Detection of partially phosphorylated forms of ERK by monoclonal antibodies reveals spatial regulation of ERK activity by phosphatases

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Abstract When cells are stimulated by mitogens, extracellular signal-regulated kinase (ERK) is activated by phosphorylation of its regulatory threonine (Thr) and tyrosine (Tyr) residues. The inactivation of ERK may occur by phosphatase-mediated removal of the phosphates from these Tyr, Thr or both residues together. In this study, antibodies that selectively recognize all combinations of phosphorylation of the regulatory Thr and Tyr residues of ERK were developed, and used to study the inactivation of ERK upon mitogenic stimulation. We found that inactivation of ERK in the early stages of mitogenic stimulation involves separate Thr and Tyr phosphatases which operate differently in the nucleus and in the cytoplasm. Thus, ERK is differentially regulated in various subcellular compartments to secure proper length and strength of activation, which eventually determine the physiological outcome of many external signals.

Key words: Signal transduction; Phosphatase; Kinase; Anti-phospho-antibody; Mitogen-activated protein kinase

1. Introduction

The response of cells to extracellular stimuli is mediated by an array of intracellular signaling pathways that relay the extracellular information to different subcellular compartments. An important component in many intracellular signaling events is the extracellular signal-regulated kinase (ERK) which belongs to the family of mitogen-activated protein kinases (MAPKs) [1–4]. The three known isoforms of ERK (ERK1, ERK1b and ERK2 [5]) are protein serine/threonine (Ser/Thr) kinases, which are phosphorylated and activated by the upstream kinases, MEK1 and MEK2 [1]. These latter kinases phosphorylate ERKs on regulatory Thr and tyrosine (Tyr) residues which are localized in the activation loop of ERK [6]. Structurally, the phosphorylation of these two regulatory residues causes a depression in the surface of the substrate binding site of ERK, which consequently positions substrates towards the γ-phosphate of ATP, and allows phosphorylation to occur [7]. These changes induce a full catalytic activity (~5 μmol/min/mg) of ERK, which is 5–6 orders of magnitude higher than its basal activity. Interestingly, ERKs are activated either when both Thr and Tyr residues are phosphorylated; phosphate incorporation into either one of them does not change their catalytic activity [8].

MEK appears to be the only kinase capable of phosphorylating the two activating residues of ERK [9]. Kinetic analysis indicated that phosphorylation of the Tyr residue of ERK precedes that of the Thr residue [10,11]. Detailed in vitro studies [11,12] revealed that phosphorylation of these two residues is non-processive, i.e. that MEK must dissociate from ERK after the first phosphorylation and rebind for the second phosphorylation to proceed. However, the two phosphorylation events are cooperative and occur almost simultaneously [13]. Thus, the amount of inactive mono-Tyr phosphorylated ERK after stimulation should be minimal, even though mono-phosphorylated species of ERK may exist due to inactivation processes. Inactivation of ERK usually occurs by dephosphorylation, and may occur by removal of phosphate from Tyr alone, Thr alone, or from both residues together [8]. In fact, protein Tyr phosphatase (PTP), Ser/Thr protein phosphatase (PP) and dual specificity phosphatase (MKPs) have been implicated in the inactivation of ERK [14–16]. Since MKPs are usually induced proteins [17], the short term dephosphorylation of ERK probably produces mono-phosphorylated ERKs.

Previously [18], we prepared a monoclonal antibody (mAb) (MAPK-YT; also referred to as mAb-YT) that specifically recognizes the phosphorylated Thr and Tyr residues of the activation loop of ERK. This antibody was successfully used to study ERK activation in various systems [18–21]. Herein, a set of mAbs against the various phosphorylation states of ERK were prepared, and together with the mAb-TY, they were used to study the inactivation of ERK. We found that the nuclear inactivation of ERK involves an initial dephosphorylation by a Ser/Thr phosphatase, whereas the inactivation of ERK in the cytoplasm is initiated by a Tyr phosphatase(s). Thus, the activation of ERK after mitogenic stimulation is regulated in both a temporal and spatial manner by different phosphatases.

