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Phosphorylation of KLC1 modifies interaction with JIP1 and abolishes the enhanced fast velocity of APP transport by kinesin-1

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ABSTRACT In neurons, amyloid β-protein precursor (APP) is transported by binding to kinesin-1, mediated by JNK-interacting protein 1b (JIP1b), which generates the enhanced fast velocity (EFV) and efficient high frequency (EHF) of APP anterograde transport. Previously, we showed that EFV requires conventional interaction between the JIP1b C-terminal region and the kinesin light chain 1 (KLC1) tetratricopeptide repeat, whereas EHF requires a novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1. We found that phosphorylatable Thr466 of KLC1 regulates the conventional interaction with JIP1b. Substitution of Glu for Thr466 abolished this interaction and EFV, but did not impair the novel interaction responsible for EHF. Phosphorylation of KLC1 at Thr466 increased in aged brains, and JIP1 binding to kinesin-1 decreased, suggesting that APP transport is impaired by aging. We conclude that phosphorylation of KLC1 at Thr466 regulates the velocity of transport of APP by kinesin-1 by modulating its interaction with JIP1b.

INTRODUCTION Amyloid β-protein precursor (APP), a type I membrane protein that is processed to form amyloid β-protein (Aβ), is deeply implicated in Alzheimer’s disease pathogenesis. The APP gene generates three main isoforms: APP695, APP750, and APP770. Although APP is ubiquitously expressed in many tissues, APP695 is expressed exclusively and at high levels in neurons (reviewed in Suzuki and Nakaya, 2008; Huang and Mucke, 2012). One of the most important functions of APP in neurons is as a cargo receptor for kinesin-1, a conventional kinesin, which was first identified as an anterograde molecular motor in squid giant axon (Vale et al., 1985). Functional kinesin-1 forms a tetramer comprising two kinesin heavy chains (KHCs) and two kinesin light chains (KLCs), and plays an essential role in anterograde transport of many cargoes, including membrane vesicles, organelles, and RNA. This transport function is especially critical in neurons, which form huge numbers of connections with each other via extended neurites (reviewed in Hirokawa et al., 2010; Bentley and Banker, 2016). Kinesin-1 is the most extensively analyzed anterograde motor of the kinesin superfamily of proteins (KIFs); accordingly, many of its biophysical properties, including generation of mechanical force driven by ATP hydrolysis, are well understood (reviewed in Lu and Gelfand, 2017). Furthermore, several recent studies have demonstrated the importance of...
Phosphorylation of KLC1 at Ser460 specifically decreases binding of cargo receptors to kinesin-1 (Sobu et al., 2002; Taru et al., 2002). JIP1 mediates interactions between kinesin-1 and cargo receptors such as APP and apolipoprotein E receptor (APOER) (Verhey et al. 2001). In addition to connecting cargo receptors to kinesin-1, JIP1 also modulates cargo transport. Binding of JIP1 to KHC activates kinesin-1, promoting processivity, and also coordinates anterograde and retrograde transport by modulating association of vesicles with dynein, a retrograde molecular motor (Fu and Holzbaur, 2013).

Furthermore, the interaction between JIP1b and KLC1 is essential for efficient anterograde axonal transport of APP cargo, including enhanced fast velocity (EFV) and efficient high frequency (EHF) (Chiba et al., 2014a). EFV of APP anterograde transport is accomplished via conventional interaction between the JIP1b 11-amino-acid C-terminal region (C11) and the tetrastricopeptide repeat (TPR) region of KLC1. Therefore, in anterograde transport, APP cargoes connected to kinesin-1 via JIP1b move much faster than, for example, Alc1deamin (Alco) cargos bound directly to kinesin-1 in vivo or kinesin-1 on microtubules in vitro (Kawaguchi and Ishiwata, 2000; Araki et al., 2007; Chiba et al., 2014a). EHF requires a novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1, which contributes to the stable and higher-frequency anterograde transport of APP (Chiba et al., 2014a). Although it remains unclear how the interaction of JIP1b with kinesin-1 is selectively regulated to control APP cargo transport, it is obvious that temporally and regionally regulated binding of cargoes and/or adaptor proteins to kinesin-1 is required for efficient transport (Verhey et al., 2001; Fu and Holzbaur, 2013).

Previous studies suggested that phosphorylation of components of cargo–motor complexes is involved in regulation of cargo transport. For example, phosphorylation of KLC2 influences binding of vesicular cargo to kinesin-1 (Morfini et al., 2002). Phosphorylation of KLC1 at Ser460 specifically decreases binding of calystemin-1/Alc1 (Vagnoni et al., 2011), whereas phosphorylation of Alc1 at multiple seryl residues is required for its interaction with the KLC of kinesin-1 (Sobu et al., 2017). Activation of kinesin-1 by association of JIP1 with the KHC may also be modulated by phosphorylation of JIP1 by JNK (Fu and Holzbaur, 2013). However, little is known about the mechanism that regulates the interaction between JIP1 and KLC1, notwithstanding the importance of this interaction for efficient transport of APP cargo by kinesin-1 (Chiba et al., 2014a).

In this study, we found that phosphorylation of Thr466 of KLC1 regulates the interaction between KLC1 and JIP1b. Replacement of this residue with Glu to mimic phosphorylation abolished the conventional interaction between JIP1 and KLC1, resulting in reduction of the EFV of APP cargo transport mediated by JIP1b. Our findings suggest that posttranslational modification of a molecular motor can regulate the velocity of cargo transport by altering interactions with adaptor proteins.

RESULTS
Phosphorylation of KLC1, but not JIP1b, regulates the interaction between KLC1 and JIP1b
To investigate whether phosphorylation of KLC1 or JIP1b regulates the interaction between KLC1 and JIP1b, we first increased the phosphorylation level of both proteins by treating cells with okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A. COS7 cells expressing either HA-KLC1 or FLAG-JIP1b were cultured in the presence or absence of OA, and the respective cell lysates were combined to assess the association of KLC1 with JIP1b (Figure 1). In lysates of cells treated with OA, elevated phosphorylation of JIP1b was reflected by an obvious reduction in mobility in immunoblots (Figure 1A). No such decrease in mobility was apparent for HA-KLC1, but the elevated phosphorylation of this protein was confirmed in a Phos-tag–based mobility shift assay (Figure 1C).

