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Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1

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Serum and growth factors activate both the canonical extracellular signal-regulated kinase (ERK) 1/2 pathway and the ERK5/big mitogen-activated protein kinase 1 (BMK) 1 pathway. Pharmacological inhibition of the ERK1/2 pathway using PD98059 and U0126 prevents cyclin D1 expression and inhibits cell proliferation, arguing that the ERK1/2 pathway is rate limiting for cell-cycle re-entry. However, both PD98059 and U0126 also inhibit the ERK5/BMK1 pathway, raising the possibility that the anti-proliferative effect of such drugs may be due to inhibition of ERK5 or both pathways. Here we characterize the effect of the novel mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, PD184352, on the ERK1/2 and ERK5 pathways in the Chinese hamster fibroblast cell line CCl39. In quiescent cells, serum-stimulated ERK1 activity was completely inhibited by PD184352 with an IC_{50} below 1 μM, whereas ERK5 activation was unaffected even at 20 μM. Serum-stimulated DNA synthesis and cyclin D1 expression was inhibited by low doses of PD184352, which abolished ERK1 activity but had no effect on ERK5. Similarly, in cycling cells PD184352 caused a dose-dependent G_1 arrest and inhibition of cyclin D1 expression at low doses, which inhibited ERK1 but were without effect on ERK5. These results indicate that the anti-proliferative effect of PD184352 is due to inhibition of the classical ERK1/2 pathway and does not require inhibition of the ERK5 pathway.

Key words: cyclin D1, CCl39, mitogen-activated protein kinase (MAPK), MAPK/ERK kinase 5 (MEK5).

INTRODUCTION

The prototypical mammalian mitogen-activated protein kinase (MAPK) cascade, the Ras-regulated Raf → MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) 1/2 → ERK1/2 pathway, serves to link growth factor receptors at the plasma membrane to changes in gene expression in the nucleus. Several observations suggest that this pathway is important for growth factor-stimulated cell-cycle re-entry. For example, conditional mutants of Raf are sufficient to induce expression of cyclin D1, cyclin A and cell-cycle re-entry [1,2], and these responses are blocked by inhibitors such as PD98059 [3] and U0126 [4]. These inhibitors act at, or directly upstream of, MEK1/2 and are frequently referred to as 'MEK inhibitors', but this is something of a misnomer. While both drugs can inhibit MEK1 and/or MEK2 their cellular effects are almost certainly due to their ability to bind MEK1 and prevent it being activated by Raf or other MEK kinases [5–7]. Here we refer to these drugs, including PD184352, as ERK1/2 pathway inhibitors.

ERK5 [also known as big MAPK 1 (BMK1)] is a more recently identified member of the MAPK family [8,9]. In addition to the core catalytic domain, ERK5 contains a 400 amino acid C-terminal extension containing a transcriptional activation domain and a domain that interacts with the myocyte enhancer factor-2 (MEF2) family of transcription factors. In addition, ERK5 can phosphorylate some MEF2 proteins, most notably MEF2C, leading to the up-regulation of the immediate early response gene c-*jun* [10,11]. In common with ERK1 and ERK2, ERK5 requires phosphorylation at the conserved TEY motif within its activation loop for full activity [10]. ERK5 is phosphorylated and activated by a dedicated MAPK kinase called MEK5 [9,10], whereas the related MEK1 and MEK2 phosphorylate ERK1 and ERK2, but cannot phosphorylate and activate ERK5 directly. Candidate activators of MEK5 include MAPK kinase/ERK kinase kinases (MEKKs) 2 [12] and 3 [13], the Cot proto-oncogene [14] and mixed-lineage kinase (MLK)-like mitogen-activated protein triple kinase (MLTK) [15]. In addition, while Ras can activate a co-expressed ERK5 in some cells there are conflicting reports concerning the ability of Ras and Raf to activate the MEK5 → ERK5 pathway [16–19].

The ERK5 pathway can be stimulated by some stresses [10,20] but is also strongly activated by serum and many growth factors such as epidermal growth factor (EGF) and neuregulin [17,19,21]. The biological role of the pathway is still unclear, but several studies have shown that it plays an important role in promoting the G_1-to-S cell-cycle transition. For example, interfering mutants of MEK5 or ERK5 can cause cells to accumulate in G_1 [17]. Furthermore, constitutive activation of MEK5 can cause cellular transformation in co-operation with the classical ERK1/2 pathway, and interfering mutants of MEK5 and ERK5 can reverse cellular transformation [21–23].

The ability of the ERK pathway inhibitors PD98059 and U0126 to inhibit cell-cycle re-entry is consistent with a role for the ERK1/2 pathway in cell-cycle re-entry. However, recent studies have suggested that these drugs also prevent activation of the MEK5 → ERK5 pathway [19]. These observations raise the question of whether the anti-proliferative effects of such drugs are due to inhibition of the ERK1/2 pathway, the ERK5 pathway or both. PD184352, a more recently described ERK1/2 pathway inhibitor, prevents MEK1 activation with a 50% inhibitory concentration (IC_{50}) of 10–20 nM and inhibits the growth of human tumour cell lines and tumours grown as xenografts in mice [7,24,25]. Compared with many other common kinase inhibitors PD184352 appears to be very specific for MEK1/2 [7] but a recent study has shown that this drug may also inhibit ERK5 activation, albeit with reduced potency [26].

Abbreviations used: BMK1, big MAPK 1; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor-2; MEK, MAPK/ERK kinase.

