The Kinetic Mechanism of the Dual Phosphorylation of the ATF2 Transcription Factor by p38 Mitogen-activated Protein (MAP) Kinase α

IMPLICATIONS FOR SIGNAL/RESPONSE PROFILES OF MAP KINASE PATHWAYS*

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The mitogen-activated protein kinases (MAPKs) are a family of enzymes conserved among eukaryotes that regulate cellular activities in response to numerous external signals. They are the terminal component of a three-kinase cascade that is evolutionarily conserved and whose arrangement appears to offer considerable flexibility in encompassing the diverse biological situations for which they are employed. Although multistep protein phosphorylation within mitogen-activated protein kinase (MAPK) cascades can dramatically influence the sensitivity of signal propagation, an investigation of the mechanism of multisite phosphorylation by a MAPK has not been reported. Here we report a kinetic examination of the phosphorylation of Thr-69 and Thr-71 of the glutathione S-transferase fusion protein of the trans-activation domain of activating transcription factor-2 (GST-ATF2-(1–115)) by p38 MAPKα (p38α) as a model system for the phosphorylation of ATF2 by p38α. Our experiments demonstrated that GST-ATF2-(1–115) is phosphorylated in a two-step distributive mechanism, where p38α dissociates from GST-ATF2-(1–115) after the initial phosphorylation of either Thr-69 or Thr-71. Whereas p38α showed similar specificity for Thr-71 and Thr-69 in the unphosphorylated protein, it displayed a marked difference in specificity toward the mono-phosphoisomers. Phosphorylation of Thr-71 had no significant effect on the rate of Thr-69 phosphorylation, but Thr-69 phosphorylation reduced the specificity, $k_{cat}/K_M$, of p38α for Thr-71 by approximately 40-fold. Computer simulation of the mechanism suggests that the activation of ATF2 by p38α in vivo is essentially Michaelian and provides insight into how the kinetics of a two-step distributive mechanism can be adapted to modulate effectively the sensitivity of a signal transduction pathway. This work also suggests that whereas MAPKs utilize docking interactions to bind substrates, they can be weak and transient in nature, providing just enough binding energy to promote the phosphorylation of a specific substrate.

The mitogen-activated protein kinases (MAPKs)1 are a family of enzymes conserved among eukaryotes that regulate cellular activities in response to numerous external signals. They are implicated in processes ranging from pheromone responses and cell wall formation in yeast to mitogenesis, apoptosis, and stress responses in mammalian cells (1, 2). They are the terminal components of a three-kinase cascade that is evolutionarily conserved and whose arrangement appears to offer considerable flexibility in encompassing the diverse biological situations for which they are employed.

A MAP kinase cascade is generally composed of three protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase (MAPK) and a MAP kinase kinase (MAPKK) arranged in a linear hierarchical fashion as shown in Scheme 1. Although there are a large number of MAPKKKs, which become activated through a variety of mechanisms, MAPKKKs are highly specific for their conjugate MAPKK, which they activate by dual phosphorylation. In turn MAPKKs activate their conjugate MAPKs, also by dual phosphorylation, whereas MAPKs, which are the least specific kinases in these modules, phosphorylate many proteins, typically at more than one site (3). Although this simple picture is somewhat obscured by the existence of an abundance of different MAPKKKs and by the existence of MAPKK and MAPK isoforms, the three-tiered hierarchy appears to be well conserved. The kinases do not function in isolation, because protein phosphatases represent the force against which they labor to drive the steady-state concentrations of phosphorylated proteins beyond critical thresholds. There are several protein phosphatases, some such as the MAPK phosphatases are specific for certain MAPKs (4–6) whereas others such as protein phosphatases 1 (7) and 2 (8–10) dephosphorylate many cellular proteins.

Although an arrangement of kinases in a cascade such as a MAPK cascade generally leads to amplification of a signal, it also provides considerable means for control. There has been substantial interest recently in the mechanism of signal prop-

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The abbreviations used are: MAPK, mitogen-activated protein kinase; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; MKK6, mitogen-activated protein kinase kinase 6; MKK3, mitogen-activated protein kinase 3; p38α, p38 MAP kinase α; ATF2, activating transcription factor 2; ERK, extra signal regulated protein kinase; MEK4, MAP or ERK kinase kinase 4; HPLC, high pressure liquid chromatography; JNK, c-Jun N-terminal protein kinase; MS, bombarded mass fragmentation pattern of a single m/z ion derived from a MS; GST, glutathione S-transferase; ATF2(glu212), ATF2(212), and ATF2(glu212) correspond to GST-ATF2-(1–115) phosphorylated on Thr-69, Thr-71, or both Thr-69 and Thr-71.
agitation through MAPK cascades and, in particular, the generation of thresholds (11). Potential sources of thresholds in a MAPK cascade include contributions from zero-order ultrasensitivity\(^2\) (12, 13) and multistep mechanisms (12, 13), as well as other mechanisms such as the regulated translocation of proteins between organelles (14). Another potential source of ultrasensitivity is multistep phosphorylation (11). Indeed, the dual phosphorylation of MAPK by MAPKK has been shown to occur through a two-step distributive mechanism (15, 16), and theoretical calculations support the notion that this mechanism contributes to the observed switch-like behavior of the MAPK cascade responsible for the turning on and off of the cell cycle in oocytes (17). Although the generation of thresholds within signaling cascades is important, they may not always be necessary or even beneficial. For example, control over a wider range of stimulus may be more appropriate in some situations, such as when cells are exposed to certain stresses. MAPKs activate many proteins by dual phosphorylation, which is, potentially, an important source of control and which to our knowledge has not been investigated before. Because they are known to form complexes with potential substrates in cells and utilize discrete docking interactions to bind substrates, a major unanswered question is whether or not they phosphorylate multiple sites on a substrate processively. To expand our current mechanistic knowledge of MAPK cascades and in particular the MAPK enzymes and to characterize further possible mechanisms of control, we studied the kinetic mechanism of the dual phosphorylation of the glutathione S-transferase fusion protein of ATF2 (GST-ATF2-(1–115)) by the MAPK, p38\(\alpha\). This fusion protein corresponds to the trans-activation domain of ATF2 and is an excellent protein substrate for p38\(\alpha\) (18) and an excellent model for the phosphorylation of the full-length protein.

