

# Growth factor-stimulated MAP kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (MEK1)

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## Abstract

The MAP kinase module (Raf/MAPKKK-MAPKK-MAPK) has been shown to be sequentially activated after mitogenic stimulation. Here we demonstrate, by site directed mutagenesis, that MAPK is able to retrophosphorylate its own activator, MAPKK, on two threonine residues Thr-292 and Thr-386 in vitro, and that these sites are also phosphorylated in vivo. A comparison of the kinetics of serum-mediated activation of a wild-type MAPKK and of a mutant unable to undergo phosphorylation by MAPK suggests that this retrophosphorylation may be involved in a negative feedback control of the cascade in vivo.

**Key words:** MAP kinase; MAP kinase kinase; Retrophosphorylation; Feedback control; Growth control; Growth factor

## 1. Introduction

Upon stimulation by a variety of growth factors, the protein kinases, Raf and MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK), are successively activated by phosphorylation (for a review see [1]). Activation of this kinase cascade, referred to as the 'MAP kinase module', appears to be crucial for cell proliferation [2] and is probably subject to tight regulation. Since protein kinases are involved in activation, it is assumed that protein phosphatases play a role in switching off this cascade. For instance, MKP-1, a phosphatase able to specifically dephosphorylate and inactivate MAPK, has recently been identified [3]. However, this does not rule out the possibility of other means of control. Indeed it has recently been described that cAPK was able to inhibit the activation of the MAPK module by phosphorylating Raf [4]. Furthermore, the protein kinase p34<sup>cdc2</sup> has been shown to inhibit MAPK in vitro [5]. Finally MAPK is able to phosphorylate many upstream components of this cascade, such as the EGF receptor [6], the Ras nucleotide exchange factor Sos [7], Raf [8], and even its own activator MAPKK [9]. However, it remains to be demonstrated that this phosphorylation is of physiological relevance and to determine whether it is involved in the regulation of the MAPK module.

Here we show that MAPK phosphorylates MAPKK both in vitro and in vivo on two threonine residues, and that this retrophosphorylation may contribute to a feedback control mechanism.

## 2. Materials and methods

### 2.1. Materials

[ $\gamma$ -<sup>32</sup>P]ATP and the enhanced chemoluminescence (ECL) immunodetection system were from Amersham. The monoclonal antibody 12CA5, raised against a peptide from Influenza HA1 protein [10], was purchased from Babco (Emeryville, CA).

### 2.2. Mutants of MAPKK

MAPKK Chinese hamster cDNA was subcloned into the expression vector HA-pECE between the *EcoRI* sites, in frame with the hemagglutinin epitope [11] as was previously performed for MAPK [12].

Single mutants T292A and T386 were obtained directly in the HA-pECE expression vector by site-directed mutagenesis of double-stranded DNA according to the Clontech strategy [13]. The double mutant T292A/386A was constructed by exchanging the *AccI/XbaI* cassette of T292A by that of T386A. Fig. 1 illustrates the positions of the mutated forms of MAPKK used in this study.

### 2.3. Cell culture

The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection), its derivative PS120, which lacks Na<sup>+</sup>/H<sup>+</sup> antiporter (NHE1) activity [14], and corresponding transfected cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) containing 7.5% fetal calf serum, penicillin (50 U/ml) and streptomycin sulphate (50  $\mu$ g/ml). Growth-arrested cells were obtained by serum deprivation for 16–24 h.

### 2.4. Stable transfection

We used NHE1 as a selective marker and the H<sup>+</sup>-killing selection technique as previously described [2]. PS120 cells (10<sup>6</sup> cells per 10 cm plate) were co-transfected by the calcium phosphate technique with 2  $\mu$ g of pEAP expression vector (NHE1 cDNA) [15] and 20  $\mu$ g of pECE expression vector containing different constructs of MAPKK. 48 h after transfection, cells were subjected to an acid-load selection that killed non-transfected cells, usually 90–95% of the cell population. Two additional acid-load selections were applied usually at days 4 and 8 after transfection. Stable clones were either selected for analysis or mixed to provide the transfected population. The population or individual clones were passaged with application of the acid-load selection once a week.

