Fhos2, a novel formin-related actin-organizing protein, probably associates with the nestin intermediate filament

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Fhos1 is a mammalian formin-family protein, and functions as an organizer of the actin microfilament. Here we have cloned human and mouse cDNAs for a novel Fhos homolog, designated Fhos2. The messages for Fhos2 are expressed in the heart, kidney, and brain, where the Fhos1 mRNAs are not abundant. Two splice variants of Fhos2 exist in a tissue-specific manner; the longer variant Fhos2L is the major form in the heart, whereas the kidney and brain predominantly express Fhos2S that encodes a shorter protein. Over-expression of an active form of the two Fhos2 variants, as well as that of Fhos1, induces the formation of actin stress fibers in HeLa cells, suggesting that Fhos2 acts as an actin-organizing protein. Biochemical analysis using rat cardiomyoblastic H9c2 (2-1) cells reveals that endogenous Fhos2 is enriched in the intermediate filament fraction. Consistent with this, Fhos2 localizes to the nestin intermediate filament but not to other cytoskeletons, as demonstrated by staining of H9c2 (2-1) cells with anti-Fhos2 antibodies. Furthermore, Fhos2 is present in nestin-expressing neuroepithelial cells of the fetal rat brain. Thus, Fhos2 not only has the actin-organizing activity but also associates with nestin, which may imply a Fhos2-mediated link between the nestin intermediate filament and actin microfilament.

Introduction

The cytoskeleton is vital for many cellular processes, including movement, adhesion, polarity establishment, intracellular trafficking, and modulation of mechanical strength. These various tasks are performed by dynamic integration of three distinct types of cytoskeletal filament networks: actin microfilaments, microtubules, and intermediate filaments (IFs) (Fuchs & Cleveland 1998; Pollard 2003). Since the three filaments are incapable of directly binding to each other, the cooperation of the networks is organized by a diverse array of linker proteins, most of which associate with more than one filament network and coordinate the dynamics in various situations (Herrmann & Aebi 2000; Fuchs & Karakesisoglou 2001). Among such proteins are formins that function to regulate morphogenesis, cell polarity and cytokinesis in various eukaryotic cells.

The formins are structurally characterized by the presence of two conserved regions, namely the FH (formin homology) 1 and FH2 domains. The FH1 region, rich in proline residues, appears to serve as a target of the actin monomer-binding protein profilin and/or proteins containing an SH3 or WW domain (Chan et al. 1996; Imamura et al. 1997). The FH2 domain, the most conserved region among formins, locates C-terminal to the FH1 domain, and has recently been found to nucleate actin filaments (Pruyne et al. 2002; Sagot et al. 2002b). Via the two core modules FH1 and FH2, formins appear to participate in construction of actin-based structures including actin cables, contractile rings, stress fibers, lamellipodia and adherence junctions (Watanabe et al. 1997; Evangelista et al. 2002; Pelham & Chang 2002; Kobielak et al. 2004). There is also emerging evidence that formins are integrated into microtubule networks. Some formins decorate microtubule networks such as mid-bodies (Tominaga et al. 2000), spindle microtubules (Kato et al. 2001), and detyrosinated microtubules (Palazzo et al. 2001), thereby inducing localized stabilization of microtubules.

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(Kodama et al. 2003; Palazzo et al. 2004). Taken together with the observation that an active formin can induce coalignment of microfilament and microtubule arrays (Ishizaki et al. 2001), formin proteins may function as linker proteins of the actin cytoskeleton and microtubules; coordination of these filaments is considered to be important in cell movement and polarity establishment.

IFs, one of three major cytoskeletal networks in eukaryotic cells, was previously considered to be relatively stable, and to function solely to provide cellular integrity and resistance against mechanical stresses. It is, however, becoming clear that IFs undergo dynamic changes in organization during cell growth, polarization, and differentiation in conjunction with other cellular structures actin and microtubule (Helfand et al. 2004). Time-lapse studies of cells expressing GFP-vimentin clearly show that many vimentin fibrils constantly change shape and appear to assemble, disassemble, shorten and elongate throughout the cytoplasm (Yoon et al. 1998; Martyis et al. 1999). Compared with actin microfilaments and microtubules, IFs are highly diverse in their numbers, sequences, and abundances (Coulombe et al. 2001). The individual IF proteins are chemically heterogeneous, exhibiting different assembly kinetics and yielding discrete types of filaments. Nestin, an IF protein, is originally identified as a gene product that is highly expressed in the neural stem cells; this property distinguishes these cells from the more differentiated cells in the neural tube (Lendahl et al. 1990). Since the expression of nestin is restricted to progenitor cells during not only neurogenesis but also myogenesis (Lendahl et al. 1990; Sejersen & Lendahl 1993; Kachinsky et al. 1995; Vaittinen et al. 1999), nestin is used as a marker for identification of the precursor cells. However, little is known about the function of nestin at the molecular and cellular levels.

