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Purification and functional characterization of p16, the ATPase of the bacteriophage Φ29 packaging machinery

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ABSTRACT

Bacteriophage Φ29 codes for a protein (p16) that is required for viral DNA packaging both *in vivo* and *in vitro*. Co-expression of p16 with the chaperonins GroEL and GroES has allowed its purification in a soluble form. Purified p16 shows a weak ATPase activity that is stimulated by either DNA or RNA, irrespective of the presence of any other viral component. The stimulation of ATPase activity of p16, although induced under packaging conditions, is not dependent of the actual DNA packaging and in this respect the Φ29 enzyme is similar to other viral terminases. Protein p16 competes with DNA and RNA in the interaction with the viral prohead, which occurs through the N-terminal region of the connector protein (p10). In fact, p16 interacts in a nucleotide-dependent fashion with the viral Φ29-encoded RNA (pRNA) involved in DNA packaging, and this binding can be competed with DNA. Our results are consistent with a model for DNA translocation in which p16, bound and organized around the connector, acts as a power stroke to pump the DNA into the prohead, using the hydrolysis of ATP as an energy source.

INTRODUCTION

The packaging of DNA inside preformed protein shells is a common mechanism in the morphogenesis of double-stranded (ds)DNA viruses. During the packaging process, a specific DNA is condensed in a fast and processive way (~140 bp/s at 25°C) inside the viral proheads up to concentrations similar to those characteristic of DNA crystals (~500 mg/ml) (1,2). The packaging process is an endergonic reaction that requires energy provided by ATP hydrolysis, and which involves the interaction between several structural and non-structural viral components (reviewed in 3–5).

The development of *in vitro* DNA packaging systems has made it possible to learn much of what we know about this process, and the similarities observed among all the dsDNA phages suggest that they all package DNA using a basic common mechanism. DNA is packaged inside the proheads through the connector or portal protein, which is placed in only one of the 5-fold vertexes of the prohead (6,7). The three-dimensional reconstruction of the isolated connectors (8–10) and the recently published X-ray structure of the Φ29 connector protein (11) clearly shows that despite the lack of sequence homology, all functional connectors are dodecamers that share a toroidal structure enclosing a channel that runs along the oligomer. This channel is wide enough for the passage of a dsDNA molecule and there is evidence from atomic force microscopy of DNA–connector complexes (12), as well as from reconstruction of proheads during packaging (11) that strongly support this mechanism.

However, the connector is unable to package DNA by itself and it needs the help of a terminase complex. This complex shows multiple catalytic activities: it binds the specific DNA, induces staggered nicks in the concatemeric DNA to generate packable ends, and has an ATPase activity essential for DNA packaging. For several phages these proteins have been suggested to interact with the connector in the prohead mediating the interaction of the DNA with this oligomeric structure (13–16).

In the case of bacteriophage Φ29, the replication of its genome does not involve the production of concatemers, and the presence of a terminal protein (p3) covalently bound to the 5′ ends of the DNA molecule plays a fundamental role during the replication process (17). In this phage, the classical terminase complex is replaced by a viral-encoded 120-base RNA (pRNA, p for prohead or packaging) and by the product of the gene 16, the ATPase p16. The pRNA binds to the Φ29 connector assembled into the prohead but is not present in the final, mature virion (18). The extended mutational analysis of the pRNA strongly suggest that six copies of pRNA form a hexameric ring around the connector that functions during the packaging (19–22). In fact, a pRNA ring compatible with either a 6-fold (7) or 5-fold organization (11) has been directly located bound to the tip of the connector. The actual function of the pRNA ring is still unknown.

The other component of the terminase complex is the protein p16. This protein is absolutely required for the packaging of the Φ29 genome, both *in vivo* and *in vitro* (23,24) and, as in the case of the pRNA, p16 is not present in the final virion. Protein p16 is the only component of the Φ29 packaging machinery that presents an *in vitro* ATPase activity, and therefore is most probably the first molecule of the chain of energy transduction that leads to DNA transport. Until now the study of p16 has been hampered by the fact that p16 is a hydrophobic protein which aggregates in inclusion bodies when over-expressed in

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Escherichia coli (24). The classical purification methods of this protein involve several cycles of denaturation and renaturation and, in the latter process, the protein reaggregates in a concentration-dependent way (24). The difficulty of obtaining this protein in a soluble form could explain the presence in the literature of some conflicting data regarding the components required to stimulate the ATPase activity of the protein or the sequential interactions between p16 and the rest of the components of the packaging machinery (25–27).

