Common Evolutionary Origin for the Rotor Domain of Rotary ATPases and Flagellar Protein Export Apparatus

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Abstract

The V1- and F1- rotary ATPases contain a rotor that rotates against a catalytic A3B3 or α3β3 stator. The rotor F1-γ or V1-DF is composed of both anti-parallel coiled coil and globular-loop parts. The bacterial flagellar type III export apparatus contains a V1/F1-like ATPase ring structure composed of Filp homo-hexamer and Filj which adopts an anti-parallel coiled coil structure without the globular-loop part. Here we report that Filj of Salmonella enterica serovar Typhimurium shows a rotor like function in Thermus thermophilus A3B3 based on both biochemical and structural analysis. Single molecular analysis indicates that an anti-parallel coiled-coil structure protein (Filj structure protein) functions as a rotor in A3B3. A rotary ATPase possessing an F1-γ-like protein generated by fusion of the D and F subunits of V1 rotates, suggesting F1-γ could be the result of a fusion of the genes encoding two separate rotor subunits. Together with sequence comparison among the globular protein parts, the data strongly suggest that the rotor domains of the rotary ATPases and the flagellar export apparatus share a common evolutionary origin.

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Introduction

Two types of rotary ATPases are found in biological membranes; V1/V type ATPase and F1/F1 (F type ATPase) [1–4]. Evolutionary counterparts of the eukaryotic V1/V type ATPases are found in most archaea and some bacteria (often referred to as A-type ATPase). Both V1/V and F1/F1 couple ATP synthesis and hydrolysis to proton translocation across the membrane by rotation of the rotor apparatus against the surrounding stator which includes the catalytic A3B3 or α3β3 hexamer (Fig. 1a). Sequence and structural comparison of the two ATPases indicate significant homology between the catalytic subunits, but not between the subunits of the central rotor domain [5]. For example, V1/V lacks a counterpart of the rotor shaft F1-γ subunit [1,4,5].

The minimal ATP-driven rotary unit of F1/F1 is F1, which is comprised of three different proteins with a stoichiometry of α2β2γ. The F1-γ contains two distinct domains; a coiled-coil domain that penetrates the central cavity of the α2β2 cylinder from the top to the bottom, and a globular domain containing a α/β fold which makes contact with the F1-ε (Figs. 1a,b). For V1/V, the minimal rotary unit consists of the A3B3/D subunits [6]. In addition, analysis of the secondary structure of the D subunit predicts the presence of long α-helices at both the amino and carboxyl termini as found in the F1-γ (Fig. 1b, Supplementary Fig. S1a). On the basis of this, it has been suggested that the D subunit is likely to be a structural and mechanistic analog of F1-γ despite the lack of any significant sequence similarity [5]. However, F1/F1 lacks a counterpart of the V1-F subunit [1,4,5], which has a typical globular α/β fold [7] (Fig. 1c). Intriguingly, the X-ray structure of the entire V1 of T. thermophilus revealed that the central rotor subunit contains both the coiled-coil (D) and globular domains (Fγ), suggesting that the V1-F and V1-D subunits together form the counterpart of the α/β domain of F1-γ [8].

Mulkidjian et al. have proposed a scenario to explain the origin of rotary ATPases, where rotary ATPases share an evolutionary origin with the bacterial flagellar and non-flagellar type III export systems [5]. The flagellar export apparatus consists of a membrane-embedded export gate composed of FlhA, FlhB, FlhO, FlhP, FlhQ and FlhR, and a water-soluble ATPase complex consisting of FlhH, FlhD, and FlhJ (Fig. 1a, lower panel) [9]. Components of the flagellar ATPase complex, which allows the export gate to efficiently utilize proton motive force across the cytoplasmic membrane as an energy source for protein translocation [10,11], exhibit extensive structural and sequence similarity to catalytic and rotor subunits of rotary ATPases. For instance, the atomic structure of FlhD ATPase of the flagellar export apparatus is remarkably similar to the F1 β/α and V1 A/β subunits [12], Flj, which is a soluble export component protein, also shows a striking structural similarity to the coiled-coil region of F1-γ [13]. This

