Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation

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p42^{mapk} [mitogen activated protein (MAP) kinase; extracellular signal-regulated protein kinase (ERK)] is a serine/threonine-specific protein kinase that is activated by dual tyrosine and threonine phosphorylation in response to diverse agonists. Both the tyrosine and threonine phosphorylations are necessary for full enzymic activity. A MAP kinase activator recently purified and cloned has been shown to be a protein kinase (MAP kinase kinase) that is able to induce the dual phosphorylation of MAP kinase on both the regulatory tyrosine and threonine sites *in vitro*. In the present paper we have utilized MAP kinase mutants

altered in the sites of regulatory phosphorylation to show, both *in vivo* and *in vitro*, that phosphorylation of the tyrosine and the threonine can occur independently of one another, with no required order of phosphorylation. We also utilized kinase-defective variants of MAP kinase with mutations in either the ATP-binding loop or the catalytic loop, and obtained data suggesting that the activity or structure of the catalytic loop of MAP kinase plays an important role in its own dual phosphorylation.

INTRODUCTION

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that are regulated by dual phosphorylation on tyrosine and threonine residues (Ray and Sturgill, 1988). Because dual phosphorylation is required for enzymic activation, it was initially suggested that MAP kinases may play a role in the integration of signals from distinct tyrosine- and serine/threonine-specific protein kinases (Anderson et al., 1990).

The MAP kinases are activated during G₀ transitions in cells stimulated to divide (Ahn et al., 1991; Posada et al., 1991; Kahan et al., 1992; L'Allemain et al., 1992), to differentiate (Gotoh et al., 1990; Casillas et al., 1991) or to express certain differentiated functions (Ely et al., 1990; Casillas et al., 1991), and they are also activated following stimulation of oocyte maturation (Ferrell et al., 1991; Posada et al., 1991). Several members of the MAP kinase protein family have been identified, including p42^{mapk} (extracellular signal-regulated protein kinase; ERK2) (Her et al., 1991), p44^{mapk} (ERK1) (Boulton et al., 1990) and a 54 kDa MAP-kinase-related protein (Kyriakis and Avruch, 1990), as well as a possible splice variant of p42^{mapk} (Gonzalez et al., 1992). It is not yet known how or if these isoforms differ in function or regulation.

The mechanisms of activation of MAP kinases have been the subject of intense recent investigation. p42^{mapk} has been shown to be capable of a slow autophosphorylation on the site of regulatory tyrosine phosphorylation (Tyr-185 in p42^{mapk}), raising the possibility that there might exist in cells factors which could regulate this autophosphorylation activity ('autokinase enhancing factors') (Wu et al., 1991; Seger et al., 1991; Ahn et al., 1991; Robbins and Cobb, 1992). Evidence has also been presented that the tyrosine kinase p56^{lck} can directly phosphorylate this site (Ettehadieh et al., 1992). However, most recent work has concentrated on the identification (Gomez and Cohen, 1991; Adams and Parker, 1992; Alessandrini et al., 1992; Shirakabe et

al., 1992; Kosako et al., 1992), purification (Crews and Erikson, 1992; Seger et al., 1992; Matsuda et al., 1992; Wu et al., 1992) and cloning (Crews et al., 1992; Seger et al., 1992; Ashworth et al., 1992; Wu et al., 1993) of MAP kinase kinases (MAPKKs; mek1). MAPKKs appear to be dual-specificity protein kinases, capable of phosphorylating both the regulatory threonine and tyrosine residues, and hence enzymically activating p42^{mapk} and p44^{mapk} *in vitro*.

Dual-specificity kinases are a recently described class of protein kinases. In the original classification by Hanks et al. (1988), protein kinases fell into either the serine/threonine-specific class or the tyrosine-specific class, and could be distinguished on the basis of primary amino acid sequence motifs in kinase subdomains VI and VIII. Screening of cDNA expression libraries with anti-phosphotyrosine antibodies led to the discovery of protein kinases which could autophosphorylate on tyrosine, but which functioned as serine/threonine-specific protein kinases on exogenous substrates. Such so-called dual-specificity kinases include Clk, Mik1 and Spk1 (Lindberg et al., 1992). It is not at all clear whether these kinases truly are dual specificity for exogenous substrates and whether their tyrosine autokinase activity has biological significance. MAP kinase is a dual-specificity kinase in this sense. The cell cycle regulator weel has been suggested to be a dual-specificity protein kinase because its mammalian substrate, cdc2, is (similarly to MAP kinase) regulated by dual phosphorylation on adjacent tyrosine and threonine residues. Weel can autophosphorylate on serine, threonine and tyrosine, but recently it has been shown that a human weel can phosphorylate cdc2 only on tyrosine (Parker and Piwnicka-Worms, 1992). Thus MAPKK may be the only enzyme which appears to function both *in vivo* and *in vitro* as a dual-specificity kinase on an exogenous substrate.

