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Signal Integration and Activation of MAP, JNK and p38 Kinase Pathways in 293 Cells

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The need to study coordinated signaling of kinase cascades in response to a variety of extracellular stimuli is critical to advances in fundamental cell biology and disease mechanisms. The availability of highly selective phosphorylation-specific antibodies that selectively target the active form of the ERK/MAP enzymes provides a powerful new approach to unraveling the regulation of these pathways.

INTRODUCTION

Signaling in eukaryotic cells typically originates with the introduction of a particular stimulus into the extracellular space where it can activate, either directly or indirectly, a specific receptor. These stimuli can be quite diverse in nature, comprising several distinct classes of biological molecules including hormones, growth factors, cytokines, toxins, organic inhibitors, osmotic stimuli as well as ultraviolet light. Differences in the nature and consequences of signaling initiated by such diverse agents and legends results from the differential modulation of multiple signaling elements in distinct combinations. Indeed, all known effects of signaling cascades can be regulated by more than one stimulus. Moreover, overlapping pathways can be activated by different stimuli, further contributing to the regulation of signaling specificity. To define the signaling mechanisms and their role in physiological events triggered by an extracellular stimulus, it is necessary to identify the signal transducers, their interactions and the time-course in which their activity and subcellular location are modulated.

One group of enzymes under intense investigation as part of the effort to understand signaling cascades that are activated by a number of extracellular stimuli is the extracellular signal-regulated protein kinase (ERK), also referred to as the mitogen-activated protein kinase (MAP), superfamily of enzymes. This large group of protein kinases contains over a dozen members that participate in many eukaryotic regulatory pathways (1-4). These enzymes comprise at least three parallel, yet interwoven, signal transudation cascades that are differentially regulated in response to mitogens, growth factors, cytokines and various forms of stress (Figure 1). Each cascade consists of a minimum of three enzymes activated in series: a MAP kinase/ERK kinase kinase or MEKK (a MEK activator), a MAP kinase/ERK kinase or MEK (a MAP kinase activator), and a MAP kinase/ERK homologue (5).

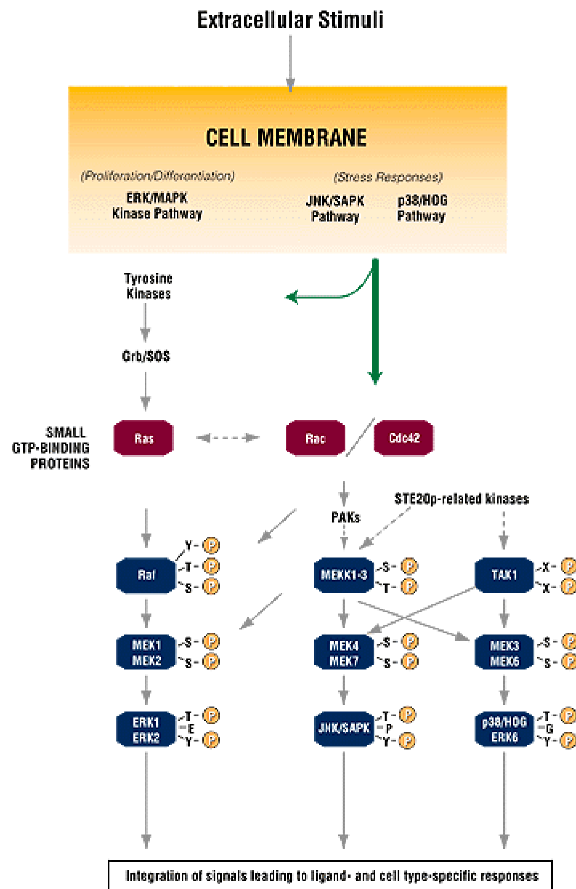

Figure 1. Activation of different ERK/MAP signaling cascades by different extracellular stimuli. The signals leading to activation of the ERK1 and ERK2, JNK/SAP and p38/HOG enzymes comprise three parallel, yet interwoven, pathways containing a three-enzyme module allowing for sequential activation. The requirement for dual-phosphorylation of the Thr-X-Tyr motif for the ERK/MAP superfamily members is indicated. Note that some stress-related signals can also activate the mitogen-stimulated cascade on the left. Broken arrows indicate interactions that are less definitively established.

