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Title	Phosphorylation of multiple sites within an acidic region of Alcadein α is required for kinesin-1 association and Golgi exit of Alcadein α cargo.
Author(s)	Sobu, Yuriko; Furukori, Keiko; Chiba, Kyoko et al.
Citation	Molecular biology of the cell, 28(26), 3844-3856 https://doi.org/10.1091/mbc.E17-05-0301
Issue Date	2017-12-15
Doc URL	https://hdl.handle.net/2115/68318
Type	journal article
File Information	Mol. Biol. Cell-2017-Sobu.pdf



Phosphorylation of multiple sites within an acidic region of Alcadein α is required for kinesin-1 association and Golgi exit of Alcadein α cargo

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ABSTRACT Alcadein α (Alc α) is a major cargo of kinesin-1 that is subjected to anterograde transport in neuronal axons. Two tryptophan- and aspartic acid-containing (WD) motifs located in its cytoplasmic domain directly bind the tetratricopeptide repeat (TPR) motifs of the kinesin light chain (KLC), which activate kinesin-1 and recruit kinesin-1 to Alc α cargo. We found that phosphorylation of three serine residues in the acidic region located between the two WD motifs is required for interaction with KLC. Phosphorylation of these serine residues may alter the disordered structure of the acidic region to induce direct association with KLC. Replacement of these serines with Ala results in a mutant that is unable to bind kinesin-1, which impairs exit of Alc α cargo from the Golgi. Despite this deficiency, the compromised Alc α mutant was still transported, albeit improperly by vesicles following missorting of the Alc α mutant with amyloid β -protein precursor (APP) cargo. This suggests that APP partially compensates for defective Alc α in anterograde transport by providing an alternative cargo receptor for kinesin-1.

Monitoring Editor

Kozo Kaibuchi
Nagoya University

Received: May 17, 2017

Revised: Sep 18, 2017

Accepted: Oct 26, 2017

INTRODUCTION

Axonal transport in neurons is largely mediated by microtubule-associated motor proteins such as kinesin superfamily proteins (KIFs) for anterograde transport and dynein for retrograde transport (reviewed in Hirokawa *et al.*, 2010). Kinesin-1/conventional kinesin, the first anterograde molecular motor to be identified (Vale *et al.*, 1985), is a heterodimer composed of two kinesin heavy chains

(KHC/KIF5) and two kinesin light chains (KLC) (reviewed in Verhey and Hammond, 2009). KHC includes a microtubule-binding motor domain with ATPase activity, and KLC has a tetratricopeptide repeat (TPR) domain that interact with cargo and cargo-mediating adaptor proteins. When kinesin-1 is not associated with cargo, it exists in an auto-inhibited state without microtubule-binding ability (Verhey *et al.*, 1998).

Alcadein/calsyntenin family proteins (Alc α /Clstn1, Alc β /Clstn3, and Alc γ /Clstn2) are neuronal type I membrane proteins that are subject to proteolytic processing, primarily by ADAM10/17, to generate a membrane-associated carboxy-terminal fragment (Alc CTF) and a secreted amino-terminal region (sAlc). The Alc CTF is then subjected to secondary cleavage within its membrane-spanning region by the γ -secretase complex (Hentsch *et al.*, 2002; Araki *et al.*, 2003, 2004; Hata *et al.*, 2009; Piao *et al.*, 2013), as is amyloid β -protein precursor (APP), to generate an intracellular cytoplasmic domain fragment (Alc ICD) and to secrete a nonaggregation-prone p3-Alc peptide that is involved in the pathology of Alzheimer's disease (AD) (Hata *et al.*, 2011, 2012; Konno *et al.*, 2011; Kamogawa *et al.*, 2012; Omori *et al.*, 2014).

The cytoplasmic domain of Alc proteins contains one or two KLC-binding tryptophan- and aspartic acid-containing (WD) motifs, with which the tetratricopeptide repeat (TPR) motifs of KLC interact

This article was published online ahead of print in MBoc in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E17-05-0301>) on November 1, 2017.

The authors declare no conflict of interest.

Author contributions: Y.S., S.H., A.C.N., and T.S. participated in the design of the study, and Y.S. and K.F. carried out all studies; K.Y. and M.K. participated in TIRF analysis, and S.H. participated in MS analysis and antibody preparation; Y.S. and T.S. conceived the study, and Y.S., S.H., A.C.N., and T.S. wrote the paper. All authors read and approved the final manuscript.

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Abbreviations used: AD, Alzheimer's disease; Alc α , Alcadein α ; APP, amyloid precursor protein; GFP, green fluorescent protein; KHC, kinesin heavy chain; KLC, kinesin light chain; TPR, tetratricopeptide repeat; WD motif, tryptophan and aspartic acid-containing sequence.

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Supplemental Material can be found at:
<http://www.molbiolcell.org/content/suppl/2017/10/30/mbc.E17-05-0301v1.DC1>

with high affinity (Araki *et al.*, 2007; Dodding *et al.*, 2011). Interestingly, the short WD motif can activate kinesin-1 in a KLC1-dependent manner (Kawano *et al.*, 2012; Yip *et al.*, 2016). Among the three Alc proteins, Alc α predominantly functions as a cargo receptor for kinesin-1. Vesicles harboring Alc α functionally associated with kinesin-1 transport various cargo substances. Alc β appears to have acquired other functions during evolution (Pettem *et al.*, 2013; Um *et al.*, 2014; Wang *et al.*, 2014), and Alc β can also interact with kinesin-1 (Kawano *et al.*, 2012). Alc γ expression may be lower in adults than expression of Alc α and Alc β (Hata *et al.*, 2011), but Alc γ also binds kinesin-1 and may be involved in memory performance and cognition (Boraxbekk *et al.*, 2015; Lipina *et al.*, 2016).

Alc α is reported to function as a cargo receptor when associated with kinesin-1 (Araki *et al.*, 2007). Either WD motif of Alc α can bind KLC1 TPR domains, and one TPR domain is able to bind two WD motifs (Zhu *et al.*, 2012). This property is thought to generate a strong interaction between Alc α and KLC with the high affinity needed to transport vesicles containing Alc α associated with kinesin-1, the Alc α cargo, in long neuronal axons (Kawano *et al.*, 2012).

Alc α forms a tripartite complex with APP mediated by cytoplasmic interaction with X11L predominantly in Golgi, and Alc α and APP cargoes are subject to respective independent anterograde transport, generally following Golgi exit (Araki *et al.*, 2003, 2004, 2007; Takei *et al.*, 2015). However, the molecular regulation of the Golgi exit of Alc α and APP remains unclear. Furthermore, an acidic region, which resides between WD1 and WD2 in the Alc α cytoplasmic region, was predicted to be intrinsically disordered by the PONDR-FIT prediction tool (Xue *et al.*, 2010a), but the role of this large acidic region in the function and/or metabolism of Alc α has not been revealed.

Together with Alc α , other membrane proteins including APP, apolipoprotein E receptor 2 (ApoER2), and a neural cell adhesion molecule (NCAM) are also known to serve as cargo and/or cargo receptors for kinesin-1 in neurons (Koo *et al.*, 1990; Kamal *et al.*, 2000; Araki *et al.*, 2007; Verhey *et al.*, 2001; Chiba *et al.*, 2014a; Wobst *et al.*, 2015). However, it remains controversial how the interaction between cargo receptors and kinesin-1 is regulated during the various stages of cargo transport, including during vesicular cargo formation at the Golgi, transportation in axons, and release in the nerve terminal. Previous reports suggest that phosphorylation of kinesin-1 and/or adapter proteins mediate the association of the cargo receptor with KLC, thereby regulating cargo binding to the motor, or motor activity directly (Chua *et al.*, 2012; Xu *et al.*, 2012; Fu and Holzbaur, 2013). A report has also suggested that association of Alc α with KLC1 is regulated by phosphorylation of KLC1 (Vagnoni *et al.*, 2011).

In the present study, we found that the cytoplasmic domain of Alc α is phosphorylated at multiple serine residues in the acidic region, and phosphorylation is required for a strong association with KLC. When unphosphorylated, Alc α fails to exit efficiently from the Golgi, and is missorted into other transport vesicles such as APP cargo vesicles, which partially compensates for the failure of defective Alc α to facilitate Golgi exit. The present results reveal the functional importance of Alc α phosphorylation at multiple sites in regulating the transport of Alc α cargo by kinesin-1 and the proper formation of post-Golgi Alc α cargo.

RESULTS

Phosphorylation of the Alc α cytoplasmic region is required for association with KLC

The cytoplasmic domain of Alc α binds directly to KLCs and includes putative phosphorylatable amino acid residues that may regulate

the interaction with KLCs. We first investigated whether the cytoplasmic domain of Alc α was subject to phosphorylation *in vivo* using membrane and cytoplasmic fractions of mouse brain homogenates. Alc α CTF (Alc α ^{816–971}), a carboxyl-terminal fragment generated by primary cleavage of Alc α , was detected in the membrane fraction, and Alc α ICD (Alc α ^{868–971} as major and Alc α ^{871–971} as minor products), a fragment endogenously generated by cleavage of Alc α CTF by γ -secretase, was recovered from the cytoplasmic fraction following immunoprecipitation and detected by immunoblotting using an anti-Alc α antibody (Figure 1A; Araki *et al.*, 2004; Hata *et al.*, 2009; Piao *et al.*, 2013). Interestingly, both endogenous fragments generated from Alc α *in vivo* migrated faster by gel electrophoresis following dephosphorylation with lambda protein phosphatase (λ PPase). These results suggest that Alc α is a neuronal phosphoprotein that is phosphorylated within the cytoplasmic region *in vivo*.

