



Detection of JNK, p38 and MAPK Enzyme Activation Using Anti-Dual-Phosphopeptide Antibodies: Coordinated Signaling of the Extracellular Signal-Regulated Protein Kinase (ERK) Superfamily

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The Extracellular Signal-Regulated Protein Kinase (ERK) superfamily is the focus of intensive study due to its widespread involvement in signaling pathways in both normal and disease states. Promega's Anti-ACTIVE™ MAPK, Anti-ACTIVE™ JNK and the soon to be released Anti-ACTIVE™ p38 pAbs provide researchers with a unique and rigorously tested panel of reagents to sort out the coordinated regulation of the ERK superfamily members. These antibodies can be used for a variety of non-radioactive applications, including Western blotting and immunocytochemistry, allowing a more accurate assessment of enzyme activation in response to a variety of extracellular stimuli.

INTRODUCTION

The Extracellular Signal-Regulated Protein Kinase (ERK), also referred to as the Mitogen-Activated Protein Kinase (MAPK), superfamily of enzymes plays a number of important roles in signal transduction in eukaryotic cells. These enzymes include the MAPK, JNK and p38 subfamilies, which comprise three interwoven signal transduction cascades (summarized in Figure 1). The MAPK, JNK and p38 kinases are the terminal enzymes in a three-kinase cascade where each kinase phosphorylates and thereby activates the next member in the sequence. A three-kinase module consists of a MAPK kinase kinase (MEKK) that activates a MAPK kinase (MEK) that, in turn, activates a member of the ERK/MAPK superfamily. MEK enzymes activate the ERK/MAPK superfamily members by phosphorylating the enzyme on both the threonine and tyrosine residue in the Thr-X-Tyr sequence within the catalytic core of the enzyme. Dephosphorylation of either residue results in inactivation of the enzyme (1), illustrating that both threonine and tyrosine phosphorylation are necessary for maximal activation. Upon activation, these kinases phosphorylate, and thus regulate, a variety of intracellular enzymes and transcription factors (2,3).

The MAPK subfamily, as exemplified by the ERK1 and ERK2 enzymes, is activated by a variety of mitogens (e.g., epidermal growth factor, platelet derived growth factor, nerve growth factor, serum; 2,3). The JNK and p38 pathways are activated by a large spectrum of stress-related stimuli (4-6). While certain stimuli are highly selective for a given pathway, other stimuli activate two or more pathways resulting in a highly coordinated series of signaling events. For example, mitogens very potently and selectively activate the Ras-Raf pathway leading to activation of the MAPK (e.g., ERK1 and ERK2) enzymes (2,3). In contrast, a variety of stress-stimuli (e.g., sorbitol) are capable of activating all three cascades. Understanding the degree to which signaling through these parallel pathways is coordinated is critical to the elucidation of their role in both normal and disease states.

More than a year ago, Promega introduced the Anti-ACTIVE™ MAPK pAb that specifically detects the activated, dually phosphorylated form of ERK1 and ERK2 (7,8). This high-affinity polyclonal antibody, which is made against a synthetic peptide encompassing residues pThr¹⁸³-Glu¹⁸⁴-pTyr¹⁸⁵ of ERK2 (9), is the only commercially available polyclonal antibody that specifically recognizes the dually phosphorylated form of the ERK1 and ERK2 enzymes. As part of Promega's commitment to developing high-quality, rigorously tested antibodies within the Anti-ACTIVE™ series, we have now introduced the second member of this product family, the Anti-ACTIVE™ JNK pAb. This antibody selectively detects the dually phosphorylated form of the JNK enzymes.