Because the *in vitro* enzymic properties of MAPKK are so unusual, we felt it was important to analyse this apparent dual specificity in detail. Using MAP kinase mutants altered in the

Abbreviations used: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MAPKK, MAP kinase kinase; DMEM, Dulbecco's modified Eagle's medium; MBP, myelin basic protein.

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sites of regulatory phosphorylation, we found that phosphorylation of MAP kinase on tyrosine and threonine residues does not require a specific order of phosphorylation. This result was obtained *in vitro* with purified enzymes and *in vivo* in cells expressing the mutant MAP kinases, suggesting that the MAPKK which has been purified and cloned can account for the *in vivo* activation of MAP kinase. In addition, we have examined the possible role of MAP kinase autophosphorylation in the dual specificity of MAPKK, and present evidence suggesting that the ability of MAP kinase to catalyse phosphate transfer plays a role in its threonine phosphorylation by MAPKK.

MATERIALS AND METHODS

Materials

The oligonucleotide encoding the epitope was purchased from Operon (Alameda, CA, U.S.A.). The nucleotide sequence is 5'-TTGGATCCTACCCATATGATGTTCCAGATTACGCTGGATCCATGGCC-3'. Dulbecco's modified Eagle's medium (DMEM) and G418 neomycin were obtained from Gibco, and [32 P] P_i and [γ - 32 P]ATP (6000 Ci/mmol) were from Du Pont-New England Nuclear (Boston, MA, U.S.A.).

Epitope tagging of MAP kinases and expression

Epitope tagging was done by insertion of an oligonucleotide encoding a peptide GLGSPYDVPDYAGSM containing an epitope (shown in **bold**) described before (Field et al., 1988) at the N-terminal side of Gly-8 of wild-type and mutant MAP kinases (T183A, Y185F, T183A/Y185F, T183F, T183E, Y185E, T183E/Y185E and K52R, where the first letter indicates the amino acid at the residue number indicated in the wild-type protein, and the second letter indicates the amino acid present at that position in the mutant protein). For expression in CCL39 hamster fibroblasts, the tagged MAP kinases were subcloned into expression vector pLNCAL7 under the control of the cytomegalovirus promoter. Transfections were performed using Lipofectin (BRL Life Technologies, Inc., Gaithersburg, MD, U.S.A.) and 10 μ g of DNAs according to the protocol provided by the company. Cells expressing the tagged MAP kinase proteins were selected by growth in DMEM supplemented with 5% calf serum plus 5% fetal calf serum containing 500 μ g/ml neomycin. G418-resistant clones were screened for expression of the tagged proteins by Western blotting of cell lysates from each clone with culture supernatant containing monoclonal antibody 12CA5, which is specific for the epitope. Clones with the highest expression levels of each tagged mutant were used for the *in vivo* 32 P labelling experiment described.

In vivo 32 P labelling of MAP kinase

For *in vivo* 32 P labelling, cells were grown in 60-mm plates to 90% confluence in selection medium before starting labelling. To start labelling, cells were depleted of serum by replacing the medium with 2 ml of phosphate-free DMEM supplemented with 5% normal serum-free medium, and were labelled by adding [32 P] P_i (2.5 mCi/ml) to each plate at the same time. After 6 h, cells were stimulated with 10% fetal calf serum for 10 min, washed twice with ice-cold phosphate-buffered saline, lysed in 0.4 ml Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 8.0, 1 mM phenylmethanesulphonyl fluoride, 2 mM sodium vanadate, 10 mM sodium pyrophosphate,

0.4 mM EDTA, 10 mM NaF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 10 mM *N*-nitrophenyl phosphate) and the lysates were cleared by centrifugation at 14000 rev./min for 10 min.

