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The Activating Dual Phosphorylation of MAPK by MEK Is Nonprocessive

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ABSTRACT: Activation of mitogen-activated protein kinases (MAPKs), also known as extracellular-signal-regulated kinases (ERKs), by MAPK/extracellular protein kinase kinases (MEKs) requires phosphorylation at two sites. The first step in MAPK activation by MEK must be the formation of a MEK·MAPK enzyme−substrate complex, followed by phosphorylation producing monophosphorylated MAPK (pMAPK). Subsequently, one of two events may occur. (1) MEK catalyzes the second and fully activating phosphorylation of MAPK, producing ppMAPK (a processive mechanism). (2) The complex of MEK·pMAPK dissociates before the second phosphorylation occurs, full activation requiring a reassociation of pMAPK with MEK (a nonprocessive or distributive mechanism). Simulations indicate that these two mechanisms predict different kinetics of MAPK activation. Specifically, the nonprocessive mechanism predicts that there will be a paradoxical decrease in the rate of MAPK activation as the MAPK concentration is increased. The present study uses p42 MAPK, also known as ERK2, and MEK1 as representatives of their respective classes of enzymes. We find that increasing the ERK2 concentration decreases the rate of activation by a mechanism which does not involve inhibition of MEK1 function. The accumulation of the active, doubly phosphorylated ERK2 (ppERK2) was directly assessed using a phosphorylation-state-specific antibody. The rate of accumulation of ppERK2 is decreased by increasing the ERK2 concentration. Therefore, the mechanism of ERK2 activation by MEK1 in vitro is nonprocessive.

The mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine-directed protein kinases which are involved in transducing the effects of numerous growth factors, signaling effects as diverse as cellular proliferation, differentiation, and apoptosis (Su & Karin, 1996; Kyriakis & Avruch, 1996). All the MAPKs require phosphorylation on threonine and tyrosine residues in a TXY motif (single-letter code; X, any amino acid), a defining property for the family. A class of homologous enzymes termed MAPK/ERK kinases (MEK) catalyze these reactions, and each MEK has activity toward both the threonine and tyrosine residues in the TXY motif. Accumulating data suggest that each of the known MEKs preferentially activates specific MAPKs (Seger & Krebs, 1995; Zanke et al., 1996). For example, MEK1 regulates the activity of MAPKs, frequently called extracellular-signal-regulated kinases (ERK1 and ERK2).

While the dual threonine/tyrosine phosphorylation of MAPKs catalyzed by MEKs is an apparently unique and critical step in signal transduction, the enzymology of these reactions remains largely uninvestigated. MEK1 purified from mitogen-treated cells phosphorylates both Thr-183 and Tyr-185 of ERK2 (Seger et al., 1992). The activation process may proceed by either a processive or a nonprocessive mechanism. Acting in a processive manner, a single molecule of MEK1 binds to ERK2, catalyzes phosphorylation of threonine 183 and tyrosine 185, and then dissociates.

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Acting in a distributive manner, a molecule of MEK1 binds ERK2, phosphorylates one residue (either Thr or Tyr of the TXY motif), and dissociates; dual phosphorylation requires that the then monophosphorylated ERK2 rebinds to a molecule of active MEK1.

The distinction between nonprocessive and processive mechanisms has several implications for the structure of the MAPK signal transduction pathways. A processive mechanism is more efficient, allowing full activation of MAPK each time a complex with active MEK occurs. The cellular consequence of this mechanism might be minimization of the lag time between activation of MEK and activation of MAPK, perhaps allowing very rapid signaling. On the other hand, Huang and Ferrell (1996) have pointed out that the nonprocessive mechanism makes the rate of MAPK activation dependent on the square of the concentration of active MEK. A squared dependency could cause the activation of MAPK to show greater sensitivity to small changes in the amount of active MEK. Alternatively, a "scaffold" protein capable of binding both MEK and MAPK could convert an inherently nonprocessive reaction to a processive one. STE5, which binds several components of the MAPK pathway for pheromone signaling in budding yeast, might function in this way (Choi et al., 1994). Mutation or deletion of STE5 disrupts pheromone responses. Despite considerable effort, no scaffold protein has been identified in any other organism, including fission yeast, making the generality of this paradigm unclear.

