Mechanistic Studies of the Dual Phosphorylation of Mitogen-activated Protein Kinase*

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Previous work on the responses of mitogen-activated protein (MAP) kinase cascade components in a Xenopus oocyte extract system demonstrated that p42 MAP kinase (MAPK) exhibits a sharp, sigmoidal stimulus/response curve, rather than a more typical hyperbolic curve. One plausible explanation for this behavior requires the assumption that MAP kinase kinase (MAPKK) carries out its dual phosphorylation of p42 MAPK by a distributive mechanism, where MAPKK dissociates from MAPK between the first and second phosphorylations, rather than a processive mechanism, where MAPKK carries out both phosphorylations before dissociating. Here we have investigated the mechanism through which a constitutively active form of human MAPK-1 (denoted MAPKK-1 R4F or MAPKK-1*) phosphorylates Xenopus p42 MAPK in vitro. We found that the amount of monophosphorylated MAPK formed during the phosphorylation reaction exceeded the amount of MAPK-1* present, which would not be possible if the phosphorylation occurred exclusively by a processive mechanism. The monophosphorylated MAPK was phosphorylated predominantly on tyrosine, but a small proportion was phosphorylated on threonine, indicating that the first phosphorylation is usually, but not invariably, the tyrosine phosphorylation. We also found that the rate at which pulse-labeled monophosphorylated MAPK became biphasorylated depended on the MAPK-1* concentration, behavior that is predicted by the distributive model but incompatible with the processive model. These findings indicate that MAPK-1* phosphorylates p42 MAPK by a two-collision, distributive mechanism rather than a single-collision, processive mechanism, and provide a mechanistic basis for understanding how MAP kinase can convert graded inputs into switch-like outputs.

The MAP1 kinases (MAPKs) are a family of protein kinases conserved across the eukaryotic kingdoms and implicated in diverse biological processes, ranging from mitogenesis, cell fate induction, and stress responses in mammalian cells through cell wall formation and mating pheromone responses in yeast (1–6). MAPKs are activated by phosphorylation of two sites, threonine and tyrosine residues within the kinase's activation loop (7, 8). In rat Erk2 these residues are threonine 183 and tyrosine 185, and they lie within the sequence TEY. Dephosphorylation of either residue inactivates the kinase, and mutant kinases with either residue replaced by a non-phosphorylatable residue are inactive or minimally active (9–13).

Examples of MAP kinases with this TEY motif, the TEY subgroup of MAP kinases, have been identified in animals, plants, fungi, and protists (6). Other MAP kinase subgroups have slightly different phosphorylation site signatures as follows: TGY for the stress activated protein kinases or Jnk proteins, TGY for the Hog1-like MAPKs, and TNY for the budding yeast Smk1 protein. However, in all cases examined, phosphorylation of the threonine and tyrosine residues has proven to be essential for activation. Thus the requirement for dual phosphorylation is conserved within and among the various MAP kinase subgroups and probably arose early in eukaryotic evolution.

The activating phosphorylations are catalyzed by a dual specificity protein kinase, a MAP kinase kinase (MAPKK). Like the MAPKs, the MAPKKs constitute an evolutionarily conserved family. Since the MAPK phosphorylation sites and the machinery for MAPK phosphorylation are highly conserved, it seems plausible that the biochemical mechanism for the phosphorylation might be conserved as well.

Two basic classes of mechanisms can be envisioned for the dual phosphorylation of a MAPK by a MAPKK. The first is a processive mechanism, sometimes colloquially referred to as a "bind and slide" mechanism. A simple processive mechanism is shown schematically in Equation 1. The active MAPKK protein collides with and binds to MAPK, phosphorylates it once, and releases, perhaps, to align the second phosphorylation site of MAPK with the active site of MAPKK, phosphorylates MAPK a second time, and then dissociates, yielding a doubly phosphorylated, active MAPK protein.

\[ \text{MAPKK} + \text{MAPK} \rightarrow \text{MAPKK-MAPK} \rightarrow \text{MAPKK-MAPK-P} \rightarrow (\text{MAPKK-MAPK-P})' \rightarrow \text{MAPKK} \rightarrow \text{MAPK} + \text{MAPK-P} \]  
\hspace*{1cm} (Eq. 1)

The other is a distributive mechanism. Active MAPKK collides with and binds to MAPK, phosphorylates it once, and releases the monophosphorylated product (Equation 2). The monophosphorylated MAPK then collides with a second molecule of active MAPKK, becomes phosphorylated a second time, and is released as MAPK-P (Equation 3).

\[ \text{MAPKK} + \text{MAPK} \rightarrow \text{MAPKK-MAPK} \rightarrow \text{MAPKK-MAPK-P} \rightarrow \text{MAPKK-MAPK-MAPK-P} \rightarrow \text{MAPKK} \rightarrow \text{MAPK} + \text{MAPK-P} \]  
\hspace*{1cm} (Eq. 2)

\[ \text{MAPKK} + \text{MAPK} \rightarrow \text{MAPKK-MAPK} \rightarrow \text{MAPKK-MAPK-P} \rightarrow \text{MAPKK-MAPK-P} \rightarrow \text{MAPKK} \rightarrow \text{MAPK} + \text{MAPK-P} \]  
\hspace*{1cm} (Eq. 3)

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The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAPK-P, monophosphorylated MAPK; MAPK-PP, bisphosphorylated MAPK; MAPKK, MAP kinase kinase; MAPKK-1*, constitutively active A32-51/S218E/S222D MAPKK-1; Thr(P), phosphothreonine; Tyr(P), phosphotyrosine.

