Thrombin inhibits Bim (Bcl-2-interacting mediator of cell death) expression and prevents serum-withdrawal-induced apoptosis via protease-activated receptor 1

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To investigate the role of thrombin in regulating apoptosis, we have used CCI39 cells, a fibroblast cell line in which thrombin-induced cell proliferation has been extensively studied. Withdrawal of serum from CCI39 cells resulted in a rapid apoptotic response that was completely prevented by the inclusion of thrombin. The protective effect of thrombin was reversed by pertussis toxin, suggesting that cell-survival signalling pathways are activated via a G_i or G_o heterotrimeric GTPase. Serum-withdrawal-induced death required *de novo* gene expression and was preceded by the rapid *de novo* expression of the pro-apoptotic 'BH3-only' protein Bim (Bcl-2-interacting mediator of cell death). Thrombin strongly inhibited the up-regulation of both Bim protein and Bim mRNA. The ability of thrombin to re-

press Bim expression, and to protect cells from apoptosis, was reversed by U0126, a MEK1/2 [MAPK (mitogen-activated protein kinase) or ERK (extracellular-signal-regulated kinase) 1/2] inhibitor, or LY294002, a phosphoinositide 3'-kinase (PI3K) inhibitor, suggesting that both the $Raf \rightarrow MEK \rightarrow ERK1/2$ and PI3K pathways co-operate to repress Bim and promote cell survival. A PAR1p (protease-activated receptor 1 agonist peptide) was also able to protect cells from serum-withdrawal-induced apoptosis, suggesting that thrombin acts via PAR1 to prevent apoptosis.

Key words: apoptosis, Bim, extracellular-signal-related kinase (ERK), protease-activated receptor 1 (PAR1), protein kinase B (PKB), thrombin.

INTRODUCTION

The serine protease thrombin is a central co-ordinator of the blood-coagulation cascade [1], but also exhibits striking effects on a variety of target cells and tissues in order to co-ordinate responses to injury. Thrombin is an extremely effective and potent growth factor for a variety of cell types, including vascular-smooth-muscle cells [2], fibroblasts [3] and some tumour cells [4]. These cellular effects of thrombin are mediated by PARs (protease-activated receptors), which are members of the G-protein-coupled-receptor superfamily [5]. The PARs have an intrinsic ligand within their extended N-terminus, which is exposed upon proteolytic cleavage. The exposed ligand then docks intramolecularly with the receptor to activate signalling [6,7]. Indeed, small, soluble peptides with sequences corresponding to the newly exposed N-terminus act as specific ligands for their cognate receptors [6,8]. Four PARs have been identified to date: PAR1, PAR3 and PAR4 are activated by thrombin, whereas PAR2 is activated by trypsin, tryptase and other coagulation factors. PAR3 cannot itself promote transmembrane signalling, but acts as a cofactor for PAR4 [1,5].

The ability of thrombin to act as a growth factor depends in large part on its ability to activate the ERK (extracellular-signal-regulated kinase) pathway [9–13]. Activated thrombin receptors couple to at least three heterotrimeric GTPases. Activation of phospholipase C is typically mediated by members of the G_q or G_i family [14], whereas activation of Rho, and subsequent effects on the actin cytoskeleton, are mediated by $G_{12/13}$ [15].

However, the ability of pertussis toxin to strongly inhibit thrombin-induced cell-cycle re-entry suggests that the primary signal driving cell proliferation is regulated by members of the G_i or G_o family [3]. Indeed, pertussis toxin has been shown to inhibit thrombin-induced activation of Ras [16] and the $Raf \rightarrow MEK$ [MAPK (mitogen-activated protein kinase) or ERK] $\rightarrow ERK1/2$ cascade [10,12]. Furthermore, selective inhibitors of the ERK1/2 pathway can inhibit thrombin-induced expression of Fos and Jun proteins, cyclin D1 expression and cell-cycle re-entry [11,12].

The CCI39 fibroblast cell line has proved a particularly informative cell system for studying the proliferative action of thrombin. Although many of the effects of thrombin, including activation of phospholipase C and inhibition of adenylate cyclase, can be mimicked by PAR1ps (PAR1 agonist peptides) [6,8], the ability of thrombin to stimulate cell proliferation cannot [8,12]. This disparity may be due to the more prolonged signalling elicited by thrombin compared with PAR1p, since sustained activation of the ERK pathway is required for cell-cycle re-entry in several fibroblast cell lines, including CCI39 cells [9,12,17], and thrombin, but not PAR1p, elicits sustained ERK activation [9,10,12].

Although the role of thrombin as a growth factor is now well defined, the influence of thrombin on cell survival is less clear. For example, thrombin and peptide agonists of the PAR1 receptor can promote cell death in some cells, including motor neurons and astrocytes [18–21], but they prevent apoptosis in other cells, including human monocytes [22], myoblasts [23] and astrocytes

Abbreviations used: Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Bim, Bcl-2-interacting mediator of cell death; Bim_S, Bim_L and Bim_{EL}, the short, long and extra-long splice variants of Bim; DEVDase, Ac-DEVD-AMC-cleaving activity; ERK, extracellular-signal-regulated kinase; FBS, fetal-bovine serum; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; PAR, protease-activated receptor; PAR1p, PAR1 agonist peptide; PI, propidium iodide; PI3K, phosphoinositide 3'-kinase; PKB, protein kinase B; PKC, protein kinase C; (Q)RT-PCR, (quantitative, real-time) reverse transcription PCR.

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[24]. The ability of thrombin to influence apoptosis would certainly be consistent with its role in co-ordinating development and the response to injury. Here we show that thrombin is an effective survival factor for CCl39 cells, preventing serum-withdrawal-induced apoptosis. In common with cell proliferation, we show that thrombin-induced cell-survival signals are mediated via the pertussis-toxin-sensitive activation of the ERK1/2 and phosphoinositide 3'-kinase (PI3K) pathways and identify the 'BH3-only' protein Bim (Bcl-2-interacting mediator of cell death) as a potential target for thrombin-mediated survival signalling.