The first and best studied enzymes of the ERK/MAP superfamily reside in the classical mitogen-activated (MAP) subfamily pathway (Figure 1). This cascade is composed of MEKK isoforms (Raff-1, B-Raff, A-Raff), MEK1 or MEK2, and ERK1 or ERK2 (5,6). The JNK and p38 enzymes comprise two additional subfamilies, whose members are potently activated by a variety of stress-related stimuli. The JNK (c-Jun N-terminal protein kinase) enzymes, also referred to as stress-activated protein kinases (Saps), are activated by a variety of stress-related stimuli (e.g., heat shock, osmotic imbalance, endotoxin, UV and protein synthesis inhibitors) and cytokines (7-10). The p38 kinase, also referred to as High Osmolarity Glycerol response kinase (HOG), is activated by a variety of stress-related stimuli, as well as cytokines and insulin (11-16). p38 and the p38-related kinases (p38alpha, p38beta, p38gamma , also referred to as p38, p38-2 and ERK6, respectively, and p38delta) represent a rapidly expanding group of enzymes that are being targeted for potential treatment of a variety of disease states (1-4).

The MAP kinases are unusual in that MEKK-mediated phosphorylation of both a tyrosine and a threonine residue, in the Thr-X-Tyr motif (Thr-Glue-Tyr for MAP, Thr-Pro-Tyr for JNK, and Thr-Gay-Tyr for p38) within the "phosphorylation lip" of each enzyme is required to stimulate their activities (17). Although dual-specificity Thr/Tyr protein phosphatases are capable of inactivating the ERK/MAP superfamily enzymes, individual Ser/Thr or Tyr phosphatases also appear to regulate their activity by dephosphorylating either core residue (18-20).

DETECTION OF MAP, JNK AND P38 ACTIVATION IN 293 CELLS

The need to study coordinated signaling of the ERK/MAP superfamily cascades in response to a variety of extracellular stimuli has become increasingly critical to our understanding of both fundamental cell biology and disease, as well as in the pursuit of new drugs with increased therapeutic value. Recent advances in synthesis of phosphopeptides and the ability to produce highly selective and high-affinity polyclonal antibodies to phosphorylated enzymes (21) is a significant breakthrough in the study of complex signal transudation cascades. Promega has introduced the Anti-ACTIVE™ series of rabbit polyclonal antibodies that preferentially recognize the dually phosphorylated active form of the MAP, JNK and p38 enzymes. These antibodies are highly effective in detecting enzyme activation in both Western blotting and immunocytochemistry applications using a variety of cell lines (e.g., CHO, FL100, NIHAU 3T3, PC12, REF52, Swiss 3T3, U373 and 293 cells) or tissue samples (16,22).

The human kidney fibroblast 293 cell line is one system in which we have studied coordinated activation of MAP, JNK and p38 enzymes by a variety of mitogenic and stress-related stimuli (see reference 24 for analysis of PC12 cells). To begin to characterize the events leading to activation of each ERK/MAP cascade, we chose stimuli known to potently activate one or more of these signaling cascades.

STIMULATION WITH SERUM

In the first set of experiments, 293 cells were grown to near confluence and then incubated for 18 hours in serum-free medium to suppress MAP activity (23). The cells then were treated with 10% serum containing medium and cell extracts were prepared for Western blot analysis. The results generated with Anti-ACTIVE™ MAP pAb (Figure 2A) illustrate that the active forms of ERK1 and ERK2 are undetectable in untreated cells (lane 1) but are easily detected after treatment with serum (lane 2). Serum contains a variety of mitogenic hormones and growth factors that potently activate the ERK/MAP cascade. As illustrated in Figure 2B, generated with a polyclonal antibody that recognizes both active and basal MAP enzyme, the levels of ERK1 and ERK2 enzyme are unaffected by the treatment (compare lanes 1 and 2). In contrast, serum treatment did not induce the activation of the JNK or p38 enzyme cascades as measured using the corresponding Anti-ACTIVE™ JNK and p38 antibodies or an immunoprecipitation-based kinase assay that uses c-Jun and ATF2 derived substrates, respectively (data not shown).

