

# Detection of ERK activation by a novel monoclonal antibody

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**Abstract** The mitogen-activated protein kinase, ERK is activated by a dual phosphorylation on threonine and tyrosine residues. Using a synthetic diphospho peptide, we have generated a monoclonal antibody directed to the active ERK. The antibody specifically identified the active doubly phosphorylated, but not the inactive mono- or non-phosphorylated forms of ERKs. A direct correlation was observed between ERK activity and the intensity in Western blot of mitogen-activated protein kinases from several species. The antibody was proven suitable for immunofluorescence staining, revealing a transient reactivity with ERKs that were translocated to the nucleus upon stimulation. In conclusion, the antibody can serve as a useful tool in the study of ERK signaling in a wide variety of organisms.

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**Key words:** MAPK; Signal transduction; Monoclonal antibody; Cellular localization

## 1. Introduction

Mitogen-activated protein kinases (MAPKs) are a family of highly homologous serine/threonine protein kinases that play a key role in the transmission of many extracellular signals (reviewed in [1–3]). The MAPKs share a common mechanism of activation which involves the phosphorylation of both threonine and tyrosine residues in a Thr-Xaa-Tyr (TXY) motif [4] positioned in their activation loop. Based on the identity of the residue in between the threonine and the tyrosine, the MAPK family can be divided into three main groups: the TEY group that includes the well-characterized ERK1 and ERK2 (p42 and p44 MAPKs; [5,6]) and also the big MAPK (ERK5; [7]); the TPY group that includes the JNK1 and JNK2 (SAPKs; [8,9]); and the TGY group that includes the p38 MAPK (HOG, RK, CSBP; [10–12]) and ERK6 [13]. Although the MAPKs are activated in response to a wide variety of extracellular stimuli, the extent of activation seems to vary between different stimuli. It is generally accepted that signals which induce proliferation and differentiation activate primarily the ERK1 and ERK2 of the TEY group, whereas agents which cause a cellular stress response mainly activate the TGY and TPY MAPKs [14].

The activation of the MAPKs is mediated by specific pro-

tein kinases that are collectively known as MAPK kinases (MAPKKs [1,15]). These MAPKKs are capable of phosphorylating both threonine and tyrosine residues in the TXY motif to cause a full activation of their downstream MAPK [15]. In spite of the extensive homology among the various isoforms of MAPK or MAPKK, in most cases the MAPKKs are specific to their downstream targets, and thereby form parallel linear pathways, both in lower organisms [16] and in mammals [1]. However, some ‘cross-talk’ between the MAPK cascades may exist in the case of the TPY and TGY kinases [17].

While the processes that lead to ERK activation are resolved, the inactivation mechanism of MAPKs is less well understood and appears to involve a battery of phosphatases. It should be noted that removal of phosphate from one of the phosphorylated threonine or tyrosine residues is sufficient to completely abolish MAPK activity [15]. Indeed, in the case of ERK1 and ERK2 the immediate inactivation process seems to involve protein serine/threonine phosphatases [18] whereas the inactivation that occurs in a somewhat delayed time course seems to be mediated by a dual specificity phosphatase specific for MAPK [19]. Thus, few minutes after treatment of cells with growth factors, doubly phosphorylated ERKs are found to co-exist along with the inactive, mono-phosphorylated and unphosphorylated forms of the ERKs.

In this paper, we describe a monoclonal anti-active ERK antibody that was generated by immunizing mice with a synthetic peptide containing 11 amino acids of the ERK activation loop, in which the threonine and tyrosine residues were phosphorylated. This antibody displays a remarkable specificity towards active ERKs and is shown to be efficient in immunofluorescence studies. In view of the structural conservation of ERK, this antibody can serve as a very useful tool in the study of signaling processes in a wide variety of species.

