Actin-related proteins in *Actinobacillus pleuropneumoniae* and their interactions with actin-binding proteins

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A group of prokaryotic actin-related proteins (PARP) with an M_r of 43 000 was detected in *Actinobacillus pleuropneumoniae*. These proteins were enriched by a depolymerization/polymerization cycle, under similar conditions to those used to polymerize muscle actin, and purified by affinity chromatography on a DNase I-Sepharose column. Three isoforms of *A. pleuropneumoniae* PARP (Ap-PARP) with pI values of 5.8, 6.15 and 6.2 were detected. Ap-PARP were recognized by four different anti-actin antibodies (one anti-muscle and three anti-cytoplasmic isoforms). Ap-PARP were also recognized by antibodies against *Anabaena variabilis* PARP (Av-PARP) and against actin-binding proteins such as α -actinin and spectrin, and also by a monoclonal antibody against heat-shock cognate protein 70 (Hsc70). Specific binding of phalloidin to Ap-PARP was detected both in permeabilized cells and *in vitro*. Purified Ap-PARP can polymerize under similar conditions to those required for skeletal muscle actin polymerization and the filaments formed appear to be decorated with myosin subfragment-1 (S1) as observed by transmission electron microscopy. The amino acid composition of Ap-PARP revealed more similarities to muscle γ -actin and the cytoplasmic β -actin isoform than to eukaryotic actin-related proteins.

Keywords: prokaryotic actin-related proteins, actin-related proteins, actin-binding proteins, cytoskeleton-like structure, *Actinobacillus pleuropneumoniae*

INTRODUCTION

Evidence from genetic and ultrastructural studies suggests that bacteria have proteins which are analogous to those that form microfilaments and microtubules in eukaryotes, and that these bacterial proteins might form a cytoskeleton-like structure with similar functions in different kinds of cells (Mayer *et al.*, 1998; Margolin, 1998; Sharpe & Errington, 1999). A major cytoskeletal-like protein of bacteria is FtsZ, which is ubiquitous in eubacteria and is also found in archaea and chloroplasts.

FtsZ localizes early at the division site to form a ring-shaped septum (Addinall *et al.*, 1996; Bauman & Jackson, 1996). This septum is required for the mechanochemical process of membrane constriction. FtsZ is a GTPase with weak sequence homology to tubulin, although its three-dimensional structure is similar to the structure of α - and β -tubulin (Lu *et al.*, 1998; Löwe & Amos, 1998; Nogales *et al.*, 1998). Chromosomal segregation requires a driving force. This problem was initially approached by genetic screening of conditional segregation mutants and revealed the involvement of MukB protein. In *Escherichia coli* the MukB protein has been demonstrated to participate in the process of chromosomal partition and it has a domain structure reminiscent of the eukaryotic motor proteins kinesin and myosin. *In vitro* studies have demonstrated that FtsZ stimulates the nucleotide turnover of MukB (Hiraga, 1993; Lockhart & Kendrick-Jones, 1998). In *E. coli*, nine proteins have been recognized as being essential for assembly of the cell-division septum. One

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Abbreviations: ABP, actin-binding proteins; ARP, actin-related proteins; CD, cytochalasin D; Hsc, heat-shock cognate protein; Hsp, heat-shock protein; PARP, prokaryotic actin-related proteins; S1, myosin subfragment-1; TRITC, tetramethylrhodamine isothiocyanate; Ap, *Actinobacillus pleuropneumoniae*; Av, *Anabaena variabilis*.

of these proteins is FtsA which co-localizes *in vivo* with FtsZ, as demonstrated by tagging each protein with green fluorescent protein (Addinall *et al.*, 1996; Ma *et al.*, 1996). FtsA has been predicted to belong to the actin family and is present in different cell compartments depending on its phosphorylation state. The phosphorylated form is in the cytoplasm and binds ATP, while the non-phosphorylated form is membrane bound and does not bind ATP. Using the sequence-pattern-search method, a correlation of FtsA with a family of ATP-binding proteins that includes DnaK, heat-shock cognate protein 70 (Hsc70), sugar kinases and actin has been found (Bork *et al.*, 1992; Sánchez *et al.*, 1994). Other actin-like proteins have been identified in different bacteria and were proposed to form a kind of cytoskeleton whose physiological role is not yet understood (Minkoff & Damadian, 1976; Neimark, 1977; Nakamura & Watanabe, 1978; Göbel, 1983; Sioud *et al.*, 1987; Barnett & Cunningham, 1992; Labbé *et al.*, 1996; Norris *et al.*, 1996; Mayer *et al.*, 1998).