We next confirmed phosphorylation of the cytoplasmic domain of Alc α in cells by prelabeling with [³²P] orthophosphate. HEK293 cells expressing the Alc α intracellular domain fragment (Alc α cyt, amino acid residues 871–971) were radiolabeled with [³²P]orthophosphate, and Alc α cyt was isolated from cell lysates by immunoprecipitation with anti-Alc α antibody to analyze phosphorylation by autoradiography, while protein expression was assessed by immunoblotting with the same antibody (Figure 1B). Alc α cyt was labeled with ³²P and the radioactive signal disappeared following treatment of immunoprecipitates with λ PPase, indicating phosphorylation of the cytoplasmic region of Alc α .

Interestingly, membrane-associated Alc α CTF isolated from the brain is highly phosphorylated, while cytoplasmic Alc α ICD released from membrane is only partially phosphorylated (Figure 1A). This suggests that phosphorylation of Alc α and its metabolic fragments may be differentially regulated *in vivo*.

We next tested whether phosphorylation was involved in the interaction with KLC1, using *in vitro* binding assays. Alc α -FLAG and HA-KLC1 were separately expressed in N2a cells, and lysates containing Alc α -FLAG were subjected to immunoprecipitation with anti-FLAG antibody using protein G Sepharose beads. Immunoprecipitated beads were treated with or without λ PPase, washed thoroughly, and combined with lysate containing HA-KLC1. After incubation, beads were collected, and washed by centrifugation, and bound proteins (IP) and lysates were analyzed by immunoblotting with anti-HA and anti-FLAG antibodies (Figure 1C). As expected, and as reported previously (Araki *et al.*, 2007; Kawano *et al.*, 2012), HA-KLC1 was recovered through binding to bead-bound Alc α -FLAG. When beads harboring Alc α -FLAG were subjected to dephosphorylation with λ PPase prior to incubation with cell lysate containing HA-KLC1, the recovery of HA-KLC1 was reduced to ~20% of that for the lysate without phosphatase treatment (Figure 1C). Decreased binding of dephosphorylated Alc α to KLC2, which is another KLC isoform expressed in neurons (Rahman *et al.*, 1998), was also observed (Figure 1D). These results indicate that phosphorylation of the Alc α cytoplasmic region is required for association with KLC.

Furthermore, this reduction was also observed for Alc γ (Figure 1E), which contains double WD motifs, as does Alc α , at least in the human protein (Araki *et al.*, 2003). In contrast, Alc β contains only a single WD motif, and this protein showed weaker binding to KLC1, indicating a lower affinity, although the WD motif peptide of Alc β can interact with the KLC1 TPR *in vitro* (see longer exposure panel of HA-KLC1 in Figure 1E; Kawano *et al.*, 2012). Regulation of the interaction with kinesin-1 by phosphorylation within the Alc cytoplasmic region is likely to be a feature of Alc α and perhaps Alc γ . Because the role of Alc α as a kinesin-1 cargo is better studied than

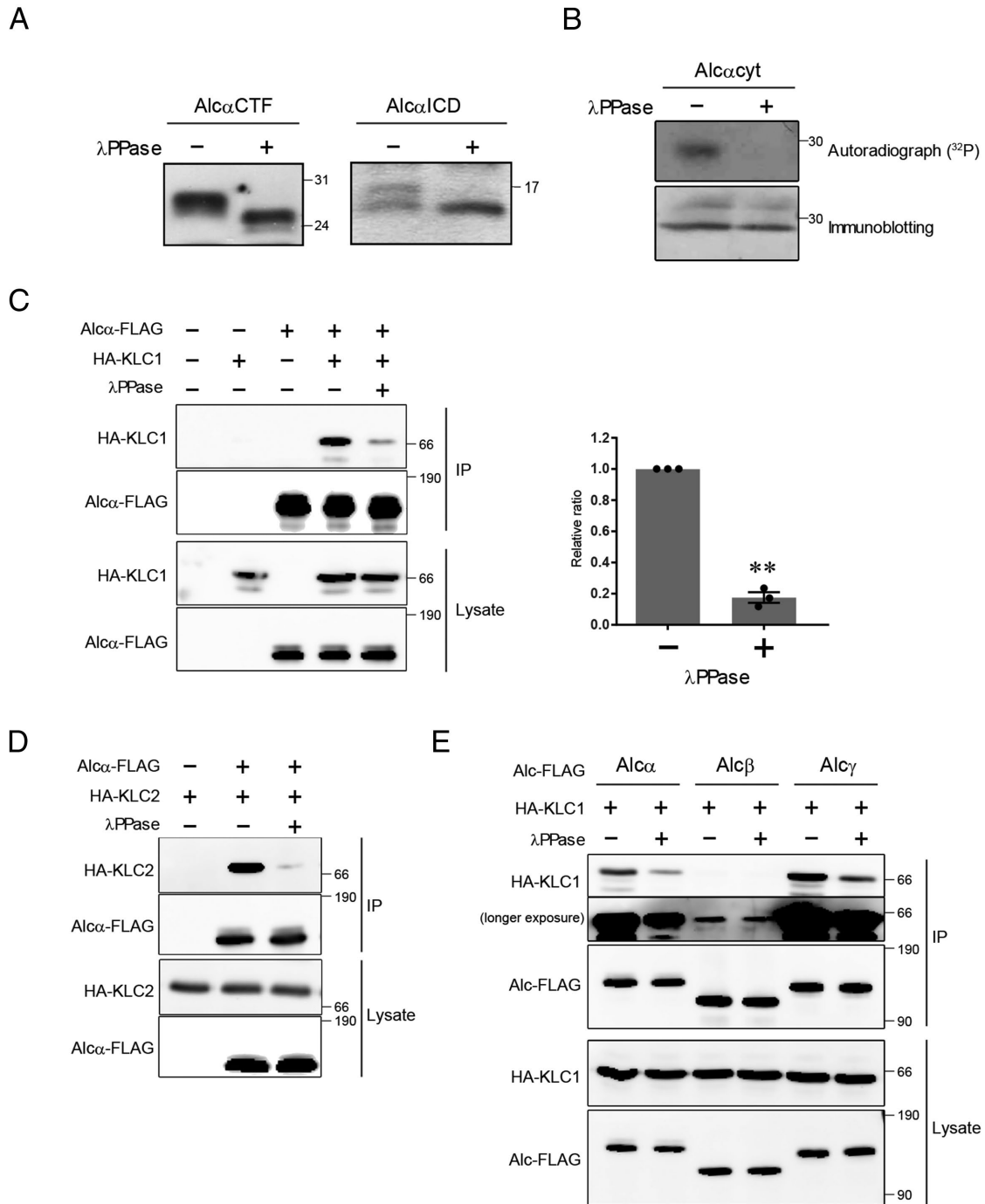


FIGURE 1: Phosphorylation of Alcadin α (Alc α) cytoplasmic region and interaction with kinesin light chain (KLC). (A) Endogenous Alc α CTF (left) and Alc α ICD (right) in mouse brain were treated with (+) or without (-) λ protein phosphatase (λ PPase) and analyzed by immunoblotting with UT195 antibody. (B) HEK293 cells expressing Alc α cyt were cultured in the presence (prelabeling) or absence (immunoblotting) of [³²P]orthophosphate. Alc α cyt recovered by immunoprecipitation was separated by SDS-PAGE and detected by autoradiography (upper) or immunoblotting with UT83 antibody (lower). (C) In vitro binding of HA-KLC1 and Alc α -FLAG (IP) was analyzed by immunoblotting along with lysates (left). The intensities of the bands corresponding to HA-KLC1 bound to Alc α -FLAG with (+) and without (-) λ PPase treatment were compared. Levels are indicated relative to the ratio without treatment (assigned a value of 1.0; right). Statistical significance was determined by Student's *t* test (means $\pm$ SE, *n* = 3, ***p* < 0.01). (D) In vitro binding of HA-KLC2 and Alc α -FLAG (IP) was analyzed by immunoblotting as described in C. Experiments were performed in duplicate. (E) Alc α -FLAG, Alc β -FLAG, and Alc γ -FLAG were separately expressed in N2a cells. The association of HA-KLC1 and Alc-FLAG proteins (IP) was analyzed by immunoblotting as described in C. The second-row panel shows the results following a longer exposure. Protein marker sizes are shown (kilodaltons).

that of Alcy (Araki *et al.*, 2007; Kawano *et al.*, 2012), and the expression of Alcy is lower than that of Al α in mice (Hintsch *et al.*, 2002) and maybe in humans (Hata *et al.*, 2009), we performed further analysis to identify phosphorylation sites in the cytoplasmic domain of Al α .

Phosphorylation within the acidic region of Al α regulates the association with KLC1

The Al α cytoplasmic domain, Al α ^{871–971}, is composed of 101 amino acids and includes many potentially phosphorylatable Ser, Thr, and Tyr residues. Five serine and threonine residues are located near the membrane region that includes the WD1 motif (residues 891–900); Thr907 and Tyr908 are located near the C-terminus of the NP sequence; eight serine and threonine residues are located within the acidic region (residues 909–957); and Ser967, Thr968, and Ser970 and Tyr971 are located in the WD2 motif (residues 962–971) (Araki *et al.*, 2003; Figure 2A).

To narrow the search for potential phosphorylation sites regulating the interaction with KLC1, we first constructed five carboxy-terminal FLAG-tagged Al α mutants: Δ N, lacking the juxtamembrane region (residues 874–902) that includes WD1; TYAA, with Ala

substituted for Thr907 and Tyr908; Δ AC, lacking the acidic region; SYAA, with Ala substitutions for Ser970 and Tyr971; and STAA, with Ala substitutions for Ser967 and Thr968 (Figure 2A). Using these mutated Al α -FLAG proteins and the wild-type (WT) protein, the impact of λ PPase treatment on the association with HA-KLC1 was examined. The Δ N, TYAA, SYAA, and STAA Al α -FLAG mutants all showed decreased binding with HA-KLC1 following treatment with λ PPase, as did the WT protein, while Δ AC Al α -FLAG showed an equivalent ability to associate with HA-KLC1 regardless of protein phosphatase treatment (Figure 2B), indicating that phosphorylation site(s) regulating the association with KLC1 are located within the acidic region.

