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Detection of phosphorylated mitogen-activated protein kinase in the developing spinal cord of the mouse embryo

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ABSTRACT

Global understanding of the proteome is a major research topic. The comprehensive visualization of the distribution of proteins *in vivo* or the construction of *in situ* protein atlases may be a valuable strategy for proteomic researchers. Information about the distribution of various proteins under physiological and pathological conditions should be extremely valuable for the basic and clinical sciences.

The mitogen-activated protein kinase (MAPK) cascade plays an essential role in intracellular signaling in organisms. This cascade also regulates biological processes involving development, differentiation, and proliferation. Phosphorylation and dephosphorylation are integral reactions in regulating the activity of MAPKs. Changes in the phosphorylation state of MAPKs are rapid and reversible; therefore, the localizations of physiologically phosphorylated MAPKs *in vivo* are difficult to accurately detect. Furthermore, phosphorylated MAPKs are likely to change phosphorylated states through commonly used experimental manipulations.

In the present study, as a step toward the construction of *in situ* phosphoprotein atlases, we attempted to detect physiologically phosphorylated MAPKs *in vivo* in developing spinal cords of mice. We previously reported an improved immunohistochemical method for detecting unstable phosphorylated MAPKs. The distribution patterns of phosphorylated MAPKs in the spinal cords of embryonic mice from embryonic day 13 (E13) to E17 were observed with an improved immunohistochemical method. Phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and phosphorylated c-Jun N-terminal kinase 1/2 (p-JNK1/2) were strongly observed in the marginal layer and the dorsal horn from E13 to E17. Our results suggest that p-ERK1/2 and p-JNK1/2 play critical roles in the developing spinal cord. Constructing phosphoprotein atlases will be possible in the future if this work is systematically developed on a larger scale than we presented here.

1. Introduction

After the completion of the Human Genome Project, understanding the proteome has become a major global research topic. The comprehensive visualization of the distribution of proteins *in vivo* or the construction of *in situ* protein atlases may be a valuable strategy for proteomic researchers. Furthermore, the presentation of *in situ* protein modifications, such as phosphorylation under physiological and pathological conditions, should provide important information that will be extremely valuable to the clinical sciences [1].

The mitogen-activated protein kinase (MAPK) cascade plays an essential role in intracellular signaling in organisms. This cascade also regulates biological processes involving development, differentiation, and proliferation [2]. Extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), p38 and extracellular signal-regulated kinase 5 (ERK5) are members of the MAPK superfamily.

ERK1/2 regulates development, differentiation, proliferation, cell cycle regulation, and survival [2–6]. JNK1/2 and p38 are activated by environmental stress [3,7]. ERK5 is strongly activated by epidermal growth factor (EGF) and nerve growth factor (NGF) [8] and is part of a MAPK signaling pathway that is required for EGF-induced cell proliferation and cell cycle progression [9]. ERK5 also reportedly regulates nerve cell survival and neural differentiation [10,11].

Phosphorylation and dephosphorylation are integral reactions in regulating the enzyme activity of MAPKs. The phosphorylation state of MAPKs can be changed rapidly and reversibly [1,12,13]. The local balance between protein kinase and protein phosphatase

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activities determines the phosphorylation state of MAPKs. Furthermore, phosphorylated MAPKs are likely to change phosphorylated states by commonly used experimental manipulations for immunohistochemical staining, including anesthetics, formalin exposure, and nerve injury [1,12,13]. Therefore, it is a very challenging task to accurately detect physiologically phosphorylated MAPKs *in vivo*. We have previously reported an improved immunohistochemical method for detecting unstable phosphorylated MAPKs [12,13]. Before constructing a phosphoprotein atlas, we attempted to detect *in vivo* physiologically phosphorylated MAPKs in developing spinal cords of mice using this improved method.