Immunoprecipitation of the tagged proteins

The tagged proteins from 0.4 ml of the cleared lysates were immunoprecipitated with 10 μ l of monoclonal antibody 12CA5 ascites (BABCO, Berkeley, CA, U.S.A.) at 4 °C for 2 h on a rotating shaker. A 200 μ g portion of rabbit anti-mouse IgG (Jackson Laboratories, Bar Harbor, ME, U.S.A.) preincubated with 100 μ l of 50% Protein A-Sepharose beads (Pharmacia, Piscataway, NJ, U.S.A.) was added to each immunoprecipitation reaction at the same time. The immunocomplexes were washed twice with 0.8 ml of the lysis buffer and twice with 0.8 ml of Tris-buffered saline, resuspended in 60 μ l of 2 $\times$ electrophoresis sample buffer and boiled for 5 min. The immunoprecipitated proteins were resolved by SDS/PAGE, transferred to Immobilon (Millipore, Bedford, MA, U.S.A.) and exposed to film. Anti-phosphotyrosine Western blotting was done essentially as described (L'Allemand et al., 1992). After the primary antibody reaction, the signal was detected using horseradish peroxidase-linked goat anti-rabbit IgG and developed with enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL, U.S.A.). For anti-epitope blotting, the filter was stripped, blocked in phosphate-buffered saline containing 5% dry milk and reprobed with the 12CA5 culture supernatant. Horseradish peroxidase-linked sheep anti-mouse IgG was used to detect the signals. Blotting of MAP kinase was performed with the monoclonal antibody 1B3B9, raised in this laboratory against p42 MAP kinase, and sold by Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Immunocomplex MBP kinase assay

For each reaction, 20 μ l of rabbit anti-(MAP kinase) antiserum (TR2) was preincubated with 200 μ l of 50% Protein A-Sepharose beads for 1 h on ice. The beads were washed once with Tris-buffered saline then added to 0.4 ml of whole-cell lysate at 4 °C for 2 h. The immunocomplexes were washed twice in the lysis buffer, twice in Tris-buffered saline and once in a final wash buffer (20 mM Hepes, pH 7.8, 10 mM magnesium acetate). The beads were resuspended in 100 μ l of the final wash buffer and a 30 μ l portion was used for the assay. The kinase reaction was performed as described before (Wu et al., 1991).

Purification of MAPKK

MAPKK was purified essentially as described in Wu et al. (1992). Rabbit muscle (500 g) was homogenized in buffer containing 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM benzamide, 0.1% β -mercaptoethanol and 1 mM phenylmethanesulphonyl fluoride at pH 7.0. The homogenate was centrifuged and the supernatant was subjected to batch adsorption in 250 ml of DE52 equilibrated in homogenization buffer containing 25 mM Tris, pH 7.0, at 4 °C. The flow-through was collected, adjusted to pH 8.8 and applied to Fast Flow Q equilibrated with the same homogenization buffer at pH 8.8. The resin was washed with 25 mM NaCl plus the homogenization buffer and eluted with 300 mM NaCl in the homogenization buffer. The NaCl concentration of the eluate was brought up to 1 M and it was adsorbed to phenyl-Sepharose. The phenyl-Sepharose was washed with the same buffer without NaCl and eluted with 60% ethylene glycol. The eluate was then diluted and applied to Mono

Q at pH 8.8. The fractions containing MAPKK activity were pooled, dialysed and further purified sequentially by a second Mono Q column at pH 7.5, gel filtration, and finally through Superose 12.

***In vitro* MAPKK reaction**

A 3 μ g sample of the purified recombinant MAP kinase proteins was used in each reaction. The reactions were started by adding 25 μ l of $1.6 \times$ 'hot ATP mix' [final concentrations: 10 mM magnesium acetate, 1 mM dithiothreitol, 50 mM β -glycerol phosphate, 1 mM EDTA, 85 μ M sodium vanadate, 100 μ M ATP (25 c.p.m./fmol) and 6 μ l of the MAPKK purified from rabbit muscle cells (Wu et al., 1992)] to the recombinant substrates at 30 °C for 1.5 h. To stop the reactions, 40 μ l of $2 \times$ electrophoresis sample buffer was added to each tube and the mixtures were boiled for 5 min. Samples of 75 μ l were loaded on to a 10% polyacrylamide gel. Phosphorylated proteins were transferred to Immobilon and exposed to Kodak X-AR film for 45 min. The bands corresponding to the MAP kinase proteins were excised from the filter for phosphoamino acid analysis (L'Allemain et al., 1992).

