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Localization of PTP-FERM in Nerve Processes through Its FERM Domain[1]

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PTP-FERM is a protein tyrosine phosphatase (PTP) of *Caenorhabditis elegans* containing a FERM domain and a PDZ domain. Here we report the characterization of PTP-FERM and the essential role of its FERM domain in the localization of PTP-FERM in the worm. There are at least three alternatively spliced PTP-FERM isoforms, all of which contain a band 4.1/FERM domain, a PDZ domain, and a catalytic domain. PTP-FERM possessed phosphatase activity. PTP-FERM was expressed predominantly in neurons in the nerve ring and the ventral nerve cord. PTP-FERM was found in the nerve processes and to be enriched in the perimembrane region. Studies using various deletion mutants revealed that the FERM domain was essential and sufficient for the subcellular localization. These results suggest the essential role of the FERM domain in the function of PTP-FERM in the neurons of *C. elegans*.

Key Words: **protein-tyrosine-phosphatase; FERM domain; nerve processes; *C. elegans*.**

There is a group of protein tyrosine phosphatases (PTPs) possessing a domain called band 4.1 or FERM (band four-point-one, ezrin, radixin, moesin homology) domain. Five mammalian PTPs, namely, PTPH1 (1), PTPMEG (2), PTPD1/PTP-RL10/rPTP2E (3–5), PTP36/PTPD2/Pez (3, 6, 7), and PTP-BAS/hPTP1E/PTPL1/FAP-1 (8–11), belong to this band 4.1-superfamily PTP. In addition to the band 4.1/FERM domain, PTPH1 and PTPMEG have a PDZ domain, while PTP-BAS/hPTP1E/PTPL1/FAP-1 have five PDZ domains.

The band 4.1/FERM domain was originally found in the members of the band 4.1 and ERM (ezrin, radixin, moesin) family cytoskeletal proteins. In the case of ERM, this domain associates with membrane-spanning molecules such as CD44 and thereby mediates linkage between plasma membrane and actin cytoskeletons (12). It has been reported recently that the N-terminal regions of FAK and JAK tyrosine kinases are divergent members of the classical band 4.1-domain family (13). Through band 4.1/FERM domains, FAK and JAK associate with PDGF- and EGF-receptors (14) and cytokine receptors (13), respectively. Conservation of the band 4.1/FERM domain in a group of PTPs suggests the importance of these domains in the function of the PTP.

To understand the function of band 4.1-superfamily PTPs, it is important to identify the physiological substrates. A substrate-trapping mutant of PTPH1 identified VCP/p97/CDC48 as a specific substrate of PTPH1 (15). VCP/p97/CDC48 is an AAA (ATPase associated with different cellular activities) family ATPase and plays a critical role in organelle membrane fusion. This observation is interesting because the phosphorylation of CDC48 is important for the cell cycle progression in yeast. It is possible that PTPH1 may exert its effect on cell growth through VCP/p97/CDC48.

Besides VCP/p97/CDC48, many other molecules are reported to associate with band 4.1-superfamily PTPs. PTPH1 associates with the adaptor 14-3-3β molecule in a serine phosphorylation-dependent manner (16). The band 4.1/FERM domain of PTPD1/PTP-RL10/rPTP2E but not that of PTP36/PTPD2/Pez can bind to KIF1C, a kinesin-like protein involved in the vesicle transport (17). PTPMEG interacts with glutamate receptor δ2 and ϵ1 subunits through its PDZ domain (18). This interaction enhances Fyn-mediated tyrosine phosphorylation of glutamate receptor ϵ1 (18). PTP-

Abbreviations used: PTP, protein tyrosine phosphatase; FERM, band four-point-one, ezrin, radixin, moesin homology; GST, glutathione *S*-transferase; kb, kilobase; nt, nucleotide; aa, amino acid.

