FEBS Letters 580 (2006) 5797–5801

Phosphorylation of Numb regulates its interaction with the clathrin-associated adaptor AP-2

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Received 4 September 2006; revised 14 September 2006; accepted 18 September 2006

Available online 27 September 2006

Edited by Sandro Sonnino

Abstract **Numb is thought to participate in clathrin-dependent endocytosis by directly interacting with the clathrin-associated adaptor complex AP-2, although the underlying mechanisms are unknown. Numb is also known to be phosphorylated at Ser^{264} *in vitro* and *in vivo*. Here, we found that Numb is phosphorylated *in vitro* by Ca^{2+}/calmodulin-dependent protein kinase I on Ser^{283}. This phosphorylation was also observed in transfected COS-7 cells, indicating its physiological relevance. Pull-down experiments showed that the phosphorylation of Numb impaired its binding to the AP-2 complex and simultaneously recruited 14–3–3 proteins *in vitro*. Based on experiments using Numb mutants, both the initial phosphorylation of Ser^{264} and the subsequent phosphorylation of Ser^{283} are sufficient to abolish the binding of Numb to AP-2 and to promote the interaction with 14–3–3 protein. These findings suggest a novel mechanism for the regulation of Numb-mediated endocytosis, namely through direct phosphorylation.**

Keywords: Numb; Phosphorylation; AP-2 complex; Endocytosis

1. Introduction

Numb was originally identified as a gene involved in the determination of cell fate during peripheral and neural development in *Drosophila* through inhibition of Notch signaling [1,2]. During the asymmetric division of neural precursor cells, Numb is segregated to one of the daughter cells [3–5]. Recent studies in mice have demonstrated that Numb and a related protein, Numblike (Numbl), play redundant but crucial roles in maintaining neural progenitor cells during neurogenesis [6–8].

The Numb family proteins structurally resemble an adaptor or scaffold protein and contain a phosphotyrosine binding (PTB) domain [9], a proline-rich carboxy terminal region containing several putative Src homology 3 domain-binding sites [10], and an Eps15 homology domain-binding motif [11]. Indeed, Numb interacts with components of the endocytic machinery including Eps15 and α-adaptin, which is a subunit of the AP-2 complex and a major component of clathrin-coated pits, suggesting a general role for Numb in the endocytic process [11–14]. A recent study has shown that Numb interacts with collapsing mediator protein-2 via the PTB domain, regulating Numb-mediated endocytosis at the growth cone [15]. Also, Numb is implicated in dendritic spine morphogenesis in cultured hippocampal neurons [16]. Numb and Numbl are phosphorylated at Ser^{264} and Ser^{304}, respectively, *in vivo* and *in vitro* by multiple Ca^{2+}/calmodulin-dependent protein kinases (CaM-Ks), resulting in the recruitment of 14–3–3 proteins [17]. Most recently, Dho *et al.* reported that the membrane localization of Numb is dynamically regulated by G protein-coupled receptor activated phospholipid hydrolysis and protein kinase C dependent phosphorylation [18].

Despite this information, neither the precise role nor the regulation of Numb in the endocytic process has been explored. In the current studies, we examined the role of phosphorylation in the regulation of Numb. We found that site-specific phosphorylation regulates the interaction of Numb with the AP-2 complex and 14–3–3 proteins.

2. Materials and methods

2.1. Materials

GST-rat Numb and GST-rat Numb fragment (residues 238–304) with a His_6 tag at its C-terminus were constructed, expressed in *Escherichia coli* JM109, and purified as previously described [17]. GST-rat Numb mutants including Ser264Ala and Ser264/283Ala were constructed by site-directed mutagenesis (GeneEditor™; Promega) and PCR, respectively. The nucleotide sequences of all constructs used in this study were confirmed by an ABI377 automated sequencer (PE Biosystems, Foster City, CA). Recombinant wild-type rat Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaM-K) Iα and wild-type rat Ca^{2+}/CaM-dependent protein kinase kinaseα (CaM-KKα) were expressed and purified as previously described [19,20]. Recombinant rat CaM was expressed in *E. coli* BL-21 (DE3) using pET-CaM (kindly provided by Dr. Nobuhiro Hayashi, Fujita Health University, Toyoake, Japan) and purified by phenyl-Sepharose column chromatography [21]. Recombinant CaM-KIα was activated by phosphorylation with CaM-KKα as described previously [17] and stored at −80°C until use. Both anti-α-adaptin (α-subunits of AP-2, Clone100/2) antibody and anti-β-adaptins (β1 subunit of AP-1 and β2 subunit of AP-2, Clone100/1) antibody were obtained from Sigma. Anti-Numb and anti-14–3–3η antibodies were purchased from Upstate Biotechnology,