Determining the processivity of MEK activity toward MAPK has at least two important implications. First, detecting a nonprocessive mechanism in vitro fulfills a necessary condition for one model of signal amplification in the MAPK pathway [as suggested in Huang and Ferrell (1996)]. Second, the strategy of screening for putative scaffolding proteins as factors that enhance the rate of MAPK activation (rather than by sequence similarity to STE5) is validated by detecting a nonprocessive mechanism (Scott et al., 1995).

The experiments presented here distinguish between processive and nonprocessive activation mechanisms in vitro using purified MEK1 and ERK2. Kinetic models based on these mechanisms make testable predictions of the dependencies on the ERK2 concentration. The data presented here are consistent with a nonprocessive model of activation.

MATERIALS AND METHODS

Recombinant rat ERK2 was expressed and purified as described in Wu et al. (1991). The expression vector for the constitutively active MEK tagged at the amino terminus with a hexahistidine sequence was a gift of N. Ahn (University of Colorado, Boulder, CO). The protein is similar to that previously described (Mansour et al., 1994). A bacterial system expressing hexahistidine-tagged, doubly phosphorylated, active ERK2 was a gift of M. Cobb (University of Texas, Southwestern Medical Center, Dallas, TX). The constitutively active MEK and active ERK2 were purified by metal affinity chromatography essentially as described in Gardner et al. (1994) with the following modifications: lysis was performed by direct probe sonication without detergent; a commercially available mixture of protease inhibitors was used (Complete, Boeringher-Mannheim, Mannheim, FRG); and chromatography used Talon metal affinity resin (Clontech, Palo Alto, CA). Preparations were greater than 95% pure as judged by Coomassie-stained SDS−PAGE gels. Wild-type, doubly phosphorylated, active MEK1 was obtained from Upstate Biotechnology (Lake Placid, NY).

Polyclonal anti-ERK2 serum (TR12) is described in Her et al. (1993). Antibody to tyrosine 185/threonine 183 phosphorylated ERK2 (Anti-ACTIVE) and reagents for detection using enhanced chemiluminescence were obtained from Promega Corporation (Madison, WI).

All enzymes and other reaction components were equilibrated for 5 min at 30 °C in a reaction buffer containing 20 mM HEPES (pH 8), 1 mM DTT, 10 mM $MgCl_2$, and 1 mM EGTA. To start the reactions, 25 μL of reaction buffer containing ATP (with or without MBP) was mixed with 20 μL of reaction buffer containing MEK1 and ERK2. The final concentration of ATP was 100 μM in all reactions (with or without [γ-^{32}P]ATP to 10 cpm/fmol) with MBP at a nominal concentration of 100 μM (see below). The reactions were stopped by transferring samples to tubes containing an equal volume of 2× SDS sample buffer and boiling. ERK2 and MBP were resolved (15% SDS−PAGE) and visualized with Coomassie stain, and ^{32}P incorporation was quantitated by scintillation counting of excised bands. MBP, high-purity grade from Sigma, is a mixture of molecular masses. The highest-molecular mass form (18 kDa) accounts for about 60% of the total protein as judged by Coomassie staining; the incorporation of ^{32}P into MBP was assessed using the highest-molecular mass band. No more than 2% of the total ATP and MBP, and 0.02% of the ERK2, was exhausted in the course of any reaction.

