Characterization of an Evolutionarily Conserved Metallophosphoesterase That Is Expressed in the Fetal Brain and Associated with the WAGR Syndrome*

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Among the human diseases that result from chromosomal aberrations, a de novo deletion in chromosome 11p13 is clinically associated with a syndrome characterized by Wilms’ tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR). Not all genes in the deleted region have been characterized biochemically or functionally. We have recently identified the first Class III cyclic nucleotide phosphodiesterase, Rv0805, from Mycobacterium tuberculosis, which biochemically and structurally belongs to the superfamily of metallophosphoesterases. We performed a large scale bioinformatic analysis to identify orthologs of the Rv0805 protein and identified many eukaryotic genes that included the human 239FB gene present in the region deleted in the WAGR syndrome. We report here the first detailed biochemical characterization of the rat 239FB protein and show that it possesses metallophosphodiesterase activity. Extensive mutational analysis identified residues that are involved in metal interaction at the binuclear metal center. Generation of a rat 239FB protein with a mutation corresponding to a single nucleotide polymorphism seen in human 239FB led to complete inactivation of the protein. A close ortholog of 239FB is found in adult tissues, and biochemical characterization of the 239AB protein demonstrated significant hydrolytic activity against 2’,3’-cAMP, thus representing the first evidence for a Class III cyclic nucleotide phosphodiesterase in mammals. Highly conserved orthologs of the 239FB protein are found in Caenorhabditis elegans and Drosophila and, coupled with available evidence suggesting that 239FB is a tumor suppressor, indicate the important role this protein must play in diverse cellular events.

With the advent of large scale genome sequencing efforts along with more sophisticated methods of genetic mapping, a number of loci have been identified that are associated with human disease. Intriguingly, many genes identified in these loci remain uncharacterized at the biochemical and functional level, and although current annotation can provide a general prediction of the function of some proteins, understanding finer aspects of their regulation require individual analysis at a single gene level.

Chromosome 11p13-14 has been extensively studied because of its association with various tumors, among them lung and bladder cancers (1, 2) as well as several developmental disorders such as the WAGR syndrome (Wilms’ tumor, aniridia, genitourinary anomalies, mental retardation (3–5). Extensive studies on this contiguous gene syndrome and association of 11p13 interstitial deletions with the WAGR syndrome suggest that this region contained a cluster of developmentally significant genes (6–9). Sequence analysis of this locus showed that the region chr11p13-14 contains several genes (WT1, BDNF, KCNA4, FSHB, 239FB, PAX6, and RCN1), and our inspection of available sequenced mammalian genomes indicated a shared synteny, highlighting the evolutionary conservation of this locus.

Association of WT1 and PAX6 genes with Wilms’ tumor and aniridia (9–12), respectively, suggested that the other genes in this region could be involved in the development or function of the central nervous system, and therefore, deletion of these genes could be responsible for the mental retardation seen in WAGR patients. Based on extensive physical and genetic mapping combined with clinical and cytogenetic data, high resolution integrated maps have been constructed for chromosome 11p13-14 region (13–18). The 239FB gene is present between the FSHB and PAX6 genes, and earlier studies have shown that the chromosomal locus of 239FB gene is present within a deletion interval that had been associated with the mental retardation phenotype of WAGR syndrome (19). 239FB mRNA is predominantly expressed in the fetal brain (20), and the high degree of similarity between the predicted protein sequence of 239FB and two Caenorhabditis elegans cDNA clones showed an extensive evolutionary conservation of this protein (21). Although the function of the 239FB protein remains unknown, the distinctive expression of the gene in the fetal brain and the presence of an “ancient conserved region” (21) in this gene suggest its role in the development of the nervous system.

In current databases (Genecards), the 239FB gene has been annotated as a metallophosphoesterase (MPE), and a close

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4 The abbreviations used are: MPE, metallophosphoesterase; CthPnkp, C. thermocellum polynucleotide kinase-phosphatase; DLS, dynamic light scattering; SNP, single nucleotide polymorphism; GST, glutathione S-transferase; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; PDE, phosphodiesterase.
ortholog of this protein, called 239AB, is expressed in the adult brain (21). Neither protein has hitherto been characterized. Metallophosphoesterases are a large group of enzymes that catalyze a variety of diverse reactions. All members contain five blocks of residues and within these blocks are found 11 invariant residues, DXGDX_{GNH}(E/D)XHX_{GHXH}, and the structural motif conserved in metallophosphoesterases is the $\beta$-$\alpha$-$\beta$-$\alpha$-$\beta$-fold (22-24). The superfamily of metallophosphoesterases is made up of two functionally and structurally well characterized groups, phosphomonoesterases and phosphodiesterases. Whereas phosphomonoesterases have been divided into protein Ser/Thr phosphatases, purple acid phosphatases, and 5'-nucleotidases, diesterases are categorized into phospholipases, 2',3'-cyclic nucleotide phosphodiesterases, UDP 2',3'-diamyloylglucosaminidase, and Class III phosphodiesterases (25, 26). The amino acid residues involved in coordinating the dimetal center are located at the carboxyl end of the parallel $\beta$-strands of the fold (23, 24). Structural and biochemical analysis of these proteins have shown the metal requirements ($Fe^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}$) at the dimetal nuclear center for activity (27–30). Despite the large number of MPEs that have been biochemically characterized, the function of these proteins in the organism remains enigmatic, and no natural substrate has been identified for many of them (23, 31, 32). Moreover, it is not readily possible to classify the MPEs as either monoesterases or diesterases by inspection of the sequences of the proteins.

Recently, we have biochemically and structurally characterized the Rv0805 gene from Mycobacterium tuberculosis, which to date is the only identifiable cyclic nucleotide phosphodiesterase in Mycobacterium tuberculosis (24, 33). The Rv0805 protein is a Class III cyclic nucleotide phosphodiesterase, which are enzymes found almost exclusively in bacteria. The catalytic core of Rv0805 is distantly related to the calcineurin-like phosphatases, and a unique substrate binding pocket was identified by computational docking (24). During our attempts to identify orthologs of this gene in different organisms, we were surprised to see that a number of genes similar to Rv0805 protein could be identified in eukaryotes and among them were the 239FB and 239AB genes. In the present study we have cloned the full-length cDNAs of the 239FB and 239AB genes from rat brain (21). Neither protein has hitherto been characterized.

