Identification of a cysteine residue important for the ATPase activity of *C. elegans* fidgetin homologue

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Abstract   Based on the amino acid alignment, *Caenorhabditis elegans* F32D1.1 was identified to be a homologue of the mammalian fidgetin. We produced and purified the F32D1.1 protein by using a baculovirus-expression system. F32D1.1 has an ATPase activity, which is sensitive to *N*-ethylmaleimide. K_m and V_{max} for the ATPase activity of F32D1.1 were estimated to be 0.44 mM and 225 nmol/mg/min, respectively. When the cysteine at the position of 368 was mutated to alanine, the ATPase activity was greatly decreased; V_{max} was decreased to one-sixth, while K_m remained similar. These results suggest that the unique position of cysteine 368, located immediately downstream of the Walker A motif, plays an important role in the ATP hydrolysis process of *C. elegans* F32D1.1 protein.
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Keywords: ATPase associated with diverse cellular activities protein; Fidgetin; Cysteine; *N*-Ethylmaleimide; ATP hydrolysis; *Caenorhabditis elegans*

1. Introduction

The mouse mutation fidget was isolated in 1943 [1]. Morphologically, the fidget mice have small eyes and lower penetrance skeletal abnormalities. Behaviorally, the mice are characterized by a side-to-side head-shaking and circling motion [2,3]. The gene fidgetin, responsible for the fidget mutation, was isolated and found to encode a member of AAA (ATPases associated with diverse cellular activities) family of proteins [4]. AAA proteins are involved in a variety of cellular activities including membrane fusion, protein folding and unfolding, proteolysis, and assembly and disassembly of protein complexes [5]. The AAA family proteins contain conserved ATPase domains, typically spanning 200–250 amino acid residues, referred to as AAA modules, that consist of three motifs, Walker A and B and SRH (second region of homology) [5,6]. Structural studies have revealed that AAA proteins usually form ring-shaped oligomers [7–10]. The ATP binding pocket is created at the interface between protomers and the three motifs are all essential for ATP hydrolysis [11,12]. In particular, residues of the SRH can contribute to two adjacent ATP binding pockets. These data taken together have suggested an intersubunit catalysis mechanism of ATP hydrolysis [11].

Some of the AAA proteins are now known to be involved in genetic diseases. Mutations in peroxins Pex1p and Pex6p cause peroxisomal disease, such as Zellweger syndrome, neonatal adrenoleukodystrophy and infant Refsum disease [13]. Mutations in paraplegin and spastin result in hereditary spastic paraplegia [14,15]. More recently, it was reported that inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutations in valosin-containing protein/p97 (VCP/p97) [16]. Although the function of fidgetin has not yet been elucidated, fidgetin is the first AAA protein to be implicated in mammalian development [4].

In this study, we focused on the *Caenorhabditis elegans* homologue of fidgetin and characterized its ATPase activity. Through mutational analysis, we found that a specific cysteine was required for full ATPase activity.

2. Materials and methods

2.1. Plasmid construction

The entire open reading frame for F32D1.1 was amplified by PCR using the cDNA clone yk61d2 (a generous gift from Dr. Y. Kohara, National Institute of Genetics, Japan) as a template and the following primers: 5′-TATGCTCGAGGAATGTATTCTCCAAAACGGG-3′ (sense, *Xho*I site underlined) and 5′-AGAACCCGGGTCAA-CGGGATATTGAAGGAGG-3′ (antisense, *Sma*I site underlined). The PCR product was digested with *Xho*I and *Sma*I and cloned into the expression vector pAcHLT-B (BD Biosciences) to make an N-terminal His6-tagged construct. Site-directed mutagenesis was carried out by using QuickChange II XL Site-Directed Mutagenesis Kit (Strategene). Primers used for mutagenesis are listed with the mutated codons underlined: C368A 5′-GGAAAAACAATGATCGGGAGAGCTGT-CGCTTCCCAGTGCAAAG-3′ (sense), 5′-CTTTGCACTGGGAAG-CGACAGCTCTCCCGATCATTGTTTTTCC-3′ (antisense); C373A 5′-GGGAGATGTGTCGCTTCCCAGGCCAAAGCCACTTTCTTC-AATATTTCAGC-3′ (sense), 5′-GCTGAAATATTGAAGAAAGTG-GCTTTGGCCTGGGAAGCGACACATCTCCC-3′   (antisense); C527A 5′-GGAGCTGACATGAGGCAATTGGCCACTGAAGCA-GCGATGGGTCC-3′ (sense), 5′-GGACCCATCGCTGCTTCAGT-GGCCAATTGCCTCATGTCAGCTCC-3′ (antisense); and T360C 5′-CTATTCGGACCACCAGGATGCGGAAAAACAATGATCGG-G-3′ (sense), 5′-CCCGATCATTGTTTTTCCGCATCCTGGTGG-TCCGAATAG-3′ (antisense). All of the mutations were confirmed by sequencing.

