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Correlation between the structure and biochemical activities of FtsA, an essential cell division protein of the actin family

Manuel Sánchez, Alfonso Valencia[1], María-José Ferrándiz, Chris Sander[1] and Miguel Vicente[2]

Departamento de Biología Celular y del Desarrollo, CIB, Consejo Superior de Investigaciones Científicas, C/Velázquez 144, 28006 Madrid, Spain and [1]Protein Design Group, European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg, Germany
[2]Corresponding author

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Cell division protein FtsA, predicted to belong to the actin family, is present in different cell compartments depending on its phosphorylation state. The FtsA fraction isolated from the cytoplasm is phosphorylated and capable of binding ATP, while the membrane-bound form is unphosphorylated and does not bind ATP. A variant of the protein FtsA102, in which the nucleotide binding site was destroyed by mutagenesis of a highly conserved residue predicted to be needed for the binding, does not bind ATP. Another variant, FtsA104, cannot be phosphorylated because the predicted phosphorylatable residue has been replaced by a non-phosphorylatable one. This protein although unable to bind ATP *in vitro*, is able to rescue the reversible *ftsA2*, the irreversible *ftsA3* and, almost with the same efficiency, the *ftsA16* amber alleles. Consequently, phosphorylation and ATP binding may not be essential for the function of FtsA. Alternatively they may have a regulatory role on the action of FtsA in the septator.

Key words: ATP binding/cell division/*Escherichia coli*/FtsA/phosphorylation

Introduction

Control of cell division in bacteria is a complex process that has been extensively analysed at the morphological and genetic levels. FtsA is one of the key proteins required for septation in *Escherichia coli* that is coded by a gene found in the main division and cell wall gene cluster (*dcw* cluster) at 2.5 min of the genetic map (Ayala *et al.*, 1994). FtsA is present in low amounts [150 molecules per cell (Wang and Gayda, 1992)], but it is essential for cell division in *E.coli*. Further, *Bacillus subtilis* lacking FtsA divides less efficiently and cannot sporulate (Beall and Lutkenhaus, 1992). The action of FtsA occurs late in the division cycle (Tormo *et al.*, 1980) and requires DNA replication for its proper function (Tormo *et al.*, 1985a). It forms part of the structure of the septum where an altered form, FtsA3 produced at 42°C, creates a septation block, even at the permissive temperature (Tormo and Vicente, 1984), that needs the Lon protease for its removal (Dopazo *et al.*, 1987; Pla *et al.*, 1993). Genetic evidence proves that FtsA interacts with PBP3, the penicillin binding protein essential for septum formation (Tormo *et al.*, 1986).

Using a novel sequence-pattern search method it was proposed that FtsA belongs to the actin, Hsc70 and hexokinase family, whose members contain an ATP binding site (Bork *et al.*, 1992). We report here evidence supporting that indeed FtsA is able to bind ATP, an activity present only when it is phosphorylated. The intracellular localization of FtsA and its ATP binding capacity are both related to its phosphorylation state. These findings can be correlated with genetic and physiological observations on this cell cycle protein and suggest some possibilities for explaining its action in cell division.

