

Detection of MAP Kinase Activation Using Anti-ACTIVE™ MAPK pAb



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Promega's Anti-ACTIVE™ MAPK pAb provides a novel and effective method for the study of complex signaling pathways, including activation of the mitogen-activated protein kinase (MAPK) enzymes ERK1 and ERK2. In this update, we demonstrate the utility of this antibody in Western blotting and immunocytochemistry.

Introduction

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated protein kinases (p44/ERK1 and p42/ERK2), play an important role in signal transduction in all eukaryotic cells. MAPKs represent the convergence point for many signaling pathways and they, in turn, modulate a variety of cellular events (1-4; see also the review in this issue). Activation of enzymes of the MAPK superfamily (ERKs, JNKs and p38) requires the dual phosphorylation of these enzymes on both the Thr and Tyr residue in the Thr-X-Tyr (TXY) consensus sequence within the catalytic core of the enzyme (5-7). In the case of ERK1 and ERK2, the intervening amino acid is a Glu residue, thus forming the TEY consensus sequence in which both residues can be phosphorylated by the MAP kinase kinase (MEK) enzymes MEK1 and MEK2 (1).

Recent observations have led to the identification of an increasing number of dual-specificity protein phosphatases capable of inactivating the ERK enzymes by dephosphorylating both the phosphoThr and phosphoTyr residues (8). However, it is also known that individual Ser/Thr or Tyr protein phosphatases can regulate ERK activity by dephosphorylating only one of the two phosphorylated residues, inactivating the enzyme (9,10). The relatively high abundance and high specific activity of these Ser/Thr or Tyr protein phosphatases argues for the importance of this mechanism for regulating the activity of ERK enzymes.

To overcome the problems inherent in studying ERK activation, Promega has developed an antibody to a dually phosphorylated synthetic peptide, encompassing residues Thr¹⁸³ and Tyr¹⁸⁵ of p42/MAPK2/ERK2 (3), corresponding to the active form of the ERK1 and ERK2 enzymes. This antibody is purified initially using a negative adsorption step to remove antibody that recognizes the nonphosphopeptide. This is followed by positive selection (affinity purification with the dually phosphorylated peptide) for antibody that preferentially recognizes the active enzymes. The Anti-ACTIVE™ MAPK pAb therefore provides more accurate results compared to antibody reagents that target only the phosphorylated tyrosine residue, irrespective of the phosphorylation state of the threonine (and thus the active state) of the MAPK enzyme catalytic core.

The Anti-ACTIVE™ MAPK pAb is highly effective in detecting ERK activation. In Western blotting applications, this antibody has been used to analyze the state of ERK phosphorylation in a variety of cell lines, with high signal-to-noise ratios and dramatic (10- to 80-fold) stimulation of MAPK activation as compared to the untreated controls. The detection of less than 250pg of active ERK2 enzyme (11,12) illustrates the high sensitivity of the antibody. In this report, we demonstrate two scenarios in which we have used the Anti-ACTIVE™ MAPK pAb to effectively detect the activation of ERK enzymes.

Western blotting

In this experiment, we studied the effects of glucose and forskolin treatment on ERK enzyme activity in INS1 cells. This established rat beta cell line, which is highly differentiated and responsive to glucose, is used to study glucose-regulated insulin secretion as a model for normal blood glucose homeostasis (13,14). Glucose and other insulin secretagogues are known to stimulate the MAP kinase pathway, although such effects are reported to be small (14). Forskolin is an activator of adenylate cyclase, which elevates cyclic AMP levels, and produces a pleiotropic response (15,16). To examine MAP kinase activation, INS1 cells were either left untreated or treated with glucose alone, forskolin alone, or a combination of glucose and forskolin. Extracts of treated and untreated cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (Figure 1). Blots were first probed with the Anti-ACTIVE™ MAPK pAb to detect active ERK1 and ERK2 enzymes (Panel A). The blots then were stripped and reprobed with a pan ERK polyclonal antibody (Panel B). The pan ERK blot is an important control that illustrates the total amount of ERK enzyme (active and basal forms) in each lane, allowing accurate interpretation of the signals obtained with the Anti-ACTIVE™ MAPK pAb. The results illustrate the synergistic effects of forskolin and glucose on the activation of ERK enzymes, suggesting that some of the pleiotropic responses induced by insulin secretagogues are likely mediated through potent activation of ERK1 and ERK2 enzymes.

