

Research article

Identification of protein tyrosine phosphatase IB and casein as substrates for I24-v-Mos

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Abstract

Background: The mos proto-oncogene encodes a cytoplasmic serine/threonine-specific protein kinase with crucial function during meiotic cell division in vertebrates. Based on oncogenic amino acid substitutions the viral derivative, I24-v-Mos, displays constitutive protein kinase activity and functions independent of unknown upstream effectors of mos protein kinase. We have utilized this property of I24-v-Mos and screened for novel mos substrates in immunocomplex kinase assays in vitro.

Results: We generated recombinant I24-v-Mos using the baculovirus expression system in *Spodoptera frugiperda* cells and demonstrated constitutive kinase activity by the ability of I24-v-Mos to auto-phosphorylate and to phosphorylate vimentin, a known substrate of c-Mos. Using this approach we analyzed a panel of acidic and basic substrates in immunocomplex protein kinase assays and identified novel in vitro substrates for I24-v-Mos, the protein tyrosine phosphatase IB (PTPIB), alpha-casein and beta-casein. We controlled mos-specific phosphorylation of PTPIB and casein in comparative assays using a synthetic kinase-inactive I24-v-Mos mutant and further, tryptic digests of mos-phosphorylated beta-casein identified a phosphopeptide specifically targeted by wild-type I24-v-Mos. Two-dimensional phosphoamino acid analyses showed that I24-v-mos targets serine and threonine residues for phosphorylation in casein at a 1:1 ratio but auto-phosphorylation occurs predominantly on serine residues.

Conclusion: The mos substrates identified in this study represent a basis to approach the identification of the mos-consensus phosphorylation motif, important for the development of specific inhibitors of the Mos protein kinase.

Background

Mos belongs to a small family of cytoplasmic protein serine/threonine kinases having oncogenic activity [1,2]. It is highly expressed in germ cells but barely detectable in a variety of somatic tissues [3–5]. Studies in *Xenopus*

oocytes have established a role for c-mos in a) initiation of the maturation process and the meiosis I / meiosis II transition and b) in metaphase II arrest in mature oocytes [6–12]. In mouse c-Mos is apparently not required for in-

itiation of maturation, however, like in *Xenopus* it is absolutely essential for the metaphase II arrest [13,14].

The 124-v-mos oncogene represents one of several transforming gene isolates of the moloney murine sarcoma virus [15,16] and shows unique constitutive protein kinase activity and enhanced transforming activity when compared to other v-Mos proteins or to c-Mos [2,17–19]. The transforming mechanism of Mos involves signalling through the MAP kinase pathway as phosphorylation of MEK by c-Mos has been demonstrated [20–23] and mapping analyses have shown that Mos and Raf phosphorylate identical sites on MEK [16,24]. The upstream events of the Mos/MEK/MAPK signalling cascade have not as yet been identified. In earlier studies we have shown that an activating mechanism of c-Mos is likely to involve a conformational change which is mimicked when a single amino acid is exchanged in the α -helix C loop of the kinase domain (Arg145-Gly) resulting in constitutive active c-Mos [19]. Recently Fisher and co-workers proposed an activating mechanism of c-Mos by sequential association with Hsp70 and Hsp90, in addition to phosphorylation [25,26].

Presence of the activating Arg145-Gly amino acid substitution in 124-v-Mos does not change kinase specificity but is sufficient for constitutive kinase activity [19]. Hence the kinase activity of 124-v-Mos is independent of upstream effectors and we have used this oncogenic Mos derivative to identify substrates for the Mos protein kinase *in vitro*. Using the baculo virus expression system we have expressed active 124-v-Mos protein kinase, as demonstrated by its ability to auto-phosphorylate, predominantly on serine residues, and to phosphorylate vimentin *in vitro*. We have analysed a panel of acidic and basic substrates in immunocomplex protein kinase assays and identified two novel *in vitro* substrates for 124-v-Mos, the protein tyrosine phosphatase 1B and α/β -casein.