Anti-ACTIVE™ JNK is a polyclonal antibody generated against a synthetic, dually phosphorylated peptide containing the pThr¹⁸³-Pro¹⁸⁴-pTyr¹⁸⁵ motif of JNK2. The final product undergoes an extensive multistep affinity purification procedure to ensure that the antibody preparation is specific to the activated (i.e., dually phosphorylated) form of the targeted JNK enzymes. Indeed, selective treatment of the active, dually phosphorylated MAPK or JNK enzymes with either a Ser/Thr protein phosphatase or a Tyr protein phosphatase, results in loss of both enzymatic activity of these kinases and recognition by Promega's dual-phosphorylation-specific antibodies. A third antibody in this series, Anti-ACTIVE™ p38 pAb, which recognizes the active forms of this subfamily by virtue of the dually phosphorylated pThr-Gly-pTyr sequence, will be introduced in the near future. This set of Anti-ACTIVE™ pAbs provides researchers with a unique and dynamic panel of reagents to investigate the coordinated regulation of pathways involving the

ERK/MAPK superfamily members.

NOMENCLATURE AND ISOFORMS

The complexity of the ERK/MAPK signal transduction pathways and the diversity of organisms in which these systems have been studied have resulted in an equally daunting nomenclature for the proteins involved (see reference 8 for more detail). The term MAPK was originally chosen to indicate Microtubule-Associated Protein Kinase in order to describe a new class of protein kinases that could associate with cytoskeletal elements upon activation (2,3). Later the MAPK designation became synonymous with Mitogen-Activated Protein Kinase. The p44 ERK1 and p42 ERK2 enzymes, the most studied members of this subfamily, phosphorylate a variety of proteins including the c-Jun and ELK-1 transcription factors. Subsequently, other proteins containing the conserved Thr-X-Tyr motif were identified; however, these enzymes were poorly activated by mitogens but more potently activated by various stress-related stimuli. JNK (c-Jun-N-Terminal Kinase), also known as SAPK (Stress Activated Protein Kinase), phosphorylates a number of proteins including the transcription factors c-Jun and SAP-1a. Thus, both MAPK and JNK contribute to the formation of active AP1 transcription factor complexes (4-6). The major isoforms of JNK are p46 JNK1, p54 JNK2 and p49 JNK3 (10), although as many as ten splice variants have been identified (11). The p38 kinase was originally termed CSBP, for Cytokine Suppressive Anti-Inflammatory Drug Binding Protein. In yeast, p38 is referred to as HOG (High Osmolarity Glycerol Response). While SAPK1 refers to the JNK1 isoform of JNK, SAPK2 is not synonymous with the JNK2 isoform; it is another designation for p38 (8,12). The p38 isoforms are referred to as p38, p38 beta, p38 gamma and p38 delta, which show only slight differences in mobility when analyzed by SDS-PAGE (13). The p38 enzymes phosphorylate a variety of cellular substrates including the ATF family of transcription factors (14).