When HA-KLC1 in lysates of cells treated with OA was combined with FLAG-JIP1b in lysates of cells treated with or without OA, the ability of KLC1 to bind JIP1b decreased significantly, as demonstrated by communoprecipitation with anti-FLAG antibody. By contrast, binding of KLC1 to JIP1 was not diminished when FLAG–JIP1b in lysates of cells treated with or without OA was combined with HA-KLC1 in lysates of cells not treated with OA. In contrast, the association with HA-KLC1 with FLAG–JIP1b was strengthened in lysates from cells treated with OA (Figure 1, A and B). These results suggest that phosphorylation of KLC1 suppresses the interaction between KLC1 and JIP1b, whereas phosphorylation of JIP1b promotes it. Because phosphorylation of KLC1 had a major suppressive effect on the interaction with JIP1b, we further analyzed the effect of this modification.

To confirm that phosphorylation of KLC1 suppresses the interaction with JIP1b, and dephosphorylation of KLC1 restores the association, we performed in vitro pull-down assays with GST–JIP1b51–707 fusion protein, which lacks the amino-terminal half of JIP1b but includes all domains and motifs required for the interaction with KLC1 (Chiba et al., 2014a). FLAG-KLC1 protein purified from lysates of cells treated with OA bound less strongly to GST–JIP1b51–707 than FLAG-KLC1 from lysates treated without OA (Figure 1D). However, when lysates of cells treated with OA were treated with λ phosphatase (PPase), the amount of FLAG-KLC1 bound to GST–JIP1b51–707 was comparable to that in samples not treated with OA (Figure 1, D and E). These results clearly indicate that OA increases the phosphorylation level of KLC1 in cells and that phosphorylation of KLC1 suppresses the interaction with JIP1b.

The suppressive effect of OA on the binding of KLC1 to JIP1b was not complete. This is because the interaction between KLC1 and JIP1b requires multiple subdomains, involving a conventional interaction between the JIP1b C11 region and the KLC1 TPR region, as well as a novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1 (Chiba et al., 2014a). The partial inhibition of KLC1–JIP1b binding by KLC1 phosphorylation suggests that either, but not both, of these interactions is regulated by KLC1 phosphorylation. To explore this idea further, we sought to identify the phosphorylation site(s) of KLC1.

Detection of phosphorylated amino acids in KLC1 in cells treated with OA
To determine which amino acids of KLC1 are phosphorylated, we performed multidimensional LC/MS-based analysis. First, we immunoprecipitated FLAG–KLC1 from lysates of cells treated with OA and resolved the isolated protein by SDS–PAGE. The KLC1 band was cut out and subjected to in-gel tryptic digestion. Because we could not exclude the possibility that phosphorylation occurs at
When conventional interaction between KLC1 and JIP1b is impaired, the association between KLC1 and JIP1b persists due to a novel interaction between the coiled-coil domain of KLC1 and the central region of JIP1b, as we reported previously (Chiba et al., 2014a). Those results suggest that phosphorylation at one or more residue(s) negatively regulates the conventional interaction between KLC1 and JIP1b, consistent with our observation that phosphorylation of KLC1 attenuates, but does not completely inhibit, the interaction with JIP1b (Figure 1).

Identification of the phosphorylation sites of KLC1 that attenuate the association with JIP1b

LC-MS/MS analysis revealed several novel phosphorylatable residues in the sixth TPR region. To determine the residue(s) whose phosphorylation regulates the association with JIP1b, we next performed binding studies of proteins harboring amino acid substitutions at each of the candidate positions. Multiple sites, and because identification of the phosphorylation site(s) might be affected by heterogeneity within the pool, we performed a second round of separation: after extraction from the SDS gel, peptides were further fractionated, with or without phosphopeptide enrichment, by the immobilized metal affinity chromatography (IMAC) and/or TiO2 methods. The peptides from each fraction were subjected to analysis and the results were compiled. This analysis identified 17 residues of KLC1 as candidate phosphorylation sites (Figure 2A). Nine of the 17 residues (Ser460, Ser462, Thr464, Thr466, Ser515, Ser521, Ser524, Tyr532, and Ser534) were located in the sixth TPR motif or the C-terminal region and were thus potentially involved in the interaction with JIP1b C11. For example, three (Ser460, Thr462, and Thr464) were phosphorylated in the tryptic fragment 458-V-D-S-P-T-V-T-T-T-L-K-468; representative MS spectra are shown in Figure 2B. This analysis indicated that multiple residues within the sixth TPR motif and the C-terminal region are subject to phosphorylation.
Ala or Glu were examined separately, because a previous study showed that phosphorylation at Ser460 does not modulate the KLC1–JIP1 interaction (Vagnoni et al., 2011). Ala substitutions at all five residues did not alter KLC1 binding to JIP1b, nor did the S460A mutation alone (Figure 3B, left, Ala mutant). In contrast, Glu substitution of residues Thr462, Thr464, and Thr466 significantly weakened the KLC1–JIP1b association, whereas Glu substitution of Ser460 had no significant effect (Figure 3B, left, Glu mutant). These results suggest that phosphorylation of one or more of the three Thr residues regulates the interaction between JIP1b and KLC1. To identify the phosphorylation site responsible for binding, we examined KLC1 mutants in which Thr462, Thr464, and Thr466 were individually replaced by Glu (Figure 3B, right). Of the single-replacement mutants, only Thr466Glu (T466E) significantly decreased the interaction with JIP1b, as did substitution of all three residues (T462E/T464E/T466E) (Figure 3B, right), strongly suggesting that phosphorylation of KLC1 at Thr466 is primarily responsible for regulating the interaction with JIP1b. We noted that HA-KLC harboring T466E tended to be expressed in cells at lower levels than the other versions of the protein (Lysate in Figure 3B, right). FLAG-JIP1b expressed in the absence of the binding partner KLC1 also tended to be unstable (unpublished data). Both proteins in their unbound state may therefore be susceptible to proteolytic degradation in cells, in contrast to the bound form of KLC1 with JIP1b.

A recent study showed that Asn343 in the TPR domain of KLC1 is located within a binding pocket of the 11-amino acid C-terminal region of JIP1b (C11), which contains Tyr705 (Zhu et al., 2012), a residue that is critical for the conventional interaction with KLC1 (Chiba et al., 2014a). The N343S mutation decreases the binding affinity to JIP1b, as determined by thermodynamic parameters (Zhu et al., 2012). Therefore, we compared the importance of the Thr466 residue of KLC1 for the interaction with JIP1b with that for the JIP1b-binding ability of KLC1 with the Asn343Ser mutation (N343S). As shown in Figure 3C, KLC1 N343S exhibited a very weak association with JIP1b, as expected. Interestingly, KLC1 T466E also exhibited a weaker association with JIP1b than KLC1 wild type (WT). In contrast, the T466A mutation did not alter the strength of binding to JIP1b.