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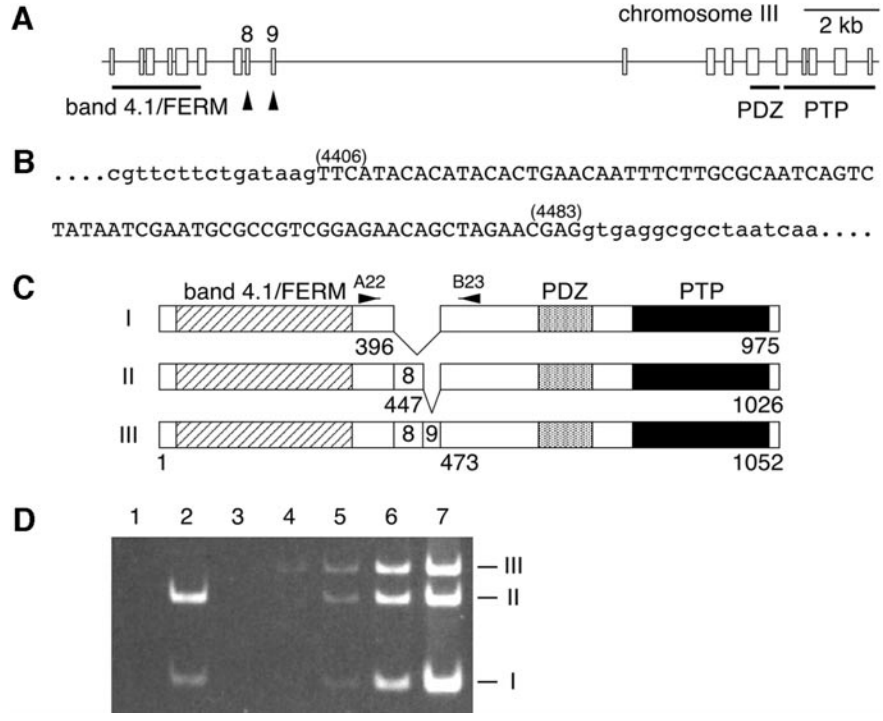

FIG. 1. Structure of the *ptp-ferm* gene and its products. (A) Exon structure of the *ptp-ferm* gene. The structure is based on genome sequence data (Z36237, Z48241), EST data (yk417b1, yk129c1, yk133g6, yk227f7, and yk287g3), and our own sequence analysis of PTP-FERM cDNA. Exons are indicated by boxes and introns are indicated by lines. The arrows show alternatively spliced exons. (B) The sequence of exon 9. The exon sequence is shown in uppercase letters and the intron sequence is shown in lowercase letters. (C) Schematic drawings of PTP-FERM isoforms produced by alternative splicing of exons 8 and 9. Band 4.1/FERM, band 4.1/FERM domain; PDZ, a PDZ domain; PTP, a phosphatase domain. (D) Expression of PTP-FERM isoforms in the worm. RT-PCR was performed using total RNA from worms of mixed age (lane 2). The locations of PCR primers, A22 and B23, are shown in C as arrows. This primer set produces 192, 342, and 424-bp PCR fragments from cDNA of isoforms I, II, and III, respectively. In lane 1, the RNA sample was treated exactly as lane 2 except that no reverse transcriptase was added in the RT step (negative control). Lane 3, no sample (negative control). In lanes 4 to 7, various amounts of cDNA mixture that contained 10 (lane 4), 100 (lane 5), 1000 (lane 6), or 10000 (lane 7) molecules of each isoform were used as positive controls. The position of each isoform was shown on the right.

BAS/hPTP1E/PTPL1/FAP-1 has five PDZ domains, which have the capacity to associate with BP75, a bromodomain containing protein (19), IκBα, an inhibitor of transcription factor NFκB (20), LIM containing proteins such as zyxin-related protein-1 (ZRP-1) (21, 22) and RIL (23), human CD95/FAS (11, 24, 25), neurotropin receptor p75 (26), and GTPase-activating protein (GAP) toward Rho (PARG-1) (27). Of these binding molecules some may be downstream effectors or upstream regulators of PTPs. However, the physiological significance of these interactions remains to be elucidated.