The acidic region is located between the two KLC-binding motifs (WD1 and WD2) and contains eight serine and threonine residues. We first confirmed phosphorylation in the acidic region using MALDI-TOF/MS. To detect phosphorylated peptides, the Al α acidic region peptide NH₂-Met plus Al α ^{909–950}-FLAG peptide was expressed in N2a cells, purified from lysates using anti-FLAG antibody, and analyzed by MALDI-TOF/MS (the amino acid sequence of Al α ^{909–950} is shown in the upper part of Figure 2C). Following a

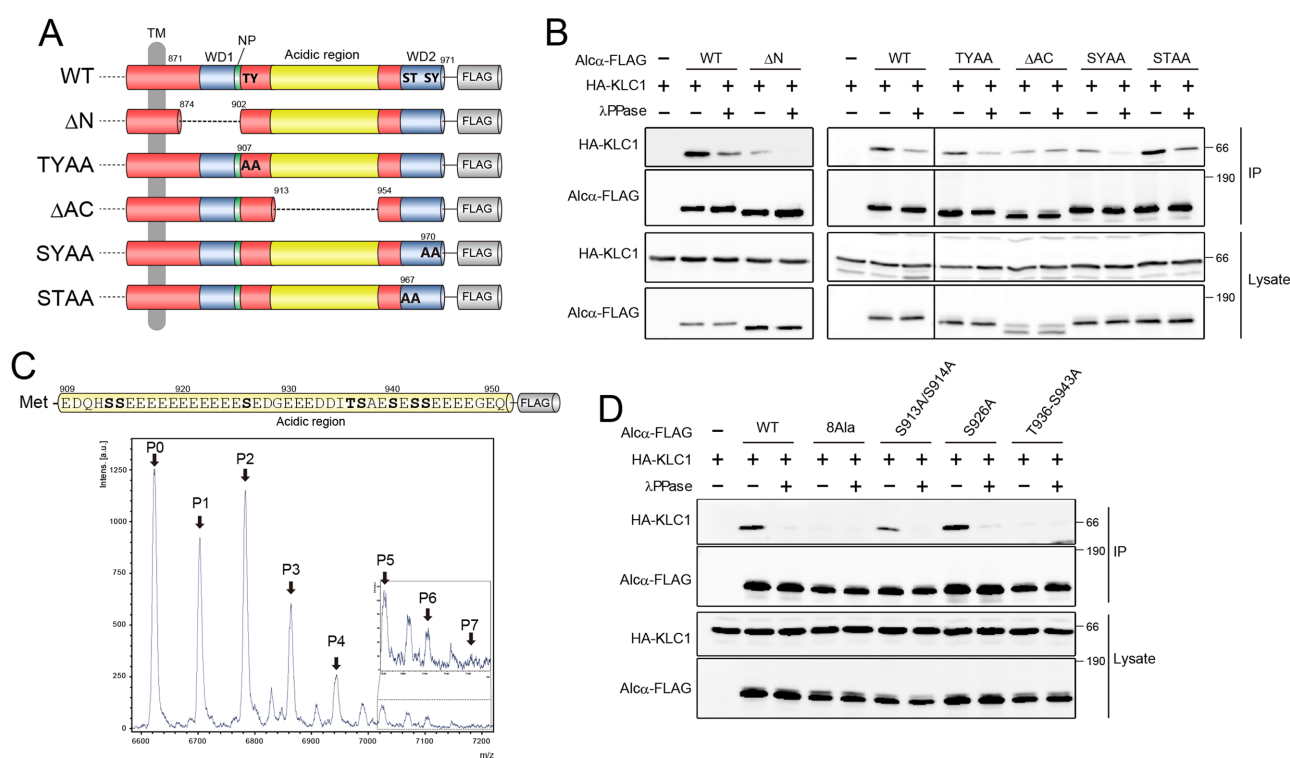


FIGURE 2: Phosphorylation of Al α within the cytoplasmic acidic region. (A) Schematic structure of Al α mutants used in this study. WT, wild-type Al α ; Δ N, Al α lacking the juxtamembrane region containing WD1; TYAA, Al α with Ala substitutions at Thr907 and Tyr908; Δ AC, Al α lacking the acidic region; SYAA, Al α with Ala substitutions at Ser970 and Tyr971; STAA, Al α with Ala substitutions at Ser967 and Ser968. TM, transmembrane region. (B) Phosphatase sensitivity of indicated Al α -FLAG proteins on the association with HA-KLC1 was examined by immunoprecipitation with lysates of N2a cells separately expressed Al α -FLAG proteins and HA-KLC1 as described in Figure 1. (C) The carboxyl-terminal FLAG-tagged Al α acidic region (Al α ^{909–950}) with an amino-terminal methionine was expressed in N2a cells and analyzed by MALDI-TOF/MS to assess phosphorylation. A representative MS spectrum is shown, and the *m/z* of phosphorylated Met+Al α ^{909–950}-FLAG is indicated by arrows (P1–P7) alongside the peak of nonphosphorylated peptide (P0). Areas of the spectrum that include peptides with five to seven phosphorylated amino acids are enlarged in the inset. The amino acid sequence is shown with phosphorylatable serine and threonine residues (bold letters). (D) The impact of λ PPase treatment on the association with HA-KLC1 was assayed with Al α -FLAG mutants containing alanine substitutions at the indicated serine and threonine residues within the acidic region as described in Figure 1. WT, wild-type Al α ; 8Ala, Al α with Ala substitutions at eight serine and threonine residues; S913A/S914A, Al α with Ala substitutions at Ser913 and Ser914; S926A, Al α with Ala substitution at Ser926; T936-S943A, Al α with Ala substitutions at Thr936, Ser937, Ser940, Ser942, and Ser943. Protein size markers are shown (kilodaltons).

single nonphosphorylated peptide, peptides containing from one to seven phosphorylated residues (+80 Da/phosphorylation) were detected, as shown in representative MS spectra (Figure 2C). This indicated that at least seven of the eight residues in the acidic region were phosphorylated in vivo.

To determine which serine and/or threonine residues regulate KLC1 association in a phosphorylation-dependent manner, we next constructed Al α -FLAG mutants harboring four types of amino acid substitution and assayed the impact of λ PPase treatment on the association with HA-KLC1. We prepared an Al α -FLAG "8Ala" mutant in which all eight Ser/Thr residues were replaced with Ala, the S913A/S914A mutant, the S926A mutant, and the T936-S943A mutant with five of the Al α ⁹³⁶⁻⁹⁴³ Ser/Thr residues replaced with Ala (Figure 2D). The binding of the 8Ala and T936-S943A mutants to KLC1 was lost, and as a result no effect of λ PPase treatment was found. Meanwhile, the S913A/S914A and S926A mutants behaved comparably to WT Al α -FLAG. These results indicate that phosphorylation at serine and/or threonine residues occurs within the carboxyl-terminal part of the acidic region, at Thr936, Ser937, Ser940, Ser942, or Ser943, to regulate the association with KLC1.

Multiple-site phosphorylation within the Al α acidic region is required for strong association with KLC1

To determine which of the five residues regulates the association with KLC1, Al α -FLAG mutants harboring single Ala substitutions (Thr936, Ser937, Ser940, Ser942, and Ser943) were generated. While replacing all five residues with Ala abrogated the impact of dephosphorylation, Al α harboring single Ala substitutions at any of the five positions behaved comparably to WT Al α (Figure 3A, left).

We next analyzed the interaction of KLC1 with Al α harboring the double Ala substitutions T936/S937 and S942/S943 and the triple Ala substitution S940/S942/S943. The T936A/S937A mutant showed decreased association with KLC1 following phosphatase treatment, as did WT Al α . The S942A/S943A double mutant also showed diminished binding to KLC1 following phosphatase treatment, but to a lesser extent than observed for the WT protein. The S940A/S942A/S943A triple mutant attenuated the association with KLC1 regardless of λ PPase treatment (Figure 3A, right).

Next, we confirmed that the triple Ala substitution of Ser940, Ser942, and Ser943 attenuated the association with KLC1 in cells. Al α -FLAG mutants harboring all eight Ala substitutions (8Ala), five Ala substitutions within the T936-S943 region (5Ala), three Ala substitutions (S940A/S942A/S943A), and two Ala substitutions (S942A/S943A) were coexpressed with HA-KLC1 and recovered by immunoprecipitation with anti-FLAG antibody as described above (Figure 3B, left). Al α 8Ala, Al α 5Ala, and Al α S940A/S942A/S943A all showed significantly decreased association with HA-KLC1 compared with WT Al α . The S942A/S943A double mutant bound to KLC1 less strongly than WT Al α , but the decrease in binding was not significant (Figure 3B, right).

We next investigated whether the phosphorylation of Al α at S940/S942/S943 regulates the association with KLC1 within the kinesin-1 motor. Lysates from cells expressing Al α WT or Al α 3A together with HA-KLC1 and myc-KHC were immunoprecipitated with anti-KHC antibody. The amount of Al α 3A recovered by immunoprecipitation was decreased, even though the same amount of KLC1 was recovered (Figure 3C). We also investigated the interaction of KLC1 with a potential phosphomimetic mutant of Al α , Al α S940E/S942E/S943E (Al α 3E) (Supplemental Figure S1). Al α 3E associated with KLC1 at a level that was significantly less

than that for Al α WT, but that was not significantly different from that for the Al α 3A mutant. This suggests that the triple Glu substitution for Ser940, Ser942, and Ser943 does not mimic the phosphorylation state of Al α . This may result from the fact that side chains of phosphorylated amino acids are different from acidic amino acids in terms of their chemical structure (Bah and Forman-Kay, 2016). Taken together, these results suggest that phosphorylation at three serine residues (S940, S942, and S943), located close together within the acidic region, is required for strong association with kinesin-1.