These results demonstrate that the association between KLC1 and JIP1b is more complex than suggested by a previous model (Verhey et al., 2001), in which binding is mediated by an interaction between JIP1b C11 and the tetratricopeptide repeat (TPR) of KLC1. This conventional interaction is required for the EFV of anterograde transport of APP cargo by kinesin-1 in axons (Chiba et al., 2014a). If

FIGURE 2: Identification of KLC1 phosphorylation sites by LC-MS analysis. (A) Phosphorylation sites of KLC1. Multidimensional sample preparation, including SDS–PAGE separation, phosphopeptide enrichment, and LC-MS/MS analysis, was performed for phosphorylated peptides from FLAG-KLC1 purified from OA-treated COS7 cells. A total of 17 phosphorylation sites were identified in the purified KLC1 samples. (B) Representative MS/MS spectra of a tryptic peptide, KLC1458–468. Spectra with MS/MS fragmentation of peptide 458–468 are shown: NH2-V-D-S-P-T-V-T-T-L-K-COOH (first row, nonphosphopeptide), NH2-V-D-pS-P-T-V-T-T-L-K-COOH (second row, peptide phosphorylated at Ser460), NH2-V-D-S-pP-T-V-T-T-L-K-COOH (third row, peptide phosphorylated at Thr462), and NH2-V-D-S-P-T-V-pT-T-L-K-COOH (fourth row, peptide phosphorylated at Thr464).

HA-KLC1 variants harboring the indicated alanine or glutamic acid substitutions for residues Ser460, Thr462, Thr464, Thr465, and Thr466 in the sixth TPR region were expressed in Neuro 2a (N2a) cells along with FLAG-JIP1b, and the proteins were coimmunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IP) and cell lysates (Lysate) were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies (Figure 3). Taking into account the possibility of phosphorylation at multiple residues, we first examined KLC1 mutants harboring multiple Ala or Glu substitution at all five residues (Figure 3A). KLC1 mutants in which Ser460 was replaced with N343S exhibited a very weak association with JIP1b, as expected. Interestingly, KLC1 T466E also exhibited a weaker association with JIP1b than KLC1 wild type (WT). In contrast, the T466A mutation did not alter the strength of binding to JIP1b.

These results demonstrate that the association between KLC1 and JIP1b is more complex than suggested by a previous model (Verhey et al., 2001), in which binding is mediated by an interaction between JIP1b C11 and the tetratricopeptide repeat (TPR) of KLC1. This conventional interaction is required for the EFV of anterograde transport of APP cargo by kinesin-1 in axons (Chiba et al., 2014a). If
Phosphorylation of KLC1 at Thr466

To demonstrate that KLC1 is phosphorylated at Thr466 in neuronal tissues in vivo and to reveal the phosphorylation state of KLC1 in association with JIP1b, we developed a phosphorylation state-specific antibody against Thr466 of KLC1 (anti-pKLC1). Affinity-purified antibody was tested for phosphorylation state specificity by an enzyme-linked immunosorbent assay (ELISA). In these experiments, the indicated amounts of antigen peptide KLC1462–470 phosphorylated at Thr466 were reacted with purified antibody along with nonphosphorylated KLC1 kinase-catalyzed reaction, and the PKLC1 harboring T466E or N343S, which both affect the conventional interaction with JIP1b, can maintain a weak association with JIP1b (Figure 3C). Accordingly, we investigated the JIP1b interactions of the KLC1 TPR region, which lacks the N-terminal coiled-coil region (KLC11–202) and the C-terminal tail region (KLC1497–542) (Figure 3A), harboring T466E, T466A, or N343S (Figure 3D). Again, KLC1 TPR harboring T466A retained the association with JIP1b, as did the WT KLC1 TPR region. In contrast, KLC1 TPR harboring T466E or N343S completely lost the ability to interact with JIP1b (Figure 3D). This result clearly shows that Thr466 of KLC1 is critically involved in regulation of the interaction with JIP1b and that a negative charge at position 466 (due to either amino acid substitution or phosphorylation) abolishes the conventional association between the JIP1b C11 and the KLC1 TPR region.

Phosphorylation of KLC1 at Thr466

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Next, we investigated whether the anti-pKLC1 antibody recognized the intact KLC1 protein. Because we anticipated that the level of KLC1 phosphorylated at Thr466 would not be constitutively high, we treated N2a cells expressing FLAG-KLC1 with OA to increase the phosphorylation level. Cell lysates were subject to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates (IP) were analyzed by immunoblotting with anti-HA and anti-FLAG antibodies. Representative blots are shown. (C) Immunoprecipitation of KLC1 and JIP1b. HA-KLC1 (WT) and HA-KLC1 protein harboring the Thr466Glu, Thr466Ala, or Asn343Ser substitution were expressed in N2a cells with (+) or without (-) FLAG-JIP1b, and KLC1 binding to JIP1b was analyzed as described in B. A representative blot is shown along with a blot for actin (as a loading control). (D) Immunoprecipitation of KLC1 TPR and JIP1b. HA-KLC1 TPR (WT) and HA-KLC1 TPR proteins harboring the Thr466Glu, Thr466Ala, or Asn343Ser substitution were expressed in N2a cells with (+) or without (-) FLAG-JIP1b, and KLC1 TPR-binding to JIP1b was analyzed as described above. A representative blot is shown along with a blot for actin. (B–D) Numbers indicate protein size markers (in kilodaltons).
Figure 4: KLC1 is phosphorylated at Thr466 in the brain, and the phosphorylation level within the kinesin-1 complex increases with aging. (A) Specificity of the anti-pKLC1 [pThr466] antibody. Phosphorylation state specificity of anti-pKLC1 [pThr466] antibody (anti-pKLC1 antibody; 0.05 μg IgG) was examined by ELISA using the indicated amounts of KLC154–172 peptides with (circle) or without (triangle) phosphate at Thr466, and an unrelated phosphopeptide, APLP2732–740, phosphorylated at Thr736 (square). Immunoreactive bands were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody, and tetramethylbenzidine was used to colorimetrically detect antigen–antibody complex by measuring OD415. (B) Detection of FLAG-KLC1 phosphorylated at Thr466. N2a cells expressing FLAG-KLC1 were treated with OA (400 nM) for 3 h and lysed for immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were treated with (+) or without (-) λ protein phosphatase (PPase) and protein phosphatase (PPase) and immunoblotting with anti-pKLC1 (top) and anti-FLAG (bottom) antibodies. The immunoprecipitates were treated with (+) or without (-) λ protein phosphatase (PPase) and analyzed by immunoblotting with anti-pKLC1 (top) and anti-FLAG (bottom) antibodies. The immunoprecipitates were treated with (+) or without (-) OA as described in B. FLAG-KLC1 was recovered by immunoprecipitation with anti-FLAG antibody and analyzed by immunoblotting with anti-pKLC1 (top) and anti-FLAG (bottom) antibodies. (D) Reduced phosphorylation of KLC1 in complex with JIP1b. To determine the phosphorylation state of KLC1 at Thr466 within a complex associated with JIP1b, FLAG-KLC1 and HA-JIP1b or FLAG-JIP1b and HA-KLC1 were coexpressed in N2a cells and treated with OA (400 nM) for 3 h, cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitated HA-JIP1b and FLAG-KLC1 or HA-KLC1 and FLAG-JIP1b were analyzed by immunoblotting with anti-pKLC1, anti-KLC1, and anti-JIP1 antibodies (left panel). A linear relationship between band intensities of KLC1 phosphorylated at Thr466 and those of KLC1 was observed in a dose-dependent manner of immunoprecipitates (×1-, ×2- and ×4-fold) when FLAG-KLC1 was expressed in cells (circle), and the level of KLC1 phosphorylated at Thr466 was shown when HA-KLC1 was expressed in cells (diamond) (middle panel). Phosphorylation level, as a proportion of overall amount of HA-KLC1 coimmunoprecipitated with FLAG-JIP1b, was estimated and compared with the level of FLAG-KLC1 recovered by immunoprecipitation with anti-FLAG antibody; the result is expressed as a ratio (pKLC1/ KLC1) (right panel). Statistical analysis used Student’s t test (n = 4; ***, p < 0.01).

