Phosphorylation of neurofilament heavy chain side-arms by stress activated protein kinase-1b/Jun N-terminal kinase-3


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SUMMARY

Neurofilaments comprise three subunit proteins; neurofilament light, middle and heavy chains (NF-L, NF-M and NF-H). The carboxy-terminal domains of NF-M and NF-H form side-arms that project from the filament and that of NF-H contains multiple repeats of the motif lys-ser-pro, the serines of which are targets for phosphorylation. The level of phosphorylation on the lys-ser-pro repeats varies topographically within the cell; in cell bodies and proximal axons, the side-arms are largely non-phosphorylated whereas in more distal regions of axons, the side-arms are heavily phosphorylated. Here we show that stress activated protein kinase 1b (SAPK1b), a major SAPK in neurones will phosphorylate NF-H side-arms both in vitro and in transfected cells. These studies suggest that SAPK1b targets multiple phosphorylation sites within NF-H side-arms. Additionally, we show that glutamate treatment induces activation of SAPK1b in primary cortical neurones and increased phosphorylation of NF-H in cell bodies. This suggests that glutamate causes increased NF-H phosphorylation at least in part by activation of stress activated protein kinases.

Key words: Stress activated protein kinase, Jun N-terminal kinase, Neurofilament heavy chain, Amyotrophic lateral sclerosis, Neurodegenerative disease

INTRODUCTION

Neurofilaments are the major intermediate filament proteins of neurones and in most adult neurones comprise three subunit proteins, neurofilament light chain (NF-L), neurofilament middle chain (NF-M) and neurofilament heavy chain (NF-H). In common with other members of the intermediate filament protein family, NF-L, NF-M and NF-H all contain a central alpha-helical rod domain that can form coiled coil oligomers, an amino-terminal head domain that is believed to be involved in the regulation of filament assembly, and carboxy-terminal domains (see Lee and Cleveland, 1996). The carboxy-terminal domains of NF-M and NF-H are longer than that of NF-L and form side-arms that project from the filament and appear to form crossbridges between adjacent neurofilaments and between neurofilaments and other axoplasmic components (Hirokawa, 1982). These NF-M/NF-H side-arms are phosphorylated and that of NF-H is particularly heavily phosphorylated. Much of the phosphate in the NF-H side-arm domain is located within a multi-phosphorylation repeat (MPR) domain that contains the motif lys-ser-pro (KSP) which is repeated between 43 and 51 times in mammals (Julien et al., 1988; Lees et al., 1988). Although the serines of the KSP repeats are heavily phosphorylated in axons, they are largely non-phosphorylated in perikarya and more proximal regions of axons (Julien and Mushynski, 1982; Carden et al., 1985; Lee et al., 1988; Elhanany et al., 1994; Sternberger and Sternberger, 1983; Lee et al., 1987; Nixon et al., 1994b). The level of this phosphorylation in axons makes NF-H one of the most phosphorylated proteins known.

The role of NF-H side-arm phosphorylation is not properly understood but it may provide a mechanism for regulating the rate of transport of neurofilaments down the axon. Neurofilaments are transported down the axon by slow axonal transport (see for review Nixon, 1998) and many studies have shown that increased phosphorylation of NF-H side-arms correlates with a slowing of transport (see for example Watson et al., 1991; Nixon et al., 1994a; Archer et al., 1994). Indeed, overexpression of NF-H in transgenic mice leads to a slowing of neurofilament transport (Marszalek et al., 1996).

Abnormal perikaryal accumulations of neurofilaments containing phosphorylated NF-H side-arms are seen in several neurodegenerative diseases (see for review Trojanowski and Lee, 1994) including motor neurone disease or amyotrophic lateral sclerosis (ALS) (Hirano, 1991; Leigh and Garafolo, 1995). Understanding the mechanisms that regulate NF-H side-arm phosphorylation are thus relevant to normal neuronal biology and also to pathogenic mechanisms in some disease states. The processes that lead to motor neurone demise in ALS are not properly understood but a body of evidence suggests that glutamate toxicity might be part of the degenerative process (see for example Rothstein et al., 1990; Shaw, 1994; Roy et al., 1998; Lin et al., 1998). However, how glutamate toxicity might lead
to alterations in NF-H side-arm phosphorylation is not clear. Here, we demonstrate that stress activated protein kinase 1b (SAPK1b) (also known as Jun N-terminal kinase-3-JNK-3) will phosphorylate NF-H side-arms and that in primary cortical neurones, glutamate treatment will both activate SAPK1b and induce perikaryal hyperphosphorylation of NF-H side-arms.