To further confirm the phosphorylation of Al α at Ser940, Ser942, and Ser943, we attempted to prepare a phosphorylation state-specific antibody that recognizes endogenous Al α phosphorylated at these serine residues. Antibody was raised against a peptide antigen that included phosphorylated Ser940, Ser942, and Ser943. The specificity of this phosphorylation state-specific antibody (anti-pAl α) was examined using the corresponding Al α Ala substitution mutants. Al α -FLAG mutants harboring triple alanine substitutions (S940A/S942A/S943A, 3A) and single alanine substitutions (S940A, S942A, or S943A) were expressed in N2a cells along with WT Al α -FLAG (WT), and immunoprecipitates recovered with anti-FLAG antibody were analyzed by immunoblotting using anti-pAl α and anti-FLAG antibodies (Figure 3D). Almost the same amount of WT and the mutant proteins were detected by anti-FLAG antibody. However, only WT Al α -FLAG (pAl α), but not the 3A mutant, was detected using the anti-pAl α antibody. Moreover, the anti-pAl α antibody weakly recognized Al α S940A (containing phosphorylatable S942 and S943 residues), Al α S942A (containing phosphorylatable S940 and S943 residues), and Al α S943A (containing phosphorylatable S940 and S942 residues). The pAl α antibody therefore appears to strongly recognize Al α phosphorylated at all three sites (Ser940, Ser942, and Ser943), but only weakly when phosphorylated at any two of the three sites.

Next, we examined phosphorylation of endogenous Al α at Ser940, Ser942, and Ser943 in brain tissue. Al α was recovered from mouse brain lysates by immunoprecipitation with anti-Al α UT195 antibody, and immunoprecipitates were treated with (+) or without (-) λ PPase and analyzed by immunoblotting using UT195 and pAl α antibodies (Figure 3E). Anti-pAl α antibody successfully detected pAl α , and this signal disappeared following treatment with λ PPase, indicating that Al α is phosphorylated in vivo at Ser940, Ser942, and Ser943 and/or at any two of three serine residues in brain tissue.

The Ser940, Ser942, and Ser943 residues are conserved in Al α from humans, mice/rats, and zebrafish, along with the conserved WD1 and WD2 motifs (for comparison, see Supplemental Figure S2), suggesting that phosphorylation at these serine residues is conserved in Al α among species. We explored whether these serine residues of Al α are conserved within the acidic region of Al γ . There are several serine residues in the acidic region of Al γ . Ser922 of human Al γ , Ser917 of mouse/rat Al γ , and Ser911 of zebrafish Al α likely correspond to Ser940 of human Al α , Ser950 of mouse/rat Al α , and Ser950 of zebrafish Al α , respectively (Supplemental Figure S2A). However, it is difficult to identify serine residues that might functionally correspond to Ser940, Ser942, and Ser943 of Al α within the Al γ acidic region (51% amino acid identity). Moreover, distinct from human Al γ , the entire WD2 motif was not present in mouse/rat, suggesting that Al γ may not play a major role as a kinesin-1 cargo receptor in mouse/rat. The WD2 motif of zebrafish Al γ may bind KLC1 because the minimum sequence (-L/M-X-W-D/E-) for KLC-binding is retained (Kawano *et al.*, 2012),

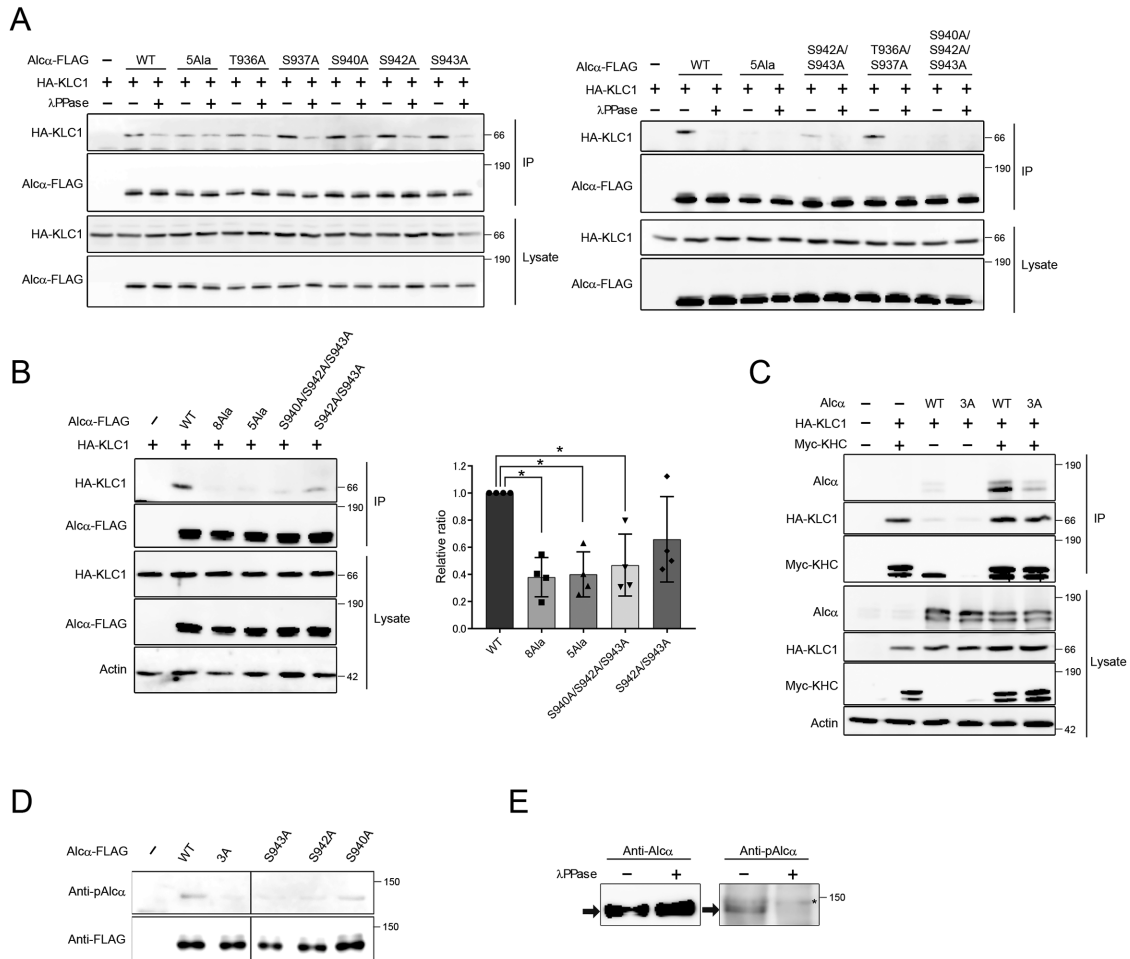


FIGURE 3: Identification of multiple phosphorylation sites. (A) The impact of λ PPase treatment on the association with HA-KLC1 was assayed with Alca-FLAG containing a single alanine substitution at Thr936, Ser937, Ser940, Ser942, or Ser943 (left), double Ser942Ala/Ser943Ala and Thr936Ala/Ser937Ala mutations, or triple Ser940Ala/Ser942Ala/Ser943Ala substitutions (right). WT Alca-FLAG and Alca-FLAG with five alanine substitutions (5Ala) were also tested. In vitro binding assays were performed as described in Figure 1, and representative results are shown. (B) Coimmunoprecipitation of Alca-FLAG mutants with HA-KLC1 (left). The band intensity of bound HA-KLC1 was standardized with the band intensity of Alca-FLAGs and levels calculated as the ratio relative to the amount bound to WT Alca (given a value of 1; right). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post hoc test (means $\pm$ SE, $n = 4$, $*p < 0.05$). (C) WT Alca-FLAG or Alca3A (S940A/S942A/S943A)-FLAG was expressed in N2a cells with HA-KLC1 and myc-KHC. The immunoprecipitates with anti-KHC antibody (IP) and lysates were analyzed. Representative results are shown. (D) WT and mutant Alca-FLAG with the indicated alanine substitutions separately expressed in N2a cells were immunoprecipitated with anti-FLAG antibody and analyzed with anti-pAlca and anti-FLAG antibodies. Representative results are shown. (E) Alca immunoprecipitated from mouse brain lysates were analyzed by immunoblotting with anti-Alca UT195 and anti-pAlca antibodies. The arrow indicates Alca, and the asterisk indicates a nonspecific product. Protein size markers are shown (kilodaltons).

but proline residues at positions 932 and 936 in zebrafish Alcy may alter the motif conformation to affect KLC-binding (Supplemental Figure S2A). *Caenorhabditis elegans* and *Drosophila melanogaster* have only one Alcadin gene, and the predicted proteins possess a simple WD2 motif composed of the -L-E-W-D- sequence, which is essential and a minimum sequence for KLC1-binding (Kawano *et al.*, 2012; Sanger *et al.*, 2017), and also have an obvious acidic region including serine residues that are distinct in position from the serine residues of vertebrate Alca (Supplemental Figure S2B). Taken together, functional regulation in cargo-motor interaction by phosphorylation at serine residues within the acidic region of Alca may have been acquired in vertebrates during evolution.

