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Differences in c-Jun N-terminal kinase recognition and phosphorylation of closely related stathmin-family members

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ABSTRACT

The stathmin (STMN) family of tubulin-binding phosphoproteins are critical regulators of interphase microtubule dynamics and organization in a broad range of cellular processes. c-Jun N-terminal kinase (JNK) signalling to STMN family proteins has been implicated specifically in neuronal maturation, degeneration and cell stress responses more broadly. Previously, we characterized mechanisms underlying JNK phosphorylation of STMN at proline-flanked serine residues (Ser25 and Ser38) that are conserved across STMN-like proteins. In this study, we demonstrated using *in vitro* kinase assays and alanine replacement of serine residues that JNK phosphorylated the STMN-like domain (SLD) of SCG10 on Ser73, consistent with our previous finding that STMN Ser38 was the primary JNK target site. In addition, we confirmed that a JNK binding motif (^{41}KKKDLSL^{47}) that facilitates JNK targeting of STMN is conserved in SCG10. In contrast, SCLIP was phosphorylated by JNK primarily on Ser60 which corresponds to Ser25 on STMN. Moreover, although the JNK-binding motif identified in STMN and SCG10 was not conserved in SCLIP, JNK phosphorylation of SCLIP was inhibited by a substrate competitive peptide (TI-JIP) highlighting kinase-substrate interaction as required for JNK targeting. Thus, STMN and SCG10 are similarly targeted by JNK but there are clear differences in JNK recognition and phosphorylation of the closely related family member, SCLIP.

1. Introduction

The stathmin (STMN) family of microtubule-destabilizing phosphoproteins are characterized by the presence of STMN-like domains (SLDs) that bind two α/β tubulin heterodimers to promote the disassembly of tubulin polymers [1,2]. The archetypal member of this family, STMN, is a 17 kD cytoplasmic protein that is ubiquitously expressed and contributes broadly to the regulation of cell proliferation, migration and survival [3,4]. Other members of the STMN family include superior cervical ganglia-10 (SCG10, STMN2), SCG10-like protein (SCLIP, STMN3) and RB3 (STMN4), which is alternatively spliced into two isoforms RB3′ and RB3″ [5–7]. STMN family members share up to 70% sequence homology with highest conservation within tubulin-binding SLD domains [8]. In addition, the STMN-like proteins, such as SCG10 and SCLIP, contain an N-terminal extension beyond their SLD with conserved palmitoylated cysteine residues (Fig. 1A) that result in their localization on membrane vesicles, particularly in neurons, and that likely contribute to functions distinct from those of cytoplasmic STMN [9–11].

STMN and STMN-like protein interactions with tubulin subunits are regulated by the phosphorylation of serine residues located within their SLD domains [8]. STMN Ser16, Ser25, Ser38 and Ser63 phosphorylation negatively regulate STMN-tubulin binding leading to STMN inactivation [12]. These four phosphorylated residues are also highly conserved in STMN-like proteins with the phosphorylation of the corresponding residues of SCG10 and SCLIP similarly directly antagonizing tubulin binding in *in vitro* assays [13]. STMN serine residues are phosphorylated by multiple kinases and this largely depends on the specific stimulus and cellular context [3,8]. In this manner, STMN integrates and relays signals from complex intracellular signalling pathways to effect changes in the microtubule cytoskeleton. We recently demonstrated that the proline-flanked STMN Ser25 and Ser38 residues are major targets of the c-Jun N-terminal kinase (JNK) particularly in response to extracellular abiotic stress stimuli such as chemotoxic stress with

Abbreviations: JBD, JNK binding domain; JNK, c-Jun N-terminal kinase; SCG10, superior cervical ganglia-10; SCLIP, SCG10-like protein; SLD, stathmin-like domain; STMN, stathmin.

