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MNB/DYRK1A Phosphorylation Regulates the Interactions of Synaptojanin 1 with Endocytic Accessory Proteins

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Abstract

MNB/DYRK1A is a proline-directed serine/threonine kinase implicated in Down syndrome (DS). In an earlier screening, two proteins from adult rat brain, one 100-kDa and the other 140-kDa, were found to be prominently phosphorylated by the kinase. The 100-kDa protein was previously characterized as an isoform of dynamin 1. In this study, we identified the 140-kDa protein as synaptojanin 1 (SJ1). MNB/DYRK1A phosphorylates SJ1 at multiple sites and produces complex behaviors in binding to amphiphysin 1 and intersectin 1 (ITSN1). However, the phosphorylation has little effect on the phosphatidylinositol phosphatase activity of SJ1. These results suggest that MNB/DYRK1A is involved in regulating the recruitment activity but not the phosphatase activity of SJ1. Our findings may be especially important in the etiology of DS because MNB/DYRK1A, SJ1, and ITSN1 are all located at or near the region of human chromosome 21, which is postulated to be involved in the disease.

Keywords

Down syndrome; human chromosome 21 genes; protein-protein interaction; intersectin; amphiphysin

Introduction

Minibrain kinase (MNB) was originally identified as a gene essential to the neuronal proliferation of Drosophila [1]. The ortholog of Drosophila MNB, termed dual specificity tyrosine-phosphorylation regulated kinase 1A (MNB/DYRK1A), was subsequently characterized in many organisms [2-6]. Similar to Drosophila, MNB/DYRK1A is involved in the early development of the central nervous system of vertebrates [6,7]. However, the expression of MNB/DYRK1A is persistent throughout adulthood in selected brain regions [4,8-10], indicating that MNB/DYRK1A is also important for specific functions in the adult brain. The human MNB/DYRK1A gene is mapped to the Down syndrome critical region (DSCR) of human chromosome 21 [3-5], and the protein level of MNB/DYRK1A is elevated in DS brains and in Ts65Dn mouse [10] (Dowjat, W. K., personal communications), an animal DS model [11]. MNB/DYRK1A and calcineurin (protein phosphatase 2B) could form a regulatory circuit in controlling the phosphorylation states of many proteins [12]. The overexpression of MNB/DYRK1A and DSCR1, a DSCR gene encoding a specific calcineurin

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inhibitor [13,14], has been shown to raise levels of phosphorylated NFAT, disrupt its signaling pathway, and produce DS-like phenotypes in mice [15].

We previously detected two prominent MNB/DYRK1A phosphorylated proteins, one 100-kDa (Sub-100) and the other 140-kDa (Sub-140), in adult rat brain by a solid-phase assay [16,17]. Sub-100 has been purified and identified as dynamin 1xa (DY1xa) (either 1aa or 1ba isoforms) [16,17]. DY1xa is a GTPase known to be involved in clathrin-mediated endocytosis and synaptic vesicle recycling [18]. MNB/DYRK1A phosphorylation at S857 in the proline-rich domain (PRD) of DY1xa differentially regulates the interactions of DY1xa [16,17] with several endocytic accessory proteins [19]. Recently, amphiphysin 1 (AMPH1), a BAR (Bin-Amphiphysin-Rvsp) domain containing endocytic accessory protein [19], was also identified as an MNB/DYRK1A substrate initially through sequence comparison [20]. MNB/DYRK1A phosphorylates AMPH1 primarily at S293 in PRD and inhibits the binding of AMPH1 to endophilin [20]. In this study, we provide evidence to show that Sub-140 is synaptojanin 1 (SJ1). SJ1 is a brain-enriched phosphoinositide phosphatase [21] involved in regulating clathrin-mediated endocytosis and synaptic vesicle recycling [22]. Similar to DY1 and AMPH1, MNB/DYRK1A phosphorylation controls the interactions of SJ1 with endocytic accessory proteins.