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2.2. Purification of proteins

Plasmids encoding the recombinant proteins were used to transfect Sf9 cells together with helper virus (BD Biosciences) and thus recombinant baculoviruses were obtained. Sf9 cells (300 ml culture) were infected with each recombinant virus and were harvested and suspended in 10 ml of lysis buffer (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 1% Triton X-100, 10% glycerol, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μM pepstatin A, 40 μM leupeptin, 1.6 μM aprotinin, 30 μM E-64, and 160 μM bestatin). After 1 h incubation on ice, cell lysate was cleared by ultracentrifugation ($100\,000 \times g$) for 1 h at 4 °C.

Soluble fractions thus obtained were incubated with Ni^{2+}–NTA agarose (Qiagen) for 1 h at 4 °C. The Ni^{2+}–NTA agarose resin was washed with buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.02% Triton X-100, and 10% glycerol) containing 5 mM imidazole and then with buffer A containing 20 mM imidazole. The His_6-tagged recombinant F32D1.1 proteins were finally eluted with buffer A containing 100 mM imidazole. Eluted fractions thus obtained were analyzed by SDS–PAGE visualized with Coomassie brilliant blue staining. Protein concentration was determined by using BCA Protein Assay Kit (Pierce).

2.3. In vitro ATPase assay

ATPase activity was measured by determining the amount of inorganic phosphate formed upon ATP hydrolysis as a complex with malachite green and ammonium molybdate [17]. The reaction was started by the addition of ATP and stopped by the addition of malachite green followed by citrate solution. After 1 h incubation at 30 °C, absorbance at 660 nm was measured. The amount of phosphate released was calculated from a standard curve using KH_2PO_4.

3. Results and discussion

3.1. C. elegans F32D1.1 is highly homologous to fidgetin

A BLAST search using the amino acid sequences of the mouse fidgetin as a query was carried out. Proteins showing high homology to it from human, mouse, fly, yeast and C. elegans were analyzed using CLUSTAL W and a phylogenetic tree was constructed. Fidgetin of human and mouse, fidgetin-like 1 of human and mouse, F32D1.1 of C. elegans and CG3326 of Drosophila melanogaster were located on the same branch (Fig. 1A). The amino acid sequence alignment of their AAA domains is shown in Fig. 1B. These results clearly indicate that F32D1.1 of C. elegans is a homologue of fidgetin. It is interesting to note that mammals, human and mouse, possess a second fidgetin, fidgetin-like 1, while C. elegans and D. melanogaster possess only a single fidgetin. Based on the presence of some unusual amino acid substitutions in conserved motifs, including an important substitution, aspartic acid to serine, in the Walker B box, it has been suggested that fidgetin may lack ATPase activity, but might instead regulate the function of other AAA family proteins [4]. Therefore, we focused on the biochemical characterization of F32D1.1 from C. elegans.

3.2. Expression and purification of C. elegans fidgetin homologue

The entire open reading frame of F32D1.1 was cloned into the baculovirus expression vector pAcHLT-B as described in Section 2. From this construct, F32D1.1 protein (594 amino acid residues) is produced as an N-terminal His_6-tagged recombinant protein. Most of the F32D1.1 protein was found in the soluble fraction after ultracentrifugation (data not shown). After purification on Ni^{2+}–NTA agarose, the F32D1.1 obtained was greater than 90% pure as judged by SDS–PAGE analysis (Fig. 2A). Typically, 0.6 mg of purified

F32D1.1 protein was obtained from a 300-ml culture of infected Sf9 cells.