(E) Coimmunoprecipitation of KLC1 TPR with JIP1b. HA-KLC1 TPR (see Figure 3A) was expressed in N2a cells with FLAG-JIP1b, lysed, and subjected to immunoprecipitation with anti-HA or anti-FLAG antibody. Precipitated KLC1 TPR, pKLC1 TPR, and JIP1b were detected by immunoblotting with the indicated antibodies. (F) Elevated phosphorylation of KLC1 of kinesin-1 in aged brain. Brain lysates were prepared from young (2-mo-old) and aged (22-mo-old) mice and subjected to immunoprecipitation with anti-KHC antibody and identical amounts of normal IgG (5 μg). The immunoprecipitates (IP) and lysates (20 μg protein) were analyzed by immunoblotting with anti-KHC, anti-KLC1, anti-pKLC1, and anti-JIP1 antibodies. Asterisk (*) indicates nonspecific products. Representative blots are shown. (B–F) Numbers indicate protein size markers (in kilodaltons).
We speculated that, in comparison with free KLC in cytoplasm, KLC in the kinesin-1 heterotetramer associating with JIP1b is likely more dephosphorylated; this idea was supported by the observation that the phosphomimetic substitution of Glu for Thr466 weakened the interaction between KLC1 and JIP1b (Figure 3). To explore this possibility further, we compared the phosphorylation level of KLC1 bound to JIP1b with that of total KLC1, including free KLC1 molecules. For this purpose, N2a cells transiently coexpressing FLAG-KLC1 and HA-JIP1b or FLAG-JIP1b and HA-KLC1 were cultured in the presence of OA. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitated KLC1, pKLC1, and JIP1b were detected by immunoblotting with the appropriate antibodies (Figure 4D, left). Band intensities of KLC1 and pKLC1 were calculated as the ratio relative to the amount of the furthest left lane (given a value of 1.0), and the levels of KLC1 were normalized to the levels of pKLC1 to show the relationship between KLC1 and pKLC1 levels (Figure 4D, middle). The phosphorylation level of FLAG-KLC1 increased linearly with the amount of FLAG-KLC1 even when HA-JIP1b was coexpressed (circles in the middle panel). When HA-KLC1 was coinmunoprecipitated with FLAG-JIP1b using anti-FLAG antibody, the relative ratio of pKLC1 to KLC1 was significantly lower (Figure 4D, right) compared with the relative ratio observed following immunoprecipitation of FLAG-KLC1.

The result suggests that unphosphorylated KLC1 associates preferentially with JIP1b, although KLC1 can interact with JIP1b via the novel interaction between the KLC1 coiled-coil region and the JIP1b C11 and KLC1 TPR regions, irrespective of KLC1 phosphorylation at Thr466 (Figure 3, C and D). Hence, we next performed experiments using FLAG-KLC1 TPR and HA-KLC1 TPR, which lack the coiled-coil region involved in the novel interaction (Figure 4E). The level of KLC1 TPR phosphorylated at Thr466 in association with JIP1b was negligible (see the rightmost lane of Figure 4E) in comparison with the level of total KLC1 TPR, including free KLC1 TPR, isolated with anti-HA antibody. In other words, phosphorylation of KLC1 at Thr466 suppressed the conventional interaction with JIP1b.

Using the anti-pKLC1 antibody, we investigated whether KLC1 is phosphorylated at Thr466 in brain tissues in vivo. To this end, we isolated kinesin-1 from cerebrum lysates of young (2 mo) and aged (22 mo) mice by immunoprecipitation with anti-KHC antibody; an identical amount of nonimmune normal immunoglobulin G (IgG) was used as a negative control. Immunoprecipitates and lysates were analyzed by immunoblotting with anti-KHC, anti-KLC1, anti-pKLC1, and anti-JIP1 antibodies. In samples from young mice, anti-KHC antibody recovered both KLC1 and JIP1, but KLC1 phosphorylated at Thr466 was undetectable. In contrast, in samples from aged mice, the recovery of JIP1 was significantly reduced but KLC1 phosphorylated at Thr466 was present (Figure 4F). Endogenous brain JIP1 showed two protein bands by immunoblotting. Both signals were JIP1 splicing variants, because the two proteins were not detected in brain lysate of JIP1 KO mice (unpublished data). This result indicates that KLC1 is phosphorylated at Thr466 in brain in vivo and that the phosphorylation level of this protein is undetectable or very low in the kinesin-1 complex associated with JIP1 in young mice. Moreover, in aged mice, the phosphorylation level was elevated, and the association of JIP1 with kinesin-1 was attenuated. The result suggests that the increased phosphorylation of KLC1 of kinesin-1 heterotetramer suppresses the conventional interaction of JIP1 with kinesin-1 in aged mice.