Simulations of the nonprocessive mechanism assumed that each phosphorylation was independent and adequately described by Michaelis−Menten kinetics. The initial conditions ($t = 0$) are

$$[\text{pMAPK}] = [\text{ppMAPK}] = 0; \quad [\text{MAPK}] = \text{total MAPK}$$

The partition functions are

$$Q_1 = [\text{MAPK}](1 + K[\text{MEK}]);$$
$$Q_2 = [\text{pMAPK}](1 + K[\text{MEK}]);$$
$$Q_3 = [\text{ppMAPK}](1 + K[\text{MEK}])$$

The concentrations of all MEK·MAPK complexes were determined by solving these three equations simultaneously by an iterative, numerical method. The accumulation of pMAPK and ppMAPK was determined from the rate equations

$$d[\text{pMAPK}]/dt = k_{cat}[\text{MEK·MAPK}];$$
$$d[\text{ppMAPK}]/dt = k_{cat}[\text{MEK·pMAPK}]$$

where MEK·MAPK and MEK·pMAPK are enzyme−substrate complexes. ATP and Mg^{2+} were assumed to be saturating. The change in the state of the system was calculated for small time intervals (0.1 of the assumed $1/k_{cat}$). The rate of MBP phosphorylation is proportional to the ppMAPK concentration. In the simulations shown, the affinity constants of MEK for MAPK and monophosphorylated MAPK (pMAPK) were assumed to be identical, as were

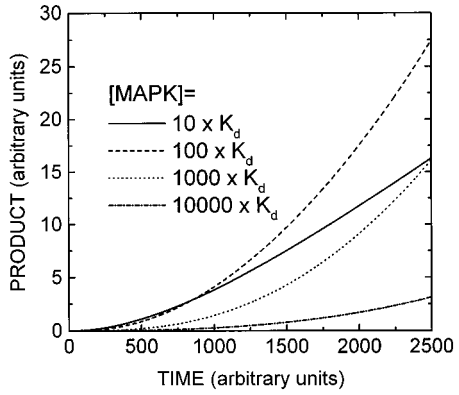

FIGURE 1: Simulation of a nonprocessive model of MAPK activation by MEK. MAPK substrates, MAPK, and active MEK are mixed when $t = 0$. Calculated curves represent product accumulation as a function of time. The MAPK concentrations are relative to the assumed dissociation constant (K_d) of MEK·MAPK. Less than 2% of the MAPK concentration is phosphorylated in any simulation.

the intrinsic rate constants for phosphorylation of MAPK and pMAPK by MEK. The computer code in Basic is available.

RESULTS

In a processive model of MAPK activation by MEK, the dual phosphorylation of MAPK proceeds in a manner indistinguishable from Michaelis−Menten kinetics. The rate of ppMAPK generation is a monotonically increasing function with respect to the MAPK concentration (data not shown). In a nonprocessive model of MAPK activation by MEK, the relationship between the rate of ppMAPK generation and the MAPK concentration is nontrivial (Figure 1). The simulation models experiments in which MBP (a substrate for ppMAPK), MAPK, active MEK1, and cofactors are mixed, allowing MEK to phosphorylate MAPK and ppMAPK to phosphorylate its substrate. The simulation shows that the rate of MBP phosphorylation, and therefore the amount of ppMAPK, depends in a nonlinear manner on the total initial MAPK concentration. For example, at early time points, the system containing 10 units of MAPK yields the greatest activity. Some time later, the system containing 100 units of MAPK yields the greatest activity. At still later times, a system with a higher initial MAPK concentration will display the greatest activity. Therefore, the nonprocessive model predicts that at any specified time there will be a total MAPK concentration which produces the maximal accumulation of phosphorylated MBP. In other words, under certain circumstances, the nonprocessive model predicts that increasing the concentration of MAPK will decrease the rate of MBP phosphorylation. Furthermore, the MAPK concentration yielding the largest accumulation of reaction product will depend on the duration of the assay.

The values of several parameters must be assumed to carry out these simulations. While these assumptions dictate the rate at which the simulated reaction proceeds, the maximum initial rate always occurs with the lowest concentration of MAPK, and at long times, the highest rate occurs with the highest concentration of MAPK (data not shown). Furthermore, the inclusion of a MEK·ppERK complex results in no relevant changes in the predictions of this simulation (data not shown).