MATERIALS AND METHODS

Cell culture and transfection

Primary cortical neurones were obtained from E16 rat embryos and cultured on glass coverslips coated with poly-D-lysine and laminin in Neurobasal medium and B27 supplement (GibcoBRL) containing 100 units/ml penicillin, 100 μg/ml streptomycin (GibcoBRL) and 2 mM glutamine. Cells were harvested for analyses 8 days after plating.

COS cells were cultured in DMEM containing 10% (v/v) foetal calf serum supplemented with 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells grown in 60 mm Petri dishes were transfected with 6 μg of each plasmid using a Promega CaPO4 transfection kit according to the manufacturer’s instructions and harvested for analyses 48 hours later.

For expression of NF-H in COS cells, a rat NF-H cDNA (Chin and Liem, 1990) cloned into the expression vector pMT2 was utilised (Guidato et al., 1996). To express stress activated protein kinase 1b (SAPK1b), hemagluttinin (ha) tagged cDNAs encoding both the p46 (Liem, 1990) cloned into the expression vector pMT2 was utilised (Carboni et al., 1997, 1998). Additionally, SAPK1b was detected using a SAPK1b selective antibody (Insight Biotechnology) and transfected SAPK1b from COS cells was immunoprecipitated using antibody 12CA5 (Roche Molecular Biochemicals) to the ha tag. cdk5 and GSK-3 assays were also performed as previously described (He et al., 1995; Guidato et al., 1996; Brownlees et al., 1997) using antibodies C8 (Santa Cruz Biotechnology) and TPK1 (Affiniti) to immunoprecipitate cdk5 and GSK-3, respectively. Histone H1 was used as a substrate for cdk5 and phosphopeptide GS1 substrate (He et al., 1995) used for GSK-3. 40-100 μg of cell lysate (identical amounts for each experiment) were used in the immunoprecipitations. For SAPK and cdk5 assays, samples were run on 10% (w/v) acrylamide SDS-PAGE gels; for GSK-3 assays, samples were resolved on 25% (w/v) acrylamide SDS-PAGE gels as described (He et al., 1995; Brownlees et al., 1997). Gels were analysed using a FUJIX BAS1000 phosphorimagier equipped with BAS Reader and Tina 2.07 and by autoradiography.

In vitro phosphorylation of recombinant NF-H side-arm fragments by SAPK1b

Sequences encoding fragments of NF-H MPR side-arm domain were as previously described (Bajaj and Miller, 1997). These were cloned into the GST-fusion vector pGEX3x (Pharmacia) as BamHI fragments. Fragment 1 spans the complete NF-H MPR domain (residues 504-895) and contains all 52 KSP repeats; fragment 2 spans the latter part of the MPR domain (residues 768-895) and fragment 3 spans the complementary, amino-terminal portion of the MPR domain (residues 504-768). Recombinant protein fragments 1, 2 and 3, expressed in Escherichia coli BL21 were purified and cleaved from GST with Factor Xa (Boehringer Mannheim) essentially as described (Pharmacia protocols).

For in vitro phosphorylation of recombinant Fragments 1, 2 and 3 proteins, 40-120 picomoles of each fragment were phosphorylated by recombinant rat SAPK1b (Kyríakis et al., 1994) at 30°C in a final volume of 20 μl as described previously for phosphorylation of tau (Reynolds et al., 1997). Reactions were stopped by addition of SDS sample buffer and the samples analysed by SDS-PAGE and autoradiography.

RESULTS

SAPK1b is one of the major stress activated kinases expressed in the nervous system; it is present in both brain, spinal cord and the peripheral nervous system (Mohit et al., 1995; Martin et al., 1996; Carboni et al., 1997, 1998). Additionally, SAPK1b is known to be mechanistically involved in glutamate-mediated excitotoxicity (Yang et al., 1997). We therefore studied NF-H phosphorylation in COS cells transfected with NF-H either alone, or in co-transfections with SAPK1b. In vitro SAPK1b assays (using (GST)-c-jun as a substrate) of these transfected cells following immunoprecipitation of the transfected kinase using antibody 12CA5 to the ha tag revealed that SAPK1b was active in the cells but that further activation could be achieved by treatment with stress agents such as anisomycin (Fig. 1) or sorbitol (data not shown). These observations are similar to those previously reported (Kyríakis et al., 1994).