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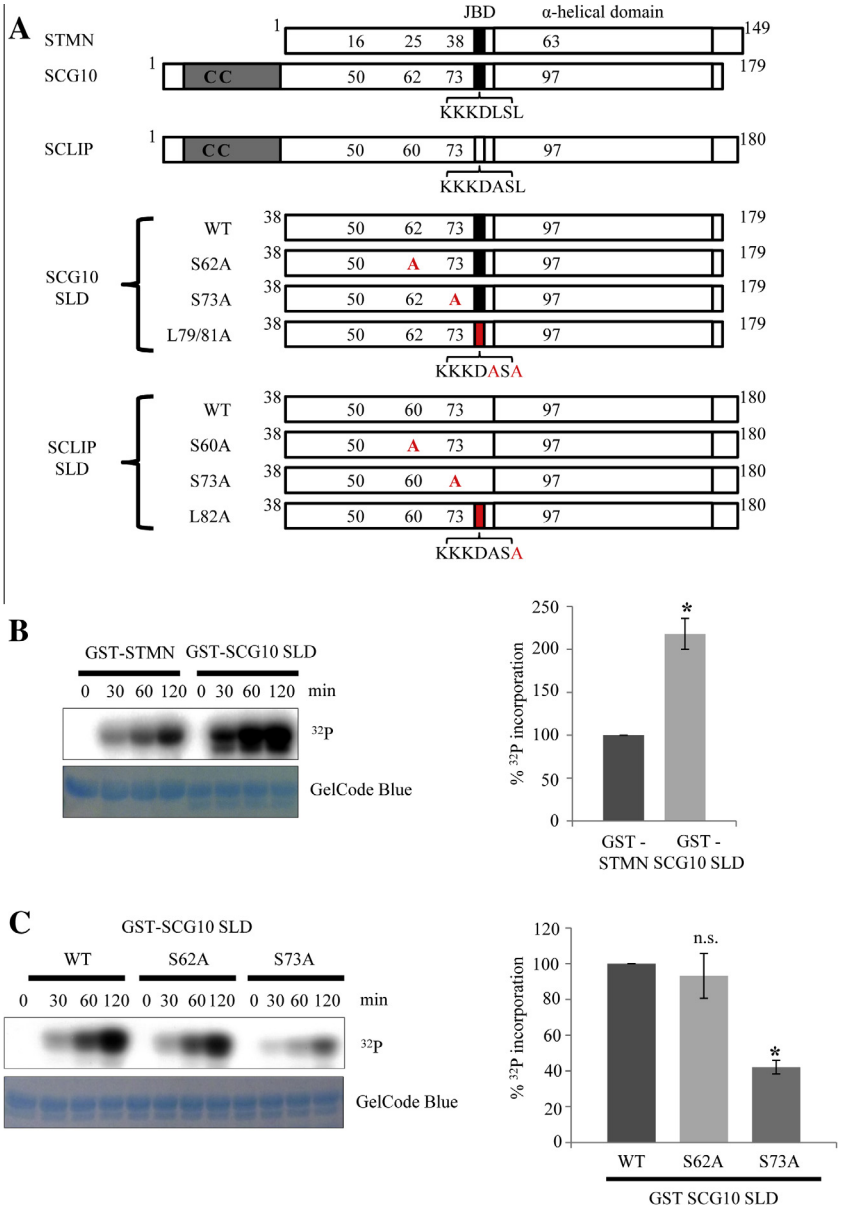

Fig. 1. JNK phosphorylates SCG10 predominantly on Ser73. (A) Schematic representation of STMN family members and mutant constructs. Alanine replacement of conserved serine phosphorylation sites or leucine residues potentially involved in kinase interactions are indicated. Tubulin-binding α-helical regions are shaded in light gray and N-terminal extensions on SCG10 and SCLIP shaded in dark gray. (B) GST-STMN or GST-SCG10 SLD were phosphorylated with active JNK1 (10 ng) for the durations indicated. (C) GST-SCG10 SLD phosphorylation mutants (Ser62Ala or Ser73Ala, 10 μg) were incubated with active JNK1 (10 ng). Wild-type GST-SCG10 SLD was included for comparison. Autoradiograph and GelCode Blue stained gels are representative of three independent experiments. Graphs depict cerenkov counts of excised bands from the assays at the 120 min time point expressed as a percentage of appropriate controls (GST-STMN or GST-SCG10 WT). Values are mean ± S.E. ($n = 3$, $^{*}P < 0.05$).

arsenite, changes in osmolarity, and temperature [14,15]. Furthermore, JNK-mediated phosphorylation of SCG10 in the developing central nervous system has been shown to be important in the regulation of neurite extension, neuronal differentiation and neurogenesis [16,17]. These findings indicate that JNK targeting of STMN-like proteins is critical for neuronal development.

The JNK members of the mitogen-activated protein kinase (MAPK) family comprise three members (JNK1, 2 and 3) that belong to group of proline-directed kinases that include cyclin-dependent kinases. Originally characterized as stress-responsive kinases with pro-apoptotic functions [18,19], JNK family members target a wide repertoire of cytoplasmic and nuclear targets to regulate a broad range of cellular processes including cellular proliferation and differentiation [20]. The regulation of JNK substrate selectivity is directed in part by protein interaction mediated through a consensus JNK-binding motif (R/K_{2-3}-X_{1-6}-L/I-X-L/I) present on JNK target proteins [20]. We recently identified STMN residues ^{41}KKKDLSL^{47} as the JNK-binding motif required for JNK-mediated phosphorylation [14]. Although these residues are largely conserved in other STMN family members (i.e. ^{75}KKKDLSL^{81} for SCG10 and ^{76}KKKDASL^{82} for SCLIP), it is unknown whether similar mechanisms are involved in JNK recognition and targeting of STMN-like proteins.

In this study, using established *in vitro* kinase assays, we evaluated the mechanism of JNK targeting of SCG10 and SCLIP in comparison to the archetypal family member STMN. We demonstrate that the JBD required for JNK recognition is conserved in SCG10 but not SCLIP and discovered variations in the specific serine residues preferentially targeted by JNK between highly

conserved STMN-like proteins. Thus, JNK targets multiple STMN family members and the mechanism is largely conserved although specific differences in kinase recognition and serine phosphorylation revealed may translate to distinct biological outcomes.

2. Materials and methods

2.1. Plasmids

Wild type STMN and the SLDs of SCG10 (aa 38–179) and SCLIP (aa 38–180) were cloned into pGEX-6P-1-GST vector. Phosphorylation-deficient (S → A) and JNK-binding motif (L → A) mutants (Fig. 1A) were generated by site-directed mutagenesis and Dpn1 digestion according to the Quickchange protocol (Stratagene). Briefly, 10 ng DNA template, 1X Pfu buffer with $MgSO_4$ (20 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton® X-100 and 100 μg/ml nuclease-free BSA), 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTP mix and 1.0 unit Pfu DNA polymerase (Promega) were prepared to a final volume of 50 μl and amplified by PCR. Template plasmids were digested with Dpn1, PCR products transformed into *Escherichia coli* (XL10-Gold, Stratagene) and specific mutations confirmed by DNA sequencing.

