

Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway

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Abstract Antibodies that recognise the active phosphorylated forms of mitogen-activated protein kinase (MAPK) kinase 5 (MKK5) and extracellular signal-regulated kinase 5 (ERK5) in untransfected cells have been exploited to show that the epidermal growth factor (EGF)-induced activation of MKK5 and ERK5 occurs subsequent to the activation of ERK1 and ERK2 in HeLa cells. The drugs U0126 and PD184352, which prevent the activation of MKK1 (and hence the activation of ERK1/ERK2), also prevent the activation of MKK5, although higher concentrations are required. Our studies define physiological targets of the MKK5/ERK5 pathway as proteins whose phosphorylation is largely prevented by 10 μ M PD184352, but unaffected by 2 μ M PD184352. Surprisingly, 2 μ M PD184352 prolongs the activation of MKK5 and ERK5 induced by EGF or H₂O₂, indicating negative control of the MKK5/ERK5 pathway by the classical MAPK cascade. Our results also indicate that ERK5 is not a significant activator of MAPK-activated protein kinase-1/RSK in HeLa cells. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The classical Ras-dependent mitogen-activated protein kinase (MAPK) cascade plays a key role in triggering the proliferation of some cells and the differentiation of others. The conversion of Ras to its active GTP-bound state leads to the sequential activation of the protein kinases Raf, MAPK kinase 1 (MKK1, also called MEK), and extracellular signal-regulated kinases 1 and 2 (ERK1, ERK2). ERK1/ERK2 then modulate the functions of proteins that are critical for proliferation and differentiation.

The uncontrolled activation of the classical MAPK cascade, caused, for example, by the overexpression or mutation of growth factor receptors and/or the mutation of Ras to constitutively active forms, is a cause of cell transformation and cancer. Two compounds, PD98059 and U0126, suppress activation of the MAPK cascade by binding to MKK1, thereby preventing its activation by Raf [1,2], and reverse the phenotype of Ras-transformed cell lines [3]. Moreover, PD184352, a more potent compound that acts in the same way [2], inhibits

the growth of human colon cancers implanted into mice, without obvious toxic side effects [4]. These observations suggest that inhibitors of the classical MAPK cascade might be useful for the treatment of some types of cancer.

Recently, a potential problem in using PD98059 and U0126 to identify physiological roles of the MAPK cascade has arisen, with the report that they also inhibit the activation of ERK5, another MAPK family member [5]. ERK5 is activated by MKK5 [6] in response to epidermal growth factor (EGF) [7] via a separate Ras-dependent pathway [5]. ERK5 can also be activated in response to osmotic and oxidative stress [8]. MKK5 is not activated by Raf [9] but by co-transfection with MEKK3 [10] or MEKK2 [11]. However, whether these and/or other protein kinases activate MKK5 in vivo has yet to be established. These findings raise the question of whether the effects of PD98059 and U0126 on cells, which have been reported in over 2000 papers, are caused by inhibition of the classical MAPK cascade or by inhibition of the MKK5/ERK5 cascade.

In this paper, we have studied the effects of PD98059, U0126 and PD184352 on the MKK5/ERK5 cascade in HeLa cells, by using phospho-specific antibodies capable of detecting the endogenous MKK5 and ERK5 proteins. We demonstrate that all three compounds are weaker inhibitors of the MKK5/ERK5 pathway than the classical MAPK cascade. Interestingly, PD184352 blocks activation of the classical MAPK cascade at concentrations where the activation of MKK5 and ERK5 is unaffected. This finding should aid the identification of the physiological substrates of ERK5 and has led us to discover that the classical MAPK cascade exerts a negative influence on the activation of MKK5 and ERK5 in HeLa cells.

2. Materials and methods

2.1. Materials

EGF, microcystin-LR, and reagents for cell culture were purchased from Life Technologies (Paisley, UK), PD98059 and U0126 from Calbiochem (Nottingham, UK) and hydrogen peroxide and 12-*O*-tetradecanoyl phorbol 13-acetate (from Sigma (Poole, UK)). 'Complete' protease inhibitor cocktail tablets were from Roche (Lewes, Sussex, UK). Precast 4–12% Bis-Tris gradient SDS polyacrylamide gels were from Invitrogen. All the peptides used in this study were synthesised by Dr G. Bloomberg (University of Bristol, UK).

2.2. Cell culture, stimulation and cell lysis

HeLa cells were obtained from the European Tissue Culture Collection and maintained in 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum and 2 mM L-glutamine.