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Figure 1. Structure of rotary ATPases and rotors. (a) Schematic model of prokaryotic F\textsubscript{o}F\textsubscript{1} (left) and V\textsubscript{o}V\textsubscript{1} (right). Rotor subunits (D, F, V\textsubscript{1}-FliH, FliI, and FliJ) are presented in brown. Peripheral stators (EG and V\textsubscript{1}-a in V\textsubscript{o}V\textsubscript{1}, b, d, and F-a in F\textsubscript{o}F\textsubscript{1}) are presented in sky blue. Schematic model of the flagellar type III export apparatus (lower). The export apparatus consists of a proton-driven helical coiled coil formed by the N- and C-terminal regions of the \( \gamma \) subunit penetrating into the central cavity of the \( \alpha_{2}\beta_{3} \) ring. These findings indicate that the type III export system has a \( \gamma \)-like and \( \gamma_{1} \)-F structure and share a common evolutionary origin.

Here we aimed to explore the evolutionary relationship between the rotor domains of the V\textsubscript{o}V\textsubscript{1} and F\textsubscript{o}F\textsubscript{1} and the type III export system. FliJ formed chimeric complex with A\textsubscript{B}\textsubscript{3} of V\textsubscript{o}V\textsubscript{1} and promoted their ATPase activity. Single molecular analysis indicates anti-parallel coiled coil structure (FliJ structure) protein functions as a rotor axis. These results strongly suggest that the FliJ structure proteins are the ancestral subunit of the rotor subunit of rotary ATPases. We also discuss the evolutionary relationship between globular domain of F\textsubscript{1}-c and V\textsubscript{1}-F.

Methods

Proteins

The A\textsubscript{B}\textsubscript{3}JL and A\textsubscript{B}\textsubscript{3}DF expression plasmids were constructed from His-tagged V\textsubscript{1} (A\textsubscript{B\textsubscript{3}H-10/C28S/S232A/T235S/C255A/C508A}3B\textsubscript{C264S}D(E48C/Q55C)F) as described in Figs. S3a, S4a. The A\textsubscript{B}\textsubscript{3}JL (A\textsubscript{B\textsubscript{3}H-10/C28S/S232A/T235S/C255A/C508A}3B\textsubscript{C264S}JL), A\textsubscript{B}\textsubscript{3}DF, and A\textsubscript{B}\textsubscript{3} were expressed in E. coli and the expressed enzymes purified by Ni\textsuperscript{2+}-affinity chromatography (Qiagen) followed by ion exchange on a RESOURCE Q column (GE healthcare) [6]. The purified His-tagged enzymes were biotinylated at two cysteines using 6-[\textsuperscript{N}-\textsuperscript{2}-maeleimide;ethyl]-N-piperazinylamide[hexyl-D-biotinamide (Dojindo, Kumamoto, Japan). The bound ADP in each enzyme was partially removed by successive EDTA-heat treatment [18].

FliJ was expressed in BL21(DE3)pLysS and purified as described previously [13]. FliJ(C32T/I61C/I67C) was created by QuikChange mutagenesis (Stratagene), expressed in BL21(DE3)pLysS, biotinylated and purified as the same protocol used for FliJ(C32T/I61C/I67C) [13]. A\textsubscript{B}\textsubscript{3} was expressed and purified as previously described [15]. The A\textsubscript{B}\textsubscript{3}FliJ complex was reconstructed from the purified FliJ and A\textsubscript{B}\textsubscript{3}. FliJ and A\textsubscript{B}\textsubscript{3} were mixed and incubated overnight at 23°C. The mixture was applied to a HiLoad Superdex 200 column in 20 mM MOPS-NaOH pH 7.0 and 100 mM NaCl. The fraction containing the A\textsubscript{B}\textsubscript{3}-FliJ complex was pooled and stored for future use.