Materials and Methods

Protein purification

GST-MNB/DYRK1A, GST-ITSN1(SH3) (all five SH3 domains), and GST-AMPH1(SH3) were purified from the *E. coli* expression system by glutathione affinity chromatography as previously described [16]. Sub-140 was purified from adult rat brains exactly as Sub-100 for the first few steps [16]. Mono Q separates Sub-100 (0.18–0.24 M NaCl) and Sub-140 (0.26–0.32 M NaCl) into two distinct fractions. Sub-140 fractions were pooled and then subjected to two runs of FPLC-Mono S chromatography. The Mono S column was eluted with 0- 0.6 M NaCl gradient in a buffer containing 25 mM MES (pH 6.5) and 25 mM NaCl. Sub-140 was eluted from column with 0.30-0.39 M NaCl. Affinity purification of SJ1 was performed as follows. Rat brain Triton extract was prepared similarly as described [17]. The extract (20-30 ml) was mixed with 2 ml (bed volume) Affi-Gel 15 (BioRad) containing immobilized GST-AMPH1(SH3) at 4 °C for 2 hours. After washing extensively with a buffer containing 25 mM Tris-HCl, pH7.4, 150 mM NaCl, and 1% Triton, the bound proteins were eluted first by 5×1 ml fractions of 0.5 M MES (pH 4.6) followed by another 5×1 ml fractions of a buffer containing 0.1 M MES, pH 4.6 and 1M NaCl. The MES alone eluents contain SJ1 and DY1 mixture and the second batch eluents contain predominantly SJ1. If necessary, the eluents were pooled and dialyzed against 50 mM MES, pH6.5, 25 mM NaCl, and 5% glycerol at 4 °C overnight. The dialysis precipitated virtually all of the DY1. Subsequently, SJ1 was concentrated in an Amicon Centriprep P10 and stored at −70 °C until use. Covalently linked GST-AMPH1(SH3) was prepared by coupling 5 mg GST-AMPH1(SH3) to 2 ml Affi-Gel 15 as suggested by the manufacturer.

Clone construction

GST-ITSN1(SH3) (residues 691 to 1570 of mouse ITSN1), was constructed by PCR using EseL1 clone [23] as the template and oligonucleotides cctgaattcgaagagagagccaagccggaaatg and caagcggccgctcactggctggggtccatgtctgtgg, respectively as the 5′ and 3′ primer. The 1.5 kb amplicon was cloned into vector pGEX4T through EcoR I and Not I sites. All clones were verified by DNA sequencing.

Phosphorylation assays

Solid-phase MNB/DYRK1A phosphorylation was performed as previously described [16]. The stoichiometry of SJ1 phosphorylation was determined by incubating 1.5 μg SJ1 in 30 μl solution containing 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 50 μM [γ-^{32}P]-ATP (1.34 Ci/mmole) and indicated amounts of GSTMNB/DYRK1A for 30 min at 30 °C as described previously [17]. Phosphorylated SJ1 was separated by SDS-PAGE and stained with Coomassie blue. The SJ1 band was then cut off and quantified by a scintillation counter for the stoichiometry calculation. SJ1 used for the pull-down binding and phosphatase assays was phosphorylated exactly as described above, except with cold ATP.

Pull-down binding assay

SJ1 protein binding was determined by the pull-down assay using glutathione-resin immobilized GST-AMPH1(SH3) and ITSN1(SH3-ABCDE) as described previously [16,17]. The anti-SJ1 antibody obtained from BD Biosciences (cat # 612248) was used for detecting SJ1 throughout the pull-down assays.