3.3. Biochemical parameters for ATPase activity of F32D1.1

In order to demonstrate that the purified C. elegans F32D1.1 possesses an ATPase activity, purified F32D1.1 (0.6 μg) was incubated with ATP at 30 °C for 30 min in a final volume of 25 μl, which contained 20 mM Tris–HCl (pH 7.5), 25 mM K-glutamate, 2 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol (DTT) and 3 mM ATP. An ATPase activity of 30 nmol/mg protein/min was measured, indicating that the purified F32D1.1 protein has an ATPase activity. It is well known that mutations in the Walker A or B motif of AAA proteins abolish the ATPase activity [5]. In order to eliminate the possibility that the observed activity is due to the contamination, we prepared mutant proteins of Walker A and B motifs as the way the wild-type protein was purified. We found that these mutant proteins had no detectable ATPase activity as expected (data not shown). Therefore, the purified F32D1.1 protein indeed has an ATPase activity.

Next, we determined the optimum conditions for ATPase activity. ATP hydrolysis occurred dose-dependently (data not shown). With 0.6 μg of protein, ATP hydrolysis occurred linearly up to 60 min of incubation (data not shown). To determine the optimal reaction temperature, ATPase assays were carried out at 25, 30, 37, and 42 °C. As shown in Table 1, maximum activity was found at 25 and 30 °C. Since C. elegans dwells in the soil and is usually maintained under 25 °C, all the reactions described below were therefore carried out at 25 °C.

The effects of pH, salt, Mg^{2+}, DTT, BSA, and Triton X-100 on ATPase activity were subsequently examined (Table 1). F32D1.1 was found to prefer alkaline conditions. It should be noted that VCP and NEM (N-ethylmaleimide)-sensitive fusion factor (NSF), members of AAA family proteins, also have alkaline pH optimum [18,19]. The F32D1.1 ATPase activity was found to be high under the low salt conditions and to be a nearly 2-fold higher rate with 25 mM KCl than with NaCl and K-glutamate. High ATPase activity under the low salt conditions is a similar character of VCP [18]. Mg^{2+} ion was found to be essential. DTT was also required for ATPase activity with maximal effect at 5 mM. However, background values also increased as the concentration of DTT increased. Another reducing reagent 2-mercaptoethanol (30 mM) stimulated the ATPase activity without the increase in background. BSA enhanced ATPase activity in the range of 0.5–1.0 mg/ml. Triton X-100 at a final concentration of 0.2% showed the highest activity.