Several reports have described the phenotypic changes of cell lines upon overexpression of some of these PTPs. Recently, we have demonstrated that the overexpression of PTP36/PTPD2/Pez in HeLa cells inhibits cell growth and induces the changes in the cytoskeleton (28). Overexpression of PTPH1 and PTP-MEG has been reported to suppress cell growth (15, 29). It is also reported that the overexpression of PTPH1 reduces T cell antigen receptor signaling (30). Elevation of PTP-BAS/hPTP1E/PTPL1/FAP-1 in a human cell line partially inhibits CD95/FAS-induced apoptosis (11). Because characterization of these phenotypes has been mainly based on overexpression experiments of wild type PTPs, the physiological function of the band 4.1-superfamily PTPs remains to be determined.

Studies of band 4.1-superfamily PTPs in model organisms such as *C. elegans* may facilitate characterization of the functions and effectors/regulators of the PTP. Therefore we analyzed the structure and expression of PTP-FERM, a band 4.1-superfamily PTP of *C. elegans*, and found that the FERM domain is essential for its localization in the nerve processes.

MATERIALS AND METHODS

Materials. Overlapping cosmid clones covering the entire PTP-FERM genome (C48D5, C44H11, DF12, and C32A3) were kindly provided by Dr. Alan Coulson, The Sanger Center. PTP-FERM cDNA clones (yk417b1, yk129c1, yk133g6, yk227f7, and yk287g3) were a gift from Dr. Yuji Kohara, National Institute of Genetics, Japan. Anti-Flag antibody (M2) and anti-GFP antibodies were purchased

from Eastman Kodak Co. (New Haven, CT) and Clontech Laboratories, Inc. (Palo Alto, CA), respectively.

Construction of expression plasmids and transfection. The expression plasmid, 95.P1, containing the *ptp-ferm::ptp-ferm-gfp* chimeric mini gene was constructed as follows. A 7 kb 5′ genomic fragment of *ptp-ferm* (nt −5880–1145) was generated by PCR using the C48D5 cosmid as a template and primers, 5′-aagcatgcagttcctctagccaatcgcataa-3′ and 5′-aacccgggtagaattggaacctcgtaaactcgtc-3′. The DNA fragment was digested by *Sph*I and *Sma*I, and cloned into the *Sph*I/*Sma*I site of pPD95.77, a *gfp* reporter vector developed by A. Fire at the Carnegie Institution of Washington. The resulting plasmid, pPD95/PTP-FERM/GFP1, was digested with *Bam*HI/*Bal*I and ligated to a PTP-FERM cDNA fragment. The resulting plasmid, 95.P1, was used to express PTP-FERM-GFP in the worm. Expression plasmids encoding various mutants of PTP-FERM-GFP were constructed by replacing the PTP-FERM cDNA fragment of 95.P1 with cDNA fragments encoding PTP-FERM mutants. Plasmids (0.1 μg/ml) were injected into the gonad of *C. elegans* and the worm lines that have the plasmid DNA as extrachromosomal arrays have been established. At least two independent lines were established and analyzed for each DNA construct.

For expression in mammalian cells, cDNAs for PTP-FERM and its mutants were subcloned into modified versions of the pEF-BOS mammalian expression vector containing EGFP or Flag-epitope-tag. 293T cells were transiently transfected by the calcium phosphate coprecipitation method as previously described (31).

Reverse transcriptase-PCR (RT-PCR). To study the expression of PTP-FERM isoforms, RT-PCR was performed. cDNA was synthesized from total RNA of mixed age worm with random hexamer primers. PCR was performed at 95°C for 12 min, and then for 30 cycles at 95°C for 30 s and 64°C for 1 min, using AmpliTag Gold (PE Biosystems) and the primers, A22 (5′-GCAACACACAATCAATTGATTCATCCCG-3′) and B23 (5′-GACGTAGAGGTACATGCCATGCTCGGA-3′).

Immunoblotting and immunofluorescence studies. Immunoblotting and immunofluorescence studies were performed as previously described (28).

