Interaction of Ash/Grb-2 via its SH3 domains with neuron-specific p150 and p65

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We found that 180 kDa, 150 kDa (p150), 110 kDa, 100 kDa and 65 kDa (p65) proteins comprise the major Ash/Grb-2-binding proteins in bovine brain. Among these proteins, 180 kDa and 100 kDa proteins have already been identified as Sos and dynamin respectively. Here, p150 and p65 were affinity-purified with glutathione S-transferase–Ash fusion protein and their partial amino acid sequences were determined. Analysis showed p150 and p65 to be new proteins. These two proteins bind to both the N-terminal SH3 domain and the C-terminal SH3 domain of Ash. It was found that p150 and p65 are expressed predominantly in brain, although Ash is widely distributed in all tissues examined by Western blots. Immunohistochemical staining of rat brain showed p150 and p65 to be localized in a variety of neurons in the cerebellum and hippocampus, with p65 being especially concentrated in the nerve terminal. When the Ash-binding-motif peptide of the epidermal growth factor receptor was used to detect complexes formed with Ash *in vivo*, 180 kDa, 150 kDa, 110 kDa, 100 kDa and 65 kDa proteins were also bound; this shows that these proteins form complexes with Ash in brain. In addition, p150 and p65 co-immunoprecipitated with Ash. All these results suggest that Ash may function as a regulator of synaptic vesicle transport through dynamin, p150 and p65.

INTRODUCTION

Ash/Grb-2 [1,2], which is composed of one SH2 and two SH3 domains, has been found to link the signal between receptor tyrosine kinase and Sos (Ras GDP/GTP exchange protein), resulting in Ras activation. In this case, the SH3 domain of Ash binds to the C-terminal proline-rich region of Sos [3–9], whereas the SH2 domain binds to tyrosine phosphorylation sites in a number of proteins such as the epidermal growth factor (EGF) receptor, Shc, IRS-1 and Syp [10–15]. Thus, it is demonstrated that Ash is the adaptor molecule that physically associates with Sos, allowing ligand-activated receptor tyrosine kinase to modulate Ras activity. It has been shown that microinjection of the antibody against Ash into cells abolishes the organization of actin stress fibres [16], which is controlled by Rho and Rac proteins [17,18], in addition to the signals leading to Ras activation, suggesting that there are other downstream Ash signals.

Dynamin is a GTPase that is required for synaptic vesicle endocytosis, as indicated by its similarity to the product of the *Drosophila shibire* gene [19,20]. It binds to the Ash SH3 domain through its C-terminal proline-rich region [21–23]. The binding of dynamin to Ash stimulates GTPase activity and the complexes are found to co-immunoprecipitate.

Later, when Ash-binding proteins in rat brain were investigated, two other brain-specific proteins, synapsin I and p145, were found to bind the Ash SH3 domain [24]. Synapsin I is a synaptic vesicle-associated nerve terminal protein that interacts with actin and is thought to mediate the interactions of synaptic vesicles with the presynaptic cytomatrix. On the other hand, a fourth Ash-binding protein in brain, p145, is present exclusively in neurons and co-localizes with dynamin, suggesting important roles in endocytosis [25].

All these results suggest that Ash may play a crucial role in the regulation of endo/exocytosis of synaptic vesicle. Therefore, to elucidate the roles of Ash in endo/exocytosis, we attempted to clarify the downstream signallings of Ash in brain. We purified proteins bound to the Ash SH3 domains from bovine brain, and found that 180 kDa, 150 kDa, 110 kDa, 100 kDa and 65 kDa proteins comprise the major Ash-binding proteins in bovine brain. According to the partial amino acid sequences of these proteins, p150 and p65 seemed to be new proteins. p150 and p65 proteins are found to be expressed exclusively in brain and localize in nerve terminals. Moreover, these proteins associate preferentially with Ash rather than phospholipase C γ1 (PLC γ1) or phosphatidylinositol 3-kinase (PI 3-kinase) *in vivo*.

EXPERIMENTAL

Antibodies

Polyclonal anti-(Ash/Grb-2) antibody was prepared by the methods described previously [21]. Polyclonal anti-Sos antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.). Anti-dynamin antibody was prepared according to the methods of Noda et al. [26]. Anti-(PLC γ1) and anti-(PI 3-kinase 85 kDa subunit) antibodies were prepared by the methods described previously [27,28]. Anti-p150 and anti-p65 antibodies were prepared by injecting the protein bands into rabbits after Ash-binding proteins were separated by SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (ATTO Co., Tokyo). These antibodies were purified from sera with strips of PVDF membranes containing 150 kDa or 65 kDa protein.

Purification of Ash-binding proteins from bovine brain

Recombinant glutathione S-transferase (GST)–Ash fusion protein [21] coupled to glutathione–agarose (Pharmacia, LKB Biotechnology) was used to affinity-purify Ash-binding proteins

Abbreviations used: PLC γ1, phospholipase C γ1; PI 3-kinase, phosphatidylinositol 3-kinase; GST, glutathione S-transferase; DIFP, di-isopropyl fluorophosphate; PVDF, polyvinylidene difluoride; EGF, epidermal growth factor.