2. Materials and methods

2.1. Buffers and cells

Buffer A: 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; EGF, epidermal growth factor; FCS, fetal calf serum; BSA, bovine serum albumin; TEY peptide, 11-mer peptide derived from the activation loop of ERK; PT-, PY-, NP- and DP-TEY, Thr-, Tyr-, non- and doubly phosphorylated TEY peptide, respectively; Tyr, tyrosine; Ser, serine; Thr, threonine; mAb, monoclonal antibody; pAb, polyclonal antibody

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EDTA, 1 mM DTT, 1 mM sodium orthovanadate. Buffer H: buffer A containing 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 2 μg/ml pepstatin. Chinese hamster ovary cells stably transfected by ErbB1 (CHO.E) were a generous gift from Dr. Y. Yarden (The Weizmann Institute of Science, Rehovot, Israel). CHO.E cells were grown in 50% DMEM/50% F12 supplemented with 10% fetal calf serum (FCS).

2.2. Antibody development

mAbs were raised against peptides containing the 11 amino acid HTGFLTEYVAT corresponding to the ERK activation loop either Thr-phosphorylated (PT-TEY), Tyr-phosphorylated (PY-TEY) or non-phosphorylated (NP-TEY). For immunization, the peptides were conjugated to KLH using glutaraldehyde. 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 enzyme-linked immunosorbent assay (ELISA) on a bovine serum albumin (BSA) conjugate of the above peptides (peptide-BSA), and by Western blotting of cell extracts. The following clones were selected: ERK-YNP (termed mAb-155) was originated against the NP-TEY peptide; ERK-PT115 (mAb-115) was originated against the PT-TEY peptide; and ERK-PY193 (mAb-193) was originated against the PY-TEY peptide. In this study, we used the ascitic fluid of these and of the MAPK-YT (mAb-TY) clones (~4 mg/ml). All the above antibodies are commercially available from Sigma.

2.3. ELISA and dot blot

Antibody specificity was determined by ELISA and dot blot. Wells in a 96 well plate were coated overnight with 2 μg/ml of peptide-BSA conjugates and washed. After incubation with dilutions of each antibody (1 h), the wells were washed, incubated with peroxidase-conjugated secondary antibodies (1 h) and developed according to the manufacturer's instructions. For competitive ELISA, the antibody binding to the peptide-BSA conjugate was competed with 1 ng/ml–100 μg/ml (1 h) of non-conjugated NP-TEY, PT-TEY, PY-TEY, or doubly phosphorylated (DP-TEY) peptides, as well as with doubly phosphorylated peptides of JNK (pTPpY) or p38 MAPK (pTGpY). For dot blot, different peptide-BSA conjugates were spotted on a nitrocellulose membrane. The membrane was blocked by BSA and blotted with antibodies followed by secondary antibodies conjugated with alkaline phosphatase. The staining was developed by NBT/BCIP.

2.4. Western blot analysis

CHO.E cells were grown in 10 cm plates and serum-starved (0.1% FCS) for 16 h. After stimulation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and once with ice-cold buffer A. Cells were harvested as previously described [22] with 0.3 ml of buffer H followed by sonication (2×7 s, 40 W) and centrifugation (15 000×*g*, 15 min, 4°C). The supernatants, which contained cytosolic proteins, were collected and aliquots from each sample (20 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting. The different phosphorylation states of ERK were detected by probing the blots with mAb-TY, mAb-115, mAb-155, mAb-193 or αGPY (Zymed). To separate Thr phosphorylated ERK from non-phosphorylated ERK, 0.4% bisacrylamide was used instead of 0.8% when casting the SDS–polyacrylamide gel. Total ERK was detected with 7884 polyclonal antibody (pAb 1:20 000; [23]). The blots were developed with alkaline phosphatase or horseradish peroxidase-conjugated anti-mouse or anti-rabbit Fab antibodies (Jackson).

