Volume 306, number 1, 17–22 FEBS 11257 July 1992

Ordered phosphorylation of p42^{mapk} by MAP kinase kinase

Timothy A. J. Haystead, Paul Dent, Jie Wu, Clare M.M. Haystead and Thomas W. Sturgill

Department of Pharmacology, Health Sciences Center, Box 448, University of Virginia, Charlottesville, VA 22908, USA

Received 7 May 1992; revised version received 22 May 1992

Preparation of milligram amounts of [^{32}P]p42^{mapk}, phosphorylated at Tyr^{185} or diphosphorylated at Tyr^{185}/Thr^{183}, for use as specific protein phosphatase substrates is described. Tyr- but not Thr-phosphorylated p42^{mapk}, accumulates when ATP is limiting. Furthermore, Tyr^{185}-phosphorylated p42^{mapk} exhibits an apparent 10-fold decrease in apparent K_m (46.6 ± 6.6 nM) for MAP kinase kinase compared to that for the dephospho form (~476 nM). We conclude that Tyr^{185} precedes Thr^{183} phosphorylation, and that this is prerequisite, dramatically increasing the affinity of p42^{mapk} for MAP kinase kinase.

Recombinant p42^{mapk}; Skeletal muscle; MAP kinase kinase

1. INTRODUCTION

Mitogen-activated protein kinase (MAP kinase) was originally identified as a protein serine/threonine kinase which is acutely activated in response to insulin and other growth factors whose receptors are tyrosine kinases [1]. The enzyme was subsequently shown to be identical to pp42, a protein which becomes phosphorylated on tyrosine residues in response to a variety of mitogenic agents [2]. Indeed, it has been established that activation of the enzyme is dependent upon dual phosphorylation on tyrosine and threonine residues [3]. Since the determination of its amino acid sequence, p42^{mapk} is now known to belong to a gene family which encodes several related protein kinases which can autophosphorylate on tyrosine residues [4–6]. Recently, several groups have described a protein factor that is also activated by these hormones and effectors and which specifically induces the phosphorylation of p42^{mapk} at the regulatory Tyr^{185} and Thr^{183} sites [7–9]. There is now a substantial body of evidence to indicate that this factor is a dual specificity protein kinase (a MAP kinase kinase) activated by protein phosphorylation in response to insulin and mitogens [7–9].

We are particularly interested in the mechanism by which p42^{mapk} is inactivated by protein phosphatases. In fully differentiated cells treated with insulin or growth factors p42^{mapk} is rapidly activated and then deactivated by one or more unidentified protein phosphatases. In vitro, it has been shown that dephosphorylation of Tyr^{185} by the protein tyrosine phosphatase, CD45, or dephosphorylation of Thr^{183} by the serine/threonine phosphatase PP- 2A, results in essentially complete inactivation of p42^{mapk} [10,11]. However, treatment of intact cells with the tumor promoter okadaic acid, a serine/threonine protein phosphatase inhibitor, results in a sustained and potent activation of the enzyme. This suggests that PP- 2A plays a key role in the attenuation of MAP kinase activity in vivo [11]. To characterize the protein phosphatases regulating p42^{mapk}, we have developed a unique procedure for the preparation of milligram quantities of the enzyme either exclusively phosphorylated at Tyr^{185}, or diphosphorylated on Tyr^{185} and Thr^{183}, using recombinant p42^{mapk} and a preparation of MAP kinase kinase from rabbit skeletal muscle. In the course of developing this procedure, we observed that MAP kinase kinase phosphorylates and activates p42^{mapk} in a highly ordered fashion. Phosphorylation of Tyr^{185} precedes that of Thr^{183}, increasing the affinity of p42^{mapk} for phosphorylation of the latter residue by MAP kinase kinase.

Abbreviations: MAP kinase, mitogen-activated protein kinase; RT, retention time; p42^{mapk}, 42 kDa MAP kinase; PP-2A, protein phosphatase 2A; PTPase, protein tyrosine phosphatase; MBP, myelin basic protein.

Correspondence address: T.A.J. Haystead, Department of Pharmacology, Health Sciences Center, Box 448, University of Virginia, Charlottesville, VA 22908, USA. Fax: (1) (804) 982 3878.