### 2.5. Immune complex kinase assays

**2.5.1. Phosphorylation of MAPKK by MAPK.** Quiescent CCL39 cells expressing the p44 isoform of MAPK tagged with the hemagglutinin epitope (HA-MAPK) were stimulated in HEPES-buffered DMEM with 20% serum for 5 min at 37°C. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in Triton lysis buffer

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(50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM  $\beta$ -glycerophosphate, 200  $\mu$ M Na-orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM *p*-nitrophenyl phosphate, 4  $\mu$ g/ml aprotinin, 1% Triton X-100) for 15 min at 4°C. Insoluble material was removed by centrifugation at 12,000  $\times$  g for 5 min at 4°C. Cell lysates were incubated with the 12CA5 antibody pre-adsorbed to protein A-Sepharose coated beads for 2 h at 4°C. The same procedure was followed for preparing the substrate, MAPKK tagged with the hemagglutinin epitope (HA-MAPKK, WT or mutant) from non-stimulated PS120 cells. Immune complexes were washed three times with Triton lysis buffer and the beads with the HA-MAPK and HA-MAPKK immune complexes were mixed and washed once with kinase buffer (20 mM HEPES, pH 7.4, 20 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM dithiothreitol, 10 mM *p*-nitrophenylphosphate). MAPKK phosphorylation was assayed by resuspending the pellet in 50  $\mu$ l of kinase buffer containing 50  $\mu$ M of [ $\gamma$ -<sup>32</sup>P]ATP (5,000 cpm/pmol). The reaction mixture was incubated for 30 min at 30°C. The samples were heated at 95°C for 5 min and proteins separated by SDS-PAGE (10% gels) [16]. The gel was then dried and subjected to autoradiography.

**2.5.2. MAPKK activity.** Quiescent PS120 cells expressing different isoforms of HA-MAPKK were stimulated for the indicated times (1 min to 3 h) with 20% serum. Immunoprecipitation was performed as described above. The substrate, HA-MAPK, was immunoprecipitated from resting CCL39 cells as described above. Beads containing HA-MAPKK and HA-MAPK were mixed and kinase assays was performed as described above.

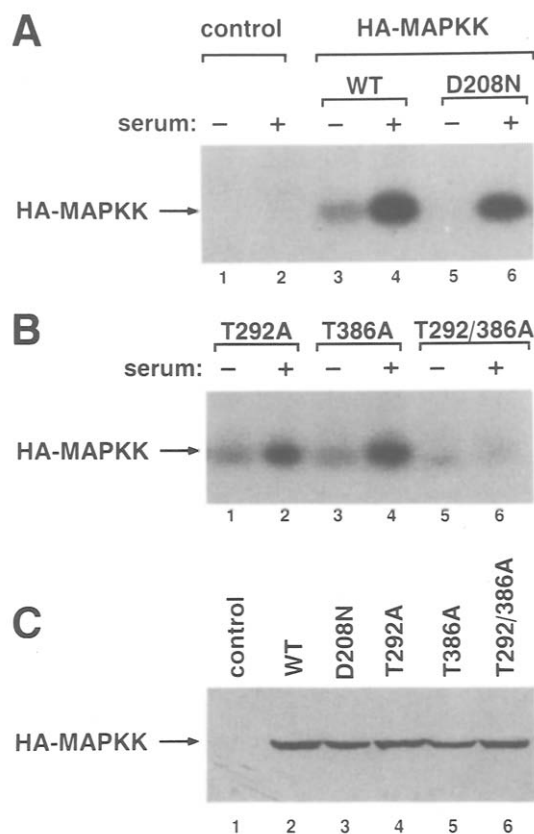
## 2.6. In vivo phosphorylation

Quiescent cells in 10 cm plates were labelled for 12 h at 37°C in phosphate-free DMEM medium containing 500  $\mu$ Ci/ml [<sup>32</sup>P]orthophosphate. Cells were stimulated by addition of 20% serum for 15 min at 37°C, washed with cold PBS and lysed in lysis buffer. After centrifugation at 12,000  $\times$  g for 5 min at 4°C, the lysates were pre-cleared for 1 h at 4°C with preimmune rabbit serum and 1 h with preimmune mouse serum pre-adsorbed on protein A-Sepharose. The lysates were then incubated for 2 h at 4°C with the 12CA5 antibody pre-adsorbed on protein A-Sepharose. Immune complexes were washed four times with Triton lysis buffer, twice with lysis buffer without NaCl, twice with lysis buffer containing 0.5 M LiCl and an additional two times with normal lysis buffer. Beads were then resuspended in Laemmli sample buffer, heated at 95°C for 5 min and the proteins were separated by SDS-PAGE (10% gels). The resolved proteins were then transferred to nitrocellulose (Hybond C, Amersham) and the membrane subjected to autoradiography for 30 min. The labelled proteins were then digested with trypsin (Sigma) and the tryptic phosphopeptides separated on phosphocellulose plates as previously described [17].

