Autoinhibitory Regulation of TrwK, an Essential VirB4 ATPase in Type IV Secretion Systems

Alejandro Peña, Jorge Ripoll-Rozada, Sandra Zunzunegui, Elena Cabezón, Fernando de la Cruz, and Ignacio Arechaga

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Type IV secretion systems (T4SS) mediate the transfer of DNA and protein substrates to target cells. TrwK, encoded by the conjugative plasmid R388, is a member of the VirB4 family, comprising the largest and most conserved proteins of T4SS. In a previous work we demonstrated that TrwK is able to hydrolyze ATP. Here, based on the structural homology of VirB4 proteins with the DNA-pumping ATPase TrwB coupling protein, we generated a series of variants of TrwK where fragments of the C-terminal domain were sequentially truncated. Surprisingly, the in vitro ATPase activity of these TrwK variants was much higher than that of the wild-type enzyme. Moreover, addition of a synthetic peptide containing the amino acid residues comprising this C-terminal region resulted in the specific inhibition of the TrwK variants lacking such domain. These results indicate that the C-terminal end of TrwK plays an important regulatory role in the functioning of the T4SS.

Type IV secretion systems (T4SSs) translocate DNA and protein substrates across the cell envelope of bacteria to a wide number of eukaryotic and prokaryotic target cells (1, 2). Conjugative T4SSs are used by bacteria to mediate the transfer of DNA and proteins to conjugation recipient cells, resulting in the widespread transmission of antibiotic resistance genes among pathogenic bacteria (3, 4). Other T4SSs are used by several plant and human pathogens, such as Agrobacterium tumefaciens, Helicobacter pylori, Bordetella pertussis, Brucella suis, etc., to deliver virulence-related effectors to eukaryotic target cells (5–7).

T4SSs are macromolecular assemblies composed of 11 maturing pair proteins (VirB1 to VirB11) and a coupling protein (VirD4) that span inner and outer bacterial membranes. Three of these proteins, VirB11, VirB4, and VirD4, are ATPases that energize DNA and protein substrate transfer as well as pilus assembly (8–10). VirB4 proteins are the largest and most evolutionarily conserved proteins in T4SS (11) and are essential for virulence and for plasmid transfer (12). They contain Walker A and B NTP binding motifs and have been suggested to energize substrate translocation across the T4SS (13). VirB4 and VirB11 ATPases have been shown to influence the disposition of VirB2, the T4SS membrane-integrated pilin (14). Recently, we demonstrated that TrwK, the VirB4 homologue in the R388 conjugative system, is able to hydrolyze ATP in the absence of potential substrates (8). The ability to hydrolyze ATP by VirB4 proteins has been later confirmed for other VirB homologues (15) (16).

Little is known about the atomic structure of VirB4 proteins. Based on computer predictions using the atomic coordinates of the coupling protein TrwB of plasmid R388 as a template, a model of the C terminus (residues 426–787) of A. tumefaciens VirB4 was created (17). This finding suggested the possibility that VirB4 subunits might assemble as higher order homodimers and work as docking sites for substrate transport. TraB, the VirB4 homologue in the conjugative plasmid pKM101, also assembles in hexameric form in solution, although it is dimeric when extracted from the membranes (15). Recently, structural studies by small angle x-ray scattering (SAXS) of the membrane-extracted dimeric form of TraB have provided insights into the size and form of this protein (18).

Here, we used a bioinformatic approach to generate a model of the C-half domain of TrwK_R388. Secondary structure predictions of TrwK and TrwB revealed the presence of three α-helices in the C terminus that are conserved in all VirB4 proteins but absent in VirB. Therefore, we decided to generate truncated variants of TrwK where these α-helical structures were sequentially removed and their in vitro and in vivo properties were analyzed. Enzymatic analysis of these mutants revealed that removal of the C-terminal α-helices of TrwK induced a large increase in ATP turnover relative to wild-type TrwK. Interestingly, this ATPase increment could be specifically reverted upon addition of an exogenous peptide consisting of the amino acid residues Gly^{402}-Val^{423} of the C terminus of TrwK. The results suggest that the C-terminal end of VirB4 proteins plays a key functional regulatory role in the biological activity of T4SS.