2. MATERIALS AND METHODS

2.1. *Purification of recombinant p42^{mapk}*

Approximately 10 mg of homogeneous p42^{mapk} was isolated from 6 l of cultured *E. coli* BL21(DE3)[pET-MK] following similar protocols to that described by Wu et al. [12] with some modifications. To prepare the enzyme for anion-exchange chromatography, the 50% ethylene glycol eluate from phenyl-Sepharose was bound to 20 ml of DE52 (Whatman) equilibrated in buffer A (50 mM Tris-HCl, pH 7.31, at 4°C, 1.5 mM EGTA, 1 mM dithiothreitol). The protein was step-eluted in a minimal volume (~40 ml) of buffer A containing 1 M NaCl. The eluate was dialysed overnight against 4 l of buffer A, and applied to a Protein-Pak Q AP-1 (Waters) anion-exchange column (1.0 × 10 cm) equilibrated in buffer A. The column was developed as indicated

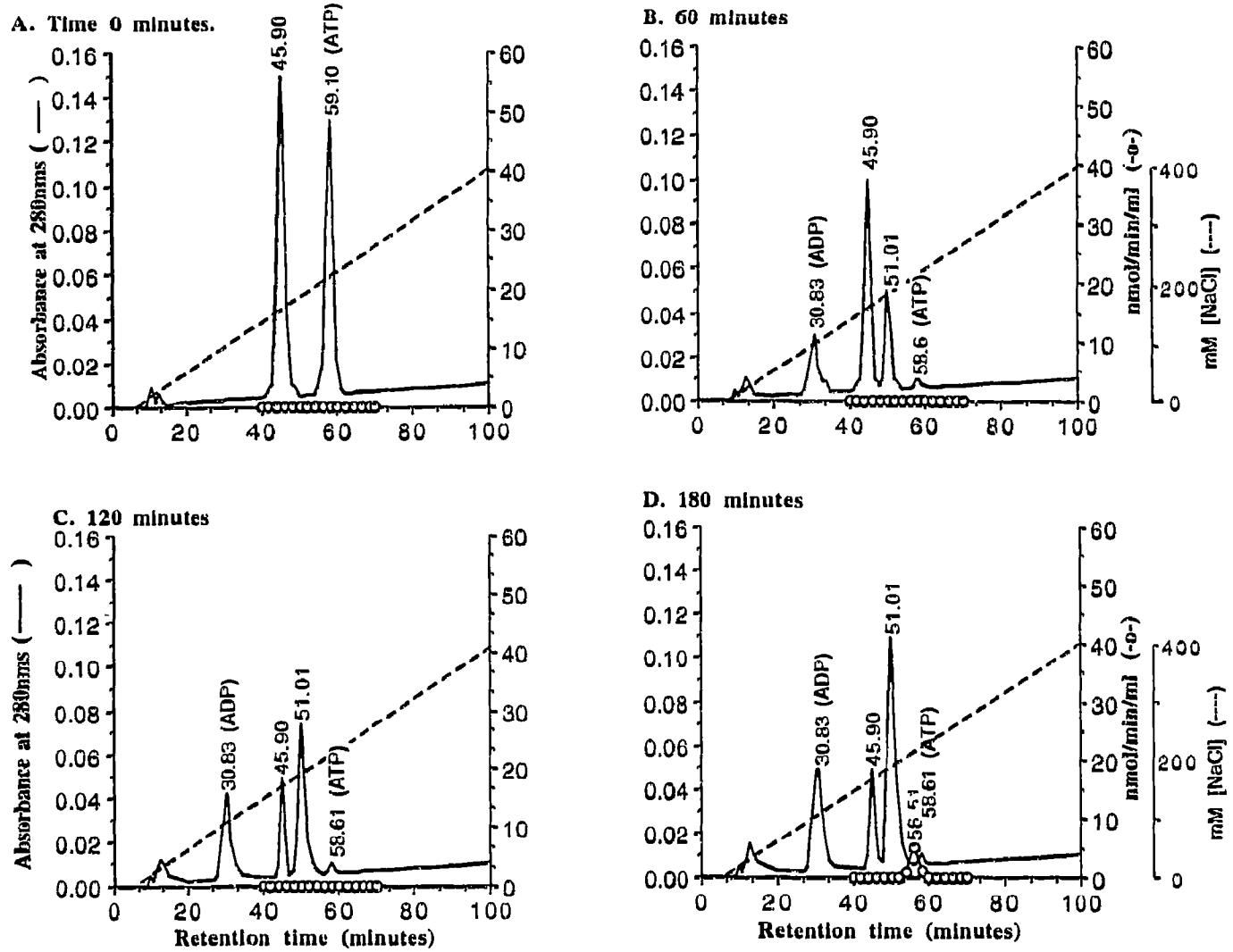

Fig. 1. Retention times of dephospho and Tyr[185] p42mapk on anion-exchange FPLC. 3.0 mg of pure $p42^{mapk}$ was incubated in buffer B (50 mM β-glycerophosphate, pH 7.31, 1.5 mM EGTA, 1 mM dithiothreitol, 0.15 mM sodium orthovanidate, 1 μM okadaic acid) with 500 U of MAP kinase kinase in the presence of 200 μM [γ-^{32}P]ATP (200 cpm/nmol)/5 mM $MgCl_2$. At each indicated time point the reaction mixture was supplemented with additional ATP and 0.1 of the reaction mixture was applied to the column. The column was developed with the indicated salt gradient (----) at a flow rate of 1.0 ml/min (2.0 ml fraction size); the absorbance profile (—) and MPB kinase activity (O) are indicated.

(see legends) and column fractions were assayed for $p42^{mapk}$ by their ability to be activated by MAP kinase kinase (see below for assay conditions). Anion-exchange chromatography resolved 2 significant peaks of $p42^{mapk}$ designated as Peak 1 and 2 (Rt ~40 and 56 min, respectively). Neither peak was active. The identity and purity (~90%) of the MAP kinase in Peaks 1 and 2 were confirmed by SDS-PAGE and Western blotting [12]. Although