EXPERIMENTAL

Materials

Cell culture reagents were purchased from Invitrogen. LY294002 and CPP32 (caspase 3) substrate Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) were from Calbiochem. U0126 was purchased from Promega. [γ -³²P]ATP was purchased from Amersham. The following antibodies were used throughout this study: ERK1 was prepared in house [12]; phospho-ERK1/2, total ERK1/2, total PKB (protein kinase B), [phosphoserine-473]PKB and cleaved caspase 3 (17 kDa fragment) were from Cell Signalling Technology/New England Biolabs; Bcl-2 and Bad were from Santa Cruz Biotechnology; Bcl-x_L was from Pharmingen; Bim was from Chemicon. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Unless otherwise stated in the text, all other chemicals were purchased from Sigma.

Cell culture

CCl39 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 4.5 mg · ml⁻¹ glucose, 100 units · ml⁻¹ penicillin, 100 µg · ml⁻¹ streptomycin, 2 mM glutamine and 10% (v/v) FBS (fetal-bovine serum). For serum starvation, cells judged to be 50–60% confluent were washed once in serum-free medium and then placed in fresh serum-free medium with the indicated dose of thrombin, other test agonists/inhibitors or the appropriate vehicle control for the times indicated in the Figure legends.

Western blotting

At the required times, cells were washed in cold PBS and harvested on ice in TG lysis buffer [20 mM Tris (pH 7.5)/137 mM NaCl/1 mM EGTA/1% (v/v) Triton X-100/10% (v/v) glycerol/1.5 mM MgCl₂/1 mM Na₃VO₄/1 mM PMSF/20 µM leupeptin/10 µg · ml⁻¹ aprotinin/50 mM NaF] and processed for Western blotting as described previously [12,25].

Immunocomplex ERK1 assays

Following stimulation, cells were lysed and ERK1 was immunoprecipitated and assayed as described previously [12]. In addition, ERK1/2 activation was also assessed by the appearance of the phosphorylated, activated forms of the enzymes after Western blotting with phospho-specific antibodies.

Analysis of cell-cycle profiles and apoptosis

Cell death was assayed by the appearance of DEVDase (Asp-Ac-DVDE-AMC-cleaving activity), sub-G₁ DNA content or annexin V staining as described previously [25].

Real-time RT-PCR (reverse transcription PCR)

Total mRNA was isolated from CCl39 cells using the guanidinium thiocyanate method [26]. Typically, RT reactions were carried out using 1 µg of RNA and random hexamers in a 20 µl reaction volume following the protocol supplied with the Taqman[®] RT reagents (Applied Biosystems). For hamster *Bim* we used 5'-TAAGGCAATCTCAGGAGGAACCT-3' as the forward primer and 5'-AGATTCGTTGAACTCGTCTCCAA-3' as the reverse primer. These primers are common to Bim_S, Bim_L and Bim_{EL} (the short, long and extra-long splice variants of Bim). For hamster β -actin we used 5'-CCGGATGCAGAAGGAGATCA-3' as the forward primer and 5'-GCCACCGATCCACACAGAGTA-3' as the reverse primer.

RESULTS

Thrombin promotes cell survival during serum withdrawal

To determine whether thrombin could act as a survival factor, sub-confluent cycling CCl39 cells were washed in serum-free medium and then placed in fresh serum-free medium in the presence or absence of thrombin. Cells placed in fresh complete medium (10% FBS) served as a control and apoptosis was assessed by three independent assays. Serum-withdrawal-induced cell death is a caspase-dependent process in CCl39 cells, since it can be inhibited by the broad-specificity caspase inhibitor zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone) [25]. Consequently we first examined the effect of thrombin stimulation on caspase activation during serum withdrawal by assaying CCl39 cell extracts for their ability to cleave the synthetic caspase substrate Ac-DEVD-AMC (a DEVDase assay). Serum starvation caused a robust increase in DEVDase activity that was apparent within 6 h, maximal after 12 h, and then declined. The presence of thrombin completely inhibited the appearance of this serum-withdrawal-induced DEVDase activity (Figure 1A, left panel). Furthermore, thrombin was also able to prevent the appearance of the cleaved, activated form of caspase 3 (Figure 1A, right panel).

We also examined two downstream caspase-dependent markers of cell death. Exposure of CCl39 cells to serum-free medium for 24 h caused the appearance of cells with sub-G₁ DNA ($28.9 \pm 5.3\%$, mean $\pm$ S.D. of five determinations), whereas cells serum-starved in the presence of thrombin exhibited no sign of cell death ($3.5 \pm 1.7\%$, significant decrease from serum-free value by Student's *t* test, $P < 0.0001$) (Figure 1B, upper panel). To confirm that this cell-death response was apoptotic, we assayed the characteristic appearance of phosphatidylserine on the outer leaflet of the plasma membrane by staining intact cells with FITC-annexin V and propidium iodide (PI) (Figure 1B, lower panel). Serum withdrawal resulted in the appearance of annexin V-positive, PI-negative cells in the lower right quadrant ($43.9 \pm 9.4\%$, mean $\pm$ S.D. of $n = 19$ determinations), and this was strongly inhibited by thrombin ($4.4 \pm 1.4\%$, significant decrease from serum-free value by Student's *t* test, $P < 0.0001$). Cells in fresh 10% FBS exhibited low levels of annexin V staining. These results indicate that thrombin is a survival factor for CCl39 cells, preventing the activation of caspases and the appearance of downstream caspase-dependent markers of apoptosis following serum withdrawal.