p38\(\alpha\) (19, 20) is involved in relaying stress-related signals in mammalian cells. It is regulated by two MAPKKs termed MAP kinase kinase 3 (MKK3) and MAP kinase kinase 6 (MKK6) (21, 22), which phosphorylate the activation loop of p38\(\alpha\) twice, once on a tyrosine and once on a threonine. Several activators of one or both of these enzymes have been identified that include MEKK4 (MAP or ERK kinase kinase) (23–25), apoptosis-stimulated kinase (26, 27), and transforming growth factor-\(\beta\)-activated kinase (28–30). The basic region-leucine zipper protein activating transcription factor 2 (ATF2) is a substrate of p38\(\alpha\) and other stress-activated MAPKs. It is a DNA-binding protein that forms a homodimer or heterodimer with c-Jun, binds to cyclic AMP-response elements (CREs), and stimulates CRE-dependent transcription of genes. Recently, it was also shown to be a histone acetyltransferase, specific for histones H2B and H4 (31). Increases in its transcriptional activity (32), its acetyltransferase activity (31), and its cellular stability (33) are associated with the dual phosphorylation of ATF2 on Thr-69 and Thr-71.

We found that the kinetic mechanism for the dual phosphorylation of the two residues within GST-ATF2-(1–115), Thr-69 and Thr-71, occurs by a two-step mechanism where p38\(\alpha\) dissociates from the protein after each phosphorylation event. The kinetics of this process is predicted to have important implications for the activation of ATF2 by p38\(\alpha\) in \textit{vivo} and highlights the potential that two-step distributive mechanisms have for controlling the amplitude sensitivity of signal transduction pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trizma base was purchased from EM Reagents (Gibbstown, NJ) and ammonium carbonate from Fisher. All other buffer components and chemicals were obtained from Sigma. Qiagen Inc. (Santa Clarita, CA) supplied nickel-nitriilotriacetic acid-agarose. GST fusion proteins were purified with glutathione-agarose from Sigma. Kinase assays were conducted with special quality ATP from Roche Molecular Biochemicals and \(\gamma\)-\(^{32}\)P-ATP from ICN (Costa Mesa, CA). Proteins were isolated on Optipan nitrocellulose membrane from Schleicher & Schuell and digested with sequencing grade trypsin from Roche Molecular Biochemicals. Plasmids used to express GST-ATF2-(1–115) (18) and His-p38\(\alpha\) (35) have been reported previously.

**Expression and Purification of Rat p38 MAP Kinase —**Activated mouse p38\(\alpha\) (19, 20) was expressed in \textit{Escherichia coli} (BL21 DE3 pLys S) with an N-terminal hexahistidine tag (34) to facilitate its purification, essentially according to the method of Khokhlatchev et al. (35). This method typically yields –0.25 mg of enzyme per liter of starting culture with a specific activity of 970 nmol/min/mg (250 \(\mu\)M ATP and 50 \(\mu\)M GST-ATF2-(1–115)).

**Expression and Purification of GST-ATF2-(1–115) —**The GST-ATF2-(1–115) construct was expressed as a glutathione S-transferase fusion protein in \textit{E. coli} (BL21 DE3 pLys S) and purified essentially according to the method of LoGrasso et al. (18) with an additional high resolution Mono-Q anion exchange chromatography step. Protein fractions from a glutathione-agarose column (obtained by following standard protocols (18)) were applied to a Mono-Q HR 5/5 chromatography column pre-equilibrated with buffer B (50 mM Tris-HCl, pH 7.5, 0.1% (by volume), 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM N-tert-sulfonyl-phenylalanine chloromethyl ketone, 1 mM benzamidine, 2 mM EDTA, 2 mM EGTA). The applied protein was fractionated using a stepwise NaCl gradient (0–50 mM NaCl per ml) at a flow rate of 0.8 ml/min and eluted as a single peak centered at 0.11 mM NaCl. Selected aliquots were concentrated in a Centricon-10 (Amicon, Bedford, MA) at 0 °C to 3 mg/ml. Electrospray mass spectrometry confirmed the identity of the protein as GST-ATF2-(1–115) with a molecular mass of 39,655 Da.