Results

Three tryptic 124-v-Mos peptides include target sites for auto-phosphorylation

We have expressed 124-v-Mos with the baculovirus system in Sf9 insect cells and immunopurified 124-v-Mos using the anti-Mos N13 antiserum [19]. As a control, a Mos-unrelated protein, a synthetic kinase-inactive construct of PKC, PKC γ ^{K380R}[27], was expressed in Sf9 cells. Mos kinase assays, completed in the presence of [γ -³²P]ATP, were resolved using SDS-PAGE and the Coomassie blue staining of the protein gel showed visible amounts of immunopurified 124-v-Mos (fig. 1B, arrowhead). The corresponding autoradiograph in figure 1A demonstrates that 124-v-Mos is expressed as a constitutive active protein kinase indicated by its ability to auto-phosphorylate *in vitro*. Further, a parallel kinase reaction was used for phos-

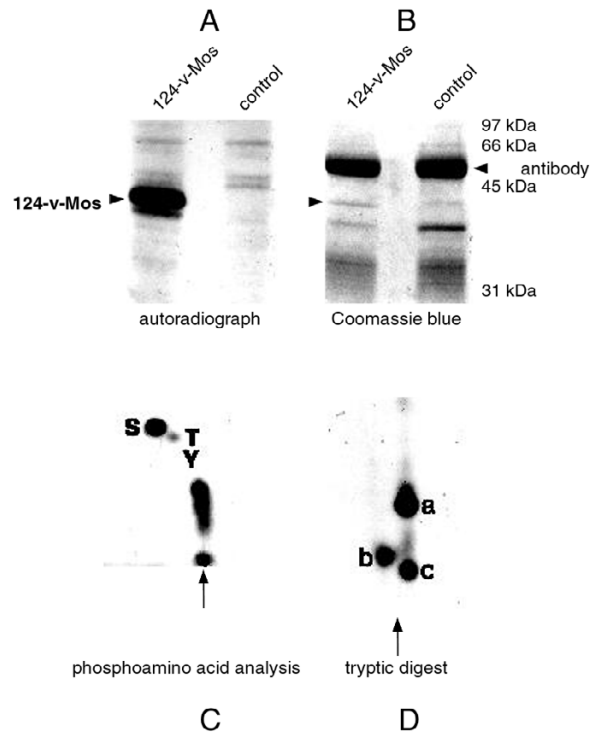


Figure 1
Constitutive kinase activity of immunopurified 124-v-Mos from baculovirus expressing Sf9 insect cells. Auto-phosphorylation of immunopurified 124-v-Mos expressed in Sf9 cells is shown in B (Coomassie stained 10% SDS-PAGE) and A (corresponding autoradiograph). Parallel 124-v-Mos kinase assays were subjected to a two-dimensional phosphoamino acid analysis (C) or a tryptic digestion followed by a two-dimensional resolution (D). Arrowheads indicate the origin of sample application in (C,D) and the position of 124-v-Mos (A,B).

phoamino acid analyses which confirmed that 124-v-Mos auto-phosphorylation occurred predominantly on serine residues (fig. 1C) and a two-dimensional resolution of a tryptic digest of auto-phosphorylated 124-v-Mos showed that three tryptic peptides include auto-phosphorylation target sites (fig. 1D), demonstrating that auto-phosphorylation occurs on multiple sites of the Mos protein [28].

124-v-Mos phosphorylates vimentin but not tubulin *in vitro*

Initially, we tested the kinase activity of 124-v-Mos using previously identified Mos substrates. It has been shown that 124-v-Mos, derived from mos-transformed fibroblasts, phosphorylates vimentin *in vitro* [29] and as presented here in figure 2C, *in vitro* kinase assays using immunopurified 124-v-mos from Sf9 insect cells showed strong vimentin phosphorylation. In contrast, tubulin