The prokaryotic actin-related proteins (PARP) from *Anabaena* species and *E. coli* share epitopes with smooth muscle actin (Guerrero-Barrera *et al.*, 1995, 1996), and actin-like proteins from *Synechocystis* spp. share antigenic reactivity with distinct antibodies that recognize actin regions known to interact with proteins cross-linking actin filaments (Usmanova *et al.*, 1998). *Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia is a Gram-negative bacterium that has been intensively investigated. Special attention has been paid to *A. pleuropneumoniae* virulence factors and the production of toxin-neutralizing antibodies to protect pigs against porcine pleuropneumonia (Haesebrouck *et al.*, 1997; Prideaux *et al.*, 1999). However, no studies have been done on the cell biology of this pathogen. To study the putative cytoskeleton of *A. pleuropneumoniae*, we searched for the presence of molecules which behave like actin. Here we report on the purification and characterization of three actin-related proteins (ARP) present in *A. pleuropneumoniae*. These proteins show similar behaviour to eukaryotic actin in several of their biochemical and immunological properties.

METHODS

Materials. All reagents were analytical grade from Sigma, except for ampholytes and Sepharose 4B which were from Pharmacia Biotech.

Bacteria. *A. pleuropneumoniae* strain 35 (serotype 1) was isolated from a lung abscess of a pig with acute porcine pleuropneumonia (Negrete-Abascal *et al.*, 1994). Cultures were maintained on brain heart agar (Difco) with 10 µg NAD ml⁻¹. Bacteria for extracts were grown to stationary phase in brain heart infusion broth with 10 µg NAD ml⁻¹ for 48 h at 37 °C with constant shaking (150 r.p.m.). *A. pleuropneumoniae* cells from a 500 ml culture were harvested and washed twice with PBS (0.145 M sodium chloride, 0.15 M sodium phosphate, pH 7.4) by centrifugation at 10000 g at 4 °C for 10 min. Cells were washed five times by centrifugation with cold acetone (−20 °C) and dried to a powder at room temperature.

***A. pleuropneumoniae* PARP (Ap-PARP) depolymerization and polymerization.** The method of Pardee & Spudich (1982) was used with some modifications. Acetone-dried cells (500 mg) were resuspended in 500 µl protease inhibitor cocktail (62.5 mM Tris/HCl, pH 7.4, 5 mM diisopropyl fluorophosphate, 2 mM *N*-ethylmaleimide, 10 mM *p*-hydroxymercuribenzoate, 5 mM tosyl-L-lysine chloromethyl ketone and 2 µM leupeptin). This cell suspension was diluted in 10 ml actin depolymerization buffer (2 mM Tris/HCl, pH 7.4, 0.2 mM Na₂ATP, 2 mM CaCl₂ and 0.5 mM β-mercaptoethanol) and incubated for 2 h at 4 °C with gentle stirring. Following incubation, the suspension was centrifuged at 25000 g at 4 °C for 30 min and the pellet was discarded. A range of 1–5 mM Na₂ATP was used to optimize Ap-PARP polymerization. PARP were polymerized by adjusting the supernatant to contain 50 mM KCl, 2 mM MgCl₂, 5 mM EGTA and 5 mM Na₂ATP (polymerization buffer A) and gently stirring for 2 h at 37 °C. The preparation was then centrifuged at 180000 g for 1.5 h at 4 °C and the supernatant was discarded. The pellet contained polymerized Ap-PARP filaments. Ap-PARP filaments were solubilized in 100 µl 1% SDS, 2% β-mercaptoethanol, 4 M urea. Precipitation according to Wessel & Flügge (1984) resulted in a detergent-free Ap-PARP pellet.

Ap-PARP purification by binding to DNase I. Ap-PARP from the detergent-free pellet were purified by affinity chromatography on a DNase I column (Guerrero-Barrera *et al.*, 1996).

Ap-PARP purification by electroelution. The detergent-free Ap-PARP pellet was also fractionated by preparative Tricine/SDS-PAGE (15% gels) as described by Schägger & von Jagow (1987). Part of the gel was used to identify Ap-PARP by Western blot analysis with different antibodies against actin and actin-binding proteins (ABP). The rest of the gel was stained with Coomassie blue and the corresponding PARP bands were cut out and electroeluted in an Electro-Eluter model 422 (Bio-Rad). The electroelution buffer was 50 mM NH₄HCO₃, 0.1% SDS. Electroelution was performed for 5 h at 10 mA per tube as described in the Bio-Rad manual.