2.2. Recombinant protein purification

Plasmid DNA was transformed into competent BL21 Codon Plus *E. coli* and protein expression induced with 100 mM IPTG (4 h, 37 °C, 200 rpm). Active JNK was produced by co-transfection of pBACPAK9-GST-JNK1, pBACPAK9-MKK4 and pBACPAK9-MKK7 with BACPAK6 baculoviral DNA into *Spodoptera frugiperda 9* insect cells. GST-tagged proteins were obtained via batch purification using Glutathione Sepharose 4B beads (GE Life Sciences) and, where required, GST-tag removed by cleavage with Precision Protease (GE Life Sciences).

2.3. In vitro kinase assay

Purified recombinant protein kinase substrates (10 μg) were incubated with active JNK (10 ng) and [γ-^{32}P]ATP (1 μCi/reaction) in a kinase reaction buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2 \cdot 6H_2O$, 40 μM ATP, 20 mM β-glycerophosphate and supplemented with 25 μM Na_3VO_4 and 100 μM DTT). Reactions were conducted at 30 °C for the indicated durations and terminated by the addition of Laemmli sample buffer. Samples were resolved on SDS–PAGE and stained with GelCode Blue Stain reagent (Thermo Scientific) to confirm protein loading. ^{32}P-labelled phosphate incorporation was visualized by autoradiography and quantitated by Cerenkov counting.

2.4. Statistical analysis

All experiments were repeated at least three times and values are presented as mean ± SEM. Values were compared using unpaired student's *t*-tests. *P*-values <0.05 were considered significant.

3. Results

3.1. JNK1 phosphorylates SCG10 predominantly on serine 73

We previously defined STMN as a JNK substrate [14] and the JNK-mediated phosphorylation of SCG10 has been shown to be involved in neurite outgrowth and degeneration [16,21–23]. Here we directly compared JNK targeting of comparable levels (10 μg) of SCG10 SLD and STMN proteins in *in vitro* kinase assays with recombinant active JNK1 incubated with either GST-STMN or GST-SCG10 SLD as substrates. This revealed higher phosphorylation levels judged by incorporation of ^{32}P for the SCG10 SLD protein. Specifically, after 120 min incubation with active JNK1, ^{32}P incorporation into GST-SCG10 SLD was approximately twofold greater when compared to the ^{32}P incorporation recorded for GST-STMN (2.18 ± 0.18-fold change, Fig. 1B). Therefore, despite 74% sequence similarity and conserved target sites, SCG10 appears to be preferentially phosphorylated by JNK *in vitro*.

The SCG10 SLD contains four conserved serine residues (Ser50, Ser62, Ser73 and Ser 97) that negatively regulate tubulin interaction [13]. SCG10, Ser62 and Ser73 are proline-flanked and can be phosphorylated by MAPK and cyclin-dependent kinases [13]. We had previously reported that conserved proline-flanked STMN Ser38 was preferentially targeted by JNK *in vitro* and in mammalian cells [14]. To determine the specific serine residues targeted by JNK, we performed alanine substitution of SCG10 on Ser62 and Ser73 (SCG10 SLD Ser62Ala or Ser73Ala, Fig. 1A) and determined JNK targeting of these phosphorylation mutants biochemically. Alanine substitution of Ser62 did not significantly alter JNK-mediated phosphorylation of SCG10 SLD (Fig. 1C). In contrast, JNK-mediated phosphorylation of SCG10 SLD Ser73Ala mutant was substantially reduced (42.1 ± 3.8% of compared to SCG10 SLD) (Fig. 1C). This indicates that SCG10 Ser73 is the primary target site required for maximum JNK phosphorylation. SCG10 Ser73 is the conserved serine equivalent to STMN Ser38. Thus, our findings indicate that SCG10 and STMN are similarly targeted by JNK *in vitro*.

3.2. Identification of JNK-binding domain required for SCG10 phosphorylation

Previously, we defined a region on STMN (^{41}KKKDLSL^{47}) as a consensus kinase docking motif (JBD), found on JNK substrates, and demonstrated that this was required for efficient JNK phosphorylation of STMN [14]. To investigate the requirement of kinase docking, we first determined the effect of a substrate competitive peptide, TI-JIP (RPKRPTTLNLF), derived from the binding motif of the JIP1 JNK scaffold protein, on JNK-mediated phosphorylation of STMN proteins. STMN phosphorylation by JNK was potently inhibited in the presence of TI-JIP (78.5 ± 0.5% compared to control, Fig. 2A) in agreement with our previous findings [14]. Similarly, JNK phosphorylation of SCG10 SLD was markedly reduced in the presence of TI-JIP (Fig. 2B). Quantitative analysis revealed GST-SCG10 SLD phosphorylation (120 min) was inhibited by 87.9 ± 0.8% in comparison to a vehicle control (Fig. 2B). This indicated a mechanism of JNK targeting that required kinase docking on a JBD motif present on SCG10.