EXPERIMENTAL PROCEDURES

Cloning of TrwK and Mutants—The DNA of R388 gene trwK was amplified by PCR and cloned into a pET3a expression vector (Novagen, Madison, WI). The trwK_1–772, trwK_1–787, and trwK_1–801 mutants were generated by PCR using the same forward primer (5’-TATCATATG GGCGCAATTGAAA-
TCCG) and the reverse primers 5′-TTTGGATCT-CAAGTCTACCATCGA, 5′-AAAGATCCCGGTGCCGAC- CAGCAC and 5′-TTTGGATCCGACTCGAATAAGT, respectively. The relevant DNA fragments were digested with NdeI and BamHI restriction enzymes and ligated into the corresponding sites in the MCS of vector pET3a (or in vector pET28a in the case of TrwK_1–772). Plasmid DNAs were used to transform Escherichia coli strain C41(DE3) (19).

Antibodies and Reagents—A polyclonal antibody recognizing TrwK was raised by injecting purified TrwK mixed with incomplete Freund’s adjuvant in New Zealand White rabbits. The primary anti-TrwK antiserum was affinity purified using antigen immobilized on nitrocellulose filters as described in Ref. 20. Donkey anti-rabbit IR-Dye 800 CW was purchased from LI-COR Biosciences, GE). TrwK-containing fractions were collected in two steps, applied to a HisTrap HP (5 ml) column (Amersham Biosciences, GE). After incubation in ice for 30 min, an equal volume of deionized water with 5 mM EDTA was slowly added. The mixture was sonicated and centrifuged for 10 min at 50,000 g and then at 200,000 g (10 min) to eliminate any possible inclusion body. Finally, membranes were obtained by centrifugation for 30 min at 220,000 × g. The pellet (membrane fraction) was resuspended in a buffer consisting of 50 mM Tris, pH 7.6, 0.5 mM PMSF, 5 mM EDTA, 2% SDS, and clarified by sonication and centrifugation at 220,000 × g. Protein samples were run in a SDS-PAGE, transferred to a nitrocellulose filter and incubated with anti-TrwK rabbit antiserum. Images were obtained after incubation with an IRDye anti-rabbit IgG (goat) antibody conjugate using an Odyssey scanner (Li-Cor Biosciences).

ATP Hydrolysis Assays—TrwK_1–772, TrwK_1–877, and TrwK_1–801 ATPase activity was measured by a coupled enzyme assay (24). To analyze ATP concentration dependence, TrwK mutants were incubated in 150 μl of ATP assay buffer, consisting of 50 mM Pipes pH 6.45, 75 mM potassium acetate, 5% (w/v) glycerol, 10 mM magnesium acetate, 1 mM potassium chloride, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, 60 μg/ml pyruvate kinase, 60 μg/ml lactate dehydrogenase, and 0.1–10 mM ATP. Reactions were started by the addition of TrwK. Activity was measured by the decrease in NADH absorbance at 340 nm for 15 min at 37 °C in a UV-1603 spectrophotometer (Shimadzu).

Molecular Modeling—An atomic model of TrwB was generated by molecular threading using the protein homology and recognition engine Phyre (25). A model of the C-half of TrwK (residues 413–772: Fig. 1) was obtained using the atomic coordinates of TrwB (1e9r.pdb) (26) as template. Based on the hexameric structure of TrwB, a model of TrwK hexamer was built using the UCSF Chimera package (27).

RESULTS

Structural Comparison between TrwB and TrwK Proteins—A bioinformatic analysis of full-length TrwK amino acid sequence (823 aac) was used to generate a model of the C-half domain of the protein (residues 413–772: Fig. 1). The model created by molecular threading of TrwK sequence on
a template consisting of the atomic structure of TrwB coupling protein (1e9r.pdb) (26) is very similar to a previously reported model of a TrwK homologue, the VirB4 protein of *A. tumefaciens* (17). In both cases, the template was TrwB, an hexameric integral membrane protein encoded by the conjugative plasmid R388 that is involved in the movement of the relaxosome DNA-binding complex toward the T4SS (28). TrwB belongs to the RecA protein-like family (26) and it displays a DNA-dependent ATPase activity (10, 29, 30). Likewise, TrwK, also encoded by plasmid R388, is also able to hydrolyze ATP (8).