2.5. Immunofluorescent staining

Serum-starved (0.1% FCS, 40 h) CHO.E cells were treated with epidermal growth factor (EGF) (50 ng/ml) for up to 15 min. Subsequently, the cells were washed with PBS and fixed in 3% formaldehyde in PBS for 20 min. Following additional washes with PBS, the cells were permeabilized with methanol at −10°C for 10 min. Staining was performed as previously described [24] by 45 min incubation with pAb 7884, or the mAbs-TY, 115, 155 or 193, followed by 45 min incubation with appropriate secondary antibodies conjugated to Cy3 or FITC. Fluorescence was viewed with a fluorescent microscope (Zeiss).

3. Results and discussion

3.1. Preparation and characterization of anti-phospho-ERK antibodies

In order to study the mechanism of ERK phosphorylation and dephosphorylation in vivo, a set of mAbs which could distinguish between the different phosphorylation states of ERKs was developed. The antigens used to generate these mAbs were 11 amino acid peptides derived from the activation loop of ERK2 (residues 178–188) with no incorporated phosphate (NP-TEY), mono-phosphorylated on Thr (PT-TEY) or mono-phosphorylated on Tyr (PY-TEY). Three mAbs were obtained: ERK-YNP (referred to as mAb-155), ERK-PT115 (referred to as mAb-115) and ERK-PY193 (referred to as mAb-193), which were raised against NP-TEY, PT-TEY and PY-TEY, respectively.

In a direct ELISA (Fig. 1A), in which BSA-conjugated peptides were used as a recognition matrix, mAb-155 recognized the Thr-phosphorylated peptide (PT-TEY-BSA) 10-fold

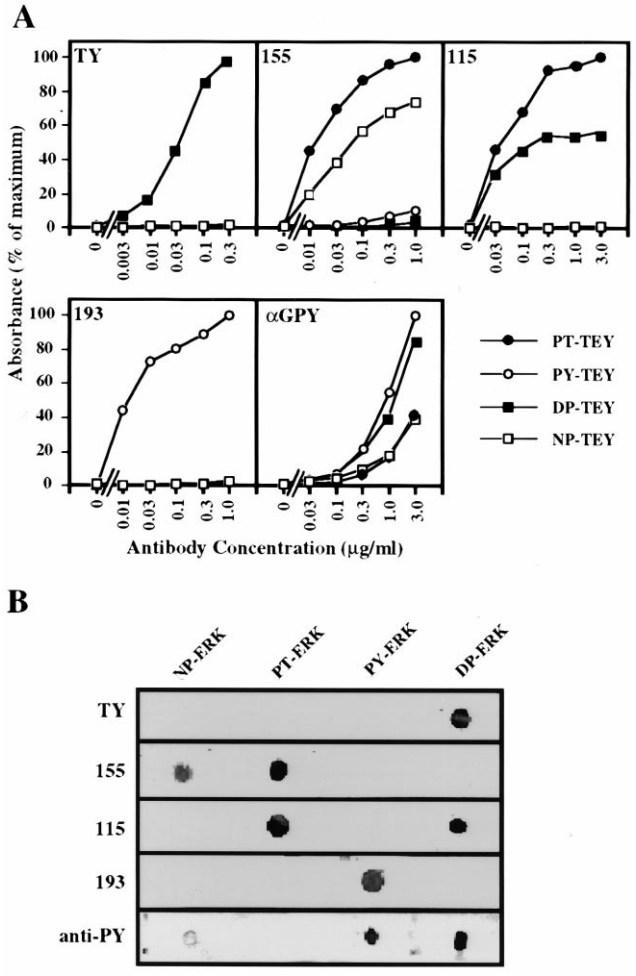

Fig. 1. Specificity of the anti-phospho-ERK antibodies using ELISA and dot blot assay. A: 96 Well plates were coated with the appropriate BSA-conjugated peptides: PT- ●, PY- ○, DP- ■ or NP- □ TEY (1 μg/ml), followed by incubation with the various antibodies. The binding was detected with peroxidase-conjugated goat anti-mouse antibody. B: The same BSA-conjugated peptides as in A were spotted on a nitrocellulose membrane. The membrane was blocked by 2% BSA and incubated with different antibodies (TY, 155, 115, 193 or GPY) followed by alkaline phosphatase-conjugated second antibodies. These results were reproduced three times.