SCG10 amino acid sequence curation identified residues 75–81 (^{75}KKKDLSL^{81}) and 129–137 (^{129}KMAEEKLIL^{137}) as regions that conform to a consensus JBD motif of R/K_{2-3}-X_{1-6}-L/I-X-L/I. To identify the SCG10 JBD, we substituted specific leucine residues critically require for kinase interaction with alanine (SCG10 SLD Leu79/81Ala or SCG10 SLD Leu135/137Ala) to generate JBD mutants. Our *in vitro* kinase assay results indicate that JNK1-mediated phosphorylation of the SCG10 SLD Leu79/81Ala mutant was significantly reduced compared to the wild type SCG10 SLD (Fig. 2C). Quantitative analysis shows ^{32}P incorporation into the SCG10 SLD Leu79/81Ala mutant was reduced to 57.8 ± 9.8% compared to wild type SCG10 SLD. In contrast, JNK1-mediated phosphorylation of the SCG10 SLD Leu135/137Ala mutant was not significantly different from that of the wild type SCG10 SLD (Fig. 2C). Our findings confirm that JBD motif of STMN (^{41}KKKDLSL^{47}) is conserved in SCG10 (^{75}KKKDLSL^{81}) and similarly required for efficient JNK targeting of SCG10.

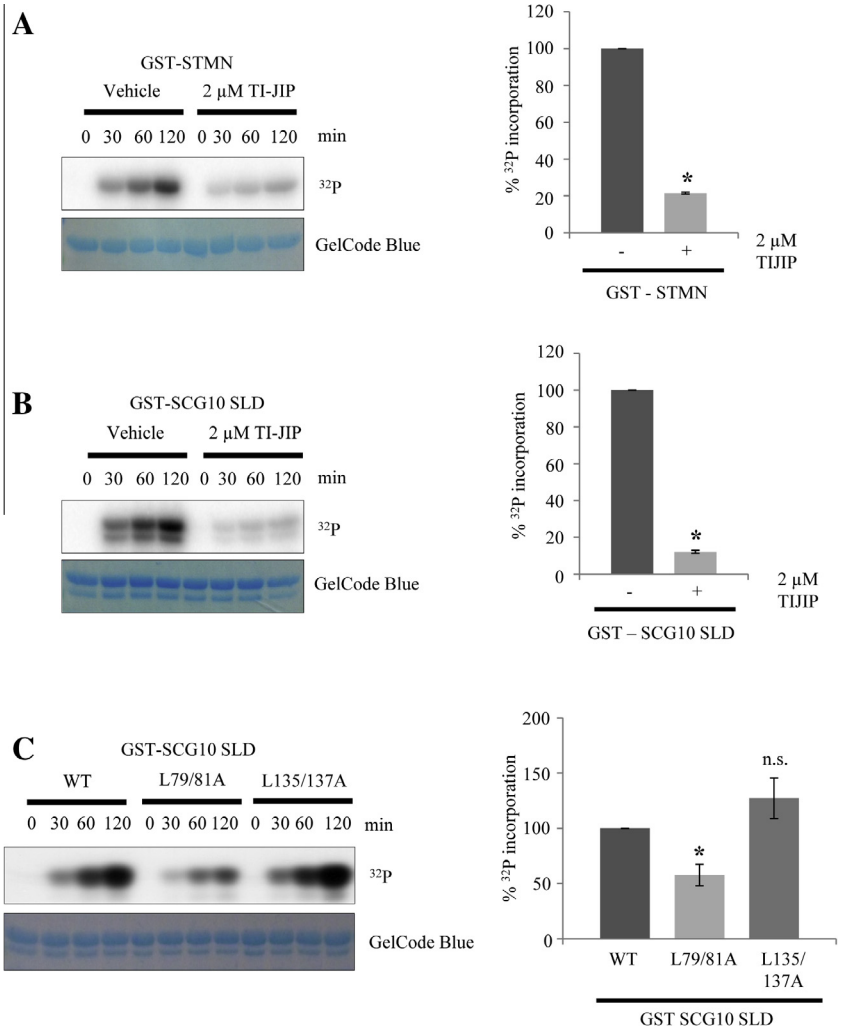

Fig. 2. Identification of conserved JNK-binding domain (JBD) on SCG10 required for phosphorylation. (A) GST-STMN, (B) GST-SCG10 SLD were phosphorylated with active JNK1 (10 ng) in the presence of TI-JIP (2 μM) for times indicated. Vehicle control (DMSO) was included as a negative control. (C) GST-SCG10 SLD mutated in putative JBD regions (Leu79/81Ala or Leu135/137Ala, 10 μg) were incubated with active JNK1 (10 ng, 0–120 min) and compared to wild-type GST-SCG10 SLD. Autoradiograph and GelCode Blue stained gels are representative of three independent experiments. Graphs depict cerenkov counts of excised bands from the assays at the 120 min time point expressed as a percentage of appropriate controls (GST-STMN or GST-SCG10 WT). Values are mean ± S.E. ($n = 3$, $^{*}P < 0.05$).