As previously reported by us and others (Guidato et al., 1996; Bajaj and Miller, 1997; Chin and Liem, 1990; Sun et al., 1996), NF-H side-arms are largely non-phosphorylated following transfection into COS cells and fibroblasts and this facilitates analyses of changes in phosphorylation induced by activation of particular protein kinases. We chose to analyse NF-H side-arm phosphorylation using the phosphorylation

Indirect immunofluorescence

Primary cortical neurones were fixed in 4% (w/v) paraformaldehyde in PBS for 20 minutes, permeabilised in 0.1% (w/v) Triton X-100 in PBS for 10 minutes and then processed for immunofluorescence. Following blocking with 5% (w/v) foetal bovine serum/0.2% (w/v) Tween-20 in PBS for 1 hour, cells were probed with primary antibodies diluted in blocking solution. Antibodies were detected using goat anti-mouse and goat anti-rabbit IgG coupled to Oregon Green or Texas Red (Molecular Probes). Cells were mounted in Vectashield (Vector Labs).

In vitro kinase assays

In vitro SAPK assays from cells were performed essentially as described using (GST)-cJun(1-79) (Stratagene) as a substrate (Watson et al., 1998). Endogenous SAPK1b from cortical neurones was immunoprecipitated using a polyclonal SAPK1b selective antibody (Insight Biotechnology) and transfected SAPK1b from COS cells was immunoprecipitated using antibody 12CA5 (Roche Molecular Biochemicals) to the ha tag. cdk5 and GSK-3 assays were also performed as previously described (He et al., 1995; Guidato et al., 1996; Brownlees et al., 1997) using antibodies C8 (Santa Cruz Biotechnology) and TPK1 (Affiniti) to immunoprecipitate cdk5 and GSK-3, respectively. Histone H1 was used as a substrate for cdk5 and phosphopeptide GS1 substrate (He et al., 1995) used for GSK-3. 40-100 μg of cell lysate (identical amounts for each experiment) were used in the immunoprecipitations. For SAPK and cdk5 assays, samples were run on 10% (w/v) acrylamide SDS-PAGE gels; for GSK-3 assays, samples were resolved on 25% (w/v) acrylamide SDS-PAGE gels as described (He et al., 1995; Brownlees et al., 1997). Gels were analysed using a FUJIX BAS1000 phosphorimagier equipped with BAS Reader and Tina 2.07 and by autoradiography.
SAPK1b/JNK3 and NF-H phosphorylation

Fig. 1. SAPK1b assays from transfected COS cells showing γ³²P incorporation into GST-c-jun(1-79) substrate. (−) and (+) refer to absence or inclusion of antibody 12CA5 to the ha tag used in the immunoprecipitations to isolate active kinase. The first two lanes are mock transfected cells, the second two lanes are cells transfected with SAPK1b and the final two lanes are SAPK1b transfected cells treated with 1 µg/ml anisomycin for 30 minutes. (A) Autoradiograph and (B) the corresponding Coomassie staining of the gel to show equal amounts of substrate in the reaction mix.

Fig. 2. Cellular phosphorylation of NF-H side arms in transfected COS cells. Track 1, mock transfected cells; track 2, cells transfected with NF-H alone; track 3, cells transfected with NF-H + p54 SAPK1b; track 4, cells transfected with NF-H and treated with 1 µg/ml anisomycin for 30 minutes; track 5, cells transfected with NF-H + p54 SAPK1b and treated with 1 µg/ml anisomycin for 30 minutes; track 6, cells transfected with NF-H + GSK3β; track 7, cells transfected with NF-H + cdk5/p35; track 8, rat brain. Similar results were obtained with the p46 isosform of SAPK1b (data not shown).