APP anterograde transport by kinesin-1 complex including phosphomimetic KLC1

JIP1 is an essential factor for the EFV of APP axonal transport by kinesin-1, and the velocity of APP anterograde transport is reduced in JIP1-deficient neurons (Chiba et al., 2014a). The velocity of APP fast transport in the absence of JIP1 is similar to that of kinesin-1 moving on microtubules in vitro (Kawaguchi and Ishiwata, 2000) or that of the fast transport of Alcα cargo connected directly to KLC1 (Araki et al., 2007; Kawano et al., 2012). Therefore, JIP1b plays an important role in the connection of APP to kinesin-1, which contributes to the EFV and the EHF of APP anterograde transport.

To investigate the roles of Thr466 of KLC1 in APP anterograde transport by kinesin-1, we used differentiating CAD cells (mouse catecholaminergic neuroblastoma). These cells can elongate neurites, permitting examination of anterograde cargo transport by kinesin-1 (Araki et al., 2007). First, we confirmed that the enhanced fast transport of APP cargoes mediated by JIP1b occurred in the differentiating CAD cells, as observed in primary cultured neurons (Chiba et al., 2014a). Cells expressing APP-EGFP, with or without small interfering RNA (siRNA) against JIP1 (siJIP1) vector, were analyzed for anterograde transport of APP cargo in neurites (Figure 5, A and B; see Supplemental Movies 1 and 2); the knockdown efficiency of siJIP1 is shown in Supplemental Figure S2. The expression level of JIP1 decreased by 38% in cells treated with siJIP1 from that in cells without siJIP1. Approximately 60% of the APP cargoes were transported in an anterograde manner, whereas 10% were transported retrogradely and 30% were stationary (Figure 5A). When JIP1 expression was knocked down, the velocity of anterograde transport of APP decreased (1.57 ± 0.56 µm/s, Figure 5B; see also Supplemental Movie 2) relative to the control (2.03 ± 0.77 µm/s, Figure 5A; see also Supplemental Movie 1), and the difference in velocity was statistically significant (p < 0.0001). The knockdown of JIP1 expression also increased retrograde transport of APP to 18%, and the increase was significant (p = 0.04; compare bar graphs in Figure 5, A and B). These results indicate that JIP1 promotes the EFV of APP anterograde transport along with preserving EHF of APP anterograde transport in differentiating CAD cells, as it does in primary cultured neurons. The reduced velocity of APP cargo transport was restored by expression of wild-type JIP1b, an siRNA-resistant form (2.08 ± 0.82 µm/s, Figure 5C; see also Supplemental Movie 3) (p < 0.0001), but not by expression of mutant JIP1bR Y705A (1.60 ± 0.50 µm/s, Figure 5D; see also Supplemental Movie 4), which inhibits the conventional interaction between the JIP1b C11 and KLC1 TPR regions, resulting in reduced velocity (Chiba et al., 2014a). Expression of WT HA-JIP1b or HA-JIP1b Y705A was not knocked down by expression of JIP1b siRNA (Supplemental Figure S2).

Expression of WT JIP1b favors the EFV and decreases the frequency of APP retrograde transport by 12%, although this decrease was not significant (p = 0.45; compare Figure 5C with Figure 5B). Expression of a mutant JIP1b Y705A showed a trend of decreased frequency of APP retrograde transport by 11%, as did expression of wild-type JIP1b (compare Figure 5D with Figure 5C). This small effect (though significant) of the knockdown, yet lack of significant rescue, may be a reflection of the lower efficiency of knockdown in CAD cells as compared with knockout in neurons (see Chiba et al., 2014a). These observations largely support our previous report that the conventional interaction between the JIP1b C11 and KLC1 TPR regions regulates EFV of APP anterograde transport by kinesins-1 (Chiba et al., 2014a). Taken together, these findings confirmed that at least the velocity of anterograde transport of APP is increased by JIP1b in neurites of differentiating CAD cells, and that the interaction between the JIP1b C11 region, including Tyr705, and the KLC1 TPR region is essential for this increase in velocity, as previously shown in primary cultured neurons (Chiba et al., 2014a).
We next sought to determine the function of residue Thr466 of KLC1 in APP cargo transport by kinesin-1 in differentiating CAD cells. For this purpose, we expressed APP-EGFP in differentiating CAD cells expressing siRNA-resistant FLAG-tagged WT KLC1 (FLAG-KLC1<sup>WT</sup>) or mutant KLC1 harboring Thr466Glu or Thr466Ala (FLAG-KLC1<sup>T466E</sup> or T466A) in the presence of siKLC1 and siKLC2 to deplete endogenous KLCs. We confirmed that endogenous KLC1 and KLC2 were knocked down by the siRNA in CAD cells, whereas expression of siRNA-resistant FLAG-KLC1<sup>WT</sup> proteins persisted (Supplemental Figure S3). Upon depletion of KLCs (KLC1 plus KLC2) in cells, almost all APP cargoes stalled (i.e., became stationary) in the neurites with fewer retrograde transporting vesicles (Figure 6A; see also Supplemental Movie 5). This result supports the previous conclusion that APP is transported anterogradely by kinesin-1 (Araki et al., 2007).

Expression of WT FLAG-KLC1<sup>WT</sup> rescued the EFV transport (2.39 ± 0.82 µm/s) in cells depleted of endogenous KLCs (Figure 6B; see Supplemental Movie 6), indicating that moving APP cargoes were largely transported by kinesin-1, including KLC1<sup>WT</sup>. APP cargoes were transported anterogradely at a velocity of 2.07 ± 0.85 µm/s in cells expressing the phosphomimetic mutant FLAG-KLC1<sup>T466E</sup> (Figure 6C; see Supplemental Movie 7), significantly slower than in cells expressing FLAG-KLC1<sup>WT</sup> (Figure 6B, Supplemental Movie 6; p < 0.0001). Average velocity (2.45 ± 0.83 µm/s) of anterograde transport of APP cargo in cells expressing FLAG-KLC1<sup>T466A</sup> was similar to that in cells expressing FLAG-KLC1<sup>WT</sup> (Figure 6B, Supplemental Movie 6; p < 0.0001). Average velocity (2.45 ± 0.83 µm/s) of anterograde transport of APP cargo in cells expressing FLAG-KLC1<sup>WT</sup> was similar to that in cells expressing FLAG-KLC1<sup>WT</sup> and FLAG-KLC1<sup>T466A</sup>, consistent with a previous report that conventional interaction between the JIP1b C11 and KLC1 TPR regions is required for the EFV of APP cargoes by kinesin-1, but not for the EHF of anterograde transport of APP cargoes (Chiba et al., 2014a). Furthermore, it is reasonable that the conventional interaction between the JIP1b C11 and KLC TPR regions would be suppressed by replacement of Thr466 with Glu, creating a negative charge analogous to phosphorylation of KLC1 at Thr466, but not by replacement with Ala.