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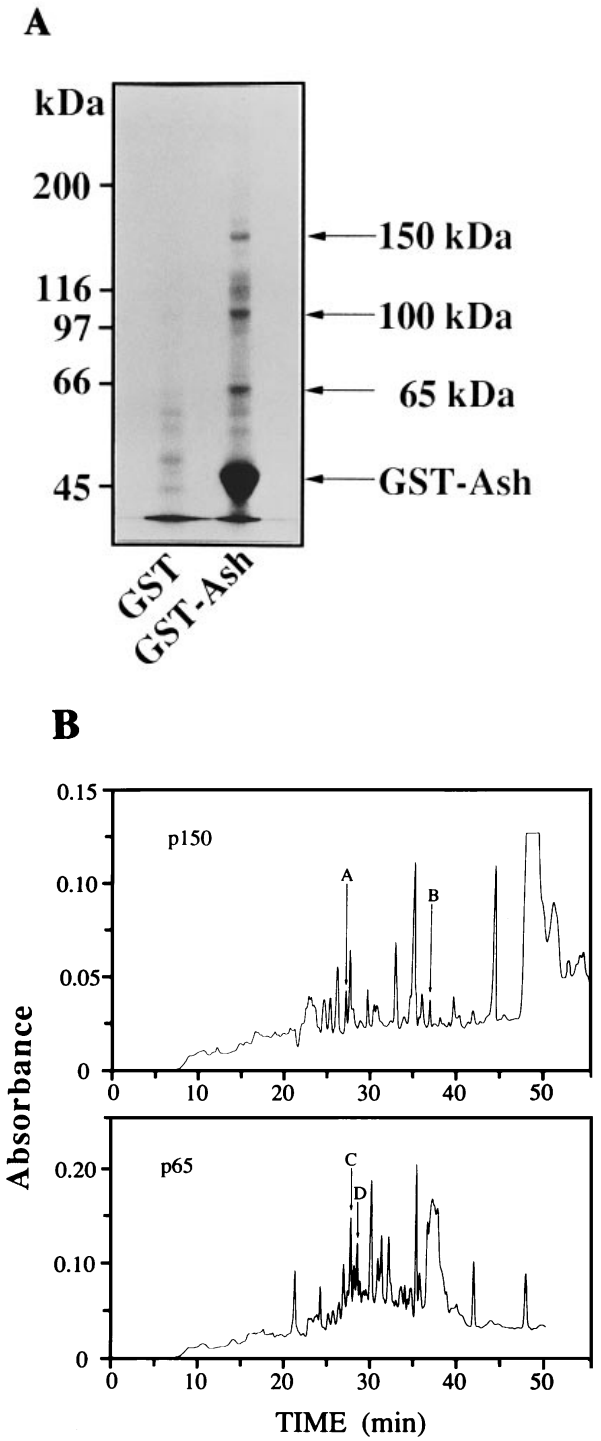

Figure 1 Partial amino acid sequences of p150 and p65

(**A**) Proteins bound to the GST–Ash affinity column. Bovine brain cytosol fractions were applied to a GST–Ash affinity column and the bound proteins were eluted with glutathione. The proteins were separated by SDS/PAGE and stained with Coomassie Brilliant Blue. (**B**) C18 reverse-phase column chromatography and determination of partial amino acid sequences of p150 and p65. Protein bands of p150 and p65 transferred to PVDF membranes were digested with lysylendopeptidase. The resultant peptides were separated by C18 reverse-phase column chromatography. Absorbance was measured at 215 nm. Four peaks from each protein were analysed using a peptide sequencer. Among them, two peptides (peaks A and B) from p150 and two peptides (peaks C and D) from p65 could be sequenced. Other peptides could not be determined for amino acid sequences because some peaks consisted of mixtures of peptides and others did not afford enough signal to sequence. Determined amino acid sequences are as follows: A, DGARSVSRTIQNNFFD; B, QEAIDVLLLGNTLNSDLA; C, NPEITTNRFYGPQINNISHT; D, RRDPPNGPNLPMATV.

from the cytosolic fractions of bovine brains. Bovine brains (1.5 kg) were homogenized in a Waring blender in Tris/HCl (pH 7.4) containing 1 mM EDTA, 0.1 mM PMSF, 0.1 mM diisopropyl fluorophosphate (DIFP), 1 μg/ml leupeptin and 1 μg/ml aprotinin. The homogenates were centrifuged at 100000 ***g*** for 1 h and the supernatants were used as bovine cytosol fractions for purification of Ash-binding proteins. To remove endogenous GST from the brain cytosol fractions, the supernatants were first applied to a glutathione–agarose column. The flowthrough fractions were next applied to a column of GST–Ash–glutathione beads. The column was washed with 20 mM Tris/HCl (pH 7.4) containing 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DIFP, 1 % (v/v) Triton X-100 and 0.5 M NaCl and then eluted with 20 mM Tris/HCl (pH 7.4) containing 50 mM glutathione. Ash-binding proteins eluted from the column were separated by SDS/PAGE, transferred to PVDF membranes and the membranes were stained with Ponceau S. Protein bands at 150 kDa and 65 kDa were cut out.

Partial amino acid sequences of p150 and p65

Membrane pieces containing p150 or p65 were digested with lysylendopeptidase. The resultant peptides were separated on a reverse-phase C18 column with a 0 to 60 % gradient of acetonitrile in 0.1 % trifluoroacetic acid. Amino acid sequences of several purified peptides were determined with an Applied Biosystems Sequencer.