Phosphatase assay. The fusion proteins between glutathione *S*-transferase (GST) and full-length PTP-FERM (type I) or PTP-FERM/C901S were produced and purified as previously described (32). The catalytic activities of GST-PTP-FERM and GST-PTP-FERM/C901S were assayed using *p*-nitrophenyl phosphate (p-NPP) as a substrate (32).

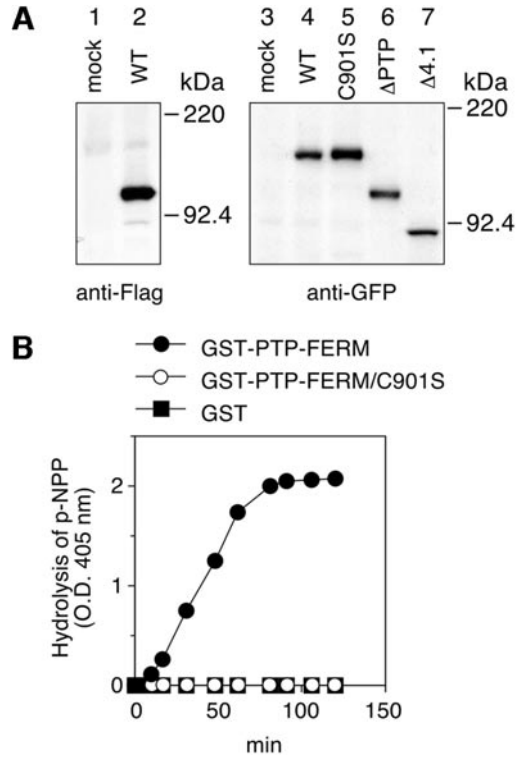

FIG. 2. PTP-FERM (type I) is a phosphatase of 110 kDa in size. (A) Expression of PTP-FERM (type I) and its mutants in a human cell line. Flag-tagged or GFP-tagged wild type PTP-FERM (lanes 2 and 4) and its mutants (lanes 5–7) were transiently expressed in 293T cells. Cell lysates were prepared and analyzed by immunoblotting experiments using anti-Flag (lane 1–2) and anti-GFP (lane 3–7) antibodies. Approximately 110 kDa Flag-PTP-FERM was detected (lane 2). GFP is about 240 aa long and the expected size of PTP-FERM-GFP was 134 kDa (lane 4). (B) Catalytic activity of PTP-FERM. Wild type PTP-FERM (type I) and PTP-FERM/C901S were expressed as GST fusion proteins in *Escherichia coli*, purified and catalytic activities were measured using *p*-nitrophenyl phosphate (p-NPP) as a substrate. GST-PTP-FERM (1 pmol), GST-PTP-FERM/C901S (2 pmol), and GST (100 pmol) were used.

RESULTS

PTP-FERM Is a C. elegans PTP with a Band 4.1/FERM Domain and a PDZ Domain

PTP-FERM was a putative PTP predicted from computer analysis of the *C. elegans* genome (Z36237, Z48241). Though PTP-FERM was originally called as PTP-1 in the database, there was another *C. elegans* phosphatase which is also called as PTP-1. To avoid the possible confusion, we call the phosphatase as PTP-FERM in this study.

Corresponding cDNA sequences were found in the EST database at the National Institute of Genetics, Japan. We sequenced several cDNA clones (yk417b1, yk129c1, yk133g6, yk227f7, and yk287g3) and found a new exon, exon 9 (nt 4406–4483) (Figs. 1A and 1B), as well as three alternatively spliced products (Fig. 1C). Type III, the longest form, contains exons 1–18, while type II and type I lack exon 9 and both exons 8 and 9, respectively. The predicted ORFs of type I, II, and III encode proteins of 975, 1026, and 1052 amino acids, respectively. All three forms contain a band 4.1/FERM domain, a PDZ domain, and a PTP domain. The expression levels of these isoforms were compared by the competitive RT-PCR. Both type I and II PCR products were amplified well, while the type III product was undetectable (Fig. 1D). The expression level of type III product could be much lower than that of other isoforms or the type III product could be expressed only in a small number of cells.