### MATERIALS AND METHODS

All nucleotides as well as the colorimetric substrates p-nitrophenyl phosphate, bis(p-nitrophenyl) phosphate, p-nitrophenyl thymidine 5'-monophosphate, p-nitrophenyl phenolphosphonate, and p-nitrophenylphosphorylcholine were obtained from Sigma-Aldrich. Malachite green reagent was purchased from MP Biomedicals.

**Bioinformatic Analysis for Identification of ICC/CpdA-like Proteins**—The metallophosphoesterase superfamily encompasses a large number of protein sub-families that have in common a core seven-residue bimetal binding active site that catalyzes the hydrolysis of various phospho-, mono-, and diester substrates. The Pfam data base of protein families groups all these proteins by a single hidden Markov model (PF00149) built on 332 seed sequences. A cyclic nucleotide phosphodiesterase from Escherichia coli (product of the ICC/CpdA gene) is a metallophosphoesterase, and the Rv0805 is similar to this protein (33). As we aimed to identify ICC-related sequences, we relied on the established subclassifications of highly related proteins in the Clusters of Orthologous Groups data base (www.ncbi.nlm.nih.gov/COG). Eight proteins of 332 Pfam seed sequences matched to Cog2129 (ICC-related) using RPS-BLAST. These sequences were aligned using PROBCONS and the alignment used to generate a new hidden Markov model that would identify only ICC-related phosphodiesterases and prevent all other metallophosphoesterases from being enlisted in the desired inventory of ICC-like proteins. A HMMER (34) search of the non-redundant data base of proteins revealed the presence of ~130 eukaryotic ICC-like proteins (supplemental Fig. 1 and Table I) among a total of 505 hits and identified two human proteins, 239AB and 239FB.

**Expression and Purification of 239FB**—RNA was isolated from fetal (20 days of gestation) and adult rat brain using TRI reagent (Sigma) as per the manufacturer’s protocol and subjected to first strand cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega). The rat 239FB full-length coding region was amplified using 239FBwdMun1 and 239FBrs primers on the fetal brain cDNA (supplementary Table II). PCR was performed with DeepVent DNA polymerase (New England Biolabs), and the purified PCR product was digested with MunI and XhoI and cloned into the EcoRI and XhoI sites of pBKS II vector (Invitrogen). The sequence of the cloned fragment was identical to accession number NM_198778. For expression of the protein, a clone was generated using a forward primer containing an Nco1 site and cloned as an NcoI-XhoI fragment into pPROExHT-C to generate plasmid pPRO-239FB1–294. This allowed expression in E. coli of the 239FB protein with a hexahistidine tag. The D65A, H67A, H67R, D86a, E89A, N117A, H118A, H213A, G252H, H254A mutations were generated on the pPRO-239FB1–294 plasmid using a single oligonucleotide-based mutagenesis protocol (35). The sequences of the oligonucleotides used for mutagenesis are provided in supplemental Table II, and all inserted were sequenced to confirm the presence of only the desired mutations (Macrogen).

Proteins were expressed in the E. coli BL21DE3 strain on induction using isopropyl $\beta$-D-1-thiogalactopyranoside (500 $\mu$m) for 20 h at 16 °C. Cells were lysed by sonication in buffer containing 50 mM Tris/HCl (pH 8.2), 5 mM $2$-mercaptoethanol, 100 mM NaCl, 10% glycerol, 1 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride followed by centrifugation at 30,000 × g. The supernatant was interacted with nickel-nitrilotriacetic acid beads (Qiagen), and the matrix was washed with a similar buffer. This was followed by washes with a buffer containing 100 mM Tris/HCl (pH 8.2), 5 mM $2$-mercaptoethanol, 500 mM NaCl, and 20 mM imidazole, and elution in the buffer containing 100 mM Tris/HCl (pH 8.2), 5 mM $2$-mercaptoethanol, 50 mM NaCl, and 300 mM imidazole. Purified protein was
desalted into buffer containing 50 mM Tris/HCl (pH 8.2), 5 mM 2-mercaptoethanol, 50 mM NaCl, and 10% glycerol and stored in aliquots at −70 °C until further use. For expression of 239FB fused to an N-terminal GST tag, a BamHI-XhoI fragment from the pBKS II-239FB plasmid was cloned in to the BamHI and XhoI sites of pGEX-6P3 (GE Healthcare) to generate plasmid, the pBKS II-239FB plasmid was cloned in to the BamHI and fused to an N-terminal GST tag, a BamHI-XhoI fragment from phage GST-AB, encoding the 239FB protein, was purified from E. coli BL21DE3 strain after induction with isopropyl β-D-thiogalactopyranoside (500 μM) for 20 h at 16 °C. Cells were lysed by sonication in a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride followed by centrifugation at 30,000 × g. The supernatant was applied to a Supelcosil LC-8DB (25 cm × 4.6 mm, 5 μm) HPLC column equilibrated with 20 mM (NH4)2HPO4 (pH 6.2) at a flow rate of 1 ml/min on an Agilent Technologies HPLC system. The column was calibrated with a mixture of nucleotides containing 2’,3’-cAMP, 3’,5’-cAMP, 3’,5’-cGMP, and 2’,5’-AMP. Dynamic Light Scattering experiments were performed on a DynaPro Molecular Sizing Instrument (Protein Solutions). DLS measures fluctuations in the intensity of light scattered by a macromolecular solution which can be related to its hydrodynamic radius (Rh). Samples of 239FB protein were analyzed directly after gel filtration at concentrations of ~400 μg/ml. Protein solutions were centrifuged three times at 16,000 × g for 15 min and immediately loaded into a quartz cuvette before measurement. Several measurements were taken at 277 K and analyzed using DYNAMICS Version 3.30 software (Protein Solutions). Data collection times of 10 s were used in all cases, for a minimum of 15 acquisitions.