Comparison between the atomic model of TrwB and the C-terminal half of TrwK revealed a striking structural similarity (Fig. 1). The main differences among both structures were found in their C termini. The structure of TrwB (Fig. 1, salmon) finishes by an unstructured tail. On the other hand, the model of TrwK ends in residue Glu-772. It was not possible to model the remaining 51 amino acid residues at the C terminus of TrwK since the template (TrwB) does not have equivalent residues at this position. Secondary structure prediction (31) for both proteins suggests that TrwK contains three \( \alpha \)-helices at its C-end that are absent in TrwB (Fig. 2). Interestingly, this C-terminal domain is highly conserved in all VirB4 homologues (Fig. 2 and supplemental Fig. S1). Therefore we decided to investigate if the \( \alpha \)-helices which are present in TrwK but absent in TrwB could play a role in

**FIGURE 1. Molecular modeling of TrwK.** A model of the C-half of TrwK (cyan) comprising amino acid residues Ala413 to Thr772 was generated by molecular threading using the atomic structure of the coupling protein TrwB (salmon, PDB code: 1e9r) as a template. The monomeric model of TrwK was assembled as an hexamer by fitting its coordinates with the hexameric TrwB (left) using Chimera software. Arrows pointing at the C terminus of both, the TrwK model and TrwB structure, indicate that these C-ends are formed by unstructured tails. Images were rendered with PyMol.

**FIGURE 2.** VirB4 proteins contains a C terminus consisting of three \( \alpha \)-helices that are absent in TrwB. Secondary structure predictions for TrwB and TrwK (top panel) reveals the presence in TrwK of three \( \alpha \)-helical structures that are absent in TrwB. Sequence alignment of a representative number of VirB4 proteins (bottom panel) shows that this C-terminal region is highly conserved in this family of proteins. Amino acids with 100% identity are represented by dark gray boxes. Alignment was performed using T-Coffee (55) and represented using Jalview (56).
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FIGURE 3. Isolation of TrwK-truncated mutants. Top, schematic representation of TrwK and the 3-helical structures of the C-terminal end indicated in Fig. 2. The C-terminal 3-helices were sequentially removed, thus generating three different truncated forms of the protein. Bottom, SDS-PAGE of purified proteins after gel filtration. Lane a, full-length TrwK (93 kDa); lane b, TrwK 1–801 (91 kDa); lane c, TrwK 1–787 (89.8 kDa); lane d, TrwK 1–772 (88 kDa).

FIGURE 4. Kinetic analysis of ATP hydrolysis by TrwK C-terminal-truncated variants. Isothermal titration response of TrwK and mutant variants as a function of ATP concentration monitored by a coupled enzyme assay. Data were fitted to a Hill equation in each case. The different data sets represent TrwK full-length (filled circles), TrwK 1–772 (gray filled triangles), TrwK 1–787 (gray filled squares), and TrwK 1–801 (open diamonds).

TABLE 1

| Mutant variant | V_{max} (nmoI min^{-1} mg^{-1}) | K_{app} (mM) |
|----------------|---------------------------------|--------------|
| TrwK           | 48.1 ± 1.9                      | 0.41 ± 0.07  |
| TrwK 1–772     | 467.8 ± 54.4                    | 0.49 ± 0.03  |
| TrwK 1–787     | 476.2 ± 22.6                    | 0.60 ± 0.16  |
| TrwK 1–801     | 591.5 ± 23.7                    | 0.38 ± 0.08  |

Values of \( V_{max} \) were calculated by using the modified representation of the Hill equation \( \log (\frac{v}{V_{max} - v}) \) vs \( \log (ATP) \). Values of \( K_{app} \) were obtained by direct data fitting to a Hill equation.

the different biological functions suggested for both proteins (effector transport/pilus assembly and DNA translocation, respectively).