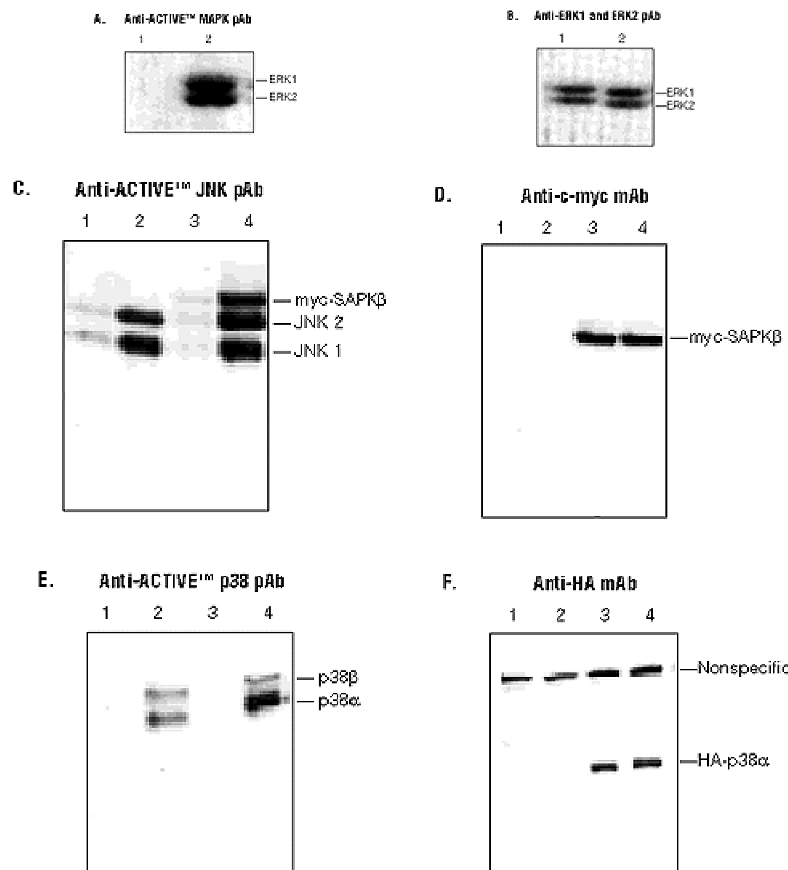

Figure 2. Western detection of the active forms of the MAP, JNK and p38 enzymes in 293 cells using Anti-ACTIVE™ pAbs. Cells extract were prepared from stimulated or unstimulated 293 cells (23). Western blots (10-20µg per lane) were developed using the antibodies described below. Chemiluminescent detection was performed using an HR.-conjugated anti-rabbit IgG secondary antibody and ECL™ Chemiluminescent Detection Reagents (Amersham). **Panels A and B:** 293 cells were grown to near confluence, incubated for 18 hours in serum-free medium and then treated for 5 minutes at 37°C with medium containing 10% serum. The Western blots were developed with either Anti-ACTIVE™ MAP pAb diluted 1:20,000 (Panel A) or with an antibody that recognizes both the active and basal forms of ERK1 and ERK2 (Panel B). Lanes 1, unstimulated; lanes 2, stimulated. **Panel C and D:** 293 cells, either transiently transfected with an expression construct encoding a myc-tagged SAPKbeta isoform or with the expression vector without the SAPKbeta cDNA, were treated with 10µg/ml anisomycin (Sigma) for 1 hour at 37°C. The Western blots were developed with either Anti-ACTIVE™ JNK pAb diluted 1:1,750 (Panel C) or with a monoclonal antibody directed against the myc-tag (Panel D). Lanes 1, vector only, unstimulated; lanes 2, vector only, stimulated; lanes 3, myc-SAPKbeta expressing, unstimulated; lanes 4, myc-SAPKbeta expressing, stimulated. **Panels E and F:** 293 cells, either transiently transfected with an expression construct encoding an HA-tagged p38 isoform or with the expression vector without the HA-p38 insert, were treated with 0.7M sodium chloride for 1 hour at 37°C. The Western blots were developed with either Anti-ACTIVE™ p38 pAb diluted 1:4,000 (Panel E) or with a monoclonal antibody directed against the HA-tagged p38 (Panel F). Lanes 1, vector only, unstimulated; lanes 2, vector only, stimulated; lanes 3, HA-p38 expressing, unstimulated; lanes 4, HA-p38 expressing, stimulated.