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Here we have set out to determine which type of mechanism describes the phosphorylation of MAPK by active MAPKK when the enzymes are present at normal physiological concentrations. We became interested in this question in the course of studies assessing the steady state responses of endogenous MAPKK-1 and p42 MAPK proteins in a highly manipulable Xenopus laevis oocyte extract system. We added various concentrations of an input stimulus, recombinant Mos (a MAP kinase kinase kinase), to the extracts, let the extracts come to a steady state, and measured the resulting MAPKK-1 and p42 MAPK activities. We found that as the cascade is descended, the kinases’ responses become progressively more switch-like. The stimulus/response curve for p42 MAPK was found to be as steeply sigmoidal as that of a cooperative enzyme with a Hill coefficient of 4–5 (14). Several mechanisms could, in principle, contribute to this remarkably abrupt response (15). One such mechanism is “multistep ultrasensitivity” (16–18), which can arise from a distributive dual phosphorylation mechanism.

As a first step toward understanding whether multistep ultrasensitivity contributes to MAPKs observed switch-like response, we set out to determine whether the mechanism of MAPK’s dual phosphorylation is processive or distributive. We phosphorylated MAPK in vitro using a recombinant active MAPKK-1 protein (here called MAPKKK-1 (19, 20)). We examined the time course of MAPK-P and MAPK-PP accumulation to see whether the amount of MAPK-P always remained smaller than the amount of MAPKK-1*, as expected if the phosphorylation mechanism were processive, or exceeded it, as could occur if the mechanism were distributive. We also examined whether the rate of conversion of MAPK-P to MAPK-PP was independent of the concentration of MAPK-1*, as expected if the phosphorylation mechanism were processive, or dependent on it, as expected if the mechanism were distributive. Our results indicate that much, and possibly all, of the dual phosphorylation of MAPK by MAPKKK-1* occurs by a distributive mechanism.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—A cDNA for a constitutively active human MAPKKK-1 (produced by replacing Ser-218 with Glu, Ser-222 with Asp, and deleting amino acids 32–51; denoted MAPKKK (ΔN3/S218E/S222D) or MAPKKK R4F (20)) was provided by Natalie Ahn (University of Colorado, Boulder) (19, 20). Since we have used only this one type of activated MAPKKK-1 in the present work, for simplicity we shall denote it MAPKKK-1*. MAPKKK-1* was expressed as a hexahistidine-tagged protein from plasmids derived from ones provided by Jonathan Cooper and Jim Posada (Fred Hutchinson Cancer Research Center, Seattle, WA) (19, 20). Hexahistidine-tagged rat Erk2/p42 MAPK was expressed in E. coli and purified by Chi-Ying Huang (Stanford University) (14) from a plasmid provided by Melanie Cobb and Tom Geppert (University of Texas Southwestern, Dallas) (13). All proteins were purified to near homogeneity by nickel chelate chromatography.

**Quantifying Levels of p42 MAPK and MAPKKK-1 in Xenopus Oocytes and Tissue Culture Cells**—Oocytes and tissue culture cells were lysed as described (22, 23). Lysates were subjected to polyacrylamide gel electrophoresis and immunoblotting using antisera provided by Catherine R. Cobb and Tom Geppert (University of Texas Southwestern, Dallas) (13). All proteins were purified to near homogeneity by nickel chelate chromatography.

**Phosphorylation Reactions**—Phosphorylation reaction mixtures consisted of MAPKKK-1* (0.1–1 μM), p42 MAPK (0.6–5 μM), ATP (100 μM, generally 1–10 μCi per reaction), NaCl (100 mM), Tris-HCl (50 mM, pH 7.0), MgCl₂ (10 mM), and 0.1% bovine serum albumin. Reaction volumes were 20–100 μL. Reactions were initiated by addition of the ATP and terminated by addition of SDS sample buffer containing excess EDTA.

Pulse-chase labeling was carried out similarly, except that carrier-free ATP was used during the pulse labeling (∼6000 Ci/mmol), and the chase was initiated by addition of unlabeled ATP to yield a final concentration of 0.5 μM.

**Phosphoamino Acid Analysis**—Proteins blotted onto polyvinylidene difluoride membranes and peptides eluted from thin layer cellulose plates were subjected to hydrolysis in 5.7 M HCl at 110 °C for 2 h (25, 26). The hydrolysates were diluted, lyophilized, and subjected to one-dimensional (pH 3.5) or two dimensional (pH 1.9 and 3.5) thin layer electrophoresis as described (25).

**Tryptic Peptide Mapping**—Blotted 32P-labeled proteins were subjected to digestion with trypsin (Worthington) as described (27), except that the blots were not allowed to dry prior to digestion (we found that this improved the recovery of tryptic peptides from the membranes); the performic acid oxidation step was omitted; and the digests were resuspended in water and lyophilized three times to remove traces of ammonium bicarbonate. Tryptic digests were subjected to one-dimensional thin layer electrophoresis at pH 8.9 (fresh 1% ammonium carbonate) for 25 min at 1000 V.

**KINETIC CALCULATIONS**

Here we derive expressions for the initial rate of MAPK phosphorylation (for either a processive or distributive mechanism), and the initial rate of conversion of monophosphorylated MAPK to bisphosphorylated MAPK (for a distributive mechanism), as functions of the total MAPK and MAPKKK concentrations. We shall use these expressions in analyzing pulse-chase data for MAPK phosphorylation (Figs. 6 and 7).