In this paper we report a simple method for the purification of soluble, functional p16 protein. We show that soluble p16 presents a nucleic acid-dependent ATPase activity under standard packaging conditions in the absence of NH_4Cl (in contrast with previously published data). We also report that the ATPase p16 binds to the proheads through the N-terminal ends of the connector or portal protein. This domain is also the pRNA binding region, and we have found that p16 interacts with the pRNA in solution in a nucleotide-dependent fashion. The data presented in this work can be integrated into the structural model of the connector assembly (7,11) and suggests a working model for the viral DNA translocation machinery.

MATERIALS AND METHODS

Cloning, over-expression and purification of the protein gp16

Gene 16 of Φ29 comprises about two-thirds of the sequence of the *Eco*RI fragments E and D (28). The Φ29 genome was partially digested with *Eco*RI and the E/D segments were isolated from agarose gel electrophoresis and ligated into the *Eco*RI site of linearized, alkaline phosphatase-treated commercial pET21 (Amp^+) vector (NovaGen). The recombinant plasmid was transformed into *E.coli* BL21 (DE3) by electroporation and the clones containing the gene 16 in the correct orientation pET21-p16 (Amp^+) were selected. To overproduce p16 in a soluble way the BL21 cells harboring the plasmid pET21-p16 (Amp^+) were transformed with the pAG (Cm^+) vector that contains the genes for the chaperonins GroEL and GroES (29). The cells harboring both vectors (Amp^+/Cm^+) were selected and grown to 10^8 cells/ml in LB containing Amp (50 μg/ml) and Cm (34 μg/ml) at 37°C. The maximum yield of soluble p16 was obtained after the induction of the chaperonins GroEL and GroES with arabinose (0.175 g/l) for 30 min at 37°C, followed by the induction of the expression of gene 16 with IPTG (5 mM) for 2 h at 30°C.

For the purification of the soluble p16, 10 g of over-expressed cells were mechanically broken, homogenized in TDS1 buffer (50 mM Tris–HCl pH 7.7, 1 mM DTT, 100 mM NaCl) and centrifuged to eliminate the cell membranes (10 000 r.p.m. in the Sorvall SS34 rotor during 10 min at 4°C). The excess of GroEL was precipitated by 10-fold dilution of the supernatant with TDS4 buffer (TDS1 plus 300 mM NaCl) supplemented with PEI (polyethilenimine) [0.16% (w/v)] for 1 h at 4°C. Then the mixture was centrifuged (8000 r.p.m. for 45 min at 4°C) and the soluble proteins concentrated into 20 ml of TBDS2 [TDS2 (TDS1 plus 100 mM NaCl) plus glycerol 5% (w/v)] by precipitation with ammonium sulfate [70% (w/v), 1 h at 4°C] and posterior centrifugation. The concentrated extract was further dialyzed against 2 l of the same buffer (overnight at 4°C). After the dialysis the extract was applied to an ionic-exchange phosphocellulose column (P-11 Watman) equilibrated with TBDS2. After washing with 5 vol TBDS2, p16 was eluted increasing step-wise the buffer salt concentration up to 400 mM (TBDS4). The fractions containing p16 were detected by SDS–PAGE, pooled and applied to a hydroxylapatite column equilibrated with TBDS4. The column was washed with step-wise increasing concentrations of phosphate buffer (PB; 0.5 M Na_2HPO_4, 0.5 M $NaHPO_4$, 1 mM DTT). After washing with 4 vol 50 mM PB and 2 vol 100 mM PB, p16 was found to elute pure at 150 mM PB. The column fractions were analyzed by SDS–PAGE and those fractions showing pure p16 by Coomassie blue and silver staining, and western blot using anti-p16 antibodies, were mixed, dialyzed overnight at 4°C against TBDS3 (50 mM Tris–HCl pH 7.7, 5% glycerol, 1 mM DTT, 0.3 M NaCl) and stored in small aliquots at –20°C.