Activation of PKC fails to mimic thrombin-induced cell survival

Thrombin stimulates phospholipase C-catalysed inositol lipid hydrolysis, resulting in the mobilization of Ca²⁺ from intracellular stores and activation of protein kinase C (PKC). Activation of PKC

Figure 1 Thrombin protects CCI39 cells from serum-withdrawal-induced apoptosis

(A) Cycling CCI39 cells were switched to serum-free (SF) medium alone or medium containing fresh 10% FBS or 100 nM thrombin for various periods of time. Whole-cell lysates were prepared and assayed for total DEVDase activity (left panel; results are means $\pm$ S.D. for $n = 3$ cell samples; Fl. U = fluorescence units) or for the appearance of the cleaved, activated form of caspase 3 by Western blot (WB; right panel). Results for the Western blot are from a single experiment representative of between three and six others. (B) Cycling CCI39 cells were switched to serum-free medium alone or containing fresh 10% FBS or 100 nM thrombin (Thr). After 24 h cultures were fixed and stained with PI to assay for sub-G₁ DNA content (upper panel) or intact cells were stained with FITC-annexin V and PI (lower panel). The data sets shown are taken from a single representative experiment. Values quoted represent means $\pm$ S.D. for percentage of cells with sub-G₁ DNA or the percentage of cells which were annexin V-positive and PI-negative and are pooled from $n = 5$ (sub-G₁) or $n = 19$ (annexin V) separate determinations.

affords protection against apoptosis in some cell types [27]. To examine the potential role of PKC in cell survival, we serum-starved cells in the presence of 100 nM PMA. In contrast with thrombin, PMA caused only a modest decrease in the serum-withdrawal-induced appearance of annexin V-positive cells (Figure 2A). However, because these assays necessitated the prolonged exposure of cells to PMA, there was a concern that PKC might be depleted by 'down-regulation' under these conditions. Therefore, as an alternative, we used a PKC inhibitor to probe the role of PKC in thrombin-induced protection. For these experiments we used the selective PKC inhibitor GF109203X, since it does not cause the concomitant activation of stress kinase pathways that is observed with some other PKC inhibitors, such

as Ro-21-8220 [28,29], and which might have compromised cell viability. As confirmation that the drug was effective, we observed that GF109203X was able to inhibit PMA-stimulated phosphorylation of ERK1/2, whereas the response to thrombin was not affected (Figure 2B). When cells were starved of serum in the presence of PMA, we again observed only a modest reduction in DEVDase activity, and this was completely reversed by treatment with GF109203X (Figure 2C). In contrast, the presence of thrombin again completely abolished serum-withdrawal-induced caspase activation, and this protection was unaffected by the PKC inhibitor (Figure 2C). Taken together, these results argue against a major role for phorbol-ester-responsive or GF109203X-inhibitable forms of PKC in thrombin-induced cell survival.

Figure 2 Thrombin-induced protection is independent of PKC

(A) Cycling CCI39 cells were switched to serum-free (SF) medium alone or containing fresh 10% FBS, 100 nM thrombin (Thr) or 100 nM PMA. After 24 h, intact cells were stained with FITC-annexin V and PI. Each data set represents the mean $\pm$ range for two cell dishes, and the results are taken from a single experiment representative of four. (B) Serum-starved CCI39 cells were pretreated with 5 μ M GF109203X (GF) or a vehicle control before stimulating with 100 nM PMA (30 min) or 100 nM thrombin (15 min) in the presence of 5 μ M GF109203X or a vehicle control. Whole-cell lysates were subjected to SDS/PAGE and blotted for total and phospho-ERK1/2. (C) Cycling CCI39 cells were switched to serum-free (SF) medium alone or containing 100 nM thrombin or 100 nM PMA in the presence or absence of 5 μ M GF109203X or vehicle control (Con). After 12 h, cells were lysed and assayed for DEVDase activity. Each data set represents the mean $\pm$ S.D. for three cell dishes, and the results are taken from a single experiment representative of three.

Thrombin-dependent survival signals are inhibited by pertussis toxin

The Raf $\rightarrow$ MEK $\rightarrow$ ERK and PI3K $\rightarrow$ PDK (phosphoinositide-dependent protein kinase) $\rightarrow$ PKB cascades are two of the major signalling pathways implicated in promoting cell proliferation and cell survival [30,31], and both pathways were activated when serum-starved CCI39 cells were stimulated with thrombin. For example, phosphorylation of ERK1/2 could be detected by immunoblot within 5–10 min of addition of thrombin, and this response persisted above basal for at least 5–7 h (Figures 3A and 3C) [12]. Phosphorylation of PKB could also be detected within

5–10 min of addition of thrombin, and this response persisted for the entire duration of the time course (Figures 3A and 3C).

It is well known that the ability of thrombin to act as a growth factor is inhibited by pertussis toxin in CCI39 cells [3]. For example, the ability of thrombin to promote [3 H]thymidine incorporation was inhibited by at least 70% by pre-treatment with pertussis toxin. (Figure 3B, left panel). Similarly, when CCI39 cells were pre-treated with pertussis toxin and then subjected to serum withdrawal, the protection provided by thrombin was reversed by 50–60% (Figure 3B, right panel). These results indicate that, in common with cell proliferation, the thrombin receptor uses G_i or G_o heterotrimeric GTPases to couple to signalling pathways that promote cell survival.

The approx. 50% decrease in thrombin-induced survival by pertussis toxin was also seen in the activation of both the ERK and PKB pathways. For example, pertussis toxin pretreatment decreased the maximum peak of thrombin-stimulated ERK1 activation at 5 min by approx. 50%, and the sustained response at 5 h by 60–70% (Figure 3C, left panel). Similarly, pertussis toxin decreased PKB phosphorylation by approx. 50% after 5 min or 5 h of thrombin stimulation (Figure 3C, right panel). Thus thrombin uses both pertussis-toxin-sensitive and -insensitive pathways to activate ERK, PKB and cell survival.