Fig. 3. In vitro phosphorylation of recombinant NF-H MPR domain fragments. Left hand panel shows a Coomassie stained gel; right hand panel is an autoradiograph of the same gel. (−) and (+) refer to absence or inclusion of SAPK1b in the phosphorylations. RM is reaction mix only without NF-H MPR substrates; F1, F2 and F3 are reactions with recombinant NF-H fragments 1, 2 and 3, respectively.
kinases in neurones, phosphorylates NF-H side-arms both in vitro and in transfected cells and appears to phosphorylate NF-H at multiple sites within the side-arm.

Recently, glutamate has been shown to activate members of the SAPK family in striatal neurones and cerebellar granule cells (Schwarzschild et al., 1997; Kawasaki et al., 1997). In situ hybridisation studies demonstrate that SAPK1b is widely expressed in the nervous system but is highly expressed in cortical neurones (Martin et al., 1996; Carboni et al., 1998). Cortical neurones are known to be sensitive to glutamate treatment (Cheng et al., 1994; Davis et al., 1995). We therefore prepared cultures of primary cortical neurones and confirmed that SAPK1b was present in these cells by western blotting (Fig. 4) and immunocytochemical labelling (Fig. 7F). Both the p46 and p54 isoforms of SAPK1b were present in these cultured neurones (Fig. 4). The immunocytochemical studies demonstrated that SAPK1b was present mainly in cell bodies but some weaker labelling was also seen in neurites (Fig. 7F). Merritt et al. (1999) have also recently shown by immunocytochemistry that SAPK1b is present in cell bodies of cortical neurones but do not describe labelling of neurites; these slight differences in findings may be due the different ages of the cultures used for experimentation and/or differences in culture conditions.

We then determined whether glutamate treatment could activate SAPK1b in the cortical neurones by performing SAPK activity assays. For all of these studies, we utilised 8-day-old cortical cultures since at this age, the cells are known to be susceptible to glutamate treatment and to express glutamate receptors (Cheng et al., 1994; Davis et al., 1995). Treatment with glutamate induced activation of SAPK1b in the cells that was maximal at approximately 30 minutes (Fig. 5). For comparison, we also investigated whether glutamate influenced cdk5 and GSK-3 activities (two further kinases that target NF-H side-arms) in the primary cortical neurones. Glutamate treatment had no noticeable effect on cdk5 activity but a decrease in GSK-3 activity was observed after 1 and 4 hours of treatment (Fig. 6).

To study the effect of glutamate treatment on NF-H side-arm phosphorylation in the cortical cultures, we treated the cells with glutamate for 30 minutes and labelled them with antibodies SMI31, RMO24 and SMI32. In untreated cells, SMI31 and RMO24 labelled NF-H in neurites but not in cell bodies (Fig. 7A,B) whereas SMI32 labelled NF-H in cell bodies but only weakly labelled or did not label NF-H at all in neurites (Fig. 7C). These observations are consistent with many previous studies which show that NF-H side-arms are phosphorylated in axons but are not phosphorylated, or are phosphorylated to a much lesser extent in perikarya (see for example Sternberger and Sternberger, 1983; Lee et al., 1987; Nixon et al., 1994b). However, following glutamate treatment, this pattern of labelling was altered such that SMI31 and RMO24 also labelled NF-H in cell bodies (Fig. 7D,E). Thus glutamate induces both activation of SAPK1b and increased phosphorylation of NF-H side arms in cortical neurones.

**DISCUSSION**

A variety of kinases have now been shown to phosphorylate NF-H side-arms. These include cdk5, glycogen synthase kinase-3 (GSK-3α and GSK-3β), the mitogen-activated protein kinases Erk1 and Erk2, and stress activated protein kinase-γ (also known as SAPK1c or JNK1) (Hisanaga et al., 1993; Lew et al., 1992; Shetty et al., 1993; Miyasaka et al., 1993; Guan...
et al., 1991; Guidato et al., 1996; Bajaj and Miller, 1997; Sun et al., 1996; Veeranna et al., 1998; Giasson and Mushynski, 1996, 1997). The KSP motifs within the MPR domain of NF-H fall into different categories and whilst the full complement of sites phosphorylated by each of these different kinases are not known, those serines within the motif KSPXK are believed to be targeted by cdk5 (Shetty et al., 1993; Hisanaga et al., 1993; Beaudette et al., 1993; Bajaj and Miller, 1997). Erk1 and Erk2 may also phosphorylate KSPXK motifs but appear additionally to target KSPXXK, KSPXXXK and KSPXXXXK motifs (Veeranna et al., 1998). Preferred targets for SAPK1c may comprise KSPXE rather than KSPXK motifs (Giasson and Mushynski, 1996).