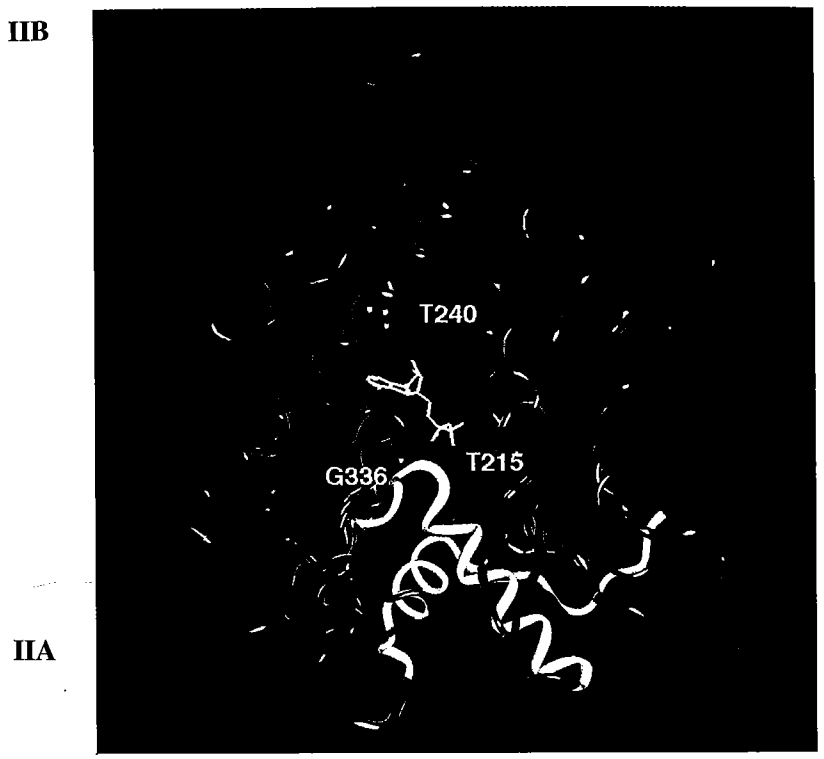
Results

Predicted structural model of FtsA and its relationship with the actin family

Structural homologies between FtsA and two other cell cycle proteins, MreB and StbA, have been predicted and relate them to a family of ATP binding proteins that include DnaK, Hsc70, actin and hexokinase (Bork *et al.*, 1992). The relationship between the FstA and the Hsc70 family is more clear through the FtsA sequence of *B. subtilis* of which ~30% is similar to the *E.coli* sequence. A 3-D model of *E.coli* FtsA has been constructed based on the multiple sequence alignment with the family. The multiple sequence alignment contains the previously reported homologous regions as detected by the pattern matching approach. The FtsA protein may then contain an overall similar topology and a quite similar ATP binding as the ATPase domains found in actin, Hsc70 and hexokinase. The homologous regions comprise seven segments corresponding to regions that are in close contact with ATP (Figure 1) (Bork *et al.*, 1992; Holmes *et al.*, 1993). These regions take up a substantial part of the sequence and belong to the regions conserved in all members of the family.

A phosphorylated form of FtsA is found in wild type cells

Two of the biochemical activities of the FtsA protein predicted in the model, i.e. the presence of phosphorylated forms and the ATP binding ability, have been tested. As predicted by analogy with DnaK, the *E.coli* Hsc70 homologue member of the family, FtsA may be present in different phosphorylation states (Rieul *et al.*, 1987; McCarty and Walker, 1991). FtsA from a wild type extract is resolved in two forms of different pIs by 2-D electrophoresis (IEF, SDS–PAGE), Figure 2A (Amster-Choder and Wright, 1990). To test whether the form with lower pI corresponds to a phosphorylated protein the same experiment was repeated but treating the extracts with alkaline phosphatase before the electrophoretic separation.