2. Materials and methods

2.1. Animals

C57BL/6J mice were originally purchased from CLEA Japan, Inc., (Tokyo, Japan). Pregnant C57BL/6J mice at the 13th, 15th, and 17th day of gestation were sacrificed by cervical dislocation, and fetuses were immediately removed by cesarean section. All animal experiment protocols were in accordance with our institution's regulations regarding animal treatment.

2.2. Antibodies for immunostaining

Anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-phospho-p38, and anti-phospho-ERK5 antibodies were obtained from Invitrogen Corporation (Carlsbad, CA). The rabbit antiserum for phosphorylated ERK1/2 (p-ERK1/2) was produced against a chemically synthesized phosphopeptide corresponding to the residues around threonine 202/185 and tyrosine 204/187 of human ERK1/2. This sequence is conserved in mouse ERK1/2. The serum was negatively preabsorbed using a nonphosphopeptide to remove antibodies reacting with nonphosphorylated ERK1/2. The final product was generated by affinity chromatography using a phosphopeptide antigen. Anti-phospho-JNK1/2, anti-phospho-p38, and anti-phospho-ERK5 antibodies were prepared using the same method as for anti-phospho-ERK1/2 antibody. The rabbit antiserum for p-JNK1/2 was produced against a chemically synthesized phosphopeptide derived from the region of human JNK1/2 corresponding to the residues around threonine 183 and tyrosine 185. The rabbit antiserum for phosphorylated p38 (p-p38) was produced against a chemically synthesized phosphopeptide derived from the region of human p38 corresponding to the residues around threonine 180 and tyrosine 182. The rabbit antiserum for phosphorylated ERK5 (p-ERK5) was produced against a chemically synthesized phosphopeptide derived from the region of human ERK5 corresponding to the residues around threonine 218 and tyrosine 220. These regions of JNK1/2, p38, and ERK5 are also conserved in human and mouse, respectively.

2.3. Preparation of tissue sections

Tissue sections were prepared according to previously described protocols [13]. After removal, the fetuses were euthanized by decapitation and immediately embedded in a freezing chamber filled with OCT mounting compound (Sakura Finetechnical Co., Tokyo, Japan), which was then immersed in liquid nitrogen. Every attempt was made to minimize the time from surgical removal of the fetuses to freezing, and the process was completed within 2 min. Fresh frozen 16-μm sections were cut using a microtome cryostat (Microtome Cryostat 4 HM550; Microm International GmbH, Walldorf, Germany) under the following conditions: room temperature, 25 °C; chamber temperature, −18 °C; and specimen temperature, −14 °C. Then, the sections were put on silanized slides (Dako, Carpinteria, CA), which were immediately fixed in 70% ethanol at 4 °C for 30 min and then dried by airflow at 25 °C for 30 min. The whole spinal cord of one individual was cut, from cervical to lumbar cord, into serial transverse sections.

2.4. Immunostaining of tissue sections

Immunostaining of tissue sections was performed as previously described [13]. In a humidified chamber, 16-μm tissue sections placed on silanized slides were incubated with a peroxidase blocking reagent (Dako) containing H_2O_2 and sodium azide for 10 min at 37 °C to block endogenous peroxidase activity. The tissue sections were incubated with nonspecific staining blocking reagent (Dako) for 30 min at 37 °C. Then, the sections were incubated overnight in a humidified chamber at 4 °C with purified rabbit polyclonal antibodies against phospho-ERK1/2, phospho-JNK1/2, phospho-p38, or phospho-ERK5, respectively. The antibodies were diluted with antibody diluent buffer containing 0.1% Tween 20 (Dako) at a 1/100, 1/50, 1/50, or 1/50 ratio, respectively. The tissue sections were incubated with the Dako EnVision + System, peroxidase (DAB) reagents, and secondary anti-rabbit antibodies conjugated with an HRP-labeled polymer (Dako) for 30 min at 37 °C. Colored products were produced by using the DAB + Liquid System (Dako) for 10 min at 37 °C. The sections were then dehydrated in an ethanol and xylene gradient and mounted.

