β-Tubulin Binds Src Homology 2 Domains through a Region Different from the Tyrosine-phosphorylated Protein-recognizing Site*

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Received for publication, February 22, 1996, and in revised form, August 14, 1996

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Sac homology 2 (SH2) domains have been demonstrated to bind tyrosine-phosphorylated proteins that participate in signaling by growth factors and oncogenes by recognizing amino acid sequences containing phosphotyrosine residue. We found that SH2 domains such as Ash/Grb2, the 85-kDa subunit of phosphatidylinositol 3-kinase, and phospholipase Cγ1 also bind β-tubulin through a different region that recognizes phosphotyrosine in vitro and in vivo. Furthermore, binding occurs even when the SH2 domain is occupied by tyrosine-phosphorylated epidermal growth factor receptors. Using deleted constructs of Ash/Grb2 SH2, we found that carboxyl-terminal β strands E and F, and α helix B (region "c") are required for binding. A synthetic peptide (FLWVVKFLNLNLVDYH) composed of region c inhibited the binding of β-tubulin to the SH2 domains of Ash/Grb2, phosphatidylinositol 3-kinase, and phospholipase Cγ1. The co-localization of SH2 proteins and microtubules is also confirmed by immunostaining. These data suggest that microtubules play important roles in the assembly of signaling molecules complexes containing SH2 proteins.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to Ash/Grb2, PI 3-kinase, and PLCγ-1 were made as described previously (22, 25, 26). Antibodies against α- and β-tubulin were purchased from Sanbio and Chemicon. Anti-EGF receptor antibody was from Transduction Labo.

Synthetic peptides corresponding to the sequence of the Ash/Grb2-binding site of the autophosphorylated EGF receptor (VTPEpYIQNSVPK) were purchased from Peptide Institute Inc. (Osaka, Japan). The Ash/Grb2 SH2 "c" region (FLWVVKFLNLNLVDYH) and control peptides (FLWVVKFLNLNLVPFPACFALWLK) were synthesized by the Fmoc (N-(9-fluorenly)methylxycarbonyl) method with a peptide synthesizer (Applied Biosystems). The termini of all peptides were deblocked.

Identification of a 55-kDa Protein Bound to GST-Ash/Grb2 as β-Tubulin—Recombinant GST-Ash fusion protein (23) coupled to glutathione-Sepharose was used to affinity purify Ash-binding proteins from lysates of NIH 3T3 fibroblasts transfected with wild-type or mutant Ash. Protein bands at 55 kDa were cut out, subjected to SDS-polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride membranes. Protein bands at 55 kDa were cut out, digested with lysylendopeptidase, and separated on a C18 reverse-phase column. We sequenced two peptides from the digested 55-kDa protein and found them (MREIvHvQAg, IREEyPDrMintFvSvMP) to be identical with the partial sequence of bovine β-tubulin.

GST Fusion Proteins—Bacterial expression plasmids coding GST fusion proteins were produced by in-frame insertion of fragments corresponding to each region. GST-PLCγ-1 SH2 expression plasmid was made by cutting the cDNA of rat PLCγ1 with XhoI and PvuII, ligating it to EcoRI linker, and inserting it into the EcoRI site of pGEX-3X. GST-p85 SH2 expression plasmid, which includes both N and CSH2 domains, was made by cutting the cDNA of human p85 with SnaI and

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1 The abbreviations used are: PLC, phospholipase C; PI 3-kinase, phosphatidylinositol 3-kinase; EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; RB, reassembly buffer (100 mM 2-(N-morpholino)ethanesulfonic acid, 0.5 mM MgCl2, 1 mM EGTA, pH 6.8); FITC, fluorescein isothiocyanate.
Tubulin purified from bovine brain was labeled with $^{125}$I using Bolton-Hunter reagent. Various concentrations of labeled tubulin were subjected to Scatchard analysis. Microtubule proteins, including tubulins and microtubule-associated proteins, were obtained from bovine brain. The crude tubulin domain of Ash/Grb2 was divided into four regions: a (amino acids 55–86), b (87–118), c (119–135), and d (136–150). GST-Ash SH2 deletion constructs were produced by in-frame insertion of a polymerase chain reaction-amplified fragment corresponding to each sequence into pGEX-3X vectors. The 5’ and 3’ primers for polymerase chain reaction were 5’-TTAGGATCCCTTA-9’TTCGTGGTAATCTACCAGCTCATT-9’G for primer a, 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer b, 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer c, and 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer d. Various deletion constructs of GST-Ash/Grb2 SH2—The SH2 domain of Ash/Grb2 was divided into four regions: a (amino acids 55–86), b (87–118), c (119–135), and d (136–150). GST-Ash/Grb2 deletion constructs were produced by in-frame insertion of a polymerase chain reaction-amplified fragment corresponding to each sequence into pGEX-3X vectors. The 5’ and 3’ primers for polymerase chain reaction were 5’-TTAGGATCCCTTA-9’TTCGTGGTAATCTACCAGCTCATT-9’G for primer a, 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer b, 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer c, and 5’-AACGGATCCCATGATGAGAACATGGATGATAG-9’TTCGTGCCTGCTGGATCATATA-9’G for primer d.