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Here, we have used the differing potency against the ERK1/2 and ERK5 pathways to determine which pathway is the target for the anti-proliferative effects of PD184352. In a CCl39 hamster fibroblast cell line stably expressing epitope-tagged ERK5, we show that PD184352 is considerably more potent against the classical ERK1 pathway than the ERK5 pathway. Furthermore, both in quiescent cells re-stimulated with serum and in cycling cells maintained in 10% foetal bovine serum (FBS), PD184352 inhibits cell proliferation at doses at which it abolishes ERK1 activity but has no effect on ERK5. Thus, we show for the first time that the ability of PD184352 to inhibit cell-cycle progression is due to inhibition of the ERK1/2 pathway, but not the ERK5 pathway. The results are considered in terms of the role of the ERK1/2 and ERK5 pathway in regulating cell proliferation.

EXPERIMENTAL

Materials

Cell culture reagents were purchased from Gibco Life Technologies. Superfect transfection reagent was supplied by Qiagen. EGF was purchased from Roche and H_2O_2 was obtained from Fluka. [γ-^{32}P]ATP was routinely purchased from Amersham. The following antibodies were used during this study. Anti-ERK1 was raised 'in house' against the peptide KELIFQETAR-FQPGAPEAP, and has been described previously [3]. Anti-FLAG M2 was supplied by Sigma Aldrich, anti-cyclin D1 and anti-p27^{kip1} by Calbiochem, anti-cyclin A by Neomarkers and anti-ERK1/2 by Transduction Laboratories. Horseradish peroxidase-conjugated secondary antibodies were provided by Bio-Rad. Unless otherwise stated in the text all other reagents were purchased from Sigma and were of the highest grade available.

Plasmids, cells and cell culture

pcDNA3-FLAG ERK5 was generously provided by J. D. Lee (Scripps Institute, La Jolla, CA, U.S.A.) and used for the stable expression of FLAG-tagged human ERK5.

Stable lines of CCl39 cells expressing FLAG-ERK5 were produced by transfection with Qiagen Superfect, limiting-dilution culture in 400 μg/ml G418 (Life Technologies) followed by ring cloning. The resulting CCl39 : ERK5 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 mg/ml glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% (v/v) FBS and 400 μg/ml G418.

Stimulations and preparation of lysates

Two discrete experimental regimens were employed. To study the transition from quiescence back into the cell cycle, cells were plated on to 10 cm dishes and grown to 70% confluence. The growth medium was replaced, where described, with serum-free Dulbecco's modified Eagle's medium for 16 h prior to treatment with 10 nM EGF, 500 μM H_2O_2 or 10% (v/v) FBS. Treatment times were as indicated in the Figure legends. To study asynchronous cycling cells, drugs were added at the indicated concentrations to cells growing in the exponential growth phase (40–50% confluent) in 10% FBS. At the end of the stimulation/treatment period cell lysates were prepared as described previously [3] and supernatant protein concentrations were measured by Bradford protein assay (Bio-Rad). Lysates were snap-frozen in liquid nitrogen and stored at -80 °C until required. In certain cases, as described in the Figure legends, cells were pre-treated with PD184352 or an equivalent of DMSO (vehicle control) prior to stimulation.

Western blotting

After stimulation as described, equal quantities of cell extracts were resolved by SDS/PAGE and transferred to Immobilon P membranes (Millipore) soaked in methanol. Membranes were blocked for at least 1 h in 0.1% (v/v) Tween-20/TBS containing 5% (w/v) powdered milk before being probed with the desired antibodies. Immune-reactive proteins were visualized with the enhanced chemiluminescence (ECL) system.

Immune complex kinase assays

Following stimulation, as described, active kinase complexes were immunoprecipitated from cell extracts normalized for protein content. The extracts were mixed, for 3 h at 4 °C, with a 10 μl bed volume of Protein A–Sepharose (ERK1) or Protein G–Sepharose (Flag-ERK5) and antibody; 5 μl of rabbit anti-ERK1 antibody (ERK1 assays), or 10 μg of mouse anti-FLAG M2 antibody (FLAG-ERK5 assays). The bead complexes were washed twice with lysis buffer and once in kinase buffer (30 mM Tris, pH 8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 25 mM β-glycerol phosphate and 0.1 mM sodium vanadate). Kinase activities were assessed by incubating the drained beads in 30 μl of kinase buffer supplemented with 10 μM ATP, 3 μCi of [γ-^{32}P]ATP and either 7 μg of myelin basic protein (ERK1 assays), or 8 μg of glutathione S-transferase (GST)-MEF2C (175-327) (ERK5 assays) for 30 min at 30 °C in a shaking incubator. Kinase reactions were terminated by boiling samples in 4 × Laemmli SDS sample buffer before resolving by SDS/PAGE. Gels were fixed and dried and incorporation of ^{32}P into respective substrates quantified by Phosphorimager (Fuji). Unless otherwise indicated, data are either combined from independent experiments or are from a single experiment on triplicate dishes, representative of at least two others giving similar results.

Flow-cytometric analysis of the cell cycle

After the indicated treatments, cells were harvested by trypsinization, washed with PBS and fixed in ice-cold 70% (v/v) ethanol/PBS for 30 min at 4 °C. Following centrifugation, the cells were resuspended in PBS containing RNase A (0.1 mg/ml) and stained with propidium iodide (50 μg/ml) for 30 min at 37 °C. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson). A single experiment, representative of three, is shown.

[^{3}H]Thymidine incorporation

Duplicate cell samples were treated as in the Figure legends followed by fixation and analysis for [^{3}H]thymidine incorporation as described previously [3]. Results are combined from two independent experiments, each in duplicate.