Electrophoretic analysis. Two methods were used for one-dimensional SDS-PAGE: the first was the Laemmli (1970) method using 10% gels; the second was the Tricine/SDS-PAGE method of Schägger & von Jagow (1987) using 15% gels. Electrophoresis was performed in a Mighty-Small II SE250 Cell (Hoefer) at 100 V for 1 or 2.5 h. Gels were stained with 0.2% Coomassie blue R250 in destaining solution (10% methanol, 10% acetic acid in water).

Two-dimensional (2D) gel electrophoresis was done according to O'Farrell (1975).

Western blotting. Ap-PARP were separated by Tricine/SDS-PAGE as described above and transferred to Immobilon-P (Millipore) or nitrocellulose membranes in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 253 mA for 45 min. The transfer buffer contained 10 mM CAPS, 0.4 mM DTT and 10% methanol (pH 11). For immunodetection, the following antibodies were used: polyclonal mouse antibody against *Anabaena variabilis* PARP (Av-PARP) and monoclonal mouse anti-rat brain actin (Guerrero-Barrera *et al.*, 1996), polyclonal rabbit anti-C-terminal actin (A 2066, synthetic peptide SGPSIVHRKCF; Sigma), monoclonal mouse anti-α-smooth muscle actin (A 2547; Sigma), polyclonal rabbit anti-profilin-γ-actin complex from calf spleen (M. Segura-Nieto & U. Lindberg, unpublished data), monoclonal mouse anti-Hsc70 (Sigma), polyclonal rabbit anti-α-actinin and polyclonal rabbit anti-spectrin (kit Cyto-2; Sigma). In all cases peroxidase-labelled protein A was used to detect antibody

binding. The immune reaction was revealed with 4-chloronaphthol in a Mini-Protein II Multiscreen Apparatus (Bio-Rad). All procedures were done at room temperature.

Phalloidin binding

Whole cells. *A. pleuropneumoniae* cells were treated with phalloidin labelled with tetramethylrhodamine isothiocyanate (TRITC) as described previously (Guerrero-Barrera *et al.*, 1996). For the assays, 10 ml of a stationary-phase culture was used. Readings were taken in a spectrofluorometer (Perkin-Elmer Luminiscence Spectrometer L5 50B) at an excitation wavelength of 550 nm and emission wavelength of 574 nm. Non-treated cells were used as negative control.

Ap-PARP. DNase I-affinity-purified Ap-PARP (100 µg) was incubated for 2 h at 37 °C in 500 µl polymerization buffer A with 50 ng TRITC-phalloidin; the drug excess was removed following precipitation of Ap-PARP using the method of Wessel & Flügge (1984). The TRITC-phalloidin filamentous Ap-PARP (F-Ap-PARP) precipitate was resuspended in 1 ml PBS. As negative control, PARP were incubated with unlabelled phalloidin, and as positive control skeletal muscle F-actin was incubated with TRITC-phalloidin. The interaction was measured with a spectrofluorometer as described above.

Electron microscopy

Negative staining of PARP. The Ap-PARP detergent-free pellet (100 µg) was resuspended in 500 µl polymerization buffer A and incubated for 2 h at 37 °C. Ten microlitres of this reaction mixture containing F-Ap-PARP was laid on nickel grids covered with Formvar and settled for 10 min at room temperature. Then the samples were fixed with 2.5% glutaraldehyde and washed with polymerization buffer A. Samples were contrasted with 0.5% uranyl acetate, pH 7.4. Skeletal muscle actin was used as positive control. The observations were made with a JEOL 2000 EX transmission electron microscope at 80 kV.

Decoration of F-Ap-PARP with myosin subfragment-1 (S1). Ten microlitres of the above polymerized F-Ap-PARP mixture was deposited on a carbon Formvar-coated nickel grid and allowed to settle for 10 min at room temperature. Then, 10 µl S1 (25 mg ml⁻¹) in polymerization buffer A (without Na₂ATP) was added before incubation overnight at 4 °C. Samples were fixed and washed, and observations were made with the electron microscope as described above.