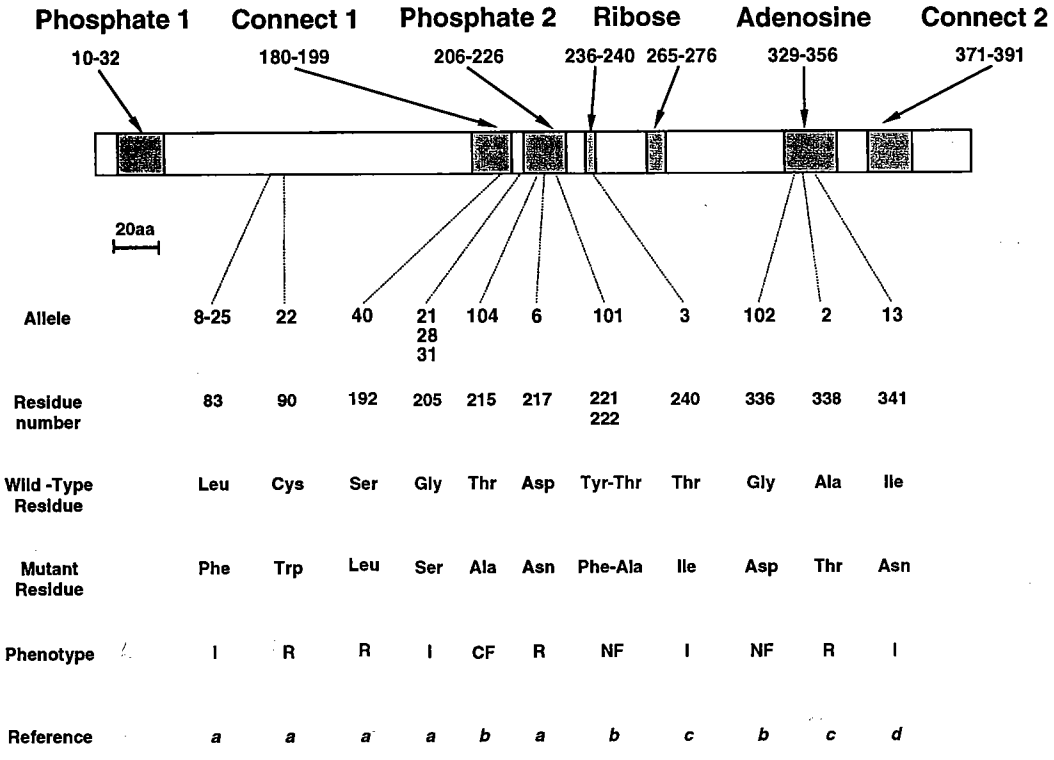


Fig. 1. Proposed structure of the FtsA protein. (**A**) A 3-D model was built for FtsA by homology with Actin/Hsc70/hexokinase family. The protein is divided into two large domains: I (right) and II (left). Each one of them comprises two subdomains: IA and IIA (down), and IB and IIB (up). The ATP molecule has been outlined in white within the binding site. The sequence motifs homologous to the actin family (Bork *et al.*, 1992; Holmes *et al.*, 1993) are marked, the two phosphate sequence motifs (red), the adenosine sequence (pink), the two ribose sequence motifs (blue) and the two connect ones (yellow). Three residues, corresponding to those altered in *ftsA3*, *ftsA102* and *ftsA104* (see below) have been highlighted with a CPK representation. (**B**) Location of *ftsA* alleles in the protein map. Sequence motifs are labelled as in (A), numbers below the domains show the residue number boundaries for each one. The allele number, its position in the molecule, the nature of the change and the phenotypic classification of each mutation are shown. I, irreversible phenotype; R, reversible phenotype (Tormo *et al.*, 1985b); NF, non-functional (the allele fails to complement *ftsA2* or *ftsA3*); CF, conditionally functional (the allele complements *ftsA2* and *ftsA3* but only partially *ftsA16*, an amber allele). (*a*) Robinson *et al.*, 1988; (*b*) this work; (*c*) Sánchez *et al.*, 1994; (*d*) Robinson *et al.*, 1991.

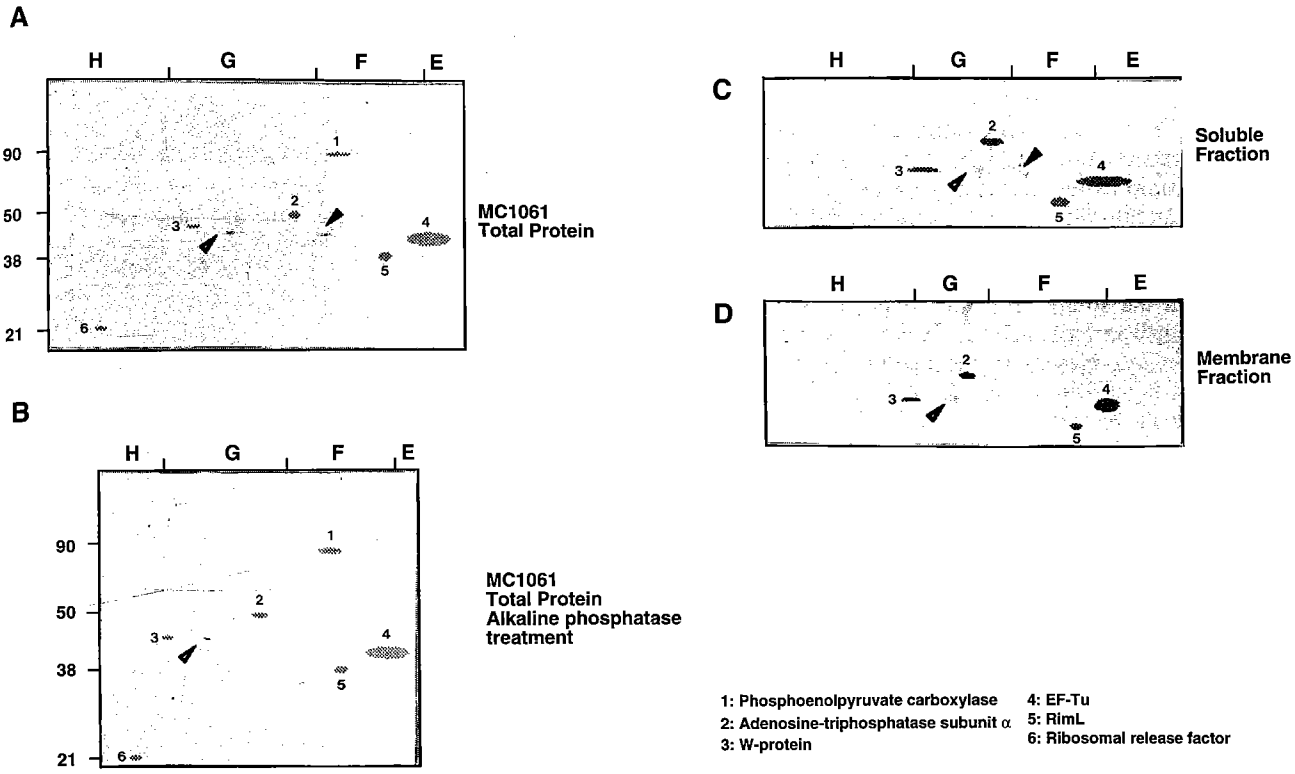


Fig. 2. Differential intracellular localization of differently phosphorylated FtsA$^+$ forms. All the frames show 2-D gel blots immunostained with anti-FtsA antiserum (see Materials and methods). (**A**) Proteins of the total cell extract from wild type *E.coli*. Arrowheads (white for the higher pI and black for the lower) mark the FtsA spots. (**B**) The same as (A) but the sample was incubated with alkaline phosphatase before loading. (**C**) Untreated soluble cell fraction. (**D**) Untreated membrane cell fraction. The four blots were stained with Ponceau red before immunostaining to reveal the total protein pattern. Proteins were identified accordingly to the protein map of Neidhart (Phillips *et al.*, 1987). The pH zones as defined by Neidhardt and the molecular weight in kDa are shown in the horizontal and vertical scales respectively. Numbered filled outlines show the position in the Ponceau red stain of some easily identifiable proteins that are named at the bottom of (D).