Removal of the 3-helices at the C Terminus of TrwK Increases the in Vitro ATPase Activity—Based on the structural homology of the C-half of TrwK with the coupling protein TrwB we designed truncated variants of TrwK where the three 3-helices at the C-end were sequentially removed. These mutants comprised amino acid residues 1–801, 1–787, and 1–772, respectively. Each mutant was purified (Fig. 3) and tested for its ability to hydrolyze ATP in the same conditions than wt TrwK. As observed in Fig. 4, removal of the C-terminal helices of TrwK resulted in a dramatic increase of the rate of ATP hydrolysis. The \( V_{max} \) of ATP hydrolysis rose from 48 nmoI min^{-1} mg^{-1} for wt TrwK to 467, 476, or 591 nmoI min^{-1} mg^{-1} for TrwK 1–772, 1–787, and 1–801, respectively. Interestingly, the \( K_{m} \) did not change significantly (Table 1), suggesting that removal of these regions did not affect the conformational structure of the nucleotide binding site (and thus its nucleotide binding affinity).

Addition of a Complementing Synthetic Peptide Reverts the ATP Turnover Increase of TrwK-truncated Variants—The increase in the ATP turnover upon removal of the C-terminal domain of TrwK opened the question if the 3-helical structure of this domain was the only requirement to exert the observed regulatory function or, on the contrary, the specific amino acid sequence of this domain was essential for such a function. Alignment of 25 VirB4 sequences (supplementary Fig. S1), belonging to distant branches of the VirB4 phylogenetic tree (11), shows a high degree of conservation in the C-terminal end (residues Gly^{802}–Val^{823} in TrwK), with a consensus sequence G-D/X-D/X-P-X-W-L/I-P/X-X-F/Y. Based on this homology we designed a synthetic peptide comprising the amino acids Gly^{802} to Val^{823} of TrwK, thus complementary to the truncated variant TrwK 1–801. The ATPase activity of all TrwK truncated variants was inhibited upon addition of this peptide to the enzyme reaction (Fig. 5). In contrast, wt TrwK, that already contains this sequence covalently bound, was unaffected. The inhibitory effect of TrwK 1–802–823 peptide did not change significantly the \( K_{m} \) value (supplementary Fig. S2), and it was specific for TrwK, as we found that the ATPase activity of TrwB and TrwD, the other two essential ATPases in T4SS, was unaffected by this peptide (data not shown). A peptide with a different amino acid sequence, equivalent to the last 20 amino acids of the R388 relaxase, TrwC, (residues Pro^{947} to Arg^{966}, see “Experimental Procedures”), used as a control, did not affect ATP turnover in any case (Fig. 5).

**TrwK C-terminal-truncated Mutants Are Unable to Complement the in Vivo Function of R388::\( \Delta trwK**—The effect of truncating C-terminal sequences of TrwK on the rate of ATP hydrolysis suggested that this region could play an important role in the regulation of TrwK function. Therefore, we studied the effect of removing this C-terminal region of TrwK on bacterial conjugation. Donor cells carrying a modified version of the conjugative plasmid R388 with a knock-out mutation in gene \( trwK \) (R388::\( \Delta trwK \)) were transformed with plas-
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FIGURE 5. Inhibition of the ATPase activity of TrwK mutant variants by addition of a synthetic peptide corresponding to the C terminus. ATPase activity of wt TrwK (filled circles), TrwK1–772 (gray filled triangles), TrwK1–787 (gray filled squares), and TrwK1–801 (open diamonds) incubated with a synthetic peptide comprising amino acids Pro947–Arg966 of TrwC (open squares), indicating that the expression of these mutants was not affecting the function of TrwK in R388 conjugation. Interestingly, experiments where both wt TrwK and the mutant variants were co-expressed within the donor cells, suggesting that either these truncated variants or wt TrwK were similar in all cases, indicating that the expression of these mutants was not affecting the function of TrwK in R388 conjugation.