Thrombin-induced cell survival requires the ERK and PI3K signalling pathways

To determine if the ERK and PI3K pathways were required for thrombin-induced survival, CCI39 cells were pretreated with U0126, a MEK1/2 inhibitor, or LY294002, a PI3K inhibitor, and then switched to serum-free medium with or without the appropriate inhibitors in the presence or absence of thrombin for 24 h. When death was assessed by annexin V staining (Figure 4A), we observed that the protective effect of thrombin was reversed by either U0126 or LY294002. In contrast, U0126 or LY294002 alone had little, if any, effect on cell death induced by serum withdrawal. Similar results were obtained when we assayed the number of cells with sub-G₁ DNA (C. J. Chalmers and S. J. Cook, unpublished work). To examine whether U0126 and LY294002 co-operated or synergized to reverse the protective effect of thrombin, we used suboptimal doses of these drugs. Although these low doses on their own had little effect (Figure 4B), the combination of these drugs did result in a synergistic effect, manifest as a partial reversal of thrombin-induced protection. Taken together these results suggest that both the Raf $\rightarrow$ MEK $\rightarrow$ ERK and PI3K signalling pathways are required, and co-operate, to transduce the survival signal(s) by which thrombin protects against serum withdrawal.

Thrombin protects against serum-withdrawal-induced, but not etoposide-induced, death

To determine whether thrombin could protect against other forms of stress, we compared the response of cells to serum withdrawal with that observed by treatment with the DNA topoisomerase inhibitor etoposide. CCI39 cells were serum-starved for 12 h or were treated with etoposide for 24 h in the presence or absence of thrombin, and whole cell lysates were assayed for DEVDase activity (Figure 5A). The results revealed that, whereas thrombin could prevent serum-withdrawal-induced caspase activation, it could not prevent etoposide-induced caspase activation. This argues for the notion that thrombin does not simply inhibit the activation of effector caspases, which are activated in response to virtually all pro-apoptotic stimuli, but rather suggests that thrombin exerts a more selective effect on the upstream components involved in the response to serum withdrawal.

Figure 3 Pertussis toxin blocks activation of ERK1/2 and PKB and inhibits thrombin-induced cell survival

(A) CCI39 cells were serum-starved and stimulated with 100 nM thrombin (SF + Thr) for the indicated times. Whole-cell lysates were subjected to SDS/PAGE and probed by immunoblot with antibodies specific for total ERK1/2 (ERK1/2), phospho-ERK1/2 (p-ERK1/2), total PKB (PKB) and [phosphoserine-473]PKB [P(S473)]. 10 m, 1, 7 and 24 mean 10 min, 1 h, 7 h and 24 h. (B) Left panel: CCI39 cells were serum-starved in presence or absence of 100 ng/ml pertussis toxin (PTx) and then re-stimulated in the presence of thrombin (Thr). DNA synthesis was assayed by [³H]thymidine incorporation ([³H]-Thy inc); results are means $\pm$ S.D. for $n=3$ cell samples taken from a single experiment representative of four others. *Inhibition by pertussis toxin was statistically significant by Student's t test, $P < 0.01$. Right panel: cycling CCI39 cells were pretreated with 100 ng/ml pertussis toxin (PTx) for 6 h and then switched to serum-free (SF) medium with or without pertussis toxin or thrombin (Thr) as indicated. Cell death was assayed by annexin V staining, and results represent the mean $\pm$ S.D. for triplicate cell samples from a single experiment representative of four. *Pertussis toxin caused a significant reversal of thrombin-induced protection by Student's t test, $P < 0.05$. (C) CCI39 cells were serum-starved in the presence or absence of 100 ng/ml pertussis toxin (PTx) and stimulated with 100 nM thrombin (Thr) for 5 min or 5 h. Left panel: ERK1 activity was assayed by immune complex kinase assay and data is expressed as fold increase over zero time control (mean $\pm$ S.D. for triplicate cell samples; 5 m means 5 min). *Inhibition by pertussis toxin was statistically significant by Student's t test, $P < 0.02$. Right panel: PKB phosphorylation was determined by blotting with anti-(total PKB) (PKB) and anti-[phosphoserine-473]PKB [P(S473)] antisera.

Figure 4 ERK and PI3K pathways are required for thrombin-induced survival

(A) Cycling CCI39 cells were switched to serum-free medium in the presence or absence of thrombin. Where indicated, U0126 (10 μ M, grey bars) or LY294002 (25 μ M, hatched bars) was included. Apoptosis was assayed by the appearance of cells which were annexin V-positive and PI-negative staining, and results are means $\pm$ S.D. for triplicate cell samples from a single experiment representative of four others. *U0126 and LY294002 both caused a significant reversal of thrombin-induced protection by Student's *t* test, $P < 0.01$. (B) Cycling CCI39 cells were switched to serum-free (SF) medium in the presence or absence of thrombin. Where indicated, U0126 (2.5 μ M, grey bars) or LY294002 (5 μ M, diagonally hatched bars) were included alone or in combination (U0 LY; horizontally striped bars). After 12 h, cells were lysed and assayed for DEVDase activity. Each data set represents the mean $\pm$ S.D. for three cell dishes, and the results are taken from a single representative experiment.

Bim is expressed in response to serum withdrawal but not in response to etoposide

Recent studies have suggested that the 'BH3-only' proteins respond to discrete forms of stress and act to initiate activation of Bax and Bak [32]. For example, PUMA (p53 upregulated modulator of apoptosis) is expressed in a p53-dependent manner following DNA damage [33]. Two BH3-only proteins, Bad and Bim, have been particularly associated with the response to withdrawal of survival factors. Bad is de-phosphorylated following withdrawal of survival factors and binds to Bcl-2 and Bcl-x_L, thereby neutralizing their protective effects [34]. In contrast with Bad, Bim is expressed *de novo* upon withdrawal of survival factors in lymphocytes [35] and neurons [36]. We compared the expression of Bim and Bad in response to serum withdrawal with that observed following treatment with etoposide. Serum starvation resulted in the rapid expression of Bim; Bim_{EL} was the major splice variant observed, with only low levels of Bim_L detected [25]. Increased expression of Bim_{EL} was apparent as early as 3 h after withdrawal of serum (Figure 5B),

preceding caspase activation (Figure 1A), and persisted for at least 24 h (Figure 6A), by which time caspase activation had peaked and downstream markers of apoptosis were apparent. In contrast, etoposide caused little if any increase in Bim expression. The expression of Bad, Bcl-2 and Bcl-x_L remained unchanged in response to serum withdrawal or etoposide (Figure 5B).