Phosphatidylinositol 5-phosphatase (PI5Pase) measurement

5-Phosphatase activity was measured by the colorimetric method as described [24] under the conditions that the Sac1 (4-phosphatase) activity was negligible. Briefly, assays were performed in 20 μl mixture consisting 0.1 M Tris-HCl (pH 7.4), 0.25% (w/v) octyl glucoside, 4 mM $MgCl_2$, 0.125 mM PI(4,5)P_2, 0.2 mg/ml BSA, and 0.15 μg phosphorylated (or control) SJ1. The reactions were allowed to proceed for 15 minutes (the reaction was linear for at least 30 minutes) at 37 °C. The entire reaction mixture was added to 80 μl pre-dispensed H_2O in 96 well plates immediately followed by the addition of 25 μl malachite green/molybdate reagent. The absorbance at 610 nm was taken after incubating for 30 minutes at room temperature and was used for calculating the amount of phosphate released from the standard curve generated with a series of known concentrations of free phosphate. Multiple ($n \geq 4$) PI5Pase assays were performed for each phosphorylated SJ1 set and the results represent the average from all data obtained from three independent phosphorylation reactions.

Results

Identification of the 140 kDa MNB/DYRK1A substrate as synaptojanin 1 (SJ1)

Similar to Sub-100 (DY1xa) (Figure 1) [16,17], we have purified Sub-140 by following the 140-kDa protein that could be phosphorylated by MNB/DYRK1A. To determine the identity of Sub-140, purified proteins were cleaved with trypsin and subjected to MALDI-TOF mass spectrometry (MS) analysis (Table 1). The analysis identified Sub-140 as rat SJ1, with an expectation value of 4.6×10^{-7} by the ProFound program. The 140 (or 145) kDa SJ1 is the brain-specific SJ1 isoform [25].

We subsequently verified the identification by MS by another method. The AMPH1 SH3 domain has been used as the affinity matrix for purifying SJ1 and DY1 [26]. Therefore, we examined whether affinity purified SJ1 was a MNB/DYRK1A substrate. Similar to results from the prior report [26], GST-AMPH1(SH3) retained primarily a 100-kDa and a 140-kDa protein from rat brain extract (Figure 1A), which were shown using immunoblotting to be DY1 and SJ1, respectively (Figures 1B and C). These proteins were then subjected to the solid-phase MNB/DYRK1A assay. Both affinity-purified SJ1 and DY1 were MNB/DYRK1A substrates (Figure 1D). This supports the conclusion that Sub-140 is SJ1. Affinity purified SJ1 was used for all subsequent assays.

MNB/DYRK1A phosphorylates native SJ1 at multiple sites

The process of solid-phase assay exposes proteins to denaturing conditions. Thus, we examined whether native SJ1 was phosphorylatable by MNB/DYRK1A. MNB/DYRK1A phosphorylates native SJ1 with the stoichiometry highly depending on the amounts of kinase used in the assay (Figure 2). This observation is similar to the MNB/DYRK1A phosphorylation of DY1 [16,17]. The stoichiometry was found to be greater than 2 if sufficient kinase was used in the reaction (Figure 2). This suggests that SJ1 contains multiple (at least three) potential MNB/DYRK1A phosphorylation sites.

The effects of MNB/DYRK1A phosphorylation on the interaction of SJ1 with endocytic accessory proteins