**Figure 5**: JIP1-dependent enhanced anterograde transport of APP vesicles in differentiating CAD cells. (A, B) APP-EGFP transport in neurites of differentiating CAD cells. APP-EGFP was expressed in CAD cells along with pSuper vector alone (A; see Supplemental Movie 1) or pSuper encoding siJIP1 (B; see Supplemental Movie 2). (C, D) APP-EGFP transport in neurites of differentiating CAD cells expressing siRNA-resistant HA-JIP1<sup>Y705A</sup>. Scale bar, 5 µm. Statistical analyses of cargo velocity distributions were performed using the Kruskal–Wallis test followed by Dunn’s multiple comparison test, and the average velocity is shown along with the SD (±SD). Statistical significance: A vs. B, p < 0.0001; B vs. C, p < 0.0001; A vs. C, not significant. Direction of moving vesicles was analyzed by the χ² test, followed by residual analysis.
To examine the KLC1 mutants in the kinesin-1 complex, we expressed WT HA-KLC1 or HA-KLC1 harboring T466E or T466A in N2a cells along with HA-JIP1b and KIF5C (KHC)-FLAG and then subjected cell lysates to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting with anti-HA and anti-FLAG antibodies (Figure 7A). Similar levels of HA-KLC1 were recovered with KIF5C (KHC)-FLAG, indicating that the immunoprecipitation recovered kinesin-1. However, the recovery of JIP1b was significantly reduced in cells expressing KLC1 T466E in comparison with cells expressing KLC1 WT or KLC1 T466A (Figure 7B). These data indicate that KLC1 T466E forms a kinesin-1 heterotetramer with KHC/KIF5C, but that its association with JIP1b is weakened by the absence of conventional interaction between KLC1 and JIP1b. The decrease in velocity of APP cargo transport by kinesin-1 in cells expressing KLC1 T466E (Figure 6C) is likely due to the impairment of the conventional interaction, which is essential for the EFV of APP cargo transport.

DISCUSSION

JIPs were first reported as cytoplasmic scaffold proteins of the c-Jun amino-terminal kinase (JNK) family (Dickens et al., 1997) and subsequently as adaptor proteins that recruit motor proteins to membrane-bound cargoes (Meyer et al., 1999; Stockinger et al., 2000; Matsuda et al., 2001; Verhey et al., 2001; Taru et al., 2002). In addition, several studies reported that JIP1 activates auto-inhibited kinesin-1 (Blasius et al., 2007; Fu and Holzbaur, 2013) and plays an important role in efficient anterograde transport of APP cargo, including the EFV and the EHF of anterograde transport by kinesin-1 (Chiba et al., 2014a). To accomplish the efficient anterograde transport of APP cargo, the interactions between JIP1 and KLC are essential: the conventional interaction between KLC1 TPR motifs and the JIP1b C11 region is required for the EFV of APP anterograde axonal transport, whereas the novel interaction between the JIP1b central region and KLC1 coiled-coil domain is required for the EHF of anterograde transport of APP (Chiba et al., 2014a) (schematically shown in Figure 8). However, the detailed regulation of the complex interactions between APP and JIP1 remains unclear.

Several studies have reported that protein phosphorylation is involved in regulating the interaction between cargoes and KIFs, including kinesin-1. Phosphorylation of KLC2 facilitates release of membrane cargoes from molecular motors at nerve terminals, but the role of KLC1 phosphorylation in regulating cargo transport is less well understood.

FIGURE 6: Phosphomimetic KLC1 T466E decreases the velocity of APP anterograde transport. (A) APP-EGFP transport in neurites of differentiating CAD cells in which KLC expression was knocked down. APP-EGFP was expressed in differentiating CAD cells in the presence of siKLC1 and siKLC2 (siKLCs; Supplemental Movie 5). (B–D) APP-EGFP transport in neurites of differentiating CAD cells in which endogenous KLC was knocked down in the presence of FLAG-KLC1 WT or FLAG-KLC1 harboring T466E or T466A. siRNA-resistant FLAG-KLC1 (B, FLAG-KLC1 WT; Supplemental Movie 6), FLAG-KLC1 harboring T466E (C, FLAG-KLC1 WT; Supplemental Movie 7), and FLAG-KLC1 harboring T466A (D, FLAG-KLC1 WT; Supplemental Movie 8) were exogenously expressed in cells. (A–D) Transport of APP cargos is depicted in movies (top left; see Supplemental Movies 5–8), and their kymographs are shown to depict all vesicle movement (bottom left). (B–D) The cumulative frequencies of velocities of anterograde transport of APP cargo are shown (middle panel; data are normalized as percentages, and average velocities are shown with the SD [±SD]). Scale bar, 5 μm. Statistical analyses of cargo velocity distributions were performed as described in Figure 5. Statistical significance: B vs. C, p < 0.0001; C vs. D, p < 0.0001; B vs. D, not significant. The proportions of anterograde (A), retrograde (R), and stationary (S) vesicles are indicated (right panel).
The key observations of this study are as follows: 1) KLC1 phosphorylation plays a major role in regulating the association between KLC1 and JIP1b; 2) KLC1 phosphorylated at Thr466 suppresses the conventional interaction with JIP1b C11 region, but does not influence the novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1; 3) the level of KLC1 phosphorylation plays a major role in regulating the association between KLC1 and JIP1b; 2) KLC1 phosphorylated at Thr466 suppresses the conventional interaction with the JIP1b C11 region, but does not influence the novel interaction between the central region of JIP1b and the coiled-coil domain of KLC1; 3) the level of KLC1 phosphorylation plays a major role in regulating the association between KLC1 and JIP1b; 4) the EFV of APP cargo transport (Chiba et al., 2014a). Even if this conventional interaction is impaired, the APP cargo can still be transported by kinesin-1 via the novel interaction of JIP1b (Figure 8), which is essential for the EFV of APP cargo transport (Chiba et al., 2014a). Using a phosphospecific antibody, we demonstrated that endogenous KLC1 is phosphorylated at Thr466 in mouse brain. Notably, another group previously reported the phosphorylation of KLC1 at Thr466 in mouse pancreas (Huttlin et al., 2010), suggesting that this phosphorylation event may regulate the association of other cargoes with kinesin-1 motors in nonneuronal cells.

