Role for Fes/Fps Tyrosine Kinase in Microtubule Nucleation through Its Fes/CIP4 Homology Domain*

We have previously demonstrated that Fes/Fps (Fes) tyrosine kinase is involved in Semaphorin3A-mediated signaling. Here we report a role for Fes tyrosine kinase in microtubule dynamics. A fibrous formation of Fes was observed in a kinase-dependent manner, which associated with microtubules and functionally correlated with microtubule bundling. Microtubule regeneration assays revealed that Fes aggregates colocalized with γ-tubulin at microtubule nucleation sites in a Fes/CIP4 homology (FCH) domain-dependent manner and that expression of FCH domain-deleted Fes mutants blocked normal centrosome formation. In support of these observations, mouse embryonic fibroblasts derived from Fes-deficient mice displayed an aberrant structure of nucleation and centrosome with unbundling and disoriented filaments of microtubules. Our findings suggest that Fes plays a critical role in microtubule dynamics including microtubule nucleation and bundling through its FCH domain.

The Fes/Fps (Fes) proto-oncogene encodes a structurally unique member of the non-receptor protein-tyrosine kinase (PTK) family (1). The Fes expression was first detected in cells of the myeloid lineage, but recent work (2) has shown that Fes exhibits a more widespread expression pattern including developing neurons and vascular endothelial cells. Transgenic mice overexpressing v-Fps showed a neurological disorder that correlated with the expression of v-Fps in the brain, and a striking bilateral enlargement of the trigeminal nerves (3). In our previous works, Fes was found to be involved in semaphorin-mediated signaling during neural development (4, 5), but the Fes function remains largely unknown.

Distinct from Src and other non-receptor PTK, Fes has a long N-terminal unique region containing Fes/CIP4 homology (FCH) domain followed by three coiled-coil domains, a central SH2 domain and a C-terminal kinase domain (6). The FCH domain was first described as a region of homology between Fps/Fes/Per PTKs and a Cdc42-interacting protein, CIP4 (7). Amino acid sequence homology searches have detected this FCH domain in numerous proteins, many of which are implicated in the regulation of cytoskeletal rearrangements (8). Although the FCH domain of CIP4 was reported to bind to microtubules, the exact role of FCH domain in Fes remains obscure.

In this study we report that Fes plays an important role in microtubule dynamics including microtubule nucleation and bundling through its FCH domain. Implications of Fes in the regulation of microtubules and neural development are briefly discussed.

EXPERIMENTAL PROCEDURES

Materials—Aprotinin, phenylmethylsulfonyl fluoride, phalloidin-TRITC, acetylated tubulin Ab, anti-α-tubulin Ab, anti-γ-tubulin Ab, and anti-FLAG rabbit polyclonal Ab were purchased from Sigma. Cy3-labeled goat anti-mouse IgG and protein A-Sepharose were from Amersham Biosciences. Wistar rats were from Japan SLC, Inc. hemagglutinin-probe (Y-11) and anti-focal adhesion kinase Ab (C-20) were from Santa Cruz Biotechnology. Anti-phosphotyrosine monoclonal Ab (4G10) and anti-Fes rabbit polyclonal Ab were from Upstate Biotechnology. Anti-GFP monoclonal Ab was from Clontech. Alexa Fluor 647 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-rabbit IgG were from Molecular Probes. Colcemid was purchased from Nacalai Tesque.

Immunoprecipitation and Immunoblotting Procedures—Mouse brains or cultured cells were lysed in the lysis buffer (20 mM Tris/HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μM Na3VO4) at 4 °C. The lysates (1 mg of protein) were clarified by centrifugation at 100,000 g for 10 min and immunoprecipitated with the appropriate Ab. Immunoprecipitates were washed three times with lysis buffer, once with 10 mM HEPES/NaOH, pH 8.0, containing 0.5 mM NaCl and finally with 10 mM HEPES/NaOH, pH 8.0. Immunoprecipitates were boiled with SDS-PAGE sample buffer for 3 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore), followed by detection with the appropriate Ab as described previously (9).