The results in Figure 2B show that the lower pI (phosphorylated) form is absent from the phosphatase-treated sample. When wild type FtsA is overproduced, additional forms of different pIs are observed (results not shown). By analogy with DnaK the Thr215 residue, located in the phosphate 2 loop in the vicinity of the gamma phosphate of ATP, could be the main phosphorylation substrate. In Hsc70 this residue is very close to the phosphates, which makes it a very likely substrate for autophosphorylation; actin, which is not a phosphorylation substrate, contains valine at the equivalent position (Flaherty *et al.*, 1990; McCarty and Walker, 1991; Bork *et al.*, 1992). The lower pI form is absent from overproduced FtsA104 (Figure 3B), in which a nonphosphorylable Ala replaces Thr215 (Figure 1B). To find whether this protein is active in cell division complementation studies of strains containing *ftsA2*, *ftsA3* or *fts16* have been done. A total of 100 colonies of each strain transformed with plasmid pMFV3 containing *ftsA104* have been checked. All colonies obtained from *ftsA2* and *ftsA3* are viable both at 30 and 42°C and were normal sized, except for 10% of longer or filamentous cells. Of the 100 *ftsA16* transformants all were viable at both temperatures but 15 colonies were filamentous at 42°C, the remainder were normal-sized cells at this temperature. The FtsA104 protein can then complement both the *ftsA2* (reversible) mutation and the *ftsA3* (irreversible) mutation and it can rescue, with almost the same efficiency, the *ftsA16* (amber) allele. A non-functional variant, FtsA101, in which the Tyr221−Thr222,

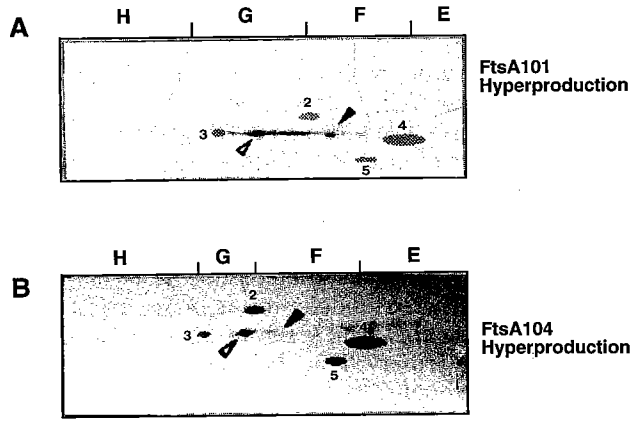


Fig. 3. Identification of phosphorylated forms of FtsA101 and FtsA104. Immunostained blots of 2-D electrophoretic separations of the total cell fraction from VIP195, a FtsA101 overproducing strain (**A**), and total cell fraction from VIP268, a FtsA104 overproducing strain (**B**). MVJ2 antisera was used to stain FtsA101, and MVM1 antisera for FtsA104. A control of a similar preparation of VIP194 (FtsA$^+$) was run in parallel for each case (not shown). Arrowheads, numbered filled outlines and pH zones as in Figure 2.

at a previously postulated phosphorylation site (Robinson *et al.*, 1987), has been changed into Phe221−Ala222, (Figure 1B) was analysed. Its resolution by 2-D electrophoresis showed a pattern similar to the overproduced wild type protein, except that the amount of the main acidic spot was slightly lower (Figure 3A). These results

rule out the proposed homology of FtsA with yeast cdc2 at this site (Robinson *et al.*, 1987).

Correlation between the phosphorylation state of FtsA and its intracellular localization

Overproduced FtsA has been described to be located in the cytoplasmic membrane facing towards the cytoplasm (Pla *et al.*, 1990). We find that, when produced at normal levels, FtsA is present both in the soluble fraction and in the cytoplasmic membrane. Results in Figure 2C and D indicate that the soluble fraction contains both forms of the protein in approximately equivalent amounts and the quantity of FtsA present in the cytoplasm of a normal *E.coli* cell may account for 60% of the total FtsA cell contents. In the membrane we can detect only the non-phosphorylated form. Due to the resolution limits of our experimental procedures and to the low amounts of FtsA protein in the cell (some 150 molecules; Wang and Gayda, 1992), we can estimate that 30% of the total amount of FtsA is found in the unphosphorylated state in the membrane. As no phosphorylated material appears in the membrane fractions we can conclude that the maximum amount of phosphorylated FtsA present in the membrane, if any, is not higher than 10% of that total.

The ability to bind ATP is only found in the phosphorylated form of FtsA

The ATP binding activity of FtsA has been tested taking advantage of its ability to be retained by ATP–agarose. Results in Figure 4A indicate that FtsA is tightly retained by ATP–agarose. The specificity of the binding was confirmed by its loss when the extracts are pre-incubated with ATP (Figure 4B) and by the absence of significant binding to AMP–agarose (Figure 4C). These results confirm the main prediction of the structural model. The ATP binding ability of FtsA differs nevertheless from that of DnaK, another prokaryotic member of the family. The DnaK binding to ATP–agarose is immediate, while the interaction of FtsA takes 90 min in the same conditions. In addition DnaK can be readily eluted from the ATP–agarose by 5 mM ATP, while FtsA remains bound.