Serum-withdrawal-induced death and Bim expression require *de novo* protein synthesis

We have recently shown that the ability of serum withdrawal to promote cell death in CCI39 cells is dependent on synthesis of new mRNA and protein [25]. For example, serum starvation for 12 h resulted in a strong increase in DEVDase activity, and this was completely inhibited by cycloheximide (Figure 5C). Cycloheximide can also prevent the activation of Bax in response to serum withdrawal [25], indicating that synthesis of a new protein is required to initiate Bax activation. BH3-only proteins are prime candidates for this role [32], so we examined the effect of cycloheximide on expression of Bim and Bad, as these proteins have previously been implicated in the response to withdrawal of survival factors [25,30–32,34–36].

Bim expression was very low in cycling cells maintained in 10% FBS, and cycloheximide had no effect on this (Figure 5D). Serum withdrawal from CCI39 cells promoted the strong expression of Bim_{EL}, and this was completely inhibited by cycloheximide. In contrast, Bad expression was not affected by cycloheximide or serum starvation, suggesting that it is a relatively stable, constitutively expressed, protein. Total levels of ERK1/2 served as a loading control. Thus *de novo* expression of Bim is an early event following serum withdrawal, but is not observed in response to DNA damage. This correlates very strongly with the almost complete requirement for new protein synthesis to initiate Bax activation and cell death during serum withdrawal [25].

Thrombin inhibits Bim expression via the ERK and PI3K pathways

Since thrombin was a very effective survival factor for CCI39 cells, we examined whether thrombin influenced Bim expression during serum withdrawal. When CCI39 cells were starved of serum in the presence or absence of thrombin, we observed that the inclusion of thrombin completely prevented the expression of Bim_{EL} protein (Figure 6A). To determine how thrombin reduces Bim levels, we used (Q)RT-PCR (real-time quantitative RT-PCR) to analyse the level of Bim mRNA. We observed that serum withdrawal resulted in a 5-fold increase in Bim mRNA, which was clearly apparent after 6 h (Figure 6B). In contrast, thrombin strongly inhibited the increase in Bim mRNA levels observed in response to serum withdrawal. For example, after 12 h of serum withdrawal, thrombin caused an $88 \pm 6.5\%$ decrease in Bim transcript levels (mean $\pm$ S.D. for three independent experiments, each assayed in triplicate). Thus thrombin acts to prevent serum-withdrawal-induced Bim expression in part by causing a substantial decrease in Bim mRNA levels.

Expression of Bim is promoted by the FKHR-L1/FOXO-3A transcription factor [35], but this is normally inactivated by PKB-catalysed phosphorylation, which excludes FKHR-L1 from the nucleus [37]. Indeed, simple inhibition of PI3K is sufficient to induce Bim expression in some cell types [25,35]. In addition, we have shown that the conditional kinase Δ Raf-1:ER* can repress Bim expression via activation of the ERK pathway [25]. To determine which pathway was being used by thrombin to repress Bim expression, we serum-starved CCI39 cells in the presence or absence of thrombin with or without U0126 or LY294002. The results of this experiment revealed that the ability of thrombin

Figure 5 Bim_{EL} is expressed *de novo* during serum-withdrawal-induced cell death

(A) CCI39 cells were either serum-starved for 12 h or treated with etoposide (30 μ M) for 24 h in the presence or absence of thrombin, and cell lysates were assayed for DEVDase activity (FLU = fluorescence units). Results are expressed as mean $\pm$ S.D. for triplicate cell samples, and similar results were observed in four experiments. *Thrombin caused a significant decrease in caspase activation by Student's *t* test, $P < 0.01$. (B) Cycling CCI39 cells were serum-starved (SF) or maintained in FBS but treated with 30 μ M etoposide (Etop) for the indicated times. The expression of Bim_{EL}, Bad, Bcl-2 and Bcl-x_L was determined by Western blotting. (C) Cycling CCI39 cells were treated with or without cycloheximide (CHX; 30 μ g/ml) and subjected to serum withdrawal. Cells maintained in FBS served as controls, and DEVDase activity was assayed. Results are means $\pm$ S.D. for triplicate cell dishes. *Cycloheximide caused a significant decrease in caspase activation by Student's *t* test, $P < 0.01$. (D) CCI39 cells were pre-treated with or without cycloheximide (CHX; 30 μ g/ml) for 30 min and then serum-starved in the presence or absence of cycloheximide for 12 h. Whole-cell lysates were then subjected to SDS/PAGE and subjected to immunoblotting with antibodies specific for Bim, Bad or total ERK1/2.

to repress Bim_{EL} expression was reversed by either U0126 or LY294002, suggesting that, like thrombin-mediated survival, both the ERK and PI3K pathways are required for thrombin to block Bim_{EL} expression (Figure 6C). It was also apparent from this analysis that LY294002 caused a partial inhibition of thrombin-induced ERK1/2 activation. Thus the requirement of PI3K in repressing Bim expression may in part reflect the use by thrombin of a PI3K-dependent pathway to optimally activate the ERK1/2 pathway. Bim_{EL} resolved as a phosphoprotein in 10% FBS, whereas the newly expressed Bim_{EL} following serum withdrawal was hypophosphorylated, in agreement with previous observations [25]. Thrombin promoted phosphorylation of Bim_{EL}, and this was inhibited by U0126 and partially by LY294002 (Figure 6C).

PAR1p mimics thrombin in promoting cell survival

PAR1 receptor-selective agonist peptides mimic many of the biochemical effects of thrombin in CCI39 cells, but fail to mimic

thrombin-stimulated cell cycle re-entry, because they cause only a transient activation of the ERK1/2 pathway [8,9,12]. To assess the effects of selective activation of the PAR1 receptor on cell survival, we used a nonamer PAR1 receptor agonist peptide (SFLLRNPND, referred to as PAR1p).