3. Results

3.1. Localization of p-ERK1/2 in the spinal cord

The distribution of p-ERK1/2 in the spinal cord was examined. On E13, almost the entire marginal layer was intensely stained with anti-p-ERK1/2 antibody throughout the level of the spinal cord (Fig. 1A-1–A-3). In the marginal layer, strong immunoreactive dots were observed. p-ERK1/2 immunoreactivities were observed as dots or fibers in the dorsal and ventral horns, which were divided by sulcus limitans (Fig. 1A-1 and A-2). The intensity of immunoreactivities in the ventral horn was lower than that in the dorsal horn. p-ERK1/2 immunoreactivities were low in the floor plate and scarce in the roof plate (Fig. 1A-2) throughout the level of the spinal cord (Fig. 1A-1–A-3). The ependymal layer was not stained with p-ERK1/2 throughout the level of the spinal cord (Fig. 1A-1–A-3).

On E15, the entire marginal layer was intensely stained with p-ERK1/2 throughout the level of the spinal cord (Fig. 1B-1–B-3). Higher p-ERK1/2 immunoreactivities were observed as dots in the marginal layer. p-ERK1/2 immunoreactivities were observed as dots or fibers in the dorsal horn and the dorsal side of the mantle layer. Fibrous p-ERK1/2 immunoreactivities sometimes appeared in the ventral horn (Fig. 1B-2). p-ERK1/2 immunoreactivities were scarce in the posterior median septum and low in the area around the anterior median septum throughout the level of the spinal cord (Fig. 1B-1–B-3). On E15, the ependymal layer was not stained with p-ERK1/2 throughout the level of the spinal cord (Fig. 1B-1–B-3).

On E17, the entire marginal layer was stained with p-ERK1/2 throughout the level of the spinal cord (Fig. 1C-1–C-3). p-ERK1/2 immunoreactivities were observed as dots and fibers in the dorsal horn and the dorsal side of the mantle layer and sometimes as fibers in the ventral horn (Fig. 1C-2). p-ERK1/2 immunoreactivities were scarce in the posterior median septum and very low in the area around the anterior median septum throughout the level of the spinal cord (Fig. 1C-1–C-3). The ependymal layer was not stained with p-ERK1/2 throughout the level of the spinal cord (Fig. 1C-1–C-3). The distribution of p-ERK1/2 in the developing spinal cord is summarized in Table 1.

