Interaction of the SH2 Domain of Fyn with a Cytoskeletal Protein, β-Adducin*

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Fyn is a Src family tyrosine kinase expressed abundantly in neurons and believed to have specific functions in the brain. To understand the function of Fyn tyrosine kinase, we attempted to identify Fyn Src homology 2 (SH2) domain-binding proteins from a Nonidet P-40-insoluble fraction of the mouse brain. β-Adducin, an actin filament-associated cytoskeletal protein, was isolated by two-dimensional gel electrophoresis and identified by tandem mass spectrometry. β-Adducin was tyrosine phosphorylated by coexpression with wild type but not with a kinase-negative form of Fyn in COS-7 cells. Cell staining analysis showed that coexpression of β-adducin with Fyn induced translocation of β-adducin from the cytoplasm to the periphery of the cells where it was colocalized with actin filaments and Fyn. These findings suggest that tyrosine-phosphorylated β-adducin associates with the SH2 domain of Fyn and colocalizes under plasma membranes.

Neurons in mammalian brain express Src family tyrosine kinases including Src, Fyn, Lyn, and Yes, which have been implicated in neuronal development, neuronal network formation, and synaptic transmission. In contrast to their diverse functions, mice lacking Src, Lyn, or Yes did not show any prominent neurological phenotype, probably because they can compensate each other (1). However, loss of the Fyn gene resulted in several behavioral abnormalities accompanied by changes in the construction of the neuronal network (2–7). We have recently found that Fyn-deficient mice have longer free running periods in the circadian rhythm of locomotive activity and a disorder of neuronal distribution in the suprachiasmatic nucleus, a master circadian oscillator (8). These findings suggest that Fyn might have specific and essential functions in the brain.

One possible function of these kinases is to regulate cytoskeletal organization and cell-to-cell contact. Rearrangement of the actin cytoskeletal structure was observed in cells transformed by v-Src (9) and those deficient in C-terminal Src family kinase (CSK), the negative regulator of Src family kinases (10, 11). Cells bearing activated forms of Src family kinases exhibit lower stress fiber formation and weaker cell contacts. This may be partly the result of direct phosphorylation of cytoskeletal proteins at their tyrosine residues by these kinases.

Src family kinases form complexes with several cytoskeletal components and signaling molecules at the site of cell contacts and are implicated in neuronal development, neuronal network formation, and synaptic transmission. In contrast to their diverse functions, mice lacking Src, Lyn, or Yes did not show any prominent neurological phenotype, probably because they can compensate each other (1). However, loss of the Fyn gene resulted in several behavioral abnormalities accompanied by changes in the construction of the neuronal network (2–7).

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Wako Chemicals), anti-Fyn polyclonal antibody (FYN3, Santa Cruz), and anti-HA monoclonal antibody (clone 12CA5, Roche Molecular Biochemicals) were purchased. Anti-H9252-adducin antiserum was prepared by immunizing a rabbit with GST-H9252-adducin (amino acids 522–726) fusion protein. Horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories Inc.), horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc.), Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes), and fluorescein isothiocyanate-labeled anti-mouse IgG (ICN) were used as secondary antibodies. Rhodamine phalloidin (Molecular Probes) was used to visualize actin filaments. Other materials and chemicals were obtained from commercial sources.

**Plasmids and GST Fusion Proteins**—To prepare GST-Fyn SH2 fusion protein, cDNA encoding the SH2 domain of Fyn was subcloned into pGEX vector (Amersham Pharmacia Biotech). The fusion protein was then produced in *Escherichia coli* and purified by glutathione-Sepharose (Amersham Pharmacia Biotech) affinity chromatography. To obtain a plasmid expressing HA epitope-tagged H9252-adducin, cDNA encoding mouse H9252-adducin was inserted into the KpnI-EcoRI site of pHM6 vector. pME-SM plasmids expressing wild type and a kinase-negative form of Fyn (Lys-299Met-299) were provided by Dr. T. Yamamoto (22).