Autophosphorylation of the kinase-defective mutants

Equal amounts of the recombinant K52R and D147A proteins (based on Bradford protein assays and staining of acrylamide gels) were used for the autokinase reactions. The specific radioactivity of ATP was constant (40 c.p.m./fmol) in every reaction, with concentrations of unlabelled ATP of 10 μ M, 50 μ M, 100 μ M, 1.0 mM or 1.7 mM in a final volume of 45 μ l. Reactions were performed at 37 °C for 1.5 h (which is within the linear range), and were stopped by addition of 12.5 μ l of $4 \times$ electrophoresis sample buffer followed by boiling for 5 min.

RESULTS

***In vivo* phosphorylation of MAP kinase**

Phosphorylation on threonine-183 and tyrosine-185 are both necessary and sufficient to activate p42 MAP kinase *in vivo* (Ray and Sturgill, 1988; Anderson et al., 1990; Payne et al., 1991) and *in vitro* (Kosako et al., 1992; L'Allemain et al., 1992; Nakielnny et al., 1992; Wu et al., 1992). To determine whether the regulatory phosphorylation of these residues occurs independently or interdependently, a cDNA clone (Her et al., 1991) of murine p42 MAP kinase was mutagenized (L'Allemain et al., 1992) to alter the sites of regulatory phosphorylation either singly (T183E and Y185E) or in combination (TE/YE) to a charged non-phosphorylatable amino acid. In addition, a kinase-defective mutant was generated in which Lys-52, which is a conserved lysine in the ATP-binding loop (Knighton et al., 1991) and is believed to interact with the α -phosphate of ATP (Zheng et al., 1993), was changed to Arg (K52R). The wild-type and mutant proteins were expressed in hamster fibroblasts under the control of the cytomegalovirus promoter.

To separate the exogenous mutants from endogenous MAP kinase, an epitope derived from influenza virus haemagglutinin (Field et al., 1988) was inserted into the N-terminus of each mutant and a monoclonal antibody (12CA5) raised against the epitope was used for immunoprecipitating the tagged proteins.

Phosphorylation of MAP kinase mutants *in vivo* is shown in Figure 1(a). Cells expressing the tagged MAP kinases were serum-depleted, labelled with [32 P]P, and stimulated with fetal calf serum at the end of labelling. The tagged proteins were then immunoprecipitated with 12CA5, resolved by SDS/PAGE, transferred to Immobilon and exposed to film. Wild-type MAP kinase and the kinase-defective K52R mutant, as well as the two single-phosphorylation-site mutants T183E and Y185E, became detectably phosphorylated in response to serum. However, the double-phosphorylation-site mutant TE/YE did not become phosphorylated in response to serum, demonstrating that MAP kinase undergoes little if any phosphorylation at sites other than those originally described (Payne et al., 1991). Phosphorylation of the kinase-defective K52R mutant suggests the involvement of an upstream MAPKK in activation of p42 MAP kinase *in vivo*, as has been shown *in vitro* (Alessandrini et al., 1992; Kosako et al., 1992; Nakielnny et al., 1992; Wu et al., 1993). Phosphorylation of both the T183E and Y185E mutants supports the hypothesis that the regulatory phosphorylation events can occur independently from each other. Comparable results have been obtained using MAP kinase mutants in which Thr-183 and/or Tyr-185 were replaced with the cognate non-phosphorylatable residues Ala or Phe (results not shown). Thus a negative charge at these sites is not necessary for these independent phosphorylations.

To determine whether Tyr-185 is indeed the only tyrosine residue involved in MAP kinase activation, the proteins blotted on to Immobilon were probed with an anti-phosphotyrosine antibody (Figure 1b). The data demonstrate that mutation of Tyr-185 eliminates reactivity with an antibody against phosphotyrosine; only wild-type MAP kinase and mutants K52R and T183E reacted with the antibody, but Y185E and TE/YE (in which Tyr-185 is mutated) did not.

In a control kinase assay, endogenous p42 MAP kinase from each transfected cell culture clone was immunoprecipitated and shown to display serum-stimulated myelin basic protein (MBP) kinase activity (Figure 1c, lower panel). This result rules out the possibility that failure of TE/YE phosphorylation might be due to a failed response of the cells to serum. These data also demonstrate that none of these exogenously expressed mutants inhibits substantial activation of endogenous MAP kinase, i.e. none of them functions as a dominant negative mutant at the expression levels obtained in this system.