Cell proliferation assays

Asynchronous cell proliferation was measured by plating cells in 24-well tissue-culture plates at a density of 1×10^4/ml. Following attachment, cells were treated, in triplicate, with increasing doses of PD184352 for 72 h. After the designated growth period, relative cell numbers were quantified by fixing cultures with acetic acid/methanol (1:3, v/v) followed by staining with 0.1% (w/v) Crystal Violet/PBS and solubilizing the resulting stained cells with 10% (v/v) acetic acid. Absorbance was read at 590 nm on a spectrophotometer (Cecil Instruments).

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Statistical analysis

Where indicated statistical analysis was performed by Student's *t* test.

RESULTS

Characterization of CCl39:FLAG-ERK5 cells

We assayed endogenous ERK1 activity using antibodies described previously [3]. In CCl39 cells we have not found any qualitative differences in activation of ERK1 and ERK2 to date, suggesting that assay of ERK1 provides a good representation of both classical ERK pathways. To study the regulation of ERK5, clonal CCl39 cell lines expressing full-length FLAG-tagged human ERK5 were isolated (Figure 1). Throughout this study results are shown from a single clone, but similar results have been obtained in other clones. In accordance with previous observations [11,20,21], ERK5 resolved on SDS/PAGE with an apparent molecular mass of $\approx$ 120 kDa, $\approx$ 20 kDa higher than its predicted molecular mass. The activation of this epitope-tagged version was characterized by stimulating serum-starved cells with various agonists, including growth factors and stresses. Three of the most effective stimulants proved to be EGF, FBS and H_2O_2. Treatment of these cells with 10 nM EGF, 10% FBS or 500 μM H_2O_2 resulted in a transient activation of ERK5, as measured by an immune-complex kinase assay with GST-MEF2C as a substrate (Figure 2A). The time of peak activation was different for each stimulatory agent, with EGF-induced activity peaking at 5 min, FBS at 15 min and H_2O_2 at 60 min. Using these time-points we compared the activation of ERK1 and ERK5 by assaying both activities from the same triplicate cell extracts (Figures 2B and 2C). EGF caused the most robust activation of both ERK1 and ERK5 kinase activity, followed by FBS. H_2O_2 resulted in a similar level of activation of ERK5 as FBS, but did not activate ERK1 in these experiments. These increases in activity occurred in the absence of any change in levels of expression of ERK5 (Figure 2D), indicating that, in common with ERK1, the stimulation reflects an increase in the specific activity of the enzyme.

To examine the anti-proliferative effects of PD184352 and to correlate them with inhibition of the ERK1/2 or ERK5 pathways we performed two discrete studies. In the first case we examined the effect of the drug on quiescent serum-starved cells undergoing serum re-stimulation; a classical assay of cell-cycle re-entry. In addition, we examined the effect of PD184352 on asynchronous cycling cells, growing in 10% FBS.

PD184352 inhibits ERK1, but not ERK5, activation in serum-starved cells re-stimulated with FBS

The ability of PD184352 to inhibit FBS-induced activation of ERK1 and ERK5 was investigated in serum-starved CCl39:ERK5 cells. PD184352 inhibited FBS-induced ERK1 activation with an IC_{50} below 1 μM, whereas even a dose of 20 μM PD184352 was insufficient to inhibit ERK5 activity, induced in the same manner and assayed from the same cell extracts as ERK1 (Figure 3). Immunoblotting the same samples for the FLAG epitope showed that these effects occurred in the absence of any changes in total FLAG-ERK5 protein level (Figure 3B).

PD184352 inhibits FBS-induced cyclin D1 expression and cell-cycle re-entry without inhibiting ERK5

The relative importance of ERK1 and ERK5 signalling in driving cell-cycle re-entry was investigated by re-stimulating

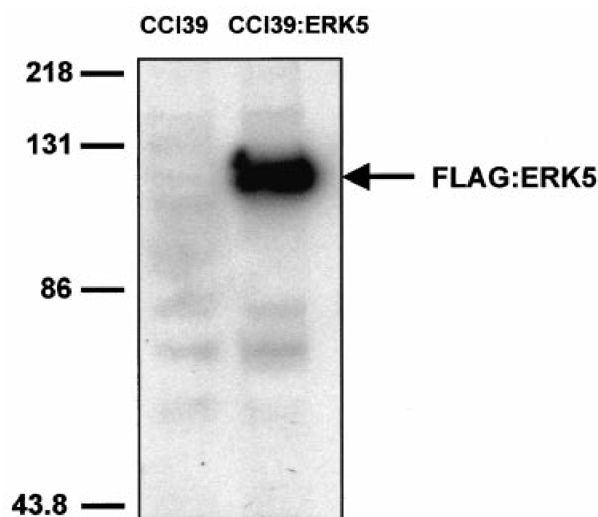

Figure 1 Expression of FLAG-ERK5 in CCl39 hamster fibroblast cells

Stable expression of FLAG-ERK5 in CCl39 cells. Following transfection, positive clones were determined by immunoblotting whole-cell lysates with the anti-FLAG-M2 monoclonal antibody. A cell lysate from the CCl39:ERK5 cell line was resolved alongside untransfected CCl39 cell lysates as a negative control.

serum-starved CCl39:ERK5 cells with FBS in the presence of varying doses of PD184352. DNA synthesis was measured by the incorporation of [^{3}H]thymidine into DNA (Figure 4A). PD184352 inhibited FBS-stimulated DNA synthesis with an IC_{50} of 0.5–1 μM, almost identical with that observed for inhibition of FBS-stimulated ERK1 activity. Doses above 2 μM completely abolished cell-cycle re-entry, but were without effect on ERK5 activity (Figure 3).