Peak 2 could be fully activated by the MAP kinase kinase, $p42^{mapk}$ in this fraction is apparently stoichiometrically phosphorylated on a tyrosine residue at a site distinct from that phosphorylated by either the kinase kinase or by MAP kinase autophosphorylation (unpublished results). Subsequent purification steps were only carried out on Peak 1. Using phenyl-Superose (Pharmacia) chromatography [12], the unphosphorylated B form of $p42^{mapk}$ was isolated and used herein. The isolated protein had no detectable activity towards MBP as determined below, but could be fully activated with rabbit muscle MAP kinase kinase.

2.2. *Preparation and assay of MAP kinase kinase*

MAP kinase kinase was partially purified from skeletal muscle using an abbreviated procedure similar to that described by Nakielny et al. [8]. MAP kinase kinase was assayed by its ability to specifically phosphorylate and activate recombinant $p42^{mapk}$. No other protein kinases phosphorylating MBP or $p42^{mapk}$ were detectable in the MAP kinase kinase preparation. Assessment of activation of $p42^{mapk}$ by MAP kinase kinase was carried out in two stages; incubation with ATP/$MgCl_2$ in the presence or absence of the MAP kinase kinase, followed immediately by rapid assay of MBP kinase activity. Activation was carried out for 10 min in 15 μl (total volume) containing 0.1 U of MAP kinase kinase, diluted $p42^{mapk}$ fraction, 1.0 mM ATP/25 mM $MgCl_2$, 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM dithiothreitol. 1 U of MAP kinase kinase is defined as the amount of enzyme that would activate dephospho-recombinant $p42^{mapk}$, resulting in the phosphorylation of MBP at a rate of 1 nmol/min. Immediately following the pre-incubation assay, 5 μl of the reaction mixture was removed and added to 20 μl of MBP kinase assay mixture containing [γ-^{32}P]ATP (200 cpm/nmol). MBP kinase activity was then measured for 5 min using conditions described previously [11].