CCI39 cells were switched to serum-free medium alone or containing thrombin, PAR1p or fresh 10% FBS for various times, and cell death was determined by the appearance of annexin V-positive cells. Thrombin again promoted cell survival, and this effect persisted for up to 48 h. In contrast, whereas PAR1p could inhibit the appearance of dead cells for up to 24 h, this protective effect was only transient and, by 48 h, the protective effect of PAR1p was completely lost (Figure 7A). Similar results were obtained when we assayed cell death by the appearance of cells with sub-G₁ DNA (C. J. Chalmers and S. J. Cook, unpublished work).

We next examined the effect of PAR1p on serum-withdrawal-induced Bim_{EL} expression. Thrombin was again able to completely prevent Bim_{EL} expression, and this effect persisted for at

Figure 6 Thrombin prevents serum-withdrawal-induced Bim expression via the ERK and PI3K pathways

CCl39 cells were serum-starved in the presence (SF + Thrombin) or absence (SF) of 100 nM thrombin for the indicated times, and whole-cell lysates were assayed for Bim expression by Western blotting (A) or by (Q)RT-PCR (B). Results are from a single experiment representative of three. (C) CCl39 cells were pre-treated with U0126 (U0) or LY294002 (LY) as indicated and were then starved of serum for 6 h in the presence or absence of 100 nM thrombin (Thr); C = control. In both cases, whole cell lysates were then subjected to SDS/PAGE and immunoblotting with antibodies specific for Bim_{EL}, phospho-ERK1/2 (P-ERK1/2), total ERK1/2 (ERK1/2) and [phosphoserine-473]PKB [P(Ser473)-PKB].

least 24 h (Figure 7B). In contrast, PAR1p was less effective at preventing the expression of Bim_{EL}. PAR1p caused a decrease in Bim levels at 12 h, but this effect was transient, so that, by 24 h, Bim_{EL} was expressed at the same level as that seen in cells serum-starved in the absence of PAR1p (Figure 7B). The total levels of Bad did not change at all during serum withdrawal and served as a useful loading control. We then compared the ability of thrombin and PAR1p to inhibit the serum-withdrawal-induced increase in expression of Bim mRNA using (Q)RT-PCR (Figure 7C). In these experiments we found that PAR1p was slightly less effective than thrombin at decreasing Bim mRNA expression; for example, thrombin caused an $88 \pm 6.5\%$ decrease in Bim mRNA levels, whereas PAR1p caused a $72.6 \pm 6.5\%$ reduction (mean $\pm$ S.D. of three independent determinations; different by

Figure 7 PAR1p mimics thrombin, but elicits only transient protection against serum withdrawal

(A) Cycling CCl39 cells were serum-starved for the indicated times in the presence or absence of fresh 10% FBS, 100 nM thrombin (Thr) or 100 μ M PAR1p. Cells were assayed for the appearance of cells staining positive for annexin V, and each data set represents the means $\pm$ S.D. for triplicate dishes of cells, taken from a single experiment representative of four. *Thrombin and FBS caused a significant decrease in cell death at 48 h compared with the serum-free control, as assessed by Student's *t* test, $P < 0.01$. (B) Cycling CCl39 cells were serum-starved in the presence (SF + Thr) or absence (SF) of thrombin or PAR1p as indicated for 12 or 24 h, and expression of Bim_{EL} and Bad was determined by Western blotting. (C) Cycling CCl39 cells were serum-starved for the indicated times in the presence or absence of fresh 10% FBS, 100 nM thrombin (Thr) or 100 μ M PAR1p and Bim mRNA levels were assayed by (Q)RT-PCR. Results are means $\pm$ S.D. of triplicate determinations from a single experiment representative of four. Con = control.

Student's *t* test; $P = 0.04$). However, this difference was small, and alone is unlikely to account for the differences seen at the protein level (Figure 7B). Thus it is possible that the difference in the ability of thrombin and PAR1p to decrease Bim levels may also reflect differences in post-translational regulation.

All these studies were performed with doses of thrombin and PAR1p that were saturating for activation of downstream signalling events in CCl39 cells. However, we did consider other possible explanations for the transient protection afforded by PAR1p. One explanation was that thrombin was utilizing a non-PAR1 receptor for sustained signalling. PAR2 is not activated by thrombin, and PAR3 has no intrinsic signalling capacity, but enhances signalling through the PAR4 receptor. However,

Figure 8 Further addition of PAR1p 12 h after serum withdrawal allows sustained protection against serum withdrawal

Cycling CCl39 cells were serum-starved for 24 or 48 h in the presence or absence of 100 nM thrombin (Thr) or 100 μ M PAR1p. In the case of PAR1p, one set of dishes received a second dose of PAR1p after 12 h (diagonally hatched bars), whereas another received additional doses of PAR1p after 12 and 24 h (horizontally striped bars). After 24 or 48 h, cultures were fixed and stained with PI to assay for sub-G₁ DNA content. The results are the mean $\pm$ range of duplicate determinations from a single experiment representative of three.

although PAR4 agonist peptides alone are effective at doses above 100 μ M, we have been unable to detect any biochemical or biological response to such peptides in CCl39 cells even at 500 μ M (C. J. Chalmers and S. J. Cook, unpublished work). Alternative explanations might include the possibility that thrombin induced a more sustained activation of the proteolysed PAR1 receptor which was not faithfully mimicked by PAR1p or that the half-life of PAR1p was 'limiting' over the 48 h of the experiment. To address the latter possibility directly, we compared the protective effect of PAR1p when added at the time of serum withdrawal with that seen when additional PAR1p was also added after 12 h or again after 24 h. When thrombin or PAR1p were added at the time of serum withdrawal, both agonists again conferred strong protection for 24 h, but PAR1p was again much less effective than thrombin at 48 h (Figure 8). In contrast, if a second dose of PAR1p was added 12 h after serum withdrawal, the PAR1p-treated cells were protected to the same extent as those treated with thrombin when assayed 48 h after serum withdrawal (Figure 8, diagonally hatched bars). A third dose of thrombin 24 h after serum withdrawal exerted no further protection over that seen with the two-dose regimen (Figure 8, horizontally striped bars). These results suggest the half-life or availability of PAR1p during the first 12 h is a major determinant of PAR1p-induced protection.