**Initial Rate of Pulse Labeling**—The initial phosphorylation of MAP kinase by MAPKKK, for a distributive mechanism, is described by Equation 4,

\[
V_o = \frac{a_1}{d_1} \frac{[\text{MAPK}] + [\text{MAPKK}]}{[\text{MAPKK}] + [\text{MAPK}] + [\text{MAPKK}]} \right) 
\]

For a processive mechanism, the reaction is identical except that the product is not released after the catalysis step. We assume that the concentration of ATP is constant and can be included in the rate constants.

If it can be assumed that the substrate concentration (MAPK) greatly exceeds that of the enzyme (MAPKK), then a steady state treatment of this reaction gives rise to the Michaelis-Menten equation. However, this assumption is not necessarily valid for the phosphorylation of MAPK by MAPKK in cells (see below) and is not valid for the *in vitro* phosphorylation reactions examined in the present study. The appropriate initial rate equation in this situation is Equation 5,

\[
V_o = \frac{1}{k_1} \left[ k_2 \left( [\text{MAPK}] + [\text{MAPKK}] + [\text{MAPKK}] \right) \right] - 4 [\text{MAPKK}] [\text{MAPKK}] 
\]

where \( k_0 \) denotes the Michaelis constant. This equation is discussed in detail elsewhere (28). Briefly, we note that (i) plots of \( V_o \) versus \([\text{MAPKK}] \) or \( V_o \) versus \([\text{MAPKK}] \) are hyperbolas; (ii) Equation 5 reduces to the Michaelis-Menten equation if it is assumed that \([\text{MAPKK}] \) is small; (iii) if neither \([\text{MAPKK}] \) nor \([\text{MAPKK}] \) is small relative to the \( k_0 \) value, then the \( V_o \) is half-maximal at a value of \([\text{MAPKK}] \) or \([\text{MAPKK}] \) greater

2 The fact that Equation 5 reduces to Michaelis-Menten equation when \([\text{MAPKK}] \) is small can be seen by expressing the square root term of Equation 5 as a binomial expansion (see Equation 5a below),

\[
(a + b)^{1/2} = a^{1/2} + \frac{1}{2} a^{-1/2} b + \cdots 
\]

where \( a = (k_0 + [\text{MAPKK}] + [\text{MAPKK}]^2) \) and \( b = 4[\text{MAPKK}] \) and truncating the second term.
than the $K_m$ value; and (iv) as $[\text{MAPKK}_{\text{tot}}]$ and $[\text{MAPK}_{\text{tot}}]$ become large, the hyperbolas defined by Equation 5 become sharper; they approach their asymptotes.

Initial Rate of Chase—The situation for the “chasing” of radiolabel from MAPK-P to MAPK-PP is more complicated, because it is necessary to factor in the presence of non-phosphorylated MAPK, which acts as a competitive inhibitor of MAPK-P for access to MAPKK. This situation is described in Equation 6,

$$a_1\text{MAPK-P}^* \frac{d\text{MAPKK}}{dt} = k_1 \text{MAPK-P}^* \text{MAPKK} \rightarrow \text{MAPK} + \text{MAPK-PP}^*$$

$$a_2\text{MAPK \rightarrow MAPK-P}$$

where the asterisks denote the pulse-labeled MAPK species.

We wish to solve for the initial rate of formation of MAPK-PP* as a function of MAPKK$_{\text{tot}}$, MAPK$_{\text{tot}}$, and the $K_m$ values. The rate of formation of MAPK-PP* is given by Equation 7,

$$d\text{[MAPK-PP*]} = k_1\text{[MAPK-P*][MAPKK]} \quad (\text{Eq. 6})$$

From the steady state assumption (see Equation 8),

$$d\text{[MAPK-PP*][MAPKK]} = a_1\text{[MAPKK]}\text{[MAPK-P*]} - (d_1 + k_1)\text{[MAPK-P*][MAPKK]} = 0 \quad (\text{Eq. 8})$$

We substitute $[\text{MAPKK}_{\text{tot}}] - [\text{MAPK}\text{-MAPKK}]$ for $[\text{MAPKK}]$ (assuming $[\text{MAPK-P}\text{-MAPKK}]$ is small relative to $[\text{MAPKK}\text{-MAPKK}]$), and $[\text{MAPK}_{\text{tot}}] - [\text{MAPK-P}\text{-MAPKK}]$ for $[\text{MAPK-P}]$, yielding Equation 9,

$$[\text{MAPK}_{\text{tot}}] - [\text{MAPK}\text{-MAPKK}] = [\text{MAPK-P}\text{-MAPKK}][\text{MAPKK}_{\text{tot}}] - [\text{MAPK}_*\text{-MAPKK}] + [\text{MAPK-P}\text{-MAPKK}][\text{MAPKK}_{\text{tot}}] - K_m\text{[MAPK-P\text{-MAPKK}]} = 0 \quad (\text{Eq. 9})$$

where $K_m$ is the Michaelis constant ($d_1 + k_1/a_1$). Solving for $[\text{MAPK-P\text{-MAPKK}}]$ yields Equation 10,

$$\frac{[\text{MAPK-P\text{-MAPKK}}][\text{MAPKK}_{\text{tot}}]}{K_m} = \frac{[\text{MAPK}_{\text{tot}}] - [\text{MAPK}\text{-MAPKK}]}{K_m + [\text{MAPK}_{\text{tot}}] - [\text{MAPK}\text{-MAPKK}]} \quad (\text{Eq. 10})$$

and so if $V_t$ is expressed as the fraction of MAPK* converted to the bisphosphorylated form per unit time, $V_t$ is given by Equation 11,

$$V_t = k_1\text{[MAPK}_{\text{tot}}] - [\text{MAPK}\text{-MAPKK}] \quad (\text{Eq. 11})$$