Serum-stimulated cyclin D1 expression is rate-limiting for cell-cycle re-entry and is regulated by the Ras-dependent ERK1/2 pathway [1,2]. ERK1/2 pathway inhibitors such as U0126 and PD98059 prevent growth factor-stimulated cyclin D1 expression [3,4], but these drugs are equally effective against the ERK5 pathway [19]. In light of the preceding results, we examined the effect of PD184352 on cyclin D1 expression by Western blotting (Figure 4B). FBS-induced expression of cyclin D1 was reduced by treatment with 0.5 μM PD184352 and completely abolished by treatment with 5 μM. Thus two markers of FBS-induced cell-cycle re-entry were completely abolished by low doses of PD184352, which inhibited ERK1 activity but had no effect on ERK5 activity.

ERK1 activity is more sensitive to inhibition by PD184352 than ERK5 activity in asynchronous cells

The preceding experiments indicated that PD184352 can inhibit cell-cycle re-entry without inhibiting ERK5 activity. However, since there is a widespread hope that ERK pathway inhibitors will prove effective against hyper-proliferative cells, we examined the effects of PD184352 on ERK1 and ERK5 activity in asynchronous cycling cells, as this seemed to be a more therapeutically relevant experimental paradigm. In common with the studies during re-stimulation of quiescent cells, the ERK5 pathway proved to be much less sensitive to inhibition by PD184352 than the ERK1 pathway. A 5 h treatment of cycling

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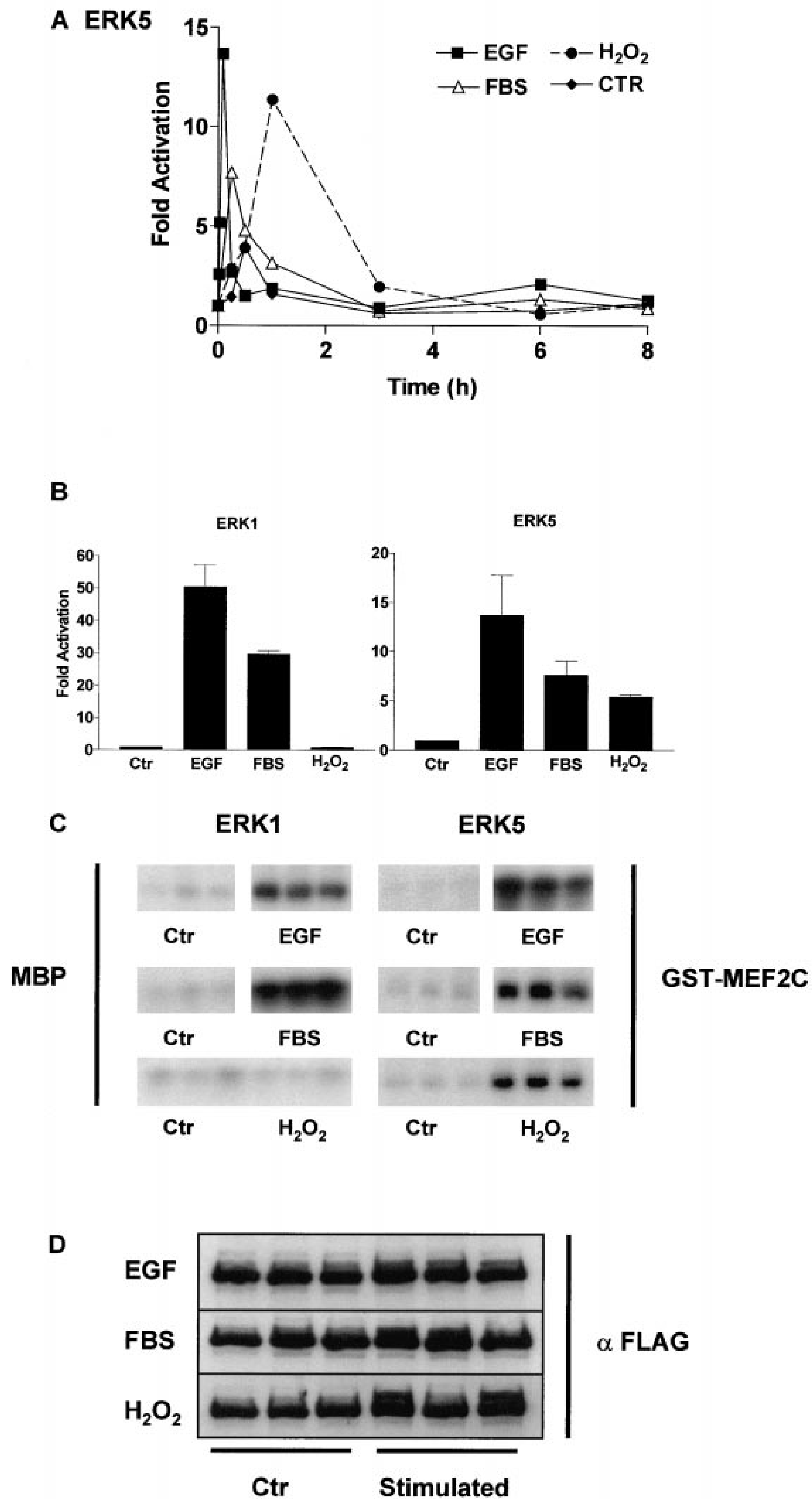