Ser460 of KLC1 is phosphorylated by extracellular signal-regulated kinase (ERK) in vitro, and this phosphorylation may regulate the binding of KLC1 to Alcα/calsthenin1 cargo (Vagnoni et al., 2011). In our LC-MS/MS analyses, we identified Ser460 as a phosphorylated residue in a trypsin-digested phosphopeptide. Because we could not clearly detect double phosphorylation at Ser460 and Thr466 residues within this phosphopeptide, we postulate that the two sites are phosphorylated in a mutually exclusive manner and may function independently at different stages of the transport process.

In this study, we did not attempt to identify the kinase that phosphorylates KLC1 at Thr466. The amino acid sequence around Thr466 may be enhanced in the brain of aging mice (Brady and Morfini, 2017). Therefore, KLC1 phosphorylation at Thr466 may also be regulated by PP2A. Moreover, it is well understood that aberrant regulation of PP2A has been implicated in altered phosphorylation of tau, which progresses Alzheimer’s disease (AD) pathology (Arif et al., 2005; Araki et al., 2007; Vagnoni et al., 2011; reviewed in Brady and Morfini, 2017). Interestingly, okadaic acid (OA) increased phosphorylation of KLC1 at Thr466. The major target of OA is protein phosphatase 2A (PP2A) which is known to be regulated by various signaling pathways in brain (Ahn et al., 2007; Andrade et al., 2017; Musante et al., 2017). Therefore, KLC1 phosphorylation at Thr466 may also be regulated by PP2A. Moreover, it is well understood that aberrant regulation of PP2A has been implicated in altered phosphorylation of tau, which progresses Alzheimer’s disease (AD) pathology (Arif et al., 2014; Wang et al., 2015). Increased phosphorylation of KLC1 at Thr466 may be enhanced in the brain of aging mice (Brady and Morfini, 2017).
AD patients and cause a further impairment of APP axonal transport in AD brain.

MATERIALS AND METHODS

Cell culture and coimmunoprecipitation

African green monkey kidney COS7 cells and mouse neuroblastoma Neuro2a (N2a) cells were cultured as described (Taru et al., 2002). To express proteins or siRNA, COS7 (−0.8 × 10⁴) or N2a (−1.0 × 10⁴) cells were transiently transfected with the indicated plasmids using Polyethyleneimine MAX (Polysciences) and cultured for 24 h in DMEM containing 10% (vol/vol) fetal bovine serum (FBS). CAD cells, a mouse brain catecholaminergic neuroblastoma cell line, were maintained in DMEM/F12 supplemented with 6% (vol/vol) calf serum (CS) (Qi et al., 1997). Cell lysates were prepared as described (Araki et al., 2007). Lysates and immunoprecipitates were subjected to immunoblot analysis using the indicated antibodies and detected using ECL reagent (GE Healthcare Bio-Sciences, Little Chalfont, UK) or Clarity Western ECL Substrate (Bio-Rad).

Animal studies were conducted in compliance with the guidelines of the Animal Studies Committee of Hokkaido University. All mice were housed in a specific pathogen-free environment in MicroVent units (Allentown).

In vitro pull-down assay

COS7 cells expressing FLAG-KLC1 were treated with or without 400 nM okadaic acid (OA; Wako Pure Chemical Industries, Osaka, Japan) for 3 h and then lysed with HBS-T buffer (20 mM HEPES pH 7.6, 150 mM NaCl, 0.5% [vol/vol] Triton X-100, 25 µg/ml chymostatin, 25 µg/ml leupeptin, and 25 µg/ml pepstatin). After centrifugation at 20,000 × g for 10 min, the supernatants were treated with or without 200 U of λ protein phosphatase (PPase; P9614; Sigma-Aldrich) for 1 h. FLAG-KLC1 was recovered from the lysates by immunoprecipitation with anti-FLAG antibody and Dynabeads Protein G (Thermo Fisher Scientific). To elute FLAG-KLC1 protein, the beads were incubated at 4°C for 30 min in HBS-T containing 0.1 mg/ml FLAG peptide (Sigma-Aldrich). GST and GST-JIP1b 351–707 beads were incubated at 4°C for 30 min in HBS-T containing 0.1% BSA and 0.05% (vol/vol) Tween-20, then were recovered with glutathione–Sepharose 4B. Input (10 ng protein) and proteins bound to the beads were analyzed by immunoblotting.

Antibodies

Anti-FLAG (M2; Sigma-Aldrich), anti-HA (12CA5; Sigma-Aldrich), anti-actin (AC-40; Sigma-Aldrich), and anti-JIP1 (B-7; Santa Cruz Biotechnology) antibodies were obtained from the indicated suppliers. Anti-KLC1 (UT109) and anti-KHC (H2) antibodies were described previously (Brady et al., 1990; Araki et al., 2007). Rabbit polyclonal antibody against KLC1 phosphorylated at Thr466 (pThr466) was raised against an antigenic peptide consisting of a Cys residue followed by mouse KLC1 462–470 containing phosphorylated Thr466 with an amidated C-terminus (C+TVTTpTLKNL-CONH₂). The antibody was purified from serum by serial affinity chromatography with the antigen and then by passage through resin conjugated with nonphosphorylated KLC1 462–470 peptide (C+TVTTpTLKNL-CONH₂), and a resin conjugated with phosphoethreonine (Sigma-Aldrich).

Phos-tag-based mobility shift assay

FLAG-KLC1 expressed in COS7 cells was recovered by immunoprecipitation with anti-FLAG M2 antibody and separated by Phos-tag SDS–PAGE (Wako Pure Chemical Industries, Osaka, Japan) (Kinoshita et al., 2006). Proteins were transferred onto nitrocellulose membrane following the manufacturer’s protocol and probed with anti-FLAG antibody.