PTP-FERM (type I) tagged by Flag (Flag-PTP-FERM) or GFP (PTP-FERM-GFP) was transiently expressed in a human 293T cell line and the cell lysates were analyzed by immunoblotting experiments. The 110 kDa PTP-FERM band of expected size was detected using an anti-Flag monoclonal antibody (Fig.

2A, lane 2). GFP is composed of about 240 amino acids and PTP-FERM-GFP was about 25 kDa larger than Flag-PTP-FERM (Fig. 2A, lane 4). These bands were undetectable in the lysates from mock-transfectants (Fig. 2A, lanes 1 and 3). Next the catalytic activity of PTP-FERM was examined. Wild type PTP-FERM (type I) was expressed as a GST fusion protein (GST-PTP-FERM) in *Escherichia coli* and purified. GST-PTP-FERM was found to have phosphatase activity (Fig. 2B). It is known that a conserved cysteine residue in the signature motif of PTP is crucial for catalytic activity. As expected, alternation of the corresponding Cys901 to Ser in PTP-FERM (GST-PTP-FERM/C901S) resulted in a complete loss of enzymatic activity (Fig. 2B).

Expression and Localization of PTP-FERM

To examine the expression and subcellular localization of PTP-FERM, we constructed a chimeric gene, *ptp-ferm::ptp-ferm-gfp* (Fig. 3A), in which a 7.1 kb 5′ genomic fragment of *ptp-ferm* (nt −5880–1232) containing exons 1–3 was fused to a cDNA encoding PTP-FERM followed by a GFP minigene. This construct was designed to express a fusion protein between full length PTP-FERM and GFP (PTP-FERM-GFP) under the regulation of PTP-FERM promoter. The construct was injected into the gonad of the worm and several lines expressing PTP-FERM-GFP were established. The expression of PTP-FERM-GFP in the worm was confirmed by immunoblotting (Fig. 3C).

PTP-FERM-GFP was visible after 2- to 3-fold stage (Fig. 3B, a). PTP-FERM-GFP was expressed predominantly in neurons in the nerve ring (Fig. 3B, c and d) and ventral nerve cord (Fig. 3B, e and f). Weaker signals were found in some muscle cells. In the neurons, PTP-FERM-GFP was enriched in the peri-membrane region in the cell bodies (Fig. 3B, b) and found in the nerve processes (Fig. 3B, e, large allows) but not in the nucleus (Fig. 3B, b).

Band 4.1/FERM Domain Is Crucial for the Subcellular Localization of PTP-FERM

We analyzed the domains involved in the subcellular localization of PTP-FERM. For this purpose, a series of PTP-FERM mutants (Fig. 4A) were produced and expressed in the worm. As described, PTP-FERM-GFP was enriched in the cell periphery of cell bodies (Figs. 3B, b, and 4B, c) and found in the nerve processes (Figs. 3B, e, and 4B, a and b). It is reported that the FERM domain is composed of three structural modules, F1, F2, and F3 (33, 34). Δ4.1 is a deletion mutant of PTP-FERM, which has a small portion of F1 but lacks both F2 and F3 (Fig. 4A). Δ4.1 was not detectable in the nerve processes (Fig. 4B, d and e) or enriched in the cell periphery (Fig. 4B, f). The localization pattern of Δ4.1ΔPDZ, another mutant lacking the FERM do-