Prior to stimulation, the cells were cultured overnight in the absence of serum. PD184352 and U0126 were dissolved in dimethyl sulphoxide

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(DMSO) at a concentration of 10 mM, and PD98059 at 50 mM. Where indicated, aliquots of these inhibitors in DMSO, or the equivalent volume of DMSO as a control, were added to the culture medium 60 min prior to stimulation. The cells were then stimulated (in the continued presence or absence of inhibitors) with the indicated agonists and lysed in ice-cold 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 10 mM sodium glycerophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μ M microcystin-LR, 'Complete' protease inhibitor cocktail (one tablet per 50 ml) and 0.1% (v/v) 2-mercaptoethanol. The lysates were centrifuged at 4°C for 5 min at 13 000 \times g and the supernatants frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined according to the Bradford method.

2.3. Expression and purification of recombinant His-ERK5

The cDNA encoding full-length ERK5 [6] was amplified by PCR from a pCMV5-HA-ERK5 vector and cloned into pFASTBAC1. This vector was then used to express and purify recombinant ERK5 preceded by six histidine residues (His-ERK5) in Sf21 cells using the Bac-to-Bac system (Life Technologies, Paisley, UK), as described previously [14].

2.4. Antibodies

A phosphopeptide corresponding to residues 214–225 of human ERK5, phosphorylated on Thr219 and Tyr221 (HQYFMT*EY*VATR, where the phosphorylated residues are marked with asterisks) was used to generate a phospho-specific ERK5 antibody, using the procedure described previously for phospho-specific c-Jun N-terminal kinase antibodies [14]. The phospho-specific ERK5 antibody also recognises phosphorylated ERK1 and ERK2. A phosphopeptide corresponding to residues 306–320 of human MKK5, phosphorylated on Ser311 and Thr315 (TQLVNS*IAKT*YVGTN) was used to generate a phospho-specific MKK5 antibody in the same way. Polyclonal anti-ERK5 antibodies were also raised in sheep against the His-ERK5 protein and affinity-purified by chromatography on His-ERK5-agarose. All antibodies were used at a concentration of 0.5–2 μ g/ml. Affinity-purified anti-sheep antibodies coupled to horseradish peroxidase were purchased from Pierce (Rockford, IL, USA) and used at dilutions of 1:5000 (phospho-specific antibodies) or 1:15 000 (anti-ERK5 antibody). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

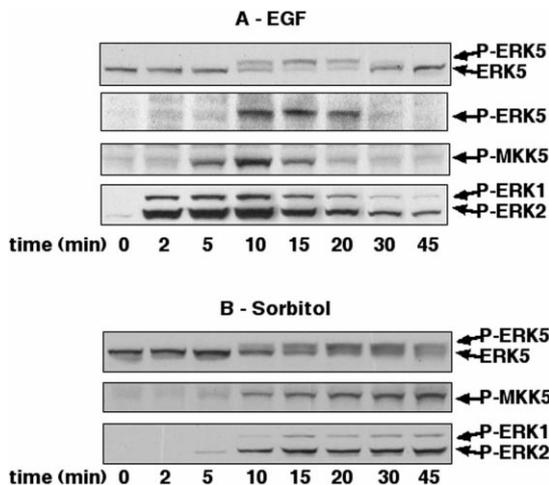


Fig. 1. Activation of the MKK5/ERK5 pathway by EGF and hyperosmotic stress. HeLa cells were serum-starved overnight, then stimulated with 100 ng/ml EGF (A) or 0.5 M sorbitol (B) for the times indicated. Phosphorylation of the endogenous MKK5 and ERK5 was then examined by immunoblotting with an antibody that recognises phosphorylated ERK5 (P-ERK5) and dephosphorylated ERK5 equally well (upper panel in A and B) and with phospho-specific antibodies that recognise the active, phosphorylated forms of ERK5, MKK5 and ERK1/ERK2. Similar results were obtained in several independent experiments.