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Abbreviations: CaM-K, Ca^{2+}/CaM-dependent protein kinase; CaM, calmodulin; LC, liquid chromatography; MS/MS, tandem mass spectrometry; GST, glutathione S-transferase

0014-5793/$32.00
doi:10.1016/j.febslet.2006.09.043

Inc (Lake Placid, NY) and Immuno-Biological Laboratories, respectively. The monoclonal antibody to Numb phosphorylated on Ser^{264} was generated as described previously [17], and the monoclonal antibody to Numb phosphorylated on Ser^{283} was generated against a synthetic phosphopeptide corresponding to residues 274–292 (CKMSPFKRQLpSLRINELPST) of rat Numb as described previously [22]. All other chemicals were obtained from standard commercial sources.

2.2. Phosphorylation of Numb in vitro

Either purified GST-Numb including wild-type and mutants or GST-Numb fragment was incubated without or with activated CaM-KI (0.5 μg/ml) at 30 °C for the indicated periods in a solution containing 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol, 2 mM $CaCl_2$, 5 μM CaM, and either 200 μM [γ-^{32}P]ATP (~1,000 cpm/pmol) for autoradiography or 200 μM ATP for mass spectrometric analysis.

2.3. Identification of phosphorylation sites in Numb by mass spectrometry

GST-Numb (~3 μg) was phosphorylated for 10 min by activated CaM-KI as described above, separated by SDS–PAGE on a 7.5% acrylamide gel, subjected to in-gel digestion with trypsin, and analyzed by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) analysis using a Micromass Q-Tof2 quadrupole /time-of-flight hybrid mass spectrometer (Micromass, Manchester, UK) interfaced with capillary reverse-phase liquid chromatography system (Micromass CapLC™ system) as described previously [23].

2.4. Expression of FLAG-tagged Numb in COS-7 cells

Transfection of either wild-type or mutants of pME-Numb-FLAG cDNAs (2 μg) into COS-7 cells (6-well plate) was carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After ~24 h, the cells were lysed with 150 μl of SDS–PAGE sample buffer and analyzed by SDS–PAGE followed by Western blotting.

2.5. Pull-down assay

Purified GST-Numb (~10–20 μg), including wild-type and mutants, or GST-Numb fragment (20 μg) was left untreated or phosphorylated with activated CaM-KI for the indicated periods as described above. After the reaction was terminated by a 5-fold dilution with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 4 mM EDTA, and 2 mM EGTA (for the time course experiment as shown in Fig. 4), the sample was incubated with 40 μl of glutathione–Sepharose overnight at 4 °C. The resin was then washed extensively with Buffer A (150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 μM microcystin LR, and 0.5 μM okadaic acid). Rat brains were homogenized with three volumes of Buffer B (Buffer A containing 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonylfluoride) and then centrifuged at 31,000 × g for 30 min. An equal volume (1 ml) of the extracts was incubated at 4 °C with ligand-coupled glutathione–Sepharose resin and then washed extensively with Buffer B. After washing the resin with Buffer A, bound Numb or Numb fragment was specifically eluted together with the interacting proteins using PreScission protease (4 units; Amersham Biosciences). To identify the Numb-interacting proteins, each eluate was analyzed by SDS–PAGE, followed by LC–MS/MS analysis as described above or by Western blotting. The MS/MS data of the eluted peptides from trypsin-digested gels were analyzed with the Mascot MS/MS Ions Search (Matrix Science) to search and assign the obtained peptides to the NCBI nonredundant data base [17] (see Supplementary data).