## 2. Materials and methods

### 2.1. Antibody development

The monoclonal anti-activated MAPK (diphosphorylated ERK1 and ERK2) (clone MAPK-YT) was raised against a diphospho peptide containing the 11 amino acid HTGFL(p)TE(p)YVAT corresponding to the phosphorylated form of the ERK activation loop. For immunization, the peptide was conjugated to KLH using the Maleimide Activated BSA/KLH Conjugation Kit (Sigma, MBK-1). BALB/c mice were immunized and their spleen cells were fused with NS-1 mouse myeloma cells. Hybridoma supernatants were screened for specific antibodies by ELISA on a BSA conjugate of the above peptide, and by Western blotting of cell extracts. One clone termed MAPK-YT, which secretes IgG1 antibodies (Sigma Immunosort TM Kit ISO-1), was selected. The antibody is now commercially available (Sigma Chemical Company).

### 2.2. ELISA assay

Antibody specificity was determined by ELISA. For this purpose,

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**Abbreviations:** MAPK, mitogen-activated protein kinase; ERK, extracellular signal responsive kinase; FCS, fetal calf serum; MBP, myelin basic protein; HA, hemagglutinin tag; EGF, epidermal growth factor; TEY peptide, 14-mer peptide derived from the activation loop of ERK and contains the regulatory threonine and tyrosine

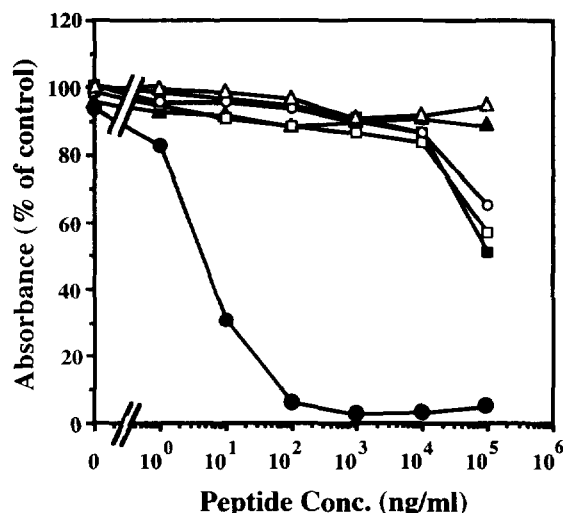


Fig. 1. Competition of MAPK-YT monoclonal antibody binding. Antibody binding to the BSA conjugated, doubly phosphorylated ERK peptide was competed by the pTEpY (●), pTEY (○), TEpY (□) and the non-phosphorylated TEY (△) peptides of ERK and with doubly phosphorylated peptides derived from the activation loops of JNK1 (■) and p38 MAPK (▲). The antibody binding was detected by ELISA as described in Section 2.

wells were coated overnight with 2 µg/ml of peptide-BSA conjugates. The assessment of MAPK-YT antibody specificity was performed by a competitive ELISA assay where antibody binding to the peptide conjugate was inhibited with 1 ng/ml–100 µg/ml of free non-phosphorylated form of the above peptide (TEY), diphosphorylated peptide (pTEpY), or each of the mono-phosphorylated peptides (pTEY, TEpY) of the ERK protein sequence. Doubly phosphorylated peptides of the activation loop from JNK (pTPpY) or p38 MAPK (pTGpY) were also used for competition. Bound antibody was detected with Peroxidase conjugated goat anti-mouse IgG antibody (Sigma).

### 2.3. Western blotting

To monitor the accumulation of doubly phosphorylated ERK, we analyzed cell extracts from stimulated and unstimulated cells. Cells were grown stimulated and lysed as previously described [20]. The cell extracts were separated on a 12% SDS-PAGE gel, and blotted onto nitrocellulose. Activated ERK was detected by probing blots with a 1:30000 dilution of the monoclonal antibody (MAPK-YT). Total ERK protein (activated and non-activated) was detected using a 1:40000 dilution of a polyclonal rabbit antibody directed towards subdomain X1 of the ERKs (Sigma; termed here general anti-MAPK). The blots were developed by Alkaline Phosphatase conjugated anti-mouse or anti-rabbit Fab (Sigma), or by horseradish peroxidase conjugated anti-mouse or anti-rabbit Fab and ECL (Amersham).