To find out if insertion of the epitope might affect MAP kinase activation, an immunocomplex MBP kinase assay was performed using the 12CA5 monoclonal antibody (Figure 1c, upper panel). The result shows that the tagged wild-type MAP kinase still possesses a regulatable kinase activity in response to serum. Interestingly, the data also show that all of the mutant MAP kinase proteins lack detectable kinase activity. Thus not only is the K52R mutant, which is defective in ATP binding, also defective in kinase activity, but the replacement of a phosphorylatable amino acid with glutamate is not able to substitute for a regulatory phosphorylation. This is the case even when only a single regulatory phosphorylation site is replaced by glutamate and the other site becomes phosphorylated in response to serum. Taken together, the data suggest that phosphorylation of both Thr-183 and Tyr-185 is required and sufficient for p42 MAP kinase activation.

The presence of MAP kinases in the immunocomplexes was confirmed by stripping the same blot and then reprobing with anti-(MAP kinase) antibody (Figure 1d). Therefore lack of phosphorylation of the TE/YE mutant (Figure 1a) and the absence of kinase activity (Figure 1c, upper panel) are not due to the absence of the MAP kinase proteins.

Figure 1 *In vivo* phosphorylation of MAP kinase mutants

CCL39 hamster fibroblasts expressing the epitope-tagged MAP kinases were labelled for 6 h with [32 P]P, and then stimulated with 10% fetal calf serum (FCS) at 37 °C for 10 min. (a) Epitope-tagged MAP kinase mutants immunoprecipitated with monoclonal antibody 12CA5 (epitope-specific) from 32 P-labelled whole cell lysates. Vec., parental CCL39 cells transfected with an empty vector; WT, wild-type transfectants; TE, T183E mutant; YE, Y185E mutant; TE/YE, T183E/Y185E double mutant; KR, K52R kinase-defective mutant, - and +, without and with FCS stimulation. (b) Anti-phosphotyrosine Western blot of the 12CA5-immunoprecipitated tagged MAP kinase proteins from the same lysates used in (a). (c) Upper panel, kinase activities of the tagged MAP kinase mutants precipitated from the same lysates used in (a) in the 12CA5 immunocomplexes. The immunocomplex kinase assays were performed using [γ - 32 P]ATP and MBP as the *in vitro* substrate. Lower panel, immunocomplex MBP kinase assays of the endogenous p42 MAP kinase activity, which was precipitated with a rabbit antiserum (TR2) specific for p42^{mapk} from FCS-stimulated cell lysates, as a control for activation of MAP kinase in the transfectants. (d) Presence of the tagged MAP kinase mutant proteins in the immunocomplexes. The epitope-tagged MAP kinase proteins were immunoprecipitated with 12CA5 and detected with a monoclonal anti-(MAP kinase) antibody (1B3B9).

Phosphorylation of MAP kinase *in vitro*

A MAPKK (mek1) has been purified and cloned in several laboratories (Crews et al., 1992; Ashworth et al., 1992; Seger et al., 1992; Wu et al., 1993). To determine whether the enzymic properties of this protein are consistent with the characteristics of the *in vivo* phosphorylation and activation of MAP kinase described above, we analysed the phosphorylation of MAP kinase mutants using purified MAPKK *in vitro*. The MAP kinase mutants were expressed in bacteria and purified to homogeneity (L'Allemain et al., 1992), and a MAPKK partially purified from rabbit muscle (Wu et al., 1992) was used to phosphorylate the recombinant proteins. The result shown in Figure 2(a) is consistent with the *in vivo* data, in that MAPKK can phosphorylate either Thr-183 or the Tyr-185 independently. Furthermore, no other sites of phosphorylation were detected in the double mutants TE/YE or T183A/Y185F.

Note that mutants T183A and T183E seem to be better substrates than Y185F and Y185E respectively. We also found the same preference for tyrosine phosphorylation on MAP kinase when using whole-cell lysate as a source of MAPKK (L'Allemain et al., 1992). Whether this is an intrinsic property of MAPKK and reflects the order of Tyr/Thr phosphorylation (Wu et al., 1991; Haystead et al., 1992) or is due to structural characteristics of the purified recombinant substrate proteins is not yet clear.