For further purification of tubulin protein from other microtubule proteins such as microtubule-associated proteins or tau, the crude microtubule proteins were polymerized and centrifuged to yield supernatant 1 and precipitate 1. Then, precipitate 1 was suspended in cold RB, sonicated briefly, cooled to depolymerize, and centrifuged to yield supernatant 2 and precipitate 2. Finally, supernatant 2 was polymerized and centrifuged to yield supernatant 3 and precipitate 3. Each supernatant and precipitate (suspended in a volume of RB equal to that of the supernatants) were separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted with antibodies against each SH2-containing protein.

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and mounted on slides with 1 mg/ml p-phenylendiamine, 90% glycerol in phosphate-buffered saline.

Localization of FITC-labeled GST-SH2 c in Microtubules—The co-localization of Ash/Grb2 SH2 c to microtubules was investigated by incubating cells with FITC-labeled GST-SH2 c in microtubule-stabilizing buffer containing 0.2% Triton X-100 (29) to permeabilize the membranes. GST-Ash SH2 c was labeled with FITC (Polysciences, Inc.) and separated from free FITC by gel filtration. The extent of labeling was checked by SDS-polyacrylamide gel electrophoresis.

RESULTS

Identification of an Ash/Grb2-binding Protein as β-Tubulin—To obtain Ash/Grb2-binding proteins, we expressed Ash/Grb2 as a GST fusion protein in Escherichia coli, immobilized it to glutathione-Sepharose, and carried out affinity purification. The cytosol fractions of bovine brain were applied to the GST-Ash/Grb2 affinity column via its SH2 domain. Ash/Grb2 and its binding proteins were stained with Coomassie Brilliant Blue (CBB), and the co-precipitated β-tubulin was detected by immunoblotting (I.B.). Ash/Grb2-binding proteins A, B, C, D, and E were revealed to be Sos, synaptotagmin, c-Cbl, dynamin, and unknown protein, respectively, by partial amino acid sequences and Western blotting (data not shown).

As shown in Fig. 2, binding of β-tubulin to GST-Ash/Grb2 immobilized to CNBr-Sepharose was inhibited by increasing amounts of αB (aa 55-86), αC, and β structuring SH2 domain of Ash/Grb2, and found that the SH2 domain could bind strongly to β-tubulin (Fig. 1A). SH2 domains of the 85-kDa subunit of PI 3-kinase (p85) and PLCγ1 could also bind to β-tubulin (Fig. 1B). This indicates that association with β-tubulin is a common characteristic conserved among SH2 domains.

To clarify whether the interaction between SH2 domains and β-tubulin is direct or not, tubulin proteins were purified from bovine brain and subjected to binding assay with SH2 domains. While β-tubulin did not bind to GST alone, a significant amount of β-tubulin was shown to bind to all SH2 domains tested (Fig. 1C).

The kinetics of the interaction between β-tubulin and SH2 domain was investigated Scatchard analysis using 125I-labeled tubulin protein, and the Kd was estimated to be 4.2 μM.

Co-immunoprecipitation of β-Tubulin with Ash/Grb2, PI 3-Kinase, and PLCγ1—Next, we analyzed the association of SH2-containing proteins with β-tubulin in vivo. Ash/Grb2, p85, and PLCγ1 were immunoprecipitated with polyclonal antibody against each protein and immunoblotted with anti-β-tubulin (Fig. 2A).

β-Tubulin binds to SH2 Domains of Ash/Grb2, PI 3-kinase, and PLCγ1—It is believed that Ash/Grb2 binds to its binding proteins via the SH2 or the SH3 domain because Ash/Grb2 is composed of only SH2 and SH3 domains. However, β-tubulin is not tyrosine phosphorylated (data not shown) and has no proline-rich motif that is likely to bind to SH3 domains. Thus, it is of great interest to identify the β-tubulin-binding domain within Ash/Grb2. To clarify which domain is responsible for the association, we used GST fusion proteins containing each domain of Ash/Grb2 and found that the SH2 domain could bind strongly to β-tubulin (Fig. 1A). SH2 domains of the 85-kDa subunit of PI 3-kinase (p85) and PLCγ1 could also bind to β-tubulin (Fig. 1B). This indicates that association with β-tubulin is a common characteristic conserved among SH2 domains.