**Protein Kinase Assay of p38\(\alpha\) —**Protein kinase assays were conducted at 27 °C in buffer C (50 mM HEPES, pH 7.4, 100 mM KCl, 2 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM EGTA), containing 10 mM \(\gamma\)-\(^{32}\)P-ATP (100–1000 cpm/pmol), 10 \(\mu\)g/ml bovine serum albumin, and 10 mM MgCl\(_2\) in a final volume of 50–100 \(\mu\)l. p38\(\alpha\) and GST-ATF2-(1–115) were preincubated for 5 min before the reaction was initiated by the addition of ATP. Aliquots (5–10 \(\mu\)l) were taken at set time points and applied to 2 \times 2 cm\(^2\) P81 cellulose paper (36). The papers were washed 3 times for 10 min in 50 mM phosphoric acid (H\(_3\)PO\(_4\)) and then in acetone and dried, and the amount of labeled protein was determined by counting the associated counts/min on a Packard Instrument Co. 1500 scintillation counter at a \(\alpha\) value of 2. The \(A_{\text{cat}}\) (1 mg ml\(^{-1}\)) of the proteins was determined by denaturing them in 6 M guanidine chloride following the method of Gill and von Hippel (37).

**Product Analysis for the Phosphorylation of GST-ATF2-(1–115) by p38\(\alpha\)–p38\(\alpha\) (42 nm) and GST-ATF2-(1–115) (25 \(\mu\)l) were incubated under the standard assay conditions in buffer C (minus 100 mM KCl) in a 250-\(\mu\)l final volume. The reaction was stopped by the addition of 250 \(\mu\)l of 0.2 M EDTA, pH 8.0. The sample was applied to a Centriprep-10 and the buffer exchanged with ammonium carbonate 25 mM, pH 8.0 (300 \(\mu\)l final volume). The protein was then incubated at 37 °C with 5 \(\mu\)g of trypsin. After 3 h, another 5 \(\mu\)g of trypsin was added, and the suspension was left for a further 12 h. After standing on ice for 10 min, the suspension was centrifuged for 10 min at 13,000 \(\times\) g. The supernatant containing >95% of the \(\gamma\)-\(^{32}\)P was removed and fractionated as described below. Fractions containing tryptic peptides were evaporated almost to

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\(^2\) Golbeter and Koshland used the term ultrasensitive (and subsensitive) to define any system that requires less (more) than an 81-fold increase in input to drive it from 10% maximal to 90% maximal activation (13).
dryness before the addition of 0.5 ml of 50% (by volume) water/aceto- 
nitrile and analyzed by electrospray mass spectrometry as described below. 

Time Course for the Phosphorylation of GST-ATF2-(1–115) by p38α—The pulse-chase experiment is composed of two parts, the pulse followed by the chase. Initially, the substrate GST-ATF2-(1–115) (16.2 μm) was incubated with p38α (24 nM) and [γ-32P]ATP (1500 cpm/fmol) (1 μCi/ml of 32P ATP (5000 cpm/ml)) in a volume of 100 μl in the standard kinase assay buffer. After 55 min, 5.4 × 10^7 cpm was incor- 
porated into the GST-ATF2-(1–115) corresponding to 1.1% of the theo-
mol of phosphate per mol of GST-ATF2-(1–115) was incorpo-
rated into the GST-ATF2-(1–115) with a rate-
constant that was significantly faster than for the remaining 
0.5 mol/mol. Steady-state Michaelis parameters were deter-
mined by the initial rate method used by LoGrasso et al. (18). Apparent 
values for substrate 
and 16.2 μm GST-ATF2-(1–115) in a 100-
ml final volume. Aliquots (10 μl) at various times were added to hot 2 × Laemmli 
buffer (60 μl Tris-Cl, pH 6.8, 2% SDS, 10% (by volume) glycerol, 0.025% (by weight) bromphenol blue) and were resolved by 10% SDS-
polyacrylamide gel electrophoresis. The samples were then transferred 
to nitrocellulose membranes, and bands corresponding to GST-ATF2-
(1–115) were identified by staining with Ponceau S (0.1% w/v in 1% 
acetic acid). After destaining (3 × 500 ml washes of water), the 
membrane was soaked in 0.5% polyvinylpyrrolidone (to prevent trypsin from 
acting as above. Control experiments were performed in the absence of a pulse, 
using ATP with a specific activity identical to that in the chase. The 
maximum incorporation into GST-ATF2-(1–115) in the control was 
2.05 × 10^6 cpm which corresponds to 3.8% of the pulse. 

Calculation of Stimulus Response Curves—Numerical integration of 
the appropriate rate equations was used to calculate the steady-state 
centricity of the species shown in Scheme 2. Calculations were 
performed on a Macintosh Powerbook Computer using KintekSim (Kin- 
tek Corp.) and the PC emulator Virtual PC 3.0 (Connectix). Each step, 
represented in Scheme 2, was modeled as a two-step reaction where enzyme and substrate combine (k_{cat}) to form a Michaelis 
complex, which partitions between dissociation (k_{d}) and product forma-

3This was calculated by accounting for the ATP (0.64 μM in 70 μl = 
4.48 × 10^{-13} mol) that remained after 55 min of the pulse phase and the 
1516-fold dilution by the cold ATP in the chase. 