Production of GST fusion proteins

GST–Ash(N-SH3) and GST–Ash(C-SH3) were prepared by the methods described previously [29]. The cDNA encoding PLC γ1 was digested with *Hin*cII and *Hpa*II, which recognize nucleotides 2204 and 2560 respectively. The resulting cDNA fragment was inserted into the pGEX-3X vector. The recombinant plasmid was transformed into the XL-I Blue *Escherichia coli* strain. GST–PI 3-kinase was prepared as an *Hin*dIII restriction fragment containing the N-terminus to residue 388 [30].

Immunohistochemical study

Wistar rats (12–14-weeks-old) were perfused under pentobarbital anaesthesia with 1 ml of 0.9 % NaCl followed by 200 ml of 4 % paraformaldehyde in PBS. The brains were removed and preserved in a fixative overnight, and then transferred into 0.1 M phosphate buffer containing 15 % (w/v) sucrose. The brains were sectioned sagittally on a freezing microtome at 50 μm. Each portion was stained immunohistochemically by the avidin–biotin–peroxidase method. Immunostaining was performed by the free-floating method and immunoreactive products were visualized with 0.05 % 3,3′-diaminobenzidine and 0.01 % hydrogen peroxide.

EGF-receptor Ash-binding-motif peptide

The tyrosine-phosphorylated peptide around the Ash-binding site of activated EGF receptor (PVPEYINQSVPK; the tyrosine in this peptide is phosphorylated) was purchased from Peptide Institute, Inc. (Osaka, Japan). The peptide (1 mg) was conjugated to CNBr-activated Sepharose (Pharmacia-LKB Biotechnology). Bovine brain cytosol fractions were applied to the peptide–Sepharose column, and the bound proteins were released from the column with SDS sample buffer.

RESULTS

Partial amino acid sequences of p150 and p65

Bovine brain cytosol fractions were applied to a GST–Ash–glutathione–agarose column to purify Ash-binding proteins. Proteins that bound to the column were eluted with glutathione and separated by SDS/PAGE. As shown in Figure 1(A), several proteins were bound to the GST–Ash fusion affinity column. In particular, 180 kDa, 150 kDa, 110 kDa, 100 kDa and 65 kDa proteins were found to be the Ash-binding proteins in bovine brain cytosol. It is likely that these proteins specifically bound to Ash but not to GST. Since the 180 kDa and 100 kDa proteins have been identified as Sos and dynamin, respectively, we tried to determine the amino acid sequences of the 150 kDa (p150) and 65 kDa (p65) proteins, because the amounts of these proteins seemed sufficient for determination. After Ash-binding proteins were subjected to SDS/PAGE and transferred to PVDF membranes, the membranes were stained with Ponceau S. The protein bands of p150 and p65 were cut out and digested with lysylendopeptidase. The resultant peptides were separated by C18 reverse-phase column chromatography. We analysed four sharp peaks from each protein by a peptide sequencer. Among them, two peptides (A and B) from p150 and two peptides (C and D) from p65 could be sequenced (Figure 1B), and we obtained amino acid sequences from p150 (A: DGARSVSRTIQNNFFD, B: QEAIDVLLLGNTLNSDLA) and from p65 (C: NPEITTNR-FYGPQINNISHT, D: RRDPPNGPNLPMATV). None of these amino acid sequences showed any sequence similarity to other proteins in the protein sequence database. Therefore, for further characterization of these proteins, we prepared antibodies against p150 and p65.

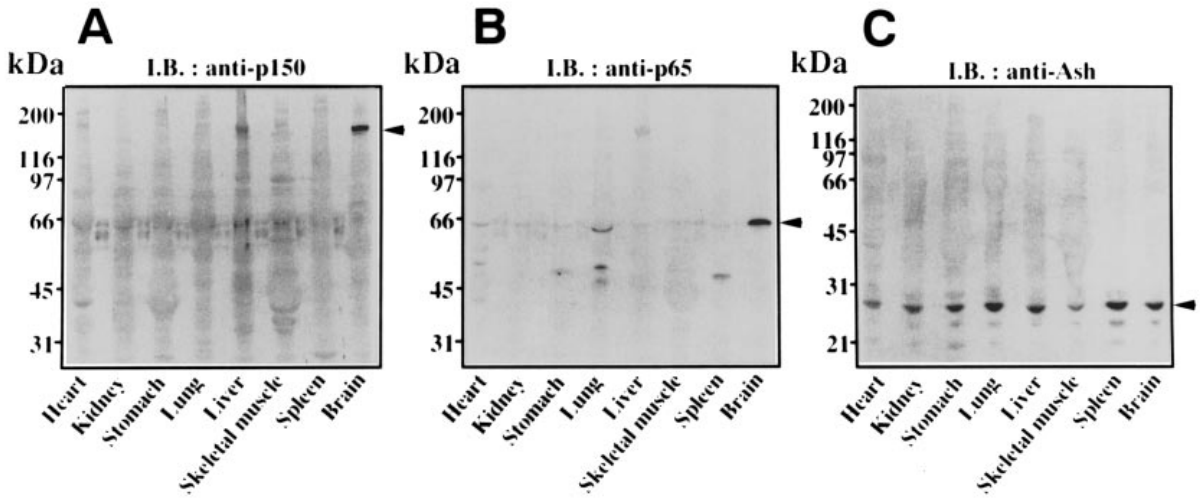

Figure 2 Tissue distribution of p150, p65 and Ash

Various rat tissues were homogenized and centrifuged at 100000 *g* for 1 h. The supernatants were subjected to SDS/PAGE and Western blots with anti-p150 (**A**), anti-65 (**B**) and anti-Ash (**C**) antibodies.