DISCUSSION

Thrombin is a growth and survival factor

We have shown that thrombin functions as an effective survival factor for CCl39 cells, preventing the activation of caspases and downstream caspase-dependent events during serum withdrawal. The signal-transduction pathways that mediate this thrombin-induced survival signal are remarkably similar to those used to promote cell-cycle re-entry. For example, inositol lipid hydrolysis and activation of PKC play a minor role in thrombin-induced cell proliferation [38], and we found that phorbol-ester-sensitive forms of PKC afforded little protection against serum withdrawal, and GF109203X had no effect on thrombin-induced survival. Thrombin promotes cell-cycle re-entry via a pertussis-toxin-

sensitive pathway, which leads to the activation of Ras and the Raf $\rightarrow$ MEK $\rightarrow$ ERK and PI3K pathways [3,8–13], and our analysis here draws very similar conclusions concerning the pathways by which thrombin promotes cell survival. Thrombin-induced cell survival was reversed by 50–60 % in pertussis-toxin-treated cells, suggesting that the major cell-survival pathways are activated via Gi-dependent and -independent heterotrimeric GTPases. In addition, thrombin activated both the ERK and PI3K pathways in a pertussis-toxin-sensitive manner (Figure 3C), and these pathways were absolutely required for thrombin-induced protection (Figure 4). Since pertussis toxin did not completely reverse this protection, this might suggest that other signal pathways, such as Rho [39], also contribute to the thrombin-mediated survival signal. However, since approx. 50 % of the ERK and PKB activation seen with thrombin was insensitive to pertussis toxin, a simpler explanation is that thrombin uses both pertussis-toxin-sensitive and -insensitive pathways to activate ERK, PKB and cell survival. Certainly constitutive activation of either the ERK or PKB pathways is sufficient to protect a variety of cells from serum withdrawal [30,31].

PAR1p, a PAR1 receptor-specific agonist peptide, mimicked many aspects of the thrombin survival signal. Indeed, in qualitative terms, PAR1p mimicked all aspects of thrombin. It promoted cell survival, and this effect was also reversed by pertussis toxin, U0126 or LY294002 (C. J. Chalmers and S. J. Cook, unpublished work). This suggests that thrombin acts via the PAR1 receptor to promote cell survival. The only notable difference between thrombin and PAR1p was the transient nature of the PAR1p pro-survival effect, which was lost after 12 h in the case of Bim expression and after 24 h in the case of downstream markers of death. This is also reminiscent of the inability of PAR1p to promote cell-cycle re-entry in serum starved CCl39 cells [8,9]. This might suggest that sustained survival signalling requires an alternative thrombin receptor. However, of the other PARs, only PAR4 would be likely to be responsive to thrombin, and we failed to detect any responses to PAR4 agonist peptides in these cells (C. J. Chalmers and S. J. Cook, unpublished work). Alternatively, since PAR1p elicits only a transient activation of the ERK pathway [9,12], it could be that the proteolysed PAR1 receptor simply provides a more sustained activation of the ERK and PI3K pathways, which is a prerequisite for both cell-cycle re-entry and to maintain cell viability. However, our results suggest a much simpler explanation. Administration of an additional dose of PAR1p, 12 h after the first during serum withdrawal (and the first dose), allowed PAR1p to elicit a sustained protective effect that matched that of thrombin for up to 48 h. This strongly suggests that the transient protective effect of PAR1p may simply be due to a poor half-life or availability of the peptide during the first 12 h of serum withdrawal. Whether this reflects proteolysis of the peptide, loss through receptor-mediated internalization or loss by non-specific binding to membranes during prolonged stimulation times, remains to be determined.

Thrombin inhibits Bim expression via the ERK and PI3K pathways

Previous studies have not addressed the mechanism by which thrombin promotes cell survival. The ability of thrombin to protect against serum withdrawal, but not etoposide, focused attention on those upstream components utilized selectively in the response to serum withdrawal. Apoptosis resulting from the withdrawal of survival factors is regulated by the Bcl-2 proteins [40]. Pro-apoptotic proteins such as Bax and Bak act to initiate death by promoting the release of apoptogenic factors from the mitochondria. Bax and Bak are 'repressed' in viable cells by their physical interaction with pro-survival proteins such as Bcl-2 and Bcl-x_L.

This balance is tipped in favour of death by the BH3-only proteins, which are activated in response to stress stimuli, bind to Bcl-2 or Bcl-x_L, and so allow Bax to promote cell death [32]. Notably, different BH3-only proteins respond to discrete forms of stress.

Two BH3-only proteins, Bad and Bim, have been implicated in death resulting from withdrawal of survival factors [30–32, 34–36, 41, 42], and it is notable that both are regulated by the PI3K/PKB and ERK pathways – the same pathways used by thrombin to protect CCI39 cells from serum withdrawal. Bad is normally repressed by phosphorylation-dependent binding to 14-3-3, the kinases responsible being PI3K-dependent PKB and ERK-dependent RSK1 (p90 ribosomal S6 kinase) [31, 34, 41, 42]. Following withdrawal of survival factors, Bad is dephosphorylated, with the result that it can now bind to Bcl-x_L and so promote apoptosis. Bim is expressed *de novo* following withdrawal of survival factors or serum from primary sympathetic neurons [36, 43], lymphocytes [35] and fibroblasts [25] and, in at least two of these systems, it is repressed by the PI3K or ERK pathways [25, 35].

