August 2004/10 January 2005; accepted 14 January 2005

Published as BJ Immediate Publication 14 January 2005, DOI 10.1042/BJ20041385