The D147A mutant has a substitution of alanine for the aspartate in kinase subdomain VI (D¹⁴⁷LKPSN). This aspartate residue is believed to be the catalytic base (Knighton et al., 1991; Zheng et al., 1993) and lies in the catalytic loop (Hanks et al., 1988). It is interesting to note that there is less phosphate incorporated into D147A than into the K52R mutant, raising the possibility that an intact catalytic subdomain of MAP kinase may be required for its full phosphorylation by MAPKK (see the Discussion section). This is further supported by the finding that heat denaturation of MAP kinase destroys its ability to function as a substrate for MAPKK (Figure 2b). In addition, a synthetic peptide corresponding to the tryptic peptide containing the MAP kinase phosphorylation site does not serve as a substrate for the purified MAPKK (results not shown).

Phosphoamino acid analysis of MAP kinase mutants phosphorylated *in vitro*

To determine which amino acids were phosphorylated by the MAPKK, phosphoamino acid analysis was performed with the mutant proteins from the above *in vitro* kinase assays. As expected, the wild-type MAP kinase (WT) was phosphorylated on both threonine and tyrosine residues (Figure 3a). Mutants T183A and T183E, with Tyr-185 the only available acceptor, were phosphorylated on tyrosine only. Mutants Y185F and Y185E were phosphorylated only on threonine. The kinase-defective K52R mutant was phosphorylated on both tyrosine and threonine (Figure 3b), consistent with the idea that MAPKK is a dual-specificity protein kinase. However, there was strikingly less phosphorylation on threonine in the case of the D147A mutant, which is predicted to be defective in catalytic properties. The data shown here, along with that from Figures 2(a) and 2(b), raise the possibility that an active kinase domain of MAP kinase may have a role in phosphorylation of Thr-183 and activation of the kinase itself.

Autophosphorylation of MAP kinase

It has been shown that p42 MAP kinase is capable of autophosphorylation on Tyr-185 (Wu et al., 1991; Seger et al., 1991;

Figure 2 Phosphorylation of recombinant MAP kinase proteins with a purified MAPKK

^{32}P autoradiographs of phosphorylated recombinant MAP kinases in *in vitro* MAPKK reactions are shown. (a) Equal amounts of recombinant MAP kinase proteins, determined by Bradford protein assays and Ponceau S staining of blots, were used as substrates for a MAPKK purified from rabbit muscle in *in vitro* kinase reactions. Mutants: TA, T183A; YF, Y185F; TAYF, T183A/Y185F; KR, K52R; DA, D147A. MAPK, MAP kinase. Phosphorylated recombinant proteins were separated on polyacrylamide gels and then transferred to Immobilon and exposed to film. (b) MAPKK assays as in a. X, purified MAPKK alone without substrate (MAP kinase); BWT, wild-type MAP kinase which has been boiled for 10 min before the reactions; KR and DA as in (a).

Robbins and Cobb, 1992), and this reaction has been characterized as being intramolecular, based on concentration independence (Wu et al., 1991). To determine whether the autophosphorylation is inter- or intra-molecular using an independent rationale, we investigated whether excess kinase-defective MAP kinase could inhibit the autophosphorylation reaction (as would be expected if it were intermolecular). The autophosphorylation of purified, recombinant, epitope-tagged, wild-type MAP kinase was measured in the presence of increasing amounts of recombinant K52R mutant. The 1.7 kDa epitope allowed separation of the tagged wild-type protein from the untagged kinase-defective mutant. No inhibition of the wild-type autophosphorylation reaction was observed, even when K52R was present in 20-fold

Figure 4 Autophosphorylation of MAP kinase: an intramolecular reaction

Competition of wild-type MAP kinase autophosphorylation with the kinase-defective K52R mutant (KR). Experiments were done by mixing 0.2 μg of the purified, recombinant, tagged wild-type (WT) MAP kinase in each reaction with increasing amounts (0, 0.1, 0.2, 0.5, 1.0 and 4.0 μg) of the recombinant KR protein, and then performing an autokinase reaction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Labelled MAP kinase proteins were separated by SDS/PAGE, and the autophosphorylated, tagged MAP kinase was detected by exposure to X-ray film (tagged p42; indicated by an arrow on the right). Also shown is the position of the autophosphorylated untagged wild-type p42 (untagged p42, arrow on the left), showing the position to which a phosphorylated KR should migrate.

excess (Figure 4). Thus the autophosphorylation of MAP kinase appears to be an intramolecular reaction by this criterion.