Figure 2 For legend see opposite page

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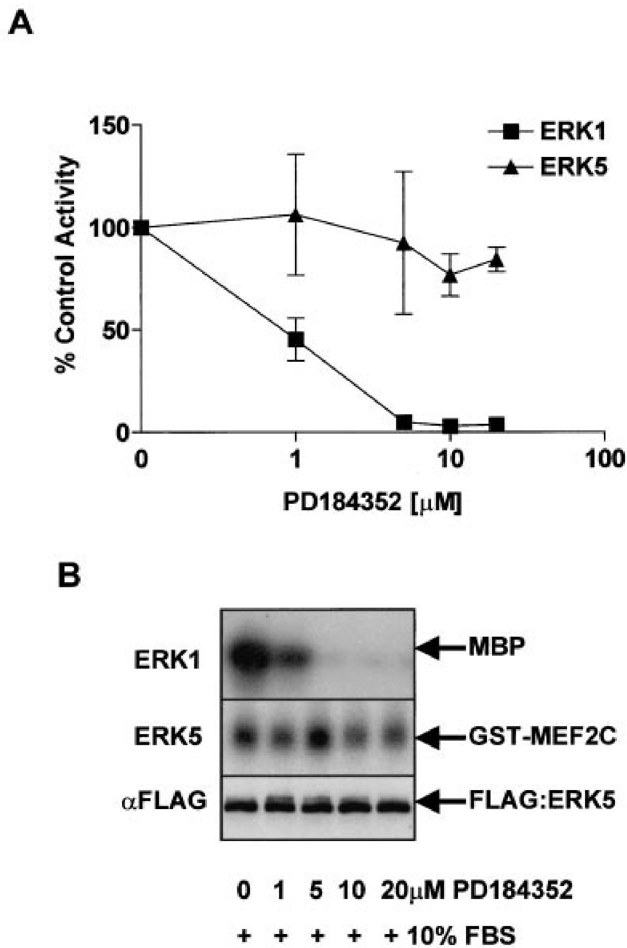

Figure 3 PD184352 inhibits FBS-induced ERK1, but not ERK5 activity, in quiescent cells

CCl39:ERK5 cells were serum-starved for 16 h prior to the addition of the indicated concentrations of PD184352, or an equivalent of DMSO vehicle control (labelled 0) for 90 min. Single dishes of cells were then stimulated with 10% FBS for 15 min and immune-complex kinase assays performed with either myelin basic protein (MBP; ERK1) or GST-MEF2C (ERK5), as a substrate. $[^{32}P]P_i$ incorporation was quantified on a Phosphorimager. (**A**) Results shown are the mean ± S.D. from three separate experiments. (**B**) The autoradiographs from one quantified assay are shown alongside an immunoblot of the same lysates with anti-FLAG antibody to confirm equal protein loading.

cells with doses of PD184352 up to 20 μM virtually abolished ERK1 activity, even at the lowest dose used (0.5 μM). However, ERK5 activity remained unaffected by even the highest dose used over this time (20 μM; Figure 5A). If the same treatments were applied for 24 h (Figure 5B), ERK1 activity was again strongly inhibited with an IC_{50} below 0.5 μM, whereas significant inhibition of ERK5 activity was only observed at the highest dose (20 μM). Interestingly, at doses of PD184352 between 0.6 and

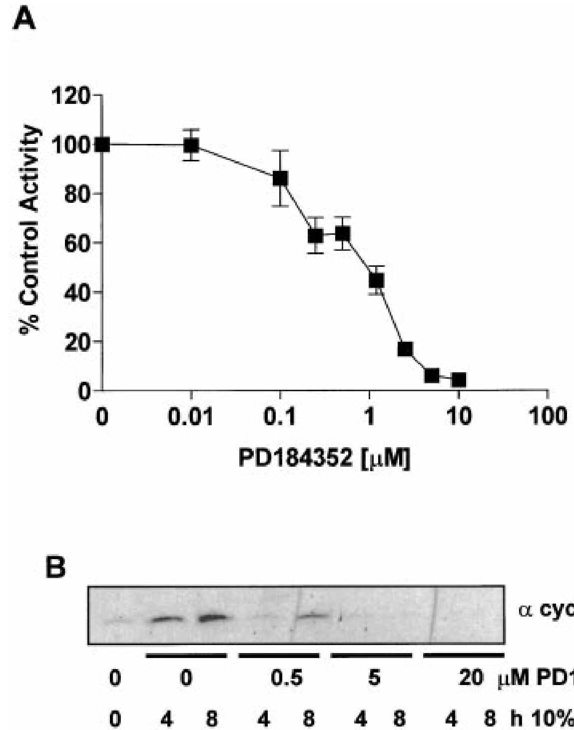

Figure 4 FBS-induced DNA synthesis and induction of cyclin D1 is inhibited by concentrations of PD184352 which abolish ERK1 activity but not ERK5

(**A**) CCl39:ERK5 cells were serum-starved for 16 h prior to re-stimulation with 10% (v/v) FBS for 18 h in the presence of the indicated concentrations of PD184352, or an equivalent of DMSO vehicle control (0 μM). DNA synthesis was assayed by addition of $[^{3}H]$thymidine for the last 6 h. Results represent the mean ± range, pooled from two experiments, each with duplicate cell samples. (**B**) Serum-starved CCl39:ERK5 cells were re-stimulated by the addition of 10% (v/v) FBS, for either 4 or 8 h, in the presence of the indicated concentrations of PD184352 or vehicle control (DMSO, 0). Equivalent amounts of cell lysate were resolved by SDS/PAGE and immunoblotted with anti-cyclin D1 antibody.

5 μM we observed a further statistically significant 2.5–3-fold activation of ERK5, which accompanied the inhibition of ERK1. Over these doses there was no effect on the expression of the FLAG-tagged ERK5, indicating that low doses of PD184352 enhance the specific activity of the enzyme. A similar observation was recently reported in HeLa cells [24], but the significance of this effect remains to be defined.