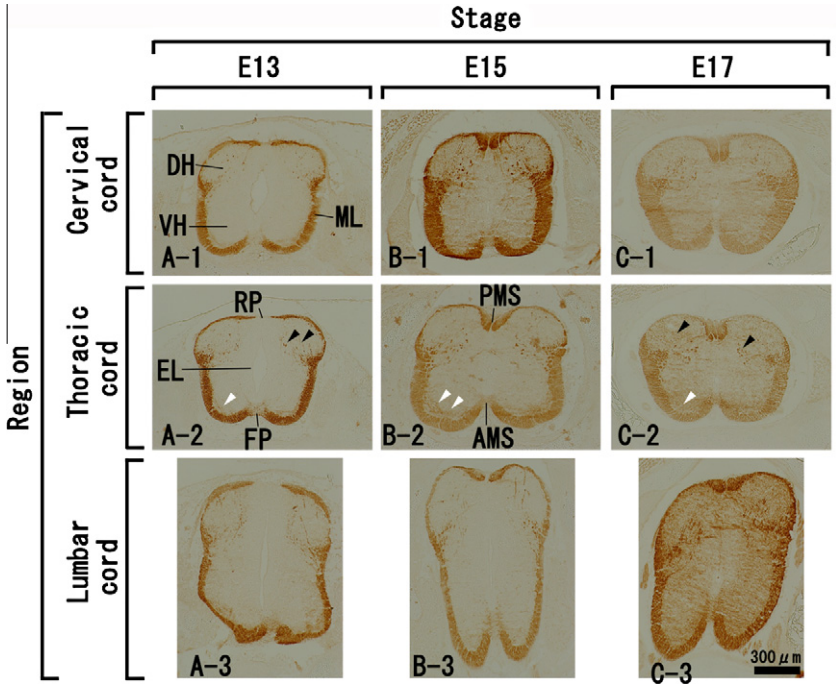

Fig. 1. Immunohistochemical staining of frozen thin sections of spinal cord with anti-phospho-ERK1/2 antibody. (A-1, -2, and -3) developing spinal cord on E13, (B-1, -2, and -3) developing spinal cord on E15, (C-1, -2, and -3) developing spinal cord on E17, (A-, B-, and C-1) cervical level of developing spinal cord, (A-, B-, and C-2) thoracic level of developing spinal cord, (A-, B-, and C-3) lumbar level of developing spinal cord. Black arrowheads indicate p-ERK1/2 immunoreactivities in the dorsal horn. White arrowheads indicate p-ERK1/2 immunoreactivities in the ventral horn. ML, marginal layer; DH, dorsal horn; VH, ventral horn; RP, roof plate; FP, floor plate; EL, ependymal layer; AMS, anterior median septum; PMS, posterior median septum.

Table 1
Spatiotemporal distribution of p-ERK1/2 and p-JNK1/2 in thoracic cord.

	E13					E15					E17				
	Marginal layer	Floor plate	Dorsal horn	Ventral horn	Roof plate	Marginal layer	Anterior median septum	Dorsal horn	Ventral horn	Posterior median septum	Marginal layer	Anterior median septum	Dorsal horn	Ventral horn	Posterior median septum
p-ERK1/2	++	±	+	+	–	++	±	+	+	–	++	±	+	+	–
p-JNK1/2	++	+	+	–	–	++	+	+	–	–	++	±	+	±	–

Immunoreactivity: ++, strong; +, moderate; ±, weak; –, none.

3.2. Localization of p-JNK1/2 in the spinal cord

On E13, almost the entire marginal layer (Fig. 2A-1) was stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2A-1–A-3). Higher p-JNK1/2 immunoreactivities were observed as dots in the marginal layer. The intensity of immunoreactivities was slightly lower than that of p-ERK1/2 on E13 (Figs. 1A-1–A-3 and 2A-1–A-3). p-JNK1/2 immunoreactivities were observed as dots or fibers in the dorsal horn and the dorsal side of the mantle layer but was very low and faint compared with those of p-ERK1/2 on E13 (Fig. 2A-1 and A-2). p-JNK1/2 immunoreactivities were very low in the floor plate and scarce in the roof plate (Fig. 2A-2). The ependymal layer was not stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2A-1–A-3). These stained patterns were very similar to those of p-ERK1/2 on E13, although p-ERK1/2 was observed slightly on the ventral horn.

On E15, the entire marginal layer was stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2B-1–B-3). In the dorsal horn, p-JNK1/2 was stained as dots (Fig. 2B-2). In the ventral horn, no p-JNK1/2 immunoreactivities were observed. p-JNK1/2 immunoreactivities were scarce in the posterior median septum and low in the area around the anterior median septum throughout the level of the spinal cord (Fig. 2B-1–B-3). The ependymal layer was not stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2B-1–B-3). These stained patterns were similar to those of p-ERK1/2 at the same stage, except that p-ERK1/2 was observed slightly on the ventral horn.

On E17, the entire marginal layer was stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2C-1–C-3). In the dorsal horn and the dorsal side of the mantle layer, p-JNK1/2 was stained as dots (Fig. 2C-2). p-JNK1/2 immunoreactivities were scarce in the posterior median septum and low in the area around the anterior median septum throughout the level of the spinal cord (Fig. 2C-1–C-3). The ependymal layer was not stained with p-JNK1/2 throughout the level of the spinal cord (Fig. 2C-1–C-3). These stained patterns were similar to those of p-ERK1/2 at the same stage.

