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Contribution of the p38^{MAPK} signalling pathway to proliferation in human cultured airway smooth muscle cells is mitogen-specific

[1,2]Darren J. Fernandes, [1,2]Claire E. Ravenhall, [1]Trudi Harris, [1]Thai Tran, [1]Ross Vlahos & *,[1]Alastair G. Stewart

[1]Department of Pharmacology, University of Melbourne, Parkville, Victoria 3010, Australia

1 We have investigated the role of p38^{MAPK} in human airway smooth muscle (HASM) proliferation in response to thrombin and bFGF. The regulation of cyclin D1 mRNA, cyclin D1, cyclin E and p21^{Cip1} protein levels, and the extent of retinoblastoma protein (pRb) phosphorylation in response to activation of p38^{MAPK} have also been examined.

2 Two distinct inhibitors of p38^{MAPK}, SB 203580 (10 μM) and SB 202190 (10 μM), prevented bFGF (0.3–3 nM)-stimulated cell proliferation, but had no effect on the response to thrombin (0.3–3 U ml^{-1}).

3 In cells incubated with thrombin or bFGF for 20 h, there was an increase in p38^{MAPK} phosphorylation in response to bFGF, but not to thrombin. Thrombin and bFGF-stimulated increases in ERK phosphorylation and cyclin D1 mRNA and protein levels were not influenced by SB 203580 pre-treatment. Similarly, cyclin E and p21^{Cip1} protein levels, measured after 20 h incubation with mitogen, did not appear to be regulated by SB 203580 (10 μM).

4 Although both thrombin and bFGF significantly increased levels of pRb phosphorylation, SB 203580 (10 μM) inhibited only bFGF-stimulated pRb phosphorylation. In addition, SB 203580 (10 μM) selectively inhibited bFGF-stimulated DNA synthesis, suggesting that the antimitogenic actions of SB 203580 on pRb phosphorylation cause cell cycle arrest at late G1 phase.

5 In conclusion, these results indicate that p38^{MAPK} is involved in bFGF-, but not in thrombin-stimulated HASM proliferation. The activation of the p38^{MAPK} pathway by bFGF, but not by thrombin, regulates the phosphorylation of pRb without influencing cyclin D1 expression.
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Keywords: p38 MAPK; bFGF; thrombin; human airway smooth muscle; proliferation; ERK; cyclin D1; cyclin E; p21^{Cip1}

Abbreviations: ASM, airway smooth muscle; bFGF, basic fibroblast growth factor; cdk, cyclin-dependent kinase; cdki, cyclin-dependent kinase inhibitor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, mitogen-activated ERK kinase; MKK, MAPK kinase; MNK1, MAPK signal-integrating kinase1; MSK1, mitogen- and stress-activated kinase1; PI3K, phosphoinositide-3-kinase; pRb, retinoblastoma protein; SAPK, stress-activated protein kinase; SB 202190, [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole]; SB 203580, [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole]

Introduction

Hyperplasia and hypertrophy of smooth muscle have a major role in the airway remodelling that thickens the airways of asthmatics (James *et al*., 1989), and the resulting increased airway smooth muscle (ASM) volume is considered to contribute to airways hyperresponsiveness (Wiggs *et al*., 1992). A number of growth factors and inflammatory mediators, known to be expressed in the inflamed asthmatic airways, are established mitogens for human cultured ASM (reviewed by Stewart, 2001).

The requirement for extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K) activation in ASM mitogenic signalling pathways has been well described (Ramakrishnan *et al*., 1998; Walker *et al*., 1998; Fernandes *et al*., 1999; Krymskaya *et al*., 1999; Page *et al*., 2000). ERK and PI3K activity are known to regulate the expression of cyclin D1, the catalytic partner for cyclin-dependent kinase 4 (cdk4) activity. The activated cyclin D1-cdk4 holoenzyme phosphorylates the retinoblastoma protein (pRb), which dissociates from E2F, leaving E2F free to stimulate the expression of factors required for progression through the G_1/S phase boundary, and for subsequent cell cycle progression through the S phase (Schulze *et al*., 1995). Cyclin E-cdk2 activity is also important in the phosphorylation of pRb, although this occurs at a later timepoint in the G1–S-phase progression. The Cip/Kip family of inhibitors (cdk inhibitors) play a dual role in the regulation of cell cycle progression. p21^{cip1} represses E2F-dependent transcription through direct binding to E2F (Shiyanov *et al*., 1996; Delavaine & La Thangue, 1999) and directly inhibits DNA replication by binding to the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ, without affecting DNA repair (Luo *et al*., 1995). In contrast, Cip/Kip proteins can also traffic/target cyclin/cdk complexes to the cell nucleus and are required for the assembly and stabilisation of the cyclin D-cdk

*Author for correspondence; E-mail: astew@clyde.its.unimelb.edu.au
[2]These authors contributed equally to this work
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complex. Thus, members of the Cip/Kip family are also important in facilitating the proliferative response (Cheng *et al*., 1999).

We recently contrasted the recruitment of mitogenic signalling pathways by thrombin and basic fibroblast growth factor (bFGF) (Ravenhall *et al*., 2000), as both of these mitogens are upregulated in asthmatic airways (Gabazza *et al*., 1999; Redington *et al*., 2001). The major conclusion obtained from this study was that the mitogenic pathways (involving extracellular-signal regulated kinase (ERK) activity and cyclin D1 levels) activated by thrombin and bFGF were different in both identity and importance for ASM mitogenesis, and were also differentially regulated depending on the concentration of the mitogen applied (Ravenhall *et al*., 2000).

Although a causal link between ERK activity and cyclin D1 levels was established in ASM stimulated with lower thrombin concentrations, at higher mitogen concentrations, cyclin D1 protein and mRNA levels were unaffected by the inhibition of ERK activity. Thus, with powerful mitogen stimulation, additional MEK1/ERK-independent signalling pathways contribute to the regulation of cyclin D1 levels and to other signals that support cell proliferation.