4These values are apparent because the assay does not distinguish 
between the phosphorylation of Thr-69 and Thr-71.
quality sequencing grade trypsin, and fractionating the phosphopeptides by reverse phase HPLC (see “Experimental Procedures”). To examine the phosphorylation of individual residues we conducted the time course shown in Fig. 1, during the course of which aliquots were taken to examine the progress of the reaction (Fig. 2, A–C). GST-ATF2-(1–115) was isolated and subjected to trypic digestion, where the recovery of the radio-labeled protein and peptides were monitored at each stage of the analysis. The overall transfer of the protein to the membrane and subsequent digestion were found to yield >90% of the initial radio-label.

Fig. 2A shows that after 1 min three trypic peaks, T1–T3, could be resolved by reverse phase HPLC. Upon mass spectrometric analysis, peak T1 was found to contain a peptide of molecular mass 1774 ± 1 Da, corresponding to residues 60–74 of ATP2, phosphorylated on two residues. T2 and T3 were found to contain a peptide of molecular mass 1694 ± 1 Da, which corresponded to a singly phosphorylated tryptic peptide 60NDSVIVADQTPPTPTR74 (where T* indicates phosphothreonine) (M = 1773.7 calculated), phosphorylated on Thr-69 and Thr-71 (Fig. 3). The peptide associated with peak T2 corresponded to the peptide 60NDSVIVADQTPPTPTR74 (M = 1693.7), monophosphorylated on Thr-69. The peptide associated with peak T3 corresponded to the peptide 60NDSVIVADQTPPTPTR74 (M = 1693.7), phosphorylated on Thr-71. In summary, both the M+ and M2+ peaks of the expected molecular mass were observed, and the fragmentation patterns of these ions are consistent with their assignment. The presence and/or absence of critical ions, which are summarized in Table I, provide strong evidence for the unambiguous assignment of each peptide.

The Kinetics of GST-ATF2-(1–115) Phosphorylation Is Not Processive—A primary goal of this work was to establish whether p38α catalyzed the phosphorylation of GST-ATF2-(1–115) with a processive (Scheme 3, upper pathway) or a two-step distributive mechanism (Scheme 3, lower pathway). ATF2 contains a putative docking sequence similar to the docking sequence termed the docking site for ERK and JNK, LXL, the DEJL-docking sequence (3), found in a number of MAPK substrates. This putative docking sequence is likely to dock on to Asp-313 and Asp-316 of p38α (41), which lies >30 Å outside of the catalytic active site (34). If this docking hypothesis is correct the DEJL sequence should form a common binding platform for both phosphorylation events. Thus a processive mechanism could be favorable, because repositioning of the threonine residues (Thr-69 and Thr-71) in the active site could be brought about through minimal reorganization of the intermediate enzyme-substrate complexes, while contact is maintained at the docking site.

With the identity of the peptides in peaks T1–T3 in hand, we were able to analyze the kinetic mechanism of p38α in more detail. Under saturating conditions, Thr-69 and Thr-71 were phosphorylated at almost identical rates (Fig. 2A), despite lying within a different sequence context. The product ratio of an enzymatic reaction is determined by the ratio of the specificity constants (kcat/KM) under all conditions (40). Therefore our analysis suggests that p38α is approximately only 1.2-fold more specific for Thr-71 than for Thr-69 in the unphosphorylated protein. This is consistent with previous observations that the specificity of MAP kinases, with respect to the sequences that surround the phosphorylation sites (SP or TP motifs), are fairly relaxed (42). By cutting out the peaks and weighing the paper we were able to determine that the concentration of the phosphorylated proteins, ATF2−69P, ATF2−71P, and ATF2−69P71P, after a 1-min incubation, were −21, 25-, and 3-fold in excess of the enzyme, p38α. This excess of the mono-phosphorylated substrates is strong evidence that the mechanism of dual phosphorylation is not processive. It strongly suggests that the mechanism is a two-step distributive mechanism, where the intermediary substrates (S*) dissociate from the enzyme faster than at least one of the following steps: nucleotide exchange, repositioning of S*, or phosphoryl transfer (Scheme 3) (15).

**Pulse-Chase Analysis of the Dual Phosphorylation of GST-ATF2-(1–115)—**The experiment described above does not rigorously prove that the formation of the mono-phosphorylated proteins ATF2−69P and ATF2−71P precedes the formation of the dual-phosphorylated product ATF2−69P71P. To provide direct evidence that both ATF2−69P and ATF2−71P are directly converted to the dual-phosphorylated product ATF2−69P71P, a pulse-chase experiment was performed. In this experiment radiolabeled ATF2−69P and ATF2−71P were first formed in the pulse phase using [γ-32P]ATP (carrier-free). During the chase phase, incorporation of unlabeled phosphate into the free site occurs to give labeled ATF2−69P71P. By monitoring both the