TABLE 2
Conjugation frequencies of R388 and trwK mutants in the presence of complementing proteins

| Plasmids in donora | Variant of TrwK in donor | Conjugation frequenciesb |
|--------------------|-------------------------|--------------------------|
| R388               | TrwK                    | 4.76 × 10⁻¹               |
| pSU4133            | R388::ΔtrwK              | <1 × 10⁻⁷                 |
| R388 + pET3a_trwK  | trwK                    | 2.21 × 10⁻²               |
| R388 + pET3a_trwK1–801 | trwK1–801               | 1.84 × 10⁻²               |
| R388 + pET3a_trwK1–787 | trwK1–787              | 1.37 × 10⁻²               |
| R388 + pET3a_trwK1–772 | trwK1–772              | 1.21 × 10⁻²               |
| pSU4133 + pET3a_trwK | trwK                   | 2 × 10⁻⁵                  |
| pSU4133 + pET3a_trwK1–801 | trwK1–801              | <1 × 10⁻⁷                 |
| pSU4133 + pET3a_trwK1–787 | trwK1–787              | <1 × 10⁻⁷                 |
| pSU4133 + pET3a_trwK1–772 | trwK1–772              | <1 × 10⁻⁷                 |

a Donor cells (E. coli K12 strain DH5α) carrying the plasmids shown in the first column were mated with strain UB1637.
b Transfer frequency of transconjugants selected in streptomycin and trimethoprim plates.

DISCUSSION

In a previous report we demonstrated that TrwK, a VirB4 homologue of the conjugal plasmid R388 was able to hydrolyze ATP (8), thus settling down an old debate on the ability of these proteins to perform such a task. These results were later confirmed in similar studies carried out with TraB, the VirB4 homologue of the conjugal plasmid pKM101 (32) and TraE of Aeromonas veronii (16). To gather further understanding of the role of ATP hydrolysis by VirB4 proteins in effector transport and/or pilus assembly, the study of its activity and regulation is essential. In the work presented here we have identified a region of TrwK that could be crucial in the regulation of its activity.

A previous model of the C-half of A. tumefaciens VirB4 (17) suggested that these proteins assemble as hexamers much like VirD4 (26) and VirB11 (33). In addition, the oligomeric state in solution of TraB of pKM101, has been shown to be predominantly hexameric, although it is dimeric when extracted from membranes (15). The A. tumefaciens VirB4 model, obtained by a bioinformatic approach, was generated using the atomic structure of TrwB, the R388 ATPase involved in DNA transfer, as a template (26). Here, we used a similar approach to create a model of the C-half of TrwK (residues 413–772). However, it was not possible to extend this model to the last 51 amino acid residues of TrwK since the template, TrwB, lacked this C-terminal region. Secondary structure predictions revealed that this C-terminal region, which is present in TrwK but absent in TrwB, consisted of three α-helices. These terminal α-helical structures seem to be a general feature in all VirB4 proteins.

Sequential removal of the three α-helices of the C-terminal domain of TrwK resulted in mutant variants with ATP hydrolysis rates over 10 times higher than those obtained with wt TrwK. The remaining kinetic parameters (Keq(app) and Hill coefficient) were not affected by these mutations suggesting that these deletions did not induce conformational changes in the nucleotide binding site. In contrast, these mutants were unable to complement the in vivo activity of TrwK in bacterial conjugation experiments, indicating that this C-terminal domain must play an essential role in conjugation. Interestingly, experiments where both wt TrwK and mutant variants were co-expressed within the donor cells, showed that expression of the mutants was not affecting the function of TrwK, suggesting that either these truncated variants are unable to associate with the wild-type protein or, if they do so, the absence of the C-terminal region in one monomer does not affect the activity of the putative heterohexamer.
The results shown here suggest that the C-terminal domain of TrwK acts as an autoinhibitory region that prevents futile ATP hydrolysis in the resting state. Interaction with specific effectors and/or with other partners of the transport machinery would produce a conformational change in this C-terminal region, resulting in stimulation of ATP hydrolysis. Similar autoinhibitory mechanisms have been found in a large variety of proteins, including kinesin (34, 35), Hsp90 (36, 37), protein kinases (38–40), Ca-ATPase (41–45), and other P-type ATPases (46–49). Moreover, in some of these cases, synthetic peptides consisting of amino acid sequences corresponding to the respective regulatory domains have been used to characterize the inhibitory mechanism of these proteins (41, 44, 45, 50). In some other cases, ATPase inhibition is carried out by a peptide encoded by a different gene, so regulation is controlled externally. That is the case of inhibition is carried out by large conformational changes of the C-terminal subunit (54), thus reg-...
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