Western Blot Analysis of 239FB—Purified 239FB protein was injected into rabbits to raise an antibody to the protein by standard procedures as described earlier (33). Rat fetal brain tissues were homogenized in homogenization buffer containing 50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 80 μM β-glycerophosphate, 1 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 100 μM sodium orthovanadate, after which samples were centrifuged at 12,000 g for 1 h at 4 °C. Aliquots were stored at −70 °C. Total brain proteins (25 μg) were fractionated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was carried out using the immunoglobulin fraction (3 μg/ml) prepared from the immune serum as described earlier (33). Blots were subsequently probed with Hsp70 antibody (Stressgen; 200 ng/ml) to normalize protein levels in the individual lanes.

Metallophosphoesterase Enzymatic Assays—Assays for various activities (phosphatase, phosphodiesterase, nuclease, phospholipase) were performed in a triple buffer system (MES, HEPES, diethanolamine, 50 mM [pH 9.0]) (37), 5 mM 2-mercaptoethanol, and 10 mM NaCl in the presence of 10 mM concentrations of the specified substrate and Mn2+ as the metal cofactor. The triple buffer system allowed a change in a wide range of pH without altering the ionic composition of the buffer. Assays were stopped by the addition of 10 μl of 200 mM NaOH, and absorbance was monitored at 405 nm. The amount of p-nitrophenol formed was estimated based on its molar extinction coefficient of 18,450 M−1 cm−1.

For kinetic analysis assays were performed with the same buffer system as described earlier with 239FB protein and different concentrations of substrates and metals (Ni2+ and Mn2+) as indicated. Each measurement was performed in triplicate for two different enzyme preparations.

Hydrolysis of single-stranded M13 DNA or double-stranded pUC19 DNA or RNA (yeast RNA type II) was assayed in 20 μl of reaction volume in buffer containing 50 mM HEPES (pH 8.0), 10 mM NaCl, 1 mM dithiothreitol, and 5 mM MnCl2 at 37 °C for 120 min in the presence of 1 μg of 239FB. The reactions were stopped by the addition of 6× loading dye, and the reaction products were analyzed on 0.8% agarose, 1× Tris acetate, EDTA gels containing 1 μg/ml ethidium bromide.

Cyclic Nucleotide Phosphodiesterase Assays—Hydrolysis of cyclic mononucleotides (2’,3’-cAMP, 3’,5’-cAMP, and 3’,5’-cGMP) was analyzed in reaction mixtures in the three buffer systems (50 mM MES, HEPES, diethanolamine (pH 9.0)), 5 mM 2-mercaptoethanol, 10 mM NaCl, and 5 mM MnCl2 with different concentrations of the specified substrate in the presence of 1 μg of 239FB and 0.1 unit of calf intestine phosphatase. The reaction was stopped after 90 min of incubation at 37 °C by the addition of 50 μl of a malachite green solution, prepared as described previously (38, 39). Absorbance was measured at 620 nm to detect the released inorganic phosphate, and the amount of released phosphate was estimated by interpolating the value to a standard curve generated with inorganic phosphate.

For analysis of the cyclic nucleotide PDE assay by HPLC, 1 μg of 239FB was incubated for 120 min with 10 μM of the specified substrate as well as metal cofactor in the buffer system mentioned above in a volume of 25 μl. The reaction was terminated by the addition of 50 μl of 20 mM (NH4)2HPO4 (pH 6.2). Aliquots from assays were applied to a Supelcosil LC-8DB (25 cm × 4.6 mm, 5 μm) HPLC column equilibrated with 20 mM (NH4)2HPO4 (pH 6.2) at a flow rate of 1 ml/min on an Agilent Technologies HPLC system. The column was calibrated with a mixture of nucleotides containing 2’,3’-cAMP, 3’,5’-cAMP, 3’,5’-cGMP, and 2’,5’-AMP.

Cloning and Expression of Rat 239AB—The full-length coding region of rat 239AB was amplified using 239ABfw and 239ABrvs primers and Deep Vent polymerase (supplemental Table II) from cDNA prepared from the adult rat brain. Single 3’-A overhangs were added to the PCR product using TaqDNA polymerase and then ligated in to pGEMT Easy vector (Promega). The clone was sequenced and subcloned as a BamHI and NotI fragment into the BamHI and XhoI sites of pGEX-5X2 (GE Healthcare) to generate plasmid pGEX-5X2–239AB, encoding 239AB fused to an N-terminal GST tag.

GST-FB and GST-AB proteins were expressed in E. coli BL21DE3 strain after induction with isopropyl β-D-thiogalactopyranoside (500 μM) for 20 h at 16 °C. Cells were lysed by sonication in a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride followed by centrifugation at 30,000 × g. The supernatant was interacted with GSH beads, and the matrix was washed with a similar buffer. This was followed by washes with a buffer con-
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taining 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, and 0.1% Triton-X. Subsequently, beads were washed with a buffer containing 50 mM Tris/HCl (pH 8.2), 1 mM dithiothreitol, 100 mM NaCl, 2 mM EDTA, and 10% glycerol, and stored at 4 °C. Protein was estimated by addition of Bradford reagent to the beads suspension.

RESULTS

Identification of 239FB as an Ortholog of the Rv0805 Gene—During our earlier studies on cyclic nucleotide metabolism in bacteria, we identified and characterized both structurally and biochemically the Rv0805 3′,5′-cAMP PDE from M. tuberculosis (24, 33). This protein hydrolyzed cAMP in vitro and in vivo and, therefore, may contribute to cAMP homeostasis in mycobacteria. Given the unique features of Rv0805 and the availability of structural information, we sought to identify similar proteins in other genomes. The MPE family encompasses several subfamilies of homologous proteins grouped based on their substrate specificities. Here we have narrowed down our searches to identify as many members possible of the subfamily that could hydrolyze 3′,5′-cAMP and -cGMP. Using a combined RPS-BLAST and hidden Markov model approach, we found that although not all genomes contain Rv0805-like proteins, these genes nonetheless seem to have a wide phyletic distribution. The majority of proteins identified were of bacterial or archaeal origin (supplemental Table I). This was expected because eukaryotic cyclic nucleotide phosphodiesterases belong to a phylogenetically unrelated group of proteins, the class I PDEs (40). However, we were intrigued to find some orthologs in eukaryotes, including mammals (Fig. 1; supplemental Table I and Fig. 1). Of the 505 genes identified, 287 were found in bacteria, 82 in archaeobacteria, 135 in eukaryotes, and a single gene in a virus.