Amino acid composition. The electroeluted Ap-PARP were hydrolysed in 6 M HCl for 24 h at 110 °C under vacuum and the amino acid composition was determined as described by Bidlingmeyer *et al.* (1984).

RESULTS AND DISCUSSION

The presence of PARP in *A. pleuropneumoniae* was investigated as an initial step in the study of its putative cytoskeletal structure. To identify and characterize ARP, the following five classical criteria for actin identification in eukaryotic cells were used.

1. Enrichment by one depolymerization/polymerization cycle and purification by binding to DNase I

One of the ways to obtain and enrich functional eukaryotic actin is through depolymerization/polymerization cycles. This method was used with *A. pleuropneumoniae* cell extracts, and a polypeptide band

with an M_r of 43 000 in SDS-PAGE was enriched as shown in Fig. 1(a), lane 3. This M_r is similar to that of skeletal muscle actin (Fig. 1a, lane 1). Using this method it was possible to identify and purify Ap-PARP from cell extracts that had polymerization capability similar to eukaryotic actin. The Ap-PARP were also enriched from the cell-free culture filtrate of exponential-phase viable cells (data not shown), apparently as a product of secretion from the bacteria.

The specific binding of DNase I to the majority of eukaryotic actins is the most common criterion used to identify this conserved protein family (Lazarides & Lindberg, 1974). PARP from several bacterial species (Guerrero-Barrera *et al.*, 1996), including Ap-PARP, have this property. The purity of Ap-PARP obtained after affinity chromatography on a DNase I column was observed by two-dimensional electrophoretic analysis (Fig. 1b). The physiological significance of the actin-DNase I interaction is unknown, but it has been exploited as a method for actin purification. The Ap-PARP-DNase I interaction also provides a one-step purification procedure. Ap-PARP bind to DNase I under the same conditions as muscle G-actin.

2. Biochemical characterization

(a) M_r and pI. Ap-PARP have an M_r of 43 000 (Fig. 1a, b), distinct from that reported for other actin-like proteins, e.g. 60 000 for *Streptococcus pyogenes* (Barnett & Cunningham, 1992) and *E. coli* (Guerrero-Barrera *et al.*, 1996), and 56 000 for *Synechococcus* spp. (Labbé *et al.*, 1996). The Ap-PARP displayed three isoforms with pI values of 5.80, 6.15 and 6.20 (Fig. 1b). These isoforms are more acidic than PARP from *Anabaena variabilis* and *E. coli* (Table 1), but less acidic than actins from other species (Garrels & Gibson, 1976; Herman, 1993). The Ap-PARP pI values are closer to those of Arp2 and Arp3 reported by the ExPASy WWW server (April, 1998). Arp1 is closer in pI, structure and amino acid sequence to skeletal muscle actin isoform (Herman, 1993; Schroer *et al.*, 1994; Mullins *et al.*, 1996).

(b) Amino acid composition. The amino acid composition of Ap-PARP reveals closer similarities to actin from different sources than ARP from *Saccharomyces cerevisiae* and *Acanthamoeba castellanii* (Table 2). On the other hand, there are clear differences in the glycine content. The level of glycine is relatively high in Ap-PARP as opposed to actin isoforms and ARP (Table 2). It is possible that the high glycine content of Ap-PARP might partially be caused by contamination during its extraction, although the electrophoretic method used to separate Ap-PARP contains Tricine buffer. Tricine is a derivative of glycine (Schägger & von Jagow, 1987) and we cannot rule out the possibility that some glycine could be generated during the electrophoresis, which might interact with the protein. The complete amino acid composition of PARP will be certified when either the primary protein sequences or the encoding region of the genes becomes known.

Comparison of Ap-PARP amino acid composition

Fig. 1. Electrophoretic pattern of Ap-PARP in (a) one and (b) two dimensions. (a) Tricine/SDS-PAGE (15 % gel) of skeletal muscle actin (lane 1) and Ap-PARP enriched by a depolymerization/polymerization cycle (lane 3). Lane 2, molecular mass markers. (b) Isoelectric focusing (IF) and SDS-PAGE (SDS) (10 % gel) of DNase-I-purified Ap-PARP showing the following pI values: (i) 5.80, (ii) 6.15 and (iii) 6.20. Lane 1, molecular mass markers.