Thus, the optimum conditions for F32D1.1 ATPase activity were determined to be as follows: the reaction is carried out at 25 °C for 30 min in 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 30 mM 2-mercaptoethanol, 1 mg/ml BSA, 0.2% Triton X-100, and 3 mM ATP with 0.6 μg of F32D1.1 in a 25-μl reaction mixture. Using ATP concentrations from 0.1 to 3 mM, a Lineweaver–Burk plot was made (Fig. 2B). K_m and V_{max} for the ATPase activity of F32D1.1 were estimated to be 0.44 mM and 225 nmol/mg/min, respectively. These values are comparative with a K_m of 0.62 mM and V_{max} of 300 nmol/mg/min for the rat liver p97/VCP [20] and with a K_m of 0.33 mM and V_{max} of 520 nmol/mg/min for the recombinant p97/VCP [18]. As discussed previously
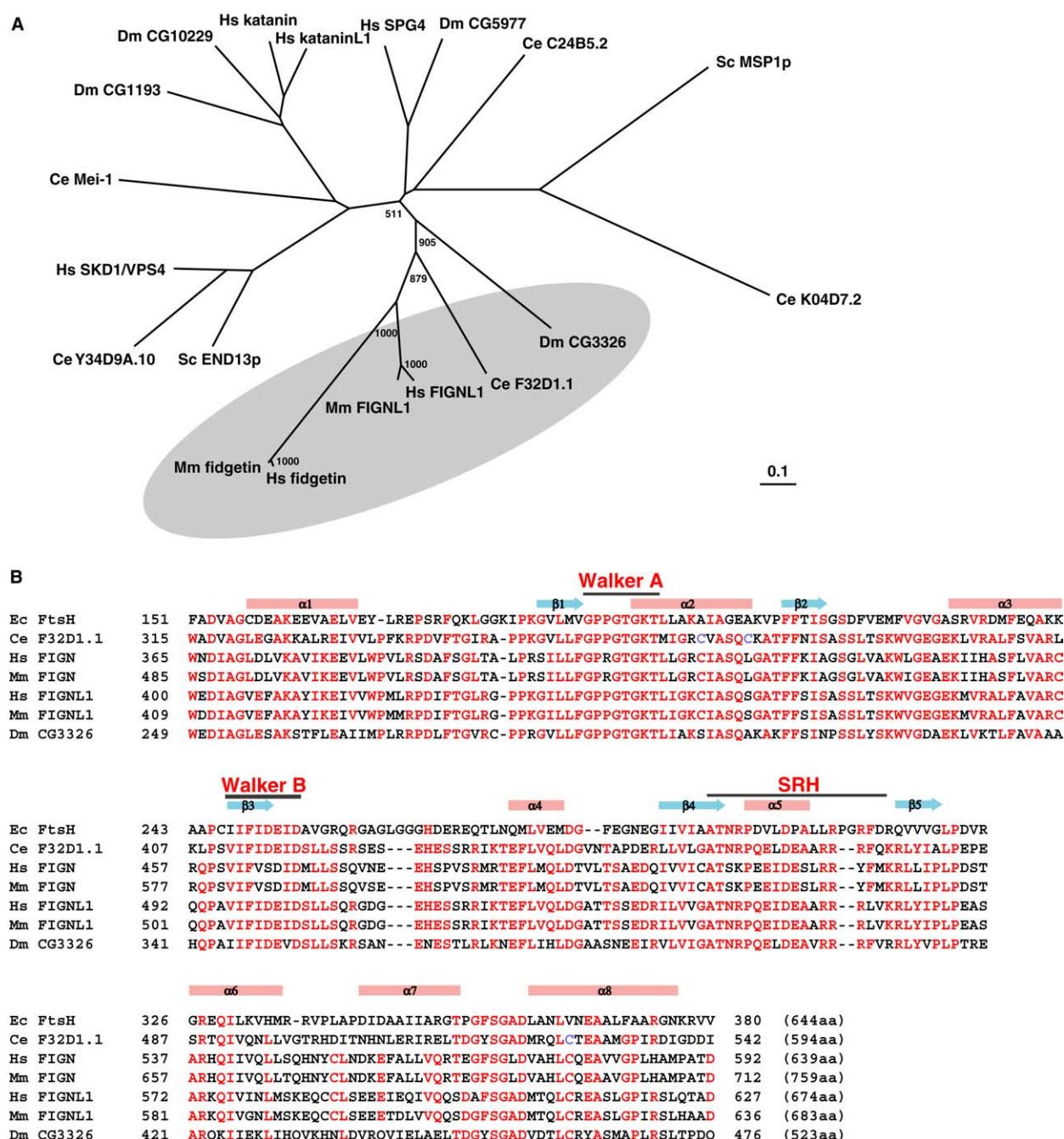
Fig. 1. Comparison of the amino acid sequences of the fidgetin containing subgroup of AAA proteins. (A) A phylogenetic tree of proteins of the fidgetin containing subgroup of AAA family proteins. The tree was constructed using CLUSTALW and TreeView software with the amino acid sequences of the following proteins: Ce F32D1.1 (Accession No. NP504197), Hs fidgetin (NP060556), Mm fidgetin (NP068362), Hs FIGNL1 (fidgetin-like 1, EAL23899), Mm FIGNL1 (fidgetin-like 1, AAH52415), Dm CG3326 (AAF51127), Hs SPG4 (spastin, CAB60208), Ce C24B5.2 (AAM29664), Dm CG5977 (AAF56223), Hs SKD1/VPS4 (NP037377), Ce Y34D9A.10 (AAK29883), Sc END13p (P52917), Hs katanin (katanin p60, AAC25114), Hs kataninL1 (katanin p60 subunit A-like 1, AAH00612), Ce Mei-1 (NP871793), Dm CG10229 (AAF52059), Dm CG1193 (AAF515955), Sc MSP1p (NP011542), and Ce K04D7.2 (P54815). Ce; *C. elegans*, Hs; *Homo sapiens*, Mm; *Mus musculus*, Dm; *Drosophila melanogaster*, Sc; *Saccharomyces cerevisiae*. Part of BOOTSTRAP data is also shown on the tree. (B) The amino acid sequence of AAA domains of fidgetin homologues from *C. elegans* (F32D1.1), *H. sapiens* (fidgetin and fidgetin-like 1), *M. musculus* (fidgetin and fidgetin-like 1), and *D. melanogaster* (CG3326). The amino acid sequence of *E. coli* FtsH is shown as a reference for α helices and β sheets. Walker A, Walker B and SRH are shown on top of the sequence. Four or more identical amino acids in one position are red. Three cysteine residues (C368, C373 and C527) of F32D1.1 are purple. Gaps are indicated by dashes.

[18,19], it is consistent with the notion that AAA family members all possess weak ATPase activities.