## 3. Results

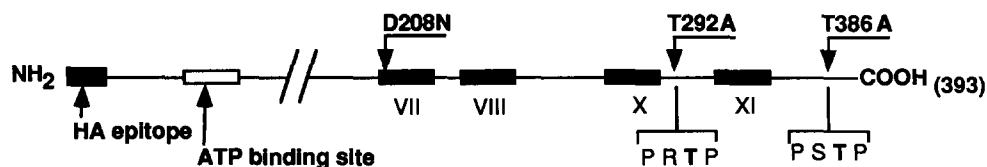
### 3.1. MAPK is able to phosphorylate its activator MAPKK

In order to investigate whether MAPKK is indeed a target for MAPK phosphorylation, we stably expressed in fibroblasts either the p44 isoform of MAPK or a form of MAPKK, both tagged with the hemagglutinin epitope (HA-MAPK or HA-MAPKK). Fig. 2A shows that HA-MAPK immunoprecipitated from stimulated cells with



**Fig. 2.** Retrophosphorylation of MAPKK by MAPK PS120 cells were stably transfected with the vector alone (control), the wild-type form of MAPKK (WT) tagged with the hemagglutinin epitope (HA-MAPKK) and different MAPKK mutants (D208N, T292A, T386A and T292/386A). CCL39 cells expressing MAPK tagged with the hemagglutinin epitope (HA-MAPK) were arrested in G<sub>0</sub> and stimulated (+) or not (-) by 20% serum for 5 min. (A) HA-MAPK was immunoprecipitated with the antibody directed against the HA epitope (anti-HA antibody) and its ability to phosphorylate MAPKK was assayed by incorporation of <sup>32</sup>P on HA-MAPKK immunoprecipitated from quiescent cells with the anti-HA antibody. Different forms of HA-MAPKK were used as a substrate: WT or D208N mutant. (B) Mutants of the consensus sites of phosphorylation by MAPK were used as MAPK substrates: T292A, T386A and T292/386A. Note that the phosphorylation is reduced by 2 fold in single mutants when compared to the wild-type. (C) Levels of expression of the different isoforms of MAPKK were analysed by Western blot with the anti-HA antibody.

the anti-HA antibody is able to phosphorylate an inactive HA-MAPKK immunoprecipitated from quiescent cells with the anti-HA antibody (lanes 3 and 4). This phosphorylation is specific as it does not occur when



**Fig. 1.** Schematic representation of the Chinese hamster MAP kinase kinase tagged with the hemagglutinin epitope (HA-MAPKK). The position of the mutations introduced and the kinases subdomains VII, VIII, X and XI were indicated.

cells are transfected with the vector alone (Fig. 2A, lanes 1 and 2). Secondly, it can not be attributed to auto-phosphorylation of MAPKK since an inactive mutant of MAPKK, mutated on the aspartic acid involved in phosphate transfer (D208N) is phosphorylated by MAPK to the same extent as the wild-type MAPKK (Fig. 2A, lanes 5 and 6).

### 3.2. MAPKK is retrophosphorylated by MAPK on Thr-292 and -386 *in vitro*

The peptidic sequence of the Chinese hamster MAPKK shows two putative consensus sites for MAPK phosphorylation (PXTP), Thr-292 and -386 (Fig. 1). Furthermore MAPKK has been shown to be phosphorylated mainly on threonine residues by MAPK [9]. Hence, we specifically mutated each threonine (T292A and T386A) individually or concomitantly (T292/386A) and stably expressed these mutants in fibroblasts. To distinguish between these mutated forms of MAPKK and the endogenous wild-type form, we also tagged these mutants with the HA epitope. They were then analysed for their ability to be retrophosphorylated by MAPK in the same way as mentioned above. The single mutants T292A or T386A also undergo retrophosphorylation by MAPK but this phosphorylation was decreased about 2 fold when compared to that of the wild-type MAPKK (Fig. 2A, lanes 2,3 and Fig. 2B, lanes 1–4). In contrast, the double mutant T292/386A was no longer able to be phosphorylated by MAPK (Fig. 2B, lanes 5 and 6). This lack of phosphorylation was not due to a conformational change reducing the ability of the protein to be expressed, or to be immunoprecipitated

since Western blotting with the anti-HA antibody (Fig. 2C) revealed that the level of expression of the different forms of HA-MAPKK was identical. These results indicate that both sites (Thr-292 and -386) of MAPKK are equally employed *in vitro* for MAPK phosphorylation and that the phosphorylation of one site is independent of the other.