**Animals**—Homozygous Fyn-deficient (−/−) mice were produced by inserting lacZ into the SH2, SH3, and tyrosine kinase domains of Fyn in the cells of an embryonic stem cell line (TT2) (3). Homozygous Fyn-deficient (−/−) mice were obtained by crossing heterozygous (+/−) mice. The genotype was analyzed by polymerase chain reaction (4). In the experiments that compared wild type (+/+) with homozygous (−/−) mice, wild type (+/+) or homozygous Fyn-deficient (−/−) mice, solubilized with RIPA buffer (RIPA lysate), and incubated with glutathione-Sepharose coupled with GST (lanes 1 and 2) or GST-Fyn SH2 fusion protein (lanes 3 and 4). Associated proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody. Positions of molecular mass markers are shown on the left. The arrow indicates the SH2-associated phosphoprotein analyzed. B, two-dimensional PAGE of Fyn SH2-associated phosphoproteins. Fyn SH2-associated proteins prepared from wild type mouse brains were resolved by isoelectric focusing with immobilized pH gradient gels in the first dimension followed by SDS-PAGE in the second dimension. After electrophoresis, proteins were transferred to a nitrocellulose membrane followed by detection using anti-phosphotyrosine antibody (anti-pY). Arrows indicate the positions of the Fyn SH2 associated-phosphoproteins, PI3, PI5, and PI7. The direction of the pH gradient is shown at the top. C, protein distribution patterns of RIPA lysate resolved by two-dimensional PAGE. 20 μg of the RIPA lysate was separated by two-dimensional gel electrophoresis as described above and then stained with silver. The right panel is a higher magnification image of the boxed area of the left panel. Arrows and arrowheads indicate the protein spots overlapped with PI5 and PI7, respectively. D, the protein spot overlapped with PI5 was excised, digested with *Achromobacter* protease I, and four peptides were sequenced by tandem mass spectrometry.
Preparation of Tissue and Cell Extracts—Brains of mice were removed quickly after decapitation and homogenized in TNE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/liter aprotinin, 10 mg/liter leupeptin, and 5 mM mercaptoethanol) at a ratio of 10:1 (v/w). After standing for 30 min, the homogenate was centrifuged for 30 min at 48,000 × g, and the pellet was collected. The pellet was washed once with TNE buffer and solubilized in a quarter-volume of RIPA buffer (TNE buffer supplemented with 0.1% SDS and 0.5% deoxycholate). After centrifugation for 30 min at 48,000 × g, the supernatant (RIPA lysate) was transferred to fresh tubes, and the protein concentration was measured by the method of Bradford with bovine serum albumin as a standard.

Cos-7 cells were plated on six-well plates coated with poly-L-lysine at a density of 3.0 × 10⁶ cells/cm², cultured for 24 h, and transfected with 5 μg of DNA using LipofectAMINE (Life Technologies, Inc.). After 24 h, cells were washed twice with phosphate-buffered saline and solubilized with RIPA buffer for 20 min on ice. The lysate was centrifuged at 12,000 × g for 30 min at 4 °C to obtain a clear supernatant.

Pull-down and Immunoprecipitation Assays—For the pull-down assay, lysates were incubated with glutathione-Sepharose bound to GST-Fyn SH2 or GST for 3 h at 4 °C with rotation. After washing seven times with TNE buffer containing 500 mM NaCl, proteins were eluted with 1 mM glutathione. For the immunoprecipitation assay, cell lysates were incubated with protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h, centrifuged to remove nonspecific materials, and the supernatant was incubated with the respective antibodies for 2 h followed by protein G-Sepharose for 1 h. The immunoprecipitate was washed with TNE buffer and analyzed by immunoblotting.

Two-dimensional Gel Electrophoresis and Mass Spectrometry—Proteins were separated by isoelectric focusing with immobilized pH gradients for the first dimension. Dried immobilized pH gradient gels (Immobiline pH 3-10NL, Amersham Pharmacia Biotech) were rehydrated with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, and 0.2% Pharmalyte) containing sample proteins for 12–16 h at room temperature and then focused at 10.5 kilovolt hours for 7-cm gels or 19 kilovolt hours for 13-cm gels. For the second dimension, the gels were equilibrated with equilibration buffer (50 mM Tris-HCl, pH 7.4, 7 M urea, 2 M thiourea, 20 mM dithiothreitol, 2% SDS) and applied onto 8% SDS-polyacrylamide gels.

Proteins were excised from Coomassie Brilliant Blue-stained gels and digested with *Achromobacter* protease I. The resultant peptides were separated by reversed-phase HPLC on a C18 column and subjected to electrospray ionization-MS/MS (Q-TOF, Micromass, Manchester, U. K.) for peptide sequencing, as described previously (23). The MS/MS spectra were interpreted by SeqMS, a software aid for de novo sequencing by MS/MS (24), and the sequence tags obtained, together with the molecular masses, were subjected to MS-Tag (25) for data base searching.
**RESULTS**

**Immunoblotting**—Proteins were separated by SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (T-TBS), incubated with primary antibodies for 2 h, washed with T-TBS, incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h, and washed with T-TBS. Immunoreactivity was visualized with an enhanced chemiluminescence system (PerkinElmer Life Sciences).