In considering a potential role for Bad or Bim, we made use of the fact that actinomycin D and cycloheximide strongly inhibit serum-withdrawal-induced death in CCI39 cells [25]. More specifically, new gene expression is required to initiate Bax activation following serum withdrawal [25]. This argues against a role for Bad in serum-withdrawal-induced Bax activation and cell death, since it is a relatively stable protein and was constitutively expressed (Figures 5B and 5D). In contrast, Bim is rapidly expressed *de novo* in response to serum withdrawal (Figures 5B and 5D) and can kill CCI39 cells when ectopically expressed [25], suggesting that Bim, but not Bad, is more likely to promote activation of Bax and cell death in CCI39 cells following serum withdrawal. Certainly, there is a strong correlation between the ability to block Bim expression and to block apoptosis during serum withdrawal. For example, thrombin was able to completely inhibit Bim expression and elicited a sustained protective effect, whereas a single dose of PAR1p caused only a transient and modest decrease in Bim expression and elicited only a transient protective effect. In addition, the ability of thrombin to inhibit Bim expression, like the cytoprotective effect, was reversed by U0126 or LY294002.

Three signal pathways have been implicated in the regulation of Bim expression. In neurons, the JNK (c-Jun N-terminal kinase)/c-Jun pathway is required for Bim mRNA expression following withdrawal of NGF (nerve growth factor) [36], but this pathway appears to play little, if any, role in fibroblasts [25]. In lymphocytes, Bim mRNA expression is promoted by the FKHR-L1/FOXO-3A transcription factor, which is normally repressed by PKB-catalysed phosphorylation; consequently inhibition of PI3K or withdrawal of survival factors is sufficient to induce Bim expression in these cells [35]. Our analysis here indicates that thrombin uses both the PI3K and the ERK pathways to repress Bim expression, since either LY294002 or U0126 was able to reverse the repression induced by thrombin. A role for the ERK1/2 pathway in repressing Bim expression is not entirely unprecedented. In fibroblasts, Bim expression is repressed by either the PI3K or ERK pathways, since selective inhibitors of each are sufficient to induce expression of Bim mRNA and Bim protein, and selective activation of the ERK1/2 pathway with the conditional kinase Δ Raf-1:ER* blocks Bim expression independently of PKB phosphorylation [25]. Nonetheless, this represents the first demonstration that a growth and survival factor uses the ERK1/2 pathway to repress Bim.

The relative importance of the PI3K pathway compared with the ERK pathway was difficult to gauge, since LY294002 caused a partial inhibition of thrombin-induced ERK1/2 activation

(Figure 6C), suggesting that some of the effect of LY294002 in reversing Bim repression may be due, in part, to inhibition of both the ERK1/2 and PI3K pathways. It is well known that PI3K inhibitors can prevent activation of ERK1/2 by certain growth factors. In some cases this may reflect a role for PI3K in signalling to Ras, whereas in others it may simply reflect the role of PI3K in intracellular trafficking of the activated receptor that may be required for optimal downstream signalling to the ERK pathway. In contrast, U0126 reversed thrombin-induced repression of Bim without inhibiting PKB phosphorylation, suggesting that the ERK1/2 pathway at least can act independently of PI3K to inhibit Bim expression. Indeed, we found that inhibition of the ERK pathway enhanced PKB phosphorylation, suggesting that activation of the ERK pathway may antagonize PKB phosphorylation, possibly to allow fine tuning of mitogenic and survival signalling (C. R. Weston and S. J. Cook, unpublished work).

The ability of thrombin to prevent the increase in Bim mRNA is consistent with thrombin acting indirectly on Bim by inhibiting the transcription of the *Bim* gene, though we cannot presently rule out a possible effect on mRNA stability. Although FOXO-3A has been shown to play a role in regulating *Bim* gene transcription downstream of PKB [35], it is possible that the ERK pathway may also contribute to thrombin-mediated inhibition of Bim mRNA levels, since U0126 is sufficient to induce Bim mRNA levels [25]. In addition, it is clear that Bim_{EL} resolves as a phosphoprotein in response to stimulation with thrombin. This phosphorylation of Bim_{EL} correlated with activation of the ERK1/2 pathway, since it was reversed by U0126. LY294002 also caused a partial reversal of phosphorylation, but this seems to reflect the fact that PI3K activity is required for optimal thrombin-induced ERK1/2 activation in these cells (Figure 6C). We have recently shown that activation of the ERK1/2 pathway is necessary and sufficient to promote the phosphorylation and proteasomal degradation of Bim_{EL} [44]. Thus thrombin may also exert a direct effect on Bim_{EL} itself by stimulating the ERK-dependent phosphorylation and turnover of the protein. Interestingly, this mechanism may account for the inability of a single dose of PAR1p to cause a sustained down-regulation of Bim protein levels. Although PAR1p was slightly less effective than thrombin at reducing Bim mRNA levels (Figure 7C), it seems unlikely that this alone would account for the substantial difference seen at the protein level (Figure 7B). Since it is well known that PAR1p causes only a very transient activation of ERK1/2 compared with the sustained response seen with thrombin [9, 12], this suggests that ERK-dependent protein turnover may make a significant contribution to the relative abilities of thrombin and PAR1p to decrease Bim_{EL} levels.

In summary, our results reveal that thrombin is a very effective survival factor, using the same signalling pathways – ERK and PI3K – to prevent serum-withdrawal-induced Bim expression and cell death as it does to promote cell-cycle re-entry. The ability of PAR1p to mimic this survival signalling suggests that thrombin acts in large part via the PAR1 receptor. The more transient protection afforded by PAR1p probably reflects a poor half-life of the peptide during prolonged stimulation and serves to underline the importance of persistent signalling in the maintenance of viability as well as cell-cycle re-entry in immortalized cells. Thrombin is the most effective growth factor in CCI39 cells and is almost unique in being able to maintain CCI39 cells in asynchronous culture in the presence of only minimal medium supplements [36]. Whereas this is no doubt due to the strong, persistent proliferative signal, it is also probably a reflection of an equally persistent survival signal.

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