### 3.3. Thr-292 and -386 are also phosphorylated *in vivo*

To determine if these sites were also phosphorylated *in vivo*, we compared the phosphopeptide maps of the wild-type MAPKK and T292/386A mutant. Cells expressing either the wild-type form of HA-MAPKK or the T292/386A mutant were arrested for 16 h in the presence of [ $^{32}$ P]orthophosphate, then stimulated or not by 20% serum for 15 min. Immunoprecipitated HA-MAPKK (WT or T292/386A mutant) was subjected to digestion by trypsin, and phosphopeptides were resolved by two-dimensional analysis (see section 2). The comparison between the WT and T292/386A mutant of MAPKK, isolated from non-stimulated or stimulated cells, shows that the mutant lacks phosphopeptides 6 and 7 (Fig. 3). This result suggests that MAPKK is also phosphorylated *in vivo* on these two sites, probably by the endogenous MAP kinases.

### 3.4. MAPKK retrophosphorylation by MAPK is involved in a negative feedback control

To assess if this retrophosphorylation plays a role in MAPKK regulation, we compared the time-course of growth factor-stimulated MAPKK (WT vs. T292/386A mutant). Fig. 4 depicts the experiment comparing the

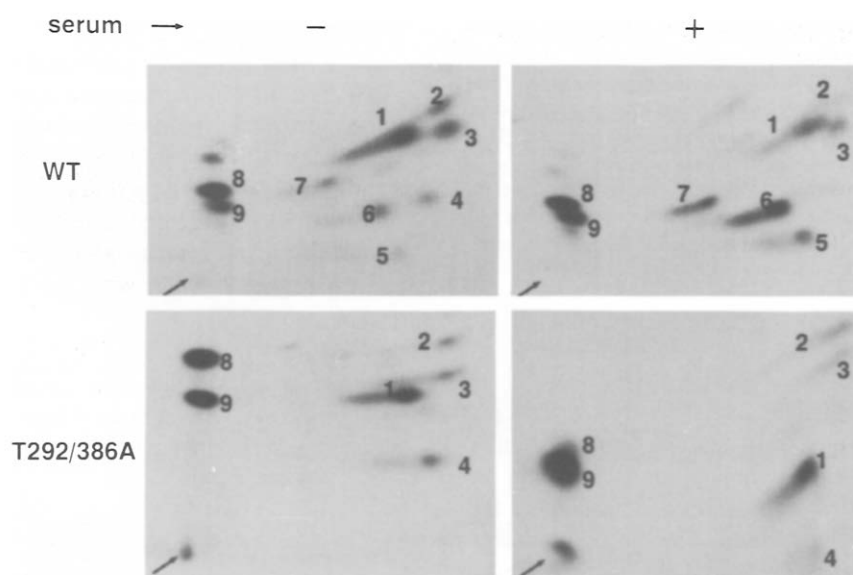
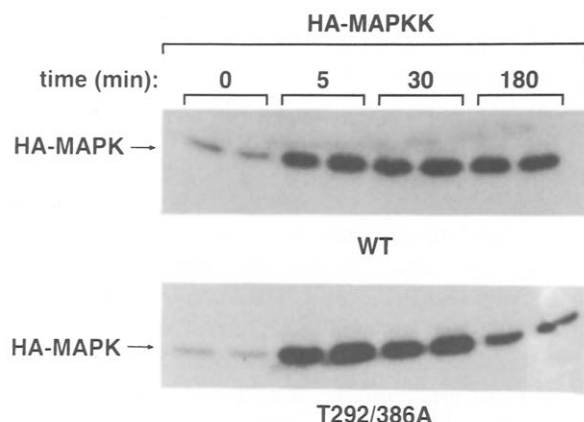


Fig. 3. *In vivo* phosphorylation of wild-type HA-MAPKK or T292/386A mutant. PS120 cells expressing wild-type HA-MAPKK or T292/386A mutant were arrested in G<sub>0</sub> in the presence of [ $^{32}$ P]orthophosphate and stimulated by 20% serum for 15 min. HA-MAPKK was immunoprecipitated with the anti-HA antibody and blotted to a nitrocellulose membrane. Tryptic digestion was performed as described in section 2 and phosphopeptides were resolved in two dimensions by electrophoresis (horizontal axis) and by chromatography (vertical axis). The major phosphopeptides are numbered (1 to 9) and the origin of migration is shown by an arrow. Autoradiograms for WT MAPKK correspond to an overnight exposure whereas those for T292/386A correspond to 3 days exposure.