The interactions of SJ1 with endocytic accessory proteins are known to be regulated by phosphorylation [27]; therefore, effects of MNB/DYRK1A phosphorylation on the interactions of SJ1 to other endocytic accessory proteins were examined. Since the level of SJ1 phosphorylation is highly dependent on the amounts of MNB/DYRK1A used in the reaction (Figure 2), SJ1 was phosphorylated with various amounts of kinase and subjected to the pull-down binding assay using GST-AMPH1(SH3) and GST-ITSN1(SH3). The level of SJ1 phosphorylation was subsequently inferred from the empirically determined values as plotted in Figure 2 and compared to the binding results. Unphosphorylated SJ1 binds to both GST fusion proteins (Figure 3) but not to GST alone (data not shown). MNB/DYRK1A phosphorylation produces a complex pattern of interaction between SJ1 and these proteins (Figure 3). Low levels of phosphorylation, corresponding to roughly 1 mole of phosphate incorporation (with 50 ng kinase), enhanced the SJ1 binding to AMPH1 (Figure 3A). However, the same level of phosphorylation did not appear to affect the binding to ITSN1 (Figure 3B). A slight increase in the phosphorylation level to between 1 and 2 moles of phosphate incorporation (with 100 ng kinase) drastically reduced the SJ1 binding to both AMPH1 and ITSN1. Interestingly, a further increase in the level of phosphorylation to greater than 2 moles of phosphate incorporation (with $\geq$ 200 ng kinase) restored the binding of SJ1 to both AMPH1 and ITSN1. These results demonstrate that the interactions of SJ1 with AMPH1 or ITSN1 are both regulated by MNB/DYRK1A in a phosphorylation level-dependent manner and each interaction appears to have a unique profile. We have performed and obtained similar binding results with either pure SJ1 or SJ1-DY1 mixture. The change in the protein binding (Figure 3) cannot be attributed to a direct competition from GST-MNB/DYRK1A used in phosphorylation because the kinase does not form a stable complex with SJ1 (data not shown). Furthermore, the anti-SJ1 antibody does not differentiate MNB/DYRK1A-phosphorylated (at all levels) and unphosphorylated SJ1 (data not shown), thus it rules out the possibility that the change in the binding is caused by the different sensitivity of the anti-SJ1 antibody to the phosphorylated samples.

The effects of MNB/DYRK1A phosphorylation on the phosphatidylinositol 5-phosphatase (PI5Pase) activity of SJ1

SJ1 was similarly phosphorylated as described above and then analyzed for PI5Pase activity using PI(4,5)P_2 as the substrate. Under the assay conditions, the affinity-purified unphosphorylated SJ1 had an hydrolytic activity of 7.73±0.84 mole PI(4,5)P_2/SJ1/min (Figure 4). MNB/DYRK1A phosphorylation exerted a small but measurable increase in SJ1's PI5Pase activity. The moderate increase in the activity appears to be correlated with the levels of phosphorylation (Figure 4).

Discussion

The 140 (145) kDa SJ1 isoform is highly enriched in nerve terminals [21,25,28]. Similar to DY1, SJ1 is directly associated with an array of accessory proteins known to participate in

endocytic synaptic vesicle cycling through its PRD [19]. The interactions of SJ1 with endocytic accessory proteins are regulated by phosphorylation [27]. Cdk5 has previously been shown to phosphorylate SJ1 at a single S1144 site and cause the reduction in SJ1 binding to AMPH1 and endophilin [29]. The phosphorylation was also found to reduce the PI5Pase activity of SJ1 [29]. In this study, we present evidence to support the conclusion that MNB/DYRK1A functions as another SJ1 kinase.

Unlike Cdk5, MNB/DYRK1A phosphorylates native SJ1 at multiple sites. The level of phosphorylation is highly dependent on the amounts of input kinase (Figure 2). This phenomenon is likely to be due not only to multiple MNB/DYRK1A phosphorylation sites but also the low stability of MNB/DYRK1A *in vitro* [16]. For DY1xa, MNB/DYRK1A preferentially phosphorylates S857 and inhibits DY1xa binding to AMPH1 [16,17]. However, an additional phosphorylation at S795 achieved by excess kinase could reverse the effects of the first site-phosphorylation and induce biphasic AMPH1 binding characteristics [16,17]. Based on the stoichiometry of phosphate incorporation and the triphasic protein binding profile, we hypothesize that MNB/DYRK1A phosphorylates at three SJ1 sites with distinct rates. Phosphorylation at the fastest site can be achieved with a low concentration of kinase and accounts for effects, such as the AMPH1 binding enhancement. Each successive phosphorylation at the following site reverses the effects conferred by the previous phosphorylation and produces the triphasic binding phenomenon. In contrast to the protein-protein interactions, MNB/DYRK1A phosphorylation generates only a small enhancement effect on PI5Pase activity (Figure 4). These results suggest that MNB/DYRK1A phosphorylation controls the recruitment/partition of SJ1, rather than its enzymatic activity.