The ATPase activity of sea urchin katanin was 217 nmol/mg/min in the presence of its substrate microtubule, which was 10-fold higher than that without substrate [21]. The ATPase activity (735 nmol/mg/min) of *Escherichia coli* FtsH was also enhanced 2-fold in the presence of the substrate σ^{32} [22]. It can be speculated that the ATPase activity of
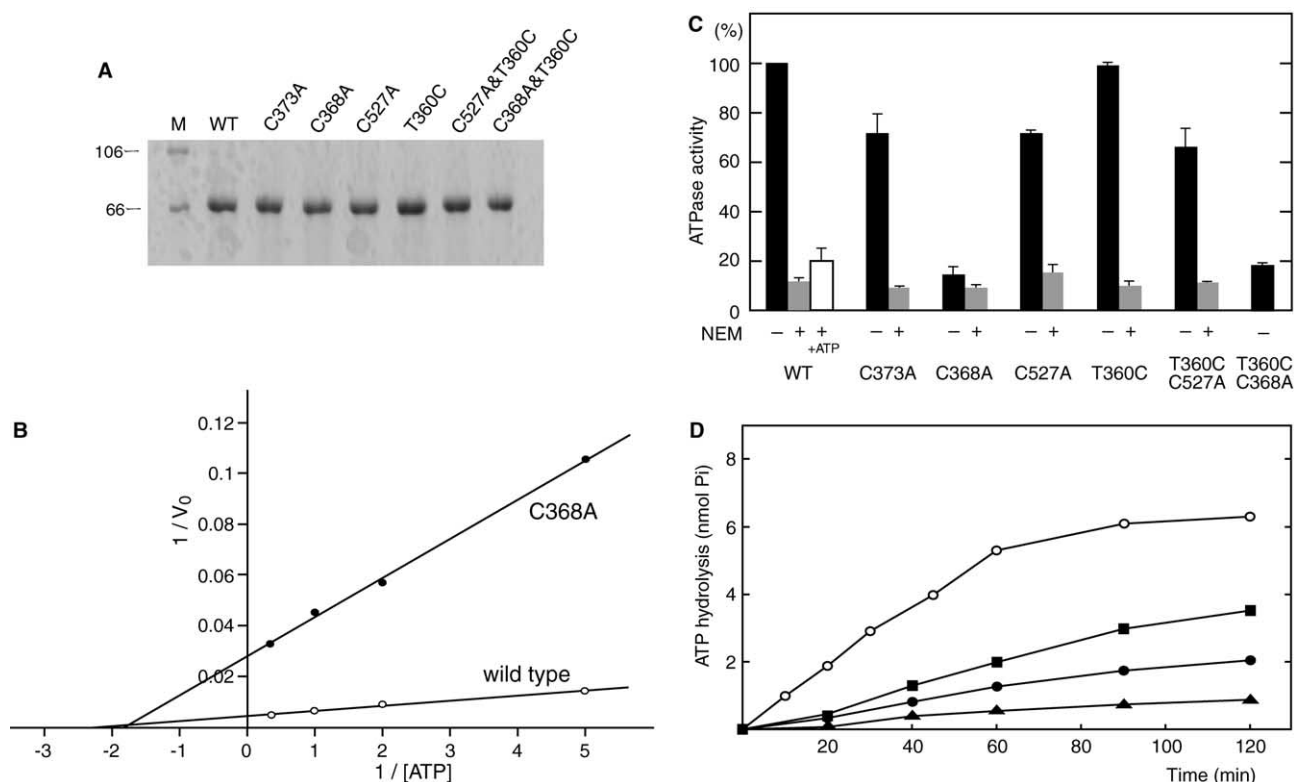
Fig. 2. (A) Purification profile of wild-type and mutant proteins. Wild-type and mutant proteins were purified as described in Section 2. Equal amounts (2 μg) of proteins were subjected to 10% SDS–PAGE followed by Coomassie brilliant blue staining. (B) Lineweaver–Burk plot of F32D1.1 ATPase activity. Each point is the average of at least two experiments. V_0, initial rate of reaction. (C) Comparison of ATPase activity of wild-type and mutant proteins. Purified wild-type and mutant F32D1.1 proteins were used in the optimized ATPase assays in the absence (filled bar) or presence (gray bar) of 0.1 mM NEM. Pretreatment of wild-type F32D1.1 with 0.1 mM NEM in the presence of 3 mM ATP was also carried out (open bar). Activities were taken from the average of at least two independent experiments. Relative activity (with S.D.) is presented with the activity detected in the wild type without NEM as 100%. (D) Kinetic study of C368A mutant. Purified proteins were used in the 25-μl reaction of the optimized ATPase assay condition using 0.6 μg of wild-type protein (open circle) and 1.2, 0.6, and 0.3 μg of C368A mutant (closed square, closed circle, and closed triangle, respectively). The data represent an average of two independent experiments.