We now need to express $[\text{MAPK}\text{-MAPKK}]$ in terms of $\text{MAPKK}_{\text{tot}}$ and $\text{MAPK}_{\text{tot}}$. This can be accomplished by assuming $[\text{MAPKK}\text{-MAPKK}]$ is at steady state (see Equation 12). Then

$$a_1\text{[MAPK][MAPKK]} - (d_2 + k_2)\text{[MAPK][MAPKK]} = 0 \quad (\text{Eq. 12})$$

It follows that (see Equation 13)

$$[\text{MAPKK}\text{-MAPKK}] + [\text{MAPK}_{\text{tot}}][\text{MAPKK}_{\text{tot}}] = 0 \quad (\text{Eq. 13})$$

where $K_m = (d_2 + k_2)$. Using the quadratic equation to obtain an expression for $[\text{MAPK}\text{-MAPKK}]$ yields Equation 14,

$$[\text{MAPKK}\text{-MAPKK}] = \frac{1}{2}([\text{MAPKK}_{\text{tot}}] + [\text{MAPK}_{\text{tot}}]) - \frac{\sqrt{[\text{MAPKK}_{\text{tot}}] + [\text{MAPK}_{\text{tot}}] + 4[\text{MAPKK}_{\text{tot}}][\text{MAPK}_{\text{tot}}]}}{2} \quad (\text{Eq. 14})$$

The relevant choice of sign is the minus sign. Combining Equation 14 with Equation 11 yields an expression for $V_t$ in terms of $\text{MAPKK}_{\text{tot}}$, $\text{MAPK}_{\text{tot}}$, $K_m$, and $K_m$ (see Equation 15),

$$V_t = \frac{[\text{MAPKK}_{\text{tot}}] + [\text{MAPK}_{\text{tot}}] - 4[\text{MAPKK}_{\text{tot}}][\text{MAPK}_{\text{tot}}]}{2([\text{MAPKK}_{\text{tot}}] + [\text{MAPK}_{\text{tot}}] - 4[\text{MAPKK}_{\text{tot}}][\text{MAPK}_{\text{tot}}])} \quad (\text{Eq. 15})$$

Equation 15 is used below to test whether our data for the initial rate of conversion of MAPK-P to MAPK-PP as a function of the MAPKK$_{\text{tot}}$ concentration are consistent with a distributive model where MAPKK dissociates fully from MAPK-P.

Steady State Modeling—Mathematica 2.2.2 was used to numerically solve the rate equations for MAP kinase phosphorylation and dephosphorylation. The stimulus/response curves shown in Fig. 1 were obtained from the calculated steady-state responses. Similar calculations are described in detail elsewhere (14).

RESULTS

Distributive Phosphorylation and Sigmoidal Stimulus Response Curves—Kinetic arguments show how multistep ultrasensitivity can arise from dual phosphorylation, and why a distributive mechanism is essential for it. Suppose MAPK's dual phosphorylation occurs by a distributive mechanism, and the second phosphorylation is slower, or at least not much faster, than the first. Then consider how the steady state amount of active (bisphosphorylated) MAPK will increase as the level of active MAPKK is increased in the face of a constant amount of MAPK phosphatase. The first increments of MAPKK will produce substantial amounts of MAPK-PP but very little MAPK-PP; the presence of large amounts of non-phosphorylated MAPK compete with the MAPK-P for access to MAPKK. Thus the first phosphorylation site acts like a buffer that must be at least partially filled before substantial amounts of bisphosphorylated MAPK can be produced.

As the buffer fills, the amount of MAPK-P grows and the amount of nonphosphorylated MAPK drops; the MAPK-P competes increasingly successfully for access to MAPKK, and so for a while each increment of active MAPKK produces a progressively larger increment of MAPK-PP. Eventually the amount of MAPK-P begins to drop, and the production of MAPK-PP plateaus. The result is an S-shaped stimulus/response curve (Fig. 1, solid line). In contrast, a processive mechanism (or a distributive mechanism with a very rapid second step) would yield a typical Michaelian hyperbolic stimulus/response curve (Fig. 1, dashed line). The first increment of active MAPKK would produce the largest increment of MAPK-PP, and each successive increment of MAPKK would produce a progressively smaller increment.

As a distributive mechanism for the dual phosphorylation of MAPK could translate into a sigmoidal, switch-like stimulus/response curve. This motivated our experimental tests of whether the dual phosphorylation of MAPK occurs by a processive or distributive mechanism.

Preliminary Experimental Studies—Since it was possible that different phosphorylation mechanisms predominated in different concentration regimes, we began by determining the concentrations of p42/p44 MAPK and MAPKK-1 in X. laevis oocytes and in various cell lines. MAPK and MAPKK-1 immu-
The ultrasensitive stimulus/response curve is that predicted for a system consisting of MAPKK-1*. p42 MAP kinase, and a MAP kinase phosphatase, under the assumption that the phosphorylation of p42 MAP kinase occurs by a two-step, distributive mechanism as shown in Equations 2 and 3. The $K_{\text{app}}$ value for the first phosphorylation was taken to be 300 nM². The $K_{\text{app}}$ value for the second phosphorylation was taken to be 46 nM (33). The $K_{\text{app}}$ values for the two dephosphorylation reactions were taken to be 300 nM, based on measurements of the apparent $K_{\text{app}}$ of the MAPK phosphatases present in crude Xenopus oocyte extracts (C.-Y. F. Huang and J. E. Ferrell, Jr., unpublished data). The rate equations for MAP kinase phosphorylation and dephosphorylation were set up and solved numerically as described (14).