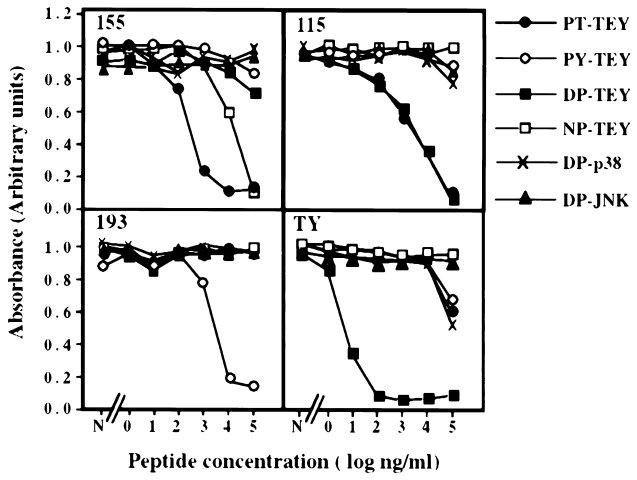

Fig. 2. Specificity of the anti-phospho-ERK antibodies using competitive ELISA. 96 Well plates were coated with the appropriate BSA-conjugated peptides (PT-TEY for 155 and 115, PY-TEY for 193 and DP-TEY for TY; 1 μg/ml each). The various antibodies were added (50 ng/ml) together with the indicated amount of the PT- ●, PY- ○, DP- ■ or NP- □ TEY peptides and with DP peptides derived from the activation loops of p38 MAPK **X** and of JNK ▲ (N: no peptide). The binding was detected with peroxidase-conjugated goat anti-mouse. The results here are from a representative experiment that was reproduced five times.

better (50% binding: 0.016 μg/ml) than non-phosphorylated peptide conjugated to BSA (NP-TEY-BSA, 50% binding: 0.085 μg/ml). mAb-115, mainly recognized the PT-TEY-BSA (50% binding: 0.04 μg/ml) and mAb-193 specifically recognized the Tyr-phosphorylated form of the peptide (PY-TEY-BSA, 50% binding: 0.015 μg/ml). A pAb that recognizes phosphorylated Tyr residues in general (αGPY) recognized both PY-TEY-BSA (50% binding: 0.5 μg/ml) and DP-TEY-BSA (50% binding: 0.7 μg/ml), both of which have phosphates incorporated to their Tyr residue.

Similar results to those obtained with the ELISA were also obtained in a dot blot assay (Fig. 1B). As previously reported [18], mAb-TY recognized only DP-ERK-BSA. MAb-155 recognized mainly PT-ERK-BSA, but also NP-ERK-BSA, mAb-115 recognized both PT-ERK-BSA and DP-ERK-BSA, mAb-193 recognized only PY-ERK-BSA and finally, the GPY antibody recognized both PY-ERK-BSA and DP-ERK-BSA. Thus, this set of five antibodies (TY, 155, 115, 193 and αGPY) appears to be capable of distinguishing between all the possible phosphorylation states of the TEY peptides.

The antibodies were further characterized by examining the ability of various peptides to compete with the antibodies for binding to BSA-conjugated peptides in a competitive ELISA (Fig. 2). As previously reported, the binding of mAb-TY to the DP-TEY-BSA was abolished only by the DP-TEY peptide [18]. mAb-155 was not as specific, and in competitive inhibition assays of its binding to PT-TEY-BSA, the IC_{50} of PT-TEY was about 0.5 μg/ml whereas the IC_{50} of NP-TEY was 6 μg/ml, indicating that mAb-155 appears to recognize PT-TEY best, but may interact also with NP-ERK. Another antibody that demonstrated specificity for PT-TEY was mAb-115, which differs from the mAb-155 in that both PT-TEY and DP-TEY inhibited its binding to PT-ERK-BSA with almost the same IC_{50} (∼5 μg/ml). Therefore, mAb-115 appears to recognize phosphorylated Thr in TEY peptides independent of whether the adjacent Tyr residue is phosphorylated. In addition to the two anti-PT-ERK antibodies, we examined mAb-193 and found that its binding to PY-TEY-BSA was specifically displaced only by PY-TEY (IC_{50} of 3 μg/ml), which indicated its specificity for the mono-phosphorylated PY-TEY. The unique specificity of the antibodies (Table 1), which can distinguish between the different phosphorylation states of ERK, should make them a useful tool in the study of ERK regulation upon extracellular stimulation.