Our previous studies have shown that cdk5/p35 and GSK-3α/β phosphorylate different, or overlapping KSP repeats within the NF-H side arm (Guidato et al., 1996; Bajaj and Miller, 1997). Thus, cdk5/p35 phosphorylates NF-H such that it co-migrates with NF-H from brain on SDS-PAGE whereas GSK-3α/β phosphorylation induces only a partial shift in electrophoretic mobility (Guidato et al., 1996; Sun et al., 1996; Bajaj and Miller, 1997; see also Fig. 2, tracks 6 and 7). Likewise GSK-3α/β but not cdk5/p35 phosphorylation generates the RMO24 epitope (Guidato et al., 1996; Bajaj and Miller, 1997; see also Fig. 2, tracks 6 and 7). SAPK1b phosphorylates NF-H so that it co-migrates with NF-H from brain which is characteristic of cdk5 but also generates the RMO24 epitope, a feature of GSK-3α/β phosphorylation. The RMO24 epitope requires phosphorylation of at least five consecutive KSP repeats (Clark and Lee, 1991). SAPK1b also phosphorylated recombinant NF-H MPR fragments in vitro; MPR fragments containing the consensus cdk5 sites (KSPXXK motifs) but also the corresponding fragment that does contain these motifs were both phosphorylated (cdk5 phosphorylates only the KSPXXK containing fragment – our unpublished data). Together, these cell transfection and in vitro studies demonstrate that SAPK1b phosphorylates multiple serines within the NF-H MPR domain.

Accumulations of neurofilaments that label with antibodies that detect phosphorylated epitopes in the MPR domain of NF-H side-arms are a pathological feature of several neurodegenerative diseases (for review see Trojanowski and Lee, 1994) including ALS (Hirano, 1991; Leigh and Garafolo, 1995). Since numerous studies have shown that phosphorylation of NF-H side-arms correlates with a slowing of neurofilament transport (see for example Watson et al., 1991; Nixon et al., 1994a; Archer et al., 1994), these accumulations may be due at least in part to increased NF-H side-arm phosphorylation. Excitotoxic mechanisms involving glutamate have been suggested to be part of the neurodegenerative process for these diseases (see for example Leigh and Meldrum, 1996; Rothstein, 1996; Guo et al., 1999) and this is particularly the case for ALS where defective glutamate metabolism is seen including abnormalities to the glial glutamate transporter EAAT2 (Shaw, 1994; Rothstein et al., 1990; Lin et al., 1998). However, there is currently little evidence to connect glutamate toxicity with changes in NF-H side-arm phosphorylation. Our findings presented here, which demonstrate firstly that SAPK1b phosphorylates NF-H side-arms, secondly that glutamate causes activation of SAPK1b in neurones and finally, that glutamate treatment of neurones induces perikaryal phosphorylation of NF-H side-arms, together suggest that activation of stress activated protein kinases, and in particular SAPK1b, might be mechanistic in ALS and other neurodegenerative diseases. However, our results do not exclude the possibility that other kinases that are activated in response to glutamate might also contribute to increased phosphorylation of NF-H side-arms (see Schwarzchild et al., 1999). It is noteworthy though, that stress activated protein kinases have recently been implicated with changes in neurofilament phosphorylation in diabetic...
neuropathy, a further neurodegenerative disease (Fernyhough et al., 1999). Inhibitors of SAPKs may therefore prove to be therapeutic for these disorders.

Glutamate had no noticeable effect on cdk5 activity but did induce a downregulation of GSK-3 activity in the cortical neurons. We have previously shown that glutamate causes dephosphorylation of the microtubule-associated protein tau in cortical neurons (Davis et al., 1995) but the mechanisms linking glutamate with tau phosphorylation were not apparent. GSK-3α/β is a cellular tau kinase (Lovestone et al., 1994, 1999 Wagner et al., 1996; Muñoz-Montaño et al., 1997; Hong et al., 1997) and so these earlier observations of ours may be due to a downregulation in GSK-3 activity. Future investigations should therefore include analyses of the molecular events linking glutamate and GSK-3 activity.

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REFERENCES