Fhos1 belongs to a novel subclass of formins, and is possibly involved in transcription from the serum response element (Westendorf 2001). We have recently shown that the active form of Fhos1 induces the formation of actin stress fibers and localizes to the actin-based structures via a direct F-actin binding of a region N-terminal to the FH1 domain (Takeya & Sumimoto 2003). Here we have cloned cDNA encoding a novel homolog of Fhos1, designated Fhos2. Fhos2 in an active form is also capable of inducing actin stress fibers, indicative of Fhos2 as an actin-organizing protein. Immunofluorescence staining of rat cardiomyoblastic H9c2 (2–1) cells reveals that Fhos2 probably associates with the nestin IF, and the identification of Fhos2 as an IF-associated protein is supported by biochemical analysis. Moreover, we demonstrate that Fhos2 is expressed in nestin-containing neural stem cells such as the neuroepithelial cells of the fetal rat brain. The association of the actin-organizing formin Fhos2 with the nestin IF raises the possibility that Fhos2 might integrate nestin filaments into the actin network.

Results
Cloning of cDNAs encoding mouse and human Fhos2
A search of human genome databases with human Fhos1 gene mapped on chromosome 16q22 (Westendorf et al. 1999) revealed that highly homologous sequences exist on a different locus (chromosome 18q12). Using the novel sequences, we searched human EST databases to obtain several clones. On the basis of the clones, we synthesized probes and obtained clones encoding partial sequence of human Fhos2 (hFhos2) cDNA by screening a human fetal brain cDNA library. The longest clone and cDNA clones from RT-PCR, and 5′RACE enabled us to obtain a full-length human Fhos2 (hFhos2: #AB084087) cDNA. Fhos2 encoded 1422 amino acids containing FH1 (corresponding to amino acids 827–858) and FH2 (amino acids 880–1316) domains (Fig. 1B).

To obtain a full-length mouse Fhos2 (mFhos2), we screened a mouse brain cDNA library with probes based on the EST clones homologous to human Fhos2. Two major transcripts resulting from alternative splicing were cloned: the long isoform (mFhos2L: #AB078608) encoded 1578 amino acids, while the short isoform (mFhos2S: #AB078609) had an in-frame deletion of 453 bp and encoded 1427 amino acids, which was 91% identical to hFhos2 (Fig. 1A). In comparison with an amino acid

Figure 1 Structure of Fhos2 and other formin-related proteins. (A) Deduced amino acid sequences of human and mouse Fhos2 (hFhos2 and mFhos2) in comparison with those of human and mouse Fhos1 (hFhos1 and mFhos1). Identical amino acid residues are indicated by asterisks. Shaded are the FH2 domains, and boxed is the sequence that is inserted in the long form of mFhos2L. (B) Schematic presentation of the structures of Fhos proteins. The homology in the indicated regions of Fhos proteins is expressed as a percentage of the number of identical amino acid residues to that of all the residues. The black boxes indicate the FH1 domains, and the gray boxes stand for the FH2 domains. The hatched box in mFhos2L represents the sequence that is truncated in mFhos2S. (C) Branches of the formin family. To build a phylogenetic tree, the FH2 domains of the formin proteins were aligned using the Clustal W program (Thompson et al. 1994) and the unrooted tree was constructed using the neighbor-joining method with the program Treeview (Page 1996). Abbreviations used for species names are Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Mm, Mus musculus; and Hs, Homo sapiens.
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A

B

C
sequence of full-length mouse Fhos1 (#AB041045), which we also cloned in the present study by screening of cDNA libraries from mouse B cells and from the spleen, the N-terminus (amino acids 1–328) and the C-terminus (amino acids 917–1578: including the FH1 and FH2 domain) of mFhos2L were 61.8% and 52.1% identical to the corresponding regions of mFhos1, respectively (Fig. 1B). Using the FH2 domains of formin homology proteins from different species, we constructed an evolutionary tree (Fig. 1C). Fhos proteins, which are also found in Caenorhabditis elegans and Drosophila melanogaster, are highly conserved among species, and therefore constitute a subclass distinguished from the most characterized subclass Diaphanus-related formins.