STIMULATION WITH ANISOMYCIN

In the second set of experiments, 293 cells were either transiently transfected with an expression construct encoding a myc-tagged SAPKbeta isoform (corresponds to the JNK1 enzyme) or with the expression vector without the myc-SAPK insert. The cells then were either untreated or treated with anisomycin, a protein synthesis inhibitor isolated from *Streptomyces*. Anisomycin provides a stress stimulus that potently activates the JNK/SAPK enzymes (i.e., JNK1 and JNK2), as illustrated by Western detection using Promega's Anti-ACTIVE™ JNK pAb (Figure 2C). Note that the myc-tagged SAPKbeta enzyme runs at a higher molecular weight as compared to JNK1 or JNK2. As illustrated in Figure 2D, a Western blot developed with a mouse monoclonal antibody to the myc tag on the recombinant enzyme, the levels of the SAPKbeta enzyme are unaffected by the treatment (compare lanes 3 and 4). Western analysis with the Anti-ACTIVE™ p38 pAb revealed that treatment of 293 cells with anisomycin also activates p38 (data not shown), consistent with the view that both JNK and p38 belong to this group of stress-activated protein kinases. In contrast, treatment of the cells with anisomycin did not highly activate the MAPK subfamily members, ERK1 or ERK2 (data not shown).

STIMULATION BY OSMOTIC SHOCK

In the third set of experiments, 293 cells were transiently transfected either with an expression construct encoding an HA-tagged p38alpha isoform or with the expression vector without the H/A-p38 insert. The cells then were either left untreated or treated with 0.7M sodium chloride, which served as another form of stress stimulus by presenting the cell with an osmotic (hypertonic) shock. As illustrated in Figure 2E, this treatment potently activates the p38/HOG cascade. As shown in Figure 2F, a Western blot developed with a mouse monoclonal antibody to the HA tag of the recombinant protein, the level of the transfected p38 enzyme is unaffected by the treatment (compare lanes 3 and 4). Western blotting with the Anti-ACTIVE™ JNK pAb indicated that hypertonic conditions also activate the JNK/SAPK enzymes (data not shown), which is consistent with the view that both p38 and JNK belong to this group of stress-activated protein kinases. However, in contrast to treatment with anisomycin, use of hypertonic sodium chloride also activated the MAPK subfamily members, ERK1 and ERK2 (data not shown). This latter result supports the increasingly recognized observation that certain stimuli (e.g., osmotic shock, endotoxin, ultraviolet light and certain cytokines) are capable of activating all three ERK/MAPK cascades (Figure 1) and emphasizes that coordinated regulation of these complex signaling cascades is important in determining the physiological response to stimuli within the extracellular environment (11-16,24).

SUMMARY

The need to study coordinated signaling of the ERK/MAPK superfamily cascades in response to diverse extracellular stimuli is critical to our understanding of both fundamental cell biology and the pursuit of new drugs to control complex disease states. The recent availability of highly selective dual phosphorylation-specific antibodies that selectively target the active form of the ERK/MAPK enzymes provides an important advance toward an increased understanding of how these pathways are regulated. The results presented here illustrate the high signal-to-noise ratio and selectivity obtained with each Anti-ACTIVE™ pAb, which reveal activation by all three stimuli tested (see also reference 24). The absence of a slower electrophoretic migration resulting from phosphorylation of the ERK/MAPK superfamily illustrates the unreliability of a gel shift to detect activation of these proteins, and underscores the utility of using the Anti-ACTIVE™ pAbs to detect activation of the MAPK, JNK and p38 enzymes.

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Ordering Information

Product	**Size[a]**	**Cat.#**
Anti-ACTIVE™ MAPK pAb	15µg	V6671
Anti-ACTIVE™ JNK pAb, Rabbit, (pTPpY)	40µl	V7931
	120µl	V7932
Anti-ACTIVE™ p38 pAb, Rabbit, (pTGpY)	100µl	V2901
	300µl	V2902
Donkey Anti-Rabbit IgG (H+L), HRP	60µl	V7951
Donkey Anti-Rabbit IgG (H+L), AP	60µl	V7971

[a]*When used at the recommended dilutions, Cat.#s V6671, V7932, V2902, V7951, V7971, will generate 600ml of blotting solution (60 blots) and Cat.#s V7931 and V2901 will generate 200ml of blotting solution (20 blots).*

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