Table 1. Comparison of Ap-PARP with other PARP, ARP, actin and Hsp70 (Hsc70) from different species

Protein	$10^{-3} \times M_r$	pI		
		(1)	(2)	(3)
<i>A. pleuropneumoniae</i> PARP	43	5.80	6.15	6.20
<i>E. coli</i> PARP*	60	6.60	6.65	6.70
<i>Anabaena variabilis</i> PARP*	45	6.40	6.50	6.80
Hsp70 (Hsc70)†	70	5.37	5.49	5.53
ARP‡	48–60	5.40	5.50–6.23	5.30–5.82
Actin§	43–48	(α) 5.40	(β) 5.42	(γ) 5.44

* Guerrero-Barrera *et al.* (1996).

† (1) Human Hsc70, (2) chicken Hsp70 and (3) bovine Hsc70 (ExPASy WWW server, April, 1998).

‡ (1) Arp1, (2) Arp2 and (3) Arp3 (ExPASy WWW server, April, 1998).

§ Garrels & Gibson (1976); ExPASy WWW server (April, 1998).

through a search on the ExPASy WWW server (April, 1998) revealed no homology with cytoplasmic bacterial proteins. Similarities were seen with the glucose-1-phosphatase of *E. coli* and with several pilus chaperone proteins like PapD, AfaB and YopN from *Yersinia pseudotuberculosis*. The search also revealed similarity with several muscle proteins such as α -skeletal muscle actin and α -smooth muscle actin from different animal species, and skeletal muscle myosin light-chain and troponin-C/-I from cardiac and skeletal muscle. No homology was seen with ARP.

(c) Toxin binding. Phalloidin binding to polymerized actin (F-actin) is a biochemical characteristic that all actins share. This is a stoichiometric interaction of one molecule of phalloidin to two actins. This toxin stabilizes actin filaments and prevents its depolymerization by agents such as DNase I, ultrasonication and proteolytic degradation (Vandekerckhove *et al.*, 1985; Miyamoto *et al.*, 1986; Sheterline *et al.*, 1998). This binding is inhibited by cytochalasin D (CD), a toxin which binds both to monomeric actin and to the barbed end of actin filaments. CD binds at a stoi-

Table 2. Comparison of the amino acid composition of Ap-PARP with that of β - and γ -actin and ARP

Data for actins and ARP are from the ExPASy WWW server (April, 1998). ND, Not determined.

Amino acid	PARP	β -Actin	γ -Actin	<i>Saccharomyces cerevisiae</i> Arp2	<i>Acanthamoeba castellanii</i> Arp2	<i>Acanthamoeba castellanii</i> Arp3
Asx	7.67	8.30	8.24	9.28	9.28	7.96
Glx	14.49	10.70	10.10	10.05	10.05	10.54
Ser	7.25	6.40	6.65	4.12	4.12	5.15
His	1.57	2.40	2.40	1.30	1.30	2.57
Gly	19.53	7.50	7.71	6.70	6.70	7.02
Thr	3.16	6.70	6.65	4.12	4.12	5.15
Ala	5.25	7.70	7.98	5.67	5.67	6.32
Pro	5.04	5.00	5.00	4.90	4.90	6.01
Tyr	2.41	4.00	3.72	3.87	3.87	3.40
Arg	3.47	4.80	5.32	6.44	6.44	5.40
Val	7.25	5.90	5.32	9.02	9.02	7.96
Met	1.26	4.30	4.26	3.35	3.35	2.34
Cys	ND	1.60	1.86	2.61	2.06	1.87
Ile	4.00	7.50	7.71	5.41	5.41	7.26
Leu	7.56	7.50	7.44	9.02	9.02	5.39
Phe	2.74	3.20	3.20	5.67	5.67	4.92
Lys	7.35	5.00	5.00	7.47	7.47	6.79

Table 3. Spectrofluorometric detection of CD and phalloidin binding to permeabilized *A. pleuropneumoniae* cells

Sample	Emission (λ_{574}) (excitation λ_{550})
Cells incubated with TRITC-phalloidin	75.01
Cells preincubated with phalloidin and then incubated with TRITC-phalloidin	0.35
Cells incubated with CD	0.29
Cells preincubated with CD and then incubated with TRITC-phalloidin	18.70

Table 4. Spectrofluorometric measurement of CD and phalloidin binding to F-Ap-PARP