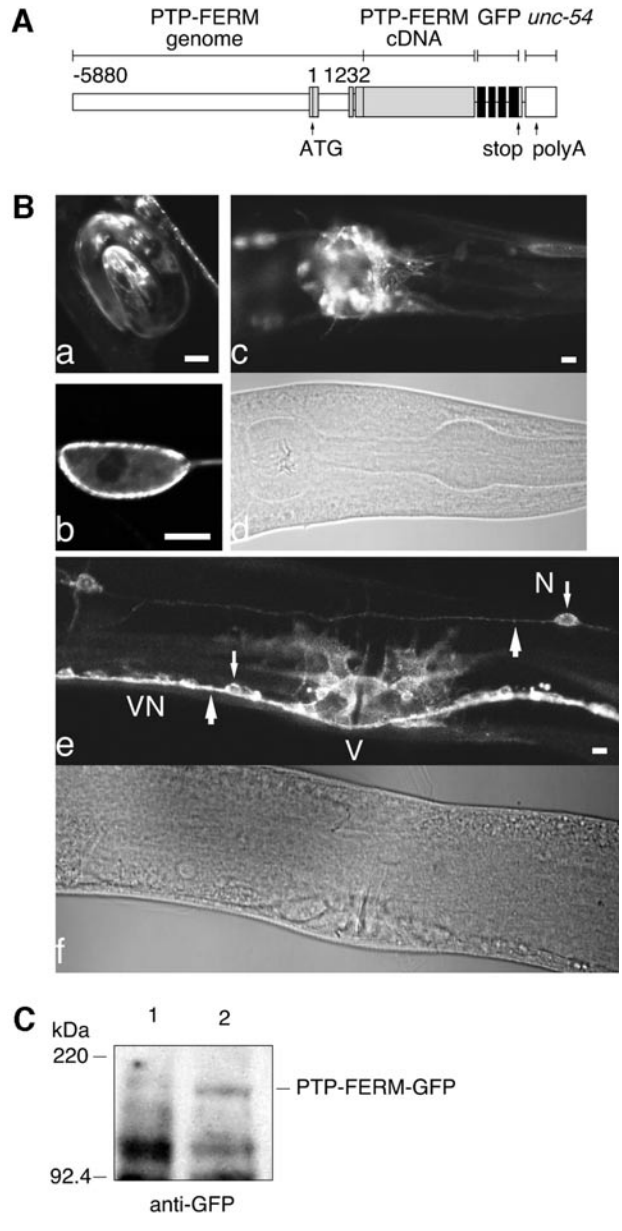

FIG. 3. Expression of *ptp-ferm::ptp-ferm-gfp* transgene in *C. elegans*. (A) A schematic drawing of *ptp-ferm::ptp-ferm-gfp* chimeric minigene. A *ptp-ferm* genomic fragment containing exon 1–3 (nt −5880–1232) was fused to a cDNA encoding PTP-FERM (type I) and a GFP minigene that has introns followed by the 3′ untranslated region of *unc-54*. The construct is designed to express a fusion protein, PTP-FERM-GFP, under the regulation of PTP-FERM promoter. (B) Fluorescence micrograph of PTP-FERM-GFP expression. Representative results from four independent worm lines that have *ptp-ferm::ptp-ferm-gfp* as an extrachromosomal array are shown. The small arrows show cell bodies and the large arrows show nerve processes. b is a confocal microscopic image of a neuron. Images d and f are bright field images of c and e, respectively. N, neuron on the lateral side of the worm; V, vulva; VN, ventral nerve cord. Scale bars, 5 μm. (C) Immunoblotting study of PTP-FERM-GFP. Lysates were prepared from N2 worms (lane 1) or a line that has *ptp-ferm::ptp-ferm-gfp* as an extrachromosomal array (lane 2) and analyzed by immunoblotting using anti-GFP antibody.