2.3. *Absorbancy of $p42^{mapk}$*

E_{280} (1%) for $p42^{mapk}$ in 50 mM Tris-HCl, pH 7.3, at 25°C was

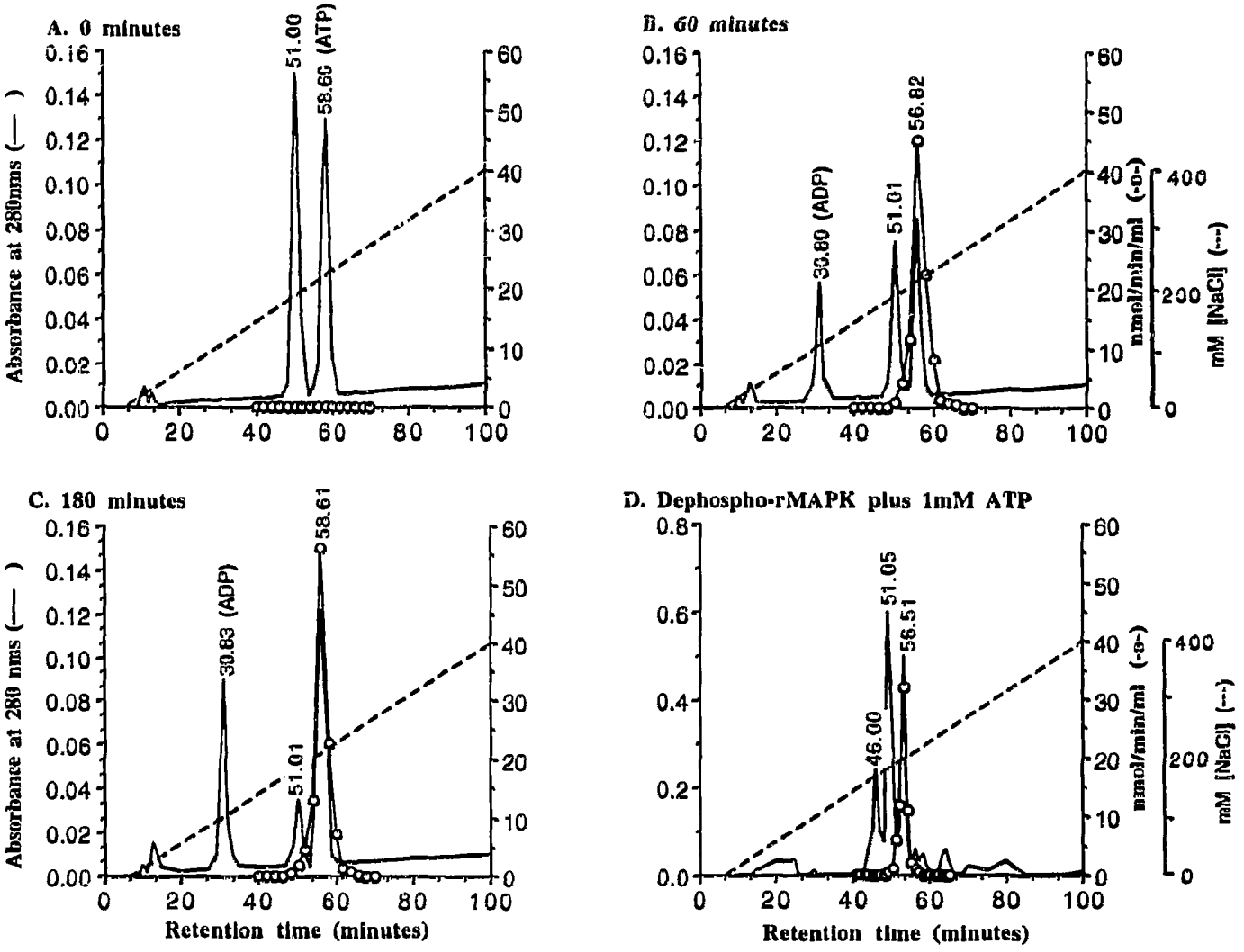

Fig. 2. Retention times of the diphospho (Tyr^{185} and Thr^{183}) $p42^{mapk}$ in relation to Tyr^{185} $p42^{mapk}$ on anion-exchange FPLC. 3.0 mg of Tyr^{185} $p42^{mapk}$ was isolated as described above and incubated in buffer B with 500 U of MAP kinase kinase in the presence of 200 μM [γ-^{32}P]ATP (200 cpm/nmol)/5 mM $MgCl_2$. At the indicated time points, the reaction was supplemented with additional ATP and 0.3 mg applied to the anion-exchange column. The column was developed as described in Fig. 1 and the absorbance profile assayed for MBP kinase activity. In Fig. 2D the enzyme reaction mixture was dialysed against buffer A to remove the ATP prior to application to the column.

determined to be 7.60. The value predicted from the amino acid composition is 7.42. In the kinetic studies, the concentration of $p42^{mapk}$ was determined by its extinction at 280 nm.