Isolation of proheads, DNAp3 and pRNA

The proheads were purified from extracts of *Bacillus subtilis* cells infected with the bacteriophage Φ29 double mutant sus14-sus16 (30) using two consecutive sucrose gradient centrifugations, as described (7). RNA-free proheads were produced by treatment of purified proheads with RNase A (0.27 mg/ml) in the presence of 7 mM EDTA for 30 min at room temperature.

The DNA used as substrate carried the terminal protein, p3 (DNAp3), and it was purified from mutant sus14, as described (31). The *B.subtilis* SpoA12 strain harboring the plasmid pUM102 was used for the *in vivo* production of Φ29 pRNA as described elsewhere (26,32).

Φ29 connectors were obtained from *E.coli* cells harboring the vector pPLc28D1. The purification of the native connectors and the connectors treated with V8 was carried out as described (33,34).

The *in vitro* system of DNA packaging

The assay of DNAp3 packaging in the defined *in vitro* system by agarose electrophoresis is based on the one previously described (35) with some modifications. Briefly, purified proheads (8 μg) and DNAp3 (1 μg) were incubated with soluble p16 (0.5 μg) in TMS buffer (50 mM Tris–HCl pH 7.7, 10 mM $MgCl_2$, 100 mM NaCl) containing ATP (1 mM) for 30 min at room temperature and the mixtures were treated with 5 μg/ml DNase I for 20 min to digest the non-packaged DNA. EDTA (10 mM) was added, and the packaged DNA was extracted from the proheads for 20 min at 70°C. The samples were treated with 500 μg/ml proteinase K for 60 min at 55°C, protein removed by phenol extraction, and run on a 0.8% (w/v) agarose gel with ethidium bromide.

ATPase activity assays

The ATPase assays were performed under optimal conditions for the *in vitro* DNA packaging reaction. p16 (0.06 nM), proheads with pRNA (1.6×10^{11} proheads, containing ~1×10^{12} pRNA molecules) and DNAp3 (0.04 pM) were mixed in TMS buffer, and the reaction was initiated by the addition of 0.5 mM ATP containing 50 μCi [γ-^{32}P]ATP. The mixture was incubated at different temperatures and the reaction stopped at 3, 6, 9, 15 and 30 min with EDTA (10 mM). Alternatively, p16 (0.06 nM) was incubated independently with nucleic acid [DNAgp3 (0.14 pM), pRNA (0.05 nM) and 5S rRNA (0.05 nM)]

or proteins (proheads with or without pRNA, 7.8×10^{10}) and connectors (3 pM), and the reactions were followed as described above. Samples were spotted onto PEI-cellulose paper (Altech Associated Inc.) and run in 1 M formic acid/0.5 M lithium chloride as described (36). The result of the hydrolysis was detected by autoradiography and quantified in a phosphorimager.

p16–prohead interaction

Φ29 proheads with or without pRNA (4 µg or 1.6×10^{11} particles) were mixed with p16 (1.7 pM) in TMS buffer with or without ATP (1 mM), in a final reaction volume of 50 µl. The mixture was incubated at room temperature for 20 min and the samples centrifuged in a 10–30% linear sucrose density gradient containing TM buffer (50 mM Tris–HCl pH 7.7, 5 mM $MgCl_2$) in the Sw55 rotor (Beckman) at 35 000 r.p.m. for 45 min at 20°C. To analyze the prohead and p16 positions, the gradients were fractionated and 75 µl of each fraction was used to cover ELISA plates (overnight at 4°C). The position of each component was detected with specific polyclonal antibodies against p16 or proheads, as described below.