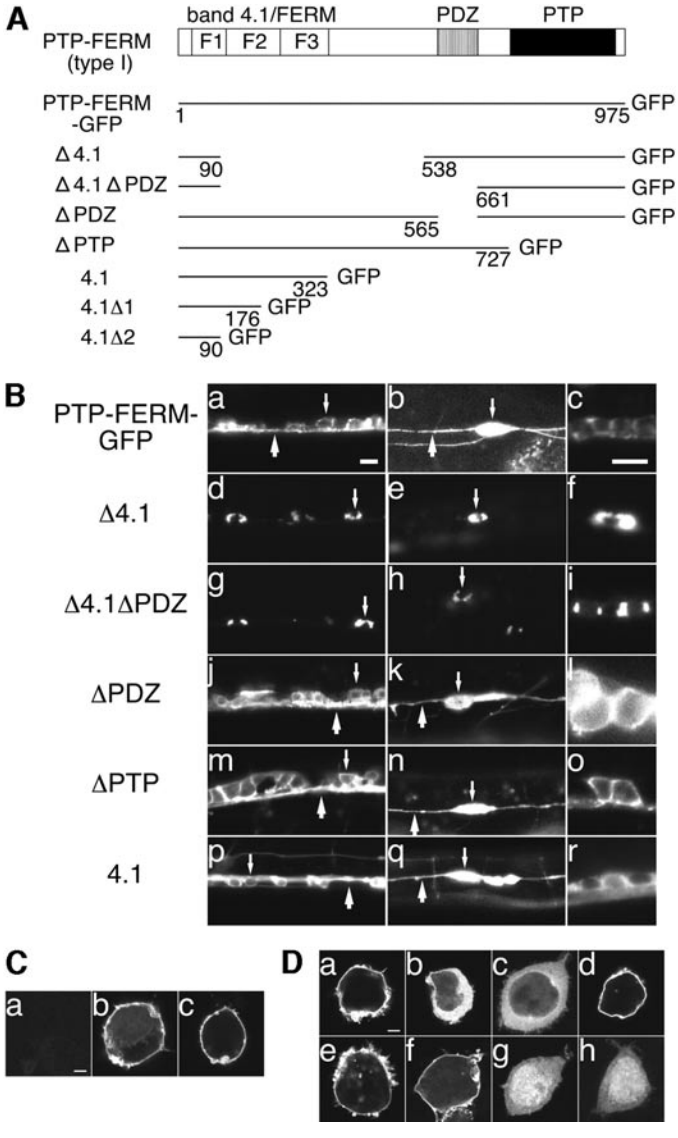

FIG. 4. Requirement of the band 4.1/FERM domain for the subcellular localization of PTP-FERM. (A) Schematic drawings of PTP-FERM mutants used in the study. The solid lines represent the portions contained in the mutants. Amino acid numbers are shown below the lines. (B) Subcellular localization of PTP-FERM and mutants in the worm. DNA constructs containing *ptp-ferm::ptp-ferm-gfp* and its mutants were injected into the gonad of worm and lines that had these DNA constructs as extrachromosomal arrays were established. a to c, PTP-FERM-GFP; d to f, Δ4.1; g to i, Δ4.1ΔPDZ; j to l, ΔPDZ; m to o, ΔPTP; p to r, 4.1. Images of the neurons in the ventral nerve cord (a, c, d, f, g, i, j, l, m, o, p, and r) or on the lateral side of the worm (b, e, h, k, n, and q) are shown. The magnification of c, f, i, l, o, and r is twofold that of others. Large and small arrows indicate nerve processes and cell bodies, respectively. Scale bars, 5 μm. (C) Confocal microscopy analysis of PTP-FERM in mammalian 293T cells. 293T cells were transiently transfected with control vector (a) or a plasmid coding for PTP-FERM-Flag (b) or Flag-PTP-FERM (c) for two days. After fixation and permeabilization, PTP-FERM was detected using anti-Flag antibody and FITC-conjugated goat anti-mouse IgG. Cells were visualized by confocal microscopy. Scale bar, 5 μm. (D) The necessity of the FERM domain for the membrane localization of PTP-FERM in mammalian 293T cells. PTP-FERM-GFP and its deletion mutants were transiently expressed in 293T cells. a, PTP-FERM-GFP; b, Δ4.1; c, Δ4.1ΔPDZ; d, ΔPDZ; e, ΔPTP; f, 4.1; g, 4.1Δ1; h, 4.1Δ2. The pattern of GFP alone is indistinguishable from that of 4.1Δ1 and 4.1Δ2 (data not shown). Scale bar, 5 μm.

main, was almost identical to that of Δ4.1 (Fig. 4B, g to i). In contrast, deletion of the PDZ domain (ΔPDZ) or PTP domain (ΔPTP) had little effect on the localization of PTP-FERM (Fig. 4B, j to o). On the other hand, the N-terminal fragment of PTP-FERM containing the FERM domain (4.1) was localized in the nerve processes (Fig. 4B, p and q) and cell periphery (Fig. 4B, r). These results demonstrate that the FERM domain is necessary and sufficient for the localization of PTP-FERM in the nerve processes and cell periphery.