### 2.4. Immunoprecipitation of ERK mutants

COS7 cells were grown in DMEM+10% fetal calf serum (FCS) in 10 cm plates were transfected (DEAE-dextran; [20]) with 10 µg of hemagglutinin (HA) tagged wild type ERK2, or the phosphorylation site mutants of ERK2, HA-T183E-ERK2 (EEY), HA-Y185F-ERK2 (TEF) or HA-T183E,Y185F-ERK2 (EEF) that were cloned in the mammalian expression vector pCDNA1 (Invitrogen). One day later, each plate was divided into two, and these were serum starved (0.1% FCS, 16 h) for additional 24 h. One plate of each pair was stimulated with 100 µM Na<sub>3</sub>VO<sub>4</sub> and 200 µM H<sub>2</sub>O<sub>2</sub> for 18 min, and the other plate served as a basal control. After stimulation, the cells were washed, lysed and the ERKs were immunoprecipitated with a monoclonal anti-HA (Antibodies Unit, WIS) using protein A-agarose (20 µl, Sigma) as described [20]. The immunoprecipitates were subjected to SDS-PAGE and Western blotting as above.

### 2.5. Determination of ERK activity

Serum starved (0.1% FCS, 16 h) NIH-3T3, Rat1 and HeLa cells were stimulated with EGF (50 ng/ml) for the desired time. After stimulation, the cells were washed, lysed and ERKs were immunoprecipitated with anti-ERK antibody (C14, Santa-Cruz Biotechnology, Inc., CA) using protein A-agarose (20 µl, Sigma). ERK activity was determined by the phosphorylation of myelin basic protein (MBP) as previously described [20].

### 2.6. Fluorescent cell staining

Serum starved (0.1% FCS, 16 h) NIH-3T3, Rat1 and HeLa cells were treated either with EGF (50 ng/ml) for 10 and 30 min or with