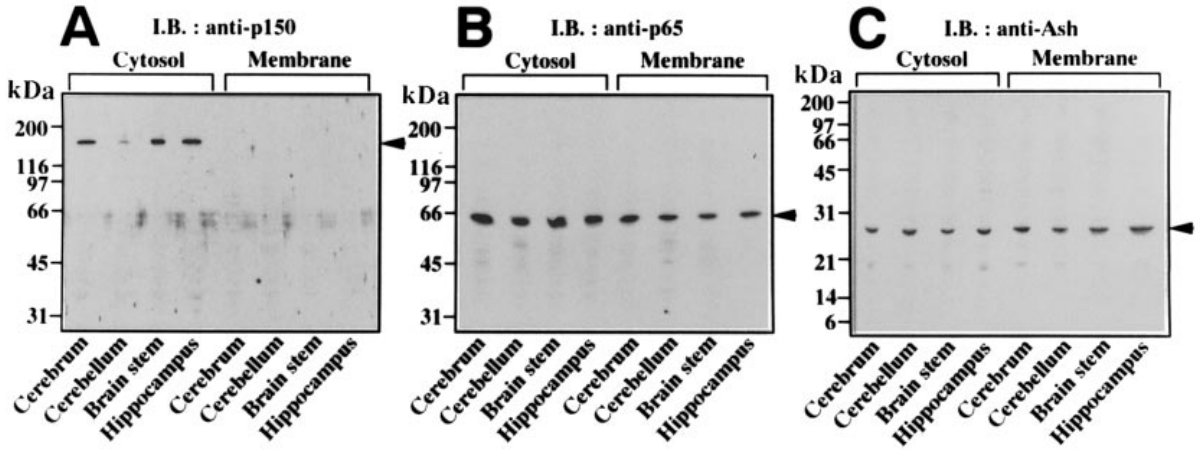

Figure 3 Distribution of p150, p65 and Ash in bovine brain

Bovine cerebrum, cerebellum, brain stem and hippocampus were homogenized and centrifuged at 100000 ***g*** for 1 h. The supernatant and precipitate fractions were used as cytosol and membranes, respectively. These samples were subjected to SDS/PAGE and transferred to PVDF membranes. The membranes were stained with anti-p150 (**A**), anti-p65 (**B**) and anti-Ash (**C**) antibodies.