3. RESULTS

3.1. *Generation of Tyr^{185} phosphorylated $p42^{mapk}$*

Dephospho $p42^{mapk}$ eluted with a retention time (Rt) of 45.90 min, and was well resolved from unreacted ATP (Rt, 59.01 min) (Fig. 1A). After 60 min of incubation with MAP kinase kinase and 200 μM ATP, approximately 25% of the $p42^{mapk}$ eluted 5 min later (Fig. 1B). The reaction rapidly exhausted the available ATP, as indicated by the disappearance of the ATP peak eluting at 59.01 min and the appearance of an ADP peak at 30.83 min. A control autophosphorylation reaction was also analyzed in which MAP kinase kinase was excluded from the reaction mixture. No $p42^{mapk}$ was detected eluting at 51.01 min in the control, even after 4 h, indicating that generation of the Rt 59.01 peak required MAP kinase kinase (not shown). To confirm that the reaction containing MAP kinase kinase was exhausting its ATP, additional ATP (200 μM, final) was added and the products analyzed after an additional 60 min of incubation (Fig. 1C). This resulted in a quantitative increase in the amount of both $p42^{mapk}$ recovered at 51.01 min and ADP recovered at 30.83 min. By 180 min, following a third addition of ATP, ~75% of the $p42^{mapk}$ had been converted to the form of $p42^{mapk}$ eluting at 51.01 min (Fig. 1D).

Significantly, when assayed for MBP kinase activity none of the major peaks of absorbance contained any

detectable active enzyme. A small peak of activity representing diphospho p42^{mapk} (see below) was detected eluting at 56.51 min after 180 min of incubation with MAP kinase kinase (Fig. 1D). However, this represented less than 1% of the total p42^{mapk} present in the incubation.

The experiment was repeated using [γ-^{32}P]ATP (200 cpm/nmol) to identify the generated peaks as specific phosphorylated forms of p42^{mapk} by phosphoamino acid analysis. Analysis of the various peaks by SDS-PAGE and auto-radiography following 180 min of incubation, revealed that the p42^{mapk} eluting at 51.01 contained >95% of the total radioactivity in the profile (data not shown). Phosphoamino acid analysis of the protein revealed that it was exclusively phosphorylated on tyrosine (Fig. 3A). Determination of the extent of tyrosine phosphorylation revealed near stoichiometric phosphorylation (~0.91 ± 0.05 mol/mol, n=3). Furthermore, two-dimensional peptide analysis revealed a single phosphorylated peptide (Fig. 3B), corresponding to Tyr^{185}-phosphorylated peptide [10,12].

We conclude that under conditions where ATP is limiting, Tyr^{185}-phosphorylated p42^{mapk} accumulates as a reaction intermediate, suggesting an ordered phosphorylation. Under these conditions, one generates little or none of the diphosphorylated (tyrosine and threonine) active p42^{mapk} as discussed below.

3.2. *Generation of diphosphorylated (Tyr^{185} and Thr^{183}) p42^{mapk}*

To produce diphospho p42^{mapk}, 6.0 mg of the dephospho enzyme was first converted to the Tyr^{185}-phosphorylated form and then purified by anion-exchange chromatography, as described above. Tyr^{185}-phosphorylated p42^{mapk} (3.0 mg) was incubated with MAP kinase kinase and ATP/$MgCl_2$ and compared with enzyme incubated with ATP/$MgCl_2$ alone (control) by anion-exchange chromatography (Fig. 2). As above, Tyr^{185}-phosphorylated p42^{mapk} and ATP eluted at 51.01 min and 58.60 min, respectively (Fig. 2A). Incubation of the Tyr^{185}-phosphorylated form with MAP kinase kinase produced a new form of p42^{mapk} eluting at 56.61 min (Fig. 2B). The proportion of enzyme eluting at this time was directly dependent upon the amount of ATP present and could be increased by further 200 μM additions of ATP (Fig. 2C). Profiles of control reactions did not significantly change for up to 4 h of incubation at 30°C. Correspondingly, as the amount of p42^{mapk} increased at 56.51 min, Tyr^{185}-phosphorylated p42^{mapk} eluting at 51.0 min decreased. The shift in Rt is consistent with phosphorylation of Tyr^{185} p42^{mapk} to the diphospho form by MAP kinase kinase. This was confirmed by phosphoamino acid analysis which demonstrated that p42^{mapk} eluting at 56.51 min contained both phosphotyrosine and phosphothreonine in equal proportions (Fig. 3C). Furthermore, phosphorylation occurred to a stoichiometry of nearly 2 mol/mol (1.85 ± 0.12 mol/mol, n=3) (Fig. 3C), generating a single tryptic phosphopeptide corresponding to Tyr^{185}/Thr^{183}-phosphorylated peptide (Fig. 3D) [10,12].