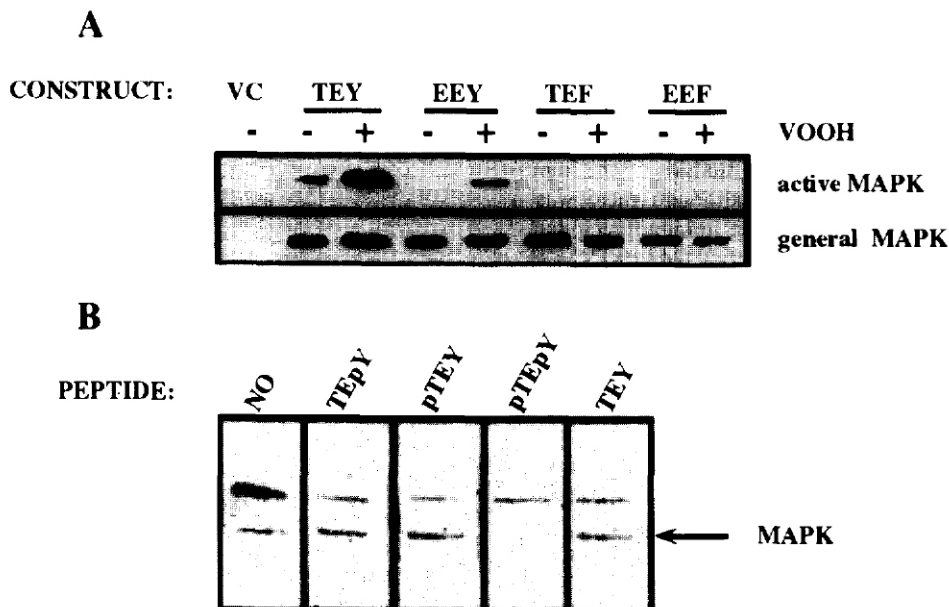


Fig. 2. MAPK-YT monoclonal antibody is specific to the active, doubly phosphorylated form of ERK2. A: COS7 cells were transfected with HA tagged wild type ERK2 (TEY), HA-T183E-ERK2 (EEY), HA-Y185F-ERK2 (TEF) or HA-T183E,Y185F-ERK2 (EEF). Each plate was divided into two and these were serum starved followed by stimulation of one of them with peroxovanadate (20 min) as described. After stimulation, the cells were lysed and the ERKs were immunoprecipitated with monoclonal anti-HA antibodies and subjected to SDS-PAGE and western blotting. B: The wild type ERK that was immunoprecipitated from the peroxovanadate activated cells was subjected to SDS-PAGE and blotted onto nitrocellulose. Separate lanes of this blot were incubated with the MAPK-YT monoclonal antibody in the presence of the pTEpY, TEpY, pTEY, TEY or no peptide (50 µg/ml each).

10% FCS for 30 min. Subsequently, the cells were washed with phosphate buffered saline (PBS) and fixed in 3% formaldehyde in PBS solution for 20 min. Following additional washes with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Co-staining was performed by 45 min incubation with the monoclonal antibody MAPK-YT (1:50) together with the general polyclonal Rabbit anti-MAPK antibody (Sigma), followed by 45 min incubation with a mixture of rhodamine conjugated goat anti-mouse IgG and FITC conjugated goat anti-rabbit IgG antibodies (both 1:200, Jackson ImmunoResearch Laboratories). Fluorescent staining was viewed with a 1024 confocal microscope (Bio-Rad Laboratories).

### 3. Results and discussion

A monoclonal antibody, termed anti-activated MAPK (diphosphorylated ERK1 and ERK2; clone MAPK-YT), was raised against an eleven amino acid peptide, derived from the activation loop of vertebrate ERKs which contained phosphorylated threonine and tyrosine residues. In ELISA, the antibody recognized strongly the doubly phosphorylated immunizing peptide and negligibly the non-phosphorylated TEY peptides (data not shown). In order to further study the specificity of the antibody, we examined the ability of the mono-phosphorylated and non-phosphorylated peptides to compete out the binding of the antibodies to the immunizing peptide antigen in a competitive ELISA assay. The only peptide that efficiently competed with antibody binding was the doubly phosphorylated peptide pTEpY ( $IC_{50} < 10$  ng/ml; Fig. 1), whereas the other peptides competed in much higher concentrations ( $IC_{50} > 100$   $\mu$ g/ml for TEpY, pTEY and TPY; Fig. 1). By the same assay we could also show that the binding of the monoclonal antibody (MAPK-YT) was not competed by doubly phosphorylated peptides derived from the TPY and TGY motif enzymes JNK1 and p38MAPK respectively (Fig. 1).

Since in ELISA the monoclonal antibody (MAPK-YT) appeared to selectively recognize the doubly phosphorylated peptide, we undertook to test whether this will also be true for the whole protein. Thus, COS7 cells were transfected with hemagglutinin (HA) tagged constructs of ERK2, or its phosphorylation site mutants. These cells were activated by peroxovanadate which is a strong general stimulator of several MAPKs [21], and ERK protein was immunoprecipitated with anti-HA antibodies. Western blot analysis of the immunoprecipitated proteins revealed that although there was a similar amount of ERK2 in each of the immunoprecipitates, the anti-active MAPK monoclonal antibody (MAPK-YT) recognized primarily the wild type ERK2 (TEY) and to some extent (3.5% of the wild type) also the T183E (EEY), but not the Y185F (TEF) or the T183E,Y185F (EEF) mutants (Fig. 2A). The recognition of ERK was efficiently competed with the doubly phosphorylated pTEpY peptide but not by the other phosphorylation site peptides (pTEY, TEpY, and TEY; Fig. 2B). These results further demonstrate the specificity of the monoclonal antibody (MAPK-YT) to the doubly phosphorylated, active form of ERK. The fact that the T183E mutant, that contains only the phosphorylated tyrosine, was recognized to some extent by the antibody is most likely due to the acidic moiety of the glutamic acid that mimics that of the phosphate in that position. Thus, the actual difference in extent of recognition between the mono-tyrosine phosphorylated and doubly phosphorylated ERKs is probably much higher than that observed in the Western blot assay, and should be closer to the difference demonstrated in the ELISA competition assays.