3.3. SCLIP is a JNK substrate

SCLIP shares 72% sequence homology with SCG10 and the SCLIP SLD is 68% identical to STMN. Importantly, the proline-flanked serine residues that are potentially phosphorylated by JNK are conserved in SCLIP (Ser60 and Ser73, Fig. 1A). In addition, heterologous expression in COS cells followed by affinity pulldown indicated an interaction between SCLIP and JNK [16]. However, it has been reported that SCLIP may not be phosphorylated by JNK [16]. Thus, we next tested the SCLIP SLD in our *in vitro* JNK assays. Interestingly, we found that SCLIP SLD was phosphorylated by JNK1 *in vitro* (Fig. 3A). JNK phosphorylation of SCLIP SLD was comparable to that of STMN albeit to marginally lower levels (79.2 ± 7.0% of STMN phosphorylation. Fig. 3A). Our results thus indicate that the SCLIP SLD can act as a JNK substrate *in vitro*.

We then performed alanine substitution of the individual proline-flanked serine residues of SCLIP SLD (Ser60Ala or Ser73Ala) and interrogated these mutants in our *in vitro* kinase assays to determine the specific residues targeted by JNK. Surprisingly, JNK-mediated phosphorylation of SCLIP SLD was not significantly altered by alanine replacement of the Ser73 (106.9 ± 6.2% compared to SCLIP SLD. Fig. 3B). This is in stark contrast to the effect of alanine replacement of the equivalent residues in SCG10 (Fig. 1C) and STMN [14]. Interestingly, alanine replacement of SCLIP SLD Ser60 resulted in significantly reduced phosphorylation by JNK1 (Fig. 3B). The phosphorylation of SCLIP SLD Ser60Ala was reduced to 45.2 ± 1.6% of levels measured for control SCLIP SLD (Fig. 3B). Thus, unlike the results for the phosphorylation of the closely related STMN and SCG10 proteins, our results indicate that SCLIP Ser60 is the primary JNK target site.

3.4. JNK recognition of SCLIP via an alternative mechanism

Our results indicate that SCLIP is phosphorylated by JNK thus raising the question of whether similar kinase docking mechanisms are involved STMN [14], SCG10 (Fig. 2) and SCLIP. Therefore, we determined the effect of the TI-JIP inhibitory peptide on JNK1-mediated SCLIP SLD phosphorylation (Fig. 4A). We found that SCLIP SLD phosphorylation was substantially decreased by TI-JIP pre-incubation (Fig. 4A). Quantitative analysis showed an 81.1 ± 0.9% decrease in JNK mediated phosphorylation of SCLIP SLD in the presence of TI-JIP inhibition compared to a vehicle control (Fig. 4A). This strongly suggests the presence of a SCLIP JBD motif that is involved in docking and phosphorylation by JNK, but the

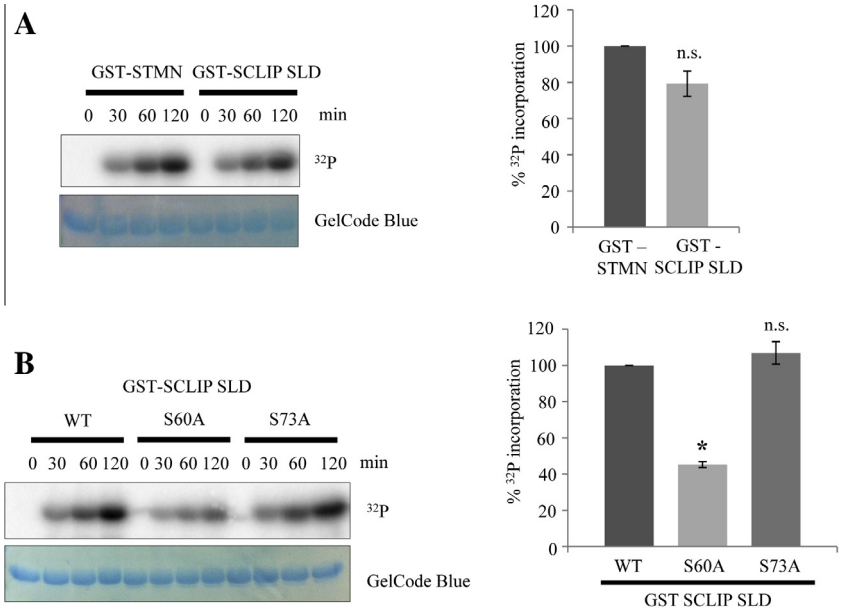

Fig. 3. SCLIP is phosphorylated by JNK *in vitro* on Ser60. (A) GST-STMN or GST-SCLIP SLD were incubated with active JNK1 (10 ng) for the durations indicated. (B) Wild-type GST-SCLIP SLD and alanine-substituted phosphorylation mutants (Ser60Ala or Ser73Ala) (10 μg) were incubated with active JNK1 (10 ng, 0–120 min). ^{32}P-labelled phosphate incorporation was visualized by autoradiography and GelCode Blue staining revealed protein loading across samples. Graphs depict cerenkov counts of excised bands from the assays at the 120 min time point expressed as a percentage of appropriate controls (GST-STMN or GST-SCLIP SLD WT). Values are mean ± S.E. (n = 3, $^{*}P$ < 0.05).