Table 1
Specificity of the anti-phospho-ERK antibodies

	NP-ERK	PT-ERK	PY-ERK	DP-ERK
mAb-TY	−	−	−	+++
mAb-155	+	+++	−	−
mAb-115	−	+++	−	++
mAb-193	−	−	+++	−
αGPY	±	±	++	++

This table summarizes the specificities of the various antibodies to the different phosphorylation states of ERK according to the different parameters used (direct ELISA, competitive ELISA, recognition of ERK mutants and gel-shift as shown in Figs. 1–3). +++ High specificity, ++, medium specificity, + low specificity, − no recognition.

3.2. Changes in ERK phosphorylation upon mitogenic stimulation of CHO.E cells

The anti-phospho-ERK antibodies were used to monitor the phosphorylation states of ERK upon mitogenic stimulation in CHO cells stably transfected with EGF receptor (CHO.E). Thus, serum-starved CHO.E cells were stimulated with EGF for various periods, and the cell lysates were subjected to SDS–PAGE followed by Western blot analysis with the various antibodies (Fig. 3). The results obtained with the anti-general ERK, pAb 7884 confirmed that essentially equivalent amounts (< 10% differences) of ERKs were loaded in each lane, allowing to interpret variations in staining intensities by the various antibodies as changes in the amount of incorporated phosphates to the appropriate Tyr and Thr residues. Up to four bands can be detected with the various antibodies, these are ERK1, shifted (phosphorylated) ERK1, ERK2 and shifted (phosphorylated) ERK2. Although only the results obtained with ERK2 will be described, similar results were obtained for ERK1.

When the cell lysates were subjected to Western blot analysis with mAb-TY, the dually phosphorylated ERK2 appeared within 2 min of EGF stimulation, peaked within 6 min and declined after an additional 9 min (Fig. 3). We then examined the pattern of phosphorylation with mAb-155, which recognizes both Thr-phosphorylated and non-phosphorylated TEY peptide. Since only the minority of ERK2 became phosphorylated, as can be judged from the small amount of shifted ERK2 (Fig. 3, 7884), it can be concluded that the upshifted band was detected by mAb-155 in a more efficient manner than the non-phosphorylated ERK2 (lower band). The accumulation of PT-ERK was therefore detected from the intensity of the shifted band. Thus, the PT-ERK2 was detected already 2 min after activation, peaked at 8 min after stimulation and decreased, in similar (or sometimes slightly slower) kinetics, to that obtained with the DP-ERK antibodies. As would be expected from this similarity, when the state of ERK phosphorylation was examined using the mAb-115, which recognizes DP-TEY and PT-TEY, the