![Fig. 1. Concentration of phosphate incorporated into GST-ATF2-(1–115) by p38α against time (●).](image-url)
disappearance of the mono-phospho forms of ATF2-(1–115), ATF2\(^{69P}\), and ATF2\(^{71P}\) and the appearance of the dual-phosphorylated product ATF2\(^{69P/71P}\), one can provide evidence for the direct conversion of the mono-phosphorylated substrates to the dual-phosphorylated product. To label GST-ATF2-(1–115) in the pulse phase, 24 nM p38\(\alpha\), GST-ATF2-(1–115) (16.2 \(\mu\)M), and MgATP (1 \(\mu\)M) were combined in a small volume and incubated for 55 min before the ATP was diluted >1500-fold by the addition of 1 mM carrier ATP. The progress of the reaction was then monitored in the same manner as the time course experiments described above. During the pulse phase, approximately equal proportions of ATF2\(^{69P}\) (0.09 \(\mu\)M) and ATF2\(^{71P}\) (0.09 \(\mu\)M) were formed as well as a small amount of the doubly labeled ATF2\(^{69P/71P}\) (less than 0.001 \(\mu\)M) (Fig.

![Fig. 2. A–C, tryptic digestion of GST-ATF2-(1–115) phosphorylated by p38\(\alpha\). Aliquots from the experiment shown in Fig. 1 were treated with trypsin and the peptides generated, separated by reverse phase HPLC at pH 6.8 (10 mM ammonium acetate), as explained under “Experimental Procedures.” Peak T1 contained a peptide of molecular mass 1773.7 corresponding to residues 60–74 of ATF2 phosphorylated on Thr-69 and Thr-71. Peak T2 contained a peptide of molecular mass 1683.7 corresponding to residues 60–74 of ATF2 phosphorylated on Thr-69. Peak T2 contained a peptide of molecular mass 1693.7 corresponding to residues 60–74 of ATF2 phosphorylated on Thr-71. D and E, ATF2\(^{69P}\) and ATF2\(^{71P}\) are substrates of p38\(\alpha\). GST-ATF2-(1–115) (16.2 \(\mu\)M) was pulse labeled under the standard kinase assay conditions with 24 nM p38\(\alpha\) and \(^{32}\)P-ATP (1000 cpm/pmol) (1 \(\mu\)M) for 55 min in a final volume of 100 \(\mu\)l. To 70 \(\mu\)l of the pulse unlabeled ATP and p38\(\alpha\) was added to a final volume of 77 \(\mu\)l and final concentrations of 0.97 \(\mu\)M and 46 nM respectively. Analysis of the reaction was performed as described in the legend.

Activation of the ATF2 Transcription Factor by p38 MAP\(\alpha\)
The two-step distributive mechanism allows for easy adaptation of sensitivity—The difference in the specificity of p38α for the mono-phosphorylated intermediates was surprising, and therefore we examined the possible consequences of such differential specificity on the activation of a substrate by a two-step distributive mechanism in vivo (Scheme 2A). To do this we modeled the two-step distributive mechanism shown in Scheme 2A and examined the effect of decreasing the specificity of the enzyme for one mono-phosphoform of the protein, S\textsuperscript{69P}, by 10-fold. This conservatively represents the decreased affinity of p38α for ATF2\textsuperscript{69P} that we observed experimentally. The parameters chosen were not chosen to be specific for p38α but were chosen to be generally representative of an MAPK. For comparison, we also modeled a processive mechanism (Scheme 2B), which is not expected to display significant intrinsic ultra-sensitivity in the absence of zero-order effects (12, 13). Initially, we assumed reasonable values for enzyme and substrate concentrations, taking into account both total protein concentration and subcellular localization. Reasonable K\textsubscript{M} values were assumed based on experimentally measured values for typical protein substrates.

In the two-step distributive mechanism (Scheme 2A) the dual-phosphorylated product, S\textsuperscript{P1P2}, is formed in two discrete steps. In step 1 the enzyme and substrate combine to give a mono-phosphorylated protein S\textsuperscript{P1}, the intermediary enzyme-protein complexes (E\textsuperscript{S\textsuperscript{P1}} or E\textsuperscript{S\textsuperscript{P2}}) then fully dissociate, before rebinding with the enzyme to give the dual-phosphorylated protein (S\textsuperscript{P1P2}). Each phosphorylation step is reversed by a protein phosphatase. In the processive mechanism (Scheme 2B) formation of S\textsuperscript{P1P2} occurs after just one enzyme-substrate collision (middle pathway) and the protein phosphatases reverse this process by the upper and lower pathways. It is assumed that S\textsuperscript{P1} and S\textsuperscript{P2} are substrates of the MAPK. Typical cellular concentrations of MAPKs, for example ERK2, are in the range of 1–2 μM, but because MAPKs are localized within cells it can be reasonably assumed that not all of the MAPK

within the limits k\textsuperscript{cat} = 0.1–0.6 s\textsuperscript{−1} and K\textsubscript{M} = 25–150 μM for this step, little differences in the fit were seen.

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**TABLE I.** Critical ions Y\textsubscript{4}–Y\textsubscript{6} (38) used to assign peptides T1–T3

| Peptide\textsuperscript{a} | Amino acid sequence | Mass of Y\textsubscript{4}\textsuperscript{72PTR74} | Mass of Y\textsubscript{5}\textsuperscript{72PTR74} | Mass of Y\textsubscript{6}\textsuperscript{65PTR74} |
|---------------------------|---------------------|-------------------------|-------------------------|-------------------------|
| T1 | T1 | 373 | 554 | 651 |
| T2 | T2 | 373 | (473)' | 571 |
| T3 | T3 | 373 | 553 | 651 |

\textsuperscript{a} See Fig. 2.