The experimental effect of increasing the total ERK2 concentration on the accumulation of phosphorylated MBP

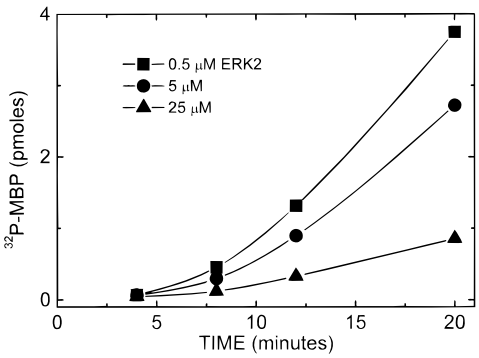

FIGURE 2: Increasing the ERK2 concentration decreases the accumulation of phosphorylated MBP. MAPK, constitutively active MEK (0.4 nM), MBP (100 μM), and Mg^{2+}/ATP are mixed when $t = 0$.

is depicted in Figure 2. The constitutively active mutant of MEK1, wild-type ERK2, and MBP were incubated with the reaction mixture, and the reactions were stopped at the times indicated. At all concentrations of ERK2, the accumulation of product shows a positive curvature, which is expected since activation of ERK2 is occurring throughout the time course. Most importantly, the accumulation of product is greatest with the lowest ERK2 concentration. Therefore, under these conditions, the accumulation of product is a decreasing function of the total ERK2 concentration. Essentially identical data were obtained using wild-type, activated MEK1 in place of mutant constitutively active MEK1 (data not shown). These data are consistent with a nonprocessive model of ERK2 activation by MEK1.

Other mechanisms which could account for the data in Figure 2 were considered and excluded. For example, the ERK2 preparation could contain an inhibitor of MEK1 function, ERK2 itself could bind to some regulatory site of MEK1 and act as an inhibitor (substrate inhibition), or ERK2 might carry out a feedback inhibitory phosphorylation of MEK1. If the decrease in ERK2 activation with increasing ERK2 concentration were due to any of these mechanisms, then the rate at which ERK2 is itself phosphorylated by MEK1 would be predicted to decrease with increasing ERK2 concentration. Consequently, the rates of ERK2 phosphorylation and MBP phosphorylation were determined in the same experiment (Figure 3). The rate of phosphorylation of ERK2 by MEK1 asymptotically approaches a maximum, suggesting that the incorporation of phosphate into ERK2 is not inhibited by high concentrations of ERK2 (Figure 3A). Furthermore, the rate of ERK2 phosphorylation is constant as a function of time (compare the rates during the first and second 15 min periods of the reaction). Also, the rate of MBP phosphorylation passes through a maximum as a function of the ERK2 concentration, as predicted by the nonprocessive model (Figure 3B). Therefore, a high ERK2 concentration does not inhibit the incorporation of phosphate into ERK2 but does inhibit the incorporation of phosphate into MBP. These data strongly argue that the decreasing rate of MBP phosphorylation with increasing ERK2 concentration is not due to inhibition of activated MEK1.

Activity toward MBP is a surrogate marker of the phosphorylation state of ERK2. The use of activity as a direct measure of the ppERK2 concentration could mask other effects of the increasing total ERK2 concentration. For example, the highest ERK2 concentration used in these assays is 26 μM, and the concentrations of two substrates,