Rotation experiments

Biotinylated FliJ(C32T/I61C/I67C) and His-tagged A\textsubscript{B}\textsubscript{3} were mixed and incubated overnight at 23°C. The A\textsubscript{B}\textsubscript{3}-FliJ mixture was applied to 5-ml polypropylene column containing Ni-NTA agarose (Qiagen), and A\textsubscript{B}\textsubscript{3}-FliJ complexes diluted 1:10 into BSA buffer and infused into the flow cell. After incubation for 15 minutes at 23°C, unbound gold spheres were washed out with buffer A. After infusion of buffer A containing 4 mM Mg-ATP, 2.5 mM phosphoenol pyruvate, and structural motif is also seen in the YscO-like protein, CT670 from Chlamydia tracomatis, a FliJ homolog of the non-flagellar type III export apparatus [14]. Interestingly, FliJ promotes the formation of a homo-hexamer of FliJ by binding to the center of the ring, facilitating the FliJ ATPase activity [9]. A very similar arrangement is observed in the F\textsubscript{o}F\textsubscript{1}-ATPase with the anti-parallel \( \alpha \)-helical coiled coil formed by the N- and C-terminal regions of the \( \gamma \) subunit penetrating into the central cavity of the \( \alpha_{2}\beta_{3} \) ring. These findings indicate that the type III export system has a \( \gamma \)-like and \( \gamma_{1} \)-F structure and share a common evolutionary origin.
0.5 mg ml\(^{-1}\) pyruvate kinase, rotation of the gold spheres were observed by a dark-field microscope (BX53, U-DCW, UPlanFLN 100x; Olympus) and recorded with a high-speed camera (ICL-B0620M-KC, IMPERX) at 0.8–1.6-ms intervals at 23°C. For the rotation assay of A\(_3\)B\(_3\)DF\(_f\) or A\(_3\)B\(_3\)JL, the biotinylated enzyme (1–5 nM) in buffer A was applied to the flow cell and incubated for a few minutes at 23°C. Streptavidin-coated magnetic beads (100–300 nm) and Ni\(^{2+}\)-NTA coated cover glasses were prepared as previously described [20,21]. Unbound enzyme was washed out with 20\(\mu\)l of buffer A. Then, 20\(\mu\)l of buffer A with 2 mg ml\(^{-1}\) BSA was infused to the flow cell and incubated for 30 s to prevent nonspecific binding. The BSA solution in the chamber was washed out with 20\(\mu\)l of buffer A. Then, 20\(\mu\)l of buffer A with 2 mg ml\(^{-1}\) BSA was infused to the flow cell and incubated for <30 s to prevent nonspecific binding. The 0.5 mg ml\(^{-1}\) BSA solution in the chamber was washed out with 20\(\mu\)l of buffer A. Then, buffer A containing streptavidin coated magnetic beads (10\(^{10}\)–10\(^{11}\) particles ml\(^{-1}\)) were infused into the flow cell and incubated for a few minutes. Unbound beads were washed out with 20\(\mu\)l of buffer A. After infusion of 80\(\mu\)l of buffer A containing Mg-ATP at the indicated concentration, 2 mM MgCl\(_2\), 2.5 mM phosphoenolpyruvate, and 0.5 mg ml\(^{-1}\) pyruvate kinase, rotation of the bead was recorded with a high-speed camera (Eclips, IN) at 1000 frames per second (f.p.s.) using a phase-contrast microscope (IX70, Olympus) with 6\(\times\)100 objective lens (N.A., 1.30, Olympus) at 23°C. Images were captured as an 8-bit AVI file. The centroid of the bead images was calculated [20,21].