PD184352 inhibits cyclin D1 expression and proliferation of cycling cells at doses that abolish ERK1 but not ERK5 activity

The relative contributions of the ERK1 and ERK5 pathways in the maintenance of asynchronous cell proliferation were investigated by examining the effect of PD184352 on cell proliferation and the expression of a number of cell-cycle regulators in cycling cells. Inhibition of cell proliferation was first studied by growing cells in the presence of varying doses of PD184352 for 72 h

Figure 2 Characterization of FLAG-ERK5 activation in CCl39 cells

(**A**) CCl39:ERK5 cells were serum starved for 16 h prior to stimulation with 10% FBS, 10 nM EGF or 500 μM H_2O_2 as described. Immune-complex kinase assays were performed with the anti-FLAG antibody and GST-MEF2C as a substrate. Phosphate incorporation was quantified on a Phosphorimager. (**B**) Using the time of maximal activation for each agonist (5 min for EGF, 15 min for FBS or 60 min for H_2O_2), triplicate dishes were stimulated and assayed for ERK1 and ERK5. The kinase activities of both ERK5 and ERK1 were quantified by immune-complex kinase assay from the same set of lysates, so the results are means ± S.D. of triplicate cell samples. (**C**) The autoradiographs from these assays and (**D**) an immunoblot with the anti-FLAG monoclonal antibody were performed on the same lysates, to confirm an equal amount of ERK5 present in each sample. Data are taken from a single experiment representative of three giving similar results. CTR, control; MBP, myelin basic protein.

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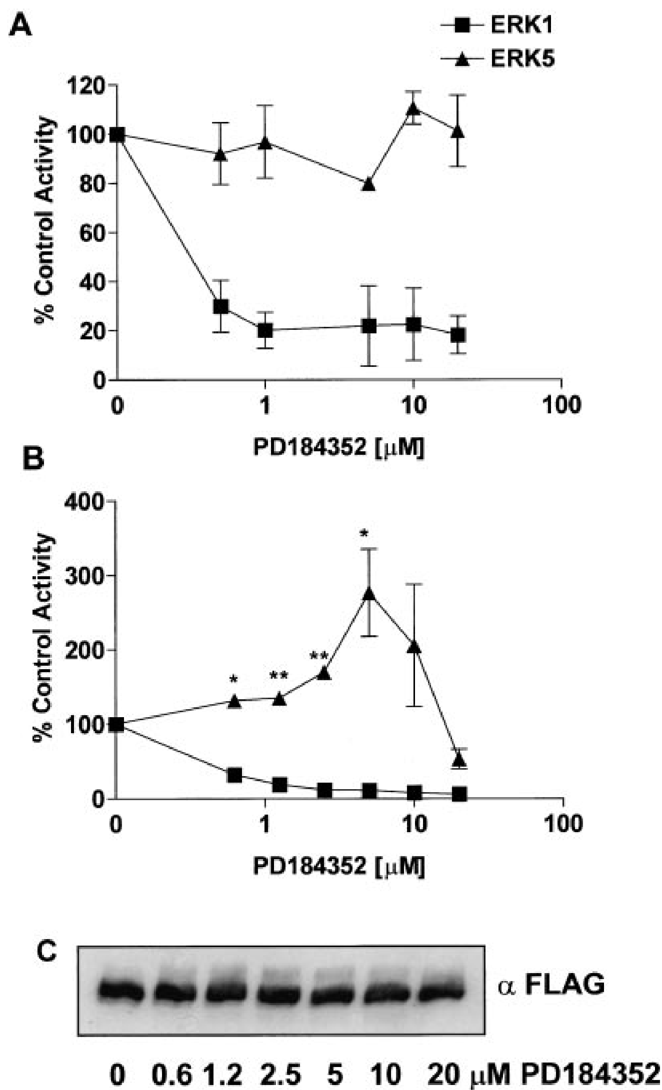

Figure 5 ERK1 activity is more sensitive to inhibition by PD184352 than ERK5 activity in asynchronous cells

Sub-confluent CCl39:ERK5 cells, growing asynchronously in 10% FBS, were transferred to media containing the indicated concentrations of PD184352. They were harvested after either 5 h (**A**) or 24 h (**B**) and the activities of ERK1 and ERK5 quantified by immune-complex kinase assay. Control cells (0 μM), were maintained in an equivalent amount of DMSO (vehicle control). Results represent means $\pm$ S.D. of triplicate cell dishes and are pooled from two experiments; a third gave similar results. $^{*}P < 0.05$, $^{**}P < 0.005$, significantly different from 0 μM control. Whole-cell lysates from the 24 h time point were separated by SDS/PAGE followed by immunoblotting with anti-FLAG, to detect FLAG:ERK5, to confirm equal protein loading. The experiment shown is representative of three giving similar results.