Ash/Grb2, PI 3-Kinase, and PLCγ1 Co-polymerize and Codepolymerize with Microtubules—We purified microtubule protein, which includes tubulins and microtubule-associated proteins, from bovine brain by two-cycle polymerization-depolymerization steps. The microtubule protein obtained was further polymerized and depolymerized by heating and cooling; then the coexistence of SH2-containing proteins and tubulin was examined by immunoblotting (Fig. 2B).

β-Tubulin and tyrosine-phosphorylated protein A, β-tubulin does not inhibit the association of the autophosphorylated EGF receptor and the SH2 domain. GST-Ash/Grb2 SH2 immobilized to beads was incubated with increasing amounts of 0, 10, and 1000 μg of tubulin purified from bovine brain and then incubated with membrane fractions from A431 cells containing autophosphorylated EGF receptor. Bound EGF receptors were detected by Western blotting with anti-EGF receptor antibody (top). GST-Ash/Grb2 SH2 was incubated with membrane fractions from A431 cells containing autophosphorylated EGF receptor (0, 100, and 1000 μg protein) prior to incubation with purified tubulin protein. Bound β-tubulin was detected by Western blotting (bottom). B, an immobilized phosphopeptide corresponding to the Ash/Grb2-binding site of the EGF receptor can precipitate β-tubulin via Ash/Grb2. A phosphopeptide corresponding to the Ash SH2-binding site of the autophosphorylated EGF receptor was immobilized to CNBr-Sepharose. Ash/Grb2 was affinity purified with this phosphopeptide column via its SH2 domain.
SH2 Domains Can Bind to β-Tubulin and Tyrosine-phosphorylated Proteins Simultaneously—SH2 domains are known to recognize amino acid sequences containing a phosphotyrosine residue. However, β-tubulin, which binds to the SH2 domain of Ash/Grb2, is not tyrosine phosphorylated (data not shown). To determine whether the association between the SH2 domain and β-tubulin is distinct from that between SH2 domains and tyrosine-phosphorylated proteins, we investigated whether β-tubulin can inhibit the association between SH2 domains and tyrosine-phosphorylated proteins and vice versa. GST-SH2 of Ash/Grb2 immobilized to glutathione-Sepharose was preincubated with purified tubulin and then incubated with membrane fractions from A431 cells, which contain autophosphorylated EGF receptors. The bound EGF receptor was detected by immunoblotting with anti-EGF receptor antibody. The SH2 domain efficiently precipitated EGF receptor regardless of the presence of bound β-tubulin and could precipitate β-tubulin when already associated with tyrosine-phosphorylated EGF receptor (Fig. 3A). Furthermore, we precipitated Ash/Grb2 from bovine brain with an immobilized peptide corresponding to the Ash/Grb2-binding motif in the autophosphorylated EGF receptor. The Ash/Grb2 bound to peptide still could associate with β-tubulin (Fig. 3B).

A Carboxyl-terminal Region of the SH2 Domain Is Resisible for Binding to β-Tubulin—To identify the β-tubulin binding site within the Ash/Grb2 SH2 domain, we constructed a series of GST-SH2 deletion mutants for tubulin binding assays (Fig. 4A). The Ash SH2 domain was divided into four regions, designated a, b, c, and d, and mutants lacking different regions were expressed as GST fusion proteins (Fig. 4A) and used for β-tubulin binding assays. GST-bcd and GST-cd, which do not contain the conserved arginine residue known to interact with phosphate in phosphotyrosine, still precipitated β-tubulin (Fig. 4B); however, GST-d did not. Finally, we found that GST-c was sufficient to precipitate β-tubulin and concluded that the SH2 c region is the β-tubulin-binding site.