\textsuperscript{b} The presence of an ion at $M_{r}$ = 373 for T1–T3 and the absence of an ion at $M_{r}$ = 472 is consistent with the absence of phosphate on Thr-73.

\textsuperscript{c} Not observed.

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FIG. 3. Mass spectrum of the dual-phosphorylated peptide T1 (NDSVIVADQTPPTP\textsubscript{PTR}). Y and B series ions are indicated (See Table I for diagnostic ions).

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Scheme 3. Possible mechanisms for the dual phosphorylation of a protein substrate, S, by a MAP kinase, E. For simplicity, the repositioning of the protein substrate within the active site of the enzyme is not shown as a separate step in this scheme. A strictly processive mechanism requires that the following inequalities, $k_{\text{ATP}} \gg k_{S^{*}}$, $k_{\text{ATP}} \geq k_{\text{SP1}}$, and $k_{\text{ATP}} \gg k_{\text{SP2}}$, are met.

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2D). The addition of 1 mM ATP resulted in the fast conversion of ATF2\textsuperscript{71P} to ATF2\textsuperscript{69P/71P} with a half-life of less than 5 min and the slower conversion of ATF2\textsuperscript{69P} to ATF2\textsuperscript{69P/71P} with a half-life of ~60 min (see Fig. 2, D–F). The rate of disappearance of peaks corresponding to ATF2\textsuperscript{69P} and ATF2\textsuperscript{71P} matched the rate of appearance of label in T1, corresponding to ATF2\textsuperscript{69P/71P}. This is consistent with the direct conversion of both ATF2\textsuperscript{69P} and ATF2\textsuperscript{71P} to the dual-phosphorylated product.

Quantifying the Kinetic Mechanism—A careful examination of the products provided insight into the biphasic kinetics shown in Fig. 1. At ~1.5 mol/mol of phosphate was incorporated (20 min, data not shown) only substrate mono-phosphorylated on Thr-69 remained. No ATF2\textsuperscript{71P} was present, because it had all been transformed to the dually phosphorylated product ATF2\textsuperscript{69P/71P}. ATF2\textsuperscript{69P} is considerably less reactive as witnessed by its presence (~3–4 μM) after 60 min (Fig. 2B). After a more extensive incubation, however, it was fully converted to the dually phosphorylated product (Fig. 2C). We can quantitatively account for the observations using the mechanism of Scheme 2A (in the absence of phosphatases), where S\textsuperscript{P1} and S\textsuperscript{P2} correspond to the two mono-phosphoforms, ATF2\textsuperscript{69P} and ATF2\textsuperscript{71P}, respectively. The line through the data in Fig. 1 was obtained by using the experimentally observed values for $k_{\text{cat(app)}}$ and $K_{\text{M(app)}}$ for all of the steps in Scheme 2A, except for the transformation of ATF2\textsuperscript{69P} to ATF2\textsuperscript{69P/71P}, whose parameters were allowed to float. The best fit\textsuperscript{5} to the data using Kinteksim gave a $k_{\text{cat}}/K_{M}$ for the transformation of ATF2\textsuperscript{69P} to ATF2\textsuperscript{69P/71P} that was 40-fold lower than $k_{\text{cat(app)}}/K_{M(app)}$. This model quantitatively accounts for the progress of phosphate incorporation into GST-ATF2-(1–115), as well as the absence of ATF2\textsuperscript{71P} after 20 min and the presence of 3–4 μM ATF2\textsuperscript{69P} (calculated 4.5 μM) after 60 min. Product inhibition cannot account for the difference in the rate of disappearance of the mono-phosphoisomers, because in the pulse-chase experiment (Fig. 2, D and E) the concentration of phosphorylated product is initially submicromolar. Furthermore, inhibition of p38α by phosphorylated products would be expected to have an equal effect on the initial turnover of both mono-phosphoisomers in the pulse-chase experiment.
present in a cell are available to phosphorylate any one substrate. Furthermore, the catalytic subunits of protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2) are also targeted to specific subcellular locations through complexation with a variety of other proteins (7, 10). Therefore, assuming a 20-fold lower concentration of the available protein phosphatases, compared with the activated MAPK, we set the phosphatase concentration at 50 nM. MAPKs and protein phosphatases typically have $K_M$ values in the 1–10 μM range for bona fide protein substrates, and therefore we used a value of $K_M = 5$ μM for all substrates. Substrate concentration was fixed at 500 nM, corresponding to the approximate concentration of several MAPK substrates, such as the p90RR isomers, in mammalian cells. Finally, a separate cycle was introduced (Scheme 2C) to account for competitive substrate inhibition of the MAPK and the phosphatases by other cellular substrates. The concentration of total cellular substrate was estimated to be ~5 μM, based on the assumption that an MAPK has between 20 and 40 substrates at cellular concentrations of 250–500 nM. In all the simulations the signal was interpreted as the concentration of the simulated signal/response curve (compare curve $D^*$ with curve $D$). A 17- and 29-fold increase in stimulus (MAPK activity) is needed to drive the concentration of the dual-phosphorylated protein, $S^{P1P2}$, from 10% maximal to 90% maximal for the distributive (line $D$ in Fig. 4) and processive (line $P$ in Fig. 4) mechanisms, respectively. In contrast a 58-fold increase in stimulus is needed to drive the same change in activity for the $D^*$ mechanism. A similar relative increase in the hyperbolic character of the curves (an increase in the control parameter $R$ (12)) was observed when the $K_M$ of the phosphatase was varied between 1 and 50 μM or the total substrate concentration varied from 1.5 to 20 μM. As mentioned above the ultrasensitivity predicted for the processive mechanism ($R = 29$) is due to contributions from zero-order ultrasensitivity (12, 13). These contributions were estimated by extrapolating to conditions where the enzymes are not saturated by substrates (12, 13).