Tissue specific expression of splice variants of mFhos2

The mRNA for mouse Fhos2 was abundant in the heart and kidney, and also in the brain but to a lesser extent (Fig. 2A, left panel); the expression pattern is similar to that of its human orthologue hFhos2 (data not shown). On the other hand, the mRNA for mFhos1 was ubiquitously expressed except for the brain (Fig. 2B). Northern blot analysis using ‘a’ fragment, corresponding to the FH2 domain of mFhos2, as a probe revealed that the electrophoresis mobility of the transcript in the heart was slightly lower than that of the transcripts in the brain and kidney (Fig. 2A, left panel). Since Fhos2S differs from Fhos2L by an internal deletion of 453 nucleotides, which results in a protein lacking 151 amino acids (401–551), we assumed that these alternatively spliced variants distribute in a tissue specific pattern. To test this possibility, we used the cDNA fragment ‘b’ whose sequence corresponds to the region within the insert sequence, and confirmed the tissue-specific expression of alternative splicing variants: the band was detected in the heart (Fig. 2C). Although the expression level of mFhos2S message in the kidney was comparable to that in the brain (Fig. 2A,C), the protein level of mFhos2S in the kidney was lower than that in the brain by the Northern blot analysis. The reason for this discrepancy presently remains unknown, but it might be caused by the protein instability or the transcriptional/translational regulation in the kidney.

Remodeling of the actin cytoskeleton induced by mFhos2 mutant proteins

Foramin homology proteins are known to play a crucial role in the regulation of the actin cytoskeleton (Evangelista et al. 2003; Wallar & Alberts 2003). Among them, Fhos1 as well as mDia is known to induce formation of actin stress fibers upon activation (Gasteier et al. 2003; Koka et al. 2003; Takeya & Sumimoto 2003). Since over-expression of full-length Fhos1 by itself does not

Figure 2 Tissue-specific expression of alternatively spliced variants of Fhos2. (A) Northern blot analysis of mRNAs for mouse Fhos2. Mouse Multiple Tissue Northern blots (Clontech) were hybridized with the DNA fragment ‘a’ (left) or ‘b’ (right) shown in Fig. 2E. (B) Northern blot analysis of mRNA for mouse Fhos1. Mouse Multiple Tissue Northern blots (Clontech) were hybridized with a cDNA fragment of mouse Fhos1, the region of which encodes amino acids 797–1019 of mouse Fhos1. (C) RT-PCR analysis of mouse Fhos2. RT-PCR with a pair of the primers ‘c’ and ‘e’ (left panel) and a pair of ‘d’ and ‘e’ (right panel). The indicated bands of the lengths of 1074, 978 and 621 bp were the products from Fhos2L, Fhos2M and Fhos2S, respectively. The identities of these bands were confirmed by DNA sequencing. For detail, see Experimental procedures. (D) Western blot analysis of mouse Fhos2 variants. Lysates of indicated tissues or cell lines were subjected to SDS-PAGE, followed by immunoblot with antibodies anti-Fhos2 (873–974) raised against GST-fusion protein comprising amino acids 873–974 of Fhos2L (left), anti-Fhos2 (C-20) raised against the C-terminal peptide (1559–1578) of Fhos2L (middle) and anti-Fhos2 (650–802) raised against GST-fusion protein comprising amino acids 650–802 of Fhos2L (right). Lysates of HeLa cells expressing Fhos2L or Fhos2S by adenovirus-mediated transfection were also analyzed by immunoblot. (E) Location of probes for Northern blot analyses, primers for RT-PCR analyses, and peptides for generation of anti-Fhos2 antibodies. The cDNA fragments ‘a’ and ‘b’ used in Northern blot analyses (A) encode the regions of amino acids 1074–1347 and 445–550 of Fhos2L, respectively. The PCR primers ‘c’, ‘d’ and ‘e’ were used in RT-PCR analyses (B). The antibodies raised against the Fhos2 peptides ‘f’ (amino acids 650–802), ‘g’ (amino acids 873–974), and ‘h’ (amino acids 1559–1578) were used in Western blot analyses (C).
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A

B

C

D

E

probe: a

probe: b

primer: c & e

primer: d & e

30 µg/lane 1x10^5 cell/lane 5x10^3 cell/lane

blot: anti-Fhos2(873–974)

blot: anti-Fhos2(C-20)

blot: anti-Fhos2(650–802)