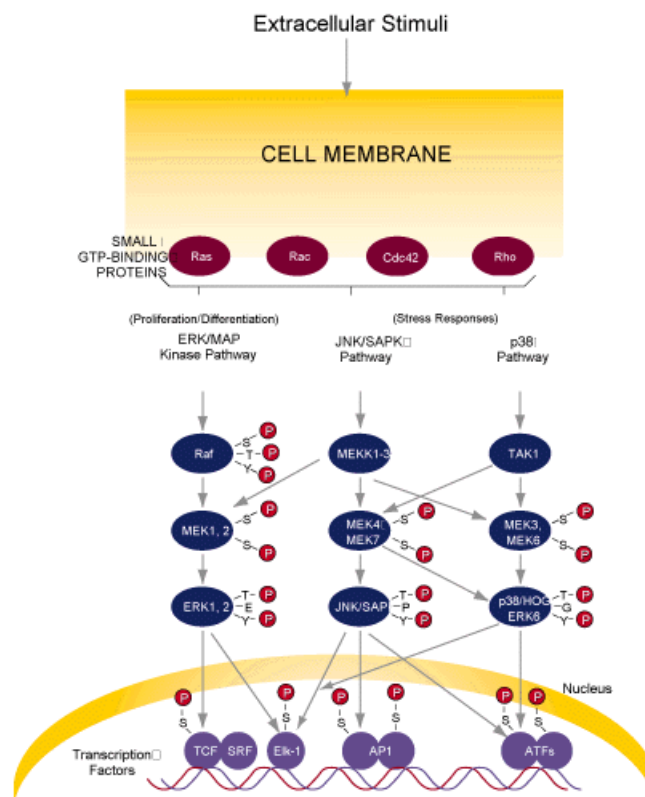


Figure 1. Parallel ERK/MAP kinase cascades involve specific ERK/MAPK superfamily enzyme modules. Each of the individual MAPK (e.g., ERK1 and ERK2), JNK and p38 cascades consists of a three-enzyme module that includes a MEKK, MEK and a ERK/MAPK superfamily member. Each cascade is activated following stimulation of cells with the appropriate extracellular signals, often involving the binding of ligands to their respective cell surface receptors. In each case, activation of intracellular signaling cascades may be restricted to the cytosol or it can extend to the nucleus, where activation of transcription factors can lead to changes in the gene expression.

REGULATION OF MAPK, JNK AND P38 ENZYMES

The specificity of the MAPK (e.g., ERK1 and ERK2) subfamily of enzymes was the first to be characterized, revealing activation by a wide variety of mitogens, growth factors and hormones, many of which initiate their signal transduction by binding to transmembrane receptors with intrinsic tyrosine kinase activity (14). The JNK/SAPK and p38/HOG pathways are activated by ultraviolet light, inflammatory cytokines (e.g., IL-1beta, TNFalpha; 14), osmotic shock agents (e.g., sorbitol; 15) and inhibitors of protein synthesis (e.g., anisomycin; 15). This large spectrum of regulators suggested that these enzymes transduce a variety of stress responses. The signals are transmitted from cell membrane-bound receptors (Figure 1) through small GTP-binding proteins, such as Cdc42 and Rac in the case of

both JNK and p38 (16), to the level of the MEK kinases (MEKKs). JNK and p38 are activated by their respective MEK kinases, MEK4/7 and MEK3/6, for which activation depends upon both the stimulus and the cell type (15,17). For example, in Jurkat leukemic T cells, JNK activation is very short-lived, and in thymocytes, activation of JNK is less apparent due to high basal levels (18). The recent observation that the classical MAPK enzymes, ERK1 and ERK2, can be activated by certain stress-stimuli (e.g., osmotic shock, shear stress and UV-light) further illustrates the complex coordination that occurs between these pathways (4-6).

Signal transduction cascades involving ERK/MAPK superfamily enzymes are also regulated by the activities of a variety of protein phosphatases. Several dual-specificity protein phosphatases have been identified that can differentially dephosphorylate the MAPK, JNK or p38 enzymes. For example, the phosphatase MKP2 acts on both MAPK and JNK enzymes, while M3/M6 acts on JNK and p38. In contrast, PAC1 acts primarily on MAPKs while MKP1, also known as CL-100, can act on all three enzymes (19,20). In addition, individual Ser/Thr (e.g., PP2A) or Tyr (e.g., PTP1) phosphatases also appear to regulate the activity of the ERK/MAPK enzymes by dephosphorylating either core residue (21-23). Thus, the cell can tightly regulate the activity of the ERK/MAPK enzymes by judicious use of different combinations of MEKs, the mono- and dual-specificity protein phosphatases and the subcellular localization of each enzyme to elicit the appropriate physiological response. This level of complexity emphasizes the need for reagents that can accurately detect the active, dually phosphorylated forms of these enzymes.