The distribution of p-JNK1/2 in the developing spinal cord is summarized in Table 1.

3.3. Localization of p-p38 and p-ERK5 in the developing spinal cord

On E13, E15, and E17, the immunoreactivities of p-p38 and p-ERK5 were below the immunohistochemically detectable limits throughout the level of the spinal cord.

4. Discussion

MAPKs easily change their activity *in vivo* because their phosphorylated state easily changes during experimental manipulation [1,12,13]. For example, several anesthetics and fixatives are

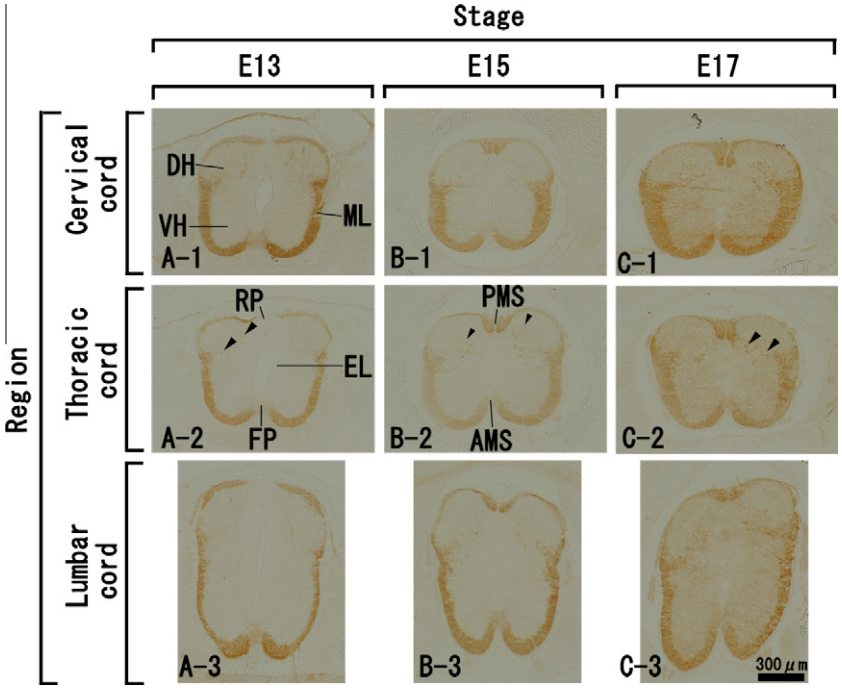

Fig. 2. Immunohistochemical staining of frozen thin sections of spinal cord with anti-phospho-JNK1/2 antibody. (A-1, -2, and -3) developing spinal cord on E13, (B-1, -2, and -3) developing spinal cord on E15, (C-1, -2, and -3) developing spinal cord on E17, (A-, B-, and C-1) cervical level of developing spinal cord, (A-, B-, and C-2) thoracic level of developing spinal cord, (A-, B-, and C-3) lumbar level of developing spinal cord. Black arrowheads indicate p-JNK1/2 immunoreactivities in the dorsal horn. ML, marginal layer; DH, dorsal horn; VH, ventral horn; RP, roof plate; FP, floor plate; EL, ependymal layer; AMS, anterior median septum; PMS, posterior median septum.

reported to have effects on the phosphorylation of MAPKs [1,12–15]. Therefore, when detecting phosphorylated MAPKs, researchers should pay attention to the fact that the phosphorylation of MAPKs is rapid and reversible *in vivo* and that artificial stimuli and/or essential experimental manipulations can change their phosphorylated states. Previously, we reported our attempts to resolve these problems, and we have applied an improved method to detect physiologically phosphorylated MAPKs *in vivo* [1,12,13].