As expected for either class of mechanism, MAPKK-1* brought about a time-dependent increase in p42 MAPK phosphorylation. The initial rate of phosphorylation was a saturable function of both the MAPK and MAPKK-1* concentrations (see below in Fig. 6, and data not shown). p42 MAPK phosphorylation generally plateaued at less than 2 mol of phosphate/mol of MAPK (Fig. 4B and data not shown), in agreement with previous reports (13, 29). Pulse-chase data indicated (see Fig. 5C, "Discussion") that there was negligible MAPK phosphatase activity present, so the plateau levels of MAPK phosphorylation reflect termination of the phosphorylation reaction rather than acquisition of a pseudo-equilibrium between the phosphorylated and non-phosphorylated forms of MAPK.

p42 MAPK autophosphorylation (wild-type and the various inactive mutants) was found to be negligible compared with MAPKK-1*-catalyzed p42 MAPK phosphorylation under the reaction conditions employed here (not shown), in agreement with previous reports (11, 13, 30–32). p42 MAPK and MAPKK-1* resolved well from each other under the SDS-gel electrophoresis conditions used here, and so MAPKK-1*-derived phosphopeptides and phosphoamino acids were not detected in the p42 MAPK bands analyzed (Fig. 2C and data not shown).

For most of the experiments described below, the K57R mutant of p42 MAPK was used to avoid possible complications from the phosphorylation of MAPKK-1* by MAPK.

**Accumulation of Monophosphorylated p42 MAPK during a Dual Phosphorylation Reaction**—Haystead and co-workers (29, 33) have reported that the threonine phosphorylation of p42 MAPK by MAPKK-1 lags substantially behind the tyrosine phosphorylation in vitro. Robbins and Cobb (34) have found that the same is true for the phosphorylation of ERK1/p44 MAPK in vivo. These findings suggest that monophosphorylated MAPK (mostly consisting of MAPK phosphorylated on...
tyrosine but not threonine) might be formed during a dual phosphorylation reaction in excess of what could be accounted for by a strictly processive mechanism.

**Tryptic Peptide Analysis as a Quantitative Assay of MAPK-P**—We set out to develop and validate a direct assay for the amount of monophosphorylated MAPK present during a phosphorylation reaction. Our first strategy was to determine what fraction of the p42 MAPK was non-phosphorylated from MAPK immunoblots (the non-phosphorylated MAPK runs with a lower apparent molecular mass than either mono- or bisphosphorylated MAPK (Ref. 10, Fig. 3A, and data not shown)) and then to determine what fraction of the phosphorylated MAPK was phosphorylated once and what fraction was phosphorylated twice by tryptic peptide mapping.

32P-Labeled p42 MAPK K57R was separated from MAPKK-1* by gel electrophoresis, transferred to a blotting membrane, excised, and subjected to exhaustive digestion with trypsin. The resulting tryptic peptides were separated by one-dimensional thin layer electrophoresis at pH 8.9 and detected by autoradiography. As shown in Fig. 2A, phosphorylated MAPK yielded two major radiolabeled spots. The first, denoted spot 1b, had the electrophoretic mobility expected for a monophosphorylated peptide, and the second, denoted spot 2, had the expected electrophoretic mobility for the bisphosphorylated peptide. Phosphoamino acid analysis of spot 1b yielded phosphotyrosine almost exclusively, consistent with its identification as a monophosphorylated peptide, and spot 2 yielded both phosphothreonine and phosphotyrosine (Fig. 2B), consistent with its identification as a bisphosphorylated peptide.

We often detected two minor spots, designated spots 1a and 1c. Spot 1a varied from being barely detectable (Figs. 2A and 3A) to being about equal in intensity to the major monophosphorylated spot, spot 1b (Fig. 5A). Phosphoamino acid analysis of spot 1a yielded predominantly phosphotyrosine (Fig. 2B), and spot 1a’s accumulation (Fig. 3A) paralleled those of spot 1b. We therefore identified spot 1a as being an alternate mobility form of the monophosphorylated peptide. The other minor spot, spot 1c, yielded predominantly phosphothreonine upon partial acid hydrolysis and consequently was identified as threonine-phosphorylated, monophosphorylated peptide (Thr(P)-peptide).

To test these identifications further, we phosphorylated a series of p42 MAPK phosphorylation site mutants (0.6 μM) with MAPKK-1* (1 μM) for 2 h, separated the MAPK from MAPKK-1* by polyacrylamide gel electrophoresis, and subjected the phosphorylated MAPK bands to tryptic mapping and phosphoamino acid analysis (Fig. 2C). Wild-type p42 MAPK (Fig. 2C, lane 1) and the catalytically inactive K57R mutant (lane 5) both yielded two major tryptic spots (spot 1b and spot 2, the putative Tyr(P)- and Thr(P)/Tyr(P)-peptides), and yielded both phosphotyrosine and phosphothreonine upon partial acid hydrolysis. The Y190F mutant (Fig. 2C, lane 2) yielded a single major spot, corresponding to spot 1c, the putative Thr(P)-peptide, and yielded only phosphothreonine upon partial acid hydrolysis. The T188V mutant (Fig. 2C, lane 3) yielded a single major spot, corresponding to spot 1b, the Tyr(P)-peptide, and yielded only phosphotyrosine upon partial acid hydrolysis. The TY/VF double phosphorylation site mutant yielded no labeled MAPK (Fig. 2C, lane 4), as did phosphorylation reactions where no p42 MAPK was included (Fig. 2C, lane 6). These findings corroborate the tryptic peptide identifications described above.