Electron cryo-microscopy and image analysis

Sample grids were prepared by applying 3\(\mu\)l of a protein solution containing the in vitro reconstructed A\(_3\)B\(_3\)J rings (150 \(\mu\)g/\(\mu\)l) onto a holey carbon grid (Quantifoil R0.6/1.3, Quantifoil Micro Tools, Jena, Germany), which had been glow discharge for 20 s before use. The grids were blotted onto filter paper for 5 s to remove excess solution, vitrified in liquid ethane at \(-196^\circ\text{C}\) using a Vitrobot (FEI, Eindhoven, Netherlands) and transferred into a liquid nitrogen storage capsule. Particle images were recorded with a 4 K \(\times\) 4 K Slow Scan CCD camera (TemCam-F415MP, TVIPS) mounted on a JEM-3200FSC electron microscope (JEOL, Tokyo, Japan), equipped with a liquid-helium cooled specimen stage, a \(\Omega\)-type energy filter and a field-emission electron gun operated at an accelerating voltage of 200 kV. Electron micrographs were collected at 50 K with a magnification of \(\times\)140,000, corresponding to 1.07 A\(\times\)pixel. Focal pairs of the micrographs were recorded at a defocus between 1.5 and 2.5 μm for the first micrograph and at a defocus between 3.5 and 5.5 μm for the second. Electron dose was set to 30 e\(^{-}\)/Å\(^{2}\) for both micrographs. The particle images were processed using the EMAN software package [22]. The focal pairs were merged using FOCALPAIR [22,23]. The defocus amplitude, envelope and noise values were determined using CTFFT [22]. Micrographs showing significant astigmatism or drift were discarded. The particle images were selected with BOXER [22] and the boxed particle images were aligned and classified using REFINET2D.PY [22].

Phylogenetic tree analysis

All sequences used in this study were aligned using the MAFFT program [http://mafft.cbrc.jp/alignment/server/] [24]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [25]. The phylogenetic tree was constructed using the Maximum-Likelihood (ML) method under the Jones-Taylor-Thornton model by using MEGA.

Figure 2. Analysis of A\(_3\)B\(_3\)J. (a) SDS-PAGE analysis of A\(_3\)B\(_3\)J and A\(_3\)B\(_3\)DF\(_f\), (b) Analysis of disassembly of A\(_3\)B\(_3\) and A\(_3\)B\(_3\)J on Native PAGE. Each complex was incubated without nucleotide, or with 1 mM MgATP at 37°C for 1 h, followed by separation by Native PAGE. (c) ATP hydrolysis activity of A\(_3\)B\(_3\) and A\(_3\)B\(_3\)J. Time courses of ATP hydrolysis catalyzed by A\(_3\)B\(_3\) (blue lines) and A\(_3\)B\(_3\)J (red lines) at 25°C and at 4 mM MgATP. The reaction was started by the addition of 20 μl of 1 μM enzyme solution to 2 ml of assay mixture.

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Figure 3. Structure of A\(_3\)B\(_3\) and A\(_3\)B\(_3\)J. Averaged cryo-EM image of A\(_3\)B\(_3\) (a) and A\(_3\)B\(_3\)J (b) are shown. Possible ribbon models of A\(_3\)B\(_3\) (c) and A\(_3\)B\(_3\)J (d) are also indicated below each EM image.

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Results

Reconstitution of A3B3J

Structural sequence alignment has previously revealed that FliJ shares some amino acid conservation with F1γ-7 [13]. The structural and sequence similarity suggests that FliJ may behave as a counterpart of rotor subunit of rotary ATPases. To test this possibility, we investigated whether FliJ binds to the center of the A3B3 ring of T. thermophila, forming the A3B3J complex. A3B3 was mixed with excess amounts of FliJ, and the mixture incubated overnight at room temperature. The mixture was applied to a gel filtration column to remove free FliJ. The retention time of the complex peak was almost identical to that of A3B3 alone (Fig. S2a). Previous studies have shown that V1 and A3B3 are resistant to ATP-induced dissociation [15] while A3B3D completely dissociates into monomeric A and B subunits by incubation with 1 mM ATP for 30 min (Fig. 2b). In contrast, the A3B3J complex is at least partially resistant to ATP-induced dissociation (Fig. 2b), indicating that the presence of FliJ stabilizes the A3B3 complex to a significant degree. ATP hydrolysis by V1 or A3B3D proceeds at a steady rate for a few minutes, then decelerates slowly, due to ADP inhibition [16]. In contrast, the ATPase activity of A3B3 decelerates rapidly, reaching a steady state rate within a few minutes owing to rapid ATP-induced dissociation of A3B3 [16]. In the case of A3B3J, the ATP hydrolysis profile is similar to that of V1 or A3B3D exhibiting continuous ATP hydrolysis activity after an initial burst phase (Fig. 2c). The turnover rate of A3B3J is ~7.0 s⁻¹, almost twice that of A3B3 (~4.0 s⁻¹). Together, these results suggest that FliJ stabilizes the A3B3 hexamer by binding to A3B3 and promotes continuous ATPase activity in a similar manner to the V1-D subunit.