Fhos2L

Fhos2S
affect the actin cytoskeleton, Fhos1 seems to be normally folded in an inactive form, which is maintained by an intramolecular interaction between the N- and C-terminal regions (Gasteier et al. 2003; Takeya & Sumimoto 2003). Disruption of the intramolecular interaction by deleting the N- or C-terminal region leads to activation of Fhos1, thereby inducing formation of stress fibers (Takeya & Sumimoto 2003). Thus we examined the influence of mFhos2 on the actin cytoskeleton by over-expressing a C-terminally deleted mFhos2L (mFhos2LΔC) and mFhos2S (mFhos2SΔC) in HeLa cells, and found that both of them caused stress fiber formation together with cell elongation (Fig. 3). mFhos1ΔC strongly localized on the actin filaments like hFhos1ΔC (Takeya & Sumimoto 2003). GFP–mFhos2SΔC appeared to localize on the actin filaments but to a lesser extent (Fig. 3). On the other hand, GFP–mFhos2LΔC, a C-terminally deleted protein of the long variant, hardly localized to the actin filaments, although it induced stress fibers. Fhos2L contains an extra insert sequence in the region N-terminal to the FH1 domain, compared with Fhos2S and Fhos1 (Fig. 1B). The insertion in the N-terminal region might prevent Fhos2L from localizing to the actin stress fibers. The importance of the N-terminal region in association with actin filaments is indicated by our previous observation that the region N-terminal to the FH1 domain of Fhos1 directly binds to F-actin and mediates the targeting of this protein to actin stress fibers (Takeya & Sumimoto 2003).

**Fhos2 localizes to the nestin IF**

To study the role of mFhos2 in regulation of the cytoskeletal network, we used confocal microscopic fluorescence immunocytochemistry with the anti-mFhos2 antibodies and examined its subcellular localization. According to the finding that mFhos2 was predominantly expressed in the heart by Western blotting (Fig. 2D), we first examined the subcellular localization of mFhos2 in H9c2-(2-1) cells, which are derived from rat embryonal cardiomyocytes (Kimes & Brandt 1976; Mejia-Alvarez et al. 1994). Double staining of these cells with the anti-mFhos2 antibodies and phalloidin showed that some populations of Fhos2 localized to actin fibers (Fig. 4A). However, the residual part displayed filamentous staining pattern different from that of actin filaments; therefore we investigated co-localization of mFhos2 with other cytoskeleton components. Double-labeling experiments with the anti-mFhos2 antibodies and an anti-β-tubulin antibody revealed that Fhos2 does...
not seem to be associated with microtubules. Next we focused on the intermediate filament (IF) proteins: desmin and nestin, which have been reported to comprise IFs in cardiomyocytes (Kachinsky et al. 1995; Capetanaki et al. 1997). A staining pattern of desmin filaments, aligned along the longitudinal axis of the cell body, was totally different from that of mFhos2, but the distribution of nestin detected with monoclonal antibody Rat 401 bore a striking resemblance to that of mFhos2 (Fig. 4A). Magnified images of cells shown in Fig. 4B clearly showed the co-localization of Fhos2 with nestin. This result was also confirmed with other anti-nestin antibodies (clone 25 and 4D4; not shown) and with the anti-Fhos2 (873–974) antibodies (Fig. 4C) and the anti-Fhos2 (C–20) antibodies (data not shown).

It has been shown that, when cells are treated with microtubule–disrupting agents such as colcemid and colchicine, the IF proteins vimentin and neurofilament (NF-L) aggregate in the perinuclear area (Osborn et al. 1980; Jacobs et al. 1982), suggesting the dependence of IF networks on microtubules. We studied the effect of microtubule disruption on the association of Fhos2 with nestin. Treatment of H9c2 (2–1) cells with nocodazole, an agent that disrupts microtubules (Liao et al. 1995), did not affect the localization of Fhos2 on the nestin IF (Fig. 4A), although the agent had a moderate destructive effect on the nestin IF (Fig. 4A). Thus the association of Fhos2 with nestin by itself seems to be independent of microtubules. Essentially the same effect was observed when colcemid or colchicine was used instead of nocodazole. We next investigated the association of mFhos2 with nestin in another type of cell. Because mFhos2 was also expressed in the brain (Fig. 2D), we utilized rat pheochromocytoma PC12 cells derived from adrenomedulla, where nestin is shown to be expressed (Bertelli et al. 2002). We studied the distribution of Fhos2 in PC12 cells treated with nerve growth factor and found that Fhos2 did localize to nestin in the undifferentiated cells and that it was also expressed in the differentiated cells in contrast to nestin (Fig. 4D). Taken together with the Fhos2 expression in adult brain (Fig. 2), Fhos2 seems to be expressed in cells missing nestin, where Fhos2 might have different functions from IF organizing. In addition we exogenously co-expressed HA–tagged mFhos2 and GFP–tagged nestin in COS7 cells and investigated their localization by recognizing each tag. As shown in Fig. 4E, both mFhos2L and mFhos2S clearly co-localized with GFP–nestin.

To further study the association between Fhos2 and nestin, we made biochemical analysis. The IF proteins are, unlike microtubules and actin microfilaments, quite insoluble in non-denaturing buffers, and the bacterially expressed full-length Fhos2 is also insoluble (data not shown). This makes it difficult to use usual methods for detection of protein–protein interactions, such as in vitro pull-down assays. Thus we performed a conventional subcellular fractionation assay (Starger et al. 1978; Aynardi et al. 1984; Zackroff et al. 1984) using H9c2 (2–1) cells that express endogenous Fhos2 and nestin; this assay is widely used for estimation of interactions with IF proteins (Starger et al. 1978; Aynardi et al. 1984; Zackroff et al. 1984). As shown in Fig. 4F, both Fhos2 and nestin are enriched in the IF fraction, but not in the actin- and tubulin-containing fraction. The finding supports the idea that Fhos2 is a nestin-associated protein.