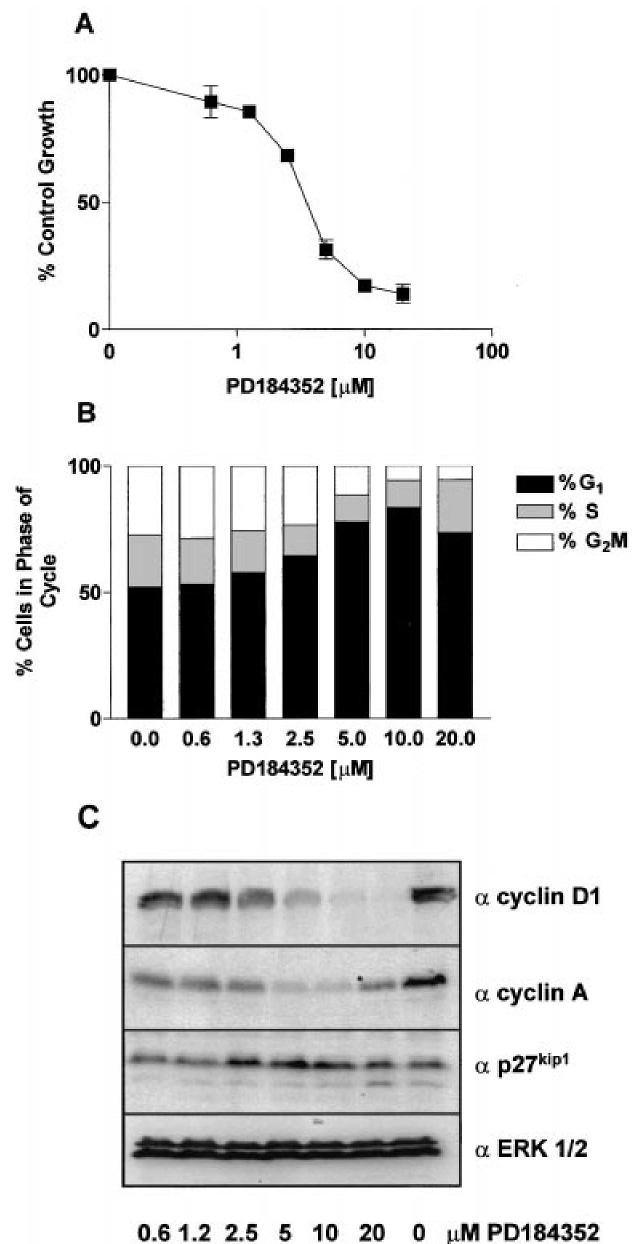

Figure 6 PD184352 induces a G_1 arrest and inhibits expression of cyclin D1 at concentrations that inhibit ERK1 but not ERK5 in cycling cells

(**A**) CCl39:ERK5 cells, growing asynchronously in 10% FBS, were treated with a vehicle control (0 μM = DMSO) or increasing concentrations of PD184352, for 72 h, and relative cell number was assayed by Crystal Violet staining. The data shown were combined from two experiments conducted in triplicate. (**B** and **C**) CCl39:ERK5 cells, growing asynchronously in 10% FBS, were cultured in the presence of the indicated concentrations of PD184352, or an equivalent of DMSO vehicle control (0 μM), for 24 h. (**B**) Cells were then stained with propidium iodide and cell-cycle distribution was analysed by flow cytometry using the Cell Quest package. Data shown are from a single experiment, conducted with duplicate cell dishes, and representative of three giving similar results. (**C**) In parallel, cells were lysed and whole-cell lysates separated by SDS/PAGE followed by immunoblotting with anti-cyclin D1, anti-cyclin A, anti-p27^{kip1} or anti-ERK antibodies.

before using Crystal Violet staining as an estimate of cell number (Figure 6A). Under these conditions PD184352 caused a dose-dependent 80–90% inhibition of cell proliferation, with half-maximal inhibition being observed at ≈ 3 μM. Because inhibition of ERK1 and ERK5 was determined following a 24 h stimulation (Figure 5B), we also used propidium iodide staining followed by flow cytometry to examine the effect of a 24 h exposure to PD184352 on the cell-cycle distribution of CCL39:ERK5 cells. In this case PD184352 caused a dose-dependent accumulation of cells in G_1; the percentage of G_1 cells increased from 52% in control populations to 83% in populations treated with 10 μM PD184352. The percentage of cells in S or G_2/M showed a concomitant decline. G_1 arrest was observed at doses of PD184352 from 1.3 μM, and the most pronounced effect was observed over the 2.5–10 μM range (Figure 6B), at which ERK5

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activity was not inhibited, but actually potentiated (Figure 5B). This dose-dependent inhibition of proliferation was mirrored in the inhibition of cyclin D1 and cyclin A expression observed when CCl39:ERK5 cells were exposed to increasing doses of PD184352 for 24 h (Figure 6C). The expression of these cyclin proteins was inhibited at concentrations of PD184352 from 2.5 μM and abolished at concentrations of 5–10 μM. A small dose-dependent accumulation of the cell-cycle inhibitor $p27^{kip1}$ was also observed in the same samples over the same concentration range. Finally, Western blots were performed on these lysates using antibodies specific for ERK1/2 and confirmed equal protein loading. Taken together, these results suggest that inhibition of the classical ERKs, ERK1 and ERK2, is sufficient to account for the anti-proliferative effect of PD184352. Indeed, in cycling cells the anti-proliferative effect of PD184352 actually correlates with an increase in ERK5 activity.

DISCUSSION

The ability of U0126 and PD98059 to antagonize cell proliferation is consistent with the proposal that the ERK1/2 pathway is required for growth factor-stimulated cell-cycle re-entry. However, the demonstration that both of these drugs also prevent activation of ERK5 [19] raised the possibility that the effects of such drugs on cell proliferation, or indeed any other cellular assay, may be due in part to inhibition of the ERK5 pathway. In this study we have addressed these issues by examining the effects of PD184352, a recently described ERK pathway inhibitor, on serum-stimulated cell-cycle re-entry and asynchronous cell proliferation.