Phosphorylation of Alca is required for proper transport of Alca-containing vesicles

Alca moves in axons by anterograde transport via binding and activating kinesin-1 (Kawano *et al.*, 2012). Because three phosphorylation sites are required for strong association with kinesin-1 (Figure 3C), we investigated whether phosphorylation at Ser940, Ser942, and Ser943 regulates the axonal transport of Alca cargo. First, WT Alca-EGFP (AlcaWT-EGFP) and Alca-EGFP harboring S940A, S942A, and S943A mutations (Alca3A-EGFP) were expressed in primary cultured mouse cortical neurons, and localization of Alca was observed, along with immunostaining of MAP2B to distinguish axons from dendrites (Figure 4A). Despite decreased association with kinesin-1 (Figure 3C), signals of Alca3A-containing

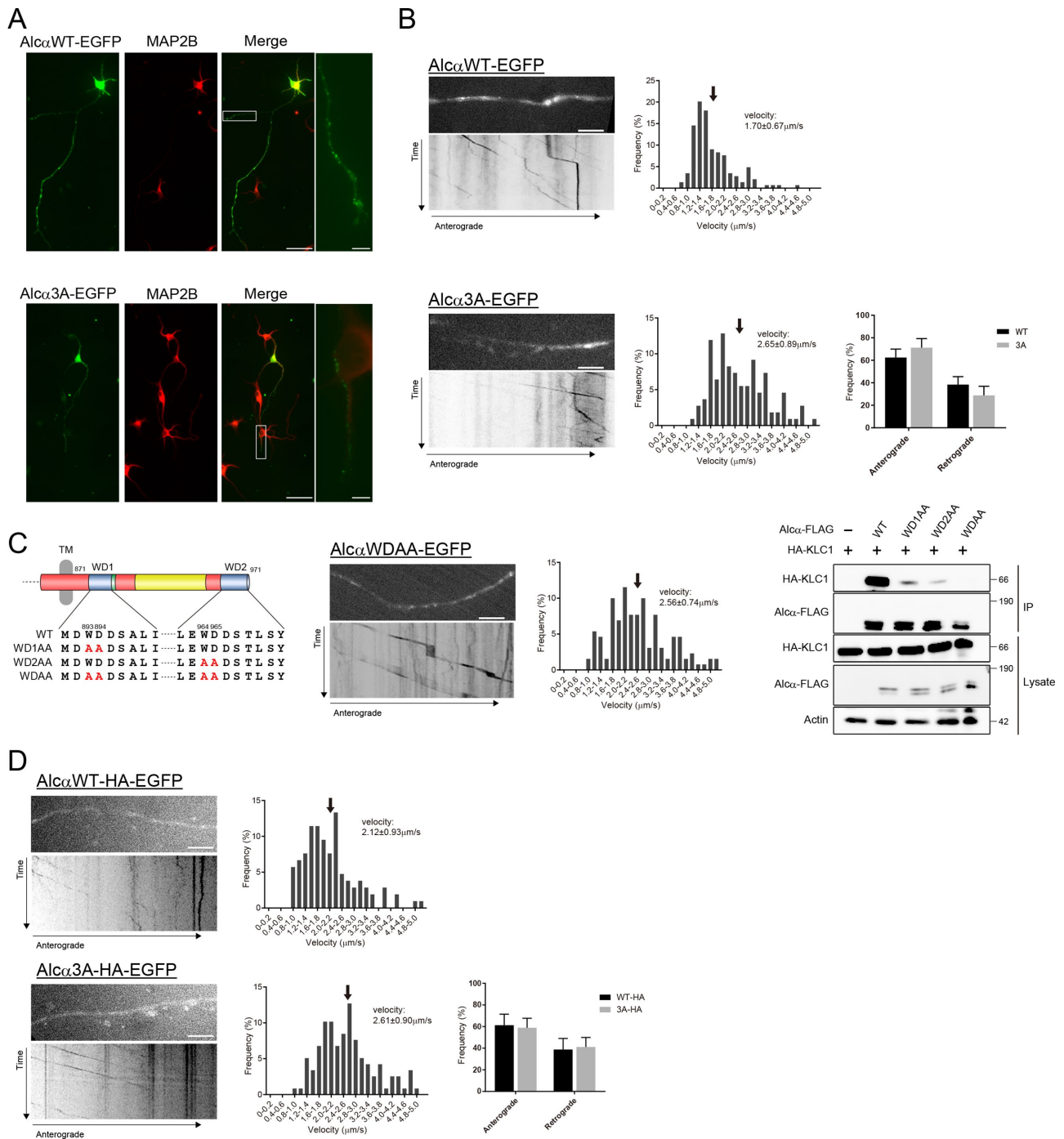


FIGURE 4: Functional analysis of the Alcα3A mutant in axonal transport by kinesin-1. (A) Primary cultured mouse cortical neurons were transfected with pcDNA3.1-AlcαWT-EGFP (upper panels) and pcDNA3.1-Alcα3A-EGFP (lower panels) at div 5. Neurons were fixed and immunostained with anti-MAP2B antibody (red) and then observed with a fluorescence microscope. Higher-magnification images of axons are shown in the panel at the far right of each set of panels. Scale bar = 50 μm and 5 μm for the higher-magnification images. (B) Primary cultured mouse cortical neurons expressing AlcαWT-EGFP (upper panels) and Alcα3A-EGFP (lower panels) were analyzed for axonal transport of Alcα cargo using a TIRF microscope. Transport of Alcα cargo is shown using movies (left upper panels; see also Supplemental Movies 1 and 2) and kymographs showing vesicle movement (left lower panels). The cumulative frequency of the velocity (middle) of the anterograde transport of Alcα cargo is shown. Data are normalized as percentages, and arrows indicate the average velocity (WT, $n = 144$; 3A, $n = 109$). Ratios for anterograde and retrograde transport of AlcαWT-EGFP (black) and Alcα3A-EGFP (gray) are shown in the panels on the right (WT, $n = 20$; 3A, $n = 21$). Statistical significance between WT and 3A was determined by Student's *t* test (means ± SE, not significant). (C) Primary cultured mouse cortical neurons expressing AlcαWDAA-EGFP were analyzed for axonal transport as described in B. The amino acid sequences of Alcα mutants used in this study are shown in the far left panel. A transport movie and kymograph (middle left panel; see also Supplemental Movie 3), and the cumulative frequency of the velocity (middle right) of anterograde transport are shown (data are normalized as percentages, and arrows indicate the average velocity). The

vesicles were detected in axons as well as Al α WT (compare the upper set of panels [WT] with the lower set of panels [3A] in Figure 4A; panels on the far right show images of the boxed axonal region). This distribution indicated that Al α 3A was still transported into axons.

Next, axonal transport of Al α vesicles was examined in living neurons using total internal reflectance fluorescence (TIRF) microscopy (Figure 4, B–D). In most known cases, impaired binding between kinesin and its cargo receptor results in disruption of the transport direction (Araki *et al.*, 2007; Vagnoni *et al.*, 2011; Chiba *et al.*, 2014a) and a decreased anterograde transport ratio in axons. However, Al α WT-EGFP (Supplemental Movie 1) and Al α 3A-EGFP (Supplemental Movie 2) were transported in similar ratios in both anterograde and retrograde directions without significant differences (Figure 4B, left for movies and kymographs and right for direction ratios), and the ratio of stationary cargoes also did not change significantly (unpublished data). However, Al α 3A-EGFP showed a significantly higher transport velocity than that for Al α WT-EGFP (Figure 4B, middle; $p < 0.0001$). The velocity of Al α WT-EGFP is consistent with the *in vitro* kinesin-1 velocity and the velocity of transport of Al α cargo by kinesin-1 in neurons determined previously (Kawaguchi and Ishiwata, 2000; Araki *et al.*, 2007). This higher velocity of Al α 3A-EGFP was also observed with the Al α WDAA-EGFP mutant (Figure 4C, middle panels; Supplemental Movie 3), which does not bind to kinesin-1 because both KLC-binding WD motifs are mutated by Ala substitutions (Figure 4C, far left panel). The average velocity of Al α WDAA-EGFP was equivalent (not significant) to that of Al α 3A-EGFP, and was significantly faster than that for Al α WT-EGFP ($p < 0.0001$). We confirmed that Al α WDAA-FLAG completely lost the ability to bind to HA-KLC1, while Al α WD1AA-FLAG and Al α WD2AA-FLAG retained reduced binding ability (Figure 4C, far right panel). Hence, it is reasonable to conclude that nonphosphorylatable Al α 3A was transported faster by a mechanism distinct from that involving direct binding to the KLC of kinesin-1.

Cytoplasmic Al α ICD, the intracellular domain fragment of Al α generated following cleavage by α - and γ -secretases, may influence the association of Al α with kinesin-1, because Al α ICD contains the KLC-interacting WD motifs. To exclude any influence of Al α ICD on Al α axonal transport, we analyzed axonal transport of the Al α -HA-EGFP mutant containing the HA-tag sequence instead of the α -cleavage sequence, which is resistant to primary cleavage by α -secretase and consequently also to secondary cleavage by γ -secretase (Maruta *et al.*, 2012) (Figure 4D). Again, Al α 3A-HA-EGFP was transported with a significantly higher velocity during anterograde transport than Al α WT-HA-EGFP ($p < 0.0001$), while the anterograde and retrograde transport ratios were almost equivalent for both Al α WT-HA and Al α 3A-HA without any statistical significance, suggesting that this difference was not due to a change in the number of Al α ICDs generated from Al α WT and Al α 3A in

cells. Again, the ratio of stationary cargoes did not change significantly (unpublished data). Thus, we speculated that nonphosphorylated Al α largely lost its ability to bind kinesin-1, and this inefficient form of Al α was instead transported by a separate axonal anterograde transport system with a velocity that is higher than that for the phosphorylation-dependent Al α cargo mechanism.