Fhos2 is expressed in neuroepithelial cells of the fetal rat brain

To explore the association between Fhos2 and nestin at the histological level, we performed immunohistochemical analysis of samples of the rat fetal brain. As shown in Fig. 5A, Fhos2 immunoreactivity is found in radial structures throughout the fetal brain. Cells facing the ventricle and radial processes extending from these cells were immunoreactive for Fhos2. The distribution pattern was similar to that of the immunoreactivity of nestin, an intermediate filament present in neuroepithelial cells. In the cerebral cortex, both immunoreactivities were localized in many radial fibers, extending from the ventricle surface to the pial surface at E16 and E20 (Fig. 5A). In the medulla oblongata, Fhos2 immunoreactivity was found in the radial processes extending from the surface of the fourth ventricle, which were also immunoreactive for nestin (Fig. 5B). Thus Fhos2 is expressed in neuroepithelial cells where it likely localizes to nestin at least partially.

Expression of Fhos2 and nestin in the fetal rat heart

Since Fhos2 is abundant in the heart (Fig. 2), we tested whether Fhos2 also associates with nestin in sections of the fetal rat heart. Fhos2 immunoreactivity is detected in the I-band structure throughout in the cardiac muscle cells (Fig. 5C). On the other hand, nestin is found in diffuse and weak expressions all over the cells. Nestin-positive cells are found in small-restricted clusters, and this distribution pattern was similar to that of Fhos2 in these restricted areas. Thus only a small amount of nestin is expressed in cardiac muscle cells; however, a part of nestin appears to co-localize with Fhos2 in these cells, although a large portion of Fhos2 does not associate with nestin.
**Discussion**

In the present study, we have cloned human and mouse cDNAs for Fhos2, a novel formin-family protein. Formins are presently considered to function as key integrators of actin and microtubule networks (Tominaga et al. 2000; Ishizaki et al. 2001; Kato et al. 2001; Palazzo et al. 2001, 2004; Kodama et al. 2003). Fhos2 as well as Fhos1 has an activity to induce the formation of actin stress fibers (Fig. 3); the actin-organizing activity is common to the formin family proteins. We also show that Fhos2 associates with the nestin-based IF in various cells (Figs 4 and 5). To our knowledge, this is the first report that has shown that a formin-family protein likely interacts with IFs. Thus it seems possible that Fhos2 forms a link between the nestin IF and actin microfilament.

Fhos2 is abundantly expressed in the heart and kidney, and also in the brain, but to a lesser extent (Fig. 2A). On the other hand, Fhos1 is almost ubiquitously expressed; however, its expression is relatively low in the heart and undetectable in the brain (Fig. 2B). The finding that tissue expressions of Fhos1 and Fhos2 are nearly complementary raises the possibility that the Fhos proteins have a common function that plays an essential role in various tissues. We also demonstrate that two major variants of Fhos2, derived from alternative splicing, exist in a tissue-specific manner at both protein and mRNA levels (Fig. 2); the longer variant Fhos2L is the major form in the heart, whereas the kidney and brain predominantly express Fhos2S that encodes a shorter protein. The variant proteins both exhibit the activity to induce actin stress fibers and localize to the nestin IF (Figs 3 and 4). On the other hand, an active form of Fhos2S is targeted to the stress fibers formed, while targeting of the corresponding form of Fhos2L is hardly observed (Fig. 3). The difference might imply a distinct function of the two isoforms of Fhos2, although it remains to be elucidated.