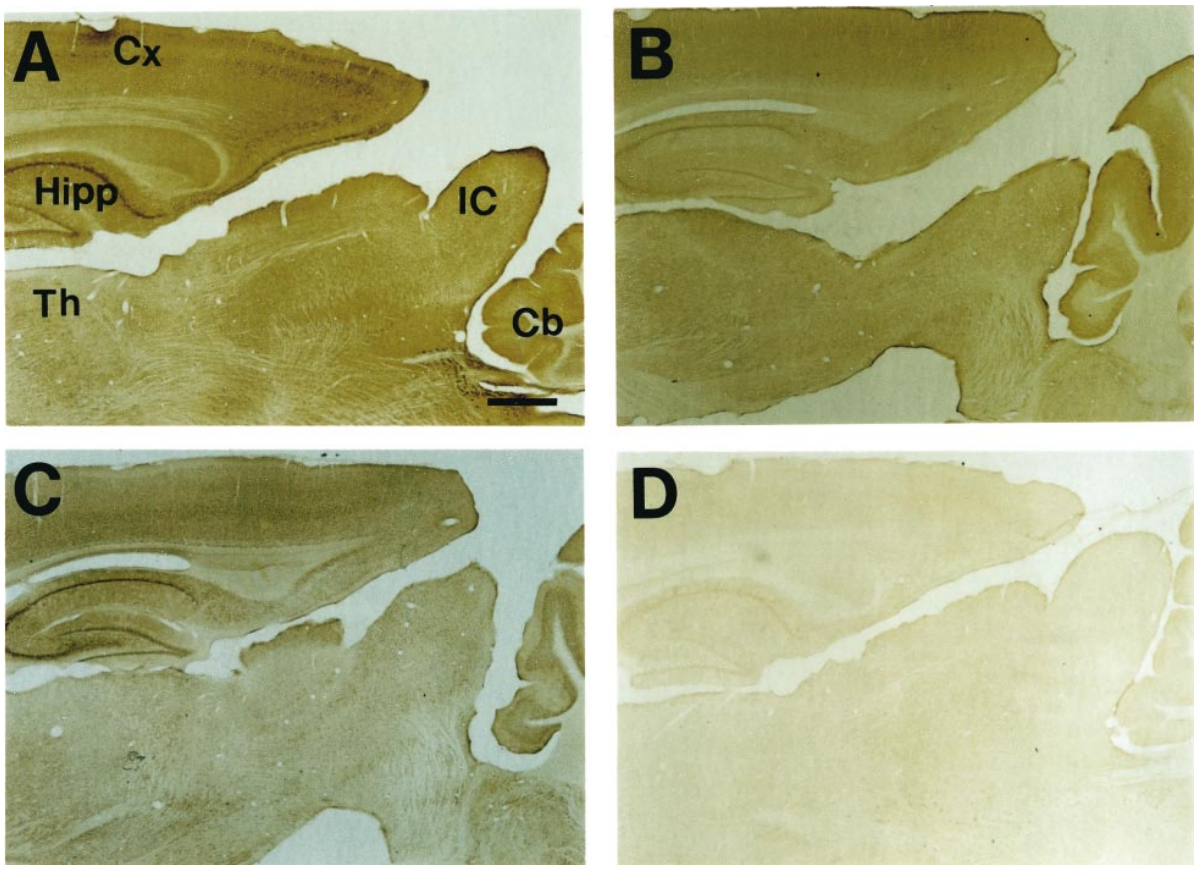

Figure 4 Immunohistochemical staining of rat brain by anti-p150, anti-p65 and anti-Ash antibodies

Sagittal sections of rat brain were stained with anti-Ash (**A**), anti-p150 (**B**) and anti-p65 (**C**) antibodies. Control staining with non-immune serum is shown in (**D**). Abbreviations: Cx, cortex; Hipp, hippocampus; IC, inferior colliculus; Cb, cerebellum; Th, thalamus. Bar in the Figure represents 1 mm.

Preparation of antibodies against p150 and p65

Antibodies against p150 and p65 were made by immunizing rabbits with PVDF membranes containing p150 and p65. The sera were affinity-purified using p150 or p65 blotted on to PVDF membranes. The anti-p150 antibody reacted with a 150 kDa band on immunoblotting of crude bovine brain homogenates and GST–Ash-binding proteins (results not shown). Similarly, the anti-p65 antibody reacted only with the 65 kDa band on immunoblotting of crude homogenate and GST–Ash-binding proteins. These results show that anti-p150 and anti-p65 antibodies are specific for the 150 kDa and 65 kDa Ash-binding proteins respectively. These antibodies also reacted with rat brain p150 and p65 (results not shown).

Tissue distribution of p150, p65 and Ash

To examine the tissue distribution of p150 and p65, various rat tissues were homogenized and centrifuged at 100000 ***g*** for 1 h. The supernatant fractions were subjected to SDS/PAGE and Western blot analyses with anti-Ash, anti-p150 and anti-p65 antibodies (Figure 2). As shown in Figure 2(A), p150 was expressed predominantly in the brain. We also detected a weak band in a blot of liver lysates. Similarly, p65 was predominantly detected in brain and slightly in lung (Figure 2B). The bands in the lower-molecular-mass area on the blots from lung and spleen lysates seem to be due to non-specific staining rather than degradative products, since they were detected even when samples were treated with SDS sample buffer immediately after tissue excision. However, Ash was expressed ubiquitously in all tissues examined (Figure 2C). These results suggest that p150 and p65 function specifically in the brain, while Ash is expressed widely. To further examine the brain distribution of p150 and p65, cerebrum, cerebellum, brain stem and hippocampus were collected from bovine brains. From these tissues, membrane (100000 ***g*** precipitate fraction) and cytosol (100000 ***g*** supernatant) fractions were separated. Results of immunoblotting of p150 and p65 are shown in Figure 3. Ash was present extensively in both the membrane and cytosol fractions of all tissues. Similarly, p65 was present in both the membrane and cytosol fractions of all tissues. However, p150 was present only in the cytosol, but the level of p150 in cerebellum was lower compared with other brain areas.

Immunohistochemical staining of p150 and p65 in rat brain

Immunohistochemical staining using affinity-purified antibodies was performed, to characterize further the subcellular localization of p150 and p65. Ash, p150 and p65 were distributed widely in brain (Figure 4). In particular, cerebral cortex, cerebellum and hippocampus were strongly positive for immunostaining by anti-Ash, anti-p150 and anti-p65 antibodies. However, there are differences in the subcellular distribution of the three proteins as revealed by higher magnification of the cerebellum and hippocampus (Figure 5). Immunoreactivity of Ash was strong in