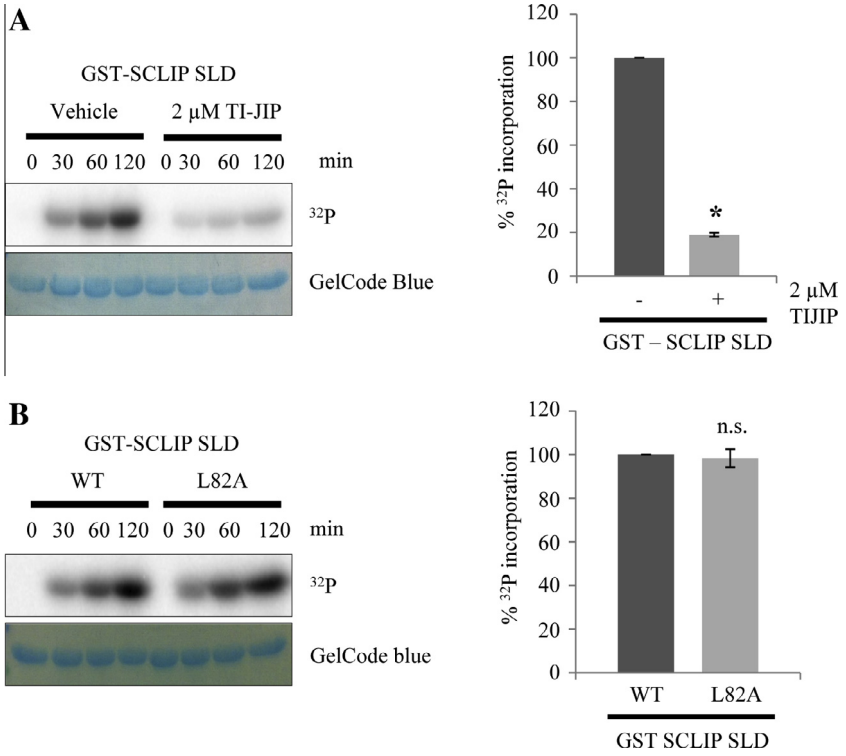

Fig. 4. Kinase-substrate interactions mediate JNK phosphorylation of SCLIP but the region involved is not equivalent to the region identified for STMN or SCG10. (A) GST-SCLIP SLD was incubated with active recombinant JNK1 (10 ng) and TI-JIP (2 μM) for the durations indicated. TI-JIP was substituted with DMSO (vehicle) as a negative control. (B) Wild-type GST-SCLIP SLD or a mutant with alanine substitution of Leu82 (Leu82Ala, 10 μg) were incubated with active recombinant JNK1 (10 ng) for the durations indicated. GelCode Blue staining revealed protein loading and ^{32}P-labelled phosphate incorporation was visualized by autoradiography. Autoradiograph and gel images are representative of three independent experiments. Graphs depict cerenkov counts of excised bands from the assays at the 120 min time point expressed as a percentage of GST-SCLIP WT. Values are mean ± S.E. (n = 3, $^{*}P$ < 0.05).

identified JBD motif (KKKDLSL) on STMN and SCG10 is only partially conserved in SCLIP. Specifically, the equivalent SCLIP sequence (^{76}KKKDASL^{82}) has an alanine in place of the first hydrophobic leucine/isoleucine residue that is involved in forming direct contact with a substrate recognition region on JNK. To determine if this partially conserved sequence functioned as a JBD, we generated a SCLIP SLD JBD mutant (Leu82Ala) and tested its effects on JNK-mediated phosphorylation. In comparison to SCLIP SLD WT,

the mutation did not reduce SCLIP SLD phosphorylation as initially predicted (Fig. 4B). Quantitative analysis showed phosphorylation of the SCLIP SLD JBD mutant (98.3 ± 4.1% of control levels) was not significantly different to control (Fig. 4B). This indicates that JNK targeting of SCLIP does not correspond with the identified JBD motif of STMN [14] and SCG10, and thus reveals that this different mechanism of targeting may drive JNK to phosphorylate SCLIP Ser60 in preference to SCLIP Ser73.

4. Discussion

STMN family proteins are highly conserved phosphoproteins that are responsible for the integration of multiple intracellular signalling pathways to regulate microtubule organization [24]. The JNK family of stress-activated protein kinases phosphorylates both nuclear and cytoplasmic substrates that are crucial for the regulation of gene expression, cell proliferation and cell viability [20]. JNK regulation of the cytoskeleton, that determines cell architecture and movement, is also well established [25]. In particular, the critical role of JNK signalling during multipolar stage transitions of neural progenitors [17], axonal degeneration [22] and cellular responses to abiotic stress [14] involve the targeting of STMN family of microtubule regulatory proteins. In this study, we have conducted biochemical analysis of JNK targeting of STMN, SCG10 and SCLIP and revealed differences in kinase recognition and phosphorylation of closely related family members.

Our *in vitro* kinase assays showed that SCG10 SLD was phosphorylated by JNK to a higher extent compared to STMN despite similar mechanisms in kinase recognition and serine residues targeted. This is in line with previous reports that SCG10 is phosphorylated to higher stoichiometry [16]. The biochemical basis for this remains unclear but, in the absence of tubulin and free of cellular context, differences in the primary sequence of STMN and the SLD of SCG10 is likely responsible. STMN is an intrinsically disordered protein [26]. Despite high sequence conservation, subtle differences may alter secondary structure that restricts the accessibility and the availability of proline-flanked serine phosphorylation sites to JNK therefore resulting in different phosphorylation efficiencies.