ROLE IN HEALTH AND DISEASE

There is a rapidly increasing yet still rudimentary understanding of the role that the ERK/MAPK superfamily of enzymes play in human health and disease. Formerly, it was thought that the ERK1 and ERK2 enzymes were involved in normal cell growth responses and that the JNK and p38 enzymes responded to a variety of stress stimuli. It now appears that this diverse family of protein kinases plays many different roles. JNK, for example, seems to function in T cell anergy (24), while p38 can be stimulated by insulin as part of neuronal growth and differentiation as well as regulation of skeletal muscle by exercise (25, 26). Similarly, the ERK1 and ERK2 enzymes are involved in normal growth and development and have also been implicated in a variety of disease states including breast cancer (27), hypertension (28), and ischemia (29). Understanding the degree to which these enzyme cascades can be maintained as distinct entities versus their ability to communicate with one another (see [Figure 1](#)) is an important step toward resolving the complexity and identifying novel drug targets.

WESTERN BLOTTING

The Anti-ACTIVE™ MAPK, JNK and p38 pAbs were developed to provide a more accurate measure of enzyme activation. To illustrate the utility of these reagents, we analyzed extracts prepared from PC12 cells (a pheochromocytoma-derived cell line) that were either untreated or treated with a variety of stimuli. In each case, when the Anti-ACTIVE™ pAbs were used to probe Western blots, there were no detectable ERK, JNK or p38 signals in lanes containing extracts prepared from untreated cells ([Figure 2A](#); lanes 1, 3, 5, 7, 9 and 11). Western analysis using the Anti-ACTIVE™ MAPK pAb clearly illustrated that the MAPK subfamily, ERK1 and ERK2, enzymes were strongly activated by nerve growth factor (NGF, a potent mitogen) (lane 2) and to a lesser degree (~30%) by osmotic shock using sorbitol (lane 4). In contrast, the JNK1 (46kDa) and JNK2 (54kDa) isoforms were strongly activated by sorbitol (lane 8) and NGF-withdrawal (data not shown) and, to a lesser degree (~50%), by anisomycin (data not shown). Finally, p38 was potently activated by both sorbitol (lane 12) and anisomycin (data not shown), but not by NGF-withdrawal (data not shown). As an additional control, blots probed with antibodies that recognize total enzyme (active and basal) for each subfamily member ([Figure 2B](#)) resulted in similar signals with both untreated and treated extracts. This illustrates that the differences in signals observed with the corresponding Anti-ACTIVE™ pAbs were not simply due to differences in the levels of each enzyme. The data demonstrate the utility of these reagents to study the differential activation of endogenous isoforms of the MAPK, JNK and p38 using non-radioactive detection.