$p38^{MAPK}$ and c-Jun amino-terminal kinase (JNK) are activated by cell stresses, including pro-inflammatory cytokines, heat, osmotic shock, UV radiation and hypoxia (Derijard *et al*., 1994; Raingeaud *et al*., 1995), and are therefore referred to as stress-activated protein kinases (SAPKs). While the signalling pathways downstream of JNK activation appear to be non-mitogenic (Orsini *et al*., 1999; Page *et al*., 1999), the $p38^{MAPK}$ signalling pathway has been implicated in the regulation of cyclin D1 levels and/or proliferation in a variety of different cell types (Lavoie *et al*., 1996; Maher, 1999; Orsini *et al*., 1999; Chen *et al*., 2000; Page *et al*., 2001; Vlahos *et al*., 2003).

The relative importance of $p38^{MAPK}$ in the signalling leading to mitogenesis is variable and appears to differ among different cell types (Maher, 1999; Kanda *et al*., 2001; Adam *et al*., 2003). In bovine ASM, the $p38^{MAPK}$ signalling pathway decreases PDGF-stimulated expression of cyclin D1 in bovine ASM (Page *et al*., 2001), suggesting that $p38^{MAPK}$ activity might decrease mitogenesis in bovine ASM. In human ASM, the role of $p38^{MAPK}$ in mitogenic signalling remains poorly defined. We observed recently that EGF-stimulated human ASM proliferation was inhibited partly by the $p38^{MAPK}$ inhibitor SB 203580 (Vlahos *et al*., 2003). In the current study, we have assessed the regulation of cyclin D1 mRNA, cyclin D1 and cyclin E protein levels, $p21^{Cip1}$ levels and the extent of pRb phosphorylation in bFGF- and thrombin-treated human ASM cells, to explore the role of the $p38^{MAPK}$ pathway in this response. Our findings suggest that $p38^{MAPK}$ is involved in the regulation of human ASM cell proliferation and pRb phosphorylation in response to bFGF, but not thrombin.

Methods

Culture of human ASM

Human ASM was cultured from macroscopically normal bronchi (0.5–2 cm in diameter) obtained from lung resection or heart–lung transplant specimens provided by the Alfred (Melbourne) and Royal Melbourne Hospitals. Cultures were prepared as previously described in detail (Tomlinson *et al*., 1995) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 0.25% v v^{-1} BSA, 2 mM L-glutamine, 100 U ml^{-1} penicillin G, 100 $\mu g\,ml^{-1}$ streptomycin, 2 $\mu g\,ml^{-1}$ amphotericin B, 1% v v^{-1} non-essential amino acids and 1% v v^{-1} sodium pyruvate, and containing fetal calf serum (FCS) (10% v v^{-1}). Cells were maintained in Falcon culture flasks and incubated (37°C, 5% CO_2) until monolayer confluence was reached and harvested weekly by exposure to 0.125% w v^{-1} trypsin and 1 mM EDTA in PBS and passaged at a 1 : 3 split ratio. Cells at passage numbers 4–15 were used for experiments, over which range of passaging there is no detectable relationship between passage number and responsiveness to growth factors or inhibitors, or to the expression of smooth muscle-specific α-actin (Stewart *et al*., 1997). Monomed A (1% v v^{-1}), a serum-free medium supplement containing insulin, transferrin and selenium, was added to all cells at the time of mitogen addition to provide progression factors that are essential for mitogenesis. The cellular composition of the cultures was determined using the expression of smooth muscle-specific α-actin and myosin as described previously (Stewart *et al*., 1997; Fernandes *et al*., 1999).

Measurement of cell proliferation

Cells were seeded into six-well plates at a density of 1.5×10^4 cells cm^{-1} and were made quiescent. As a longer period of stimulation is required for the detection of changes in cell number (Stewart *et al*., 1995b), the cells were incubated for 72 h with the appropriate mitogen. Cells were detached from the culture plate by the addition of trypsin (as described previously), washed twice (2% FCS in PBS), isolated by centrifugation ($12{,}000 \times g$, 5 min) and resuspended in 300 μl (2% FCS in PBS) before counting in a haemocytometer chamber. Cytotoxicity was measured by flow cytometric detection (Becton Dickinson FACs Calibur) of propidium iodide-stained cells. Cells were detached from the culture plate by incubation in trypsin (as described above) and incubated in propidium iodide for 15 min to enable dye access to DNA/RNA of cells lacking membrane integrity.

Measurement of DNA synthesis

DNA synthesis was measured between 24 and 28 h after mitogen stimulation, as human ASM cells enter S phase approximately 22 h after the addition of thrombin (Stewart *et al*., 1995a). Cells were subcultured into 24-well plates in a 1 : 3 split ratio at a density of 1.5×10^4 cells cm^{-2} and grown to monolayer confluence in DMEM containing 10% v v^{-1} FCS, over a 72–96 h period (5% CO_2 in air, 37°C). Quiescence was induced 24 h prior to mitogen stimulation by removing the medium, washing cells once with PBS and replacing the medium with serum-free medium (supplemented as described above). Where required, cells were treated with the $p38^{MAPK}$ inhibitor SB 203580 (10 μM) for 30 min prior to the addition of mitogen. Cells were incubated with the appropriate mitogen for 24 h (5% CO_2 in air, 37°C) then pulsed with [^{3}H]-thymidine (1 μCi ml^{-1}) for 4 h to measure the incorporation of the radiolabel into newly synthesised DNA.