Proper exit of Al α cargo from Golgi requires phosphorylation of Al α at three serine residues

We previously demonstrated the enhanced fast transport (~ 2.7 μ m/s) of APP cargo by kinesin-1, mediated by the adaptor protein JIP1 (Chiba *et al.*, 2014a). Al α tends to largely colocalize with APP through the neuronal adaptor protein X11-like (X11L)/Mint2 (Tomita *et al.*, 1999; Araki *et al.*, 2003), which is involved in the retention of membrane proteins in the Golgi apparatus (Zhang *et al.*, 2009; Saito *et al.*, 2011). The trend of colocalization of these proteins in the Golgi suggests that Al α 3A might be transported as APP cargo without direct binding of Al α to kinesin-1 when X11L dissociates APP and Al α from the ternary complex. To investigate this possibility, colocalization of Al α -FLAG and APP in axons of mouse cortical neurons was analyzed by immunostaining using anti-FLAG and anti-APP antibodies. Some of the Al α WT-FLAG-containing vesicles were colocalized with APP, but many vesicles were independent in axons (Figure 5A, upper set), consistent with a previous report showing that APP and Al α are independently transported at different velocities, with only $\sim 30\%$ colocalized in axons *in vivo* (Araki *et al.*, 2007). In contrast, a smaller proportion of Al α 3A-FLAG-containing vesicles were independent from APP, and most Al α 3A-FLAG was colocalized with APP (Figure 5A, middle set), as was Al α WDAA-FLAG (Figure 5A, lower set), which cannot bind to kinesin-1 (Figure 4C). The efficiency of colocalization was assessed, and the Pearson's correlation coefficient of Al α -FLAG and APP was significantly higher with Al α 3A and Al α WDAA than with Al α WT (Figure 5B). These results strongly indicate that Al α is not transported as typical Al α cargo unless it is phosphorylated at the S940, S942, and S943 residues and that nonphosphorylated Al α is instead transported as APP cargo, as are Al α 3A and Al α WDAA mutants without the ability to bind kinesin-1.

Some KIFs, including kinesin-1, are known to mediate Golgi exit of membrane proteins (Jaulin *et al.*, 2007; Xue *et al.*, 2010b; Yin *et al.*, 2012). The missorting of Al α 3A into atypical cargo vesicles suggests that Al α 3A is unable to form functional transport vesicles at the Golgi exit point. Because X11L is believed to regulate a closed localization of Al α and APP at Golgi (Araki *et al.*, 2004), we first confirmed that the Al α 3A mutant did not affect binding to X11L. Lysates from cells expressing Al α 3A-FLAG or Al α WT-FLAG together with HA-X11L were subjected to coimmunoprecipitation with anti-FLAG antibody (Figure 5C). Al α 3A-FLAG and Al α WT-FLAG recovered the same amounts of HA-X11L (Figure 5C, right), indicating that the Al α 3A mutant

inability of Al α WDAA to bind KLC1 was shown using immunoprecipitation assays (far right panel). Al α -FLAG with the indicated mutations in the WD motifs were coexpressed in N2a cells with HA-KLC1 and immunoprecipitated with anti-FLAG antibody (IP). A representative blot with anti-HA and anti-FLAG antibodies is shown. Protein size markers are shown (kilodaltons). (D) Primary cultured mouse cortical neurons expressing Al α WT-HA-EGFP (upper panels) and Al α 3A-HA-EGFP (lower panels) were analyzed for axonal transport of Al α cargo with a TIRF microscope. Transport of Al α cargo is shown as a movie (top left panel; see also Supplemental Movies 4 and 5) and kymographs are shown in the bottom left panel. The cumulative frequency of the velocity of the anterograde transport of Al α cargo is shown. Data are normalized as percentages, and arrows indicate the average velocity (Al α WT-HA-EGFP, $n = 105$; Al α 3A-HA-EGFP, $n = 118$; middle panel). Ratios of anterograde and retrograde transport vesicles containing Al α WT-HA-EGFP (black) and Al α 3A-HA-EGFP (gray) are shown in the panel on the right (Al α WT-HA-EGFP, $n = 13$; Al α 3A-HA-EGFP, $n = 16$). Statistical significance between WT and 3A was determined by Student's *t* test (means $\pm$ SE, not significant).

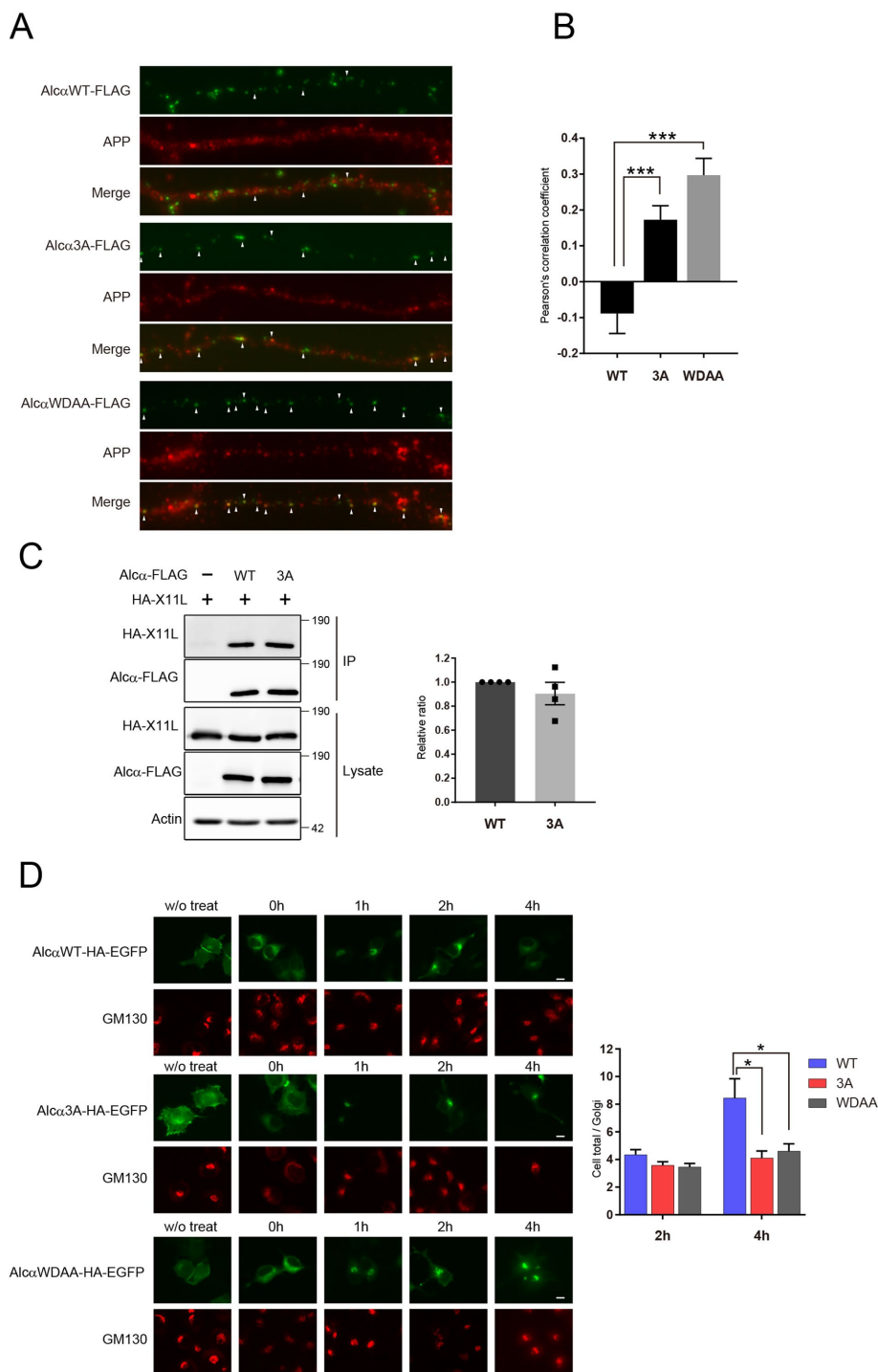


FIGURE 5: Golgi exit of Alca cargo is regulated by the multiple-site phosphorylation. (A) Primary cultured mouse cortical neurons were transfected with pcDNA3.1-AlcaWT-FLAG (upper panels), pcDNA3.1-Alca3A-FLAG (middle panels), or pcDNA3.1-AlcaWDAA-FLAG (lower panels) at div 5 and cultured for 8 h. Neurons were fixed and immunostained with anti-FLAG and anti-APP antibodies. Arrowheads indicate vesicles containing colocalized APP and Alca. Scale bar = 5 μ m. (B) Colocalization of axonal APP with AlcaWT-FLAG, Alca3A-FLAG or AlcaWDAA-FLAG was calculated using the coloc2 plug-in. Pearson's colocalization coefficients are shown. Statistical significance was determined by one-way ANOVA with Tukey's post hoc test (means $\pm$ SE, WT, n = 7; 3A, n = 7; WDAA, n = 9; *** p < 0.005). (C) AlcaWT-FLAG and Alca3A-FLAG were coexpressed in N2a cells, and immunoprecipitates with anti-FLAG antibody (IP) and lysates were analyzed by immunoblotting (left panels). The band intensity of bound HA-X11L was standardized with the band intensity of Alca-FLAG, and the level was calculated as the ratio relative to the amount bound to WT Alca (given a value of 1; right). Protein size markers are shown (kilodaltons). Statistical significance was determined by Student's t test

binds to X11L as effectively as WT Alca and suggesting that dissociation of Alca3A from X11L is equivalent to that from WT Alca at Golgi exit.