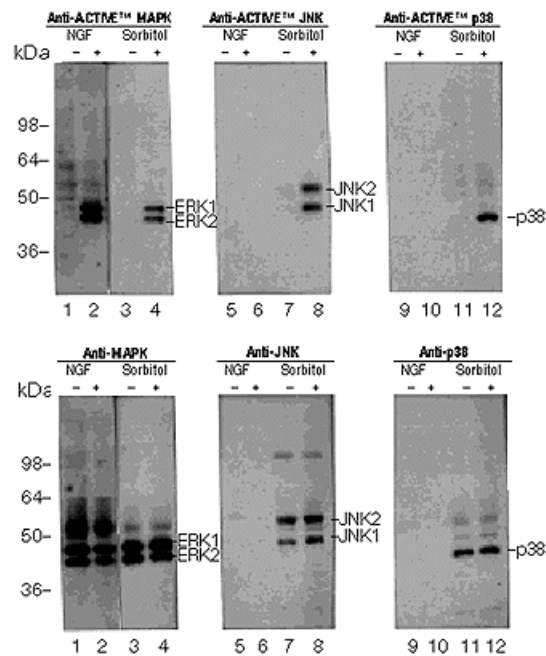


Figure 2. Detection of activated JNK, p38 and MAPK in PC12 cell extracts by Western blotting using Anti-ACTIVE™ JNK, Anti-ACTIVE™ p38 and Anti-ACTIVE™ MAPK Polyclonal Antibodies. PC12 cells were grown to 60-80% confluence in RPMI 1640 medium supplemented with 25mM HEPES, 0.5mM EGTA, 10% horse serum and 5% fetal bovine serum. Cells were either untreated, treated with 50ng/ml of nerve growth factor (NGF) for 5 minutes, or 0.5M sorbitol for 5 minutes, as indicated. Cells were harvested, homogenized and subjected to high-speed centrifugation (15). The resulting supernatant was stored at -70°C. Aliquots of each extract were analyzed by SDS-PAGE (10% gel, under reducing conditions) and transferred to a nitrocellulose membrane. The membranes were probed with either the indicated Anti-ACTIVE™ pAb (Panel A) or with corresponding antibodies that recognize both active and inactive forms of each subfamily of kinases (Panel B). For JNK, the antibody that detects both forms was generated against whole JNK enzyme from rat, while the corresponding antibodies for p38 and ERK1 and ERK2 were generated against synthetic peptides derived from the deduced amino acid sequence of each protein. The secondary antibody was a donkey anti-rabbit alkaline phosphatase conjugate. Chemiluminescent detection was performed using a Tropix Western Star™ Kit and Kodak® Bio-Max film as described by the manufacturer. Lanes: lanes 1, 5 and 9, 2µg of unstimulated PC12 cell extract; lanes 2, 6 and 10, 2µg of NGF-stimulated PC12 cell extract; lanes 3, 7 and 11, 20µg of unstimulated PC12 cell extract; lanes 4, 8 and 12, 20µg of sorbitol-treated PC12 cell extract.

Promega will offer a series of reagents that are pre-qualified for use with our Anti-ACTIVE™ pAbs. Promega's Donkey Anti-Rabbit IgG HRP-Conjugated Secondary Antibody is now available and the corresponding AP-labeled conjugate is in the final stages of development.

IMMUNOCYTOCHEMISTRY

Promega's Anti-ACTIVE™ antibodies have been used in a variety of immunocytochemistry applications to determine the level and location of active ERK/MAPK superfamily enzymes (8). Indeed, an understanding of the intracellular location of the MAPK, JNK and p38 enzymes is an important component in sorting out these signal transduction pathways.

In *Drosophila melanogaster*, the *basket* (*bsk*) gene encodes a JNK homolog essential for epithelial morphogenesis (30). Recent studies indicate that both JNK and p38 are active during larval and pupal eye development, although the staining pattern changes as the retina matures (31). This was illustrated using the Anti-ACTIVE™ JNK and the Anti-ACTIVE™ p38 Polyclonal Antibodies to immunostain the *Drosophila* pupal retina (Figure 3). The compound eye of insects contains repeated retinal units termed ommatidia. In *Drosophila*, the apical surface of each ommatidium consists of four lens-forming cone cells in the center, surrounded by two primary pigment cells (the half moon shaped structures in Figure 3). The primary pigment cells are, in turn, bordered by a hexagonal lattice of secondary and tertiary pigment cells. The point at which the three rows of secondary cells intersect marks the location of a mechanosensory "bristle," which is made up of neuronally-derived tissue. The immunocytochemistry images shown in Figure 3 were taken at 25% of full pupal development, which is before the onset of programmed cell death required for normal differentiation.

Immunocytochemistry with the Anti-ACTIVE™ JNK and p38 antibodies reveals intense staining of the cone cells in the center of the

ommatidia as well as the mechanosensory bristles and the pigment cells at apical membrane junctions. These results illustrate that the JNK and p38 enzymes are present in their active, dually phosphorylated forms in a highly restricted and temporal manner during pupal development. In addition to the distinct patterns of localization for each enzyme, the high-specificity of the Anti-ACTIVE™ JNK and p38 pAbs is supported further by the absence of staining in control immunocytochemistry experiments (data not shown) and the appropriate recognition of the JNK and p38 isoforms as demonstrated by Western analysis of *Drosophila* tissue extracts (31).