Electron cryo-microscopy analysis of A3B3J

In order to identify the location of FliJ in the A3B3J complex, we analyzed the images of frozen-hydrated A3B3J and A3B3 particles embedded in vitreous ice by electron cryo-microscopy. Since the distribution of the particle orientation was strongly biased to end-on view, we aligned and averaged the end-on images of each particle. The averaged image of A3B3 shows a hetero-hexameric ring structure with an unoccupied central hole of 2 nm in diameter (Fig. 3a), consistent with the crystal structure of A3B3 [17]. In contrast, the averaged image of A3B3J, clearly shows extra density in the interior of the central hole but located off center, close to one of the peripheral subunits (Fig. 3b). These observations strongly suggest that FliJ penetrates into the central hole of the A3B3 complex in a way similar to the D-subunit of V1-ATPase [8].

Rotary motion of FliJ in A3B3 without uni-directionality

We attempted to demonstrate the rotary motion of FliJ against A3B3 immobilizing A3B3J on a Ni-NTA-coated glass surface through His10-tags introduced at the N terminus of the A subunits, and attaching a 40-nm streptavidin-coated gold colloid (40-nm bead) to the biotin-labelled FliJ (Fig. 4a). Beads were imaged by dark-field microscopy, and beads motions were recorded on a fast-framing CCD camera at speeds up to 1,000 frames s⁻¹. We found rotating beads attached to the FliJ in A3B3. It was confirmed that gold beads observed under the microscope were attached to the A3B3J complex through FliJ because very few beads were found without A3B3J. Two to ten beads showing rotary motion were usually found in a single flow cell. However, they did not show apparent uni-directionality. For instance, both rotating beads showing clockwise or anti clockwise rotation (Fig. 4 b–e) were found. Some beads showed stepwise rotary motion (Fig. 4 f,g). The rotary motion of A3B3J was also observed without ATP in the infusion buffer but the number of beads showing rotary motion apparently decreased; one to three beads showing rotary motion were found in a single flow cell. These results indicate that FliJ in the A3B3J does not function as a perfect rotor axis. FliJ is only weakly bound to A3B3, thereby dissociating FliJ from the complex after successive gel filtration chromatography of the A3B3J complex (Fig. S2b). In agreement with this, the ATPase activity of A3B3J (7.0 s⁻¹), although significantly higher than A3B3, is much lower than that of V1 (65 s⁻¹) or A3B3D (25 s⁻¹), reflecting the relative instability of the A3B3J complex. Therefore, the low binding affinity of the FliJ for A3B3 is likely to reduce the chance of FliJ rotation being coupled with a conformational change of A3B3 by ATP hydrolysis.

Anti-parallel coiled coil structure (FliJ structure) functions as a rotor

FliJ adopts an anti-parallel coiled coil structure composed of both N- and C-terminal α-helices and is similar to that of V1-D or F1γ-7 [13]. If we assume that the rotor subunits of the rotary ATPases and the flagellar type III export apparatus share a common evolutionary origin, then the ancestral rotor subunits...
adopt an anti-parallel coiled-coil structure, that is, the FliJ structure. In order to demonstrate that the FliJ structure functions as a rotor, we constructed a gene encoding a FliJ structure (FliJ-like protein, termed JL hereafter). The JL gene encodes an anti-parallel coiled coil region of V1-D composed of both N- and C-terminal α-helices, without the additional region (52 a.a. length) between the N- and C-terminal helices (Fig. S1a). Then we constructed an expression vector encoding the JL gene along with atpA and atpB genes (Fig. S3a). The JL protein was co-expressed along with the A and B subunits in Escherichia coli and purified the A3B3JL complex to homogeneity (Fig. 2a). The A3B3JL was active as an ATPase and the V_max value of the A3B3JL is similar to that of A3B3D (Fig. S3b). Using a direct observation system similar to that described for V1 [6], the rotation of the A3B3JL was able to be visualized (Fig. 5a). We observed rotating beads attached to the A3B3JL when the flow cell was infused with buffer containing 4 mM or 10 μM ATP (hereafter [ATP]) (Fig. 5b). The results clearly indicate that the JL-protein functions as a rotor. At 10 μM [ATP], A3B3JL showed a clear stepwise rotation, pausing every 120° like F1 or V1. Interestingly, frequent backward steps were observed in rotation of A3B3JL but were seldom seen in stepwise rotation of either V1 or F1, indicating that the extra 53 amino acid residues inserted between the two helices were necessary for continuous rotation without irregular motions (Fig. 5b, inset). Taken together, we propose that an anti-parallel coiled-coil fold unit like FliJ is sufficient to function as a rotor for the ATP-driven rotary motor.