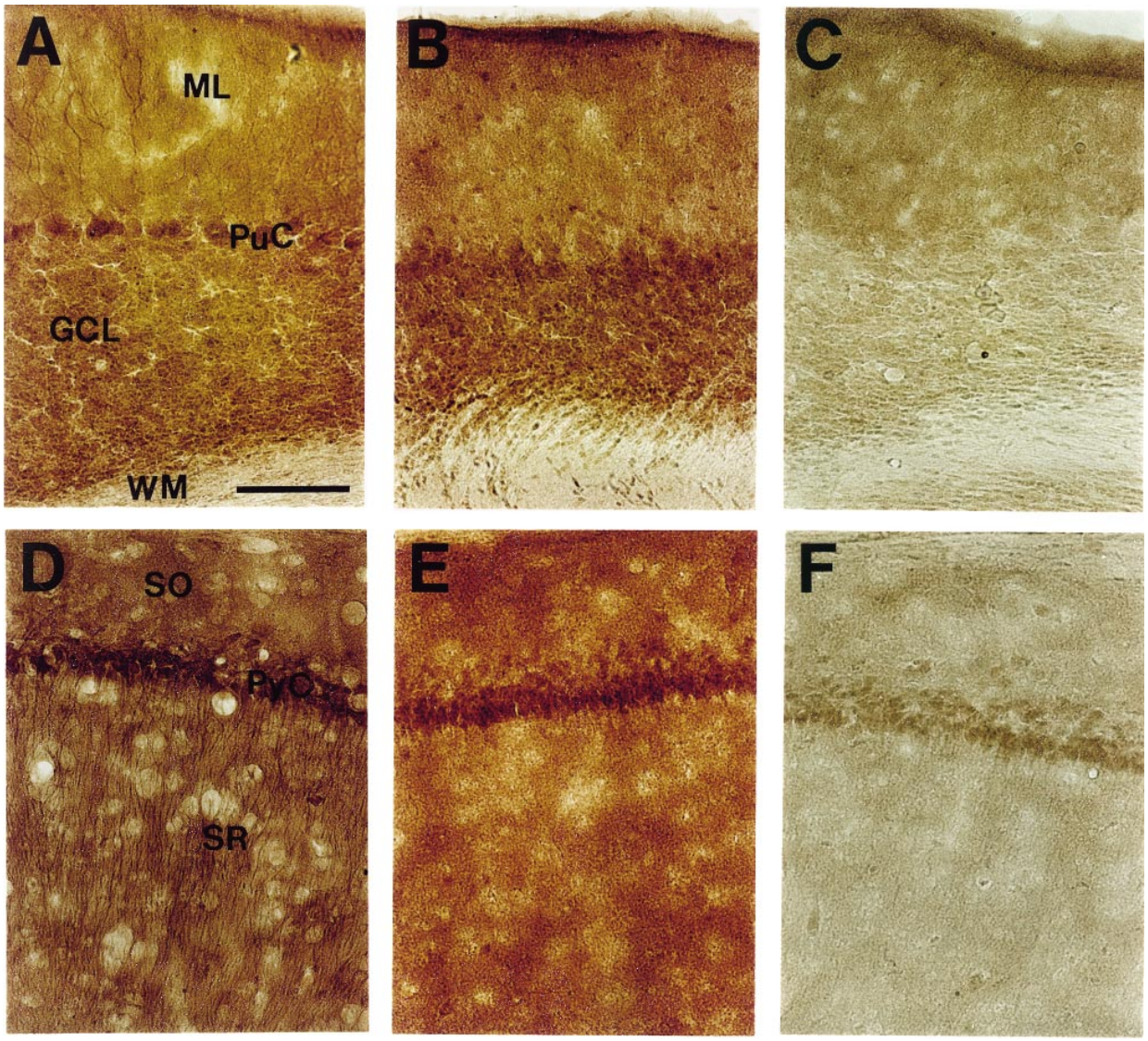

Figure 5 Immunohistochemical staining of cerebellum (A–C) and the CA1 area of hippocampus (D–E)

(**A**) and (**D**), (**B**) and (**E**), and (**C**) and (**F**) show Ash, p150 and p65 staining respectively. Abbreviations: ML, molecular layer; Puc, Purkinje cell; GCL, granule cell layer; WM, white matter, SO, stratum oriens; SR, stratum radiatam; Pyc, pyramidal cell. Bar in the Figure represents 100 μm.

cerebellar Purkinje cells with their dendritic shafts and satellite cells (Figure 5A) and hippocampal pyramidal neurons with their apical dendrites (Figure 5D). Intense immunoreactivity of p150 was observed in the cell bodies of Purkinje neurons, the molecular layer, satellite cells, basket cells and mossy fibre terminals in the cerebellum (Figure 5B). In the hippocampus, the cell bodies of the pyramidal neurons and interneurons stained heavily for p150 (Figure 5E). The immunoreactivity to p65 was weak in the cell bodies of Purkinje neurons but strong in the molecular layer, with the terminals of the parallel fibres showing possible intense immunoreactivity (Figure 5C). In the hippocampus, p65 immunoreactivity was seen in the pyramidal neurons and their dendrites (Figure 5F).

p150 and p65 bind to the SH3 domains of Ash

To examine the domain of Ash to which p150 and p65 bind, we prepared GST fusion proteins such as GST–Ash(N-SH3), GST–Ash(C-SH3) and GST–Ash(SH2). Affinity columns were prepared with these GST fusion proteins and the proteins binding to each SH domain were checked (Figure 6). p150 and p65 bound to both SH3 domains (N-terminal and C-terminal), but not to the SH2 domain. Next, to determine whether the binding of these proteins is specific to Ash SH3 domains, we examined binding to the PLC γ1 SH3 and PI 3-kinase SH3 domains. As shown in Figure 7, p150 and p65 bound preferentially to the Ash SH3 domain. On longer exposure, however, we could detect them bound to the PI 3-kinase SH3 domain, but not to the PLC γ1 SH3 domain. These results suggest the close association of p150 and p65 with the Ash SH3 domains.