## A



## B

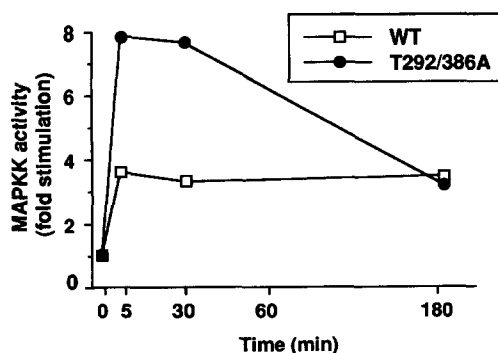


Fig. 4. Comparison of time-courses of activation of wild-type MAPKK and T292/386A mutant. (A) Cells expressing HA-MAPKK (WT or T292/386A mutant) were arrested in  $G_0$  and stimulated by 20% serum for the indicated times. HA-MAPKK was immunoprecipitated with the antibody directed against the epitope, and its activity was assayed by its ability to phosphorylate the epitope tagged form of MAPK (HA-MAPK) immunoprecipitated from resting cells with the anti-HA antibody. Each time point was performed in duplicate and the autoradiogram shown is representative of three independent experiments. (B) Relative MAPKK activities normalized to basal levels. Quantification was obtained by densitometry scanning and computer analysis of the autoradiogram.

WT and double mutant T292/386A MAPKK over a period of 3 h. Interestingly, this mutant exhibits higher kinase activity at an earlier time upon stimulation by serum growth factors (5 min) when compared to the wild-type form. After 3 h of stimulation by serum, a time at which retrophosphorylation is still evident, the kinase activity of the mutant decreases and approaches the wild-type activity (Fig. 4B). The same results were obtained with the single mutant T386A. These results support the notion that MAPK exerts a negative control on the cascade by retrophosphorylation of its activator MAPKK.

## 4. Discussion

Here we show that the p44 isoform of MAPK, among many other targets, is able to phosphorylate MAPKK, an upstream component of the activation cascade, confirming the results obtained by Matsuda et al. [9]. Mutagenesis experiments demonstrate that phosphorylation occurs both in vitro and in vivo on two threonine residues (Thr-292 and -386) which share the consensus site of phosphorylation by MAPK (PXTTP). The observation that the WT MAPKK possesses the phosphopeptides 6 and 7 (lacking in the T292/386A mutant), both under non-stimulated and stimulated conditions, may be explained by a slow turnover of phosphate on these sites.

Comparison of the in vivo activity of wild-type and the non-retrophosphorylatable form of MAPKK (T292/386A) indicates that this retrophosphorylation is inhibitory in the initial phase of activation by growth factors. This result is reminiscent of the situation observed in the yeast *Saccharomyces cerevisiae*, where the disruption of FUS3, the equivalent of MAPK, significantly enhances the activity of STE7, the homologue of mammalian MAPKK [18]. However the effect of phosphorylation of MAPKK by MAPK, although reproducible, is not so striking. A more pronounced effect should be seen upon disruption of both p42 and p44 MAPK or over-expression of a dominant negative mutant of MAPK. Interestingly the single mutant T386A/MAPKK, in common with the double mutant, displays an increased activity upon short-term stimulation by serum (data not shown). This result suggests that phosphorylation of Thr-386, a residue which is conserved in MAPKK from all the species tested, seems to be sufficient to induce a retro-control on the activity of MAPKK.

As a final point, we examined whether expression of the T292/386A mutant, which displays enhanced activity in response to mitogens, reduces the requirement for growth factors to stimulate cell proliferation. This was shown not to be the case (data not shown). However, this aspect deserves further investigation, in particular by combining mutations that constitutively activate MAPKK [11] together with the T292/386A mutation.

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# Note added in proof

During the completion of this work, Saito et al. [FEBS Letters (1994) 341, 119] identified identical phosphorylation sites on MAP kinase kinase of PC12 cells following MAP kinase activation in response to NGF.