In both experimental paradigms ERK5 activity was considerably less sensitive to PD184352 than ERK1. In quiescent cells, FBS-stimulated ERK1 activity was inhibited by doses of PD184352 below 1 μM, whereas ERK5 remained unaffected by a dose of 20 μM. Similarly, in an asynchronous cycling population of cells, ERK1 activity was completely inhibited by a 5 h treatment with doses of 0.5–1 μM PD184352, yet ERK5 activity was only inhibited after 24 h treatment and only at 20 μM, the highest dose used. A similar differential effect of PD184352 on ERK1/2 versus ERK5 activity was recently reported in EGF-stimulated HeLa cells [26], suggesting that differential sensitivity of these pathways to PD184352 may be a common feature of different cell lines.

When PD184352 was used to treat a population of asynchronous cycling cells, we observed a significant 2.5–3-fold increase in cycling ERK5 activity at doses of PD184352 from 0.6 to 5 μM. This activation, which accompanied the strong inhibition of ERK1, was highly reproducible, but was only observed after a 24 h treatment with the drug; it was not observed after serum re-stimulation. A similar observation was made in a previous study where EGF-induced MEK5 and ERK5 phosphorylation were enhanced for 20 and 10 min respectively at doses of PD184352 that inhibited the ERK1 pathway [26]. In our hands this potentiation of ERK5 was only observed during prolonged exposure of cells which were cycling, but with these caveats in mind it seems to be a reproducible observation between cell lines. This result may indicate some aspect of negative feedback from the ERK1/2 pathway to the ERK5 pathway, and experiments are currently underway to address this.

In the case of MEK1, PD184352 is not competitive for either ATP or the MAPK substrate *in vitro* and it exerts its inhibition via an allosteric mechanism [24,25]. The disparity between the concentrations of PD184352 required to inhibit the ERK5 pathway and ERK1/2 pathway may result from it interacting with MEK5 less well than it does with MEK1, thus resulting in the need for a higher concentration with which to observe downstream effects. Crystal structures of PD184352 bound to MEK1 or MEK5 may help to resolve this issue in the future and may allow a 'chemistry effort' to separate MEK5-specific determinants from the MEK1/2 inhibitory activity.

Regardless of the mechanism for these differential effects, these results strongly suggest that careful titration of PD184352 should allow selective inhibition of the ERK1/2 pathway even in those cases where the ERK5 pathway is also activated. Here we have used the differential sensitivity of the two pathways to determine which is more likely to be the target of the anti-proliferative effects of PD184352. In quiescent cells, serum-stimulated cell-cycle re-entry and cyclin D1 expression were abolished by 2.5–5 μM PD184352, while half-maximal inhibition was observed at 0.5–1 μM. Similarly, in asynchronous cells, PD184352 inhibited cyclin D1 and cyclin A expression, resulting in a G_1 arrest over the same dose range. Most importantly, all these anti-proliferative effects were observed in response to doses of PD184352 that failed to inhibit ERK5 activity. Indeed, in cycling cells optimal growth inhibition was observed at doses at which ERK5 activity actually increased, commensurate with the inhibition of ERK1. The simplest conclusion from these results is that the ability of PD184352 to inhibit cell-cycle re-entry or asynchronous cell proliferation is dependent on inhibition of the ERK1/2 pathway, but does not require inhibition of the ERK5 pathway.

It is important to emphasize that these results do not necessarily imply that ERK5 is not important in promoting or regulating cell proliferation. Indeed, there is ample evidence supporting a role for the ERK5 pathway in promoting cell proliferation. For example, dominant negative mutants of ERK5 and MEK5 can inhibit Raf-induced cellular transformation [22], and constitutively active MEK1 and MEK5 have been shown to synergize with Raf in transformation and activation of nuclear factor κB [23]. The ERK5 pathway, together with c-Jun N-terminal kinase and p38, is required for the Cot1 oncogene to fully activate the c-*jun* promoter and induce cellular transformation [14]. Other studies have described a requirement for ERK5 in the EGF-induced proliferation of MCF10-A cells [17] and shown that phosphorylation of serum- and glucocorticoid-inducible kinase (SGK) on Ser^{78}, by ERK5, is required for $G_1 \rightarrow S$ transition [27]. Finally, a recent study demonstrated that dominant negative MEK5 inhibited neuregulin-induced proliferation of MCF-7 breast carcinoma cells [21].

All of these observations suggest that the ERK5 pathway may be a rate-determining step in cell-cycle re-entry and even cellular transformation. However, our results, in which ERK1/2 is inhibited independently from ERK5, suggest that whatever function the ERK5 pathway performs to promote cell proliferation, it cannot substitute for loss of ERK1/2 activity in cells treated with low doses of PD184352. Indeed, this argues that these pathways have some discrete separate functions during cell proliferation, and this is consistent with the ability of constitutively active mutants of MEK5 to synergize with MEK1 in cell transformation [23].

In summary, our data show that when the ERK1/2 pathway is inhibited by low doses of PD184352, the ERK5 pathway is not sufficient to sustain cell proliferation. This suggests that the anti-proliferative effects of PD184352 observed in tumour cells [24,25] may, for the most part, be due to inhibition of the MEK1/2 $\rightarrow$ ERK1/2 pathway. It will be interesting to assess if the ability of an ERK pathway inhibitor to inhibit both the classical ERK1/2 and the ERK5 pathways is desirable, in terms of greater efficacy, or undesirable, in terms of greater toxicity. Obviously, a selective

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MEK5 → ERK5 pathway inhibitor would also be of great help in defining the role of the pathway in cell proliferation, or indeed any other cellular response. In instances in which both the ERK1/2 and ERK5 pathways are activated, PD184352 would seem to be the inhibitor of choice, since careful titration allows inhibition of ERK1/2 independently of ERK5.

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