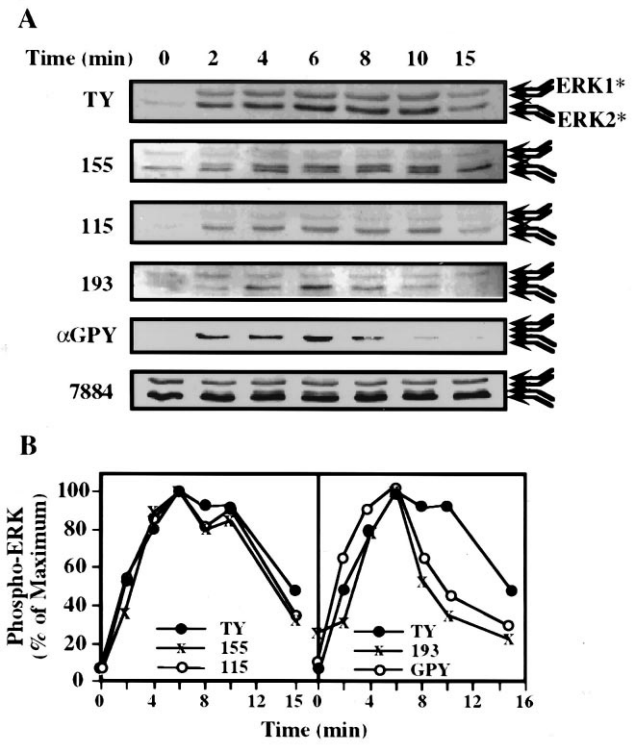

Fig. 3. Time courses of EGF-induced ERK phosphorylation in CHO.E cells. A: Serum-starved CHO.E cells were stimulated with EGF (50 ng/ml) for the indicated times. Cell lysates were then subject to Western blot analysis with the various mAbs, anti-general phosphotyrosine antibody (αGPY) or anti-general ERK antibody (pAb 7884). The places of ERK2 and its 'shifted' form (ERK2*) and that of ERK1 and its 'shifted' form (ERK1*) are indicated. B: The intensity of staining of the upper band of ERK2 by mAbs-TY, 155 and 115 (left panel) and TY, 193 and pAb αGPY (right panel) were determined as percentage of maximal intensity of each antibody. The results are from a representative experiment that was reproduced three times.

results obtained were also similar to those obtained with mAb-TY and with mAb-155 (Fig. 3, 115).

In contrast to the similar time course observed with mAb-TY, mAb-155 and mAb-115, the kinetics of phosphorylation observed with the antibodies which recognize PY-ERK (mAb-193 and αGPY) were significantly different from the kinetics of the DP-ERK2 appearance. Thus, the accumulation of PY-ERK2 upon EGF stimulation, which was detected with mAb-193, was transient, peaked at 6 min after stimulation and sharply declined to basal levels. Then, the accumulation of phosphorylated Tyr-183 in ERK2, either in the presence or the absence of phosphate on Thr-185, was detected by the general anti-phosphotyrosine antibody pAb αGPY. The kinetics of this phosphorylation appeared similar to that detected by mAb-193 and not to that detected by mAb-TY. This similarity between the profiles detected by mAb-193 and of GPY indicates that the amount of mono-phosphorylated PY-ERK produced after activation is larger than that of the dually phosphorylated ERK, and therefore PY-ERK is the main contributor to the profile obtained with the pAb αGPY.

Mono-phosphorylated PY-ERKs can be formed by two distinct mechanisms including phosphorylation by MEK, which phosphorylates Tyr-183 before it can phosphorylate Thr-185 [25], or by pSer/Thr phosphatases which dephosphorylate the phosphothreonyl residue of DP-ERK. In addition, the level of PY-ERK can be regulated by the removal of the phosphate from Tyr-183 or by phosphorylation on the adjacent Thr-185 to form a DP-ERK which is not recognized by the mAb-193. Our results show that MEK activation, under the condition used, peaks 4 min after activation, and reduces back to basal levels within 12 min (data not shown). However, since the phosphorylation of the two regulatory residues of ERK after EGF stimulation occurs almost simultaneously ([8] and data not shown), selective phosphorylation by MEK on Tyr-183 alone probably cannot account for the production of the large amount of PY-ERK described above. On the other hand, it is likely that the accumulation of PY-ERK is carried out and regulated by various phosphatases. Thus, the increase in the amount of mono-phosphorylated PY-ERK probably occurs due to removal of the phosphate from the Thr residue of the DP-ERK. The decrease in the amount of PY-ERK is most probably due to the removal of the phosphate from the Tyr-183 of ERK2 and not due to rephosphorylation by the residual activity of MEK. Therefore, the high amount of PY-ERK formed in the CHO.E cells after EGF stimulation (Fig. 3, 193) is likely to occur by constitutively active protein Ser/Thr phosphatases, which can remove the phosphate from the Thr but not the Tyr residues of ERKs, and which have been reported to play a role in the inactivation process of ERK [15]. In addition, the existence of a significant amount of mono-phosphorylated PT-ERK described above and the sharp decline in the amount of PY-ERK in the absence of significant MEK activity indicate that as suggested [26], PTP is also involved in the mechanism of ERK dephosphorylation. The accumulation of PY-ERK2 6 min after stimulation, and the slower time course of appearance of mono-phosphorylated PT, would predict that the cellular activity of this putative PTP is lower than that of the protein Ser/Thr phosphatase. However, the sharp reduction in amounts of PY-ERK2 at 8 min after stimulation suggests that an additional PTP with different mode of regulation may be involved in the later stages of ERK's dephosphorylation as will be discussed below.