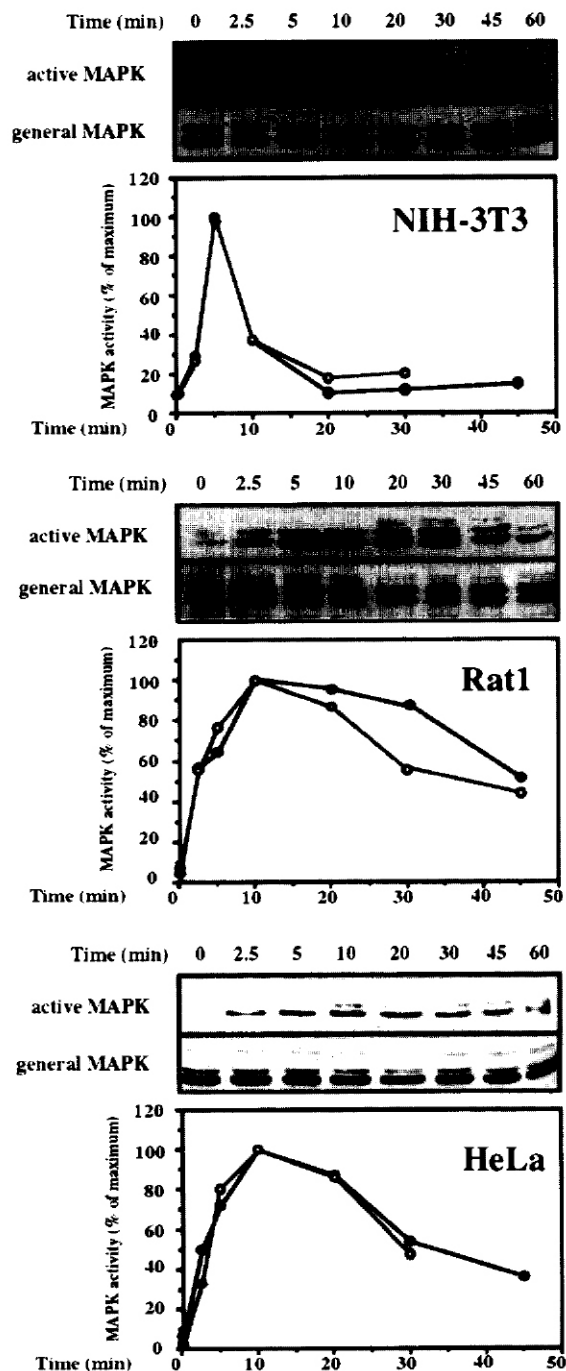


Fig. 3. Comparison of ERK activity and staining by the MAPK-YT monoclonal antibody in EGF stimulated NIH-3T3, Rat1 and HeLa cells. NIH-3T3, Rat1 and HeLa cells were stimulated with EGF (50 ng/ml) for the indicated times, harvested and lysed as described. Lysates were then subjected to a western blot analysis with the MAPK-YT monoclonal or with the general anti-MAPK polyclonal antibodies (developed by the alkaline phosphatase method), and ERK activity was assayed by phosphorylation of MBP after immunoprecipitation with anti C-terminus antibodies as described. The amount of immunoreactivity in the blots (●) or in the autoradiograms of the kinase assays (○) was determined (Bio-Rad 690 densitometer) and plotted as % of maximal activity of the separate experiments.