The FH2 domains of the mammalian formin mDia and the yeast formin Bni1p both exhibit an actin-polymerizing activity, which is considered to be involved in formation of actin fibers in vivo (Watanabe et al. 1999; Evangelista et al. 2002; Pruyn et al. 2002; Sagot et al. 2002a,b; Li & Higgs 2003). The present phylogenetic analysis of FH2 domains reveals that the metazoan formins can be classified into at least five groups: the Dia, Fmn, Daam, Frl and Fhos groups (Fig. 1C); on the other hand, the two genes for the budding yeast formins and the three genes for the fission yeast formins do not seem to belong to any of the metazoan formin groups. Thus, in spite of the distance between FH2 domains in yeast and mammals, the domains have a common function, i.e. actin polymerization (Evangelista et al. 2003; Wallar & Alberts 2003). Consistent with this, the FH2 domain of Fhos1 is required for the formation of actin stress fibers (Takeya & Sumimoto 2003); as shown in the present study, Fhos2 also have the conserved activity to form the stress fibers. The diversity of metazoan formin groups may account for the difference in the details of the polymerization mechanism: the mode of regulation, partners required for its activity, and so forth. The diversity also may be due to a function of the N-terminal region of formin groups, a region that is less conserved. Among the Fhos proteins, the N-terminal regions are remarkably well conserved (Fig. 1). The region N-terminal to the FH1 domain of Fhos1 has an activity to bind to F-actin, which activity is required for the localization of Fhos1 to actin stress fibers (Takeya & Sumimoto 2003). The corresponding region of Fhos2S also appears to be involved in the targeting to the actin fibers, since Fhos2L carrying the insertion to this region fails to localize to the structure (Fig. 3). Although the extreme N-terminal regions of Fhos1 and Fhos2 are the most conserved with about a 60% amino acid identity, the function of the highly conserved regions is presently unknown.

As shown in the present study, Fhos2 not only acts as an actin-organizing protein, but also appears to associate...
with the IF nestin in various types of cells (Figs 4 and 5). This interaction is specific to nestin; Fhos2 does not co-localize with desmin, another IF protein (Fig. 4). It remains, however, to be determined whether the binding is direct or indirect because of the difficulty of a large-scale preparation of recombinant Fhos2 and the IF protein nestin. One of the candidates might be a protein of the spectraplakin family, which can interact with all the three elements of the cytoskeleton: actin, microtubules and IFs (Roper et al. 2002; Jefferson et al. 2004). It has recently been shown that ACF7, a member of spectraplakins, may function downstream of mDia, a mammalian formin, in the signaling pathway of cell polarization (Kodama et al. 2003). However, it is presently unknown whether one of the candidate proteins indeed binds to nestin. Future studies are required for understanding of the biological role and the molecular mechanism for the interaction between Fhos2 and nestin.

Despite accumulating bodies of evidence that nestin is widely expressed during mammalian embryogenesis in a variety of embryonal tissues (Wiese et al. 2004), little is known about its role in cellular events including the cytoskeletal dynamics. The present finding of the association between Fhos2 and nestin may provide a clue for the understanding of the cellular function of nestin.

**Experimental procedures**

**Isolation of cDNA for human Fhos2**

A search of human genome database with human Fhos1 revealed that there exist some partial nucleotide sequences homologous but not identical to those of Fhos1 on the locus (18q12) different from that of Fhos1 (16q22). A search of human EST database with these sequences revealed multiple EST clones. On the basis of sequences of a human EST clone (AA338649) homologous to human Fhos1, we synthesized the two unique oligonucleotide primers 5′-GCAAGATCTTTAGGGTGGTCCCAGGTACCC-3′ and 5′-TGCACTGCCCAGGGGGATTTC-3′, where sequences from the EST clone are in italics. With the primes, details, see Experimental procedures. (B) Immunohistochemical localization of Fhos2 (left panels) and nestin (right panels) in the rat medulla oblongata at E16. Magnified views of the inserts are also shown in lower panels. (C) Immunohistochemical localization of Fhos2 (red) and nestin (green) in the rat heart. The cryo-sections were double-stained with purified rabbit polyclonal antibodies against Fhos2 (650–802) (1 : 500) and mouse monoclonal antibody against nestin (Rat401) (1 : 10), and the merged image is shown. For details, see Experimental procedures. Scale bars, 20 μm.
PCR was performed using the EST clone as a template. The PCR product of 468 nucleotides was used as a probe for screening a human fetal brain cDNA library (Stratagene). Sixteen independent positive clones were obtained from about 1 × 10^9 plaques, and the clones were sequenced in both directions. They were found to contain a stop codon near the 3’ end, but none of them contained the putative first methionine. To obtain further 5’ sequence, we used a human kidney cDNA library as a template and performed PCR with two unique oligonucleotide primers, 5’-GGTGCACACGTGGTT (primer ‘c’) and 5’-CAGGGATCCAGGGATGAG-3’ (primer ‘e’), where sequences from the positive clones are in italics. The RT-PCR products were revealed three spliced variants: the long isoform as mouse Fhos2L (GenBank™ accession no. AB078608) containing 453 bp unique insert sequences, the short isoform as Fhos2S (GenBank™ accession no. AB078609), and the minor isoform of intermediate length as Fhos2M (GenBank™ accession no. AB100088) containing 357 bp of the insert.