Under these conditions (in the absence of a competitive substrate (Scheme 2C), a processive mechanism is predicted to be essentially Michaelian (R = 75), a two-step distributive mechanism ultrasensitive ($R = 37$), and the $D^*$ mechanism subsensitive ($R = 115$). These calculations show how the sensitivity through a two-step distributive mechanism can be modulated to be either ultrasensitive or subsensitive by changes in the specificity of an enzyme for an intermediate substrate brought about by phosphorylation.

**DISCUSSION**

In this study we show that the stress-activated MAPK, p38α, dually phosphorylates the transcription factor ATF2 by a two-step (double collision) distributive mechanism and not by a one-step (single collision) processive mechanism. Our observation was surprising, because the manner that substrates are recognized by MAPKs appears to be ideal to promote a processive mechanism of substrate phosphorylation. There are two possible mechanisms of dual phosphorylation that can be distinguished by the relative order of substrate binding (Scheme 3). The upper pathway in Scheme 3 represents a processive mechanism of phosphorylation, where the protein substrate does not dissociate from the enzyme until after the second phosphoryl transfer, $k_p$, has occurred. The requirements for this mechanism are fairly stringent because nucleotide exchange, substrate repositioning, and phosphoryl transfer must all occur faster than the dissociation of the mono-phosphorylated intermediate ($S^*$) from the respective ternary or binary complexes. The lower pathway corresponds to the less stringent, two-step, distributive pathway, where $S^*$ dissociates from the enzyme after the first phosphorylation, $k_p$, and then recombines before the second, $k_p$.

MAPKs are believed to utilize substrate-docking interactions that appear to be well situated to provide a common platform for the phosphorylation of multiple residues within a substrate through a processive mechanism (3, 43, 44). By using a docking site to maintain contact with a substrate and then simply “sliding” the active site from one phosphorylation site to the next, an MAPK could phosphorylate several residues without

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*The parameter $R$ is the concentration of the stimulus that causes a 90% maximal response divided by the concentration of the stimulus that causes a 10% maximal response.*
dissociating fully from the substrate. The catalytic advantage of such a process, over a nonprocessive mechanism, while not expected to be large for the dual phosphorylation of a protein, could be significant within the context of a signal transduction cascade, where the efficient propagation of a signal through the cascade may be critical. Interestingly, it has been proposed and also examined theoretically, although not proven experimentally, that scaffold proteins facilitate the processive phosphorylation of proteins in signal transduction cascades.

ATF2 contains a putative MAPK docking site that is similar to the DEJL docking sequence, (K/R)K/R/K/RX₁₋₅−₁(1)L(1)L(1)L(1), found in many other MAPK substrates. This DEJL-like sequence, 46KHKHEMTL₅₃, found N-terminal to the phosphorylation sites, Thr-69 and Thr-71, within the trans-activation domain of ATF2, probably interacts with Asp-313 and Asp-316 of p38α and is predicted to be a primary specificity determinant. Whereas the DEJL-like domain appears to be ideally situated to promote a processive mechanism, it is clear from our data that this does not happen and that the mono-phosphorylated GST-ATF2-(1–115) dissociates from p38α with a rate constant greater than, or equal to 0.6 s⁻¹, the magnitude of GST-ATF2-(1–115) turnover. Previous studies with p38α uncovered a dependence of kₐₘₐₓ, for the phosphorylation of a peptide, on viscosity and also a small thiol effect (46). This suggests that phosphoryl transfer is not rate-limiting for the phosphorylation of a peptide substrate and that a viscosity-sensitive conformational change or a product dissociation step is rate-limiting instead. We are currently investigating the kinetic mechanism of p38α in more detail to determine the rate-limiting step for the phosphorylation of GST-ATF2-(1–115). There are several possible reasons why the mechanism of ATF2 phosphorylation by p38α is not processive. One possibility is that the putative docking interactions are not the only interactions required to form a stable p38α-ATF2 complex and that other interactions within or near the active site of p38α are also necessary. This is supported by mutagenesis experiments on p38 isoforms that showed that docking site interactions are not the only interactions that determine the specificity of the interactions between p38 isoforms and protein substrates. If other interactions are critical for complex formation and become disrupted after the first phosphoryl transfer, ATF2 dissociation might quickly ensue, leading to a distributive mechanism.