In order to further characterize the antibody, we wanted to determine whether the intensity of detection by this antibody in Western blot fully correlates with ERK activity. Therefore, NIH-3T3, Rat1 and HeLa cells were stimulated with EGF

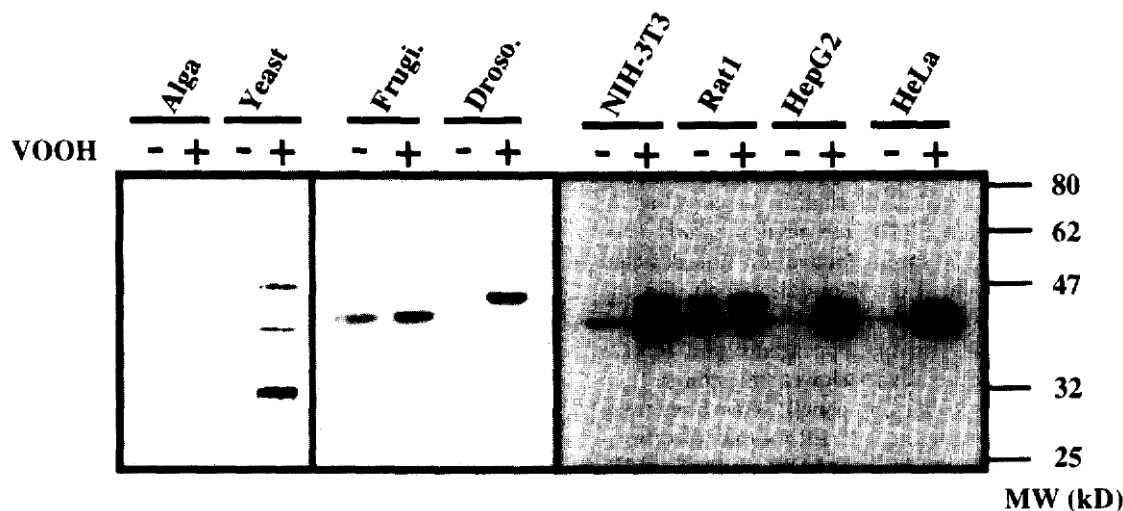


Fig. 4. Western blot analysis of cell extracts from various organisms with the MAPK-YT monoclonal antibody. Cells ( $10^6$  cells per reaction) from alga (*Dunaliella salina*), yeast (w303), insects (*Spodoptera frugiperda* Sf9 and *Drosophila* Schneider S2 cells) and mammals (NIH-3T3 (mouse), Rat1 (rat), HeLa (human), and HepG2 (human)) were either treated with the general activator VOOH (100  $\mu$ M vanadate and 200  $\mu$ M  $H_2O_2$ ; 20 min, 37°C) or left untreated. The cells were harvested, and the cell extracts were separated by SDS-PAGE as in Section 2. Active MAPK was detected using 1:30 000 dilution of the MAPK-YT monoclonal antibody and the blots were developed either by the alkaline phosphatase (alga and yeast) or by the ECL methods.

and their cytosolic extracts were examined for ERK activity towards MBP after immunoprecipitation with anti-ERK C-terminus antibody, as well as for the intensity of staining by the MAPK-YT monoclonal antibody in Western blot analysis. A direct correlation between ERK activity and the recognition by the antibody was observed in all cell types (Fig. 3), verifying that the antibody is a valid tool to determine MAPK activation in vivo. Interestingly, the time course of ERK activation by EGF varied between the three cell types. In the NIH-3T3 cells, the activation was transient, peaked at 5 min after the addition of EGF and decreased thereafter. On the

other hand, the activation of ERKs in HeLa and Rat1 cells was more prolonged; in both cells it peaked at 10 min after stimulation and remained high for the next 50 min, demonstrating a pattern of sustained activation. It was previously shown that the duration of ERK signal may determine cell fate in some cell types such as PC12 cells, where transient activation leads to proliferation, and sustained ERK activation leads to differentiation (reviewed in [3]). However, in the cells examined here, Rat1 and HeLa, the mitogenic agent EGF, caused sustained activation which did not seem to block proliferation. The role of the sustained activation in these cells