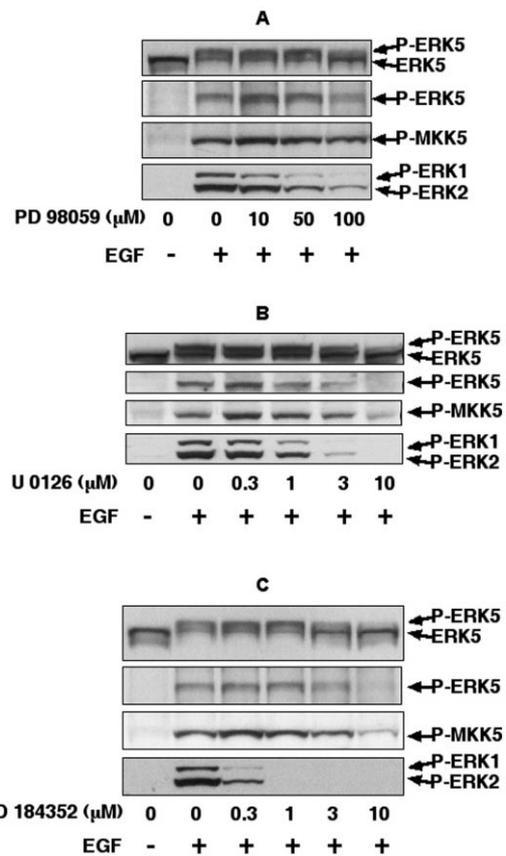


Fig. 2. Inhibitors of the classical MAPK pathway are less effective at suppressing the activation of the MKK5/ERK5 pathway in HeLa cells. The cells were serum-starved overnight, pretreated for 1 h with the indicated concentrations of PD98059 (A), U0126 (B) or PD184352 (C), then stimulated for 10 min with 100 ng/ml EGF. Following cell lysis, the phosphorylation states of the endogenous MKK5 and ERK5 were examined by immunoblotting with an antibody that recognises phosphorylated ERK5 (P-ERK5) and dephosphorylated ERK5 equally well (upper panels) and with phospho-specific antibodies that recognise the phosphorylated forms of ERK5, MKK5 and ERK1/ERK2. Similar results were obtained in several independent experiments.

3. Results

3.1. Generation of phospho-specific antibodies that recognise the activated forms of MKK5 and ERK5

In order to study the effects of PD98059, U0126 and PD184352 on the MKK5/ERK5 pathway, we first raised antibodies capable of recognising the phosphorylated forms of these enzymes (see Section 2). Although the activating phosphorylation sites on MKK5 have not yet been identified directly, by analogy with other MKK family members, they were assumed to be the serine and threonine residues that lie in positions equivalent to Ser217 and Ser221 of MKK1, the residues that are phosphorylated by Raf [12]. Similarly, the sites on ERK5 that are phosphorylated by MKK5 have not been identified directly, but are assumed to be the threonine and tyrosine residues of the Thr-Glu-Tyr motif that are present at equivalent positions to the Thr-Glu-Tyr sequence in ERK1 and ERK2 that is phosphorylated by MKK1 [6,13].

Using these antibodies, we were able to detect proteins in HeLa cell extracts that became phosphorylated in response to