Expression Constructs—Cloning of mouse Fes cDNA was performed as described previously (5). Fes cDNA tagged with FLAG epitope at the N terminus was subcloned into pCMV5 expression vectors. Sequencing of all strands of the cloned reverse transcriptase-PCR fragments were performed using an ABI-310 automated DNA sequencer. Fes kinase-negative mutant was generated by missense mutation K562R as described previously (10). Point mutation of Arg318 of Fes cDNA in SH2 domain to Lys (R482K) was performed by the site-directed mutagenesis kit from Strategene. A sense primer (5′-GGACTTCTCTGTAGTTAAGGAG-
AGCGAGGGCC-3') was used. For the FCH-domain deletion mutant Fes (amino acid residues 1–95), a sense primer 5'-GAAGATCTAACTCGGGGAGCCCTTGAAGC-3') was used. For FCH and coiled-coil domain deletion mutant Fes (amino acid residues 452–820), a sense primer (5'-GGAAGATCTAACCTTCTGAGCAGCTGG-3') was used. Both Fes and Fes kinase-negative cDNAs were subcloned into pEGFP-C1 (Clontech) for green fluorescence. FCH-domain deletion mutant, FCH, coiled-coil domain deletion mutant, and SH2 mutant were also subcloned into pEGFP-C1.

Cell Culture and Stimulation—COS-7 cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma) containing 10% fetal bovine serum. Hippocampal cultures were prepared as described previously (11). For mouse embryonic fibroblasts (MEFs), fetuses from embryonic day 12 were cut into small size and homogenized in 0.05% trypsin-PBS solution with a Dounce homogenizer. After trypsin digestion for 1 h at 37 °C, the supernatants were collected and centrifuged at 1000 × g for 5 min. The pellet was resuspended in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and plated onto 6-well plates.

Transfection of Plasmids into Cells and Treatment with Chemicals—COS-7 cells and MEFs were cultured in dishes at about 50% confluence. Expression vectors (1 µg) were mixed with serum-free medium and transfected using a LipofectAMINE Plus kit (Invitrogen). After 48 h, the medium was changed to serum-free medium for about 4 h, and COS-7 cells were then treated with colcemid (0.8 µg/ml) for 1 h at 37 °C. After washing twice with PBS, COS-7 cells were incubated with Iscove’s modified Dulbecco’s medium for 45 min at 37 °C to polymerize microtubules.

Immunofluorescence Microscopy—COS-7 cells transiently coexpressing cDNAs or hippocampal cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed three times with PBS, and blocked with 3% bovine serum albumin in PBS, all at room temperature. For double staining, the cells were incubated with appropriate Abs for 2 h at room temperature and washed three times with 0.2% Triton X-100 in PBS and then with appropriate secondary Ab (Cy3-labeled goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-mouse IgG) for 30 min. The samples were washed as before, mounted using SlowFade-Light (Molecular Probes), and analyzed using LSM510 META (Carl Zeiss).

RESULTS AND DISCUSSION

Association of Fes with Microtubules and Correlation with Microtubule Modifications—To investigate the subcellular distribution of Fes, Fes was visualized as green fluorescent protein (GFP)-tagged fusion protein (GFP-Fes) in COS-7 cells. As shown in Fig. 1A, GFP-Fes showed three characteristic expression patterns in COS-7 cells: aggregate, fibrous, and diffuse. On the other hand, GFP-Fes(k–) revealed either a diffuse or an aggregate distribution without the fibrous structures. The ratio of each expression pattern was measured in 100 Fes-transfected cells, and each percentage is shown in the panels of Fig. 1A. GFP-Fes revealed a fibrous formation at 30–40% in transfected cells but in a kinase-dependent manner. Since these fibrous Fes structures appeared very similar to microtubules, we examined whether these Fes structures colocalized with microtubules. Expectedly, immunofluorescence analysis using anti-α-tubulin Ab showed a complete colocalization of Fes with microtubules (Fig. 1B, top panels). In addition, Fes structures were also immunostained with anti-phosphotyrosine (PY) Ab (Fig. 1B, bottom panels). To confirm the association of Fes with microtubules, immunoprecipitation assay was performed. As shown in Fig. 1C, tyrosine-phosphorylated α-tubulin was detected in GFP-Fes immunoprecipitates but not in GFP-Fes(k–) immunoprecipitates. To examine whether Fes associates with microtubules in developing brain, whole lysate of neonatal rat brain was immunoprecipitated with anti-Fes Ab or normal rabbit serum as control. As shown in Fig. 1C, lower panels, tubulin was detected in Fes immunoprecipitates but not in control immunoprecipitates. In addition, colocalization of endogenous Fes with microtubules was detected in neuronal cells (see Supplemental Fig. S1). These results demonstrated that Fes colocalized and associated with microtubules in a kinase activity-dependent manner and that tubulin might be phosphorylated by Fes.