Western blot analysis

Cells were subcultured into six-well plates at a density of 1.5×10^4 cells cm^{-1}, grown to confluence and stimulated under conditions identical to those required for the measurement of cell proliferation. A stimulation period of 20 h was chosen in order to measure cell cycle protein levels late in G_1 phase before S-phase entry at approximately 22 h after mitogen addition (Stewart *et al*., 1995a). The cell protein was harvested by addition of lysis buffer (for composition and details see Fernandes *et al*., 1999; Ravenhall *et al*., 2000), the whole cell extracted protein (60 μg/lane) was separated using SDS–PAGE, transferred to nitrocellulose membranes and then the membranes were subjected to Western blotting for cyclin D1, cyclin E and p21 levels, and for phosphorylated forms of ERK, p38^{MAPK} and pRb as described previously (Fernandes *et al*., 1999; Ravenhall *et al*., 2000). Proteins were visualised on Kodak X-omat AR film after incubation with enhanced chemiluminescence reagents, then exposure levels were quantitated by laser-scanning densitometry (Molecular Dynamics Personal Densitometer, Molecular Dynamics, U.S.A.) and amounts were normalised to the levels of protein detected in control cells (fold increment over baseline). Levels of β-actin were measured to assess whether the treatments caused significant changes in protein content. No significant differences in β-actin levels were observed using the different treatments after 20 h incubation ($n=4$, fold increments over control for the various treatments were bFGF 3 nM − 1.01 ± 0.04, Thr 3 U ml^{-1} − 1.06 ± 0.06, SB 10 μM − 1.00 ± 0.11, bFGF 3 nM + SB − 1.04 ± 0.10, Thr 3 U ml^{-1} + SB − 1.03 ± 0.12).

Northern blot analyses

Cells were seeded into 75 cm^2 Falcon flasks at a density of 1.5×10^4 cells cm^{-2}, grown to confluence, serum-starved and stimulated with the appropriate mitogen for 16 h to coincide with the mid- to late-stages of the G_1 phase. Total RNA was extracted with 1 ml Trizol™ reagent according to the manufacturer's instructions. The mRNA was isolated from the total RNA using Dynabeads oligo $(dT)_{25}$, then was separated on a 1.2% formaldehyde denaturing gel and transferred to Immobilon-Ny$^+$ nylon membranes as previously described (Ravenhall *et al*., 2000). Cyclin D1 mRNA was detected by Northern hybridisation (Megaprime labelling kit, Amersham, U.K.) using a 440 bp human cDNA probe (Xiong *et al*., 1991) labelled with α-^{32}P-dCTP. The membranes were washed with standard saline citrate (SSC) and 0.1% SDS as previously described (Ravenhall *et al*., 2000), then exposed to autoradiography film. The membranes were also probed for GAPDH using a 1.3 kbp chicken cDNA probe (Dugaiczyk *et al*., 1983) and hybridised as described above. The autoradiographs were quantitated using laser-scanning densitometry (Molecular Dynamics Personal Densitometer, Molecular Dynamics, U.S.A.). Cyclin D1 mRNA levels were normalised against the levels of GAPDH mRNA to control for loading differences.

Materials

All chemicals were of analytical grade or higher. The compounds used and their sources were as follows: essentially fatty acid-free bovine serum albumin fraction V (BSA), L-glutamine, thrombin (bovine plasma), anti-smooth muscle myosin (mouse monoclonal) (Sigma, U.S.A.); amphotericin B (fungizone), human recombinant bFGF (Promega, U.S.A.); collagenase type CLS 1, elastase (Worthington Biochemical, U.S.A.); Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, U.K.); Dulbecco's 'A' phosphate-buffered saline (PBS) (Oxoid, U.K.); foetal calf serum (FCS), monomed A, penicillin-G, versene, streptomycin, trypsin (CSL, Australia); Hybond™-C supernitrocellulose membranes, α-^{32}P-dCTP (3 mCi mmol^{-1}), [^{3}H]-thymidine (5 mCi mmol^{-1}), enhanced chemiluminescence reagents, hyperfilm MP (Amersham, U.K.); anti-smooth muscle α-actin antibody (mouse monoclonal (M851)) (Dako Corporation, U.S.A.); anti-β actin antibody (mouse monoclonal AC-15) (Abcam, U.S.A.); sheep anti-rabbit IgG horseradish peroxidase-conjugated antibody (Silenus Laboratories, Australia); phospho-specific p38^{MAPK} antibody (rabbit polyclonal) (New England Biolabs, U.K.); dimethylsulphoxide (DMSO) (BDH, U.K.); X-omat film (Kodak, Australia); anti-cyclin D1 antibody (rabbit polyclonal) (Upstate Biotechnology, U.S.A.); Trizol™ reagent (Gibco BRL, Australia); Dynabeads, oligo $(dT)_{25}$ (Dynal, Norway); Immobilon-Ny$^+$ nylon membranes (Millipore, U.S.A.); SB 203580, SB 202190 (Calbiochem, Germany); cyclin E antibody (rabbit polyclonal) (Santa Cruz, U.S.A.); p21 antibody (rabbit polyclonal) (Transduction Laboratories, U.S.A.); Phospho-pRb (Ser780) antibody (rabbit polyclonal) (Cell Signalling Technologies, U.S.A.).

SB 203580 and SB 202190 were initially dissolved in DMSO to produce a stock solution of 10 mM. Immediately prior to use, SB 203580 was diluted 1 in 10 in media then added to cell supernatants to give a final concentration of 10 μM. Stock solutions of thrombin (300 U ml^{-1}) and bFGF (300 nM) were prepared in BSA (0.25% w v^{-1} in PBS).

Statistical analysis of results

All incubations for the [^{3}H]-thymidine incorporation assays were performed in duplicate. Experiments were carried out in at least four cell lines, each derived from at least four different individuals as specified. Results are presented as the mean ± standard error (s.e.m.) of n cultures. To minimise the influence of variability between tissue donors on comparisons of data, values have usually been expressed as a percentage of the response in control cells from the same plate (stimulated with Monomed A (1%) alone). Grouped data were analysed by ANOVA with Dunnet's *post hoc* comparisons to identify individual differences between responses in control cells and responses in cells stimulated with mitogens in the presence and absence of inhibitors. Significance was also identified where appropriate by the use of a paired t-test. Differences were considered to be statistically significant when the two-tailed probability was less than 0.05 with protection against multiple comparisons. All statistical analyses were performed using Graphpad Prism for Windows (Version 2.01) or Statview for Windows (Version 5).