To confirm the role of the FERM domain in the subcellular localization, we studied the distribution pattern of PTP-FERM mutants ectopically expressed in the 293T human embryonic kidney cells. The expression of PTP-FERM mutants with expected sizes was confirmed by the immunoblotting experiment (Fig. 2A and data not shown). Like in *C. elegans* neurons, PTP-FERM-GFP was enriched in the peri-membrane region (Fig. 4D, a). Either N-terminally or C-terminally Flag-tagged PTP-FERM showed almost identical distribution (Fig. 4C, b and c). The FERM domain is necessary and sufficient for the localization of PTP-FERM close to the plasma membrane in 293T cells (Fig. 4D).

DISCUSSION

PTP-FERM was a putative tyrosine phosphatase predicted from *C. elegans* genome and the cDNA projects. In this report, we showed that PTP-FERM has catalytic activity and is expressed predominantly in neurons. Furthermore, we demonstrated that the band 4.1/FERM domain is crucial for subcellular distribution of PTP-FERM in *C. elegans*.

In *C. elegans*, the FERM domain of PTP-FERM is essential and sufficient for its localization in nerve processes. One possible mechanism is the association of PTP-FERM with molecules in the nerve processes through the FERM domain. Another possibility is the association of PTP-FERM with machinery that transports PTP-FERM from the cell body to the nerve processes via the FERM domain. Interestingly, PTPD1, a mammalian PTP, is reported to associate with KIF1C, a microtubule motor protein, via its FERM domain (17).

It is reported that several mammalian band 4.1-superfamily PTPs such as PTPMEG and PTP36 associate with the cell membrane (28, 35, 36). Additionally, the association of PTPH1 with membrane structures has also been described (15, 37). E. Cuppen and colleagues has reported that the FERM domain of PTP-BL/RIP (38, 39), a mouse homologue of PTP-BAS/hPTP1E/PTPL1/FAP-1, is primarily responsible for its submembranous distribution (40). We demonstrated in this report that the FERM domain of PTP-FERM is essential for the enrichment of PTP-FERM in the peri-membrane region in the worm. In the subcellular fractionation experiment using 293T cells, PTP-FERM-

GFP and Flag-PTP-FERM were recovered in the membrane fraction, indicating the physical association of PTP-FERM with the membrane (Uchida and Ogata, unpublished observation). Though the FERM domains of several PTPs such as PTPH1 and PTP-BL/RIP are implicated in association with the plasma membrane, no membrane anchoring molecules that can bind to the FERM domain of PTP have been identified. The anchoring molecules of PTP-FERM remain to be determined too.

We found that a 5′ genomic fragment of *ptp-ferm* (nt −5880–1232) had promoter activity. Overexpression of PTP-FERM/D869A, a substrate-trapping type mutant (41) of PTP-FERM, resulted in an inhibitory effect on the worm growth (Uchida and Ogata, unpublished observation). This D to A type PTP is reported to make a stable complex with its substrate. It is therefore speculated that endogenous PTP-FERM might be involved in the regulation of worm growth through the dephosphorylation of its substrates. It is clear however that the establishment of mutant worm lines lacking PTP-FERM as well as the identification of the substrates of PTP-FERM is necessary to elucidate the physiological function of PTP-FERM.

In this study, we have clearly demonstrated the importance of the FERM domain in the localization of PTP-FERM in the nerve processes, suggesting the essential role of PTP-FERM in the neurons of *C. elegans*.

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