The immunofluorescence signals from microtubules seemed to be enhanced by colocalization with Fes structures. To confirm this observation, the effect of Fes expression on microtubule bundling activity was compared by densitometric analysis of immunofluorescence signals from normal- and Fes-colocalized microtubules immunostained with anti-α-tubulin Ab. As shown in Fig. 1D, immunofluorescence signals from microtubules colocalized with Fes were significantly enhanced, and the space between microtubules became wider than that of normal microtubules. All Fes fibrous structures observed in other cells exhibited similar results. In control cells, these strong micro-
tubule bundling was not observed (Fig. 1D). Therefore, we concluded that Fes was actively involved in the processes of microtubule bundling.

We next examined whether Fes was involved in the post-translational modifications of tubulins such as acetylation and detyrosination. Acetylated tubulins are generally found in stable microtubules. As shown in Fig. 1E, fibrous Fes structures were partially colocalized with acetylated and detyrosinated tubulins. Similar results were obtained in almost all other Fes-expressing cells. To examine whether Fes associates with acetylated tubulin, immunoprecipitation assay was performed. GFP-Fes, but not control GFP, was found to be associated with acetylated tubulin (Fig. 2B). However, this result showed a weak coimmunoprecipitation of Fes with acetylated tubulin, as with α-tubulin in Fig. 1C, suggesting that this interaction may be partial and limited. At present, it is uncertain whether Fes was actively involved in these posttranslational modifications.

**Role for Fes in Microtubule Nucleation and Effects of Various Fes Mutants on Microtubule Dynamics—**To understand the role for Fes in microtubule dynamics, COS-7 cells expressing GFP-Fes were treated with colcemid, and the relationship between Fes and microtubule dynamics was examined during the time-dependent process of microtubule regeneration. When microtubules were depolymerized by colcemid treatment, Fes existed in an aggregate form without any microtubule formation (not shown). Upon removal of colcemid to start microtubule nucleation and polymerization, microtubule nucleation of centrosomes was observed at 45 min after removal of colcemid and Fes aggregations were found to be colocalized with microtubule nucleation sites (Fig. 2A, upper panels). Thereafter, fibrous Fes structures which colocalized with newly polymerized microtubules were detected after 60 min (Fig. 2A, lower panels). To examine whether Fes associates with γ-tubulin, immunoprecipitation assay was performed. GFP-Fes, but not control GFP, was found to be associated with γ-tubulin, although at a very weak level of association (Fig. 2B). Equal amount of immunoprecipitated γ-tubulin or acetylated tubulin was confirmed by immunoblot analysis using anti-γ-tubulin Ab (not shown). To confirm the localization of Fes to microtubule nucleation sites, COS-7 cells expressing GFP-Fes were double labeled with anti-α-tubulin and anti-γ-tubulin Abs. As shown in Fig. 2C, Fes aggregates were detected at microtubule nucleation sites with γ-tubulin aggregations.

To investigate which domain of Fes was required for its localization to microtubule nucleation, effects of several deletion mutants on microtubule nucleation were examined. As shown in Fig. 2D, GFP-FCH domain fusion protein was able to localize to microtubule nucleation sites (Fig. 2D, upper panels). Interestingly, in some cells, aggregations of GFP-FCH domain were colocalized with multiple γ-tubulin aggregations (Fig. 2D, bottom panels), suggesting the close relationship between Fes FCH domain and γ-tubulin. On the other hand, FCH domain-deleted mutants never colocalized with microtubule nucleation sites. Rather, this mutant blocked normal formation of microtubule nucleation and centrosome. Surprisingly, a fibrous formation of FCH domain deleted Fes mutant was detected, but this mutant Fes showed little colocalization with microtubules (Fig. 2E). Furthermore, expression of FCH domain-deleted mutants blocked normal γ-tubulin localization at microtubule nucleation sites (Fig. 2F). How could FCH domain-deleted mutant lead to the collapse of microtubule structure? One explanation is that in addition to interfering with Fes-mediated signaling, the FCH domain-deleted mutant is also disrupting Fer signaling because Fes and Fer may perform redundant signaling functions in cells. Another explanation is that the FCH domain-deleted mutant could still bind to putative Fes substrates that were involved in microtubule formation, but without the FCH domain these substrates could not distribute to their normal subcellular location such as colocalization to microtubule bundling.
Role for Fes in Microtubule Dynamics

Table I

| Effect of various Fes mutants on microtubule dynamics |
|----------------------------------|
| Wild-type | Kinase | ΔFCH | FCH | mSH2 |
|----------------|
| Fibrous formation | + | - | + | - |
| Microtubule colocalization | + | - | - | - |
| Microtubule bundling | + | - | - | - |
| Acetylation | + | - | - | - |
| Nucleation | + | + | + | - |
| Centrosome collapse | - | - | + | - |

...bules or nucleation. Therefore, it is important to determine how this FCH domain might mediate functional modification of microtubules and which molecules interact with this domain.