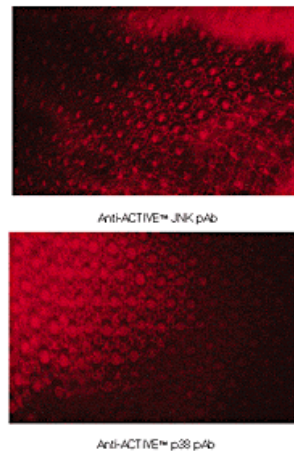


Figure 3. Immunocytochemical detection of active JNK and p38 enzymes in *Drosophila melanogaster* pupal retina using Anti-ACTIVE™ JNK and Anti-ACTIVE™ p38 pAbs. *Drosophila* pupal retinas, taken at 25% of pupal development, were fixed in 3% paraformaldehyde in PBS. The Anti-ACTIVE™ JNK and p38 pAbs were diluted 1:100 in PBS containing 10% fetal bovine serum and 0.2% Triton® X-100 (Union Carbide). Samples were incubated with the primary antibody overnight at 4°C, washed 3 times (10 minutes each at 25°C) with 0.2% Triton® X-100 and then incubated with a goat anti-rabbit Cy3 conjugate for 2 hours at 4°C. Whole mounts were visualized with a Zeiss® Axioskop® fluorescent microscope.

SUMMARY

The importance of the MAPK, JNK and p38 enzymes and the coordinated regulation that occurs between these complex signaling pathways has fueled the explosive growth of research on these pathways and the pursuit of pharmacological agents that can effectively modulate them. To meet the need for improved detection methods, Promega has developed a series of dynamic polyclonal antibody reagents referred to as the Anti-ACTIVE™ series. The Anti-ACTIVE™ MAPK, Anti-ACTIVE™ JNK and the soon to be released Anti-ACTIVE™ p38 antibodies specifically recognize the dually phosphorylated, active form of their respective members of the ERK/MAPK superfamily.

Compared to other commonly used methods, the Anti-ACTIVE™ pAbs result in a more accurate measure of enzyme activation. For example, immunoprecipitation-based kinase assays, commonly used to measure enzyme activity, can be biased by a variety of factors including (i) the typically low yields obtained with immunoprecipitation, (ii) the chance that antibody binding can influence enzyme activity, (iii) the use of nonselective substrates to measure kinase activity, and (iv) the fact that other kinases can be coimmunoprecipitated, due to their tight association, which can contribute to the resulting activity. Indeed, a series of protein kinases have been recently shown to differentially bind with high affinity to their cognate ERK/MAPK superfamily members (1). Another commonly used approach is to measure mobility shifts on gels as a measure of activation. This type of shift (observed even under denaturing and reducing conditions) can suffer from nonreproducibility and can be induced by nonproductive phosphorylation (i.e., at sites other than the TXY motif). Furthermore, such shifts tend to be even less reproducible with the JNK enzymes and do not appear to occur at all with p38. Finally, the Anti-ACTIVE™ pAbs are superior to antibodies that target only phosphorylated Threonine or Tyrosine residues, irrespective of the phosphorylation state of the other residue in the TXY motif and thus the activity, of the MAPK, JNK and p38 enzymes.

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Ordering Information		
Product	Size^a	Cat.#
Anti-ACTIVE™ JNK pAb	40µl	V7931
	120µl	V7932
Anti-ACTIVE™ MAPK pAb	15µg	V6671
Donkey Anti-Rabbit IgG (H+L), HRP	60µl	V7951

^aCat.# V7931 and V7932 contain sufficient antibody to prepare 200ml and 600ml, respectively, of Western blotting solution at the suggested working concentration (1:5,000 dilution). Cat.# V6671 contains sufficient antibody to prepare 600ml of Western blotting solution at the suggested working concentration of 25ng/ml.

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