**F1-γ like rotor derived from V1-D and V1-F**

As shown in Figure 1, the similar folds of V1-F and the globular domain of F1-γ prompted us to probe an evolutionary relationship between these two proteins, i.e. if could F1-γ be derived from two separate proteins as observed for V1-D and V1-F. To investigate the functional relationship between V1-F and globular domain of F1-γ, we constructed an expression vector of a mutant V1 containing a fusion of the genes coding for subunits D and F. The gene coding for F (atpF) was inserted between the two helical regions of the gene coding for D (atpD; see Fig. S4a) and the resulting fusion protein was termed DFf. The mutant ATPase containing the fused protein (A3B3DFf) was expressed in E. coli and purified to homogeneity (Fig. 2a). The A3B3DFf showed an ATPase activity and exhibited simple Michaelis-Menten kinetics, nearly equal to those of wild type V1, which contains separate D and F subunits in the rotor [18]. Using a direct observation system similar to that described for V1, rotation was visualized via a bead attached obliquely to the helical region in the DFf (Fig. 5c). We
observed rotating beads attached to the A3B3DFf at 4 mM or 10 μM ATP (Fig. 5d). Stepwise rotation of the A3B3DFf passing every 120° was also observed at 10 μM ATP. In this case the frequent backwards steps seen for A3B3JL were not observed (Figs. 5d, inset). Together, the identical rotation behavior and kinetic parameters of A3B3DFf confirm that the DFf fully functions as a shaft in the rotary motor. These observations indicate that the V1-F when fused to V1-D, functions in the rotor in the same way as the globular domain of F1-γ and suggest an evolutionary relationship between V1-F and V1-D and F1-γ. In other words, F1-γ could be the product of fusion of the genes encoding an ancestral helical protein similar to V1-D and an ancestral globular protein similar to V1-F.

Discussion

In this study, we have provided several lines of evidence on the functional similarity of FliJ of the flagellar type III export system to the rotor subunit in rotary ATPases. Analysis of reconstituted A3B3JL revealed that FliJ stabilizes the A3B3 hexamer by penetrating into A3B3 and promotes continuous ATPase activity in a manner similar to the V1-D subunit. Although FliJ in A3B3 does not show unidirectional rotation coupling with ATP hydrolysis due to its low binding affinity for A3B3, the JL protein is sufficient for functioning as a rotor of the ATP-driven rotary motor. Based on very recent high resolution X-ray crystal structure of V1 from E. hirae, Arai and co-workers have proposed that the interaction of the coiled-coil part of V1-D with the A subunit is essential for rotation of V1-D against A3B3 heterohexamer [26]. In their V1 structure, the globular-loop part of V1-DF is rarely in contact with the A3B3 hexamer. Our results, in which the anti-parallel coiled-coil structure is sufficient as a rotor in rotary ATPases, are consistent with their structural study.

Here we propose that V1-D, F1-γ and FliJ evolved from a common evolutionary origin. The JL protein derived from V1-D functioned as a rotor shaft (Figure 5A), strongly suggesting that FliJ maintains features of a prototype rotor. Because the rotor is composed of separate helical and globular subunits in V1/V1-γ, this rotor is an intermediate between the ancestral rotor and the F1-γ, which is a single protein containing both helical and globular domains. Therefore, we propose a possible scenario of evolutionary process of rotor apparatus in rotary motors (Fig. 6). In this scenario, F1-γ evolved from a gene fusion of genes encoding the ancestral V1-D and V1-F like proteins. However, because there is the sequence and structural diversity between V1-F and in the globular domain of F1-γ, we cannot exclude the possibility that V1-F is not ancestral gene of globular domain of F1-γ.