Another conclusion that can be drawn from our results is that the slow migrating (shifted) band of ERKs contains not only the active, dually phosphorylated form, but also the mono-phosphorylated forms of ERK2. Thus the slower migration of ERK on SDS–PAGE does not always correlate with the actual ERK activity, especially during inactivation processes as observed here (Fig. 3). Therefore, the use of the 'upshift assay' to study ERK activation may not always be accurate.

3.3. Intracellular distribution of phosphorylated ERKs

In order to study the subcellular distribution of the various phosphorylated forms of ERKs, we have used the different anti-phospho-ERK antibodies in immunohistochemical studies. The appearance of mono-phosphorylated and dually phosphorylated ERKs (Fig. 4) showed similar kinetics to that observed by the immunoblotting studies (Fig. 3), they peaked at about 5 min after stimulation and returned to the basal states within 15 min. In resting CHO.E cells, ERKs were localized in the cytoplasm, as revealed by pAb 7884 (Fig. 4). Nuclear translocation of ERK was observed 5 min after EGF stimulation and this nuclear localization was diminished 15 min after stimulation. When mAb-TY was used for immu-

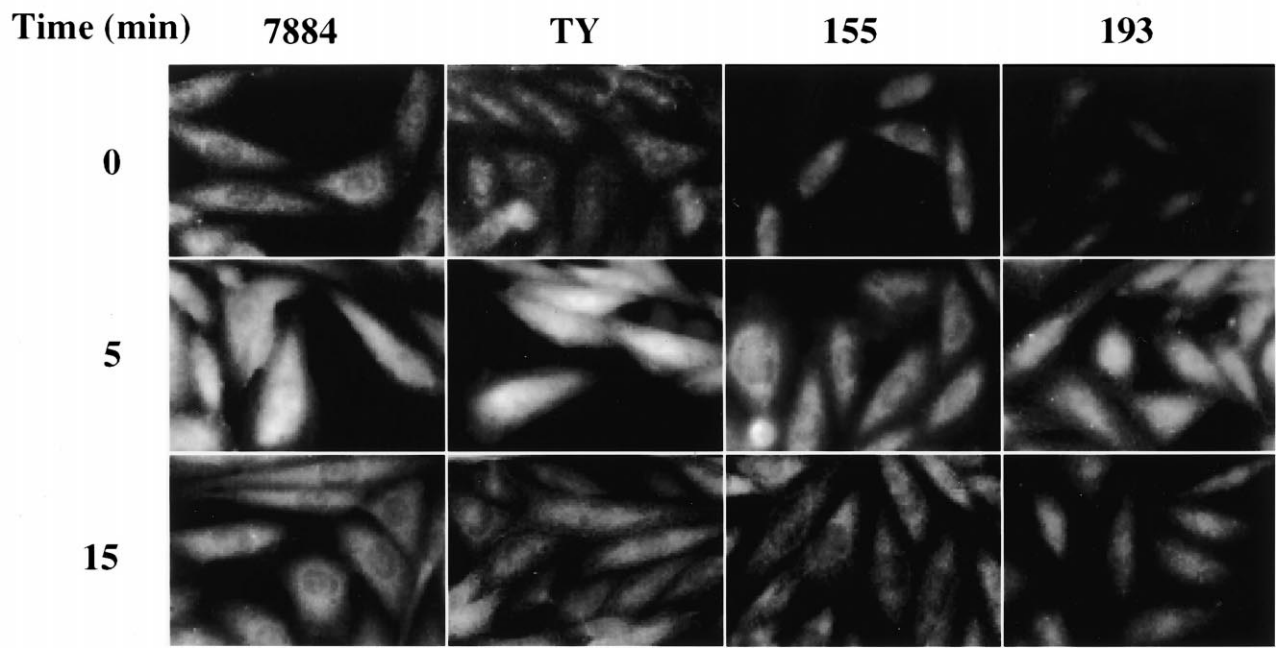

Fig. 4. Immunofluorescent staining of EGF-induced CHO.E cells with the anti-phospho-antibodies. CHO.E cells were grown on microslides for 8 h, serum-starved for 40 h and stimulated with EGF (50 ng/ml). The cells were fixed, permeabilized and stained with the various antibodies (pAb 7884 and mAbs-TY, 155 and 193; 100 μg/ml each) and Cy3-conjugated second antibodies as above. The results were reproduced twice.

nofluorescent staining in resting CHO.E cells, a faint staining was detected mainly in the cytoplasm. Stimulation with EGF (5 min) caused an increased staining with mAb-TY, in the cytoplasm and in the nucleus, whereas 15 min after stimulation, the staining intensity reduced again and was localized mainly to the cytoplasm. Detection of the distribution of PT-ERK with mAb-155 revealed that in resting CHO.E cells, a small amount of PT-ERK was detected, mainly in the cytosol. Upon EGF stimulation (5 min), the intensity of staining with mAb-155 increased, accumulating mainly in the cytosol and perinuclear regions, and the pattern of staining returned to that of resting cells 15 min after stimulation. The distribution of PY-ERK detected with mAb-193 was quite different from that of PT-ERK. It was very faint and uniformly distributed in resting cells, increased mainly in the nucleus (5 min after stimulation) and the staining returned to near-basal state 10 min later.