3. Results and discussion

Identification of phosphorylation sites in Numb – Recently, we identified Ser^{264} as a major *in vitro* phosphorylation site in rat Numb for multifunctional CaM-Ks (Fig. 1A) [17]. Phosphorylation of Ser^{264} in rat Numb is readily detected in various tissues and cultured cells, indicating its physiological relevance [17]; however, Ser^{264} is not the only site phosphorylated by CaM-Ks because residual phosphorylation of a Ser264Ala mutant is observed ([17] and Fig. 1C). Therefore, we attempted to identify the additional sites phosphorylated by activated CaM-KI *in vitro*. Mass spectrometry identified Ser^{283} as a novel phosphorylation site (Fig. 1B). In addition, an *in vitro* kinase assay (Fig. 1C) revealed that incorporation of phosphate into Numb is reduced by mutation of Ser^{264} to Ala, although residual phosphorylation can still be observed. Furthermore, double Ala mutations at Ser^{264} and Ser^{283} almost completely prevented the phosphorylation of Numb by CaM-KI. These results indicate that activated CaM-KI phosphorylates both Ser^{264} and Ser^{283} *in vitro*. It is noteworthy that both phosphorylation sites are conserved in a related protein, Numblike (Numbl in Fig. 1A).

Phosphorylation of Numb in transfected cells – To determine whether Numb is phosphorylated on Ser^{283} *in vivo*, we generated a monoclonal antibody that specifically recognizes Numb phosphorylated at this site. Although basal phosphorylation of Numb on Ser^{264} was detected with an anti-phospho-Ser^{264} antibody [17], basal phosphorylation at Ser^{283} was not readily detected in intact cells (data not shown). This could be due to a relatively low sensitivity of the antibody or a low level of basal phosphorylation in intact cells. Therefore, we analyzed the site-specific phosphorylation of Numb using COS-7 cells transfected with various constructs of C-terminal FLAG-tagged Numb, including wild-type, Ser264Ala, and Ser264/283Ala. Expression of FLAG-tagged Numb proteins was detected by Western blot analysis using anti-FLAG antibody (Fig. 2, upper panel). Western blot analysis of the extracts from transfected cells using either anti-phosphoSer^{264} (Fig. 2, middle panel) or anti-phosphoSer^{283} antibody (Fig. 2, lower panel) confirmed the specificity of each antibody and also demonstrated the intrinsic phosphorylation of rat Numb at both Ser^{264} and Ser^{283} in cultured cells.

Phosphorylation of Numb impairs its interaction with the AP-2 complex – To elucidate the physiological role(s) of Numb phosphorylation, we attempted to identify Numb-interacting proteins using a pull-down experiment with either GST-Numb fragment (residues 238–304) containing the phosphorylation sites (Ser^{264} and Ser^{283}) or GST-full-length Numb (Fig. 3A). Rat brain lysate was incubated with glutathione–Sepharose-bound GST-Numb fusion proteins that were left unphosphorylated or were phosphorylated by activated CaM-KI. After extensive washing, each resin was treated with PreScission protease to release Numb and Numb-interacting proteins (Fig. 3A). Numb-interacting proteins from each pull-down sample assay were analyzed by SDS–PAGE (Fig. 3B) and identified by mass spectrometry. In agreement with our recent finding using rat liver lysate [17], 14–3–3 proteins specifically bound to only phosphorylated GST-Numb fragment (residues 238–304). Also, similar to the pull-down experiment using GST-Numb fragment, full-length Numb bound 14–3–3 proteins in a phosphorylation-dependent manner. In contrast, unphosphorylated full-length Numb interacted with ~105 kDa proteins and ~50 kDa protein (Fig. 3B), which were identified by LC–MS/MS analysis (shown in Supplementary data) as α1 subunit, β2 subunit, α2 subunit, and μ2 subunit of the clathrin-associated AP-2 complex. This agrees with previous findings that Numb interacts directly with α-adaptin (alpha subunit) in the AP-2 complex via its C-terminal DPF motif [12]. Thus, the Numb fragment (residues 238–304), which does not contain a DPF motif, was not capable of inter-