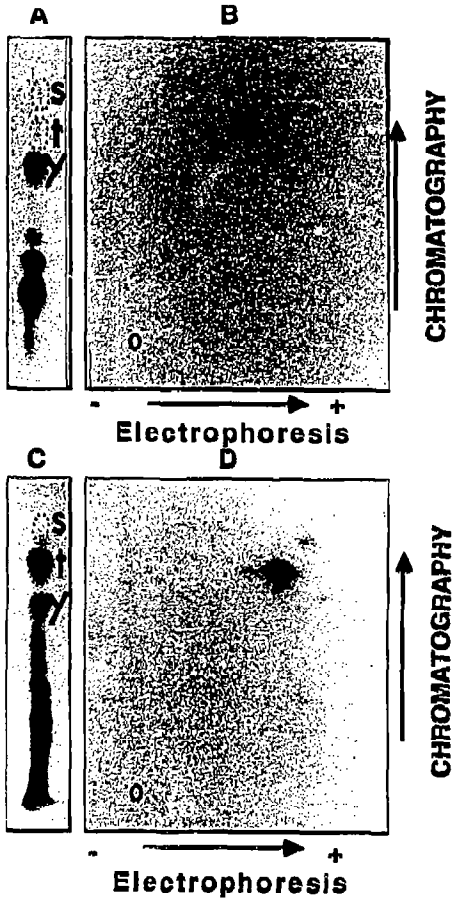

Fig. 3. Autoradiograms of phosphoamino acid and 2D tryptic phospho-peptide analysis of Tyr^{185}-phospho p42^{mapk} and Tyr^{185}/Thr^{183}-phospho p42^{mapk}. In Fig. 3A and C, 20 μl fractions from peaks of absorbance eluting at 51.01 min (Fig. 1) and 56.82 min (Fig. 2) were subjected to SDS-PAGE and Western blotting. Autoradiography of the blots revealed a single ^{32}P-labelled polypeptide of 42 kDa; this was excised and phosphoamino acid analysis carried out in the presence of phosphotyrosine, serine and threonine markers as described by Wu et al. [12]. Fig. 3B and D shows autoradiograms of 2D phosphopeptide maps following tryptic digestion of 20 μl of TCA-precipitated (25% v/v) p42^{mapk} eluting at 51.01 and 56.82 min. Conditions for 2D mapping were described previously [12].

Assay of fractions for MBP kinase activity revealed a single peak of activity which co-eluted with diphosphorylated p42^{mapk} (Fig. 2B,C). Activated p42^{mapk} displayed a specific activity toward MBP of 0.18 ± 0.086 μmol/min/mg (n=3), approximating values estimated for the native enzyme from mammalian sources [4].

In the presence of MAP kinase kinase and millimolar concentrations of ATP and $MgCl_2$, both Tyr^{185} and diphospho p42^{mapk} (Tyr^{185}/Thr^{183}) are generated in the same incubation mixture (Fig. 2D). Based on findings presented above, generation of active p42^{mapk} at high ATP is a reflection of the ordered appearance of Tyr^{185}-