ATP-concentration-dependent K52R autophosphorylation

At high concentrations of mutant K52R, a small amount of phosphorylation of the kinase-defective protein was detected. To determine whether this mutant protein retained residual autokinase activity, the purified recombinant K52R was incubated with increasing amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and the reaction products were then separated by gel electrophoresis. As shown in Figure 5, wild-type MAP kinase showed substantial autokinase activity within the range of ATP concentrations tested. The K52R mutant displayed low but detectable autokinase activity at ATP concentrations higher than 100 μM . The autokinase activity of the D147A mutant was below the level of detection under these conditions. Thus the mutant of MAP kinase that is kinase-defective because of a lesion in the ATP-binding domain could still display detectable autokinase activity at high ATP concentrations, whereas the mutant which is kinase-defective because of a lesion in the catalytic base has a lower autokinase capability.

Figure 3 Phosphoamino acid analysis of MAP kinases phosphorylated *in vitro*

(a) Bands of the phosphorylated MAP kinase proteins from the *in vitro* MAP kinase kinase reactions (Figures 2a and 2b) were cut out from Immobilon and subjected to one-dimensional phosphoamino acid analysis. pS, pT and pY indicate the positions of phosphoserine, phosphothreonine and phosphotyrosine respectively. WT, wild-type; TA, T183A mutant; YF, Y185F mutant; TE, T183E; YE, Y185E. (b) Comparisons of phosphoamino acid contents of wild-type (WT) MAP kinase and mutants K52R (KR) and D147A (DA) phosphorylated by MAPKK *in vitro*. MKK, MAPKK control without MAP kinase as substrate.

Figure 5 Autophosphorylation of kinase-defective mutants

Autophosphorylation of mutants K52R and D147A, and of wild-type (WT) MAP kinase, was carried out in the presence of increasing ATP concentrations but with the same ^{32}P specific radioactivity. K52R and D147A were present in equal concentrations, whereas the wild-type protein was used at half that concentration. The labelled proteins were separated by SDS/PAGE then transferred to Immobilon and exposed to film. MAPK, MAP kinase.

DISCUSSION

Because MAP kinases are widely expressed and are rapidly activated during cellular responses to diverse agonists, it is generally believed that they play an important role in the transmission of regulatory signals from the membrane. For this reason, it is important to understand the mechanism(s) regulating their activation. Recent studies on MAP kinase activation have centred on examination of a MAPKK. This enzyme was identified initially based on its ability to activate recombinant wild-type MAP kinase *in vitro* and to phosphorylate a kinase-defective MAP kinase mutant (Gomez and Cohen, 1991; L'Allemain et al., 1992; Nakielnny et al., 1992; Rossomando et al., 1992). The predicted amino acid sequence of MAPKK, based on the cloned cDNAs, is most similar to that of serine/threonine-specific protein kinases rather than tyrosine-specific protein kinases (Crews et al., 1992; Ashworth et al., 1992; Wu et al., 1993). Nevertheless, this enzyme is capable of catalysing the dual phosphorylation of MAP kinase on both threonine and tyrosine. In fact, the enzyme favours tyrosine phosphorylation kinetically (Haystead et al., 1992), as the tyrosine phosphorylation of the MAP kinase substrate can go nearly to completion before substantial threonine phosphorylation is detected. Thus MAPKK functions as a 'dual-specificity' protein kinase on an exogenous substrate.

Other protein kinases have been suggested to be dual-specificity kinases, e.g. MAP kinases, STY1/clk and SPK1 (reviewed in Lindberg et al., 1992). These so-called dual-specificity kinases are capable of autophosphorylating on serine/threonine and on tyrosine, but phosphorylate exogenous substrates only on serine/threonine or on tyrosine. Wee1 has been thought to be a dual-specificity protein kinase because yeast p107^{wee1} is capable of autophosphorylation on serine and tyrosine (Parker et al., 1992), and the human cdc2 is phosphorylated on Thr-14 and Tyr-15. However recent evidence indicates that the human Wee1 kinase autophosphorylates and phosphorylates cdc2 only on tyrosine (Parker and Piwnica-Worms, 1992). Therefore Wee1 appears to be a tyrosine-specific protein kinase. Thus no kinase besides MAPKK has been shown to be able directly to phosphorylate an exogenous substrate on both serine/threonine and tyrosine. Therefore MAPKKs in this sense are the only *bona fide* dual-specificity protein kinases identified so far.