Although MNB/DYRK1A contains a bipartite nucleus targeting sequence [2], MNB/DYRK1A is not a strict nuclear kinase. This is supported by the findings that a substantial fraction of endogenous MNB/DYRK1A is localized in neuronal processes and synapses in the brain [8-10,30]. In fact, MNB/DYRK1A have been colocalized with DY1, which displays similar subcellular distributions as SJ1 [21], in the growing dendritic trees of developing chicken brains [30]. Through the identification of three MNB/DYRK1A substrates among members of the endocytic accessory proteins, it is clear that MNB/DYRK1A is involved in regulating the assembly of endocytic accessory proteins. Dephosphorylation of SJ1, DY1, and AMPH1 is catalyzed by calcineurin [31]. This property would render SJ1, DY1, and AMPH1 susceptible to the influence of MNB/DYRK1A-DSCR1 gene-dosage imbalance [12,15], particularly, if the imbalance were able to provoke the phase change in protein binding. Intriguingly, both the SJ1 and ITSN1 genes are also located in human chromosome 21 at or near DSCR and the elevated expression of these proteins has been documented in either DS patients [32,33] or in Ts65Dn mice [12]. Whether or not the additional overexpression of SJ1 and ITSN1 could selectively amplify the gene-dosage imbalance effects of MNB/DYRK1A-DSCR1 would be especially worthwhile to explore.

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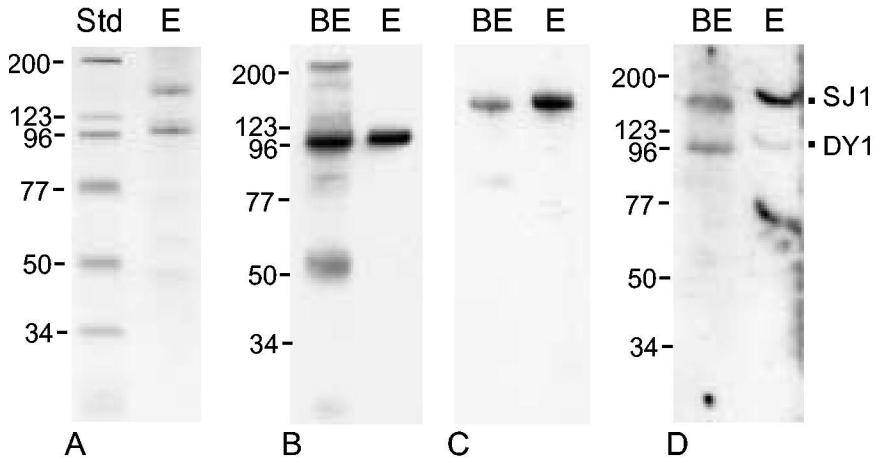

Figure 1.

Phosphorylation of affinity-purified SJ1 and DY1 by MNB/DYRK1A. SJ1 and DY1 were purified from rat brain extract by the AMPH1(SH3) affinity chromatography as described in Materials and Methods. The AMPH1(SH3) domain binds primarily a 100-kDa and a 140-kDa proteins as revealed by Coomassie blue staining (A). The eluted sample was then analyzed for (B) DY1 (using antibody Hudy-1) and (C) SJ1 (using the anti-SJ1 antibody provided by Dr. P. S. McPherson) by immunoblotting. The same samples were also subjected to MNB/DYRK1A solid-phase assay (D). BE, crude brain extract; E, column elution; Std, protein standards.

Figure 2.

Phosphorylation of native SJ1 by MNB/DYRK1A. Affinity-purified SJ1 (1.5 μg) was phosphorylated with the indicated amounts of MNB/DYRK1A under the native conditions as described in Materials and Methods. Stoichiometry of phosphate incorporation was calculated as the number of moles of phosphate per mole of SJ1. Each point represents the average of three independent phosphorylation assays.