It will be interesting to determine whether other MAPKs, which exhibit potentially tighter binding interactions, phosphorylate their substrates with a processive mechanism. There have been several reports that suggest that in some cases MAPKs can bind tightly to docking domains. For example, the binding of the MAPK ERK2 to a glutathione S-transferase fusion protein of the Ets domain transactivation factor Elk-1 (GST-Elk1) immobilized on glutathione-agarose beads was shown to be strong enough to survive multiple washes by buffer (48). It was shown that the docking site interactions were both necessary and sufficient to maintain the ERK2-Elk-1 complex on the beads. Similar results were observed for several other MAPK/substrate interactions using a similar approach (49). However, it is possible that the tight binding of MAPK-substrate complexes seen on glutathione-agarose beads are due to nonspecific binding associated with the matrix that increases the affinity of the interactions.

Although p38α showed similar specificity for Thr-71 and Thr-69 in GST-ATF2-(1–115) (Fig. 2A), it displayed a marked difference in the specificity toward the mono-phosphoisomers. This demonstrates that although local sequence is not the primary determinant of p38α specificity; it can be very important nevertheless. Cantley and co-workers (42) recently showed that ERK2 has a preference for a proline at the P-2 position, and therefore it is possible that phosphorylation of a threonine at this position can hinder binding of a substrate to p38α.

Interestingly, many MAP kinase substrates appear to be phosphorylated, at least twice by a MAP kinase, before they become activated. Numerous examples of protein conformations regulated by single phosphorylations exist in the literature, so dual phosphorylation is not required per se. Why then is dual phosphorylation so common in MAPK cascades? It has been suggested that dual phosphorylation provides an added check against the inappropriate low rate activation by another protein kinase present in the cell. This may be true for the activation of MAPK substrates, because different MAPKs, although similar in both structure and specificity, activate different proteins with different functions in vivo. Another possibility, however, is that multiple phosphorylation mechanisms (by the same enzyme) provide a simple and effective way of influencing the amplitude sensitivity of a pathway. The recent argument by Huang and Ferrell (17) that a two-step distributive mechanism can lead to an increase in ultrasensitivity of a signaling pathway is compelling and could help explain the existence of the exquisite switch-like behavior of a MAPK cascade in Xenopus oocytes. Their laboratory investigated how the variable concentration of the maturation-inducing hormone progesterone, which activates the MAPKKK, Mos, is converted into a switch-like activation of MAPK. They concluded that the observed behavior is consistent with a model where at low concentrations of progesterone the MAPK remains inactive, but upon passing through a narrow concentration threshold of progesterone the MAPK becomes fully activated (50). Sigmoidal stimulus-response curves are important because they provide a way of switching from off to on over a narrow range of input and provide a threshold that can filter out base-line noise.

Computer simulation of our experimentally observed kinetic results (Fig. 4) shows, however, that a distributive mechanism does not necessarily result in an increase in ultrasensitivity in vivo. In fact it appears to offer the potential for considerable variation in the degree of sensitivity. We observed a 40-fold reduction in the specificity of p38α toward ATF2 71P in vitro, and we used this as the basis for our calculations. Rather than model the reaction of p38α specifically, we examined the effect of decreasing the specificity of a MAPK for one of two intermediates formed as part of a two-step distributive mechanism (Scheme 2A). We assumed that the intermediates were initially formed in equal amounts from the unphosphorylated substrate but that the MAPK displays a 10-fold decrease in specificity toward one of them. The decrease in specificity was predicted to lead to a 3-fold increase in the range of MAPK activation required to drive the formation of the product from 10% to 90% maximum, assuming that the phosphatase did not discriminate between the phosphorylated products (Fig. 4). This conclusion is qualitatively independent of the level of the contribution from zero-order ultrasensitivity and illustrates how the degree of activation of a system in response to a signal could be adapted by mutations that alter the kinetic parameters of activation.

The kinetics of ATF2 activation by p38α suggests that a further increase in ultrasensitivity at this level of the pathway is unnecessary. It will be interesting to determine whether the activation of p38α displays “switch-like” ultrasensitivity in response to cell stresses or whether its response is more hyperbolic, where control is exerted over a wider range of stimulus.
It will also be interesting to compare the activation of other p38α substrates such as the transcription factors MEF2 (51) and CHOP (52), because in each case, activation is brought about by the phosphorylation of two sites that lie close together in primary sequence. It will also be fascinating to determine how the activation of ATF2 by the other subfamily of stress-activated MAPKs, the c-Jun N-terminal protein kinases (JNKs), compares to p38α. Differences in the specificity could reflect differences in the dependence of ATF2 activation on the activity of the two subfamilies.

In summary, the dual phosphorylation of protein substrates by a two-step distributive mechanism appears to offer considerable flexibility in the control of the amplitude-sensitivity of a pathway and is likely to contribute significantly to the signal/response profiles of numerous signal transduction pathways. At one extreme a distributive mechanism of multiple phosphorylation can increase the ultrasensitivity of a pathway, as is probably the case for the activation of MAPK by MAPKK in Xenopus oocytes (17). At the other extreme the same mechanism could help maintain a broader threshold, allowing the system to respond to a wider range of stimulus. These factors are likely to be important for the activation of a large number of enzymes and proteins regulated by MAPK cascades in eukaryotic organisms.

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