The majority of sequence diversity in the MPE domain was found in archaeobacteria followed by proteobacteria and actinobacteria, among the eubacterial lineages. We, therefore, selected the most diverse members among the bacterial genes to construct a representative phylogenetic tree retaining Rv0805 and a few other eukaryotic sequences to highlight their relative placement and bring out the diversification within several bacterial and archaean clades. We were surprised to notice the close relation between Rv0805 and the mammalian proteins (Fig. 1), and the availability of biochemical and structural information on Rv0805 prompted us to further investigate the possibility of the presence of class I-like cyclic nucleotide PDEs in mammalian genomes.

Two genes, namely 239FB (also referred to as C11orf8 or MPPED2) and 239AB, were identified in the human genome, and very similar genes were also detected in multiple eukaryotic genomes, including mammals (Fig. 1; supplemental Table I). The 239FB and 239AB genes have been annotated as metallophosphoesterases in genome databases, but there has been no biochemical or functional characterization of these proteins. We, therefore, set out to characterize the biochemical properties of these proteins in detail using the rat proteins as model enzymes. There are only two amino acid changes between the human and the rat 239FB proteins (at positions Ile-207 and -259 in human 239FB that are changed to Thr residues) that do not lie in the critical MPE motifs.

Biochemical Characterization of the 239FB Gene Product—The mRNA for 239FB has been shown to be expressed in the human fetal brain and not in the adult brain by Northern blot analysis (19). Reverse transcription-PCR analysis of RNA prepared from the brain of a 20-day-old rat embryo and adult rat brain indicated that the mRNA for 239FB could indeed be detected only in the fetal brain (Fig. 2A). Using the cDNA prepared from the fetal brain on day 20 of gestation, we cloned the full-length cDNA for 239FB and expressed the protein in E. coli to obtain sufficient amounts for biochemical characterization and antibody production (Fig. 2B). Western blot analysis of lysates prepared from the fetal brain indicated that the 239FB protein was indeed expressed in the tissue (Fig. 2C).

The sequence of the 239FB protein as predicted from the cDNA has all the residues that are required to classify the protein as a metallophosphoesterase (see Fig. 4). Therefore, 239FB was purified and used for biochemical analysis. Assays were performed in the presence of Mn²⁺ as a metal co-factor because many metallophosphoesterases utilize Mn²⁺ for catalysis. As shown in Fig. 3A, 239FB protein was able to hydrolyze the phosphodiester substrate bis-p-nitrophenyl phosphate but not p-nitrophenyl phosphate, a monoester. In addition, it was also able to hydrolyze p-nitrophenyl phenolphosphonate. Neither thymidine 5′-monophosphate-p-nitrophenyl ester or p-nitrophenolphosphorylcholine was hydrolyzed. These results, therefore, indicate that 239FB protein is a phosphodi-
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Rv0805, which appeared to purify with Mn$^{2+}$ and Fe$^{3+}$ bound to the protein and, therefore, showed considerable activity even in the absence of added metals during the assay (33). The $K_m$ for Mn$^{2+}$ of 239FB was found to be 1.5 mM, which is nearly 60-fold higher than that of Rv0805 (33), which accounts for the absence of bound metals to purified 239FB and, therefore, low activity without added Mn$^{2+}$ in the assay (Fig. 3B).

The $K_m$ for bis-p-nitrophenyl phosphate could not be determined as high concentrations of the substrate led to precipitation of the substrate during assays (data not shown). However, the $K_m$ for p-nitrophenyl phenylphosphonate could be measured and was found to be 10 mM (Fig. 3C). The Hill coefficient of 2 indicated cooperativity between the two active sites. Subsequently, all further assays were performed in the presence of 10–20 mM p-nitrophenyl phenylphosphonate and 5 mM Mn$^{2+}$.

We have shown earlier that Rv0805 is a very stable dimer, with dimerization enhanced in the presence of Mn$^{2+}$ and Fe$^{3+}$ (24, 33). We subjected the purified 239FB protein to gel filtration analysis and found that it eluted from the column at a position that would correspond to a protein of Mr $\sim 17,000$ (Fig. 3D). This aberrant elution from the gel filtration column could have been because of a reversible interaction of 239FB with the gel filtration matrix. We, therefore, subjected the peak fraction to DLS (Fig. 3E). The sample showed a base-line value of 1.001 and SOS value of <20%, with a bimodal size distribution (Fig. 3E). Particles with radius of gyration $\sim 3.7$ nm had an estimated molecular mass of $\sim 70$ kDa (dimer of 239FB) with low % polydispersity and, therefore, a reasonably high homogeneity in particle size. Particles of radius $>$62 were from large aggregates and were present in very low amounts, as judged by the % mass (Fig. 3E). Therefore, despite the aberrant elution from the gel filtration column, DLS indicated the dimeric nature of 239FB protein, which could also account for the Hill coefficient of 2 seen in enzymatic assays (Fig. 3C).

Esterase but shows some specificity in the phosphodiester substrates it can hydrolyze.

We observed that 239FB showed activity only in the presence of Mn$^{2+}$ and Co$^{2+}$ as the added metal, and metals such as Fe$^{2+}$, Ni$^{2+}$, and Mg$^{2+}$ that are utilized by other metallophosphoesterase did not result in the hydrolysis of substrates (data not shown). The activity of the purified protein was very low in the absence of added Mn$^{2+}$ in the assay, which is in contrast to 239FB with the gel filtration matrix. We, therefore, subjected the peak fraction to DLS (Fig. 3E). The sample showed a base-line value of 1.001 and SOS value of <20%, with a bimodal size distribution (Fig. 3E). Particles with radius of gyration $\sim 3.7$ nm had an estimated molecular mass of $\sim 70$ kDa (dimer of 239FB) with low % polydispersity and, therefore, a reasonably high homogeneity in particle size. Particles of radius $>$62 were from large aggregates and were present in very low amounts, as judged by the % mass (Fig. 3E). Therefore, despite the aberrant elution from the gel filtration column, DLS indicated the dimeric nature of 239FB protein, which could also account for the Hill coefficient of 2 seen in enzymatic assays (Fig. 3C).