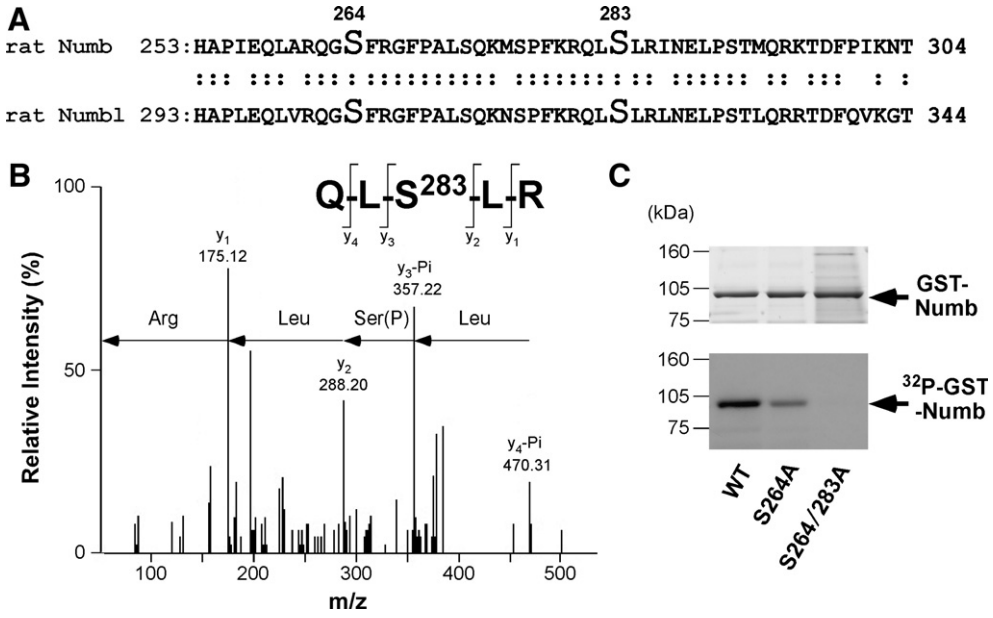

Fig. 1. *Identification of phosphorylation sites in rat Numb*. (A) Alignment of amino acid sequences of rat Numb (residues 253–304) and rat Numbl (residues 293–344). Identical residues are indicated by *colons*. (B) GST-Numb phosphorylated by activated CaM-KI for 10 min was separated by SDS–PAGE and digested with trypsin. Phosphorylation of Ser^{283} was analyzed by LC–MS/MS of the singly charged ion for the peptide containing residues 281–285. The observed y-ion fragment series generated by collision-induced dissociation are indicated by arrows. The observed fragment ions are indicated below the peptide sequence. (C) GST-fused wild-type (WT), Ser264Ala (S264A), or Ser264/283Ala (S264/283A) Numb (3 μg) was incubated with activated CaM-KI at 30 °C for 10 min with [γ-^{32}P]ATP. The samples were then subjected to SDS–PAGE and stained with Coomassie brilliant blue (upper panel) or analyzed by autoradiography (lower panel).

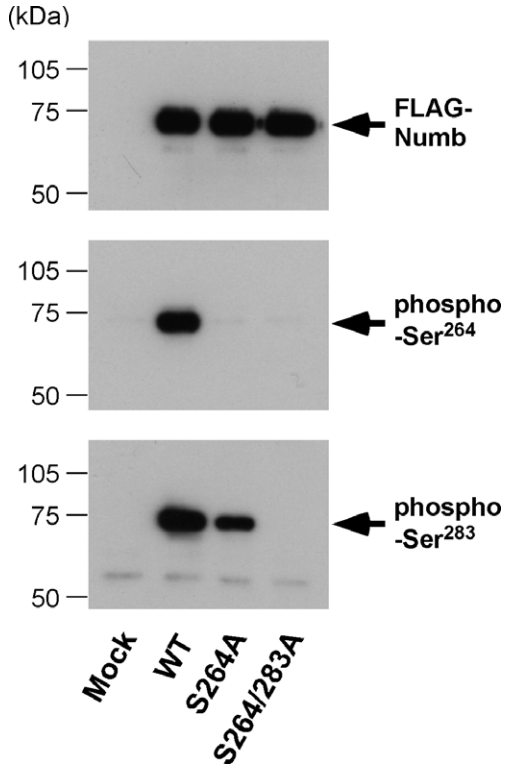

Fig. 2. *Phosphorylation of FLAG-tagged Numb in transfected COS-7 cells*. COS-7 cells expressing FLAG-tagged wild-type (WT), Ser264Ala (S264A), or Ser264/283Ala (S264/283A) Numb were lysed, and 10 μl of each sample was separated by SDS–PAGE and analyzed by Western blotting using antibodies to FLAG (upper panel), Numb phosphorylated on Ser^{264} (middle panel), or Numb phosphorylated on Ser^{283} (lower panel).