V1-F is structurally similar to CheY, a regulatory subunit of the bacterial flagellar motor, which functions to switch the direction of rotation [7]. A phylogenetic tree analysis using the Maximum Likelihood (ML) method indicated that V1-F is evolutionarily related to CheY rather than to the globular domain of F1-γ (Fig. 5S). The topology of V1-F is also more similar to that of CheY. (Fig. 5S). This indicates that V1-F and CheY share a common evolutionary origin. In contrast, the sequence and structural diversity between V1-F/CheY and in the globular domain of F1-γ can be explained. The central rotor apparatus of the two rotary ATPases contain significant structural differences. The V1 sector contains the funnel shaped C subunit (V1-c subunit), which serves as a socket for the DF rotor in V1 [1,19]. In contrast, F1-γ attaches directly onto the F1-c ring while the F1-e forms contacts with both the F1-γ and F1-c ring [1,3] (see Fig. 1a). It is possible that the differences in contact features between V1-F and the globular domain of F1-γ have promoted structural and sequence diversity of the rotors during the evolution of the two different ATPases.

In contrast to V1-DF and the F1-γ, there is little similarity between the other central rotor domain of the V1/V1-γ and F1/F1 (see Fig. 1a). The F1-e, composed of an N-terminal β-sandwich and a short C-terminal helix [1,3] shows neither sequence nor structural similarity to the equivalent V1-c (prokaryotic C subunit). Assuming that V1/V1-γ have conserved the ancestral form in the rotor apparatus, V1-c has been replaced with F1-e during evolution of F1/F1.

Supporting Information

Figure S1  Secondary structure prediction of the D subunit of T. thermophilus V-ATPase (a), the γ subunit of E. coli F1, (b) and FliJ of S. enterica (c) using PORTER: http://distill.ucd.ie/porter/. Predicted helical, sheet, and coiled regions are indicated by H, E, and C, respectively. For the γ subunit, both N- and C-terminal helices in the crystal structure (PDB: 1E79) are indicated by black lines. For the D subunit, assigned helices in the crystal structure (PDB: 3A5C) are indicated by black lines. Other regions are disordered in the crystal structure. For the FliJ, both N- and C-terminal helices in the crystal structure (PDB: 3AWJ) are indicated by black lines. The site for insertion of the F subunit in the D subunit is indicated with red characters. (DOC)

Figure S2  Analysis of ATPase complexes with gel-permeation chromatography. The mixture of A3B3 and FliJ was incubated at room temperature for overnight, and then applied onto Superdex-200 equilibrated with 20 mM MOPS (pH 7.0) and 150 mM NaCl. (a) SDS-PAGE analysis for A3B3J after successive gel-permeation chromatography. A3B3J was further applied onto gel-permeation chromatography, the resultant complex was analyzed by 15% SDS-PAGE (see lane marked 2nd). (DOC)

Figure S3  (a) Construction of the A3B3JL expression vector. (b) ATP hydrolysis activity of A3B3JL at the indicated [ATP]. (DOC)

Figure S4  (a) Construction of A3B3DFf expression vector. (b) ATP hydrolysis activity of A3B3DFf at the indicated [ATP]. (DOC)

Figure S5  Phylogenetic tree of V1-F (red circle), the globular domain of F1-γ (blue circles), and CheY (green circles). Open circles indicate genes from eukaryotes. Construction of the phylogenetic tree is described in the Methods section. (DOC)

Table S1  Structure and function of each rotor subunit of Flagellar protein export apparatus, V1/V1-γ, and F1/F1. (DOC)

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Author Contributions

Conceived and designed the experiments: KY KI. Performed the experiments: JK IT AN SN HU HK. Analyzed the data: JK IT AN SN HU HK. Contributed reagents/materials/analysis tools: KN. Wrote the paper: KY KI TM.
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