A more specific prediction is that a change in the Gly336 residue should affect the ATP binding activity. To test this prediction a mutation was constructed *in vitro* that substituted the Gly336 residue with Asp (Figure 1B). This residue, although not being a site of direct contact with ATP, is totally conserved in the family and plays an essential role in forming the loop structure of the adenosine binding domain (Bork *et al.*, 1992; Holmes *et al.*, 1993). Its change into Asp should destroy the adenine binding site. The mutated protein, FtsA102, does not bind to ATP–agarose columns (Figure 4F) and it is unable to complement the *ftsA* amber, the *ftsA2*, and *ftsA3* mutations. We cannot conclude if the loss of activity of this protein is directly due to its inability to bind ATP because the structural change introduced in the loop by the Asp is not conservative and may have affected other structural features.

As we have shown above, FtsA is present in two main different forms with different intracellular localization. The membrane fraction contains mostly unphosphorylated FtsA. To test the effect of the FtsA phosphorylation state on its ATP binding activity the soluble fraction from the

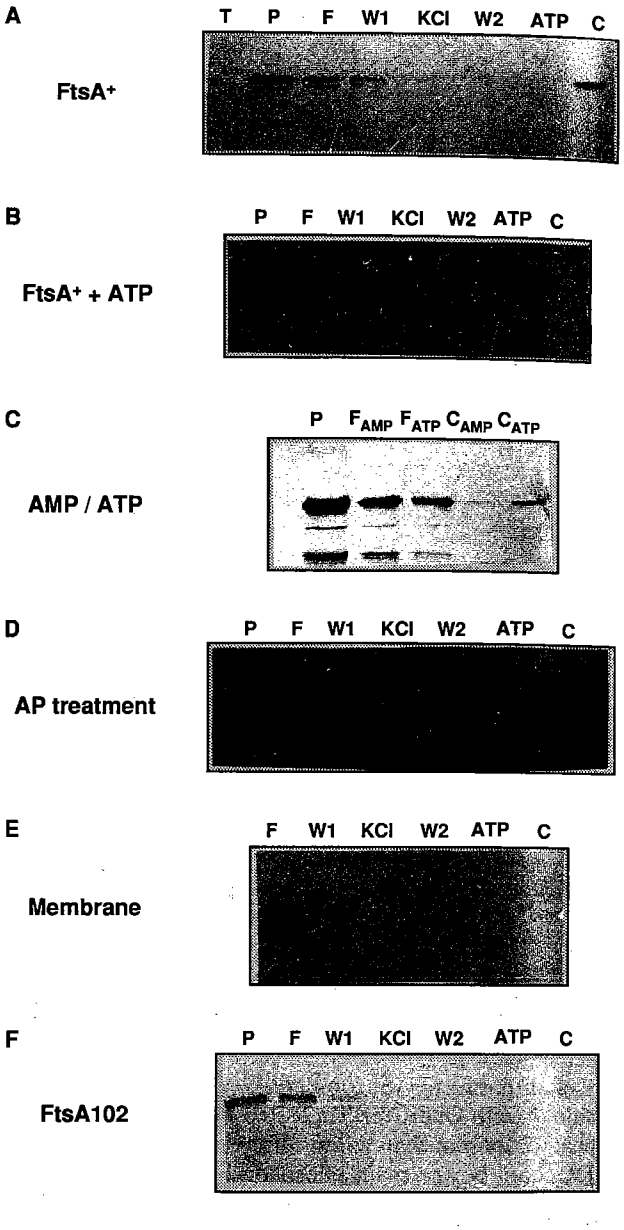


Fig. 4. Ability of FtsA to bind ATP. (**A**) Elution pattern of the soluble fraction of FtsA$^+$ overproduced in strain VIP194 that was run through an ATP–agarose column. Samples from each wash and from the columns were loaded into a 12% SDS–acrylamide gel and immunobloted with MVJ2 anti-FtsA polyclonal antibody (see Materials and methods). Each lane contained material corresponding to cells present in 0.25 units of OD_{600}. T, total cell extract from MC1061 used as marker; P, 35% ammonium sulphate fraction from the VIP194 soluble fraction; F, flow through the ATP–agarose column of the same fraction as in P; W1, first wash with imidazol buffer; KCl, wash with 1 M KCl imidazol buffer; W2, second wash with imidazol buffer; ATP, wash with 10 mM ATP imidazol buffer; C, material retained by the ATP–agarose column after all the treatments. (**B**) As (A) but the sample was pre-incubated with 10 mM ATP before its application to the column. (**C**) Retention of FtsA$^+$ in AMP–agarose column and ATP–agarose columns. P, 35% ammonium sulphate fraction from the VIP194 soluble fraction; F_{AMP}, flow through the AMP-agarose column; F_{ATP}, flow through the ATP–agarose column; C_{AMP}, material retained by the AMP–agarose column; C_{ATP}, material retained by the ATP–agarose column. (**D**) As (A) but the sample was previously run through an immobilized alkaline phosphatase column before application to the ATP–agarose column. (**E**) As (A) but using FtsA$^+$ from the membrane fraction of the overproducing strain VIP194. (**F**) As (A) but using the soluble fraction of an FtsA102 overproducing strain.

wild type was treated with alkaline phosphatase before being applied to the ATP–agarose column. Dephosphorylated FtsA does not bind to ATP–agarose (Figure 4D). Moreover, FtsA obtained from wild type membrane fractions and overproduced FtsA104, that are not phosphorylated, do not bind to the ATP–agarose column (Figure 4E and results not shown, respectively). The requirement for phosphorylation of FtsA to be proficient in ATP binding deviates from the behaviour observed in DnaK and casts doubts on the role of ATP in autophosphorylation of the Thr215 residue.