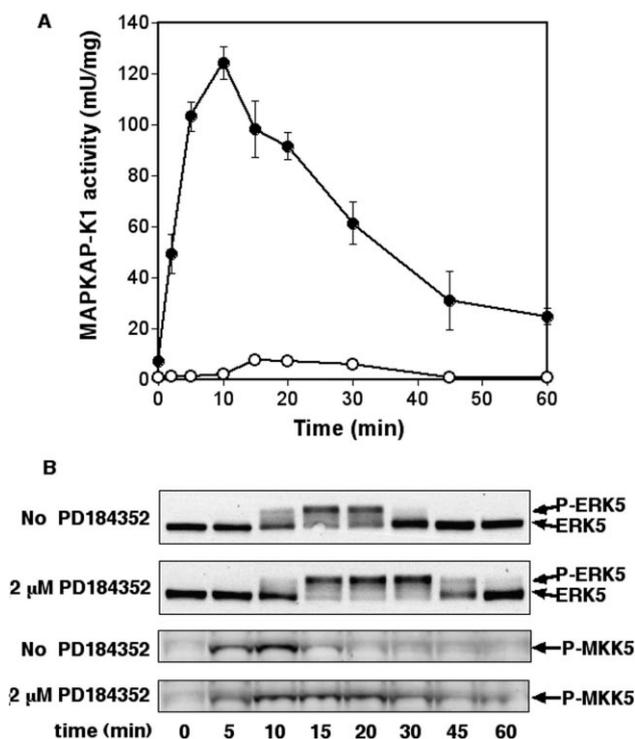


Fig. 3. Inhibition of the classical MAPK pathway causes a more sustained activation of the MKK5/ERK5 pathway after stimulation with EGF. A: HeLa cells were serum-starved overnight, pretreated for 1 h without (closed circles) or with 2 μ M PD184352 (open circles), then stimulated with 100 ng/ml EGF for the times indicated. MAPKAP-K1b (RSK2) was immunoprecipitated from the cell lysates (50 μ g protein) using an antibody raised against the peptide RNQSPVLEPVGRRSTLAQRRGIKK [1] and then assayed with the peptide Crosstide (GRPRTSSFAEG) as described [16,17]. One unit of activity (U) was that amount which catalysed the phosphorylation of 1 nmol of Crosstide in 1 min in the standard assay. The protein concentration in the extract was determined by the method of Bradford. B: Same as A, except that aliquots of the extracts were denatured in SDS, electrophoresed on SDS polyacrylamide gels and transferred to nitrocellulose. Phosphorylation of the endogenous ERK5 and MKK5 was then monitored by immunoblotting with an antibody that recognises phosphorylated ERK5 (P-ERK5) and dephosphorylated ERK5 equally well (upper two panels) and with a phospho-specific antibody that recognises the phosphorylated form of MKK5 (lower two panels). Similar results were obtained in several independent experiments.

signals (EGF and osmotic stress) that are known to activate the MKK5/ERK5 pathway and which migrated on SDS polyacrylamide gels with the molecular masses expected for MKK5 and ERK5 (Fig. 1). The phosphorylation of ERK5 could also be detected by a small decrease in its electrophoretic mobility using another antibody that recognises the phosphorylated and dephosphorylated forms of the protein equally well. After stimulation with EGF, or exposure to osmotic stress, ERK5 was transformed into a species of slightly lower electrophoretic mobility which comigrated with the band detected with the phospho-specific antibody. Similarly, an antibody that recognises the phosphorylated and dephosphorylated forms of MKK5 equally well also recognised a band in HeLa extracts that comigrated with that detected with the MKK5 phospho-specific antibody (data not shown). The activation of MKK5 occurred slightly earlier than the activation of ERK5, as expected, if MKK5 lies upstream of ERK5 in the pathway. These experiments indicate that the

phospho-proteins detected by the antibodies are indeed MKK5 and ERK5.

The amino acid sequences surrounding the phosphorylation sites on ERK1, ERK2 and ERK5 are similar. For this reason, the phospho-specific antibody that recognised ERK5 also recognised ERK1 and ERK2, allowing the activation of ERK1/ERK2 and ERK5 to be visualised simultaneously on the same immunoblot. The EGF-induced activation of ERK5 in HeLa cells was almost maximal 2 min after stimulation with EGF, but the activation of ERK5 was only maximal after 15 min (Fig. 1A). When the cells were exposed to 0.5 M sorbitol, an osmotic stress (Fig. 1B), the activations of ERK1/ERK2 and ERK5 were both maximal after 15–20 min.

3.2. Effects of PD98059, U0126 and PD184352 on the activation of MKK5 and ERK5

Pre-incubation of HeLa cells with PD98059 or U0126 inhibited the EGF-induced phosphorylation of ERK5, as expected from an earlier study in COS7 cells [5]. However, in our experiments, both inhibitors were less effective at suppressing the phosphorylation of MKK5 and ERK5 than the activation of ERK1 and ERK2, and inhibition of MKK5 and ERK5 phosphorylation by PD98059 was weak (Fig. 2A,B).

PD184352 also inhibited the phosphorylation of ERK5. However, whereas 1–2 μ M PD184352 was sufficient to block the classical MAPK cascade, as judged by suppression of the phosphorylation of ERK1/ERK2 (Fig. 2) and activation of one of their substrates, MAPK-activated protein kinase 1 (MAPKAP-K1, also called RSK) (Fig. 3A). It was necessary to increase PD184352 to 10 μ M to largely suppress the activation of MKK5 and ERK5 (Fig. 2). U0126 and PD184352 also blocked the activation of MKK5, at concentrations similar to those needed suppress the activation of ERK5 (Fig. 2).