**Isolation of cDNA for mouse Fhos1**

A database search yielded multiple mouse EST clones which are homologous to human Fhos1. Based on sequences of two homologous clones (W98506 and AA671082), we synthesized the unique oligonucleotide primers 5’-CAGGGATCCCATGGTGAAGCTTCTGGTGATG-3’ (forward primer) and 5’-GAGGAATTCAACCCCAAGGGGACAG-3’ (reverse primer), where sequences from the EST clones are in italics. With the two primers we performed reverse-transcription PCR, using total RNA as a template, which was extracted from mouse heart tissue by acid guanidinium-phenol-chloroform (AGPC) method. The PCR product of 315 nucleotides was used as a probe for screening mouse B-cell and spleen cDNA libraries (Stratagene). Seven independent positive clones were obtained from about 3 × 10^9 plaques and sequenced in both directions. The longest clone was found to encode a full-length mouse Fhos1 (GenBank™ accession no. AB041045).

**Northern blot analysis**

Mouse Multiple Tissue Northern™ blots (Clontech) were hybridized with a 32P-labeled cDNA fragment of mouse Fhos1 (corresponding to amino acids 797–1019), Fhos2 (corresponding to amino acids 1074–1347 of Fhos2L) or Fhos2L (corresponding to amino acids 445–550) under high-stringency conditions using Express Hyb™ (Clontech).

**Construction of recombinant plasmids and recombinant adenoviruses**

For expression of mouse Fhos1, Fhos2L and 2S, and their deletion mutants Fhos1ΔC (lacking amino acid residues 1086–1197), Fhos2LΔC (lacking amino acid residues 1500–1578) and Fhos2SΔC (lacking amino acid residues 1349–1427) as GFP-tagged proteins, their cDNAs were ligated to pEGFP-C1 (Clontech). The adenoviruses encoding HA–tagged Fhos2L, Fhos2S, and their mutants were constructed by Adeno-X™ Expression System (Clontech) according to manufacturer’s instructions. The full-length cDNA of mouse nestin was ligated to pEGFP-C1 (Yang et al. 2000, 2001).

**Antibodies**

Affinity-purified rabbit polyclonal antibodies specific for Fhos2 were prepared as follows. Anti–Fhos2 (650–802) and anti–Fhos2 (873–974) antibodies were raised against the respective GST–fusion
proteins comprising amino acids 650–802 and 873–974 of Fhos2L. Another anti-Fhos antibody, namely anti-Fhos2 (C–20), was raised against a synthetic peptide corresponding to amino acids 1559–1578, which sequence is identical among mouse, rat and human Fhos2 but not in Fhos1. The anti-Fhos2 antibodies were affinity purified by a HiTrap–NHS column (Amersham Bioscience) conjugated with MBP–fusion proteins corresponding to the immunizing regions or with the synthetic peptide used for immunization, respectively. Monoclonal antibodies were purchased from commercial sources as indicated; Rat 401 and clone 25 against nestin (BD Biosciences); 4D4 against nestin (Biongenesis); TUB 2.1 against β-tubulin (Sigma); DE-U-10 against desmin (Sigma); V9 against vimentin (ICN Pharmaceuticals); DA2 against neurofilament (NF-L) (Chemicon International); C4 against actin (Roche); HA.11 against hemagglutinin (COVANCE).

Immunoblot analysis
Mouse adult organs were dissected, homogenized and sonicated at 4 °C in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4) containing 1% TritonX-100, 200 µg/mL chymostatin, 200 µg/mL pepstatin A, 100 µg/mL aprotinin, 400 µg/mL leupeptin, and 200 µM phenylmethylsulfonyl fluoride (PMSF). The homogenates were spun at 8000 g for 5 min at 4 °C. The supernatants were boiled in sample buffer containing β-mercaptoethanol (β-ME), and respun at 20 000 g for 10 min 30 µg of protein was separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was probed with the anti-Fhos2 rabbit polyclonal antibodies. The blots were developed using ECL-plus (Amersham Bioscience) to visualize the antibodies.

Cells and transfection
HeLa cells and NIH3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). HeLa cells were washed once and incubated for 3 h in OPTI-MEM (Invitrogen) containing plasmids mixed with Lipofectamine (Invitrogen) and/or adenoviruses. After the addition of DMEM containing 10% FCS, cells were cultured for another 13 h. H9c2 (2-1) cardiac myoblast cell line, derived from the embryonic rat heart (Kimes & Brandt 1976; Mejia-Alvarez et al. 1994), were purchased from American Type Culture Collection (ATCC) and cultured in DMEM containing 10% FCS. Experiments were carried out using cells at passage 20 or lower; cells arrived from ATCC at passage 14. PC12 cells were plated on to poly-L-ornithine (Sigma)-coated glass coverslips and cultured in RPMI1640 with 10% horse serum and 5% FCS. To induce differentiation of PC12 cells to a neuronal phenotype, nerve growth factor (60 ng/mL) was added and cultured for 1 week.