Finally, we examined whether kinesin-1 mediates Golgi exit of Alca by direct binding. Again, cleavage-resistant AlcaWT-HA-EGFP, Alca3A-HA-EGFP, and AlcaWDAA-HA-EGFP were expressed in mouse catecholaminergic CAD cells and treated with brefeldin A (BFA), which inhibits Arf GTPase, leading to the collapse of Golgi stacks. After a 4-h treatment, the medium was changed to remove BFA, and cells were cultured for a further 4 h. AlcaWT-HA-EGFP was localized in reconstructed Golgi apparatus, as observed by immunostaining with anti-GM130 antibody, and vesicular localization in peripheral cytoplasm was again evident (Figure 5D, upper panels). At 4 h after BFA treatment, both Alca3A-HA-EGFP and AlcaWDAA-HA-EGFP, which lost all kinesin-1 binding ability, were significantly less well transported into the cell periphery, and instead remained within the Golgi (Figure 5D, left, middle and lower panels). The peripheral localization of Alca3A-HA-EGFP and AlcaWDAA-HA-EGFP at 4 h was significantly lower than that of AlcaWT-HA-EGFP (Figure 5D, right). Taken together, the results suggest that Golgi exit of Alca and/or proper formation of Alca cargo is mediated by direct binding of Alca to the KLC of kinesin-1, and this binding is dependent on multiple site phosphorylation of Alca at Ser940, Ser942, and Ser943 residues.

(means $\pm$ SE, n = 4, not significant). (D) pcDNA3.1-AlcaWT-HA-EGFP (left upper panels), pcDNA3.1-Alca3A-HA-EGFP (left middle panels), and pcDNA3.1-AlcaWDAA-HA-EGFP (left lower panels) were separately transfected into CAD cells. At 12 h after transfection, cells were treated with or without brefeldin A (BFA) for 4 h. After BFA was removed by changing the medium (0 h), cells were further cultured and fixed at the indicated times (0, 1, 2, and 4 h). Cells were immunostained with anti-GM130 antibody to identify the Golgi and observed using a fluorescence microscope along with the Alca-EGFP signal. Scale bar = 10 μ m. Golgi exit of Alca was quantified by calculating the intensity of the Alca-HA-EGFP signal within the total area of cells relative to the intensity of the Golgi area at 2 and 4 h. AlcaWT-HA-EGFP (blue; 2 h, n = 32; 4 h, n = 30), Alca3A-HA-EGFP (red; 2 h, n = 32; 4 h, n = 29), and AlcaWDAA-HA-EGFP (gray; 2 h, n = 32; 4 h, n = 23) are shown. Statistical significance was determined by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test (means $\pm$ SE, * p < 0.05).

DISCUSSION

Alc α and APP are major cargoes for kinesin-1 in neurons. However, the molecular mechanisms underlying how vesicles containing Alc α and APP are generated in cells, how they are associated with kinesin-1 as cargo for axonal transport, and how they are released from kinesin-1 at the nerve terminal remain to be resolved. Previous studies showed that Alc α /Clstn1 binds directly to the KLC of kinesin-1 during anterograde transport (Konecna et al., 2006; Araki et al., 2007), and the binding of the Alc α WD motif to the KLC1 TPR region activates kinesin-1 to initiate cargo transport (Kawano et al., 2012). One study showed that replacing Ser460 of KLC1 with Ala increases the association with Clstn1, whereas replacement with Asp decreases binding to Clstn1, suggesting that phosphorylation of KLC1 at Ser460 may regulate the interaction between the cargo and kinesin-1 (Vagnoni et al., 2011). Furthermore, our recent research revealed that phosphorylation of KLC1 at Thr466 regulates the velocity of transport of APP cargo by modulating the interaction with JNK-interacting protein 1b (JIP1b) (Chiba et al., 2017).

We report here the following novel findings: 1) Alc α is a phosphoprotein, phosphorylation occurs within the cytoplasmic region, and the phosphorylation level is reduced following cleavage of Alc α CTF by γ -secretase to generate a cytoplasmic Alc α ICD fragment; 2) dephosphorylation of Alc α significantly decreases binding to KLC1 and KLC2; 3) phosphorylation of three amino acid residues in Alc α , Ser940, Ser942, and Ser943, is required for the association with KLC; and 4) Alc α not phosphorylated at these three serine residues is unable to form a proper cargo, and this inefficient Alc α cargo receptor is transported via distinct vesicles, likely due to the compensatory function of APP.

The Ser940, Ser942, and Ser943 phosphorylation sites are located within the acidic region of the Alc α cytoplasmic domain, between the KLC-binding WD1 and WD2 motifs. The presence of two WD motifs in a cargo molecule results in a stronger interaction with KLC than occurs with a single WD motif (Zhu et al., 2012), consistent with the lower affinity of Alc β for KLC1, which possesses only one KLC-binding WD motif. The nonphosphorylated form of Alc α harboring Ala substitutions at Ser940, Ser942, and Ser943 (Alc α 3A mutant) showed significantly attenuated association with KLC. Alc α harboring double Ala substitutions at Ser942 and Ser943 (Alc α S942A/S943A mutant) also displayed diminished KLC binding, but the decrease was less than that observed for Alc α 3A, indicating that multiple phosphorylation at serine residues is required for optimum association with KLC. In Alc γ , only one serine residue, Ser922 in human Alc γ , is conserved, and this Ser922 may correspond to Ser940 of human Alc α (Supplemental Figure S2). Although it remains uncertain whether Ser922 of Alc γ is phosphorylated, phosphorylation of Ser922 and/or other Ser/Thr residues within the acidic region may regulate KLC binding.

Phosphorylation within the acidic region may change the conformation or alter the disordered structure of the Alc α cytoplasmic region that includes the two WD motifs, because regulation of disordered structure by phosphorylation has been reported for other proteins (Iakoucheva et al., 2004; Travers et al., 2015). The protein kinase(s) responsible for phosphorylating Ser940, Ser942, and Ser943 of Alc α remain unknown. However, casein kinase I (CK1) and/or II (CK2) are probable candidates. CK1 and 2 are known to phosphorylate serine residues embedded in acidic regions of DARPP-32 in neurons (Desdouits et al., 1995), and our in vitro experiments showed that at least CK2 can phosphorylate a substrate peptide containing these serine residues (unpublished observation by YS).

We expected that axonal transport of the Alc α 3A mutant may be impaired, because this mutant was almost incapable of binding to kinesin-1. However, Alc α 3A vesicles were observed in axons, and their transport velocity was higher than that of WT Alc α vesicles. Further experiments revealed that Alc α 3A was included in distinct APP cargo vesicles. Although previous observations suggested that APP and Alc α work cooperatively during Golgi exit (Ludwig et al., 2009; Takei et al., 2015), the transport of Alc α cargo vesicles is largely competitive with and independent of APP cargo vesicles after Golgi exit (Araki et al., 2007). APP cargoes are transported at a higher velocity than Alc α cargoes in neuronal axons, even though both cargoes are transported by the same kinesin-1 molecular motor (Araki et al., 2007; Chiba et al., 2014a). Additionally, endogenous Alc α cargoes are transported separately from APP cargoes, and colocalization of the two cargoes in axons is ~30% at most (Araki et al., 2007). However, in the present study, we showed that inefficient binding of Alc α to kinesin-1 results in increased colocalization within APP vesicles in axons. This nonconventional localization of Alc α is presumably due to the compensatory action of APP cargo in defective Alc α transport.

Interestingly, the Alc α 3A mutant displayed decreased Golgi exit of Alc α vesicles in undifferentiated CAD cells, which exhibit lower expression of endogenous APP than in neurons (Fragkouli et al., 2017). Several reports suggest that KIFs such as kinesin-1 play an important role in Golgi exit of cargo vesicles and/or post-Golgi transport of cargoes (Jaulin et al., 2007; Xue et al., 2010b; Yin et al., 2012). KIFs may function to form tubular structures at the Golgi exit point (reviewed in Weisz and Rodriguez-Boulant, 2009). Our finding that the Alc α 3A mutant decreases Golgi exit is consistent with the previous report on KIF function and suggests that Alc α 3A is unable to form proper Alc α cargoes. Although the exit of Alc α 3A or Alc α WDAA from Golgi was decreased, vesicles containing these mutants were still transported in axons, albeit by alternative vesicles and at a higher velocity. These results indicate that decreased binding to kinesin-1 impairs the proper formation of Alc α vesicles at the Golgi, and defective Alc α is incorporated into distinct APP cargo vesicles and possibly others.

Alc α vesicles can carry various cargo proteins, but when Alc α is not phosphorylated, these cargoes will not be transported efficiently to the required destination. Although relatively little is known about the roles of Alc α transport vesicles, the phenotypes of Alc α gene knockout animals have been reported. Axon branching is impaired in zebrafish, and spine formation is affected in mice (Ponomareva et al., 2014; Ster et al., 2014). Impaired phosphorylation of Alc α might cause these phenotypes in vivo, but this requires further investigation.