Alternatively, ELISA tests were used to detect the interaction between p16 and the proheads. 96-well microliter ELISA plates were coated by overnight incubation with p16 at a final concentration of 5 µg/ml in PBS buffer supplemented with $MgCl_2$ (5 mM). After a blocking step with 1% (w/v) BSA (1 h at 37°C), plates were incubated with proheads, either native or devoid of pRNA, at various concentrations (starting at 5 µg/ml) in PBS–5 mM $MgCl_2$ (1 h at 37°C). The specific binding of the proheads by the p16 carpet was analyzed by incubation with the polyclonal antibodies against the proheads (1:10 000) followed by peroxidase-labeled goat anti-rabbit Ig (Amersham) for 1 h at 37°C. The reaction was developed with *O*-phenylenediamine dihydrochloride (Sigma) and the reaction stopped with 2 M sulfuric acid. Wells were washed three times with PBS between assay steps.

p16–connector interaction

ELISA tests were also used to detect the interaction between p16 and the connector protein. The experiments were performed as described above, but the proheads were replaced by the same amount of connectors or connectors treated with the endoproteinase Glu-C from *Staphylococcus aureus* (protease V8) (34). The specific retention of the connector protein was detected with polyclonal antibodies against p10 (1:1000). To detect the effect of the nucleic acid in this interaction, connectors (5 µg/ml) were preincubated with increasing molar ratios of pRNA (pRNA:conector 0:1, 3:1, 10:1, 27:1) or DNAp3 (connector:DNAp3, 13:1, 4:1, 1:0), and the result of the interaction was analyzed as described above.

The interaction between p16 and the isolated connector was also tested in solution by immunoprecipitation assays. The p10-specific rabbit serum was bound to protein A–Sepharose for 60 min at room temperature and washed six times with TMS–1% NP-40 buffer. The connector protein (0.2 µg) and the ATPase gp16 (2 µg) were incubated together for 20 min at room temperature in TMS buffer containing 150 mM NaCl and the preformed matrix was used to immunoprecipitate protein p10 from the reaction after a 60 min incubation. After washing three times with 1% NP-40, 150 mM NaCl, TMS, the presence of both proteins in the immunocomplexes was detected by western blot analysis.

p16–RNA interaction

To generate a ^{32}P-radiolabeled pRNA probe, a DNA fragment of 194 bp containing the T7 RNA polymerase promoter and the 120 nt pRNA sequence was used in an *in vitro* transcription system in the presence of [α-^{32}P]GTP (400 mCi/pmol). In order to prepare an unrelated ^{32}P-radiolabeled RNA probe containing dsRNA regions, we used plasmid PNSZ (37) that generates a 240 nt RNA upon transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP (400 mCi/pmol).

RNA–protein interaction was tested as follows. Increasing amounts of the soluble p16 (or isolated native connectors, as a positive control) were mixed with a constant amount of labeled probe (0.015 pmol), incubated for 20 min at room temperature in 50 mM Tris–HCl pH 7.7, 5 mM $MgCl_2$, 300 mM NaCl, 2 mM DTT, buffer with or without ATP (1 mM) or AMP-PNP (1 mM) and filtered through a nitrocellulose filter in a dot-blot apparatus. After washing the filter with the same buffer, the proportion of the retained radiolabeled RNA probe was determined in a phosphorimager.

RESULTS

Overproduction and purification of a soluble and functional p16

The production of p16 under controlled conditions was started by cloning the Φ29 gene 16 into a commercial pET vector. The over-expression of the protein corroborated previously published results (24), showing that p16 aggregates and it is found in the insoluble fraction of the over-expressed extracts, regardless of the induction conditions (Fig. 1A, lane 4). As the chaperonin GroEL and its cochaperonin GroES help the correct folding of a wide variety of *E.coli* proteins (38), we decided to co-express these proteins with protein p16. To this end, the vector pAG (Cm^+) containing the GroEL and GroES genes (29) was transformed into the cells containing the pET-p16 (Amp^+). After testing a broad range of induction conditions, the maximum yield of soluble p16 was obtained after a previous induction of the GroEL/ES genes with arabinose (0.175 g/l) for 30 min at 37°C, followed by the induction of gene 16 with IPTG (0.5 mM) for 2 h more at 30°C. The result of the expression under these conditions is shown in Figure 1B (lane 3), where a significant amount of p16 was found in the soluble fraction.