Overexpression of Fes FCH protein also blocked normal formation of microtubule nucleation and centrosome without fibrous structures. In this regard, we should consider that the involvement of FCH domains of CIP4 and other FCH domain containing proteins with microtubules is of relevance to the Fes FCH overexpression results. Indeed, a previous work (12) has shown that truncation of Fes which retains the N-terminal domain, but lacks both the SH2 and the tyrosine kinase domain via gene targeting results in embryonic lethality. Thus, mice expressing Fes N-terminal domain showed far more severe phenotype when compared with Fes-deficient mice. This result is highly suggestive that FCH domain-containing proteins also may be involved in microtubule dynamics such as nucleation.

Coiled-coil domains-deleted Fes mutant and Fes SH2 mutant revealed either a diffuse or an aggregate distribution but no fibrous formation, same as Fes(k−) mutant (see Supplemental Fig. S2). A striking feature of Fes/Fer PTKs is their ability to form oligomers which is believed to be mediated by the coiled coil domains (13). In addition, a previous work (14) has indicated that the SH2 domain of Fes can bind to autophosphorylation sites. Therefore, intramolecular or intermolecular interactions may be required for fibrous formation of Fes. These results are briefly summarized in Table I.

Aberrant Structure of Centrosome in Fes-deficient Fibroblasts—To determine the physiological relevance of Fes in microtubule dynamics, we compared MEFs derived from wild-type and Fes-deficient mice. Light microscopic analysis indicated that Fes-deficient cells were smaller than wild-type fibroblasts (not shown). These Fes−/− and Fes+/+ MEFs were labeled with anti-acetylated tubulin Ab, and the structures of centrosome were compared. In contrast to wild-type, Fes-deficient cells displayed an aberrant centrosome structure with disoriented filaments of acetylated microtubules. Furthermore, in Fes-deficient cells, the immunofluorescence signals for acetylated microtubules were significantly decreased and microtubule bundling was also severely inhibited. We examined microtubule nucleation in these cells further by γ-tubulin immunostaining. Normal microtubule nucleation colocalized with γ-tubulin was observed in control cells, but microtubule nucleation and centrosome were not normally developed in Fes-deficient cells (Fig. 3A, right panels). These phenotypes were commonly observed in almost all MEFs derived from three independent Fes-deficient mice. Furthermore, the transfection of FLAG-tagged Fes into Fes-deficient cells restored the normal structure of centrosome with oriented and bundled acetylated microtubules (Fig. 3B). This experiment was performed independently at three times, and similar results were obtained. Thus, these results suggested that Fes was required for normal microtubule formation.

We examined whether these abnormal microtubule structures observed in Fes-deficient cells were due to an indirect effect such as cell adhesion. As shown in Fig. 3C, analysis using focal adhesion and F-actin staining showed no significant difference between wild-type and Fes-deficient cells. In addition, confocal laser microscopy scanning along the z axis revealed similar morphology between wild-type and Fes-deficient cells. These results suggested that abnormal microtubule formation of Fes-deficient cells was not due to cell adhesion. However, these findings cannot completely deny the indirect effect.

Although an abnormal microtubule structure was detected in cultured Fes-deficient cells, Fes-deficient mice have been reported to be born and developed normally without any obvious defects (15). Biochemical microtubule assembly analysis using brain lysates of wild-type and Fes-deficient mice did not show...
a significant change in microtubule polymerization activity. Since Fer is known as another member of Fes family PTKs, one possibility is that Fer may play a redundant role in microtubule dynamics during development of Fes-deficient mice. Generation of Fes and Fer double deficient mice will answer that question and reveal the physiological importance of this PTK family.