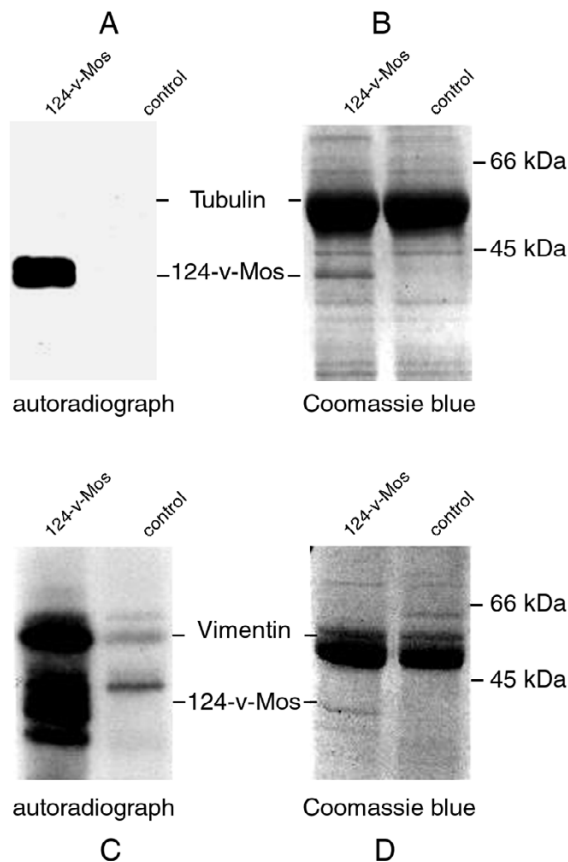


Figure 2
124-v-Mos phosphorylates vimentin but not tubulin.
 In vitro 124-v-Mos kinase assays with either vimentin (C,D) or purified tubulin from brain (A,B) as substrates were electrophoresed using 10% SDS-PAGE and Coomassie stained (B,D), the corresponding autoradiographs are shown in (A,C). Immunoprecipitates of Sf9 cells expressing the kinase-inactive PKC γ ^{K380R} were indicated as controls.

which has been shown to be phosphorylated in vivo and in vitro by Xenopus c-Mos [30] was not a substrate for 124-v-Mos in vitro (fig. 2A). We have tested tubulin purified from various organs (mouse brain, testis and spleen) either polymerised, unpolymerised or pretreated with phosphatases but in none of these states found tubulin to be phosphorylated by 124-v-Mos (data not shown).

Demonstration of alpha and beta-casein phosphorylation by 124-v-Mos

In search of further substrates for the 124-v-Mos protein kinase we tested MBP; histone HI, H2AS, H3; protamine; protaminsulphate; purified PKC- α / β II/ γ and α - and β -casein. With the exception of α - and β -casein (fig. 3A) none of these substrates were phosphorylated by 124-v-Mos

(data not shown). The possibility that factors other than 124-v-Mos in the immunoprecipitate might be responsible for the observed casein phosphorylation was eliminated by including a synthetic kinase-inactive construct of 124-v-Mos, 124-v-Mos^{K121R}[19], as a control in addition to the Mos-unrelated protein, PKC γ ^{K380R}. A comparison of background phosphorylation on β -casein in the immunoprecipitates of both controls and 124-v-Mos specific phosphorylation showed that 124-v-Mos phosphorylates β -casein 7fold relative to background (fig. 3B). Critically, a tryptic digest of phosphorylated β -casein revealed that 124-v-Mos phosphorylates a specific tryptic peptide in β -casein which shows no background phosphorylation in either controls (fig. 3C, arrowhead) strongly supporting that 124-v-Mos is able to phosphorylate β -casein. Further, a two-dimensional phosphoamino acid analysis (fig. 3D) showed that 124-v-Mos phosphorylates α - and β -casein on serine and threonine residues at a ratio of 1:1.

The protein tyrosine phosphatase 1B is a novel in vitro substrate for 124-v-Mos

Protein tyrosine phosphatases constitute a diverse family of enzymes that can be divided into several subgroups, including receptor and non-receptor PTPs [31]. The non-transmembrane protein tyrosine phosphatase PTP-1B, a major intracellular PTP is widely expressed. PTP-1B has been demonstrated to be phosphorylated on multiple sites in a cell cycle specific manner whereby mitotic hyperphosphorylation occurs, reflected by a protein mobility shift in SDS-PAGE analyses [32]. Using purified PTP-1B as a substrate, we show here that 124-v-Mos can phosphorylate PTP-1B in vitro (fig. 4A). We controlled this result by using immunoprecipitates from Sf9 cells expressing the synthetic kinase-inactive 124-v-Mos construct or purified PTP-1B alone in parallel kinase assays (fig. 4A). Other kinases such as PKC and CKII that phosphorylate PTP-1B in vitro are unable to induce a mobility shift of PTP-1B as observed in mitotic cells [32]. Likewise, as shown in figure 4B, a Mos-dependent phosphorylation did not result in a mobility shift of PTP-1B.