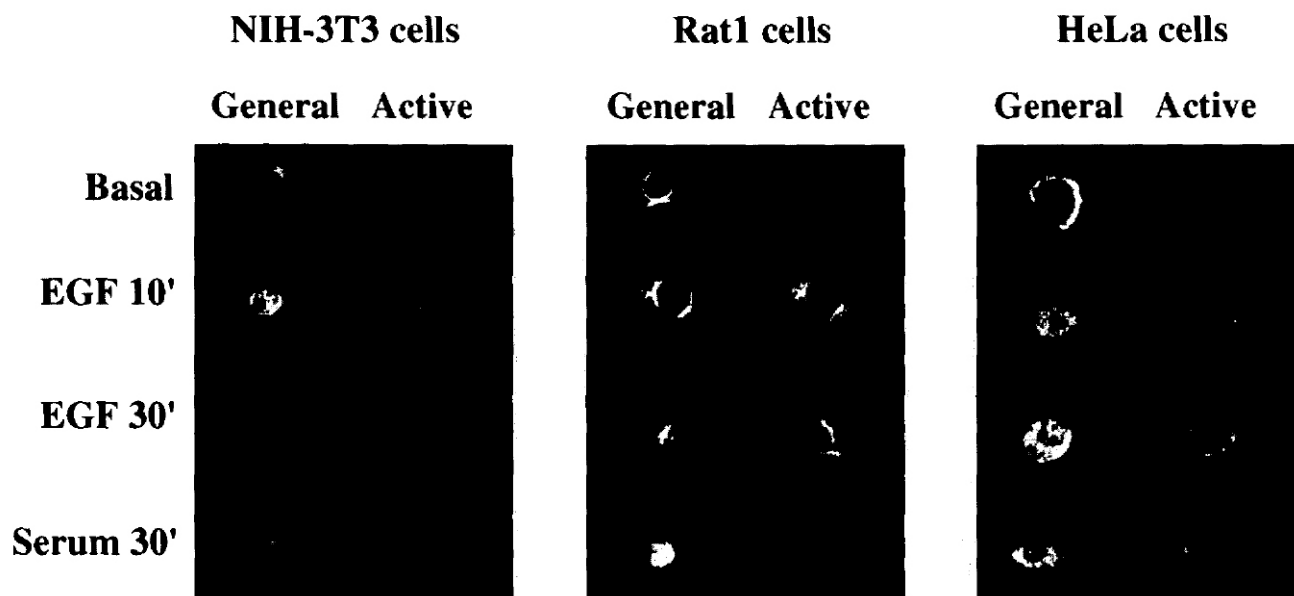


Fig. 5. Staining of unstimulated and stimulated NIH-3T3, Rat1 and HeLa cells with MAPK-YT monoclonal antibody and general anti-MAPK. NIH-3T3, Rat1 and HeLa cells were grown on microslides for 48 h, serum starved (16 h) and stimulated by EGF (50 ng/ml 10 and 30 min) or by serum (10%, 30 min). The cells were fixed, permeabilized and stained as described in Section 2. The cells were viewed by confocal microscope (objective  $\times 40$ , zoom 4). Both green (general anti-MAPK) and red (anti-active MAPK) channels of the same cells are presented (left and right sides of each panel respectively). Since each slide was stained separately, the differences in intensity between the different treatments may not be accurate.

is not known but may indicate that in these cells ERKs have additional roles in later processes.