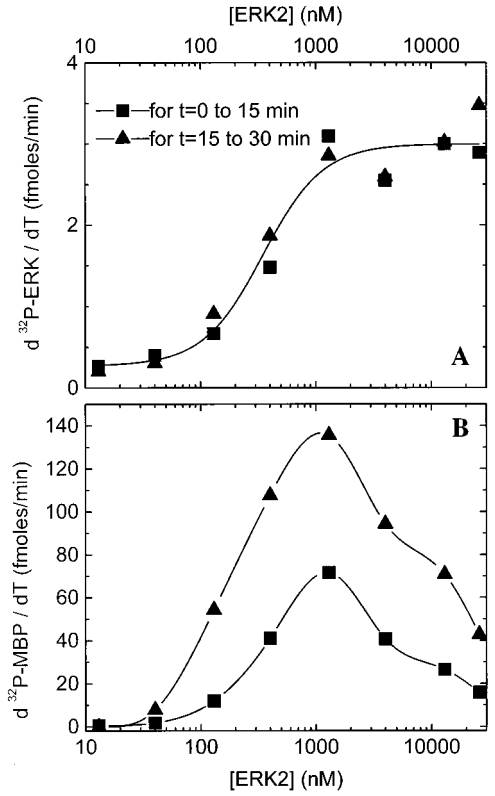

FIGURE 3: Increasing the ERK2 concentration decreases the accumulation of phosphorylated MBP but does not inhibit MEK. MAPK, constitutively active MEK (0.4 nM), MBP, and Mg^{2+}/ATP are mixed when $t = 0$. Aliquots were removed, and the reaction was quenched at 15 (squares) and 30 (triangles) min. Note that the rate of MAPK phosphorylation is the same at 15 and 30 min, while the rate of MBP phosphorylation is greater at 30 min than at 15 min. Incorporation of ^{32}P in the absence of MEK was subtracted from all values as blanks. Rates are calculated with the equations rate(15′) = (phosphate incorporated at 15′)/15 and rate(30′) = (phosphate incorporated at 30′ − phosphate incorporated at 15′)/15′.

MBP and ATP, are 100 μM. This raises the possibility that inhibition by a high ERK2 concentration is due to sequestration of substrate by ERK2. To address these issues, we directly assessed the accumulation of ppERK2, rather than inferring ppERK2 accumulation from the rate of MBP phosphorylation. A commercially available antibody, anti-ACTIVE, specific for the doubly phosphorylated state of ERK2 was used to monitor the accumulation of ppERK2. Time courses of ppERK2 accumulation at two concentrations of ERK2, incubated with and without active MEK1, confirmed the antibody's specificity for ppERK2 (data not shown). The relative specificity of this antibody for pTyr/pThr-ERK2 over either monophosphorylated species was also confirmed by treating the ppERK2 with either a protein tyrosine phosphatase (PTP1B) or a serine/threonine phosphatase (PP2A) (data not shown).

Consistent with the nonprocessive model, increasing the concentration of ERK2 20-fold resulted in a profound diminution in the amount of accumulated ppERK2 (Figure 4). Both ERK2 and ppERK2 at two concentrations (as a positive control) were incubated for 30 min with and without active MEK. Accumulation of ppERK2 was assessed using anti-ACTIVE antibody specific for doubly phosphorylated ERK2 (top), and the relative quantities of ERK2 in each lane were confirmed by reprobing the same Western blot with a polyclonal antibody to ERK2 (bottom). In the presence of

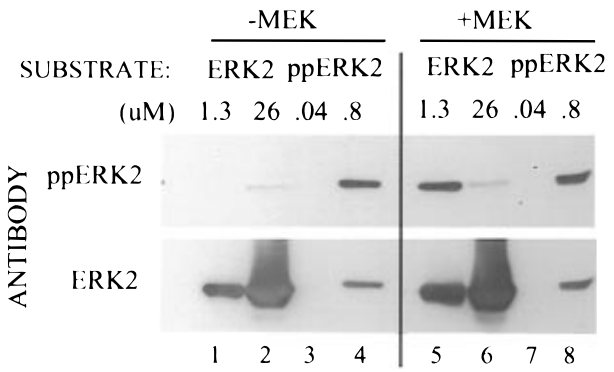

FIGURE 4: Increasing the ERK2 concentration decreases the accumulation of ppERK2. ERK2 or activated ERK2 (ppERK2) was mixed with constitutively active MEK1 (2 nM) and Mg^{2+}/ATP when $t = 0$. The reaction was stopped at 30 min by the addition of sample buffer for Western blotting with anti-ACTIVE antibody specific for doubly phosphorylated ERK2 (top) and with an anti-MAPK polyclonal antibody (bottom). [ppERK2] = 40 and 800 nM. [ERK2] = 1.3 and 26 μM.

active MEK, far more ppERK2 accumulates after 30 min at the lower total ERK2 concentration (lane 5 versus 6). The lower concentration (1.3 μM) corresponds to the concentration which yielded the greatest accumulation of phosphorylated MBP at 30 min (Figure 3B). Minimal accumulation of ppERK2 occurs in the absence of active MEK (lanes 1 and 2), and the reactivity with purified, active (hexahistidine-tagged) ppERK2 is not perceptibly altered by the presence of active MEK (lanes 4 and 8), indicating that this preparation is a good positive control. These data provide a second indication that the rate of ppERK2 accumulation is a complex function of the ERK2 concentration, clearly distinct from the simple monotonic relationship expected for a processive reaction.