Fixation and immunofluorescence staining
For the staining of F-actin, cells were washed once with PBS and fixed for 15 min at 37 °C in the same solution containing 3.7% formaldehyde. For the staining of microtubule and intermediate filament, cells were rinsed with PBS and then fixed in methanol for 10 min at −20 °C. Cells were permeabilized for 4 min in PBS containing 0.1% TritonX-100. Alternatively, cells were permeabilized for 1 min in PBS containing 1% TritonX-100 (in the case of Fig. 4D). Following three washes with PBS, cells were blocked with PBS containing 3% bovine serum albumin for 60 min. The samples were initially incubated with primary antibodies. Alexa Fluor™ 594-labeled goat anti-mouse and anti-rabbit IgG antibodies (Molecular Probes) and FITC-conjugated goat anti-mouse and anti-rabbit IgG antibodies (BIOSOURCE) were used as secondary antibodies. For F-actin staining, Texas Red-X phalloidin (Molecular Probes) was used. Images were visualized with laser confocal microscopes LSM5 PASCAL (Carl Zeiss), with the exception of those shown in Figs 3B and 5B, which were acquired by a Nikon Eclipse TE300 microscope and captured on an ORCA digital camera (Hamamatsu Photonics).

Association with an IF-rich fraction
IF-rich fractions were prepared from rat H9c2 (2-1) and mouse NIH3T3 cells, according to the method previously reported (Starger et al. 1978; Aynardi et al. 1984; Zackroff et al. 1984) with minor modifications. Briefly, cells were rinsed twice with PBS and then lyzed with a lysis buffer (PBS containing 1% TritonX-100, 0.6 mM KCl, 10 mM MgCl2, 1% β-ME, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mg/mL p-tosyl-L-arginine methyl-ester) for 3 min at room temperature. Deoxyribonuclease I (Roche) was added to a final concentration of 0.5 mg/mL, and the lysate was then centrifuged at 16000 g for 10 min at 4 °C. The resulting pellet was washed three times in PBS containing 0.1 mM PMSF and 1% β-ME, and then subjected to SDS-PAGE as an IF-rich fraction, followed by immunoblot with the anti-Fhos2 antibodies.

Tissue preparation and immunohistochemical procedure
Fetuses of SD rats were used in the present study. The day a vaginal plug was found was designated as embryonic day 0.5 (E0.5) and fetuses of E16.5 and E20.5 were used for immunohistochemical analysis. Fetuses were removed from the mother uterus under ether anesthesia, and their heads were cut off and immersed in ice-cold acid-alcohol solution (95% ethanol and 5% acetic acid) overnight. Their brains were immersed in absolute ethanol, transferred to xylene and embedded in paraffin. Five µm-thick frontal sections were cut serially on a rotary microtome (Microm H325, Heidelberg, Germany), mounted on glass slides, and air-dried on a hot plate overnight at 44 °C. After hearts were fixed, 8 µm-thick sections were cut on cryo-rotary microtome (Leica, Heidelberg, Germany), mounted on glass slides, and air-dried overnight at room temperature.

Immunohistochemical procedures were performed as previously described (Kawano et al. 1999, 2003) with minor modifications. Briefly, paraffin sections were deparaffinized in xylene, rehydrated through a descending ethanol series and rinsed in distilled water. Sections were incubated with a mouse monoclonal antibody against nestin (Rat401) (1 : 10), (American Research Products Inc, Belmont, MA, USA) or purified rabbit polyclonal
antibodies against Fhos2 (650–802) (1:500) overnight at 4 °C. Antibodies were diluted with 20 mM PBS containing 0.5% skim milk. After rinsing in distilled water for 15 min, sections were incubated for 1 h at 37 °C with peroxidase-labeled anti-rabbit or anti-mouse IgG Fab’ fragments (1:100) (Medical and Biological Laboratories, Nagoya, Japan) as appropriate. After rinsing in distilled water for 15 min, immunoreaction was visualized in 50 mM Tris, pH 7.4, containing 0.01% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide for 5–15 min at 37 °C. Mounted sections were observed and recorded using a Zeiss Axiophoto microscope equipped with a cooled CCD camera (Zeiss Axio Cam) and Zeiss Axiovision software. Cryo-sections were incubated with mouse monoclonal antibody against nestin (Rat401) (1:10) or/and purified rabbit polyclonal antibodies against Fhos2 (650–802) (1:500) overnight at 4 °C. After rinsing in PBS for 15 min, sections were incubated for 1 h at 37 °C with rhodamin or FITC-labeled anti-rabbit or anti-mouse IgG Fab’ fragments (1:500); (Chemicon International, USA). After rinsing, the sections were observed and recorded with a Zeiss LSM510 confocal microscope.

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