Results

Influence of the p38^{MAPK} inhibitors, SB 203580 and SB 202190, on bFGF- and thrombin-stimulated cellular proliferation

We have previously contrasted the effects of near-maximal and supramaximal concentrations of bFGF (0.3 and 3 nM) and

thrombin (0.3 and 3 U ml^{-1}) (Ravenhall *et al*., 2000) on the activation of the p38^{MAPK} signalling pathway, and its importance for mitogen signalling. For all experiments in this study, a concentration of 10 μM SB 203580 was used to inhibit p38^{MAPK} activation, as it has been shown to completely inhibit the activity of p38^{MAPK} and the phosphorylation of its substrate, MAPKAPK-2, in several different cell types, and SB 203580 is highly selective for p38^{MAPK} even at concentrations of 100 μM (Lee *et al*., 1994; Cuenda *et al*., 1995; Foltz *et al*., 1997; Maruoka *et al*., 2000). Inhibition of the p38^{MAPK} signalling pathway had no effect on thrombin-stimulated cell proliferation, but prevented bFGF-stimulated cell proliferation (Table 1(a)). In a separate series of experiments, the effects of another p38MAPK inhibitor SB 202190 (10 μM) (Lee *et al*., 1994) were examined. SB 202190 reduced bFGF mitogenic responses, but had no effect on the response to thrombin or on control cell numbers (Table 1(b)). The number of nonviable cells in individual incubations ranged from 3 to 6% and was not significantly affected by mitogens or by SB 20380 (10 μM). Furthermore, there was no morphological evidence (cell detachment, rounded appearance) indicative of cytotoxicity.

Effects of bFGF and thrombin on p38^{MAPK} phosphorylation

A surrogate marker of activation of p38^{MAPK} was measured by examining phosphorylation of p38^{MAPK} using an antibody that detects dual phosphorylation at Thr180 and Tyr182 (New England Biolabs). Phospho-p38^{MAPK} levels were measured in lysates from cells that had been incubated with thrombin or bFGF for 20 h. At this time, increases in p38^{MAPK} phosphorylation were observed in response to stimulation by bFGF, but not thrombin (Figure 1). Although there was a tendency for an increase in p38^{MAPK} phosphorylation in response to thrombin stimulation, it was not significant (repeated-measures ANOVA, $P=0.09$, $n=10$). Moreover, thrombin-mediated increases were observed only in approximately half of the cultures tested and were of a lesser magnitude than the responses to bFGF. To determine the time course of activation of p38^{MAPK}, we tested the actions of bFGF and thrombin on phosphorylated p38^{MAPK} after 5 min, 30 min, 2, 6 and 20 h. We found that bFGF 3 nM elevated levels of phosphorylated p38^{MAPK} at 5 min (bFGF 4.3$\pm$1.5-fold increment over basal, $n=5$, $^*P<0.05$), 30 min (bFGF 2.1$\pm$0.5-fold increment over basal, $n=5$, $^*P<0.05$), and 20 h (bFGF 4.2$\pm$1.5-fold increment over basal, $n=10$, $^*P<0.05$), but not at 2 h (2.5$\pm$1.0-fold increment over basal, $n=4$, $P>0.05$), or 6 h (1.3$\pm$0.4-fold increment over basal, $n=5$, $P>0.05$).

Table 1 Effect of (a) SB 203580 and (b) SB 202190 on the cell proliferation in cells stimulated with thrombin or bFGF for 72 h

(a)

	Cell number (fold over basal)	
Mitogen	*Control*	*SB 203580 (10 μM)*
—	1	0.98$\pm$0.05NS
Thr (0.3 U ml^{-1})	1.37$\pm$0.03*	1.26$\pm$0.08*
Thr (3 U ml^{-1})	1.45$\pm$0.08*	1.36$\pm$0.12*
bFGF (0.3 nM)	1.45$\pm$0.08*	1.15$\pm$0.06‡
bFGF (3 nM)	1.25$\pm$0.09*	1.00$\pm$0.07‡

(b)

	Cell number (fold over basal)	
Mitogen	*Control*	*SB 202190 (10 μM)*
—	1	1.11$\pm$0.05^{NS}
Thr (0.3 U ml^{-1})	1.45$\pm$0.10*	1.36$\pm$0.04*
Thr (3 U ml^{-1})	1.45$\pm$0.11*	1.50$\pm$0.07*
bFGF (0.3 nM)	1.51$\pm$0.07*	1.17$\pm$0.08*
bFGF (3 nM)	1.61$\pm$0.11*	1.18$\pm$0.05*‡

Cell number data represent the means and s.e.m. of results from at least four different cell lines, and are expressed as fold increments over the control number of cells. Increases in cell number in response to thrombin and bFGF are compared to the responses in control cells.
$^*P<0.05$ cf. control cell number (ANOVA with repeated measures identified overall significance; and individual differences were established using Dunnett's multiple comparisons test).
$^{\ddagger}P<0.05$ cf. bFGF response in the absence of SB 203580 or SB 202190.

Effect of the p38^{MAPK} inhibitor SB 203580 on thrombin- and bFGF-induced ERK phosphorylation

To determine whether activated p38^{MAPK} exerts effects on the ERK signalling pathway following thrombin or bFGF