To confirm that the c region is necessary for tubulin binding, we used a synthetic peptide (FLWVKFNSLNEIVDYH) corresponding to the sequence of the c region as a competitor of the Ash SH2 domain binding to β-tubulin. Increasing concentrations of the peptide were preincubated with bovine brain cytosol fractions, and then immobilized GST-Ash SH2 was added. Bound β-tubulin was immunoblotted with anti-β-tubulin antibody. Peptide c inhibited the association in a concentration-dependent manner (Fig. 5). We also tested the ability of peptide c to inhibit the binding of other SH2 domains to β-tubulin and found that it also inhibits the SH2 domains of p85 and PLCγ1 (Fig. 5). This indicates that the c region (including β strands E and F and α helix B) is necessary for the association. However, a peptide corresponding to the last 11 residues (FNSLNEIVDYH) within the c region (which is especially conserved among SH2 domains and can be boxed within c) did not inhibit binding. Next, to test the possibility that the binding between β-tubulin and SH2 domains occurred via hydrophobic interaction, peptides corresponding to the hydrophobic sequence within the former half of region c or a hydrophobic sequence within an unrelated protein, PLCδ1, were used for binding inhibition assay. In contrast to peptide c, these peptides did not inhibit the binding at any concentration.

Co-localization of SH2-containing Proteins with Microtubules in Vivo—To confirm the coexistence of Ash/Grb2, p85, or PLCγ1 and β-tubulin, Swiss 3T3 cells were immunostained with each antibody. Fig. 6, A–C, clearly shows that a part of the Ash/Grb2, p85, and PLCγ1 co-localizes in microtubules.

Furthermore, to examine whether SH2 c recognizes microtubules in permeabilized Swiss 3T3 cells, fluorescent-labeled GST-SH2 c was incubated with Swiss 3T3 cells in the presence of 0.2% Triton X-100. Fig. 6D shows that GST-SH2 c localizes in microtubules, suggesting that SH2 c is the tubulin-binding site.

DISCUSSION

We have demonstrated that β-tubulin binds to the SH2 domains of Ash/Grb2, PI 3-kinase, and phospholipase Cγ1. This association is direct as shown by the binding of β-tubulin to GST-SH2 fusion proteins. Furthermore, we determined that strands E and F and α helix B in the SH2 domain are required for binding. The crystal structure and nuclear magnetic resonance structure of SH2 domains revealed that the core element is an antiparallel β sheet sandwiched between two α helices. The central β sheet (strands B, C, and D) is at the core of the structure and divides the domain into two functionally distinct regions (30–33). One site, containing α helix A and one face of the central sheet, is concerned primarily with phosphotyrosine binding. The other site (strands E, F, and helix B) provides binding sites for the peptide residues immediately following the phosphotyrosine. β-Tubulin binds to SH2 domains even after the SH2 domains are occupied by tyrosine-phosphorylated proteins. This indicates that strands E and F and helix B, although important for recognizing the peptide sequence following a phosphotyrosine residue, can also bind β-tubulin simultaneously. This region is rich in hydrophobic amino acids,
binding although were then immunostained to confirm the co-localization. washed with phosphate-buffered saline, and fixed. The microtubules were incubated with 0.1 mg/ml of FITC-labeled GST-SH2 c prior to fixation to wash out cytosolic proteins that do not associate with microtubule-stabilizing buffer containing 0.2% Triton X-100 at 37 °C to SH2 domains is a common characteristic of a variety of proteins designated microtubule-associated proteins. Kapeller et al. (34) showed that PI 3-kinase and carboxyl-terminal SH2 domains, but not to a SH2 domain. However, they did not succeed in determining the precise site for β-tubulin binding. We here demonstrate that GST-Ash/Grb2 SH2 and a synthetic peptide corresponding to β strands E and F and α helix B of Ash/Grb2 SH2 also inhibit the binding of β-tubulin to SH2 domains of PI 3-kinase and PLCγ1. Moreover, we found that the synthetic peptide alone could bind to β-tubulin (data not shown). Therefore, it is likely that the c region is sufficient for binding.

In the tyrosine kinase signaling system, tubulin may play important roles in the receptor-induced endocytosis of signaling molecules through binding to SH2-containing proteins. It is also possible that microtubules regulate the assembly and disassembly of signaling molecules containing SH2 domains.

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FIG. 6. Immunofluorescence analysis shows that SH2 containing proteins co-localize with microtubules in vivo. A-C, double immunofluorescence analysis of tubulin- and SH2-containing proteins (A, Ash/Grb2; B, p85; C, PLCγ1). Swiss 3T3 cells were treated with microtubule-stabilizing buffer containing 0.2% Triton X-100 at 37 °C prior to fixation to wash out cytosolic proteins that do not associate with microtubules. Incubations with first and second antibodies were then performed. D, FITC-labeled SH2 c region co-localizes with microtubules. Cells were incubated with 0.1 mg/ml of FITC-labeled GST-SH2 c in microtubule-stabilizing buffer containing 0.2% Triton X-100 at 37 °C, washed with phosphate-buffered saline, and fixed. The microtubules were then immunostained to confirm the co-localization.