Discussion

In this study we have expressed constitutive active 124-v-Mos using the baculovirus expression system and identified novel in vitro substrates for Mos by immunocomplex kinase assays. It has been shown that 124-v-Mos from mos-transformed mouse fibroblasts phosphorylates vimentin in vitro [29] and that v-Mos is physically associated with vimentin in transformed cells [33]. We have used vimentin as a positive control for 124-v-Mos kinase assays in vitro to demonstrate protein kinase activity of baculovirus expressed 124-v-Mos (fig. 2). It is known that the kinase activity of c-Mos is regulated by cellular factors and therefore we have chosen the oncogenic variant of c-Mos, 124-v-Mos, in our study since it is independent of activat-

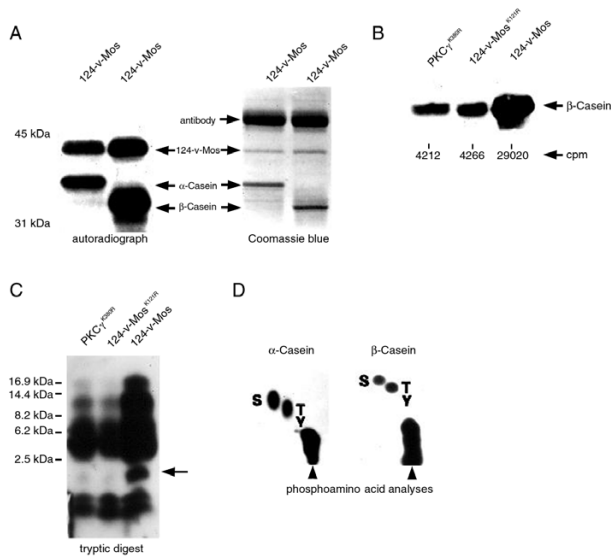


Figure 3
124-v-Mos phosphorylates α - and β -casein in vitro.
 Mos kinase assays, in the presence of α - and β -casein, were resolved using 10% SDS-PAGE; the Coomassie stained protein gel shown in 3A, right panel and the corresponding autoradiograph on the left panel. Arrowheads indicate the position of 124-v-Mos, α - and β -casein and the antibody. Using two control immunoprecipitates of Sf9 cells expressing the synthetic kinase-inactive constructs, 124-v-Mos^{K121R} or PKC γ ^{K380R}, Mos-specific β -casein phosphorylation was demonstrated in 3B and 3C: Mos kinase assays were blotted on nylon-membrane, the phospho- β -casein bands (B, arrowhead) excised and ³²P-Cerenkov counts recorded (B). Alternatively, the excised phospho- β -casein bands were digested with trypsin and electrophoresed using 16% SDS-PAGE (C). The arrowhead in 3C indicates the tryptic β -casein peptide phosphorylated by wild-type 124-v-Mos only. Further, two-dimensional phosphoamino acid analyses of 124-v-Mos phosphorylated α -casein (D, left panel) and β -casein (D, right panel) were completed, the arrowheads indicating the origins of sample application.

ing mechanisms. Recently it has been shown that Hsp70 and Hsp90 physically interact with c-Mos in *Xenopus* oocytes and are required for c-Mos activation [25,26]. Another factor controlling c-Mos kinase activity in *Xenopus* oocytes was identified by Chen and colleagues [34,35] to be CKII, a tetrameric holoenzyme composed of two catalytic α -subunits and two regulatory β -subunits [36]. In *Xenopus* oocytes c-Mos kinase activity is inhibited by binding to the C-terminus of CKII β -subunit and by overexpression of the α -subunit of CKII this effect can be neutralized suggesting a binding competition between c-Mos and the α -subunit of CKII [34]. Another protein that interacts with c-Mos in *Xenopus* oocytes is tubulin. Tubulin not only co-precipitates with c-Mos but also serves as an