Discussion

The FtsA protein has properties that correspond to those predicted by the structural model for the actin family

The structural model predicted for FtsA has been confirmed in two aspects concerning its biochemical properties, namely that it is present in different phosphorylation states and that it binds ATP. We also find a correlation between the phosphorylation state of FtsA, its ability to bind ATP and its intracellular localization. The complementation pattern of FtsA104, able to rescue efficiently two point mutations but less efficiently an amber one, suggests that the FtsA function may involve a homomolecular interaction. Interaction with other proteins is a property present in other members of the actin family (Holmes *et al.*, 1993). FtsA differs from other members of the family: its binding to ATP proceeds very slowly but it is rather tight and it lacks the ability to bind ATP when it is unphosphorylated. In contrast DnaK binding of ATP proceeds very rapidly and is readily reverted by free ATP. When unphosphorylated, DnaK retains the ATP binding ability (McCarty and Walker, 1991). The role of ATP binding to FtsA should be further investigated to find whether it is a substrate or an effector.

Correlation between phosphorylation states and intracellular localization of FtsA

A working model for the role of FtsA in cell division can be advanced: the FtsA protein would be phosphorylated in the cytoplasm where it would also bind ATP. We have no definite evidence yet to conclude if phosphorylation could either prevent association to, or promote dissociation from the cytoplasmic membrane. The observation that overproduced FtsA104 is found both in the cytoplasmic and membrane fractions suggests that phosphorylation and ATP binding are not required for the association of the protein to the membrane. Further studies on the localization of FtsA104 when produced from a single copy gene are required to corroborate this point. Anyway, as phosphorylated $FtsA^+$ cannot be detected in the membrane fraction, dephosphorylation should occur before or shortly after the association. Once in the membrane, FtsA would exert a mechanical and/or catalytic role on other elements of the septator (Vicente *et al.*, 1991). A mechanical role for FtsA, working in association with the tubulin analogue GTPase FtsZ (de Boer *et al.*, 1992; RayChaudhuri and Park, 1992; Mukherjee *et al.*, 1993; Shapiro, 1993), could also be assumed for the terminal stages of division, when the constriction of the cross-wall would create a geometry that would allow interactions between scarce proteins.

An increase in the dephosphorylated form of FtsA after alkaline phosphatase treatment, relative to the phosphorylated one, although expected, was not observed (Figure 2A and B). This could be explained if the stability of the protein dephosphorylated *in vitro* were different from the unphosphorylated form found *in vivo*. There are similar cases reported in the literature (Amster-Choder and Wright, 1990), in which alkaline phosphatase treatment of BglG does not result in an increase of the dephosphorylated form of the protein.

Possible roles for the ATP binding ability of FtsA

As FtsA104, which cannot be phosphorylated and does not bind ATP, can complement different *ftsA* alleles, including a nonsense one, it seems that phosphorylation and ATP binding are not essential for FtsA activity in septation. Once its role in the membrane has been performed, a lasting mechanism for FtsA inactivation can be predicted from the immediate cessation in cell division observed at the restrictive temperature in strain OV16, that contains an amber *ftsA* allele in a temperature-sensitive suppressor background (Donachie *et al.*, 1979). A constraint in the hinge connecting the two FtsA domains (Bork *et al.*, 1992; Holmes *et al.*, 1993) would cause a tight nucleotide binding. If, once in the membrane, FtsA were capable of undergoing a nucleotide exchange mediated by another septation protein, the trapping of the nucleotide could account for such an inactivating mechanism. The inability of the membrane-associated FtsA to bind to ATP–agarose columns could be due to an absence of binding capacity or alternatively to an irreversible nucleotide saturation of the binding pocket, perhaps associated to changes in the phosphorylation state. A plausible speculation, that different phosphorylation and ATP binding states form part of a regulatory mechanism, could be advanced. As *ftsA* expression during the cell cycle is continuous (Garrido *et al.*, 1993), and the effective period for FtsA action is discontinuous (Tormo *et al.*, 1980), phosphorylation and ATP binding could have a role in preventing any premature action of the protein on the maturing septum.