AAA family proteins may be enhanced by the substrate in general. Furthermore, the ATPase activity of Chinese hamster NSF was reported to be 59 nmol/mg/min, while it was enhanced to be 128 nmol/mg/min in the presence of the adaptor protein SNAP (soluble NSF attachment protein) [19]. An even greater level of stimulation (5–10-fold) can be seen when NSF is incubated in the presence of SNAPs prebound to SNAP receptor (SNARE) complexes [23]. Therefore, it is of great interest to identify substrate(s) and adaptor(s) for *C. elegans* F32D1.1 protein, however, it (they) remains unknown at present.

The ATPase activity reached a maximum at an ATP concentration of 3 mM (Table 2). It is reasonable to speculate that the ATPase activity can be modulated by the level of ATP within a normal physiological concentration range of ATP (0.5–4 mM) [24,25]. To determine the nucleotide specificity, NTP hydrolysis assays were carried out with ATP, GTP, CTP, and UTP at a concentration of 3 mM. High specificity of F32D1.1 for ATP was observed (Table 2). F32D1.1 did not have a hydrolysis activity towards ADP. In the presence of ATP (3 mM), different amounts of ADP were added to the reaction mixture as shown in Table 2. It was found that ADP strongly inhibits the ATP hydrolysis activity, as little ATPase activity was detected at 3-fold excess of ADP over ATP, suggesting that the F32D1.1 ATPase activity may be

tightly regulated by the hydrolyzed product ADP. It has also been reported that ADP inhibits ATPase activity of NSF and VCP [18,26].

3.4. Effects of inhibitors on ATPase activity

Addition of EDTA (10 mM) to the reaction mixture containing 5 mM MgCl$_2$ decreased the ATPase activity by 88% (Table 3). This is consistent with the result that the Mg^{2+} ion is essential for ATP hydrolysis as mentioned above (Table 1). Vanadate and NaN$_3$, a typical P-type and F-type ATPase inhibitor, respectively, slightly inhibited the ATPase activity of F32D1.1 (Table 3). Pretreatment of F32D1.1 with NEM, an SH-alkylating reagent, resulted in a concentration-dependent decline in its ATPase activity, suggesting that a free SH moiety is required for the ATPase activity. This is consistent with the effects of DTT and 2-mercaptoethanol addition listed above. Since an NEM concentration as low as 0.01 mM was effective, F32D1.1 is very sensitive to NEM. It is interesting to mention that Chinese hamster NSF and *Xenopus laevis* p97, both of which are AAA proteins, have been purified and their ATPase activities have been shown to be sensitive to NEM but not to vanadate [19,27]. Pretreatment of F32D1.1 at 80 °C for 10 min resulted in a complete loss of activity.

Table 1
Effects of factors on ATPase activity

Factor	Condition	% of ATPase activity
Temperature[a]	25 °C	97
	30 °C	100
	37 °C	81
	42 °C	20
pH[b]	7.0	52
	7.5	93
	8.0	100
	8.8	97
KCl[c]	0 mM	91
	25 mM	100
	75 mM	75
	175 mM	30
NaCl[c]	25 mM	58
K-glutamate[c]	25 mM	63
MgCl$_2$[d]	0 mM	<1
	5 mM	100
	10 mM	72
DTT[e]	0 mM	5
	0.1 mM	15
	0.5 mM	67
	1 mM	77
	5 mM	100
2-Mercaptoethanol[e]	30 mM	114
BSA[f]	0.1 mg/ml	57
	0.5 mg/ml	99
	1 mg/ml	100
	2 mg/ml	70
Triton X-100[g]	0%	60
	0.1%	72
	0.2%	100
	0.5%	62