Role for Fes in Neural Development—Transgenic mice over-expressing v-Fps showed a neurological disorder that included a striking bilateral enlargement of the trigeminal nerves (3). This result suggested an important role for Fes in neural differentiation and growth. In our previous works, Fes was found to be associated with CRMP/CRAM and involved in semaphorin-mediated signaling during neural development (4, 5). It has been recently shown that CRMP-2 binds to tubulin heterodimers and promotes microtubule assembly (16). Thus, both Fes and CRMPs are closely involved in microtubule dynamics. A previous report (17) suggested that conditions that stabilize microtubules could lead to bundle formation and allow microtubule assembly by a mechanism different from that employed by microtubule-associated proteins. This means that additional mechanisms besides the action of tau on tubulin exist to organize microtubules in the axon. One attractive hypothesis is that Fes and CRMP/CRAM may play a role in microtubule nucleation, serving as microtubule organizing center during neural development.

In summary, our findings demonstrated that Fes is involved in microtubule nucleation through FCH domain and that FCH domain is required for normal formation of microtubule nucleation and centrosome. Further studies will be needed to characterize the mechanism of microtubule dynamics mediated by Fes.

Acknowledgments—We are very grateful to Dr. D. Job for providing anti-detyrosinated tubulin Ab and to Dr. S. Jahangeer for critically reading the manuscript.

REFERENCES
1. Hanafusa, T., Wang, L. H., Anderson, S. M., Karess, R. E., Hayward, W. S., and Hanafusa, H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3009–3013
2. Haigh, J., McVeigh, J., and Greer, P. (1996) Cell Growth Differ. 7, 831–944
3. Yee, S. P., Mock, D., Maliby, V., Silver, M., Rossant, J., Bernstein, A., and Pawson, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5873–5877
4. Inatome, R., Tsujimura, T., Hitomi, T., Mitsui, N., Hermann, P., Kuroda, S., Yamamura, H., and Yanagi, S. (2000) J. Biol. Chem. 275, 27291–27302
5. Mitsui, N., Inatome, R., Takahashi, S., Goshima, Y., Yamamura, H., and Yanagi, S. (2002) EMBO J. 21, 3274–3285
6. Greer, P. (2002) Nat. Rev. Mol. Cell. Biol. 3, 278–289
7. Aspenstrom, P. (1997) Curr. Biol. 7, 479–487
8. Tian, L., Nelson, D. L., and Stewart, D. M. (2000) J. Biol. Chem. 275, 7854–7861
9. Takahashi, S., Inatome, R., Yamamura, H., and Yanagi, S. (2003) Genes Cells 8, 81–93
10. Craig, A. W., Zirngibl, R., and Greer, P. (1999) J. Biol. Chem. 274, 19934–19942
11. Inagaki, N., Chihara, K., Arimura, N., Menager, C., Kawano, Y., Matsu, N., Nishimura, T., Amano, M., and Kaibuchi, K. (2001) Nat. Neurosci. 4, 781–782
12. Hackenmiller, R., and Simon, M. C. (2002) Dev. Biol. 245, 255–269
13. Read, R. D., Lionberger, J. M., and Smithgall, T. E. (1997) Cell 89, 2283–2292
14. Hjermstad, S. J., Peters, K. L., Briggs, S. D., Glazer, R. I., and Smithgall, T. E. (1993) Oncogene 8, 2283–2292
15. Hackenmiller, R., Kim, J., Feldman, R. A., and Simon, M. C. (2000) Immunity 13, 397–407
16. Fukata, Y., Itoh, T. J., Kimura, T., Menager, C., Nishimura, T., Shiromizu, T., Watanabe, H., Inagaki, N., Iwamatsu, A., Hotani, H., and Kaibuchi, K. (2002) Nat. Cell Biol. 4, 583–591
17. Brandt, R., and Lee, G. (1994) Cell Motil. Cytoskeleton 28, 143–154
Role for Fes/Fps Tyrosine Kinase in Microtubule Nucleation through Its Fes/CIP4 Homology Domain
Shusuke Takahashi, Ryoko Inatome, Azusa Hotta, Qingyu Qin, Renee Hackenmiller, M. Celeste Simon, Hirohei Yamamura and Shigeru Yanagi

J. Biol. Chem. 2003, 278:49129-49133.
doi: 10.1074/jbc.C300289200 originally published online October 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300289200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/10/30/C300289200.DC1

This article cites 17 references, 8 of which can be accessed free at
http://www.jbc.org/content/278/49/49129.full.html#ref-list-1