In the present study, the distribution of p-ERK1/2 and p-JNK1/2 was observed strongly in the marginal layer and the dorsal horn of developing spinal cords of mice on E13, E15, and E17 but was scarce in the roof plate and the posterior median septum. p-p38 or p-ERK5 immunoreactivities were rarely detected throughout the level of the spinal cord on E13, E15, and E17. We observed that the level of spinal cord rarely affects each immunohistochemical stain patterns of four kinds of phosphorylated MAPK immunoreactivities.

The MAPKs play very important roles in various biological processes and are thought to be critical to intracellular signaling in organisms. ERK1/2, JNK1/2, p38, and ERK5 are classified as classical MAPKs. ERK1/2 is reported to control development, differentiation, proliferation, cell cycle regulation, and survival [6]. Through the signaling pathway involving ERK1/2, stromal cell-derived factor-1 alpha and Raf kinase may play a role in spinal cord neuron development [16] and dorsal root ganglion neuron development, including differentiation and axon growth [17]. Activation of ERK1/2 signaling through deleted in colorectal cancer, a receptor for netrin-1, was reported to contribute to the signaling mechanism triggered by netrin in axon outgrowth in commissural neurons of rat spinal cord [18]. JNK1/2 is a known regulator of transcription activated by several environmental stresses such as ultraviolet or cytokine exposure, and growth factor deprivation [19]. p38 is activated by environmental stress, inflammatory cytokines, or NGF [3,7]. ERK5 reportedly regulates nerve cell survival and neural differentiation [10,11]. Therefore, the MAPK cascades are expected to have critical roles in the development of the central nervous system in embryos.

In this study, we observed p-ERK1/2 and p-JNK1/2 immunoreactivities in the marginal layer on E13, E15, and E17. The marginal layer in developing spinal cords is composed of large amounts of myelin, axons, and oligodendrocytes for myelination. In oligodendrocyte progenitors, ERK1/2 is necessary for the mitogenic reaction caused by fibroblast growth factor-2 (FGF-2) [20], and FGF-2 and FGF-8 activate MAPKs in different ways. FGF-2 activates MAPKs rapidly and sustainably, whereas FGF-8 activates MAPKs weakly and transiently. This fact may explain the difference in mitogenicity of FGFs [21]. Moreover, in oligodendrocyte progenitors, insulin-like growth factor-1 (IGF-1) stimulates protein synthesis, which is important for the growth and differentiation of oligodendrocyte. Recently, the MEK/ERK pathway was revealed to be necessary for IGF-1-stimulated protein synthesis [22]. Interestingly, the distribution of p-ERK1/2 and p-JNK1/2 observed in this study was very similar to that of myelin basic protein, which is a marker of mature oligodendrocytes of adult mice [23]. The reported facts mentioned above and our data imply that p-ERK1/2 and/or p-JNK1/2 may be associated with developing oligodendrocytes, including oligodendrocyte progenitors during development of the spinal cord. p-ERK1/2 and/or p-JNK1/2 may play critical roles in the developing spinal cord.

Depending on the type of stimulation, ERK1/2 can undergo both transient and prolonged phosphorylation, resulting in different cellular responses [24,25]. In transient phosphorylation, the ERK1/2 phosphorylation peak occurs within 5–10 min and returns to baseline within about 2 h. In prolonged phosphorylation, ERK1/2 phosphorylation is sustained at high levels for at least 4 h. p-ERK1/2 immunoreactivities were observed consistently and strikingly in the marginal layer on E13, E15, and E17. This result demonstrates the probability that activation of ERK1/2 may be in a sustained manner rather than a transient manner by any mechanisms from E13 to E17.

If information about modifications such as the phosphorylation of protein was included, we may obtain more information from the protein atlases. Therefore, we consider the construction of phosphoprotein atlases to be another goal derived from the present study because the distributions of phosphorylated proteins *in vivo* have not yet been accurately detected [1,6,12,13]. The present study was performed in advance of the comprehensive representation of the spatiotemporal distribution of physiologically phosphorylated proteins *in vivo*; therefore, we think our findings will contribute to the development of modified protein atlases, especially phosphoprotein atlases.

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