Sample	Emission (λ_{574}) (excitation λ_{550})
F-Ap-PARP (200 μ g) preincubated with phalloidin and then incubated with TRITC-phalloidin	0.20
F-Ap-PARP (200 μ g) incubated with TRITC-phalloidin	6.90
F-Ap-PARP (200 μ g) preincubated with CD and then incubated with TRITC-phalloidin	0.20
Muscle actin (100 μ g) preincubated with phalloidin and then incubated with TRITC-phalloidin	0.22
Muscle actin (100 μ g) incubated with TRITC-phalloidin	8.5
Muscle actin (100 μ g) preincubated with CD and then incubated with TRITC-phalloidin	0.25

chiometry of one molecule per filament, thus inhibiting monomer exchange and resulting in depolymerization (Sheterline *et al.*, 1998). These two drugs were used to test for F-Ap-PARP both in permeabilized cells and *in vitro*. Table 3 shows the results of specific TRITC-phalloidin binding to permeabilized *A. pleuropneumoniae* cells. This interaction is blocked when these cells are preincubated with either unlabelled phalloidin or CD. The specific binding of F-Ap-PARP to

TRITC-phalloidin was also measured *in vitro* with proteins purified by DNase I affinity chromatography, using the same strategy as above. This specific interaction is inhibited by preincubation of F-Ap-PARP with either unlabelled phalloidin or CD and measured by spectrofluorometry (Table 4). As a positive control skeletal muscle F-actin was incubated with TRITC-phalloidin and this interaction was also inhibited by prior incubation with CD or unlabelled phalloidin

Table 5. Effect of various chemicals on the polymerization of *Ap*-PARP and rabbit skeletal muscle actin

Chemical	Concentration (mM)	<i>Ap</i> -PARP (200 µg)*	Actin (200 µg)*
KCl	50	+	+
CaCl ₂	2	—	—
MgCl ₂	2	+	+
EGTA	5	+	+
ATP	1–5	+	+
ATP	0.2	—	—

* +, Contributes to polymerization; —, inhibits polymerization and contributes to depolymerization.

Fig. 2. Electron micrographs of filamentous *Ap*-PARP. Negative staining of (a) rabbit skeletal muscle F-actin (the inset shows single filaments) and (b) filamentous *Ap*-PARP (the inset shows the 'amorphous' structure). Bars, 200 nm.

(Table 4). These data corroborate the observations made with permeabilized cells.

(d) ATP concentration. Table 5 shows a summary of the effects of various chemicals that inhibit or induce *Ap*-PARP polymerization. The ionic strength and ATP concentration were the same as those used to polymerize skeletal muscle actin (Spudich & Watt, 1971). The polymerization of PARP was done at twice the concentration of that used in the case of skeletal muscle actin, although PARP can also polymerize at the same concentration (data not shown).

3. F-*Ap*-PARP ultrastructural analysis

Transmission electron microscopy analysis of F-*Ap*-PARP under polymerization conditions showed two kinds of structures: one apparently similar to muscle F-actin, with filaments of 5–7 nm (Fig. 2b) (cf. muscle F-actin; Fig. 2a), and the other corresponding to an

'amorphous' network which may be due to the higher PARP concentration or to a variation in PARP filament organization (Fig. 2b, inset).

4. Binding to DNase I and S1

DNase I is an ABP which has been used to characterize the three-dimensional structure of G-actin (Kreuder *et al.*, 1984; Kabsch *et al.*, 1990). All PARP studied thus far have the capacity to bind DNase I under the same conditions as actin. This suggests that *Ap*-PARP have a similar three-dimensional DNase I binding site to actin.

Actin and myosin form one of the major motor protein complexes in all eukaryotic cells. Most of the studies on myosin interaction with actin during force generation involve the use of mammalian fast muscle myosin II or its functional proteolytic fragments S1 and HMM, together with mammalian skeletal muscle actin. Each myosin head has similar actin-binding ATPase domains but different tail regions. Also, each myosin head may bind to two adjacent molecules of actin in the filament during the cross-bridge cycle. The most stable conformation is the rigor conformation which exists at the end of the cross-bridge cycle. This conformation can be experimentally 'frozen' by removal of ATP necessary to release the myosin head (Andreev & Borejdo, 1991).