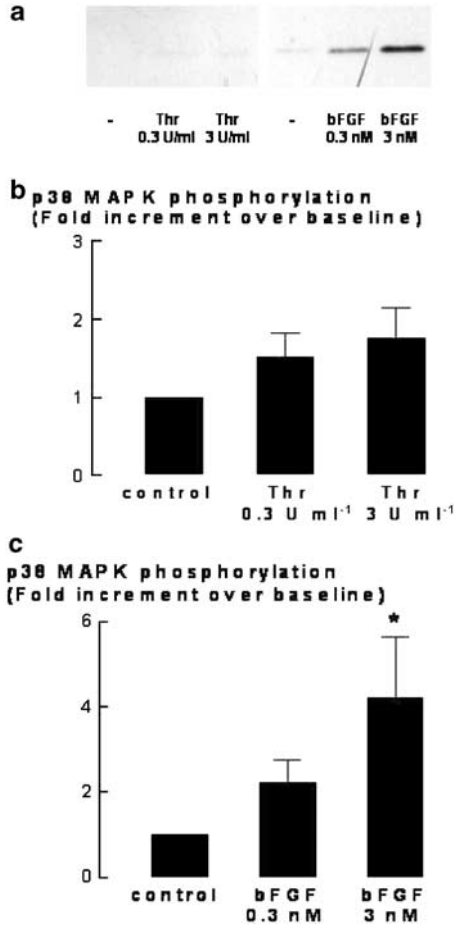

Figure 1 Phosphorylation levels of p38^{MAPK} measured by Western blot analysis in response to 20 h incubation with thrombin (0.3 and 3 U ml^{-1}) and bFGF (0.3 and 3 nM). Each histogram represents the mean and s.e.m. of results from 10 different cell lines. The mitogen-stimulated phosphorylation levels are compared to the phosphorylation levels detected in control cells. $^*P<0.05$; Differences were identified by repeated-measures ANOVA, followed by Dunnett's *post-hoc* test.

stimulation, ERK phosphorylation levels were measured in the presence and absence of SB 203580 (10 μM). Neither thrombin nor bFGF-stimulated increases in ERK phosphorylation were influenced by SB 203580 pre-treatment (Figure 2a–c). However, there was an increase in baseline ERK phosphorylation in the presence of SB 203580 (Figure 2b).

Effect of the p38^{MAPK} inhibitor SB 203580 on mitogen-stimulated cyclin D1 levels

To examine possible effects of the p38^{MAPK} pathway on cyclin D1 regulation, we measured cyclin D1 mRNA levels. At 16 h after the addition of either thrombin (0.3 and 3 U ml^{-1}) or bFGF (0.3 and 3 nM), significant increases in cyclin D1 mRNA levels were detected (Table 2). However, pre-treatment with SB 203580 (10 μM) had no effect on cyclin D1 mRNA levels increased by thrombin or bFGF (Table 2). Thrombin- (0.3 and 3 U ml^{-1}) and bFGF- (0.3 and 3 nM) induced increases in cyclin D1 protein levels were also unaffected by pretreatment with SB 203580 (10 μM) (Figure 3).

Effects of the p38^{MAPK} inhibitor SB 203580 on Cyclin E and p21^{Cip1} protein levels

The levels of p21^{cip1} detected in control cells in response to thrombin tended to increase, but there was considerable variability in the response and it did not show statistical significance (Table 3, $P>0.05$, ANOVA with repeated measures). bFGF caused a significant reduction in p21^{cip1} levels ($^*P<0.05$, ANOVA with repeated measures), but the reduction was not affected by pretreatment with SB 203580 (10 μM) (Table 3). Cyclin E protein levels measured 20 h after mitogen stimulation were less variable and did not appear to be regulated ($P>0.05$, ANOVA with repeated measures) by thrombin, bFGF or SB 203580 (Table 3).

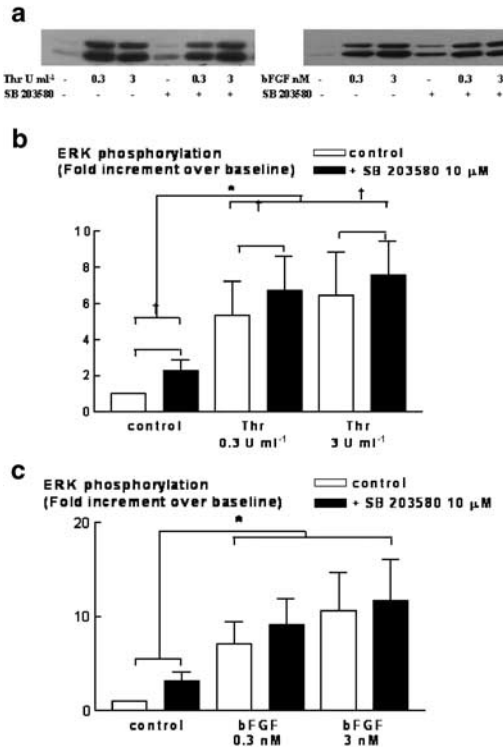

Figure 2 Increases in ERK phosphorylation measured by Western blot analysis in response to 20 h incubation with thrombin (0.3 and 3 U ml^{-1}) or bFGF (0.3 and 3 nM) in the absence and presence of SB 203580 (10 μM). (a) Representative blots and pooled data of ERK phosphorylation levels measured in response to (b) thrombin and (c) bFGF. Each histogram represents the mean and s.e.m. of results from six different cell lines. Responses are compared to ERK phosphorylation levels detected in control cells. Differences were identified using a one-way ANOVA with repeated measures, followed by Dunnett's *post-hoc* test. $^*P<0.05$ cf. control, $^{**}P<0.05$ cf. SB 203580 alone.

Table 2 Effects of SB203580 on the levels of cyclin D1 mRNA in cells stimulated with thrombin or bFGF for 16 h

	Cyclin D1 mRNA[a] *(mean fold increment control ± s.e.m.)*	
Mitogen	*Control*	*SB 203580 (10 μM)*
—	1	1.22 ± 0.21^{NS}
Thr (0.3 U ml^{-1})	1.74 ± 0.30*	1.49 ± 0.16*
Thr (3 U ml^{-1})	1.71 ± 0.22*	1.61 ± 0.24
bFGF (0.3 nM)	1.91 ± 0.57*	2.00 ± 0.40*
bFGF (3 nM)	1.97 ± 0.39*	1.95 ± 0.57*

*$P<0.05$ cf. incubation in the absence of SB 203580 (ANOVA with repeated measures identified overall significance; and individual differences were established using Dunnett's multiple comparisons test).
[a]Results are expressed as the mean ± s.e.m. of 6–8 different cell lines and normalised to the level of GAPDH detected in response to each treatment. Results are expressed as a fold increment over the mRNA levels measured in unstimulated cells. The fold increments shown above were first normalised to the corresponding GAPDH levels to account for loading and other differences, and were then derived from the ratio of cyclin D1 mRNA expression in the given condition over the expression under control conditions.