Ash-binding proteins *in vivo*

To determine whether p150 and p65 bind to Ash *in vivo*, we prepared a synthetic peptide containing the EGF receptor Ash-binding site (PVPEYINQSVPK, Y is phosphorylated). The EGF-

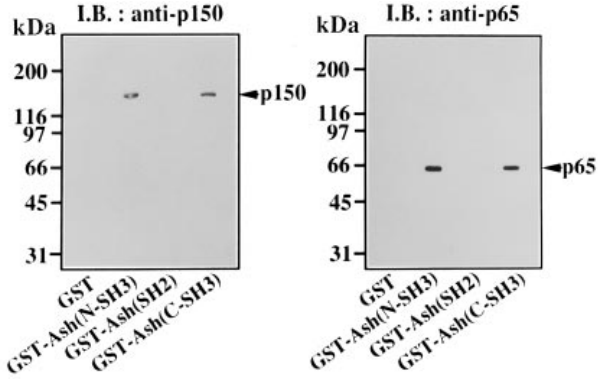

Figure 6 p150 and p65 bind to the SH3 domains of Ash

Bovine brain cytosol fractions were applied to GST–Ash(N-SH3), GST–Ash(C-SH3) and GST–Ash(SH2) beads and the bound proteins were analysed by Western blotting (I. B.) with anti-p150 and anti-p65 antibodies.

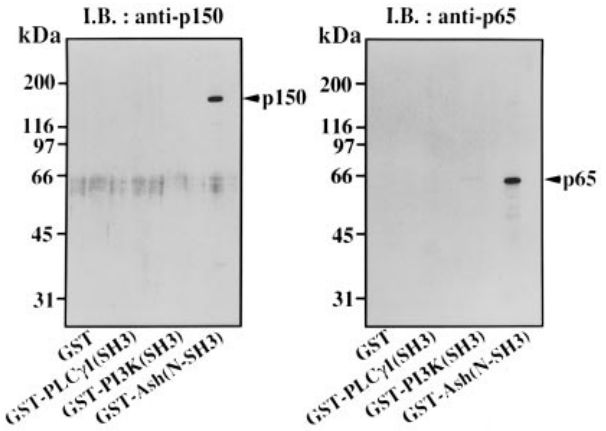

Figure 7 Specific binding of p150 and p65 to Ash-SH3

Bovine brain cytosol fractions were applied to GST–Ash(N-SH3), GST–PLC γ1(SH3), and GST–PI 3-kinase(SH3) beads and the bound proteins were analysed by Western blotting (I. B.) with anti-p150 and anti-p65 antibodies.

receptor Ash-binding-motif peptide was coupled to agarose beads, and bovine brain cytosol fractions were applied to the peptide column. The peptide can be expected to recognize Ash and bind through the Ash SH2 domain. Therefore, proteins other than Ash that absorb to this column are considered to bind through an SH3 domain of Ash. Peptide-bound proteins were separated by SDS/PAGE, transferred to PVDF membranes and stained with Coomassie Brilliant Blue (Figure 8A, left-hand lane). Various protein bands were detected with the major bands at 180 kDa, 150 kDa, 110 kDa, 100 kDa, 65 kDa, 55 kDa and 28 kDa. These proteins are comparable with those that bound to the GST–Ash affinity column except for Ash. Moreover, we found that the 180 kDa and 100 kDa proteins were Sos and dynamin respectively, using antibodies against Sos and dynamin. The 150 kDa and 65 kDa proteins were identified as p150 and p65 by their specific antibodies. These data suggest that all these proteins form complexes with Ash *in vivo*. Furthermore, we examined whether p150 and p65 co-immunoprecipitate with Ash, PLC γ1 or PI 3-kinase. Bovine brain cytosol fractions were immunoprecipitated with anti-p150 or anti-p65 antibodies, and