**Cell Staining**—COS-7 cells were plated on glass coverslips coated with poly-D-lysine at a density of 1.0 × 10⁴ cells/cm², cultured for 24 h, transfected with respective plasmids by the calcium phosphate method, and then cultured for 24 h. The cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, permeabilized with 0.2% Triton X-100 for 2 min, and blocked with blocking buffer (T-TBS containing 1% bovine serum albumin). Cells were then incubated with primary antibodies diluted in the blocking buffer followed by secondary antibodies. For visualizing the actin filaments, cells were incubated with rhodamine phalloidin together with secondary antibodies. Fluorescence images were examined using a confocal laser scanning microscope (Carl Zeiss).

**Purification and Identification of a Fyn SH2-binding Protein**—To isolate proteins that interact with the Fyn SH2 domain, Nonidet P-40-insoluble materials from mouse brains were solubilized with RIPA buffer and incubated with GST or GST-Fyn SH2 fusion protein coupled with glutathione-Sepharose beads. Several phosphotyrosyl proteins were precipitated with GST-Fyn SH2, but not with GST, indicating that these proteins interacted selectively with the Fyn SH2 domain (Fig. 1A). Among them, a 116 kDa band exhibited lower tyrosine phosphorylation in homozygous Fyn-deficient mice (−/−) than in wild type mice (+/+), indicating that the protein could have a functional relationship with Fyn tyrosine kinase.

Because the 116 kDa band might contain more than one protein, the precipitated proteins were separated by two-dimensional gel electrophoresis. As shown in Fig. 1B, three spots corresponding to the 116 kDa band were detected and termed PI3, PI5, and PI7. Compared with a silver staining pattern of RIPA lysate, PI5 and PI7 spots overlapped with horizontal arrays of protein signals (Fig. 1C), whereas PI3 did not have corresponding protein signals at detectable levels. The spot of PI7 almost overlapped that of anti-focal adhesion kinase as detected by Western blotting (data not shown), so we next tried to identify PI5 protein using MS.

The protein spot (PI5) was excised from a Coomassie-stained gel and digested with *Achromobacter* protease I. Four peptides, isolated by HPLC, were analyzed by electrospray ionization-MS/MS. The resultant spectra, through analysis by SeqMS, gave the sequence tags for each peptide. The data base search using MS-Tag, based on the sequence tags and the molecular masses, revealed the protein as mouse β-adducin with high reliability. In addition, the apparent molecular mass of β-adducin on SDS-PAGE was consistent with that reported previously (26), despite the discrepancy between the apparent (116 kDa) and calculated molecular mass of β-adducin (80 kDa). These results strongly suggested PI5 for β-adducin.

To confirm that PI5 was β-adducin, we prepared a polyclonal antibody against the GST-β-adducin (amino acids 522–726) fusion protein. It detected a single band of 116 kDa in lysates from COS-7 cells expressing exogenous β-adducin, suggesting that it reacted with β-adducin specifically (data not shown). Immunoblotting of a two-dimensional gel with this antibody showed that it reacted a single spot of 116 kDa with pI 5.6, despite the discrepancy between the apparent (116 kDa) and calculated molecular mass of β-adducin (80 kDa). These results strongly suggested PI5 for β-adducin.

To prove the functional relationship between Fyn and β-adducin, we performed the immunoblotting experiment using cell lysates treated with Fyn antisera (Fig. 2A). The data showed that the immunoblotting with anti-β-adducin had a single spot of 116 kDa in lysates from COS-7 cells expressing exogenous β-adducin, suggesting that it reacted with β-adducin specifically (data not shown). Immunoblotting of a two-dimensional gel with this antibody showed that it reacted a single spot of 116 kDa with pI 5.6, despite the discrepancy between the apparent (116 kDa) and calculated molecular mass of β-adducin (80 kDa). These results strongly suggested PI5 for β-adducin.

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was probably because a small portion of β-adducin was highly tyrosine-phosphorylated and showed an acidic shift in isoelectric focusing and lower mobility in SDS-PAGE.

To confirm the interaction of the Fyn SH2 domain with β-adducin further, the GST-Fyn SH2 fusion protein was incubated with brain lysate, and bound proteins were immunoblotted with anti-β-adducin antibody (Fig. 3). Brain homogenate was first solubilized in TNE buffer, then the insoluble materials were solubilized in RIPA buffer. β-Adducin was found in the RIPA-soluble fraction much more than in the TNE-soluble fraction. An association of Fyn SH2 and β-adducin was observed in the RIPA-soluble fraction but not in the TNE-soluble fraction. When precipitates from RIPA lysate with the GST-Fyn SH2 domain were separated by two-dimensional gel electrophoresis, β-adducin associated with Fyn SH2 domain was detected by immunoblotting (Fig. 3B).