We had reported that JNK phosphorylation of proline-flanked serine residues on STMN (Ser25 and Ser38) was required for the maintenance of interphase microtubules under such stress stimulation [14,15]. Similarly, JNK phosphorylation of conserved serine residues on SCG10 (Ser62 and Ser73) has been shown to be required for neurite outgrowth [16]. In addition, we demonstrated that *in vitro* JNK phosphorylation of SCG10 SLD was primarily at Ser73, which corresponds to STMN Ser38. Previously we showed that JNK targets STMN Ser38 preferentially [14]. This indicates that JNK targets STMN and SCG10 through similar mechanisms. Consistent with this notion, we also demonstrated that previously identified kinase recognition motif on STMN involved in JNK targeting is conserved in SCG10.

The ability of protein kinases to interact with other elements of a signalling cascade, for example upstream regulators or substrates, is highly dependent on the binding of complementary regions within the kinase to short docking motifs present on these interactors. By utilising a combination of a substrate competitive inhibitor peptide and site-directed mutagenesis, we determined that JNK targeting of SCG10 involved a kinase-substrate recognition through a motif conserved with that previously identified on STMN (KKKDLSL) [14]. We excluded a second region on SCG10 (^{129}KMAEEKLIL^{137}) that matched the JBD consensus motif (R/K$_3$-X$_{1-5}$-L/I-X-L/I) indicating that this site was not involved in JNK recognition of SCG10. Taken together our study demonstrates that JNK targeting of STMN and SCG10 is mediated through similar mechanisms. This reinforces the importance of differential intracellular localization of closely related STMN family members (i.e. SCG10 in golgi compared to cytoplasmic STMN) for compartmentalized signalling and microtubule regulation.

In broadening our analysis of STMN family proteins we tested JNK-mediated phosphorylation of the SCLIP SLD in our *in vitro* kinase assays. SCLIP shares high sequence conservation with STMN and SCG10 particularly within its tubulin-binding region. Importantly, SCLIP retains conserved proline-flanked serine residues (Ser60 and Ser73) that are potential phosphorylation targets for proline-directed kinases such as JNK [27]. Much less is known regarding the kinases that target SCLIP although it was recently reported that glycogen synthase kinase 3β phosphorylation directs the intracellular localization of SCLIP in neurons [27]. In addition, a previous study indicated that SCLIP was not phosphorylated by JNK [16]. In contrast, we found that SCLIP was phosphorylated by JNK in our *in vitro* kinase assays to a similar extent as STMN. Surprisingly, alanine replacement of potential target sites indicated that JNK phosphorylated SCLIP on Ser60 which corresponds to STMN Ser25 and SCG10 Ser62. Furthermore, we verified, through mutagenesis studies, that the JBD motif identified in STMN and SCG10 was not conserved on SCLIP. Curating the remainder of SCLIP primary sequence did reveal a sequence conforming to a consensus JBD motif. However, JNK targeting of SCLIP was inhibited with the addition of the substrate competitive peptide, TI-JIP, highlighting the presence of a kinase recognition site on SCLIP. Further studies will be required to identify the non-canonical JNK recognition motif on SCLIP. Taken together, our studies indicate that SCLIP is a substrate of JNK but the target serine phosphorylated and kinase recognition motif differs to related family members, STMN and SCG10. Although yet to be demonstrated, our results highlight a distinct possibility of JNK signalling to SCLIP within a cellular context.

Acknowledgments

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References

[1] L.D. Belmont, T.J. Mitchison, Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules, Cell 84 (1996) 623–631.
[2] L. Jourdain, P. Curmi, A. Sobel, D. Pantaloni, M.F. Carlier, Stathmin: a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules, Biochemistry 36 (1997) 10817–10821.
[3] L. Cassimeris, The oncoprotein 18/stathmin family of microtubule destabilizers, Curr. Opin. Cell Biol. 14 (2002) 18–24.
[4] B. Belletti, G. Baldassarre, Stathmin: a protein with many tasks. New biomarker and potential target in cancer, Expert Opin. Ther. Targets 15 (2011) 1249–1266.
[5] B. Antonsson, R. Lütjens, G. Di Paolo, D. Kassel, B. Allet, A. Bernard, S. Catsicas, G. Grenningloh, Purification, characterization, and *in vitro* phosphorylation of the neuron-specific membrane-associated protein SCG10, Protein Expr. Purif. 9 (1997) 363–371.
[6] S. Ozon, T. Byk, A. Sobel, SCLIP: a novel SCG10-like protein of the stathmin family expressed in the nervous system, J. Neurochem. 70 (1998) 2386–2396.
[7] A. Maucuer, J. Moreau, M. Méchali, A. Sobel, Stathmin gene family: phylogenetic conservation and developmental regulation in Xenopus, J. Biol. Chem. 268 (1993) 16420–16429.
[8] P.A. Curmi, O. Gavet, E. Charbaut, S. Ozon, S. Lachkar-Colmerauer, V. Manceau, S. Siavoshian, A. Maucuer, A. Sobel, Stathmin and its phosphoprotein family: general properties, biochemical and functional interaction with tubulin, Cell Struct. Funct. 24 (1999) 345–357.
[9] G. Di Paolo, R. Lutjens, V. Pellier, S.A. Stimpson, M.H. Beuchat, S. Catsicas, G. Grenningloh, Targeting of SCG10 to the area of the Golgi complex is mediated by its NH2-terminal region, J. Biol. Chem. 272 (1997) 5175–5182.
[10] R. Lutjens, M. Igarashi, V. Pellier, H. Blasey, G. Di Paolo, E. Ruchti, C. Pfulg, J.K. Staple, S. Catsicas, G. Grenningloh, Localization and targeting of SCG10 to the