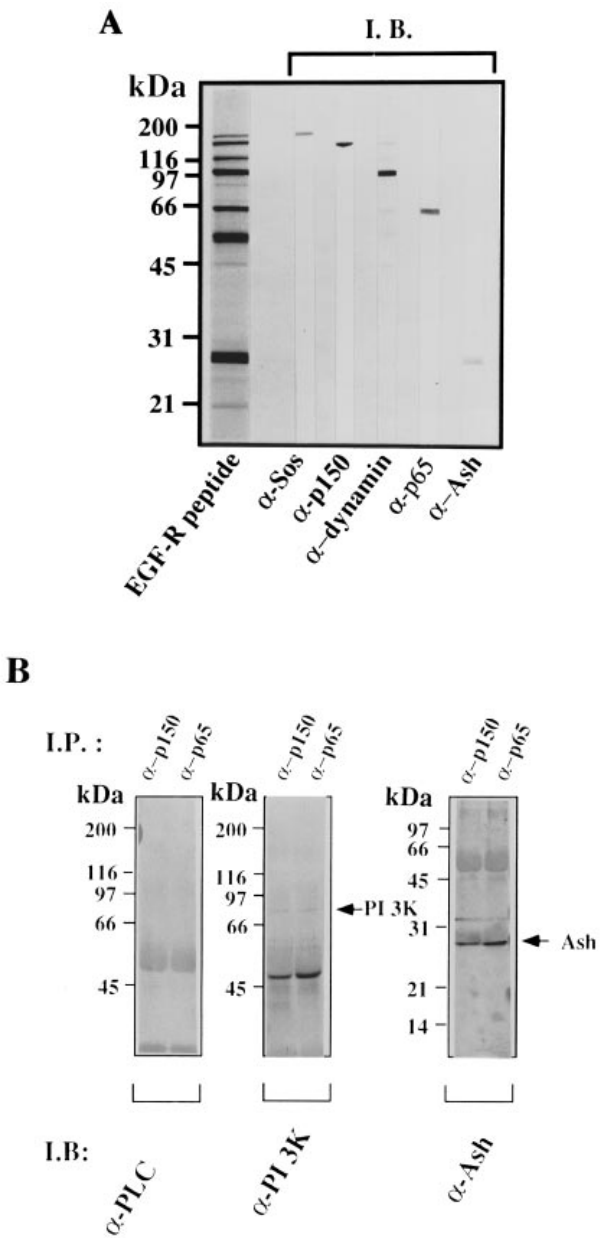

Figure 8 Proteins bound to Ash *in vivo*

(**A**) Ash-binding-motif peptide-bound proteins. Bovine brain cytosol fractions were applied to an Ash-binding-motif peptide column. Bound proteins were analysed by SDS/PAGE and Western blotting. The left-hand lane shows the Coomassie Brilliant Blue staining of the peptide binding proteins separated by SDS/PAGE. Immunoblottings were carried out using anti-Sos, anti-p150 (α-p150), anti-dynamin, anti-p65 (α-p65), and anti-Ash (α-Ash) antibodies. (**B**) p150 and p65 co-immunoprecipitate with Ash. Bovine brain cytosol fractions were immunoprecipitated (I. P.) by anti-p150 or p-65 antibody and Protein A–Sepharose. The precipitates were analysed with Western blotting (I. B.) by anti-Ash, anti-(PLC γ1) and anti-(PI 3-kinase 85 kDa subunit) antibodies. The bands around the 50 kDa area in anti-(PI 3-kinase) antibody staining are heavy chain of IgG.

the precipitates were analysed with Western blotting by anti-Ash, anti-(PLC γ1) or anti-(PI 3-kinase 85 kDa subunit) antibody. As shown in Figure 8(B), the immunoprecipitates contained large amounts of Ash and trace amounts of PI 3-kinase. On the other hand, PLC γ1 was not detected in the immunoprecipitates. These results show that p150 and p65 closely associate with Ash *in vivo*.

DISCUSSION

We demonstrated that the major proteins that bind to Ash domains are 180 kDa, 150 kDa, 110 kDa, 100 kDa, 65 kDa and 55 kDa proteins. Among them, the 180 kDa and 100 kDa proteins were found to be Sos and dynamin respectively. These proteins have already been demonstrated to bind the Ash SH3 domain through the proline-rich region. The binding of Ash to Sos causes an enhancement of the GDP/GTP exchange reaction of Ras and activated Ras. On the other hand, the binding of Ash to dynamin results in a stimulation of the GTPase activity of dynamin. Since the 55 kDa protein was found to be β-tubulin by peptide sequencing, we tried to characterize the remaining proteins, p150 and p65. Very recently, a 145 kDa protein (p145) has been found to bind Ash through its SH3 domain and to be localized in synaptic terminals [24,25]. p145 could be the same protein as our p150, because the localization and characterization of the two proteins resemble each other. In addition to p145, synapsin I has also been demonstrated to bind to the Ash SH3 domain through C-terminal proline-rich regions [24]. However, we did not detect synapsin I as a major Ash-binding protein when we used GST–Ash beads. Furthermore, even when we used the EGF receptor Ash-binding-motif peptide for the binding assay, synapsin I did not bind effectively. However, since we detected a faint band around 75 kDa from both the GST–Ash and the peptide columns, this may be synapsin I.

Here, we demonstrate that p150 and p65 preferentially associate with the SH3 domains of Ash rather than the SH3 domains of PI 3-kinase and PLC γ1, showing that these proteins mostly receive signals from Ash. In addition, these proteins are present exclusively in the nervous system. In particular, we found that p150 and p65 appeared to concentrate in nerve terminals. Taking together the fact that dynamin, p150 and p65 are localized in nerve terminals, these proteins may play important roles in the exo/endocytotic cycle of synaptic vesicles.

Synapsin I has multiphosphorylation sites and its phosphorylation is increased by the stimulus of Ca^{2+}-dependent release of neurotransmitter, such as the depolarization of synaptosomes [31]. The phosphorylation of synapsin I causes inhibition of its binding to actin and synaptic vesicles. In contrast to its effect on synapsin I, depolarization of synaptosomes causes the dephosphorylation of dynamin [32]. This opposite action may reflect participation in opposite stages of the exo/endocytotic cycle of synaptic vesicles. McPherson et al. [25] reported that p145 is dephosphorylated in parallel with dynamin after synaptosomal depolarization, suggesting that p145/p150, like dynamin, participates in synaptic vesicle endocytosis. We do not know whether p65 is phosphorylated by depolarization, but it is possible that p65, together with dynamin and p145/p150, is involved in synaptic vesicle transport. If dynamin, p145/p150 and p65 play important roles in synaptic vesicle endocytosis, Ash regulates the functions of these proteins in brain. In the nervous system, Ash forms complexes with Sos, p145/p150, dynamin, synapsin I and p65, enabling them to function in harmony. In this case, it remains to be determined whether there are any tyrosine kinases upstream of Ash and whether these tyrosine kinases generate signals through Ash, leading to collaborative functions of the exo/endocytotic cycle of synaptic vesicles.

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