acting with the AP-2 complex. Interestingly, when we used phosphorylated GST-Numb (Fig. 3C, right panel) as an affinity ligand, we could not detect the interaction of Numb with the AP-2 complex (Fig. 3B). This result was confirmed by Western blot analysis using anti-α-adaptin (α subunits of AP-2) and anti-β-adaptins (β subunits of AP-1 and AP-2) antibodies (Fig. 3C), suggesting that the phosphorylation inhibits the interaction of Numb with the AP-2 complex. Because it has been shown that the physical interaction of Numb with α-adaptin plays a role in down-regulating Notch, presumably by stimulating endocytosis of Notch [13], inhibition of their interaction by phosphorylation may involve in the dynamic regulation of Notch signaling pathway.

Phosphorylation of Ser^{264} and Ser^{283} impairs the interaction of Numb and the AP-2 complex – To examine the impact of the phosphorylation at Ser^{264} and Ser^{283} on the association of Numb and the AP-2 complex, we generated GST-Numb fusions of Ser264Ala and Ser264/283Ala and used them in a pull-down assay similar to that shown in Fig. 3. The interaction of Numb and AP-2 complex was examined by Western blot analysis using anti-α-adaptin and anti-β-adaptins antibodies. We also monitored the interaction of Numb and 14–3–3η protein since we observed that the multiple 14–3–3 isoforms bound Numb in a phosphorylation-dependent manner (Fig. 3B, [17]). All of the Numb proteins including wild-type, Ser264Ala, and Ser264/283Ala interacted with the AP-2 complex when unphosphorylated. Similar to the results shown in Fig. 3B and C, wild-type Numb rapidly (within 2 min) lost the binding ability to AP-2 complex when phosphorylated by CaM-KI. This kinetics appears to correspond well with the kinetics of phosphorylation at Ser^{264} (i.e., the phosphorylation of Ser^{264} appeared to saturate within 2 min). In contrast, even after 2 min of phosphorylation, the Ser264Ala mutant could bind the AP-2