Interaction between FtsA and other proteins of the septator

Binding of ampicillin to PBP3 is defective in cells containing thermally inactivated FtsA3 (change of Thr240 to Ile, Figure 1B) (Tormo *et al.*, 1986). This Thr is located close to the O 2′ ribose of the ATP that, together with the base binding regions, form the hydrophobic pocket for the nucleotide. The Thr240 forms part of the IIb subdomain and is very close to the interface with subdomain IIa. Theoretical analysis of the natural motion of the actin monomer (Tirion and Ben-Avraham, 1993) suggests that the correlated motion of subdomains IIa and IIb is a regulatory mechanism for the proper conformation of the base binding pocket and for the interaction with other monomers along the subdomain boundary (Kabsch *et al.*, 1990). The *ftsA3* mutation destroys part of the structure of this interdomain motion site; it is tempting to propose then an analogy between the actin binding to the actin fibre and the potential FtsA binding either to PBP3 or to another element of the septator (Vicente *et al.*, 1991) that would then interact with both proteins. Predictions from

the structural model presented here, as those already discussed, will help to fully test the details of the participation of FtsA in cell division, making it a choice model to correlate a protein structure with an essential cell function.

Materials and methods

Media and growth conditions

Luria-Bertani broth (LB) and LB agar, supplemented with antibiotics when required (50 μg/ml ampicillin), were used to grow strain MC1061 and its derivatives. Incubations were routinely done at 37°C. Strains carrying *ftsA* (ts) alleles used for complementation assays were grown in nutrient broth medium (NB) with thymine (50 μg/ml) (NBT) and antibiotics when required (50 μg/ml ampicillin) and incubated at the permissive (30°C) or restrictive (42°C) temperatures. Complementation tests were done obtaining transformants of the *ftsA* strains with plasmids harbouring specific mutations. Transformants were obtained at 30°C, isolated colonies were inoculated into NBT at 30°C and then plated into LB agar with ampicillin (incubated overnight at 30°C), and nutrient agar with thymine (incubated overnight both at 30 and 42°C). Control strains were included as follows: wild type strain; wild type strain transformed with parental plasmid, with mutated plasmids, or with *ftsA*$^+$ plasmid; mutant strains; and mutant strain transformants with the parental plasmid, or with *ftsA*$^+$ plasmid. A positive complementation result was considered when growth was visible both at 30 and 42°C in the nutrient agar with thymine (NAT) plates. In the case of positive complementation cells from both NAT plates were observed under a phase contrast microscope.

Plasmid DNA isolation, site-directed mutagenesis, and cloning procedures

Plasmid DNA isolation, cloning techniques, and transformation procedures were done as described by Sambrook *et al.* (1989). Restriction endonucleases and other enzymes were obtained from and used as recommended by Boehringer Mannheim. Agarose (D2 Pronarose) was a gift from Hispanagar S.A. Oligonucleotides JP1 and MF1 were synthesized in a Gene Assembler Plus (Pharmacia) and MS1 and MS2 were purchased from MedProbe. Site-directed mutagenesis without phenotypic selection and PCR mutagenesis were performed as described by Ausubel *et al.* (1990).

Bacterial strains and plasmids

Biochemical analysis and fractionation studies of the different FtsA forms were done in *E.coli* MC1061 [F$^-$, *araD139*, *Δ(ara-leu)7697*, *Δ(lac)X74*, *galU*, *galK*, *rpsL;* Busby *et al.*, 1982]. OV2 [F$^-$, *ilv*, *leu*, *thyA (deo)*, *his*, *ara (Am)*, *lac125 (Am)*, *galE*, *trp (Am)*, *tsx (Am)*, *tyrT (supFA81 Ts)*] and its derivatives D2 (*ftsA2*, *leu*$^+$), D3 (*ftsA3*, *leu*$^+$), and OV16 [*ftsA16 (Am) tyrT (supF-A81 (Ts)*] (Donachie *et al.*, 1976, 1979; Tormo *et al.*, 1980) were used for complementation assays. Strain OV16 was also used for pre-adsorbtion of FtsA antisera (see below).

The *ftsA101* mutation in plasmid pMSV2 was obtained by site-directed mutagenesis without phenotypic selection of the 2.3 kb *Eco*RI fragment from pJPV181, a derivative of pEMBL18 (Dente *et al.*, 1985) containing the *ftsA*$^+$ gene from pZAQ (Ward and Lutkenhaus, 1985) cloned in the *Eco*RI site in the orientation opposite to the *lac* promoter to avoid deleterious effects on bacterial growth. For this construction strain RZ1032 (*dut*, *ung*) and the oligonucleotide JP1 (5′-GCCGTTTTTGCCG-GTGGG-3′) were used. The two alleles, *ftsA*$^+$ and *ftsA101* were then recloned each as 2.2 kb *Eco*RI–*Bam*HI fragments into pBR322. The resultant plasmids pMSV5 (*ftsA*$^+$) and pMSV51 (*ftsA101*) were transformed into MC1061.

pMSV20 was obtained by deleting the 2.3 kb *Nde*I fragment of pMSV5, thus decreasing the size of the plasmid and eliminating restriction sites unwanted for subsequent PCR mutagenesis without affecting the *ftsA*$^+$ gene. pMFV1 and pMFV3 contain the *ftsA102* and *ftsA104* mutations, respectively. They were obtained by PCR site-directed mutagenesis using plasmid pMSV20 and primers MS1 (5′-AACCGATGGCGCAGCGCA-3′) and MS2 (5′-TGCGCCATCGGTTA-ATACA-3′) for *ftsA102*, and MF3 (5′-GTGGTGCAATGGATATCGC-3′) and MF4 (5′-CATTGCACCACCACCGATAT-3′) for *ftsA104*. The presence of the mutations was confirmed by DNA sequencing.