Effect of the p38^{MAPK} inhibitor SB 203580 on bFGF- and thrombin-stimulated increases in retinoblastoma phosphorylation and DNA synthesis

To assess the effect of SB 203580 on thrombin- and bFGF-stimulated actions at late G1 and S phases of the cell cycle, respectively, we assessed phosphorylation of the retinoblastoma protein at 20 h and [^{3}H]-thymidine incorporation at 24–28 h. Both thrombin (0.3 and 3 U ml^{-1}) and bFGF (0.3 and 3 nM) significantly increased levels of pRb phosphorylation (Figure 4). However, while SB 203580 (10 μM) had no effect on the level of pRb phosphorylation detected in thrombin-stimulated cells (Figure 4b), pRb phosphorylation was inhibited by SB 203580 in bFGF-stimulated cells (Figure 4c). Similarly, SB 203580 had no effect on thrombin-stimulated DNA synthesis, but reduced DNA synthesis in response to bFGF (Table 4).

Discussion

We found that bFGF activated p38^{mapk}, the activity of which appeared to contribute to mitogenic signalling independently of cyclin D1 levels. In contrast, changes in p38^{mapk} activity appeared to have no role in thrombin-induced mitogenesis.

Maher (1999) reported that bFGF stimulated a sustained increase in p38^{MAPK} activity and proliferation in 3T3 fibroblasts that was inhibited by the p38^{MAPK} inhibitor SB

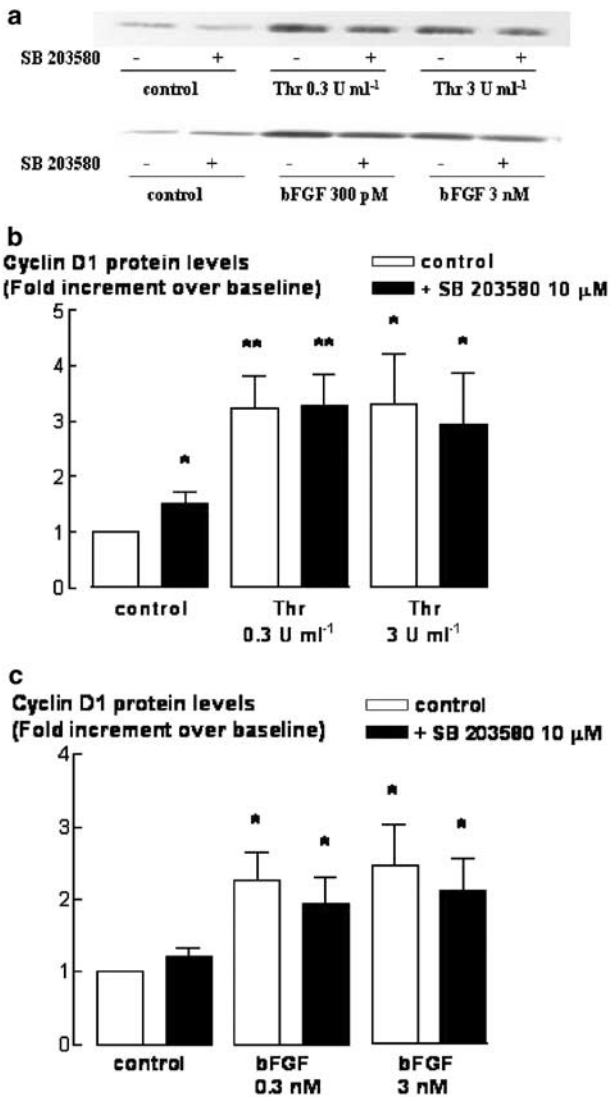

Figure 3 Cyclin D1 protein levels determined by Western blot analysis in response to 20 h treatment with thrombin (0.3 and 3 U ml^{-1}) and bFGF (0.3 and 3 nM) in the absence and presence of SB 203580 (10 μM). (a) Representative blot and pooled data of cyclin D1 levels measured in response to thrombin (b) and bFGF (c). Cyclin D1 protein levels are expressed as fold increments over the levels measured in control cells. Each histogram is representative of the mean and s.e.m. of nine different cell lines. $^{**}P<0.01$, $^{*}P<0.05$, differences were identified using a paired *t*-test. Cyclin D1 levels following mitogen stimulation are compared to those of control cells.

203580. However, serum stimulation of 3T3 cell mitogenesis was not reduced by p38^{MAPK} inhibition. In vascular smooth muscle, thrombin activates p38^{MAPK}, and SB 203580 inhibits thrombin-induced DNA synthesis, without effect on serum-stimulated DNA synthesis (Kanda *et al*., 2001). Our findings and those of Maher (1999) and Kanda *et al*. (2001) suggest that recruitment of the p38^{MAPK} pathway is mitogen-dependent in different cell types. Moreover, the differential sensitivity of the different mitogens to ERK inhibitors in human ASM cells may be explained by a separate and complementary differential contribution of the p38^{MAPK} pathway.

Incubation of HASM with bFGF, but not thrombin, appears to be associated with activation of p38^{MAPK}, as, in the presence of SB 203580, bFGF-stimulated cell proliferation was reduced. Analysis of the phosphorylation levels of p38^{MAPK} verified that bFGF increases p38^{MAPK} activation, whereas thrombin had no detectable effect. Inhibition of the p38^{MAPK} pathway had no effect on bFGF-stimulated ERK phosphorylation levels, suggesting that the effects of p38^{MAPK} on bFGF-induced mitogenesis are independent of the ERK signalling pathway, or at least occur downstream of ERK phosphorylation. SB 203580 inhibits only the p38$^{MAPK}\alpha$ and β isoforms (Kumar *et al*., 1997; Gum *et al*., 1998). While the expression of p38$^{MAPK}\gamma$ is thought to be restricted to skeletal muscle, the α, β and δ isoforms are ubiquitously expressed (Wang *et al*., 1997; Herlaar & Brown, 1999). Thus, a functional role for the p38$^{MAPK}\delta$ isoform by thrombin (or bFGF) would not be detected by the methods used in this study.