The SH2 domain is a protein motif found in a variety of signaling molecules including other members of Src family kinases. To examine the specificity of β-adducin to the Fyn SH2 domain, brain lysates were precipitated with GST fused with SH2 domains of Src, Lyn, CSK, and SHP2 (Fig. 3C). Among them, the c-Src SH2 domain had highest homology, 66%, with the Fyn SH2 domain. However, β-adducin was not precipitated with any of these fusion proteins except for the GST-Fyn SH2 domain.

**Fyn-dependent Phosphorylation of β-Adducin**—To examine whether tyrosine phosphorylation of β-adducin was dependent on the tyrosine kinase activity of Fyn, HA epitope-tagged β-adducin was cotransfected with Fyn into COS-7 cells, and its tyrosine phosphorylation level was analyzed by anti-phosphotyrosine immunoblotting. Cells transfected with wild type Fyn, but not with the kinase-negative form of Fyn, showed increased tyrosine phosphorylation in several cellular proteins (Fig. 4A). Tyrosine phosphorylation of recombinant β-adducin was observed in cells cotransfected with wild type Fyn (Fig. 4B). On the other hand, its tyrosine phosphorylation was not observed in cells transfected with β-adducin alone or those cotransfected with β-adducin and the kinase-negative form of Fyn. These observations indicate that Fyn tyrosine kinase is able to induce tyrosine phosphorylation of β-adducin.

**Interaction of Fyn and β-Adducin Depending on Tyrosine Phosphorylation**—To assess the interaction of these two proteins in vivo, Fyn was immunoprecipitated from COS-7 cells with anti-Fyn antibody, and the immune complexes were analyzed by immunoblotting with anti-β-adducin antibody. When COS-7 cells were cotransfected with β-adducin and wild type Fyn, β-adducin was coimmunoprecipitated with anti-Fyn antibody (Fig. 5A), suggesting that full-length Fyn protein could interact with β-adducin. On the other hand, interaction was not observed in cells expressing β-adducin alone or β-adducin together with the kinase-negative form of Fyn. Similar results were obtained when cell lysates were incubated with GST-Fyn SH2 fusion protein and precipitated proteins were analyzed (Fig. 5B). These results indicate that tyrosine kinase activity of Fyn is required for binding of β-adducin to the Fyn SH2 domain.

**Colocalization of β-Adducin with Fyn in COS-7 Cells**—To examine the physiological significance of the interaction of these proteins, we analyzed the localization of β-adducin expressed in COS-7 cells by immunocytochemical staining. When HA-tagged β-adducin alone was expressed, it was diffusely present in the cytoplasm of the cells as detected by anti-HA.
antibody (Fig. 6A) or anti-β-adducin antibody (data not shown). Because Src family kinases are membrane-associated proteins, Fyn immunoreactivity was highest at the plasma membrane in COS-7 cells transfected with wild type Fyn (Fig. 6E). In cells cotransfected with β-adducin and wild type Fyn, β-adducin was translocated to the cytoplasmic surface of the plasma membrane and perinuclear area (Fig. 6G). The merged image of β-adducin and Fyn immunofluorescence showed colocalization of these proteins (Fig. 6I). In contrast, in cells cotransfected with β-adducin and the kinase-negative form of Fyn, β-adducin remained in the cytoplasmic region and did not overlap Fyn. These findings are consistent with our biochemical data described above (Figs. 4 and 5).

It has been shown that β-adducin bound to the actin filaments to form spectrin-actin meshwork beneath the plasma membrane. We visualized the actin filaments of COS-7 cells expressing β-adducin with rhodamine phalloidin. When β-adducin was expressed alone, it was distributed in the cytoplasm of the cells and only partially overlapped the actin filaments (Fig. 7, A–C). However, when β-adducin was cotransfected with wild type Fyn, β-adducin was detected mostly at cortical areas of the cells and was colocalized with actin filaments (Fig. 7, D–F).

**DISCUSSION**

Src family kinases have critical roles in the signal transduction of extracellular information into cells and regulation of cytoskeletal organization. These kinases are thought to form complexes with cytoskeletal proteins and a variety of signaling molecules at the plasma membrane. The association of Src family kinases with these complexes is mostly through their SH2, SH3, and N-terminal alkylated domains (27).