S1 binds to filaments of *Ap*-PARP (Fig. 3b) in a similar fashion to skeletal muscle actin (Fig. 3a). This interaction shows that filaments of *Ap*-PARP expose similar S1-binding sites to vertebrate actin. This observation suggests that *Ap*-PARP filaments might have the ability to interact with myosin-like molecules from the bacteria *in vivo*, probably participating in intrabacterial motility, bacterial cell division and nucleoid segregation, as has been suggested by Casaregola *et al.* (1990). Although in *A. pleuropneumoniae* there have been no studies describing the presence of motor-like proteins as yet, the evidence from studies in different bacteria suggests that motor-like proteins are ubiquitous (Melby *et al.*, 1998).

MukB in *E. coli* and the structural maintenance of chromosome proteins (SMC) found in almost all bacteria, archaea and eukaryotes are involved in chromosome partition. These proteins have a domain structure reminiscent of eukaryotic motor proteins similar to kinesin and myosin. They form homodimers

Fig. 3. Decoration of filamentous actin and filamentous *Ap*-PARP with S1. (a) Rabbit muscle actin. (b) *Ap*-PARP. The arrowheads point to the microfilament decorated with S1. Bars, 100 nm.

having both DNA- and ATP/GTP-binding activities (Melby *et al.*, 1998). A construct encoding the first 342 residues of MukB (Muk342) generates a fragment with properties of the motor domain of kinesin and is capable of decorating microtubules in a similar manner to kinesin. Analysis of the stoichiometry demonstrated an interaction of one Muk342 fragment per one tubulin monomer (Lockhart & Kendrick-Jones, 1998). These results strongly suggest that MukB has a role in force generation. This type of *in vitro* experiment has not been done with F-actin filaments to see if Muk342 can interact with actin filaments in a similar manner to S1 (Fig. 3a).

Recent studies have completely overturned the classical view of chromosome segregation in bacteria. Far from being a passive process involving gradual separation of the chromosomes, there appears to exist an active, possibly mitotic-like machinery (Sharpe & Errington, 1999; Melby *et al.*, 1998). MukB and SMC proteins from *Bacillus subtilis* function in chromosome condensation and some other aspects of DNA processing (Niki *et al.*, 1992; Lockhart & Kendrick-Jones, 1998; Melby *et al.*, 1998). These findings support the idea that prokaryotes might have a cytoskeletal-like structure involved in fundamental cellular functions as in eukaryotes.

5. Immunological recognition of *Ap*-PARP by different antibodies

Ap-PARP have several properties shown above which should be sufficient to consider them as members of the actin family. To reinforce this statement, other criteria

were used, e.g. their recognition with different anti-actin antibodies and anti-ABP antibodies.

Ap-PARP obtained after one polymerization cycle were fractionated in a Tricine/SDS-PAGE gel, blotted and reacted with polyclonal antibodies against *Av*-PARP (Fig. 4, lane 2) and four different anti-actin antibodies (Fig. 4, lanes 3, 4, 5 and 8). All antibodies gave a positive reaction, showing that *Ap*-PARP have epitopes similar to those present in different actins. On the other hand, the positive recognition of *Ap*-PARP by two anti-ABP antibodies against α -actinin and spectrin, both from chicken gizzard (Fig. 4, lanes 6 and 7), suggests structural similarities to eukaryotic actin molecules as well. This cross-reaction between actin and ABP was not non-specific, since antibodies against filamin, vimentin, tropomyosin and myosin did not recognize any epitope on *Ap*-PARP (data not shown). On the other hand, the immunoelectron microscopic studies of prokaryotes of various taxonomic affiliations (archaea and bacteria) have shown a network made up of 'protein fibrils'. These fibrils are composed of proteins which exhibit cross-reactivity with the anti-actin antibody used in Fig. 4, lane 5 (C terminus of actin). The network seems to be located around the cytoplasm close to the periphery of all the cells (Mayer *et al.*, 1998). These authors propose the existence of a prokaryotic cytoskeleton that might function to preserve the shape of different prokaryotes.

Ap-PARP was also recognized by a monoclonal anti-Hsc70 antibody (Fig. 4, lane 9). This result reflects common epitopes in both proteins. Hsc70 shares a common structural fold and a nucleotide binding site with actin. It appears more likely that actin, Hsc70,

Fig. 4. Western blot of filamentous *Ap*-PARP enriched by a depolymerization/polymerization cycle. Lanes: 1, negative control reaction with protein A-peroxidase; 2-9, *Ap*-PARP recognized by anti-*Av*-PARP (2), monoclonal anti-rat brain actin (3), polyclonal anti- α -smooth muscle actin (4), polyclonal anti-C-terminal synthetic peptide (5) anti- α -actinin (6), anti-chicken spectrin (7), anti-calf spleen profilin- γ -actin complex (8) and monoclonal anti-Hsc70 (9).