Although this is the first study to examine bFGF-stimulated activation of the p38^{MAPK} pathway in human ASM cells, several previous studies have considered p38^{MAPK} activation in response to bFGF in other cell types. The p38^{MAPK} pathway and p70^{S6k} have been implicated in the bFGF-stimulated mitogenesis of oligodendrocyte progenitor cells (Baron *et al*., 2000). ERK and p38^{MAPK} pathways are required for the bFGF-stimulated proliferation and migration of endothelial cells (Tanaka *et al*., 1999). Other investigations of the role of the p38^{MAPK} in ASM cells have used mitogens including PDGF (Page *et al*., 1999), EGF, thrombin and phorbol myristate acetate (Orsini *et al*., 1999), and have suggested that these mitogens have little effect on p38^{MAPK} activation, that p38^{MAPK} is a minor regulator of cyclin D1 levels and that it does not contribute to proliferation. Furthermore, it has been suggested that the p38^{MAPK} pathway negatively regulates proliferation and may act as a 'safeguard' against excessive growth of ASM cells *in vivo* (Page *et al*., 2000; 2001). This conclusion is not consistent with our findings on bFGF and emphasises the need for caution in generalising from experimental data obtained with a limited range of stimuli.

Lavoie *et al*. (1996) first examined cyclin D1 as a potential target protein regulated by the p38^{MAPK} pathway, in a Chinese hamster lung fibroblast CCL39 cell line. While the ERK pathway was associated with increased cyclin D1 promoter activity, the expression of cyclin D1 was reduced by activation of the p38^{MAPK} kinase, MKK3 and by p38^{MAPK} itself. Conversely, inhibition of p38^{MAPK} by SB 203580 increased cyclin D1 mRNA and protein levels (Lavoie *et al*., 1996). An association between p38^{MAPK} activation and the downregulation of cyclin D1 levels has subsequently been observed in several cell types (Conrad *et al*., 1999; Terada *et al*., 1999; Awad *et al*., 2000a; Page *et al*., 2001). Furthermore, a role for p38^{MAPK} has also been identified in the post-transcriptional regulation and degradation of cyclin D1 protein, *via* the phosphorylation of Thr 286, which targets cyclin D1 protein for degradation *via* the ubiquitin proteosome degradation pathway (Awad & Gruppuso, 2000b; Casanovas *et al*., 2000). In contrast, p38^{MAPK}, together with ERK and JNK, have been associated with the induction of cyclin D1 by the proto-oncogene Neu (c-epPRbB-2) in MCF7 cells (Lee *et al*., 2000). However, as p38^{MAPK} inhibition has no effect on thrombin- or bFGF- induced increases in cyclin D1 protein or mRNA levels, we can exclude a role for cyclin D1 in the regulatory effects of the p38^{MAPK} pathway in the phosphorylation of pRb.

Given the importance of cyclin E in regulating the phosphorylation of pRb and subsequent progression to S phase of the cell cycle, we measured the effect of p38^{MAPK}

Table 3 Effects of SB203580 (10 μM) on the levels of cyclin E and p21^{Cip1} protein in cells stimulated with thrombin or bFGF for 20 h

	Cyclin E protein[a]		*p21^{Cip1} protein*[b] *(mean fold increment over control ± s.e.m.)*	
Mitogen	*Control*	*SB 203580*	*Control*	*SB 203580*
—	1	1.41 ± 0.340	1	1.45 ± 0.27
Thr (0.3 U ml^{-1})	2.02 ± 0.50	1.58 ± 0.40	3.85 ± 1.41	3.92 ± 2.08
Thr (3 U ml^{-1})	2.19 ± 0.68	2.23 ± 0.82	4.63 ± 1.97	5.17 ± 3.40
bFGF (0.3 nM)	1.47 ± 0.36	1.15 ± 0.25	0.46 ± 0.09*	0.41 ± 0.16*
bFGF (3 nM)	1.00 ± 0.25	1.11 ± 0.30	0.63 ± 0.20*	0.39 ± 0.14*

[a]Densitometry-determined levels have been normalised to the level of cyclin E in unstimulated cells (1). Control and SB203580 responses represent pooled responses from identical experimental design performed in cells incubated with either thrombin or bFGF.
[b]Densitometry-determined levels have been normalised to the level of p21^{Cip1} in unstimulated cells (1). Control and SB203580 responses represent pooled responses from identical experimental design performed in cells incubated with either thrombin or bFGF.
Results are expressed as the mean ± s.e.m. of 5–8 different cell lines.
*$P<0.05$ cf. incubation in the absence of SB 203580 (ANOVA with repeated measures identified overall significance; and individual differences were established using Dunnett's multiple comparisons test).

inhibition on cyclin E protein and pRb phosphorylation levels. Phosphorylation of pRb by the activated cyclin D1-cdk4 complex may be linked to increased levels of cyclin E protein and activation of the cyclin E-cdk2 complex, which is thought to promote further phosphorylation of pRb and dissociation of pRb from E2F (Chellappan *et al*., 1991; Matsushime *et al*., 1994; Lundberg & Weinberg, 1998). Although both thrombin and bFGF increased cyclin D1 protein levels and pRb phosphorylation, there was no corresponding increase in cyclin E protein levels. The p38^{mapk} inhibitor SB 203580 had no effect on mitogen-stimulated cyclin D1 or E levels, but there was a mitogen-dependent difference in the regulation of retinoblastoma protein. Inhibition of p38 activation had no effect on the pRb phosphorylation stimulated by thrombin, but prevented pRb phosphorylation in cells stimulated with bFGF. This observation is consistent with a role for p38^{MAPK} in promoting cell cycle progression to S phase and raises the possibility that pRb may be phosphorylated directly by members of the p38^{MAPK} family in response to bFGF stimulation. Direct phosphorylation of pRb by p38^{MAPK} and subsequent dissociation of E2F has been shown to occur independently of an increase in the kinase activities of either cyclin D or cyclin E, in Fas-stimulated Jurkat cells to augment

Table 4 Effects of SB203580 on [^{3}H]-thymidine incorporation in ASM stimulated with thrombin or bFGF for 24 h

	[^{3}H]-thymidine incorporation (mean ± s.e.m. (d.p.m.)*)*	
Mitogen	*Control*	*SB203580 (10 μM)*
—	2510 ± 1436	1118 ± 232
Thr (3 U ml^{-1})	4226 ± 1472	3850 ± 1436
bFGF (3 nM)	6675 ± 1825	2537 ± 594*

Results are mean ± s.e.m. of experiments from 4–5 different primary cultures.
*$P<0.05$ cf. bFGF response in absence of SB 203580.