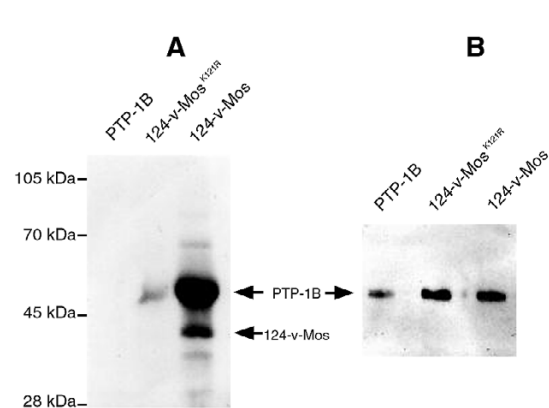


Figure 4
PTP-1B is a substrate for 124-v-Mos in vitro.
 In vitro Mos kinase assays, using purified PTP-1B as a substrate, were resolved using 10% SDS-PAGE and the autoradiograph is shown in 4A. Immunoprecipitates of Sf9 cells expressing the kinase-inactive 124-v-Mos^{K121R} variant or PTP-1B alone were included as controls (A,B). A parallel kinase assay was blotted on nylon-membrane and PTP-1B was detected (B) using the PTP-1B-specific antiserum FG6 [29], arrowheads indicate the position of 124-v-Mos and PTP-1B.

in vivo and in vitro substrate [30]. In contrast, tubulin was not a substrate for 124-v-Mos in our immunocomplex kinase studies (fig. 2A). Possibly, this indicates that a cellular factor present in *Xenopus* oocytes and co-precipitating with c-Mos might be necessary for tubulin phosphorylation by the Mos protein kinase. This factor might not interact with the v-Mos protein, be absent in Sf9 insect cells or unable to interact with v-Mos. Interestingly, we have not detected any co-precipitation of the β -subunit of CKII from Sf9 cells with 124-v-Mos in our immunoprecipitates (data not shown). However, as previously mentioned, Hsp70 is known to interact also with 124-v-Mos [26].

Having established that our recombinant 124-v-Mos protein is active in vitro, we tested a variety of molecules in immunocomplex kinase assays and identified α - and β -casein as very good substrates in vitro (fig. 3). This phosphorylation was specific to active 124-v-Mos as the overall phosphorylation on casein was significantly reduced using the synthetic kinase-inactive construct 124-v-Mos^{K121R} and more importantly, a tryptic peptide of casein was identified to be phosphorylated by 124-v-Mos only and not by either of the controls used in this study. As expected, casein phosphorylation occurred on serine and threonine residues. The Mos-specific consensus phosphorylation site has not as yet been identified and only the mos-phosphorylation sites on MAP kinase kinase have been mapped revealing them to be identical to raf-phosphorylation sites [24]. Using the mos substrates

identified in this study, it may be possible to determine the specific consensus phosphorylation site for the mos protein kinase as a basis for developing Mos-specific inhibitors.

We have also identified protein tyrosine phosphatase 1B (PTP-1B) as a substrate for 124-v-mos in vitro (fig. 4A). PTP-1B is phosphorylated on multiple sites in vivo and during mitosis becomes hyper-phosphorylated resulting in a mobility shift in SDS-PAGE [32]. Protein kinase C and CKII phosphorylate PTP-1B in vitro but neither is responsible for the observed mitotic hyper-phosphorylation in vivo [32]. We show here that likewise PTP-1B phosphorylation by 124-v-mos is insufficient to effect a mobility shift (fig. 4B). PTP-1B phosphorylation occurs on serine 386, a phosphoacceptor site for Cdc2/cyclin B in vitro and serine 352, phosphorylated by an unknown kinase. The serine 352 phosphorylation site either might not be a target for Mos in vitro or PTP-1B may be sequentially phosphorylated by multiple kinases in vivo. Interestingly, it has been shown that PTP-1B hyper-phosphorylation does not occur uniquely in mitosis but also during osmotic shock and is induced by several other stress stimuli [37]. Given that activation of c-Mos is dependent on its interaction with the heatshock proteins, Hsp70 and Hsp90, it is tempting to speculate that the Mos kinase may phosphorylate PTP-1B also in vivo.

Conclusions

The crucial biological functions of c-mos during meiosis have been analysed by antisense experiments in *Xenopus laevis* and by generating mos-deficient mice establishing mos as the main player in metaphase II arrest. In contrast, not much is known about activating mechanisms of mos and biochemical properties such as the mos-specific consensus phosphorylation site. In this study we immunopurified an oncogenic and constitutive active variant of mos, 124-v-Mos, and identified novel phosphorylation substrates, PTP1B and α - and β -casein. Our substrates represent a basis to determine the consensus mos-specific phosphorylation site and further, to analyze this phosphorylation ability functionally in vivo.