The ATPase activity of purified F32D1.1 (0.6 μg) was measured with 3 mM ATP at 25 °C for 30 min in a final volume of 25 μl reaction mixture.
Relative activity is presented with the highest activity detected in each factor as 100%.
[a] Reaction mixture contains 20 mM Tris–HCl (pH 7.5), 25 mM K-glutamate, 2 mM MgCl$_2$, 10% glycerol, and 1 mM DTT. Reaction was carried out at indicated temperature. 30 nmol/mg/min of activity is presented as 100%.
[b] Reaction mixture contains 20 mM Tris–HCl of indicated pH, 25 mM K-glutamate, 2 mM MgCl$_2$, 10% glycerol, and 1 mM DTT. 34 nmol/mg/min of activity is presented as 100%.
[c] Reaction mixture contains 20 mM Tris–HCl (pH 7.0), indicated concentration of salt, 2 mM MgCl$_2$, 10% glycerol, and 1 mM DTT. 45 nmol/mg/min of activity is presented as 100%.
[d] Reaction mixture contains 20 mM Tris–HCl (pH 7.0), 25 mM K-glutamate, indicated concentration of MgCl$_2$, 10% glycerol, and 1 mM DTT. 52 nmol/mg/min of activity is presented as 100%.
[e] Reaction mixture contains 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 1 mg/ml BSA and indicated concentration of DTT or 2-mercaptoethanol. 133 nmol/mg/min of activity is presented as 100%.
[f] Reaction mixture contains 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 1 mM DTT, and indicated concentration of BSA. 120 nmol/mg/min of activity is presented as 100%.
[g] Reaction mixture contains 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 30 mM 2-mercaptoethanol, and indicated concentration of Triton X-100. 69 nmol/mg/min of activity is presented as 100%.

Table 2
Nucleotide specificity of ATPase

Nucleotide	Concentration	% of Hydrolysis activity
ATP	0.1 mM	6
	0.2 mM	21
	0.5 mM	50
	1 mM	73
	3 mM	100
ADP	3 mM	<1
GTP	3 mM	15
CTP	3 mM	8
UTP	3 mM	<1
In the presence of 3 mM ATP		
ADP	0 mM	100
	0.3 mM	59
	3 mM	20
	10 mM	2

The NTPase activity of purified F32D1.1 (0.6 μg) was measured at 25 °C for 30 min in a final volume of 25 μl reaction mixture, which contains 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 1 mM DTT, 1 mg/ml BSA, and indicated nucleotide.
Relative activity is presented with the activity detected with 3 mM ATP (132 nmol/mg/min) as 100%.
Inhibitory effect of the addition of ADP was also analyzed.

Table 3
Effects of inhibitors on ATPase activity

Inhibitor	Concentration	% of ATPase activity
None		100
EDTA	2 mM	45
	10 mM	12
Vanadate	0.01 mM	84
	0.1 mM	84
	0.5 mM	70
NaN$_3$	2.5 mM	85
NEM	0.001 mM	93
	0.005 mM	68
	0.01 mM	33
	0.05 mM	25
	0.1 mM	23
	1 mM	19
	4 mM	16
Heat		<1

The ATPase activity of purified F32D1.1 (0.6 μg) was measured at 25 °C for 30 min in a final volume of 25 μl reaction mixture, which contains 20 mM Tris–HCl (pH 8.0), 25 mM KCl, 5 mM MgCl$_2$, 10% glycerol, 30 mM 2-mercaptoethanol, 1 mg/ml BSA, 3 mM ATP, and indicated inhibitor.
Relative activity is presented with the activity detected in the absence of inhibitor (138 nmol/mg/min) as 100%.

3.5. Construction and purification of cysteine mutants

Based on the sensitivity to NEM, F32D1.1 may have some essential cysteines that are involved in the ATPase activity of F32D1.1. The AAA domain of F32D1.1 contains three cysteine residues (C368, C373 and C527) (Fig. 1B). C368 and C373 are located immediately downstream of the Walker A motif, whereas C527 is located in the C-terminal α-helical re-

gion. C368 and C527 are well conserved among the fidgetin homologues, while C373 is found only in *C. elegans* F32D1.1 (Fig. 1B). The AAA domain of *E. coli* FtsH protein has been crystallized and its three-dimensional structure has been solved [7]. Since the AAA domains of FtsH and F32D1.1 are highly homologous (59% similarity) by sequence alignments (Fig. 1B), it is reasonably assumed that these three cysteines may be located around the ATP binding pocket. However, their precise locations should await the structural analysis of F32D1.1. To identify the reactive cysteine(s), each cysteine residue was changed to alanine by site-directed mutagenesis. Mutations at the respective positions did not alter the behavior during purification or the yield of mutant proteins. The purified proteins obtained were also greater than 90% pure as estimated by the Coomassie brilliant blue stained SDS–PAGE gel (Fig. 2A).