Because of the weak precedent for dual-specificity protein kinases, and the fact that MAP kinase can autophosphorylate on the regulatory tyrosine, Tyr-185, we wished to examine the possibility that MAPKK might exploit the kinase activity of its substrate to cause MAP kinase phosphorylation. For example,

one could imagine that MAPKK functions as a serine/threonine-specific protein kinase while stimulating the rate at which MAP kinase's tyrosine-specific autophosphorylation occurs. Since MAPKK can catalyse the dual phosphorylation of a kinase-defective MAP kinase protein (K52R), in which the MAP kinase carries a mutation in the ATP-binding loop, one might assume that full catalytic activity for threonine and tyrosine phosphorylations resides in the MAPKK. However, we have found that the K52R mutant retains residual capacity as an autokinase at high ATP concentrations (as would be expected, since the catalytic domain is intact). Thus it seemed possible that the dually specific catalytic activity of MAPKK depended in part on the catalytic competence of its substrate.

To test this possibility, we engineered a MAP kinase mutant in which Asp-147, the predicted catalytic base in the active site (Knighton et al., 1991) was converted to alanine (D147A). Such a mutant would be expected to be capable of binding ATP, but to be defective in phosphate transfer. We found, as predicted, that the D147A mutant was a considerably poorer substrate for MAPKK than was the wild-type or K52R protein. Surprisingly, the ability of MAPKK to phosphorylate on threonine (rather than tyrosine) was preferentially crippled. This was surprising because we expected that MAPKK would function as a serine/threonine protein kinase (based on its sequence) and that the catalytic domain of the MAP kinase would participate in its own tyrosine phosphorylation (based on the fact that MAP kinase autophosphorylates on tyrosine).

Thus, although MAPKK is a dual-specificity kinase with an amino acid sequence most similar to those of serine/threonine protein kinases, it appears to function more effectively as a tyrosine kinase than as a threonine kinase. This was seen also on comparison of phosphorylation rates for MAP kinase mutants in which the Thr-183 was changed to Ala or Glu versus mutants in which the Tyr-185 was changed to Phe or Glu: MAP kinase mutants lacking a site of tyrosine phosphorylation were poorer *in vitro* substrates for MAPKK than were the mutants lacking the site of threonine phosphorylation. The preference of MAPKK for tyrosine phosphorylation has been noted previously (Wu et al., 1991; L'Allemain et al., 1992; Adams and Parker, 1992; Haystead et al., 1992).

Although these results are suggestive of the notion that the MAP kinase catalytic domain participates in its own phosphorylation (especially on threonine), it is equally possible that the mutation we have introduced into the MAP kinase catalytic domain alters its structure so that it is poorly recognized by the MAPKK. Another possibility is that Tyr-185 is exposed in MAP kinase, but that Thr-183 is less accessible, and an intact catalytic

domain is necessary for MAP kinase to undergo a conformational change which makes Thr-183 fully accessible. The MAPKK has extraordinary specificity, since no other substrates have been identified, and it fails to detectably phosphorylate heat-denatured MAP kinase or a peptide corresponding to the tryptic peptide surrounding the phosphorylation site. Determination of the molecular basis for the recognition of MAP kinase by MAPKK and its ability to function as a dual-specificity protein kinase will require more detailed structural and enzymological studies.

Regardless of the detailed mechanism of dual phosphorylation, it is clear that the *in vitro* enzymology of MAPKK is consistent with the characteristics of the *in vivo* phosphorylation and activation of MAP kinase in response to agonists. Both *in vivo* and *in vitro*, the kinase-defective MAP kinase, K52R, can become phosphorylated. Removing the two identified regulatory phosphorylation sites abolishes phosphorylation completely, both *in vivo* and *in vitro*, confirming that Thr-83 and Tyr-185 are the only sites phosphorylated. Finally, phosphorylation of these two sites can occur independently, as removal of the Thr-183 by site-directed mutagenesis did not prevent phosphorylation of tyrosine and removal of Tyr-185 did not prevent phosphorylation of threonine. This result was obtained regardless of whether the sites of phosphorylation were replaced with cognate non-phosphorylatable amino acids (Phe for Tyr and Ala for Thr) or were replaced with negatively charged glutamate. Thus phosphorylation by MAPKK does not require a prior negative charge at either site of regulatory phosphorylation. These results do not exclude the possibility that there are other MAP kinase activators besides MAPKK present in cells, but they show that the MAPKK which has been purified, characterized and cloned has properties sufficient to account for the *in vivo* activation of MAP kinase.

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