The different distribution of various phosphorylated ERKs suggests a different localization of various PPs that differentially regulate ERK dephosphorylation. Since PT-ERK was detected mainly in the cytoplasm, the involved PTP should also be localized in this region. This conclusion is in agreement with the recent reports that PTP-SL, STEP [26] and PTP-ER [27] are localized in cytoplasm. Similarly, the existence of PY-ERKs in the nucleus indicates that the involved protein Ser/Thr phosphatases should be localized, at least in part, in the nucleus. An interesting possibility for the protein Ser/Thr phosphatase responsible for the accumulation of PY-ERK in the nucleus is the well characterized PP2A, which was previously shown to be localized both in the cytoplasm and in the nucleus [28]. However, it should be noted that additional phosphatases are required in each of the subcellular compartments in order to bring ERK phosphorylation back to basal levels. Therefore, after the initial dephosphorylation by protein Ser/Thr phosphatase in the nucleus, a complementary PTP is required to remove the phosphate from Tyr-185, and similarly, an additional protein Ser/Thr phosphatase is required in the cytoplasm. Thus, the temporal and spatial regulation of ERK is dependent on various phosphatases that cooperate to secure the proper length and strength of ERK activity, which eventually determine the physiological outcome of variou extracellular signalings [29].

In summary, we describe here a set of antibodies, which are shown to recognize specifically all phosphorylation states of ERK (Table 1). These antibodies can serve as a useful tool to investigate mechanisms of ERK activation and downregulation. We found that in CHO.E cells, the early phase of ERK dephosphorylation is carried out by distinct, constitutively active protein Ser/Thr and protein Tyr phosphatases. These processes are undertaken in a spatially characteristic pattern. Dephosphorylation of the phosphotyrosyl residue of the dually phosphorylated ERK is the first dephosphorylation process in the cytoplasm, whereas dephosphorylation of the phosphothreonyl residue is the initial one in the nucleus. These processes are later complemented by distinct phosphatases to secure a complete removal of phosphates from ERK, bringing it back to the basal, non-phosphorylated stage. Using the various anti-phospho-ERK antibodies, we also concluded that the slow migrating band of ERKs on SDS–PAGE gel contains not only the active, dually phosphorylated form, but also the mono-phosphorylated forms of ERK2. Thus the use of 'upshift' assay to study ERK activation may not always be accurate.

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