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Mutational Analysis of 239FB—Metallophosphoesterases contain a specific set of residues that are involved in binding either one or both of the two metals found at the catalytic site (24, 41, 42). Fig. 4A shows the alignment of the 239FB proteins with Rv0805 and Mre11, indicating residues that have been found to interact with the metals in Rv0805 and Mre11 based on crystallographic data (22, 24). We performed a systematic mutational analysis to characterize the metallophosphoesterase motif in 239FB and mutated the indicated residues to alanine to disrupt interaction with the metal ion. As shown in Fig. 4B, the D65A, D86A, N117A, and H118A mutant proteins were found to be inactive. Residues at equivalent positions in other metallophosphoesterases including Rv0805 have been found to be involved in binding the metal at site 1 (Asp-21 in Rv0805, Asp-65 in 239FB) or site 2 (Asn-97 in Rv0805, Asn-117 in 239FB) or both (Asp-63 in Rv0805 and Asp-86 in 239FB; (24, 41). Interestingly, some rat 239FB mutants remained active. Table 1 summarizes the catalytic properties of the E89A mutant (at a position equivalent to Asp-66 in Rv0805), H213A mutant (at a position equivalent to His-169 in Rv0805), and the H254A mutant (at a position equivalent to His-209 in Rv0805). The E89A mutant showed almost a similar affinity for Mn$^{2+}$ but a reduced $V_{\text{max}}$ when compared with the wild type protein, suggesting that this residue may be involved in catalysis rather than binding the metal. In contrast, the D66A mutant of Rv0805 showed a similar $V_{\text{max}}$ but a slightly higher $K_m$ for the metal than wild type Rv0805 (33). The H213A mutant protein showed a marginally reduced affinity for Mn$^{2+}$ and a lower $V_{\text{max}}$. Interestingly, the corresponding mutant of Rv0805 (H169A) was hardly altered in its catalytic activity but showed a significant increase in its allosteric behavior with respect to the metal (33). Therefore, although 239FB utilizes some of the residues that have been shown to be essential for metal binding and catalytic activity in other metallophosphoesterases, there are clearly differences with which some residues interact with the metal and regulate catalysis. In fact, this variation in enzymatic properties after mutation of residues, which by sequence alignment of MPEs are predicted to be essential for catalytic activity, is seen in a number of MPEs including Rv0805 (24, 41).

One residue that has been shown to be important for metal binding at site 2 in metallophosphoesterases is a histidine residue found in Rv0805 (His-207) and Mre11 (His-04 (22,24)). This histidine residue is replaced by glycine (Gly-252) in 239FB. To test the consequence of this substitution of the histidine to a glycine in 239FB, we generated a mutant protein where Gly-252 was replaced by histidine. The G252H mutant protein was purified (Fig. 5A, inset) and assayed. Most interestingly, we found significant activity of the mutant protein when assayed in the absence of added metals during the assay (Fig. 5A). This indicated that the single mutation of Gly-252 to a histidine residue had increased the affinity of the protein for metals significantly.
as metal co-factor was bound to the protein as purified as seen in the case of Rv0805 (33). After EDTA treatment to remove the bound metal, we measured the $K_m$ of the G252H mutant for Mn$^{2+}$, and found it to be 35 $\mu$M, nearly 50× lower than that of the 239FB wild type protein (Fig. 5B). The $V_{max}$ using p-nitrophenyl phenylphosphonate as a substrate was also higher (−60 versus −9 $\mu$mol/min/mg of protein) than that of the wild type protein (Fig. 5C). Moreover, activity could also be detected using thymidine 5'-monophosphate p-nitrophenyl ester as substrate, indicating the major effect of mutation of the Gly-252 residue in 239FB protein (Fig. 5D).

Given the higher affinity of the G252H mutant protein for Mn$^{2+}$, we hypothesized that the affinity for other metals could have increased and, therefore, could serve as co-factors in the assay. As seen in Fig. 6A, an assay performed with p-nitrophenyl phenylphosphonate in the presence of different metals showed that 239FB$_{G252H}$ could utilize a number of metals. Because Co$^{2+}$ imparts a significant color at higher concentrations which would have interfered in the colorimetric assay, we measured the $K_m$ for Ni$^{2+}$, which was ~ 30 $\mu$M, similar to that of Mn$^{2+}$ (Fig. 6B). What was striking, however, was the much reduced $V_{max}$ in the presence of Ni$^{2+}$ (−4 $\mu$mol/min/mg of protein) versus Mn$^{2+}$. This suggests that the ability to utilize the phosphodiester as a substrate is also dependent on the metal ion that is bound at the active site, and different phosphodiester could be hydrolyzed to different extents depending on the metal co-factor. Indeed, in our earlier analysis of the Rv0805 enzyme, we found that mutations that compromised the catalytic activity with bis-(p-nitrophenyl) phosphate were as active as the wild type protein in hydrolyzing cAMP (33). This, therefore, appears to be a conserved feature of metallophosphoesterases of this class.

An interesting observation was that the activity in the presence of Zn$^{2+}$ in the assay was lower than the basal activity seen with the G252H mutant in the absence of metals (Fig. 6A). As shown in Fig. 6D, Zn$^{2+}$ appears to inhibit the activity of the wild type and the G252H mutant protein with similar IC$_{50}$ values. Because the histidine residue at position 252 in the mutant protein could be present at metal binding site 2, this data could suggest that the Zn$^{2+}$ binds only at site 1.

### Hydrolisis of Cyclic Nucleotides by 239FB and 239FB$_{G252H}$—To test the ability of 239FB to cleave naturally occurring phosphodiester, we performed assays with the wild type protein using double-stranded DNA and single-stranded DNA or RNA as substrates, but none of them was cleaved by 239FB (data not shown). Moreover, the enzymes were inactive in a yeast lariat debranching assay (Ref. 43 data not shown). Neither 3′,5′-cyclic AMP or GMP nor 2′,3′-cAMP was hydrolyzed by wild type 239FB protein. We performed assays with the G252H mutant protein using naturally occurring phosphodiester substrates, as the activity of the mutant protein was so starkly different to the wild type 239FB protein. Neither DNA nor RNA was hydrolyzed by 239FB$_{G252H}$, and the protein was also inactive in a yeast lariat debranching assay. However, as shown in Fig. 7A, 3′,5′-cAMP was hydrolyzed by 239FB$_{G252H}$ with a $K_m$ of 28 mM. This $K_m$, however, was very high and nearly 100× higher than the $K_m$ for cAMP that we have reported earlier for the Rv0805 protein (33). Cyclic GMP was also hydrolyzed by 239FB$_{G252H}$ with a similar $K_m$, indicating no specificity in the nucleotide that was hydrolyzed (data not shown).