A *tac* promoter *lacI*^{q} system was used for the overproduction of FtsA$^+$ and its mutated forms, FtsA101, 102 and 104. The *ftsA*$^+$ allele contained in the 2.2 kb *Eco*RI–*Bam*HI fragment of pMSV5 was cloned in the same sites of pJF119HE (Fürster *et al.*, 1986) obtaining pMSV11. A similar procedure was followed to obtain pMSV12 containing *ftsA101* derived from pMSV51. To clone alleles *102* and *104* into pJF119HE the 1.2 kb *Eco*RI–*Bgl*II fragment from pMSV11 was replaced with the same fragments from pMFV1 (*ftsA102*) and pMFV3 (*ftsA104*) obtaining pMFV20 (*ftsA102*) and pMFV22 (*ftsA104*) respectively. These plasmids were transformed into MC1061 obtaining VIP194 (pMSV11), VIP195 (pMSV12), VIP240 (pMFV20) and VIP268 (pMFV22).

Prediction of a structural model for FtsA

A multiple sequence alignment of the known sequences of the actin family, comprising mainly the ATP binding regions, was used to provide anchor points (111 residues). Another 215 residues were aligned by direct homology between the family and FtsA. The remaining 67 residues were modelled using loops from the structural database. The model passed standard tests of normality for protein structures, but it is more reliable in the regions common to all members of the family (Bork *et al.*, 1992; Holmes *et al.*, 1993).

Two-dimensional electrophoretic protein separation and immunoblotting procedures

MC1061 cells grown at 37°C in LB medium up to 0.3 OD_{600} units were collected by centrifugation. Cell lysis and fractionation were as described by Osborn *et al.* (1972). Sample preparation and alkaline phosphatase treatment were done according to Amster-Choder and Wright (1990). Alkaline phosphatase columns were obtained from USB and used following the instructions of the manufacturers. Less than 20% of the FtsA material eluted from this type of column was found to be phosphorylated (results not shown). Two-dimensional gels were run as described by O'Farrell (1975). Samples corresponding to 4 OD_{600} units were loaded into each gel. Membrane fractions were mixed with 0.5 OD_{600} units of total protein as a marker to obtain the spot pattern by Ponceau red reversible staining. Immunoblots were developed with MVJ2, a polyclonal antiserum against FtsA (Pla *et al.*, 1990). The antiserum was previously pre-adsorbed with a total protein extract of OV16 grown at the temperature restrictive for the suppressor, as described by Sherman and Goldberg (1991). Cultures of FtsA overproducing strains, VIP194, VIP195 and VIP268, were grown to 0.1 OD_{600} units in LB containing 50 mg/ml ampicillin. 1mM IPTG (final concentration) was then added, cells were collected after 1 h and fractions for 2-D gels were prepared as described above. Samples corresponding to 2 OD_{600} units were loaded into each gel. For detection of FtsA104, immunoblots were developed with MVM1, a polyclonal antiserum against FtsA prepared and pre-adsorbed in a similar way as MVJ2. This antiserum proved more efficient in the recognition of FtsA104 than MVJ2. The blots were developed with Protein-A conjugated with peroxidase and diaminobenzidine.

ATP–agarose affinity chromatography

The FtsA$^+$ and FtsA102 proteins were overexpressed as described above under a *tac* promoter *lacI*^{q} system, except that IPTG induction was allowed for 2 h. Cells were fractionated according to Osborn *et al.* (1972). The soluble fraction was precipitated with 35% ammonium sulphate for VIP194 and VIP268 and 45% for VIP240 (*ftsA102*). The precipitate was resuspended in a volume of imidazol buffer (20 mM imidazol pH 7.8, 50 mM KCl, 5 mM magnesium acetate, 0.5 mM EDTA) calculated to contain the material derived from 100 OD_{600} units of cells in 1 ml and dialysed extensively against the same buffer. The non-dialysable fraction was divided in 0.5 ml portions and one portion loaded at 4°C on 0.5 ml ATP–agarose or AMP–agarose (Sigma) columns previously equilibrated with imidazol buffer. After 90 min each column was washed consecutively with 2 vol of the following buffers: imidazol, imidazol with 1 M KCl, imidazol and imidazol with 10 mM ATP or 10 mM AMP. After these washes the agarose in the column was resuspended in imidazol buffer up to a total volume of 1 ml, a 100 μl sample was withdrawn, and processed as other samples for SDS–PAGE (Laemmli and Favre, 1973). Membrane fractions were resuspended in 0.5% Triton X-100 in imidazol buffer and centrifuged (2 h at 35 000 *g*). The supernatant was dialysed extensively against imidazol buffer to remove the detergent (Triton alters the interaction with ATP, results not shown). The nondialysable fraction was loaded at 4°C on an ATP–agarose column as described above.

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