We purified a Fyn SH2 domain-binding protein from Nonidet P-40-insoluble components of mouse brain and identified it as β-adducin (Figs. 1–3). Adducin was originally purified from human erythrocyte membrane skeletons (28) and from brain membranes (29) as cytoskeletal proteins that regulate actin-spectrin junctions. There are three members of adducin, α-, β-, γ-adducin, which form a heteromeric protein complex comprised of either α/β subunits or α/γ subunits (29–32). α- and γ-adducins are expressed in most tissues, whereas β-adducin is in more restricted tissues such as the brain and spleen (30, 33).
Because Fyn and β-adducin were abundant in the Nonidet P-40-insoluble fraction (Fig. 3, A and B), it is possible that these two proteins form a hydrophobic complex in neuronal cells. In addition, β-adducin was not associated with the SH2 domains of other proteins including Src, Lyn, CSK, and SHP2, indicating that association with β-adducin is a specific function of the Fyn SH2 domain (Fig. 3 C).

Because the SH2 domain recognizes short peptide motifs bearing phosphotyrosine (34), it is possible that the association of β-adducin with the Fyn-SH2 domain is dependent on tyrosine phosphorylation. β-Adducin has been reported to be phosphorylated at serine and threonine residues by protein kinase A (35, 36), protein kinase C (37–39), and Rho kinase (40), but it was not known whether it is tyrosine-phosphorylated. In the present study, we showed for the first time that β-adducin is phosphorylated at its tyrosine residue (Fig. 4). In addition, the tyrosine phosphorylation levels of protein bands including β-adducin were reduced in Fyn-deficient mice (Fig. 1). Thus, it seems likely that β-adducin is one of the direct substrates of Fyn tyrosine kinase. Further studies are needed to confirm these ideas.

α-Adducin has been shown to be phosphorylated by Rho kinase at Thr-445 and Thr-480, resulting in binding to actin filaments and recruitment of spectrin (43). On the other hand, protein kinase C phosphorylates α-adducin at Ser-726 and then inhibits the activity of adducin in recruiting spectrin to actin filaments. When Madin-Darby canine kidney cells were stimulated with phorbol 12-myristate 13-acetate, spectrin was translocated from the cell-cell contact sites to the cytoplasm in parallel with the increase in serine phosphorylation of α-adducin in the cytoplasm (39). In the present study, we have shown that tyrosine phosphorylation of β-adducin resulted in translocation to actin filaments. This result suggests that in addition to serine and threonine residues, tyrosine residues are involved in determining the subcellular distribution of adducin in cells.

Tyrosine phosphorylation of β-adducin resulted in association with Fyn and translocation from the cytoplasm to the plasma membrane and promoted colocalization of β-adducin with actin filaments at the plasma membrane. It has been reported that CSK was recruited to the plasma membrane by

![Colocalization of β-adducin with actin filaments in COS-7 cells](http://www.jbc.org/)

**FIG. 7. Colocalization of β-adducin with actin filaments in COS-7 cells.** COS-7 cells were transfected with HA-tagged β-adducin with (D–F) or without (A–C) wild type (wt) Fyn. Then actin filaments were visualized with rhodamine phalloidin (red), and HA-β-adducin was visualized with anti-HA followed by fluorescein isothiocyanate-labeled anti-mouse IgG antibodies (green). Panels C and F show merged images of A and B and panels D and E, respectively. Fluorescence images were captured on a confocal laser scanning microscope.
binding to the tyrosine-phosphorylated forms of a membrane-anchored scaffold protein, CSK-binding protein, through its SH2 domain (19). In the plasma membrane, CSK could phosphorylate Src family kinases to suppress their kinase activities. Thus recruitment of signaling molecules mediated through tyrosine-phosphorylated proteins. 3. Yagi, T., Aizawa, S., Tokunaga, T., Shigetani, Y., Takeda, N., and Ikawa, Y. (1995) Nature 379, 349–354

In the brain, Fyn is concentrated in the postsynaptic density (2), nerve growth cone membranes (44, 45), and oligodendrocytes (46). Adducin is enriched at dendritic spines and growth cones of cultured neural cells (33, 39). These reports raised the possibility that Fyn and adducin interact with each other in certain regions of neurons and that they function cooperatively in neural events such as formation of neural network and synaptic transmission. β-Adducin was expressed in the abnormally positioned neurons in Fyn null (−/−) mutant mice (data not shown). It raises the possibility that β-adducin might function to arrange the suprachiasmatic nucleus and hippocampal neurons in the accurate positions. It will be of interest to examine whether mice lacking β-adducin have any abnormalities found in Fyn (−/−) mutant mice such as construction of brain networks, learning, and circadian period generation (2–8).

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