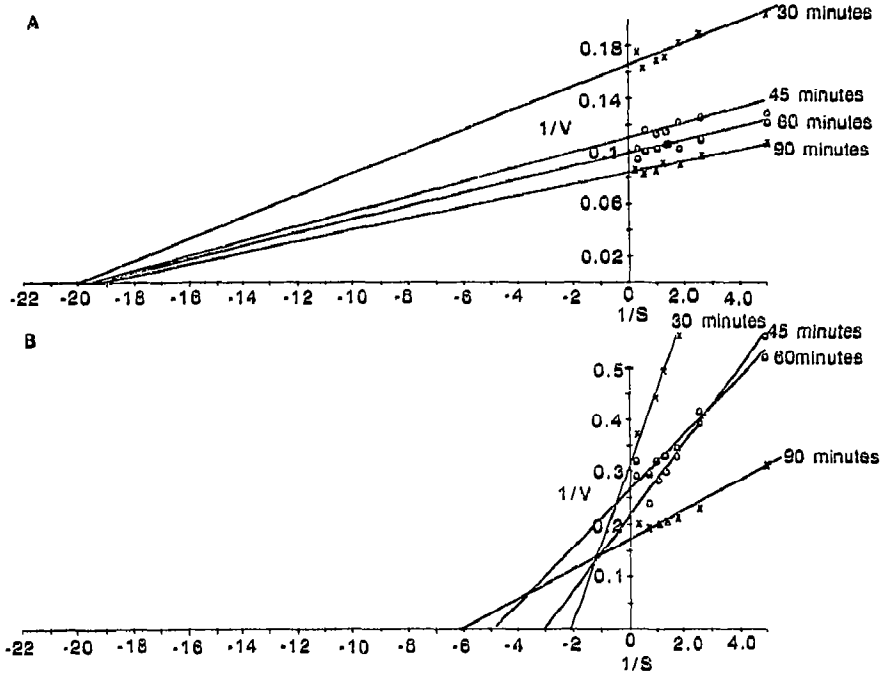

Fig. 4. Kinetic analysis of the activation of dephospho and Tyr[185]-phosphorylated p42[mapk] by MAP kinase kinase. Tyr[185]-phosphorylated p42[mapk] and dephospho p42[mapk] kinase were isolated as described in section 2. Varying concentrations of these forms of p42[mapk] were incubated in buffer B for the indicated lengths of time with 2.5 U of MAP kinase kinase and 1 mM ATP, 25 mM $MgCl_2$. 1/S is in μM, 1/V is in nmol/min/ml. (Results shown are from a single experiment, although this was repeated on several other occasions.)

phosphorylated p42[mapk] which then becomes a substrate for threonyl phosphorylation by MAP kinase kinase.

3.3. *Kinetic analysis of dephospho and Tyr[185]-phosphorylated p42[mapk]*

The relative abilities of Tyr[185]-phospho and dephospho p42[mapk] to act as substrates for MAP kinase kinase were assessed by kinetic studies (Fig. 4). The apparent K_m for activation was measured for each substrate by varying its concentration in the first incubation. Significantly, MAP kinase kinase exhibited a 10-fold increase in apparent affinity for Tyr[185] phospho p42[mapk] (K_m = 46.6 + 6.6 nM S.D.M. n = 5) in comparison to the dephospho enzyme (K_m = 476 nM) (Fig. 4A,B). Moreover, the apparent K_m for dephospho p42[mapk] progressively decreased with time of the first incubation. Note that at 30 min the apparent K_m for Tyr[185]-phosphorylated p42[mapk] was ~50 nM, whereas that for the dephosphorylated form was ~476 nM. However, after 90 min of incubation the K_m for dephospho p42[mapk] was reduced to ~166 nM. In correlation with the decrease in apparent K_m for dephospho p42[mapk], the V_{max} for MBP kinase activity increased (Fig. 4B).

4. DISCUSSION

Experiments designed to prepare p42[mapk] as a substrate for protein phosphatases inadvertently revealed important details of its mechanism of activation by MAP kinase kinase. Tyr[185]-phosphorylated p42[mapk] accumulates as a reaction intermediate under conditions of limiting ATP and has a higher affinity for MAP kinase kinase than dephospho p42[mapk]. Taken together, these findings suggest that the activation of p42[mapk] is highly ordered. p42[mapk] appears not to be a substrate for threonine phosphorylation by MAP kinase kinase until the enzyme has been phosphorylated at Tyr[185].

Ordered phosphorylation may occur as a consequence of two established mechanisms. Firstly, phosphorylation of Tyr[185] may provide a recognition determinant necessary for the phosphorylation of Thr[183]. This paradigm, as defined by Roach et al. [13] readily explains ordered phosphorylation. For example, glycogen synthase, the regulatory subunit (RII) of cyclic A kinase and the inhibitor 2 molecule of protein phosphatase 1 only become substrates for glycogen synthase kinase 3 after they have been phosphorylated by casein kinase 2 at distinct sites. Secondly, phosphorylation at Tyr[185] may induce a conformational change in p42[mapk] which exposes Thr[183], enabling it to be phosphorylated by MAP kinase kinase. This hypothesis is supported by the observation that phosphorylation of p42[mapk] on Tyr[185] induces dramatic changes in the mobility of the enzyme on phenyl-Superose (J. Wu, unpublished data). Both dephospho and diphosphorylated p42[mapk] strongly bind to the resin, and are eluted at ~37% ethylene glycol. In contrast, Tyr[185]-phospho p42[mapk] is weakly re-

tained and elutes in the wash fractions. Although phosphorylation of Tyr^{185} would be predicted to lower the hydrophobicity of the protein, a conformational change is more likely to account for this behaviour because the diphosphorylated form is as hydrophobic as the dephospho form.