trans-Golgi apparatus and growth cone vesicles, Eur. J. Neurosci. 12 (2000) 2224–2234.
[11] J. Wang, C. Shan, W. Cao, C. Zhang, J. Teng, J. Chen, SCG10 promotes non-amyloidogenic processing of amyloid precursor protein by facilitating its trafficking to the cell surface, Hum. Mol. Genet. 22 (2013) 4888–4900.
[12] G. Di Paolo, B. Antonsson, D. Kassel, B.M. Riederer, G. Grenningloh, Phosphorylation regulates the microtubule-destabilizing activity of stathmin and its interaction with tubulin, FEBS Lett. 416 (1997) 149–152.
[13] B. Antonsson, D.B. Kassel, G. Di Paolo, R. Lutjens, B.M. Riederer, G. Grenningloh, Identification of *in vitro* phosphorylation sites in the growth cone protein SCG10. Effect of phosphorylation site mutants on microtubule-destabilizing activity, J. Biol. Chem. 273 (1998) 8439–8446.
[14] D.C. Ng, T.T. Zhao, Y.Y. Yeap, K.R. Ngoei, M.A. Bogoyevitch, c-Jun N-terminal kinase phosphorylation of stathmin confers protection against cellular stress, J. Biol. Chem. 285 (2010) 29001–29013.
[15] Y.Y. Yip, Y.Y. Yeap, M.A. Bogoyevitch, D.C. Ng, cAMP-dependent protein kinase and c-Jun N-terminal kinase mediate stathmin phosphorylation for the maintenance of interphase microtubules during osmotic stress, J. Biol. Chem. 289 (2014) 2157–2169.
[16] T. Tararuk, N. Ostman, W. Li, B. Björkblom, A. Padzik, J. Zdrojewska, V. Hongisto, T. Herdegen, W. Konopka, M.J. Courtney, E.T. Coffey, JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length, J. Cell Biol. 173 (2006) 265–277.
[17] N. Westerlund, J. Zdrojewska, A. Padzik, E. Komulainen, B. Björkblom, E. Rannikko, T. Tararuk, C. Garcia-Frigola, J. Sandholm, L. Nguyen, T. Kallunki, M.J. Courtney, E.T. Coffey, Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate, Nat. Neurosci. 14 (2011) 305–313.
[18] J.M. Kyriakis, J. Avruch, Pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine, J. Biol. Chem. 265 (1990) 17355–17363.
[19] H. Le-Niculescu, E. Bonfoco, Y. Kasuya, F.X. Claret, D.R. Green, M. Karin, Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death, Mol. Cell. Biol. 19 (1999) 751–763.
[20] M.A. Bogoyevitch, B. Kobe, Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases, Microbiol. Mol. Biol. Rev. 70 (2006) 1061–1095.
[21] H. Xu, D.N. Dhanasekaran, C.M. Lee, E.P. Reddy, Regulation of neurite outgrowth by interactions between the scaffolding protein, JNK-associated leucine zipper protein, and neuronal growth-associated protein superior cervical ganglia clone 10, J. Biol. Chem. 285 (2010) 3548–3553.
[22] J.E. Shin, B.R. Miller, E. Babetto, Y. Cho, Y. Sasaki, S. Qayum, E.V. Russler, V. Cavalli, J. Milbrandt, A. DiAntonio, SCG10 is a JNK target in the axonal degeneration pathway, Proc. Natl. Acad. Sci. USA 109 (2012) E3696–E3705.
[23] S. Neidhart, B. Antonsson, C. Gilliéron, F. Vilbois, G. Grenningloh, S. Arkinstall, c-Jun N-terminal kinase-3 (JNK3)/stress-activated protein kinase-beta (SAPKbeta) binds and phosphorylates the neuronal microtubule regulator SCG10, FEBS Lett. 508 (2001) 259–264.
[24] A. Sobel, Stathmin: a relay phosphoprotein for multiple signal transduction?, Trends Biochem Sci. 16 (1991) 301–305.
[25] M. Gelderblom, S. Eminel, T. Herdegen, V. Waetzig, c-Jun N-terminal kinases (JNKs) and the cytoskeleton–functions beyond neurodegeneration, Int. J. Dev. Neurosci. 22 (2004) 559–564.
[26] S. Honnappa, W. Jahnke, J. Seelig, M.O. Steinmetz, Control of intrinsically disordered stathmin by multisite phosphorylation, J. Biol. Chem. 281 (2006) 16078–16083.
[27] S. Devaux, F.E. Poulain, V. Devignot, S. Lachkar, T. Irinopoulou, A. Sobel, Specific serine-proline phosphorylation and glycogen synthase kinase 3β-directed subcellular targeting of stathmin 3/Sclip in neurons, J. Biol. Chem. 287 (2012) 22341–22353.