Materials and Methods

Protein expression and in vitro immunocomplex protein kinase assays

The construction and isolation of recombinant baculoviruses expressing active 124-v-Mos and the synthetic kinase-inactive variant of 124-v-Mos, 124-v-Mos^{K121R}, is described in detail elsewhere [19]. According to the standard procedure published by Summers & Smith [38], recombinant proteins were expressed at 27°C in Sf9 cells for 48 hrs. and mos was immunopurified using the anti-Mos N13 antiserum as stated in [19]. Mos kinase assays were carried out in 50 μ l kinase reaction buffer (10 mM HEPES

pH 7.3, 150 mM NaCl, 0.1% Triton X-100, 2 mM DTT, 15 mM MnCl₂, 5 mM MgCl₂, 2.5 mM β -glycerophosphate, 2.5 mM NaF, 20 μ M ATP/ 10 μ Ci [γ -³²P]ATP), incubated for 20 min. at 25°C and stopped by the addition of Laemmli buffer. For in vitro substrate kinase assays, 2 μ g of substrate was added to each kinase reaction. Phosphoproteins were resolved using 10% SDS-PAGE, Coomassie stained, dried and compared with the corresponding autoradiograph. Immunodetection of western blots were performed using the ECL system and protocol (Amersham).

Substrates for in vitro immunocomplex kinase assays

α - and β -casein (dephosphorylated, bovine origin) were purchased from Sigma and vimentin from Roche. Purified PTP-1B and the PTP-1B-specific antiserum FG6 were provided by N. Tonks, Cold Spring Harbor [32]. Tubulin was purified from either mouse brain, testis or spleen by F. Propst, Vienna.

Two-dimensional phosphoamino acid analyses

Two-dimensional phosphoamino acid analyses were completed according to Boyle and colleagues [39]. Briefly, phosphoproteins were separated using SDS-PAGE, blotted on nylon-membrane and the desired protein bands were excised. The membrane strips were washed sequentially with 100% methanol and water and the phosphoproteins hydrolysed for 60 min. at 110°C in 5.7 N HCl. The hydrolysed samples were lyophilised, resuspended in 2.5% formic acid, 7.8% acetic acid and mixed at 15:1 with a non-radioactive amino acid standard (1 mg/ml of each phospho-serine, -threonine, -tyrosine; Sigma). Finally, samples were spotted on thin-layer chromatography plates and separated in two dimensions using the HTLE-7000 apparatus and manufacture's procedure (Two-Dimensional Peptide Mapping And Phosphoamino Acid Analysis, Featuring The Hunter Thin Layer Plate Electrophoresis System. B. Boyle & T. Hunter, C.B.S. Scientific Company, Del Mar, USA). First dimension: 20 min. electrophoresis at 0.8 bar, 1 kV in 2.5% formic acid, 7.8% acetic acid. Second dimension: 16 min. at 0.8 bar, 1.3 kV in 5% acetic acid, 0.5 % pyridine. The phosphoamino acids were fixed for 10 min. at 65°C and the standard non-radioactive amino acids visualised by spraying the chromatography plates with 0.25% ninhydrin followed by incubation for 15 min. at 65°C. The phosphoamino acids were located by comparing the autoradiograph with the stained standard amino acids.

Tryptic digests and one- or two-dimensional separation of tryptic phosphopeptides

According to Boyle and colleagues [39] phosphorylated proteins were proteolytically digested with trypsin by incubating twice for 2 hrs. at 37°C, on each occasion with 10 μ g trypsin (Promega, modified trypsin, sequencing grade)

in 200 µl 50 mM NH₄HCO₃ and a two-dimensional separation of tryptic phosphopeptides was completed using the HTLE-7000 apparatus and manufacture's protocol: electrophoretic separation was performed on thin layer chromatography plates for 25 min. at 0.8 bar and 1 kV, followed by conventional chromatography in 39.25% n-butanol, 30.25% pyridine, 6.1% acetic acid. One-dimensional separation of tryptic phosphopeptides was achieved using 16% SDS-PAGE according to Schägger and von Jagow [40].

List of Abbreviations used

Sf9, *Spodoptera frugiperda* cell line; MAPK, mitogen-activated protein kinase; MEK, MAP and erk kinase; Hsp, heat-shock protein; PTP, protein tyrosine phosphatase; MBP, myelin basic protein; PKC, protein kinase C; CKII, casein kinase II.

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