To detect the products of hydrolysis of 3′,5′-cAMP, we separated the

### Activities of mutant 239FB proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mn$^{2+}$ $K_m$</th>
<th>Mn$^{2+}$ $V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.5 ± 0.2</td>
<td>8 ± 0.4</td>
</tr>
<tr>
<td>E89A</td>
<td>2.3 ± 0.3</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>H213A</td>
<td>3.4 ± 0.2</td>
<td>4.4 ± 0.03</td>
</tr>
<tr>
<td>H264A</td>
<td>2.7 ± 0.4</td>
<td>9.3 ± 0.2</td>
</tr>
</tbody>
</table>

**FIGURE 5. Activity of 239FB$_{G252H}$ mutant protein.** A, assays were performed at 37°C for 15 min with 30 mM p-nitrophenyl phenylphosphonate in the presence or absence of Mn$^{2+}$ (5 mM) using 200 ng of wild type (WT) or 239FB$_{G252H}$ mutant proteins as specified. The purity of the 239FB$_{G252H}$ protein has been shown as an inset. B, 239FB$_{G252H}$ protein was treated with 5 mM EDTA for 1 h at 4°C, and then assays were performed in the presence of a fixed concentration of p-nitrophenyl phenylphosphonate (30 mM) and the indicated concentrations of Mn$^{2+}$. C, 239FB$_{G252H}$ was assayed in the presence of 5 mM Mn$^{2+}$ and indicated concentrations of p-nitrophenyl phenylphosphonate. D, 239FB$_{G252H}$ was assayed in the presence of 5 mM Mn$^{2+}$ and indicated concentrations of thymidine 5'-monophosphate-p-nitrophenyl ester. All values represent the mean ± S.E. (n = 4).
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Effect of metals on enzymatic activity of 239FB. A, reaction mixtures containing 30 mM p-nitrophenyl phenylphosphonate and 5 mM concentrations of the specified metal cofactor were incubated with 200 ng of 239FB or 239FB_G252H proteins for 15 min at 37 °C. WT, wild type. B, EDTA-treated 239FB_G252H protein was assayed in the presence of fixed concentrations of p-nitrophenyl phenylphosphonate (30 mM) and the indicated concentrations of Ni²⁺. C, 239FB_G252H was assayed in the presence of 5 mM Ni²⁺ and indicated concentrations of p-nitrophenyl phenylphosphonate. D, wild type 239FB or 239FB_G252H were assayed using 30 mM p-nitrophenyl phenylphosphonate and 5 mM Mn²⁺ in the absence and presence of the indicated concentrations of Zn²⁺. All values represent the mean ± S.E. (n = 4).

A Single Nucleotide Polymorphism in the 239FB Gene Inactivates the Protein—The role of the 239FB protein in the brain remains enigmatic despite its widespread occurrence and high degree of evolutionary conservation. We looked for single nucleotide polymorphisms in the gene in available databases (www.ncbi.nlm.nih.gov/projects/SNP) and interestingly found only one SNP reported in the coding sequence of the protein. This SNP is very rare (2 of 112 samples) and found only as a heterozygous condition. Interestingly, the SNP would convert His-67 to an arginine residue. His-67 is predicted to be one of the metal binding residues, and therefore, a mutation at this site could dramatically alter the activity of the protein. We, therefore, generated the H67A and H67R mutations in rat239FB and purified the mutant protein to study the effect of this SNP on enzymatic activity (Fig. 8A, inset). The H67A mutant protein had a reduced affinity for Mn²⁺ but higher V_max in comparison to the wild type protein (Fig. 3, A and B). Most interestingly, the 239FB_H67R mutant protein was totally inactive (Fig. 8A). The occurrence of this SNP in a heterozygous condition could also indicate that lower levels of active 239FB would be present during brain development, and the very rare occurrence of the SNP may suggest the absolute requirement for some functional 239FB protein during development.

Biochemical Activity of the 239AB Protein—The 239AB protein is highly conserved and is ~80% similar to the 239FB protein (Fig. 9A). Histidine 284 is present at a position equivalent to Gly-252 in the 239FB protein, and given the interesting biochemical properties of the G252H mutant protein, we decided to investigate the biochemical properties of the 239AB protein. The cDNA was amplified from the adult brain RNA and cloned with protein bound to the beads and used as a comparison fragment containing GST, as seen on Western blotting with GST antibodies (data not shown). We were unable to elute the GST-AB protein from the beads, so we performed assays with protein bound to the beads and used as a comparison similarly immobilized GST-239FB protein. As shown in Fig. 9C, GST-239AB protein was a phosphodiesterase and could cleave p-nitrophenyl phenylphosphonate and bis p-nitrophenyl phosphate to a higher extent than GST-239FB. In addition, GST-239AB could cleave p-nitrophenyl thyridine...
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5′-monophosphate, whereas GST-FB showed a slight activity with the choline diester substrate. Neither GST-239AB nor GST-239FB showed any monooesterase activity. GST-239FB could also cleave 2',3'-cAMP to both 2'- and 3'-AMP, as did 239FBG252H (Fig. 7D).

Therefore, in conclusion we have shown that the highly conserved family of 239FB-like proteins comprises of metallophosphodiesterases. Their endogenous substrates in the cell remain to be identified, and the reason why these genes are so highly conserved during evolution remains unknown.