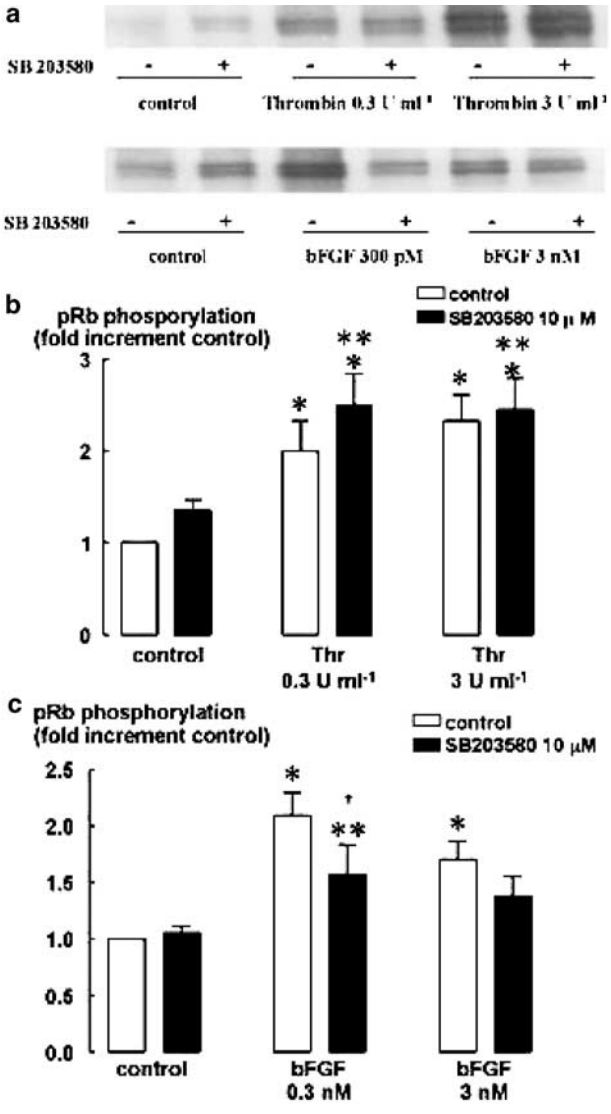

Figure 4 Increases in pRb phosphorylation detected by Western blot analysis in response to 20 h incubation with thrombin (0.3 and 3 U ml^{-1}) or bFGF (0.3 and 3 nM) in the absence and presence of SB 203580 (10 μM). (a) Representative blots and pooled data showing pRb phosphorylation levels in response to stimulation by (b) thrombin or (c) bFGF. Levels of pRb phosphorylation are expressed as fold increments over the levels detected in control cells. Each histogram is representative of the mean and s.e.m. of eight different cell lines. Differences were identified using a one-way ANOVA with repeated measures, followed by Dunnett's *post hoc* test. *$P<0.05$ cf. control, **$P<0.05$ cf. SB 203580 alone. $^{\dagger}P<0.05$, SB 203580-treated cells compared with the corresponding level in non-pretreated cells under the same incubation conditions.

apoptosis (Wang *et al*., 1999). Increased expression or activity of inhibitors of cdk such as $p21^{cip1}$, $p27^{kip1}$ or the INK family comprise alternative targets for the cyclin D/E independent regulation of pRb phosphorylation by $p38^{MAPK}$. However, there was no effect of SB 203580 on either thrombin- or bFGF-stimulated $p21^{Cip1}$ levels. Moreover, the high variability in $p21^{cip1}$ levels in response to thrombin between cultures suggests that modification of $p21^{cip1}$ levels at 20 h plays little role in thrombin-stimulated mitogenesis. The potential involvement of other cdk inhibitors cannot be excluded.

The relationship between $p38^{MAPK}$ activity after 20 h stimulation (indicative of persistent $p38^{MAPK}$ late G1-phase activation) and increases in cell number at 48 h appears complex. One might have predicted that the higher level of phosphorylation of p38MAPK stimulated by the highest concentration of bFGF (3 nM) would have stimulated a further increment in proliferation (or DNA synthesis) relative to the lower 0.3 nM dose of bFGF. However, other signal transduction systems may be more or less important at these different concentrations, confounding any simple concentration–response relationship for the bFGF $p38^{MAPK}$ effect and hence the effect of the inhibitors. It is also possible that the maximum contribution from the $p38^{MAPK}$ pathway is achieved by the 0.3 nM bFGF concentration and that the further phosphorylation and activity of $p38^{MAPK}$ is irrelevant to its role in proliferation.

Our results indicate that $p38^{MAPK}$ contributes to and is required for bFGF- but not thrombin-stimulated cell proliferation. Although both thrombin and bFGF increase levels of both cyclin D1 protein and mRNA, this key mitogenic signal does not appear to be regulated by the $p38^{MAPK}$ pathway. Moreover, $p38^{MAPK}$ is not responsible for the ERK-independent maintenance of cyclin D1 levels stimulated by thrombin ($3\,U\,ml^{-1}$) or bFGF (3 nM)(Ravenhall *et al*., 2000). Our findings indicate that a pharmacological approach to regulation of ASM proliferation will require an appreciation of the heterogeneity in the use of different intracellular signalling pathways by different mitogens.

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