hexokinase, glycerol kinase, and perhaps the prokaryotic cell cycle proteins (FtsA, MreB, StbA) and *E. coli* phosphatases, are the product of divergent evolution from a common ancestor of a single-domain molecule (Flaherty *et al.*, 1991; Bork *et al.*, 1992; Sánchez *et al.*, 1994; Kabsch & Holmes, 1995). The results from the Western blot analysis with *Ap*-PARP compared with data obtained from other PARP (Guerrero-Barrera *et al.*, 1996) are shown in Table 6.

Since Minkoff & Damadian (1976) reported actin-like proteins in prokaryotes, this has been confirmed by others (Rosenbusch *et al.*, 1976; Neimark, 1977; Beck *et al.*, 1978; Nakamura & Watanabe, 1978; Sioud *et al.*, 1987; Barnett & Cunningham, 1992; Labbé *et al.*, 1996; Guerrero-Barrera *et al.*, 1996). However, there are no sequence data available on either the proteins or the gene to reveal a relationship with actin. Preliminary Southern blot studies of *A. pleuropneumoniae* DNA hybridization with actin probes of *Taenia solium* were negative (data not shown) (probes from Campos *et al.*, 1990). Similar results were obtained for *S. pyogenes* (Barnett & Cunningham, 1992). In the case of the complete genome sequence of *Haemophilus influenzae*, *Mycoplasma genitalium* and *E. coli*, actin sequences were not found (Fleischmann *et al.*, 1995; Wahl *et al.*, 1994; Koonin *et al.*, 1996). Although there is an absence of data at the molecular level showing DNA homology between PARP and actin, the biochemical evidence suggests that PARP are ancestral actin-like protein candidates. It is possible that although homology of both the gene and the protein of PARP to eukaryotic actin could be low, its three-dimensional structure could be similar, as in the case of FtsZ and tubulin. FtsZ and tubulin have a common structural core with identical folding (including 10 β -strands surrounded by 10 α -helices) (Nogales *et al.*, 1998; Löwe & Amos, 1998).

Other cytoskeleton-like components have been found in prokaryotic cells; e.g. myosin-like protein from *E. coli* (Nakamura & Watanabe, 1978), M protein in *S. pyogenes*, which has amino acid sequence homology with coiled-coil proteins similar to tropomyosin and myosin-like proteins (Manjula & Fiscetti, 1986; Manjula *et al.*, 1985, 1991; Mische *et al.*, 1987; Barnett & Cunningham, 1992), and α -actinin-like proteins in cyanobacterium *Spirulina platensis* (Usmanova *et al.*, 1998). Thus, PARP as well as ABP might have existed before the emergence of eukaryotic cells. It is clear that many more cytoskeletal-like proteins in bacteria remain to be found and studied at the biochemical, genetic and

Table 6. Recognition of PARP by monoclonal and polyclonal antibodies against actin and ABP

Antibody	PARP*		
	<i>Ap</i>	<i>Av</i>	<i>Ec</i>
<i>Av</i> -PARP	+	+	+
C-terminal synthetic peptide	+	+	+
α -Smooth muscle actin	+	—	—
Brain actin	+	+	+
Calf spleen profilin- γ -actin complex	+	+	+
Chicken gizzard α -actinin	+	—	—
Chicken spectrin	+	—	—
Bovine brain Hsc70	+	ND	+

*Data for *Anabaena variabilis* (*Av*) and *E. coli* (*Ec*) are from Guerrero-Barrera *et al.* (1996). ND, Not determined.

subcellular level to understand their organization and participation in the dynamics of the prokaryotic cell.

ACKNOWLEDGEMENTS

We thank the Electron Microscopy Unit of the Cell Biology Department of the National Center of Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN). We thank Professor Uno Lindberg and Dr Roger Karlsson for their helpful comments, and June Simpson for her criticisms of the manuscript. We thank Aurora Verver for the amino acid composition analysis. We also thank Magda E. Reyes and Esteban Molina for technical assistance. A.L.G. was supported by the credit scholarship No. 85546 from the National Mexican Council for Science and Technology (CONACyT). This work was supported by CONACyT grants 1462-N9207 (M.G.) and 4096P-N (M.S.).

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Received 22 June 1999; accepted 22 July 1999.