DISCUSSION

The results described in this report show the presence of Class III PDEs in eukaryotes, and inspection of all the 62 hits identified from Metazoa revealed that these proteins are orthologs of the 239FB and 239AB proteins. Rat 239FB or 239AB do not show any monooesterase activity, perhaps indicating that all the proteins identified in our bioinformatic analysis are phosphodiesterases. 239AB protein is restrictive in its biochemical properties, showing no activity with p-nitrophenyl thymidine 5′-monophosphate or p-nitrophosphorylcholine. This selectivity is seen in some MPEs such as the Clostridium thermocellum polynucleotide kinase-phosphatase (CthPnkp (41)), which cannot hydrolyze diesters of thymidine or choline. In sharp contrast to CthPnkp, however, 239FB shows no detectable activity with the 2',3' or 3',5' forms of cAMP or cGMP but robust activity with p-nitrophosphorylphosphonate, as is seen in CthPnkp (41). In terms of products formed from cyclic nucleotide hydrolysis, 239FBG252H protein hydrolyzes 2',3'-cAMP to a mixture of 2' and 3' and 3',5'-cAMP to 3'-AMP. This is in contrast to that seen for the potato tuber MPE, which hydrolyzes 2',3′-cAMP to 3′-AMP and 3′,5′-cAMP to 3′-AMP and 5′-AMP (44).

The metal ion specificities of different MPEs are also varied. For example, Rv0805 (33) as well as E. coli YfcE (23) show an absolute requirement for Mn2+; whereas MJ0936 (32) and CthPnkp (41) require either Ni2+ or Mn2+ for significant activity. In contrast to these MPEs, 239FB can utilize either Mn2+ or Co2+ for activity, but 239FB is characterized by a low affinity for Mn2+, which appears to be because of the absence of a His residue at position 252. Alanine substitution of most of the metal binding residues in 239FB resulted in a complete loss of phosphodiesterase activity, in contrast to the observation that mutation of corresponding residues in other MPEs decreased the affinity of the protein for the metal but did not abolish activity completely (33). Mutation of Asp-65 in 239FB showed a complete loss of PDE activity as was seen in CthPnkp (41). Interestingly, in Rv0805, mutation of the corresponding residue (Asp-21) resulted in increased cooperativity with respect to the metal without changing the apparent K_m (33). In reported crystal structures of MPEs such as Rv0805, E. coli YfcE, A- phosphatase, and MJ0936, the equivalent Asp residue coordinates the metal in site 1 (23, 24, 32, 33, 45).

FIGURE 7. Cyclic AMP phosphodiesterase activity of 239FBG252H. A, kinetic parameters of G252H (1μg) for 3',5′-cAMP hydrolysis were measured using 5 mM Mn2+ and indicated concentrations of 3',5′-cAMP as substrate. Released Pi was estimated as described under “Materials and Methods” by a malachite green assay. The assay shown is representative of assays performed three times. B, profile of standard nucleotides in HPLC analysis. A mixture of 5 nmol of each nucleotide was fractioned on an HPLC column using a solvent system described under “Materials and Methods.” C, assays were performed using 239FBG252H protein (1μg) in the presence of 5 mM Mn2+ with 10 μM of 3',5′-cAMP as substrate, and products were analyzed by HPLC. D, assays were performed using 239FBG252H protein (1μg) in the presence of 5 mM Mn2+ with 10 μM of 2',3′-cAMP as substrate, and products were analyzed by HPLC. Data shown is representative of duplicate determinations with assays performed twice.

TABLE 2
Hydrolysis of 3′,5′-cAMP by 239FBG252H mutant protein in the presence of different metals

<table>
<thead>
<tr>
<th>Metal</th>
<th>3′-AMP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>99.7 ± 2.6</td>
</tr>
<tr>
<td>Manganese</td>
<td>1049 ± 79.5</td>
</tr>
<tr>
<td>Nickel</td>
<td>2365.8 ± 119.2</td>
</tr>
<tr>
<td>Cobalt</td>
<td>494.5 ± 56.3</td>
</tr>
<tr>
<td>Iron</td>
<td>1360 ± 175.7</td>
</tr>
<tr>
<td>Magnesium</td>
<td>524.5 ± 74.2</td>
</tr>
</tbody>
</table>

Therefore, in conclusion we have shown that the highly conserved family of 239FB-like proteins comprises of metallophosphodiesterases. Their endogenous substrates in the cell remain to be identified, and the reason why these genes are so highly conserved during evolution remains unknown.
The H67A mutant in 239FB showed an increase in $V_{\text{max}}$ but a 4-fold lower affinity for metal, suggesting its role in coordination with metal binding site 1 as seen in Rv0805 and CthPnkp. However, the H254A mutant did not show a significant difference in the affinity for metal or catalytic properties, suggesting that 239FB is more tolerant to alterations at this position in the active site. Very interestingly, the corresponding residue in this position is not present either in CthPnkp or in H9261-phosphatase (41). Structures of Rv0805 (24), E. coli YfcE (23), and MJ0936 (32) show coordination of the imidazole group of this histidine.

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**FIGURE 9.** Characterization of the 239AB protein. A, multiple sequence alignment of representative orthologs of 239FB/AB protein family in eukaryotes. Shaded residues represent those characteristic of MPEs and identical in all sequences. R. nor, Rattus norvegicus; H. sap, Homo sapiens; G. gal, Gallus gallus; X. tro, Xenopus tropicalis; D. rer, Danio rerio; D. mel, D. melanogaster; P. tet, Paramecium tetraurelia. B, purified GST-239AB or GST-239FB fusion proteins (2 g) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R dye. C, Western blot analysis of purified 239FB (10 ng) and GST-239AB fusion protein (250 ng) with antibodies to 239FB protein. D, catalytic activity of GST-239AB and GST-239FB with different substrates. Reactions were performed with 30 mM concentrations of each substrate in the presence of 5 mM Mn$^{2+}$ and 1 g of GST-239AB or GST-239FB for comparison. BisPnPP, bis(p-nitrophenyl) phosphate; pNPPP, p-nitrophenyl phenylphosphonate; TmPP, thymidine 5'-monophosphate-p-nitrophenyl ester; pNPP, p-nitrophenylphosphate; pNPPC, p-nitrophenylphosphorylcholine. E, assays were performed using GST-239AB protein (1 g) in the presence of 5 mM Mn$^{2+}$ with 10 mm 2',3'-cAMP as substrate, and products were analyzed by HPLC. Data shown are representative of assays performed twice.
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with the metal ion present in site 1, although no mutational analysis data are available on any of these enzymes to demonstrate the importance of this residue in these proteins.