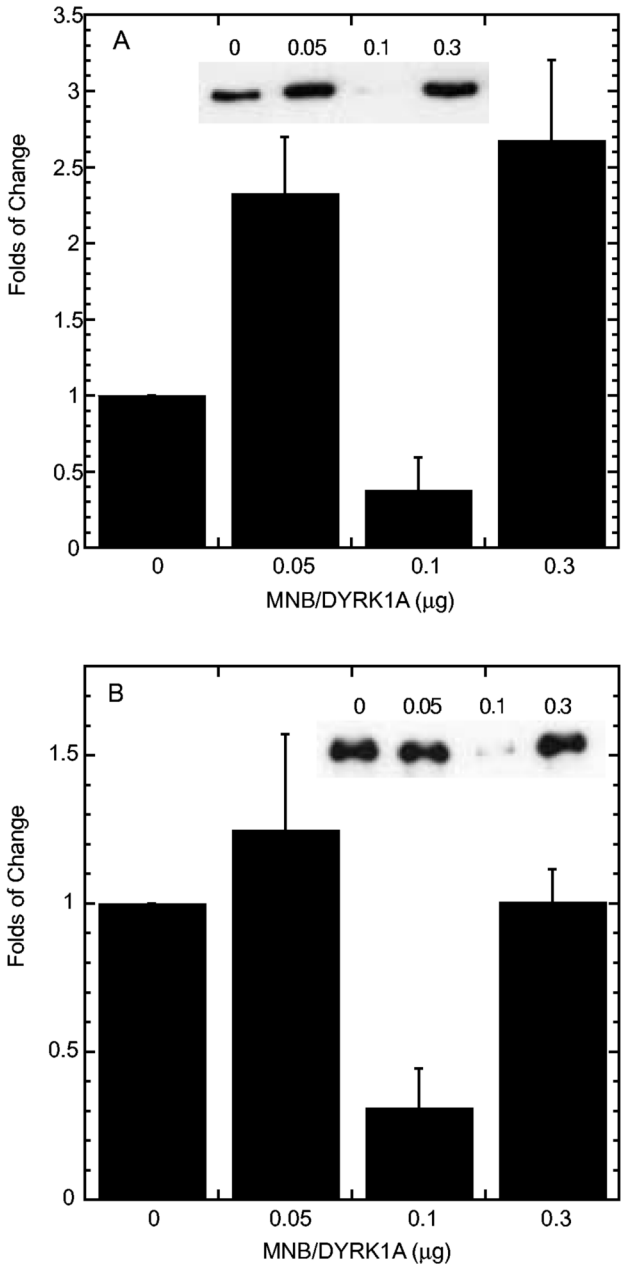

Figure 3.

The effects of MNB/DYRK1A phosphorylation on the binding of SJ1 to AMPH1 and ITSN1. Affinity-purified SJ1 (1.5 μg) was first phosphorylated with the indicated amounts of MNB/DYRK1A under the native conditions and analyzed for binding to (A) AMPH1(SH3) and (B) ITSN1(SH3) domains by the pull-down assay. The data were normalized to the unphosphorylated SJ1 before plotting and were the average of three independent trials. A representative of each binding assay (insert) was shown.