Stoichiometry of KLC1 phosphorylation

N2a cells expressing FLAG-KLC1 were treated with or without okadaic acid (400 nM) for 3 h, and FLAG-KLC1 was recovered by immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were probed with anti-KLC1 (UT109) and anti-pKLC1 antibodies, followed by an ECL-Plus detection system (GE Healthcare Bio-Sciences) and quantitated on a LAS-4000 mini (Fujifilm, Tokyo, Japan). Indicated amounts of KLC1 154–172 peptide (C+LKYYYYDDISPEDKDDSS-CONH₂) and phosphorylated KLC1 154–172 peptide (C+TVTTpTLKNL-CONH₂) were spotted on nitrocellulose membrane with a Bio-Dot microfiltration apparatus (Bio-Rad), probed with anti-KLC1 (UT109) and anti-pKLC1 antibodies, and quantitated as described above. For each peptide, arbitrary luminescence units were used to draw a standard curve. Amounts of KLC1 and pKLC1 were calculated based on the standard curves, and the ratio of KLC1 phosphorylation was determined.

ELISA

A 96-well ELISA plate (#9018; Corning) was coated at 4°C overnight with KLC1 162–470 peptide (C+TVTTTLKNL-CONH₂), phosphorylated KLC1 162–470 peptide (C+TVTTpTLKNL-CONH₂), or an unrelated phosphopeptide, APLP2 732–740 (NH₂-DPMLpTPEER+C-CONH₂; residue numbers correspond to the APLP2 763 isoform). The wells were washed with PBS and coated with blocking buffer (PBS containing 1% bovine serum albumin (BSA) and 0.05% [vol/vol] Tween-20) for 1 h at room temperature. After removal of the blocking buffer, anti-KLC1 antibody (0.05 µg IgG) in PBS containing 0.1% BSA and 0.005% (vol/vol) Tween-20 was added, and the plates were incubated at 37°C for 1 h. The wells were washed with PBS, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare Bio-Sciences, Little Chalfont, UK) at 37°C for 1 h. The plate was washed with PBS, and substrate solution (TMB Microwell Peroxidase Substrate System; Kirkegaard & Perry Lab.) was added to the wells. Following incubation at room temperature for 5 min, stop solution (1 M H₃PO₄) was added to the wells, and the absorbance at 415 nm was measured on a microplate reader.

Total internal reflectance fluorescence microscopy analysis

For imaging, CAD cells were plated at 1 × 10⁶ cells/well in an eight-well glass-bottom chamber and transfected with Lipofectamine 2000 (Thermo Fisher Scientific) for 10 h. Transfected cells were differentiated by subsequent culture in serum-depleted DMEM/F12 for 48 h. Vesicular transport in processes was observed using a total internal reflectance fluorescence (TIRF) microscope system (C1; Nikon, Tokyo, Japan) equipped with a CCD camera (Cascade 650; Photometrics). The velocity of anterograde transport was analyzed quantitatively as described (see the Supplemental Information of Araki et al., 2007). Statistical analysis of cargo velocity distributions was performed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test, and average velocities (displayed as histograms) are shown with standard deviations (±SD). The direction of moving vesicles was analyzed by the χ² test followed by residual analysis. Cargo vesicles present in the TIRF area, i.e., within ~100 nm of the plasma membrane, were counted using the “count objects” function of Metamorph 6.1 (Molecular Devices). The proportion of anterograde and retrograde vesicles as a percentage of total vesicles, shown as “frequency,” was calculated as described (Araki et al., 2007).
Kymographs of moving vesicles in axon and traces of individual vesicles undergoing anterograde transport were assembled using the application Kymomaker (Chiba et al., 2014b; open-access tool at www.pharm.hokudai.ac.jp/shinkei/Kymomaker.html).

LC-MS/MS analysis

COX57 cells expressing FLAG-KLC1 were treated with 400 nM OA for 3 h and then lysed. FLAG-KLC1 was immunoprecipitated from lysates with anti-FLAG antibody. The immunoprecipitated proteins were resolved by SDS–PAGE, and the bands corresponding to KLC1 were excised and treated with trypsin (Promega). An aliquot (10%) of the digest was directly analyzed with LC-MS/MS. Most of the remainder (80%) of the digest was applied to PHOS-select ion affinity gel (IMAC; Sigma-Aldrich) to enrich for phosphopeptides, and the eluate was dried and reconstituted in 2% acetonitrile/0.1% formic acid for LC-MS/MS analysis in positive-ion mode on an Orbitrap mass spectrometer (Thermo Fisher Scientific). The flow-through fraction from IMAC was subsequently applied to TiO2 resin to enrich it for residual phosphopeptides, and the bound peptides were eluted for LC-MS/MS analysis. Samples were injected into a Waters Symmetry C18 trapping column (300 µm i.d. × 1 cm length), desalted for 5 min at a flow rate of 15 µL/min, and then separated by in-line gradient elution on a 75 µm i.d. × 15 cm Waters BEH C18 column with 1.7 µm particles at a flow rate of 300 nL/min (Waters). Conditions were as follows: linear gradient from 2% to 10% B over 30 s, followed by 10% to 35% B over 60 min, at a flow rate of 300 nL/min; mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The mass spectra were processed by Proteome Discoverer 1.3, searched against the NCBI nr database using the Mascot searching algorithm (Matrix Science), and finally compiled in Scaffold 3 for data export.

Immunoprecipitation of endogenous kinesin-1

The cerebrum and cerebellum were dissected from 2- or 22-mo-old mice (C57/BL6) and subjected to homogenization in eight volumes of buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 0.5% [vol/vol] Triton X-100, 25 µg/ml chymostatin, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 1 mM Na2VO4, 1 mM NaF, and 1 µM microcystin-LR) followed by centrifugation at 20,000 × g for 15 min. After an additional centrifugation at 200,000 × g for 30 min, 5 mg protein was incubated with anti-KHC antibody (H2, 5 µg) or the same amount of normal IgG for 12 h. The antibodies were recovered with Dynabeads Protein G (Thermo Fisher Scientific) for analysis.

Plasmids

Plasmids constructed in vector pcDNA3 or pcDNA3.1, including expression plasmids for JIP1b and KLC1, were described previously (Taru et al., 2002; Araki et al., 2007). Mutant plasmids were prepared by PCR and subcloned into pcDNA3 or pcDNA3.1 (Invitrogen). pcDNA3.1(+)/KHC-FLAG was described (Kawano et al., 2012), siRNA against mouse KLC1 and KLC2 were described previously (Araki et al., 2007), siRNA against mouse JIP1b was designed to target nucleotides 1914–1934.

REFERENCES


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