Mutation of site 2 metal binding residue N117A in 239FB resulted in a complete loss of activity, as has been observed in the case of mutants of similar residues of Rv0805 as well as CthPnkp (33, 41). In 239FB the mutation of the H213A residue of metal binding site 2 results in a 70% decrease in $V_{\text{max}}$ and a 2-fold decrease in affinity for Mn$^{2+}$, whereas in Rv0805 there is a more modest 30% decrease observed in $V_{\text{max}}$ and CthPnkp shows complete loss of activity (41), suggesting subtle differences in the placement of the functional groups in the active sites of these enzymes. For example, the H118A mutant of 239FB showed a complete loss of activity in contrast to the 3-fold increase in $V_{\text{max}}$ of the corresponding mutant of CthPnkp (41).

The Gly residue at position 252 distinguishes 239FB from many characterized MPEs which contain histidine at this position. Because mutations of this histidine residue in Rv0805 and CthPnkp reduced the $V_{\text{max}}$ drastically (41), we attempted to introduce a His residue at this position in 239FB. Interestingly, this mutation was a gain-of-function mutation, demonstrating increased activity with $p$-nitrophenyl phenylphosphate and allowing the mutant enzyme to utilize a variety of different substrates, such as $p$-nitrophenyl thymidine 5’-monophosphate, 2’,3’ or 3’,5’ forms of cAMP, and 3’,5’-cGMP. This increased activity may have resulted from the higher affinity of 239FB$_{G252H}$ for Mn$^{2+}$, as the apparent $K_m$ of 239FB$_{G252H}$ for Mn$^+$ is ~35 μM, comparable with that seen for Rv0805 (apparent $K_m$ ~ 21 μM) (33), lower than that reported for the metal cofactor in the E. coli 5’-nucleotidase (apparent $K_m$ ~ 90 μM) (46) but higher than that of E. coli YfcE (apparent $K_m$ ~ 5.5 μM) (23). 239FB$_{G252H}$ can also utilize Co$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, and Ni$^{2+}$ for catalysis. Interestingly, the highest activity with cAMP was observed in presence of Ni$^{2+}$ metal cofactor and not with Mn$^{2+}$, suggesting that the metal bound at the active site can also direct the positioning of different substrates in an optimum manner.

The fact that the 239FB protein maps to the WAGR locus indicates the importance of this protein in the developing brain. WAGR was the first syndrome mapped to a contiguous gene deletion. The 239FB gene is found on chromosome 11 band p14 and is ~200 kilobases in size. Alternative transcripts are reported in NCBI, but both contain 7 exons, with a variation seen at the 5’ end of the transcript. Northern blot analysis using human brain RNA showed the highest level of expression of 239FB RNA in the cortex, especially the motor, frontal, and visual cortex (20), with two splice variants showing differential regional expression. A recent report has also shown that although the zebra fish ortholog of 239FB gene is expressed in the brain during development, the amphioxus ortholog was expressed in tissues outside the nervous tissue (47).

Although the WAGR locus is rich in several putative genes, studies so far have focused extensively on PAX6 and WT1. Both of these genes have been shown to be important for eye and kidney development, and deletion or mutations in these genes are responsible for a number of abnormalities in the development (48, 49). Very interestingly, both of these gene products are transcription regulators and, thus, may play a potent role in regulating the expression of other genes present at this locus (10). Most interestingly, this locus is under weak levels of negative selection, as five gene loci, WT1, PAX6, RCN1, ELP4, and 239FB, are among the 481 ultraconserved regions present in human genome, which are defined as regions where >200 bp are 100% identical in humans, rat, mouse, chicken, and dog genomes (50). Moreover, 239FB is one of only 24 instances where an ortholog of an ultraconserved element could be partially traced back by sequence similarity as far as *Ciona intestinalis*, *Drosophila melanogaster*, or *C. elegans*. These ultraconserved elements of the human genome are most often located in genes involved in RNA processing or in the regulation of transcription and development (50). The data described in the current study do not show a direct action of the 239FB protein on RNA, but it is conceivable that it could associate with RNA in the presence of additional proteins found in the brain. It is interesting to note that the base pairs coding for the His-67 residue are present in the 200 bp of the ultraconserved region.

The 239FB protein is interesting in that it is predicted to have a role as a tumor suppressor. In an early study, microcell-mediated transfer of chromosome 8 (where the 239FB gene is located) into breast cancer cell lines reduced the tumorigenicity and anchorage independent growth of the cells. Among the seven genes that were identified as candidate tumor suppressors, chr11orf8, or the 239FB gene, was up-regulated in revertant microcell hybrids relative to the parent breast cancer cell line (51). Most interestingly, 239FB is down-regulated in >50% of breast cancer tumors, and ~20% of lymph node metastases showed lower levels of 239FB expression when compared with normal breast tissue (51). Perhaps the phosphodiesterase activity of 239FB should have an important role to play in cancer cell growth.

A single nucleotide polymorphism in the 239FB gene is reported at His-67. The results described in this study show a complete loss of 239FB phosphodiesterase activity as a result of the H67R substitution. The occurrence of this SNP at a very low frequency suggests that this complete loss-of-function mutation of 239FB in human populations cannot be tolerated in the homozygous state.

Many of the MPEs have been well characterized biochemically, but their biological substrates remain elusive. The same seems to be true for the 239FB protein. The 239FB protein has a low affinity for the metal and also demonstrates very restricted substrate specificity. This suggests us two possibilities. First, the enzyme may need additional interacting proteins to show full activity, and we are currently pursuing this line of investigation. Alternatively, 239FB may be an enzyme that is losing its activity to play the role of a scaffolding protein, as is seen in the case of the vacuolar protein sorting protein Vps29p (52). Vps29p forms a complex with Vps26p and Vps35p, and the crystal structure of this complex has revealed the classical MPE-fold seen in Vps29p (52). Purified Vps29p shows no esterase activity despite possessing residues required for metal binding. A helix from Vps35p completely covers the MPE active site in Vps29p, indicating that perhaps the requirement for the bound metals in Vps29p is to stabilize the conformation of the protein and not for catalytic activity. Perhaps this is also true in
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the case of 239FB and related proteins. We anticipate that the role of the 239FB protein could be fundamental in that highly conserved orthologs are present in organisms such as C. elegans and Drosophila, suggesting that genetic manipulation in these organisms could throw light on the function of this unusual MPE.

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