DISCUSSION

Processivity reflects the relative rates at which the MEK·pERK2 complex dissociates compared to the rate at which the MEK·pERK2 complex gives rise to ppERK2. The nonprocessive mechanism demonstrated here indicates that the dissociation rate of the MEK·pERK2 complex is faster than the rate of phosphorylation of the second site. Analysis of these data indicates that the dissociation is at the very least 5 times as fast as the catalytic rate (W. R. Burack and T. Sturgill, unpublished results).

Given two sites for phosphorylation, phosphorylation may proceed by an ordered mechanism. There is evidence that tyrosine 185 is phosphorylated first (Haystead et al., 1992). The issues of processivity and ordered phosphorylation are separable and distinct aspects of the activation mechanism. The simulations performed here yield the same qualitative results regardless of whether the phosphorylation reactions are presumed to be randomly or strictly ordered (data not shown).

The in vivo mechanism of MAPK activation by MEK may differ from that observed in vitro using purified MEK1 and ERK2. The *Saccharomyces cerevisiae* protein STE5 has been shown to bind both a yeast ERK (FUS3) and MEK (STE7) with sufficient affinity to allow purification of the complex on glycerol gradients (Choi et al., 1994). A scaffold protein might stabilize a complex of active MEK and MAPK so that the complex has a lifetime longer than the k_{cat}, causing activation to occur processively. Therefore, STE5 and

possible mammalian proteins with analogous functions may convert the in vitro MAPK activation from nonprocessive to processive. No mammalian homologs of STE5 have been reported, although a factor which enhances the rate of MAPK activation by MEK has been purified (Scott et al., 1995). But even without STE5, STE7 and FUS3 have been shown to form a complex in vitro (Bardwell et al., 1996). However, this complex must dissociate for MAPK to be phosphorylated by the MEK in vitro. Although the role of this nonproductive complex is unclear, it may provide a means of coupling specific MEK and MAPK isozymes to each other (Bardwell & Thorner, 1996).

Signal amplification is frequently invoked as the raison d'etre for the cascade of sequentially activated protein kinases. While the in vivo degree of amplification remains unknown, the sensitivity of the MAPK pathway in *Xenopus* oocyte extracts has been carefully examined and analyzed (Huang & Ferrell, 1996). The simulation presented here is entirely distinct from that presented by Huang and Ferrel (1996) which models a steady-state situation in a complex mixture. In the absence of phosphatases for reversing activation, the steady state invoked by Huang and Ferrel cannot exist. Here, the kinetics of the initial phase of activation (pre-steady state) are simulated and assessed with purified components. Regardless, the signal amplification observed in oocyte extracts is consistent with the nonprocessive mechanism defined here for purified MEK1 and ERK2 in vitro.

The finding that MEK1 activates ERK2 by a largely nonprocessive mechanism has several implications both for those intimately concerned with these enzymes and for the wider field of signal transduction enzymology. A technical but perhaps very useful comment is related to a published report and several informal reports: it is difficult to obtain full phosphorylation of MAPK with MEK in vitro (Haystead et al., 1992). Our data suggest a reason for this difficulty: under certain conditions, MEK activity is largely devoted to just the first phosphorylation event and only rarely phosphorylates the second site. Using less MAPK in these reactions may then give better results. For the nonprocessive mechanism to form the basis of signal amplification, MAPK in vivo must be freely diffusible with respect to MEK, and MEK freely diffusible with respect to its activator. This requirement for free diffusion seems to be inconsistent with the finding of stable long-lived complexes of a MEK activator, MEK, MAPK, and the scaffolding protein, STE5 (Choi et al., 1994). Regardless, the characterization of the in vitro activation as nonprocessive suggests a role for scaffolding proteins in modulating the kinetics of signal transduction.

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