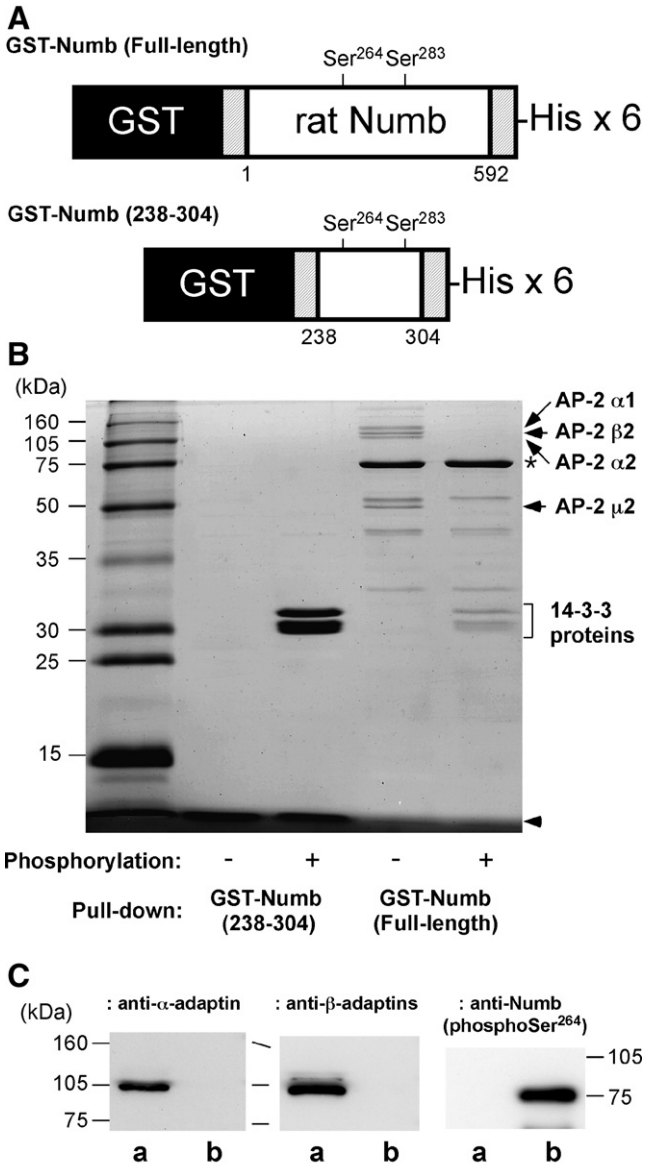

Fig. 3. *Phosphorylation of Numb regulates its interaction with the AP-2 complex*. (A) Schematic representation of fusion protein used for the pull-down assay, including His-tagged GST-full-length Numb and GST-Numb fragment (residues 238-304). The phosphorylation sites (Ser^{264} and Ser^{283}) are indicated. Shaded boxes indicate the location of the recognition sequence (Leu–Glu–Val–Phe–Gln↓Gly–Pro) for PreScission protease. (B) Rat brain extract was subjected to a pull-down assay using either GST-Numb fragment (residues 238–304) or GST-full-length Numb (20 μg) that was left untreated (−) or was phosphorylated with activated CaM-KI (+) for 90 min. The Numb-binding proteins were eluted by cleavage of the Numb proteins from GST with PreScission protease. Eluted samples (30 μl) were separated by SDS–PAGE and stained with Coomassie brilliant blue. Eluted proteins from unphosphorylated GST-Numb-coupled resin (second lane from right) had molecular masses of ~105 kDa and ~50 kDa and were identified by LC–MS/MS analysis as the α1, β2, α2, and μ2 subunits of adaptor complex AP-2 (indicated by arrows; see also Supplementary data). The arrowhead and asterisk indicate cleaved Numb fragment (residues 238–304) and full-length Numb, respectively. 14–3–3 proteins are also indicated. A left lane shows the molecular weight marker. (C) Samples eluted from glutathione–Sepharose resin coupled with either GST-Numb (lane a) or phosphorylated GST-Numb (lane b) as shown in (B) were separated by SDS–PAGE and analyzed by Western blotting using antibodies to α-adaptin (left panel), β-adaptins (middle panel), or Numb phosphorylated on Ser^{264} (right panel).

complex, but after 10 min, AP-2 binding was significantly reduced, indicating that phosphorylation at other sites impaired AP-2 binding to Numb. Given the kinetics of this phosphorylation, we suspected that it was at Ser^{283}. This was confirmed by the fact that binding of the Ser264/283Ala mutant to the AP-2 complex was unaffected even after 10 min of phosphorylation.

In addition, we monitored the recruitment of 14–3–3η protein to Numb. We found that the interaction of 14–3–3η with wild-type Numb gradually increased until 10 min. This did not correlate with the saturation of phosphorylation at Ser^{264}, which occurred within 2 min, indicating that the subsequent phosphorylation at Ser^{283} also promoted the recruitment of 14–3–3η protein. Indeed, we detected the association of 14–3–3η with the Ser264Ala mutant after 10 min of phosphorylation. This interaction of Numb with 14-3-3 was abolished by mutation of Ser^{283} to Ala. Based on the results shown in Fig. 4, phosphorylation-dependent binding of 14–3–3 protein to Numb appears to coincide with the impairment of AP-2 binding. These results raise two possible mechanisms for the regulation of Numb-AP-2 interaction by phosphorylation: (i) phosphorylation-dependent recruitment of 14–3–3 protein to Numb may generate a stable complex that cannot interact with the AP-2 complex; and (ii) phosphorylation of Numb at either