It should be also noted that small differences between the rate of ERK1 and ERK2 activation was detected in some of the cell lines. Thus, in NIH-3T3 cells, the activation of ERK1 appeared to precede that of ERK2 (2.5 min) whereas the activation of ERK2 remained high slightly longer (Fig. 3, upper panel). In HeLa cells ERK2 was predominantly activated and only some ERK1 specific activation was detected 10–45 min after stimulation (Fig. 3, middle panel). On the other hand, in Rat1 cells the activation of both isoforms occurred simultaneously, but a gradual appearance of a 46 kDa band was detected that occurred in a slower time course than ERK1 and ERK2 (Fig. 3, bottom panel). This band may correspond to the poorly characterized ERK4 that was previously identified through the use of anti-MAPK antibodies [22].

Since the TEY activation motif of MAPKs is conserved throughout evolution, we examined the ability of the antibody to specifically recognize the active form of MAPK of various organisms. Indeed, enhanced intensity of bands around 40 kDa was observed upon peroxovanadate activation of yeast, insects, and mammalian cells (but not the alga *Dulania*; Fig. 4). In yeast, three proteins were detected by the antibody, two of them at 40 and 48 kDa, are probably MAPKs [16], whereas the nature of the lower molecular weight band is not known. As expected [23], in insect cells only one form of MAPK was recognized by the antibody, whereas in mammalian cells, several bands were detected, that were also recognized by general anti-MAPK antibody (not shown). Thus, in NIH-3T3 cells the MAPK-YT monoclonal antibody detected the well known 42 and 44 kDa MAPKs (ERK2 and ERK1) that were also detected in Rat1 cells, in addition to a 46 kDa band that was detected after longer exposure. However, in the human cells HeLa and HepG2, the position of the bands was at 42 and 43 kDa. This difference in migration could be either due to changes in primary sequence of ERK1 that were not previously reported, or due to different post-translational modifications other than the phosphorylation of the TEY motif.

Another property of the MAPK-YT monoclonal antibody was its ability to stain active ERK by immunocytochemical techniques. Thus, stimulated NIH-3T3, Rat1 and HeLa cells were stained with both the MAPK-YT monoclonal antibody and the general polyclonal anti-MAPK antibody (Fig. 5). As expected [24], the staining of non-stimulated cells with the general anti-MAPK antibody was detected primarily in the cytosol, which turned nuclear (at least in part) upon stimulation. However, the staining with the anti-active MAPK monoclonal antibody (MAPK-YT) did not follow that of the general ERKs. In the basal state, some weak staining was detected in the cytosol of the three cell lines, which enhanced in intensity upon activation. In spite of the massive translocation of ERKs to the nucleus, appreciable nuclear staining was observed in NIH-3T3 and HeLa cells only 10 min after EGF stimulation but not 30 min after EGF or serum treatment of the same cells, and not at all in Rat1 cells. These results, which were corroborated by cellular fractionation and activity assays (Rubinfeld and Seger, unpublished), may indicate that in these cells, active ERK is translocated to the nucleus shortly after stimulation and this is followed by a rapid inactivation of ERK. Therefore, the sustained activation obtained in HeLa and Rat1 cells (Fig. 3) is probably due to slower inactivation in the cytosol and can not be attributed to

the nuclear MAPK phosphatases observed in other cell lines [19,25]. The mechanism of this down-regulation and its exact localization are not clear as yet.

In summary, we report here on the development of a novel, monoclonal antibody which specifically identifies the active (doubly phosphorylated) but not the inactive (mono- or non-phosphorylated) forms of ERKs. This antibody has been used here to show transient activation of ERK in the nucleus, and in a separate study [26] to study MAPK activation during *Drosophila* development. Therefore, the MAPK-YT monoclonal antibody can serve as a convenient and useful tool to study MAPK activation in many organisms.

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