Finally, to test whether tryptic peptide analysis was suitable for quantitative determination of mono- and bisphosphorylated MAPK amounts, we assessed the recovery of soluble tryptic peptides from the blotted MAPK bands for the wild-type and phosphorylation site mutant proteins. Our concern was that...
the mono- and bisphosphorylated peptides, or phosphothreonine- and phosphotyrosine-containing peptides, might not be recovered with equal efficiency. However, we found that the recovery efficiencies were high enough to imply that recovery of all of the MAPK phosphopeptides was essentially complete (97 ± 2% (S.D.) for p42 MAPK K57R, 87% for wild type p42 MAPK; 91% for p42 MAPK Y190F; 91% for p42 MAPK T188V; n = 12 for the K57R protein and n = 1 for the others). This finding validated tryptic analysis as a quantitative method for assessing MAPK phosphorylation.

**Accumulation of Monophosphorylated MAPK in Excess of the**

**Fig. 4. Threonine phosphorylation of p42 MAPK K57R lags behind tyrosine phosphorylation.** MAPKK-1* (165 nM) was mixed with p42 MAPK (0.8 or 5 μM) and [γ-32P]ATP. Aliquots were taken at various times and subjected to partial acid hydrolysis and two-dimensional thin layer electrophoresis. A, autoradiograms of phosphoamino acid analyses showing the time course of tyrosine and threonine phosphorylation, taken from one experiment. B, quantitative data pooled from two independent experiments. C, accumulation of monophosphorylated p42 MAPK. The data shown in triangles represent lower bounds for the amount of monophosphorylated p42 MAPK, calculated from: [MAPK-P] = plateau concentration of Tyr(P)–MAPK × (Tyr(P)–MAPK % of plateau - Thr(P)–MAPK % of plateau)/100. Data are shown as averages ± S.E. (n = 4 for the 10, 20, and 80 min time points; n = 2 for the other time point). Where no error bar is visible, the standard error was smaller than the size of the data point.

**Fig. 5. Pulse-chase analysis of p42 MAPK K57R phosphorylation.** p42 MAPK was pulse-labeled for 5 min with carrier-free [γ-32P]ATP and various concentrations of MAPKK-1* and was then chased with 0.5 mM unlabeled ATP for the lengths of time shown. A, tryptic digests of 32P-MAPK at various times during the chase, showing the conversion of monophosphorylated (mono-phos) MAPK (lower bands) to bisphosphorylated (bis-phos) MAPK (top band). One of two similar experiments. B, phosphoamino acid analysis of pulse-labeled p42 MAPK K57R (left panel) and p42 MAP kinase after 5 min of chase with 0.5 mM unlabeled ATP (middle panel). C, quantitative data pooled from two independent experiments. The top plot shows that the total amount of 32P-labeled p42 MAPK remained fairly constant throughout the chase. The bottom plot shows how the time course of the chase varied with the MAPKK-1* concentration. MAPKK-1* concentrations were 1 μM (○), 0.5 μM (□), 0.2 μM (△), and 0.1 μM (●). Data are shown as averages and ranges (n = 2). Where no error bar is visible, the range was smaller than the size of the data point.
Amount of Added MAPK-K57R—We used immunoblotting and tryptic mapping to determine how much non-, mono-, and bisphosphorylated p42 MAPK K57R was present as a function of time during a phosphorylation reaction. Fig. 3 shows the primary data from one such experiment (Fig. 3A) and the averaged results of two such experiments (Fig. 3B). Tryptic digests of p42 MAPK that had been phosphorylated by MAPKK-1* for short periods yielded only monophosphorylated peptides (Fig. 3). The Tyr(P)-peptide predominated, but a small amount of the Thr(P)-peptide, accounting for about 9% of the total monophosphorylated peptide, was also detectable (Fig. 3A). The estimated concentration of MAPK-P present in the reaction mixture quickly exceeded the amount of MAPKK-1* present (165 nM) (Fig. 3B). The bisphosphorylated peptide began to accumulate at a rate of about 60 nm/min after a lag of about 5 min (Fig. 3B).

These findings indicate that much or all of the MAPK dissociated from MAPKK-1* after undergoing its first phosphorylation. These results are consistent with a distributive mechanism and indicate that MAPK phosphorylation does not occur exclusively by a processive mechanism.

A Lower Bound for MAPK-P from Phosphoamino Acid Analysis—We set out to corroborate this conclusion by independent means. We reasoned that the time course of MAPK tyrosine and threonine phosphorylation could be used to calculate lower bounds for the amount of monophosphorylated p42 MAPK present in a phosphorylation reaction.

In vitro phosphorylation reactions were carried out with MAPKK-1* present at a concentration of 0.165 μM and p42 MAPK K57R present at concentrations of 0.8 and 5 μM. As shown in Fig. 4, A and B, the threonine phosphorylation of p42 MAPK lagged substantially behind the tyrosine phosphorylation, in agreement with previous reports on the phosphorylation of MAPK in vitro (29, 33) and in vivo (34). The amounts of tyrosine-phosphorylated MAPK (Tyr(P)-MAPK) and threonine-phosphorylated MAPK (Thr(P)-MAPK) plateaued within 30 min at the lower MAPK concentration and within 80 min at the higher.