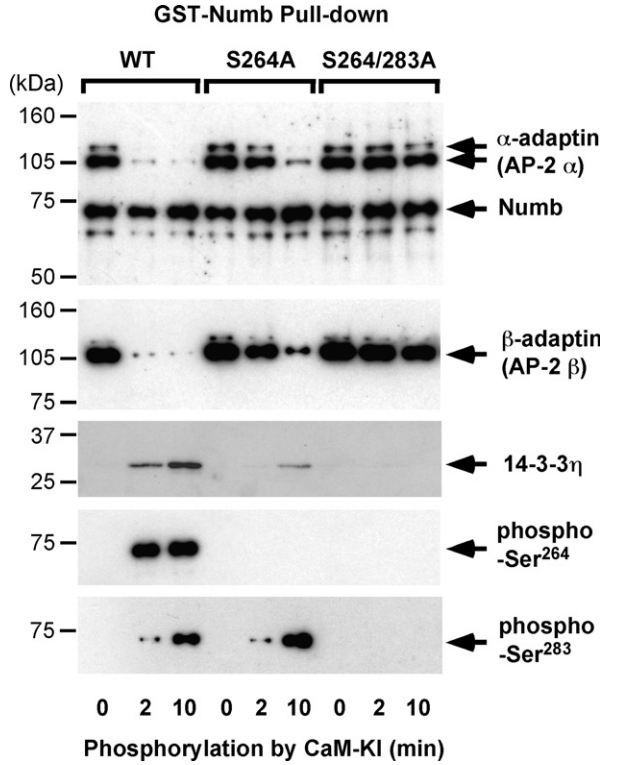

Fig. 4. *Phosphorylation of Ser^{264} and Ser^{283} impairs the interaction of Numb with the AP-2 complex*. Rat brain extract was subjected to a pull-down assay using GST fusions of full-length wild-type (WT), Ser264Ala (S264A), or Ser264/283Ala (S264/283A) Numb (10 μg) that was left untreated (0 min) or phosphorylated with activated CaM-KI for 2 or 10 min. Numb-binding proteins were eluted by cleavage of the Numb proteins from GST using PreScission protease, essentially as described in Fig. 3. Numb-interacting proteins were analyzed by Western blotting using antibodies to-α-adaptin and Numb (top panel), β-adaptins (second panel from top), or 14–3–3η (third panel from top). Phosphorylation of Ser^{264} (second panel from bottom) and Ser^{283} (bottom panel) was also examined using site-specific phosphoNumb antibodies.

Ser^{264} or Ser^{283} may be sufficient to inhibit the binding of Numb to the AP-2 complex by a mechanism independent of the recruitment of 14–3–3.

In summary, we demonstrated here that the interaction of Numb with the AP-2 complex is dynamically regulated by phosphorylation of Numb in its central portion. In contrast, Numb interacts with α-adaptin, a component of the AP-2 complex, through a C-terminal DPF motif [12] and not the central region containing Ser^{264} and Ser^{283}. We also show that the phosphorylation of either Ser^{264} or Ser^{283} in the central region of Numb recruits 14–3–3 proteins, which coincides with the inhibition of the AP-2 binding or the dissociation of AP-2 from Numb. These findings suggest that phosphorylation-dependent binding of 14–3–3 protein may regulate AP-2 binding to Numb.

The interaction of Numb with α-adaptin is involved in Numb-mediated asymmetric cell division in *Drosophila* [13]. A recent study also indicated that Numb is colocalized with AP-2 at substratum plasma membrane punctate and cortical membrane-associated vesicles and that protein kinase C-dependent phosphorylation in the central region of Numb regulates the translocation of Numb from the cortical membrane to the cytosol [18]. The interaction of Numb with the endocytic component has shown to be involved in axonal growth in hippocampal neurons through endocytosis of L1, a neuronal cell adhesion molecule [15]. These previous findings indicate that the function of Numb can be regulated by its interaction with the AP-2 complex. Our current results extend these findings and establish for the first time that the Numb-AP-2 interaction can be controlled by direct phosphorylation. It can be speculated that the phosphorylation of Numb plays an important role in physiological functions of Numb, including cell fate determination during neurogenesis or axon growth during neural development. Therefore, to further understand the function of Numb in endocytosis, additional experiments on the spatial and temporal regulation of Numb phosphorylation are needed.

Acknowledgements: We thank Takaki Nimura (Kagawa University) for his excellent technical assistance. This work was supported in part by Grant-in aid for Scientific Research 17570115 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to H.T.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.09.043.

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