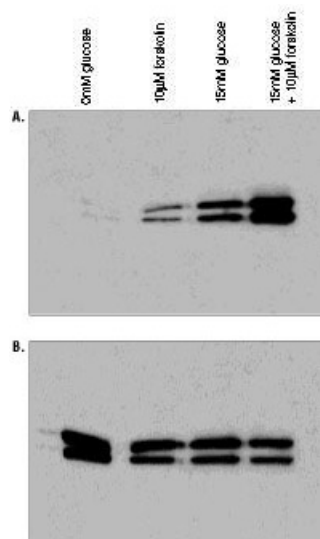


Figure 1. Western blot detection of ERK1 and ERK2 activation in INS1 cells using Anti-ACTIVE™ MAPK pAb. INS1 cells were grown to near confluence in RPMI medium supplemented with 10% fetal bovine serum and 11mM glucose. Cells were then washed three times in serum- and glucose-free medium and incubated for 1 hour at 37°C in wash medium. Cells were then treated for 30 minutes as indicated. Protein was extracted from each group of cells and 15µg aliquots were analyzed by SDS-PAGE (under reducing conditions), followed by transfer to nitrocellulose and chemiluminescent detection. These results were obtained using a 1:500 dilution of primary antibody; however, conditions were not optimized and equivalent results may be obtained at the suggested antibody concentration of 25ng/ml (11). **Panel A** indicates the level of active MAPK enzyme detected using Anti-ACTIVE™ MAPK pAb. **Panel B** illustrates total ERK levels as detected using a pan ERK pAb, which recognizes active and basal forms of the ERK enzymes.

Immunocytochemistry

In the second scenario, we studied *in situ* activation of ERK enzymes in the rat embryo fibroblast cell line, REF52. We incubated REF52 cells in the presence or absence of Platelet-Derived Growth Factor (PDGF) and examined the kinetics of activation and the pattern of subcellular localization of ERK enzymes by performing immunocytochemistry using the Anti-ACTIVE™ MAPK pAb and a fluorescein-labeled goat anti-rabbit secondary antibody (Figure 2). In the absence of stimulation, extremely low background signals are obtained (Panel A). In contrast, stimulation of these cells with PDGF for 15 minutes resulted in clear and potent activation of ERK enzymes (Panel B). We observed that maximum ERK activation occurred after approximately 15 minutes, when the enzymes are predominantly extranuclear (Panel B). After 15-30 minutes the enzymes are located throughout the cytoplasm and in the nucleus, and by 30-45 minutes the enzymes have largely been inactivated, resulting in a return to background fluorescence levels (data not shown).

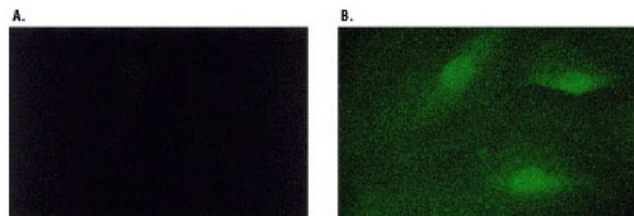


Figure 2. Use of fluorescence immunocytochemistry to detect ERK enzyme activation in REF52 cells using Anti-ACTIVE™ MAPK pAb. Nearly confluent REF52 cells were incubated at 37°C for 48 hours in medium containing 0.1% serum to suppress MAPK activity. Cells were then treated for 15 minutes with either serum-free medium as a control (Panel A) or medium containing 10ng/ml PDGF (Panel B). Cells were fixed in 10% paraformaldehyde for 10 minutes at room temperature, and permeabilized in 100% methanol for 10 minutes at -20°C. Following a blocking step with 1% BSA in PBS, cells were incubated with 2.5µg/ml (1:200 dilution) of the Anti-ACTIVE™ MAPK pAb for 1 hour, washed three times with 1mg/ml BSA and 0.05% NP-40 in PBS and incubated with a fluorescein-labeled secondary antibody. Cells were washed as before and images were captured using a Zeiss fluorescent microscope.

Summary

Promega's Anti-ACTIVE™ MAPK pAb is a highly effective reagent for studying ERK activation in a variety of immunological applications including Western blotting and immunocytochemistry. This reagent displays high signal-to-noise ratios and sensitivity,

allowing induction of MAPK activity to be clearly detected. The ability to obtain highly sensitive, selective and non-radioactive detection of MAPK activation illustrates the utility of this approach to dissect complex signal transduction cascades.

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Ordering Information

Product	Size	Cat.#
Anti-ACTIVE TM MAPK pAb	15µg*	V6671

*Sufficient for generating 600ml of Western blotting solution at the suggested working concentration of 25ng/ml.

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