The amounts of Tyr(P)-MAPK and Thr(P)-MAPK present as a function of time, as measured with respect to their plateau levels (Fig. 4B), were used to calculate lower bounds for the concentration of monophosphorylated MAPK present (Fig. 4C). As shown in Fig. 4C, MAPK-P clearly accumulated to concentrations that exceeded the amount of MAPKK-1* present. This was particularly striking in the 5 μM p42 MAPK phosphorylation reaction (Fig. 4C, bottom panel) but was also apparent when the more physiological concentration of p42 MAPK (0.8 μM) was used (Fig. 4C, top panel). Thus, phosphoamino acid analysis corroborated the conclusion that most of the MAPK that is phosphorylated once by MAPKK-1* dissociates from it, rather than remaining bound. The dual phosphorylation of p42 MAPK cannot be exclusively processive.

Dependence of the Conversion of Mono- to Bisphosphorylated MAPK on the MAPKK-1* Concentration—Demonstrating that most of the MAPK dissociates from MAPKK-1* after its first phosphorylation is not equivalent to demonstrating that most of the p42 MAPK-PP arises through a distributive mechanism, since not all of the MAPK-P goes on to become MAPK-PP under the reaction conditions used here. Therefore, a kinetic test was devised to allow us to determine whether the MAPK-PP that is formed has been produced by a distributive or a processive mechanism.

If MAPK is phosphorylated distributively, then the initial rate of conversion of pulse-labeled MAPK-P to MAPK-PP should depend upon the concentration of MAPKK-1*. If MAPK is phosphorylated processively, then the rate of conversion should be independent of the MAPKK-1* concentration. We therefore set out to assess the MAPKK-1* dependence or independence of this conversion, using pulse-chase methods to allow any effects of MAPKK-1* on the rate of conversion of MAPK-P to MAPK-PP to be distinguished from effects of MAPKK-1* on the rate of production of MAPK-P.

p42 MAPK K57R (0.6 μM) was pulse-labeled for 5 min with various concentrations of MAPKK-1* (0.1–1 μM) and carrier-free [γ-32P]ATP. Aliquots were taken and subjected to phosphoamino acid analysis, which yielded phosphotyrosine almost exclusively (Fig. 5B, left-hand panel), and tryptic mapping, which yielded monophosphorylated peptides (Fig. 5A).

Excess non-radioactive ATP (0.5 mM) was then added, and we monitored the rate at which monophosphorylated 32P-MAPK was converted to bisphosphorylated 32P-MAPK by tryptic mapping. Fig. 5A shows the primary data from one such experiment; Fig. 5C shows the averaged data from two. For the two highest concentrations of MAPKK-1* used (0.5 and 1 μM), conversion of 32P-MAPK from MAPK-P to MAPK-PP was rapid and extensive, reaching maximal levels within 5 min. At 0.2 μM MAPKK-1*, the conversion was slower, reaching about 70% maximal in 5 min, and was less extensive. At 0.1 μM MAPKK-1* the conversion was slower still, being about half-maximal at 5 min, and again was less extensive. Thus the initial rate of the chase (as well as the plateau level of bisphosphorylation) was found to be dependent on the MAPKK-1* concentration, and there was no evidence of a MAPKK-1*-independent component of the phosphorylation. These findings imply that much or all of the bisphosphorylated MAPK arose by a distributive phosphorylation mechanism.

Two assessments were made to test the validity of the pulse-chase analysis. First, we carried out phosphoamino acid analysis of the peptide after its nearly quantitative conversion from the monophosphorylated form to the bisphosphorylated form (Fig. 5B). We found that the [32P]phosphothreonine content of the peptide did not rise, indicating that addition of the nonlabeled ATP chase did succeed in squelching the radiolabeling of the MAPK. This finding also rules out the possibility that MAPK’s threonine phosphorylation arose from transfer of phosphate from phosphotyrosine rather than from ATP. The second test of the validity of the pulse-chase analysis was an assessment of the total amount of radiolabel incorporated in MAPK through the chase period. The total label was found to remain constant (Fig. 5C, top graph), indicating that there was negligible turnover of MAPK phosphates or MAPK protein on the time scale of this experiment.

Saturability of MAPK Pulse and Chase Labeling—An additional test of the hypothesized distributive phosphorylation mechanism was made by examining the saturability of the pulse and chase labeling. The Michaelis constant for the phosphorylation of purified p42 MAPK-P by MAPKK-1 (a situation that forces a distributive mechanism) has been measured to be 46 nM (33). If MAPKK dissociates fully from MAPK between the first and second phosphorylations of a dual phosphorylation reaction, as could occur in a distributive mechanism, then the Michaelis constant for the second phosphorylation (Km(2)) should be the same as that measured for the phosphorylation of free monophosphorylated p42 MAPK by MAPKK (46 nM). If MAPKK-1* dissociates only partially from p42 MAPK (that is, if the MAPKK-1* that carries out the first phosphorylation has “privileged” access to MAPK for the second phosphorylation) then the rate at which it associates with MAPK (a2) would be

Likewise, if the reaction stops before all of the MAPK-P is converted to MAPK-PP, then the plateau level of MAPK-PP should be MAPKK-1*-dependent according to the distributive model and MAPKK-1*-independent according to the processive model.
expected to be substantially higher than that for the bulk of the MAPKK-1*, and so the Michaelis constant \( \left( K_{m2} = (d_2 + k_e)(a_2) \right) \) would be expected to be substantially lower than 46 nm.