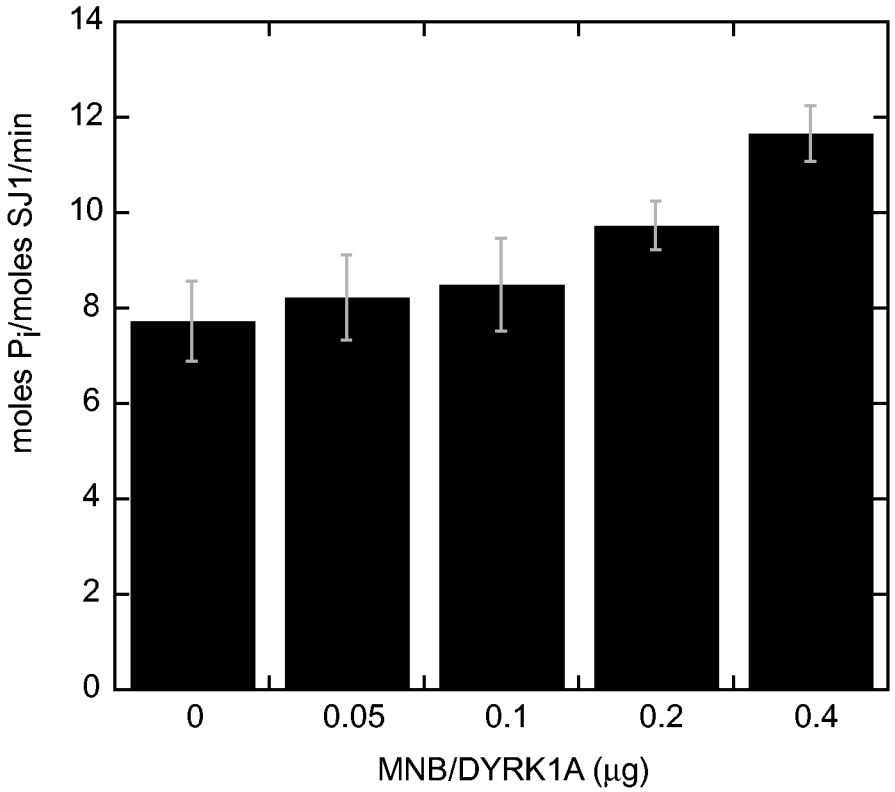

Figure 4.

The effects of MNB/DYRK1A phosphorylation on the PI5Pase activity of SJ1. Affinity-purified SJ1 (1.5 μg) was first phosphorylated with the indicated amounts of MNB/DYRK1A under the native conditions. One-tenth of the phosphorylated SJ1 was then subjected to PI5Pase assay by using PI(4,5)P_2 as the substrate. The PI5Pase activity was calculated as moles of phosphate released per mole of SJ1 per min.

Table 1

MALDI-TOF MS analysis of trypsin-digested Sub-140[†]

Measured Mass	Computed Mass	Error (Da)	Residue		Missed Cut	Sequence
			Start	To		
1151.624	1151.618	0.006	98	107	0	VTSTEFISLR
1193.546	1193.546	0.000	274	284	0	GFEANAPAFDR
1287.727	1287.755	−0.028	56	67	0	VLDAYGLLGVLR
1303.660	1303.688	−0.028	568	578	1	LAGIQEFQDKR
1426.703	1426.720	−0.017	1138	1152	0	EFGGVGAPPSPGVTR
1482.764	1482.807	−0.044	13	25	0	LDPPPFSLIVETR
1513.760	1513.777	−0.017	555	567	0	NQTLTDWLLDAPK
1568.796	1568.825	−0.029	158	169	0	FFWNQSLHLHLK
1620.834	1620.854	−0.020	390	404	0	TNSVQAFLGLEMLAK
1747.992	1747.961	0.031	13	27	1	LDPPPFSLIVETRHK
1764.987	1765.009	−0.022	405	420	0	QLEALGLAEKPQLVTR
1789.867	1789.951	−0.084	837	851	0	ILYTWTPGTLLHYGR
1821.004	1821.024	−0.020	884	901	0	EVIAVQGPPDGTVLVSIK
1930.895	1930.888	0.007	358	373	0	FLDYGFFYFDGSAVQR
1969.895	1969.985	−0.090	748	764	0	QQNWDSLIAGDQLINQK
1993.016	1993.016	−0.000	251	268	0	GSVPLFWEQPGLQVGSHR
2024.044	2024.109	−0.065	9	25	1	IYHKLDPPPFSLIVETR

[†]Measured peptides : 24; Matched peptides : 17; Min. sequence coverage: 17%