Taken together, our findings allow us to hypothesize that Alc α recruits and strongly binds kinesin-1 following multiple site phosphorylation of serine residues in its cytoplasmic acidic region, which facilitates the formation of tubular membrane structures that include Alc α and its cargo functionally associated with kinesin-1 (Figure 6). Although our results do not directly demonstrate whether Alc α located in the Golgi is highly phosphorylated, they revealed that WT Alc α binds strongly to kinesin-1, unlike the Alc α mutant harboring alanine substitutions at Ser940, Ser942, and Ser943, and confirmed that endogenous Alc α is phosphorylated at these multiple serine residues, which regulates binding to kinesin-1. Prior to the association of phosphorylated Alc α with kinesin-1, Alc α and APP are thought to dissociate from a ternary complex including X11L by an unknown mechanism (Figure 6). One possible regulatory process in the release of APP from X11L may be the phosphorylation of X11L (Sakuma et al., 2009). However, it is not known if phosphorylation of X11L contributes to release of Alc α . Nevertheless, the present

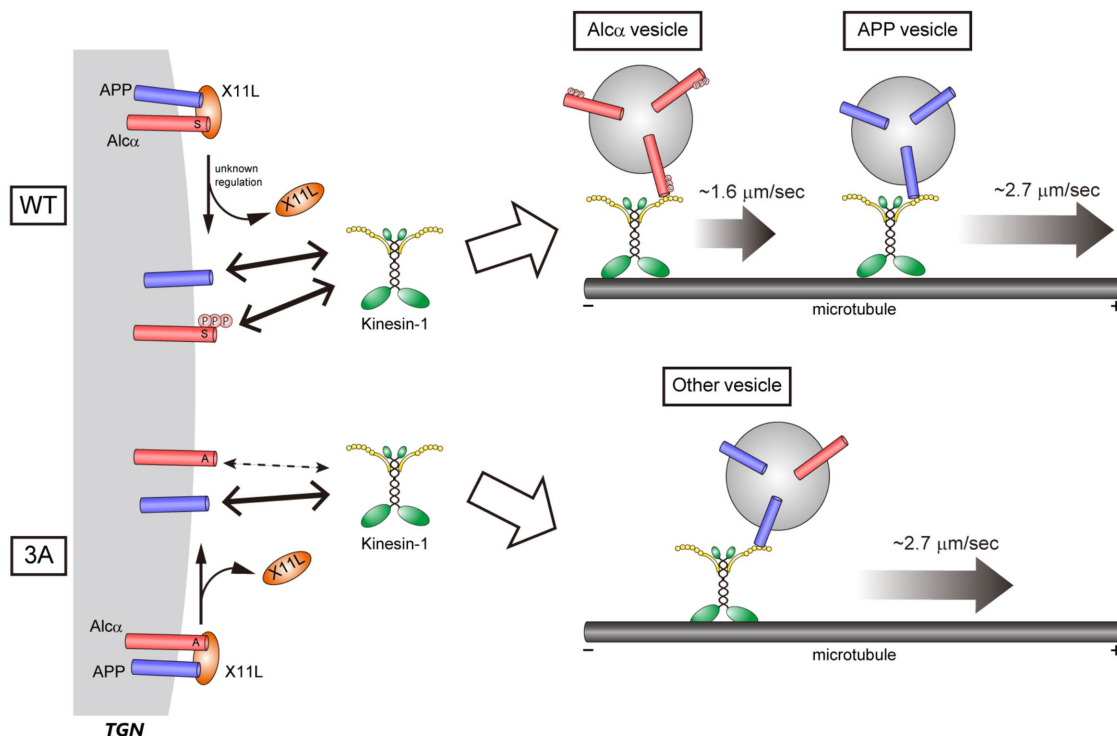


FIGURE 6: Role of Alca phosphorylation in Golgi exit of Alca vesicles by kinesin-1. Schematic representation of the possible regulation of Alca vesicle formation and transport by phosphorylation of Alca at Ser940, Ser942, and Ser943 is shown. Alca and APP are thought to colocalize in the Golgi by binding to X11L, and both membrane proteins dissociate from X11L by an unknown mechanism but still reside close to each other in the Golgi. Alca residing at the Golgi is phosphorylated at three serine residues within the cytoplasmic acidic region that is located between the WD1 and WD2 motifs. This phosphorylation enhances the association with kinesin-1 and the formation of vesicles containing Alca. The resultant Alca vesicles are transported in an anterograde manner by kinesin-1 at the correct velocity ($\sim 1.6 \mu\text{m/s}$) for proper Alca cargo, in which Alca is functionally associated with kinesin-1 (upper panel). APP vesicles are also formed independently and transported in an anterograde manner by kinesin-1 at the correct velocity ($\sim 2.7 \mu\text{m/s}$) for proper APP cargo. When Alca is not phosphorylated at the three phosphorylatable serine residues (due to substitution with alanine), the resultant inefficient Alca cannot bind kinesin-1 and fails to exit from the Golgi by forming its own cargo. However, some Alca does manage to exit from the Golgi following incorporation into other vesicles containing APP cargo. When Alca is included in APP cargo vesicles, the resultant vesicles are transported by APP-associated kinesin-1 in an anterograde manner at a higher velocity (lower panel; enhanced fast transport velocity of $\sim 2.7 \mu\text{m/s}$) that is more typical of APP cargo transport by kinesin-1 (Chiba *et al.*, 2014a).

findings broaden our understanding of the function and regulation of cargo–motor protein interactions and provide insight into the mechanism of proper cargo formation.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal anti-Alca UT195 antibody was raised to the same antigen as was used to generate UT83 (described previously in Araki *et al.*, 2003). A polyclonal phosphorylation state-specific anti-pAlca antibody was raised against the antigen peptide NH₂-Ala-Glu-pSer-Glu-pSer-pSer-Glu-Glu-Glu-Lys-Lys-Cys-CONH₂. Further information can be found in the Supplemental Methods Appendix.

Plasmids

The original plasmids pcDNA3 and pcDNA3.1 containing Alca and Alca ICD were described previously (Araki *et al.*, 2007). These were mutated using PCR and subcloned into pcDNA3.1. Details of plasmid construction are described in the Supplemental Methods Appendix.

Prelabeling of cells

HEK293 cells expressing the cytoplasmic region of Alca (Alca^{871–971}/Alcacyt) were labeled for 3 h at 37°C with [³²P]orthophosphate as described previously (Suzuki *et al.*, 1994). Alcacyt was immunoprecipitated and incubated with or without ≥ 400 units of lambda protein phosphatase (λ PPase; P9614; Sigma-Aldrich) and analyzed by autoradiography followed by Tris-glycine-buffered SDS–PAGE. A detailed description of the procedures can be found in the Supplemental Methods Appendix.

Detection of phosphorylated Alca, Alca CTF, and Alca ICD in mouse brain

For detection of endogenously generated Alca CTF and Alca ICD (Figure 1A), subfractionation of mouse brain lysate was performed as described (Nakaya and Suzuki, 2006) and analyzed by immunoblotting followed by Tris-tricine-buffered SDS–PAGE using 10% (wt/vol for Alca CTF) and 17.5% (wt/vol for Alca ICD) polyacrylamide gels (Schägger, 2006). A detailed description of the procedures can be found in the Supplemental Methods Appendix.

Cell culture and primary cultured neurons

Cell culture and transfection are described in the Supplemental Methods Appendix.

Primary cultures of mouse cortical neurons were prepared and cultured as described previously (Chiba *et al.*, 2014a). All animal studies were conducted in compliance with the guidelines of the Animal Studies Committee of Hokkaido University.

Binding assay for Alca-FLAG and HA-KLC1

N2a cells were transfected with pcDNA3.1-Alca-FLAG or pcDNA3.1-HA-KLC1 plasmid and cultured for 24 h. Cells were lysed in HBS-T buffer. Cell lysates containing Alca-FLAG were subject to immunoprecipitation with anti-FLAG M2 antibody and protein G Sepharose beads (GE Healthcare Bio-Sciences, Little Chalfont, UK), and immunoprecipitates were treated with or without λ PPase (≥ 200 U) at 30°C for 3 h. Beads were washed and then combined with cell lysate containing HA-KLC1 and incubated with rotation. Immunoreactive proteins were quantified using an LAS-4000 mini (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy analysis and quantification of fluorescence intensity

At 12 h after transfection, CAD cells were treated with 2 μ g/ml brefeldin A (BFA) for 4 h. After washing with medium containing 100 μ g/ml cycloheximide, cells were further incubated for the indicated time and immunostained with anti-GM130 antibody. Primary cultured mouse cortical neurons (in vitro, day 5) were transfected with plasmids by the calcium phosphate method and cultured for 8 h. Cells were fixed and immunostained with indicated antibodies. Fluorescence images were acquired with a fluorescence microscope (BZ-710X; Keyence, Osaka, Japan), and quantitative analysis and co-localization efficiency were analyzed with Fiji/ImageJ and the Colco2 Fiji plug-in (ImageJ-Fiji-ImgLib; <http://Fiji.sc> or <http://imageJ.net>).

Total internal reflectance fluorescence/TIRF microscopy analysis

At in vitro day 4 (div 4), primary cultured mouse cortical neurons were transfected with plasmids and cultured for 16 h. The cells were observed using a TIRF microscopy system (C1; Nikon, Tokyo, Japan) with a charge-coupled device camera (Cascade 650; Photometrics) in an incubation chamber supplied with 5% CO₂ at 37°C. The velocity of anterograde transport was analyzed quantitatively as described in the Supplemental Information of a previous report (Araki *et al.*, 2007). The average velocity (indicated with an arrow in the histograms) $\pm$ SD is shown. Statistical analysis of cargo velocity distributions was performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test or Mann–Whitney tests. Kymographs of moving vesicles were assembled using KymoMaker (Chiba *et al.*, 2014b). The direction of moving vesicles was further followed by residual analysis. The proportion (percentage) of anterograde and retrograde vesicles relative to the indicated total number of vesicles is shown as the frequency, as described previously (Araki *et al.*, 2007).

ACKNOWLEDGMENTS

We thank George S. Bloom (University of Virginia) and Sam Gandy (Mount Sinai School of Medicine) for the kind supply of antibodies. This work was supported in part by Grants-in-Aid for JSPS Research Fellow 16J04020 to Y.S. and 15J02220 to K.C. and Grants-in-Aid for Scientific Research 15K18854 to S.H. and 262930110 and 16K14690 to T.S. from the Ministry of Education, Culture, Sports, Science

and Technology (MEXT) in Japan, by National Institutes of Health Grant AG047270 to A.C.N., by the Strategic Research Program for Brain Sciences from the Japan Agency for Medical Research and Development (16dm0107142h0001, 17dm0107142h0002), and by a Grant-in-Aid for Scientific Research on Innovative Areas—Platforms for Advanced Technologies and Research Resources “Advanced Bioimaging Support” to T.S.

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