3.6. Identification of an important cysteine residue in ATP hydrolysis

As is clearly evident in Fig. 2C, there is a substantial decline in ATPase activity when cysteine 368 is mutated to alanine; however, mutations of the other cysteines lead to only nominal decreases in activity. When the NEM sensitivity of the ATPase activity was checked for the cysteine mutants, the C373A and C527A mutant proteins remained sensitive to NEM modification. K_m and V_{max} for the ATPase activity of the C368A mutant were estimated to be 0.55 mM and 36 nmol/mg/min, respectively (Fig. 2B). Compared to those of wild-type protein as described above, V_{max} was greatly decreased (about 1/6), while the affinity for ATP remained similar. Kinetic studies of the C368A mutant revealed that ATP hydrolysis was dose- and time-dependent fashions, but the overall activity was lower than that of the wild-type protein (Fig. 2D). These results indicate that C368 may be present within the catalytic site/nucleotide binding pocket of F32D1.1. Based on the conservation of cysteine at position 368 in F32D1.1 (Fig. 1B), fidgetin and fidgetin-like 1 proteins but not *D. melanogaster* CG3326 are likely to be sensitive to NEM.

Mammalian P-glycoproteins that are plasma membrane proteins possess two nucleotide-binding domains and show NEM-sensitive ATPase activity [28,29]. Mutational analyses revealed that cysteine residues (GNSG**C**GKS and GSSG**C**GKS) in the Walker A motifs of both nucleotide-binding domains are crucial for the NEM sensitivity [28,29]. Among AAA family proteins, it has been shown that VCP and NSF possess NEM-sensitive ATPase activity [18,30,31], although the residue conferring the NEM sensitivity has not been determined. Interestingly, the ATPase activity of Sec18p, an NSF homologue of *Saccharomyces cerevisiae*, was unaffected by NEM [32]. Based on amino acid sequence alignment, it is proposed that this is because Sec18p has a threonine in the Walker A motif instead of the cysteine found in mammalian NSF [33]. F32D1.1 showed the NEM-sensitive ATPase activity as shown above, however, the position of its cysteine is unique compared to those of P-glycoproteins, that is, C368 of F32D1.1 is located immediately downstream of the Walker A motif and not within it. In order to elucidate the functional difference between these two positions of cysteine, we therefore introduced a cysteine residue (T360C) in the Walker A motif on the C368A mutant to make it mimic P-glycoproteins. Site-directed mutagenesis was carried out and expression and purification

were carried out as for wild-type F32D1.1 protein (Fig. 2A). Introduction of cysteine residue into the Walker A motif of F32D1.1 did not affect the ATPase activity (Fig. 2C). The T360C mutant was still NEM sensitive (Fig. 2C). The T360C C368A mutant protein showed similar ATPase activity as that of C368A mutant protein (Fig. 2C). These results suggest that in the case of F32D1.1, T360C cannot functionally overcome the C368A mutation and therefore the function of cysteine at position 368 is different from that at 360.

It has been reported that if pretreatment of P-glycoproteins with NEM was carried out in the presence of ATP, the ATPase activity was not abolished [28,29]. Even though F32D1.1 was pretreated with NEM in the presence of 3 mM ATP, ATPase activity was still greatly decreased (Fig. 2C). These results together suggest that functions of cysteines at both positions are different to each other.

3.7. Conclusions

We have demonstrated that *C. elegans* F32D1.1, a fidgetin homologue, possesses ATPase activity comparable to those of other AAA family proteins reported previously. Mutational analysis of cysteine residues reveals that the cysteine residue (C368), immediately downstream of Walker A motif, plays an important role in the ATP hydrolysis process. It is also possible that C368 could be involved in the conformation of the ATP binding site and therefore affect the site structure without being directly involved in catalysis.

F32D1.1 knockdown in *C. elegans* by RNA interference method resulted in the defect of gonad formation and the sterile phenotype (Yamanaka et al., unpublished). These results suggest that the ATPase activity of F32D1.1 is required for at least the development of gonad. The ATPase activities are sometimes enhanced by the addition of substrates [20–23]. The identification of such substrate(s) is important for our understanding of not only biochemical property but also cellular function of F32D1.1. Determination of the higher order structure of F32D1.1 and analysis of the structural basis of the interaction between F32D1.1 and its substrate also remains to be addressed.

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