Autophosphorylation is unlikely to contribute significantly during the in vitro activation of $p42^{mapk}$. The recombinant enzyme undergoes intramolecular autophosphorylation on Tyr^{185}, but not Thr^{183}, in the absence of MAP kinase kinase [5,12]. This autophosphorylation reaction requires hours to achieve significant stoichiometry (10–20%). Furthermore, MAP kinase kinase phosphorylates a kinase-defective $p42^{mapk}$ on both regulatory sites, excluding autophosphorylation as a requirement [8,16,17] for activation by MAP kinase kinase. The results do not preclude the existence of an unidentified autophosphorylation-enhancing factor.

Although these studies have provided details of the activation mechanism, the goal was to obtain $p42^{mapk}$ specifically phosphorylated as a substrate for protein phosphatases. The ability of okadaic acid to cause a sustained activation of $p42^{mapk}$ is paradoxical because the enzyme can still be potentially inactivated by tyrosine phosphatases in vivo. The question remains as to how okadaic acid prevents Tyr^{185} dephosphorylation of $p42^{mapk}$ in vivo. Dephosphorylation of $p42^{mapk}$ may be ordered with Thr^{183} preceding Tyr^{185}. This mechanism allows for a priming effect for rephosphorylation for rapid activation of $p42^{mapk}$ by the activator. Indeed, we have observed a large and sustained activation of MAP kinase kinase after treatment of intact cells with okadaic acid (T.A.J. Haystead and P. Dent, unpublished results). Alternatively, okadaic acid could act indirectly to inhibit Tyr^{185} dephosphorylation by a specific $p42^{mapk}$ PTPase. A prerequisite for addressing these questions is identification of the protein phosphatases which inactivate $p42^{mapk}$ in vivo.

Acknowledgements: This work was supported by monies from the Lucille P. Markey Foundation (to T.A.J.H.) and the American Cancer Society (to T.A.J.H.) and BE69D (to T.W.S.). P.D. is supported by an American Cancer Society Grant (BE69D). Thank you to Rod Biltonen for helpful discussions.

REFERENCES

[1] Sturgill, T.W. and Wu, J. (1991) Biochem. Biophys. Acta 1092, 350–357.
[2] Rossomando, A.J., Payne, D.M., Weber, M.J. and Sturgill, T.W. (1989) Proc. Natl. Acad. Sci. USA 86, 6940–6943.
[3] Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J., Shabanowitz, J., Hunt, D.F., Weber, M.J. and Sturgill, T.W. (1991) EMBO J. 10, 885–892.
[4] Boulton, T.G., Yanacopoulos, G.D., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M. (1990) Science 249, 64–67.
[5] Segar, R., Ahn, N.G., Boulton, T.G., Yancopoulos, G.D., Panayotatos, E.R., Radziejewska, E., Ericson, L., Bratlien, R.L., Cobb, M.H. and Krebs, E.G. (1991) Proc. Acad. Natl. Sci. USA 88, 6142–6146.
[6] Lindberg, R., Quinn, A. and Hunter, T. (1992) Trends Biochem. Sci. 17, 114–119.
[7] Ahn, N.G., Segar, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K. and Krebs, E.G. (1991) J. Biol. Chem. 266, 4220–4227.
[8] Nakielny, S., Cohen, P., Wu, J. and Sturgill, T. (1992) EMBO J. 11, in press.
[9] Matsuda, S., Kosaka, H., Takenaka, K., Moriyama, K., Sakai, H., Akiyama, T., Gotoh, Y. and Nishida, E. (1992) EMBO J. 11, 973–982.
[10] Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Nature 343, 651–653.
[11] Haystead, T.A.J., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H. and Krebs, E.G. (1990) J. Biol. Chem. 265, 16571–16580.
[12] Wu, J., Rossomando, A., Her, J.H., Del Vecchio, R., Weber, M. and Sturgill, T. (1991) Proc. Natl. Acad. Sci. USA 88, 9508–9512.
[13] DePaoli Roach, A.A. (1984) J. Biol. Chem. 259, 12144–12152.
[14] Posada, J., Sanghera, J., Pellech, S., Aerbersold and Cooper, J.A. (1991) Mol. Cell Biol. 11, 2517–2528.
[15] Gomez, N. and Cohen, P. (1991) Nature 353, 170–175.
[16] Rossomando, A., Wu, J., Weber, M.J. and Sturgill, T.W. (1992) Proc. Natl. Acad. Sci. USA 89, in press.
[17] L'Allemain, G., Her, J.-H., Wu, J., Sturgill, T.W. and Weber, M.J. (1992) Mol. Cell Biol. 12, 2222–2229.