3.3. Activation of the MKK5/ERK5 pathway is inhibited by the classical MAPK cascade

Although the activation of ERK5 and MKK5 induced by EGF was transient, we found that activation was more pro-

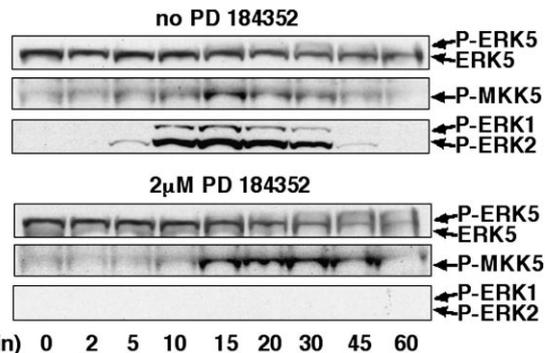


Fig. 4. Inhibition of the classical MAPK pathway allows a more sustained activation of the MKK5/ERK5 pathway by hydrogen peroxide. HeLa cells were serum-starved overnight, pretreated for 1 h without or with 2 μ M PD184352, then stimulated with or without 1 mM hydrogen peroxide for the times indicated. Phosphorylation of the endogenous MKK5 and ERK5 was then examined by immunoblotting with an antibody that recognises phosphorylated (P-ERK5) and dephosphorylated ERK5 equally well (upper panel), an antibody that recognises the phosphorylated form of MKK5 (middle panel) and an antibody that recognises the phosphorylated form of ERK1/ERK2 (lower panel). Similar results were obtained in several independent experiments.

longed if the cells were incubated with 2 μ M PD184352 (Fig. 3B). Thus the classical MAPK cascade has a negative effect on the EGF-induced activation of the MKK5/ERK5 pathway. However, the effect is not EGF-specific, because the activation of MKK5 and ERK5 induced by H₂O₂, an oxidative stress, was also more prolonged in the presence of 2 μ M PD184352 (Fig. 4).

4. Discussion

Nishida and co-workers [5] reported that the drugs PD98059 and U0126 inhibited the EGF-induced activation of transfected ERK5 in COS7 cells at concentrations similar to those which suppressed activation of the classical MAPK cascade. In this paper, we confirmed that PD98059 and U0126 inhibited activation of the endogenous ERK5 in HeLa cells, but only at higher concentrations than those required to prevent the activation of ERK1/ERK2. This was also true for PD184352, a more potent inhibitor of the classical MAPK cascade [4]. We have reported previously that PD98059, U0126 and PD184352 suppress activation of the classical MAPK cascade in cell-based assays by binding to MKK1, thereby preventing the phosphorylation and activation of MKK1 by Raf and other upstream activators [1,2]. In the present study, we showed that the same three drugs prevent the activation of ERK5 by suppressing activation of the endogenous MKK5 in HeLa cells (Fig. 2). Since the catalytic domains of MKK5 and MKK1 show 46% amino acid sequence identity, it seems likely that these compounds also exert their effects by binding to MKK5, thereby preventing its phosphorylation and activation.

The activation of ERK1 and ERK2 was completely suppressed by incubating HeLa cells with 1–2 μ M PD184352, at which concentration the activation of MKK5 or ERK5 was not reduced (Figs. 2 and 3). The activation of MKK5 and ERK5 was only suppressed at 10 μ M PD184352. These observations may facilitate identification of physiological substrates of ERK5. Moreover, we found that blocking the activation of ERK1 and ERK2 by incubating HeLa cells with 2 μ M PD184352 prolonged the activation of MKK5 and ERK5 by either EGF (Fig. 3B) or hydrogen peroxide (Fig. 4). These observations demonstrate that one or more components of the classical MAPK cascade have a negative effect on activation of the MKK5/ERK5 pathway. To our knowledge, this is the first evidence of ‘crosstalk’ between these two MAPK cascades.

The growth factor-induced activation of MAPKAP-K1 (also called RSK) is thought to be mediated *in vivo* by ERK1/ERK2, because its activation is prevented by PD98059 [1]. It has been reported that MAPKAP-K1/RSK

can also be activated by co-transfection with ERK5 and a constitutively active mutant of MKK5, suggesting that MAPKAP-K1/RSK might be activated by the MKK5/ERK5 pathway as well as, or instead of, the MKK1-ERK1/ERK2 pathway [15]. However, we found that the EGF-induced activation of MAPKAP-K1 was blocked by prior incubation of the cells with 2 μ M PD184352 (Fig. 3A), even though the activation of ERK5 was just as high and more prolonged under these conditions. Thus ERK5 makes a negligible contribution to the EGF-induced activation of MAPKAP-K1 in HeLa cells.

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