We calculated the expected initial rate for the chasing of radiolabel from mono- to bisphosphorylated p42 MAPK as a function of the MAPKK-1* concentration, using Equation 15 (see above) and making the assumption that \( K_{m2} \) was 46 nm. The expected initial rate of the chase was calculated to be half-maximal at a MAPKK-1* concentration of about 0.1 \( \mu \)M (just over twice the \( K_{m2} \) value of 46 nm), and to be nearly maximal by an MAPKK-1* concentration of 0.5 \( \mu \)M. The experimentally determined initial rates (Fig. 6B) were found to be in good agreement with the expected dependence.

For comparison, the Michaelis constant for the initial phosphorylation of p42 MAPK by MAPKK-1* has been estimated to be 300 nm.\(^5\) Based on this value, the theoretical initial rate of MAPK pulse labeling was calculated from Equation 5 to be half-maximal by an MAPKK-1* concentration of about 0.6 \( \mu \)M (about twice the \( K_{m1} \) value) and to deviate only slightly from a straight line over a concentration range of 0 to 1 \( \mu \)M. As shown in Fig. 6A, the experimental data were found to agree well with the expected dependence.

Thus, the rate of the second phosphorylation of MAPK's dual phosphorylation was found to saturate at a MAPKK-1* concentration consistent with the \( K_{m} \) value obtained by assessing the phosphorylation of purified MAPK-P by MAPKK-1*. Therefore, the MAPKK-1* that carries out the second phosphorylation of the dual phosphorylation reaction behaves like bulk MAPKK-1* and not like an enzyme with privileged access to MAPK. These findings suggest that MAPKK-1* dissociates completely from MAPK after the first phosphorylation and provide quantitative support for the distributive mechanism.

**DISCUSSION**

The Dual Phosphorylation Occurs by a Distributive Mechanism—Here we present three lines of evidence that argue that the dual phosphorylation of p42 MAPK by MAPKK-1* proceeds through a two-collision, distributive mechanism rather than a single-collision processive mechanism. First, the amount of monophosphorylated MAPK formed exceeded the amount of MAPKK-1* present, which should not be the case if the phosphorylation occurs exclusively by a processive mechanism. This was demonstrated by tryptic peptide analysis (Fig. 3) and corroborated by phosphoamino acid analysis (Fig. 4). Second, the rate at which pulse-labeled monophosphorylated MAPK becomes bisphosphorylated was found to be dependent on the MAPKK-1* concentration (Fig. 5). This behavior is consistent with a distributive mechanism but conflicts with a strong prediction of the processive model. Third, the \( K_{m} \) value for the second phosphorylation of the dual phosphorylation reaction was found to be very similar to the reported \( K_{m} \) value for the phosphorylation of free monophosphorylated MAPK by MAPKK-1 (Fig. 6B). This result suggests that MAPKK-1 dissociates fully from MAPK between the first and second phosphorylations.

The Significance of a Distributive Mechanism—A distributive mechanism has important implications for the fidelity of MAPK signaling. The specificity\(^6\) of MAPK activation should be greater under a distributive mechanism than under a processive mechanism, since activation depends upon two independent MAPKK binding events rather than one. If MAPK can become inappropriately phosphorylated (e.g. by some MAPKK-1*-like enzyme, such as a JNK kinase) and MAPK's sensitivity is the rate at which it becomes bisphosphorylated was found to be dependent on the MAPKK-1* concentration (Fig. 5). This behavior is consistent with a distributive mechanism but conflicts with a strong prediction of the processive model. Third, the \( K_{m} \) value for the second phosphorylation of the dual phosphorylation reaction was found to be very similar to the reported \( K_{m} \) value for the phosphorylation of free monophosphorylated MAPK by MAPKK-1 (Fig. 6B). This result suggests that MAPKK-1 dissociates fully from MAPK between the first and second phosphorylations.

Barondi and co-workers (35, 36) have demonstrated that a Saccharomyces cerevisiae MAPKK, Ste7p, forms high affinity (\( K_{1} \approx 5 \) nm) complexes with the MAPKs it regulates, Kss1p and Fus3, and have presented evidence that the complex must dissociate before Ste7p can phosphorylate Kss1p/Fus3 (and vice versa). If such a complex is also formed between vertebrate MAPKK-1 and p42/p44 MAPK and is important for the subsequent phosphorylation of MAPK by MAPKK, then the specificity of MAPK signaling could be increased still further, again at the cost of some sensitivity.

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\(^5\) N. Ahn, personal communication.
A distributive mechanism for the phosphorylation of p42 MAPK implies that MAPK, in principle, give rise to an ultrasensitive or switch-like steady state response through the phenomenon of multistep ultrasensitivity. The experimentally observed response of MAPK in Xenopus oocyte extracts is, in fact, highly switch-like (14). It should be possible now to determine the extent to which the experimentally observed switch-like response depends upon the distributive dual phosphorylation mechanism described here.

Aspects of the Mechanism That Remain to Be Explored—Each phosphorylation reaction in the dual phosphorylation of MAPK by MAPKK is a two-substrate, two-product (Bi Bi) reaction. We have not yet explored what type of Bi Bi reaction mechanism (for example, sequential or ping-pong, ordered or random) accounts for either step. It will be of interest to pursue this question, in part to compare the enzymology of MAPK with that of other protein kinases, and in part because elucidating these mechanisms could explain why the overall mechanism is distributive rather than processive. For example, if MAPK-P must dissociate from a MAPKK-MAPK-P-ADP complex before ADP can dissociate, it would imply that the overall mechanism must dissociate from a MAPKK

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