In order to purify p16, the high amount of over-expressed GroEL was first discarded by precipitation with PEI at 400 mM NaCl. After the concentration of the soluble proteins, the extract was applied to an ionic-exchange phophocellulose chromatography. Protein p16 elutes from the column after increasing the NaCl concentration to 400 mM (Fig. 1C, lanes 2–6). Fractions containing p16 were further purified using hydroxylapatite chromatography, where p16 protein eluted at 150 mM phosphate concentration (Fig. 1D, lanes 4–8). Fractions containing pure p16 were pooled, dialyzed and stored at –20°C. The average yield of each purification was 3 mg of soluble p16 per 10 g of cells. The purified protein was further identified as the Φ29 p16 protein by N-terminal sequencing (28).

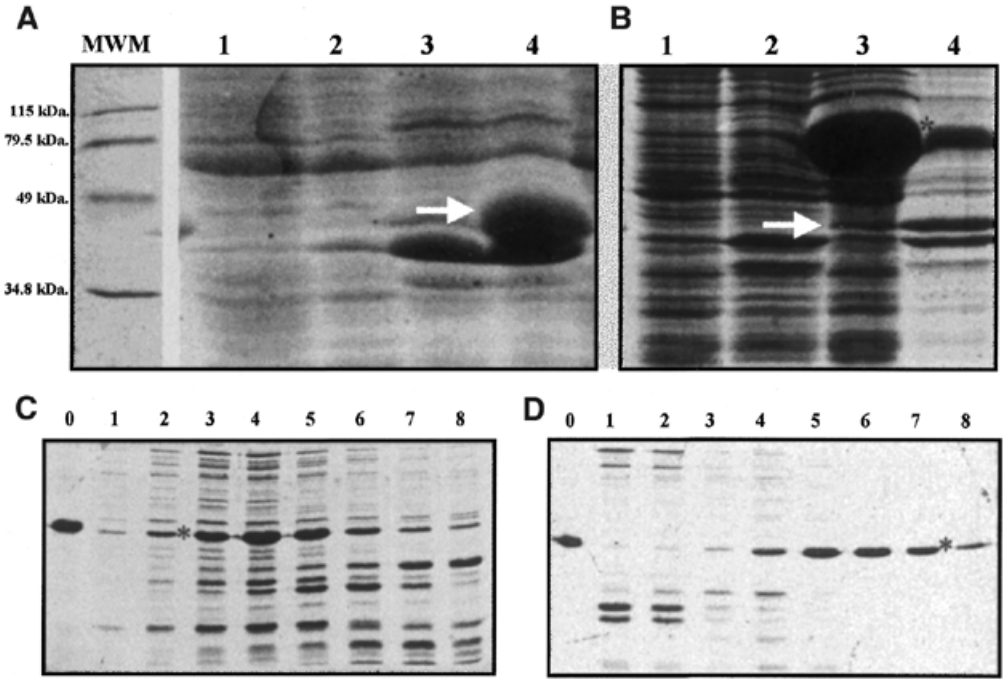

Figure 1. Overproduction and purification of a soluble p16. (**A**) Protein p16 was cloned into a pET vector and the over-expressed protein was found in the insoluble fraction. Lanes 1 and 2, supernatant and pellet of the non over-expressed extracts, respectively. Lanes 3 and 4, supernatant and pellet of the over-expressed extracts, respectively. p16 is marked with a white arrow. (**B**) The co-expression of the chaperonins GroEL/ES lead to the partial solubilization of p16. Lanes 1 and 2, supernatant and pellet of the non-over-expressed extracts, respectively. Lanes 3 and 4, supernatant and pellet of the over-expressed extracts, respectively. The soluble p16 is marked with an arrow, and GroEL with an asterisk. (**C**) After removal of the excess GroEL, the concentrated over-expressed soluble extract was loaded into an ionic-exchange phosphocellulose chromatography column (P-11) previously equilibrated with TBDS2 buffer. Lanes 1–6 show that the protein elutes from the resin after increasing the buffer NaCl concentration up to 400 mM. The soluble p16 is marked with an asterisk. (**D**) The soluble purified protein elutes from the hydroxylapatite column at 150 mM phosphate buffer concentration. Lanes 0 in (C) and (D) show a molecular weigh marker of 36 kDa. The proteins were analyzed by polyacrylamide gel electrophoresis and stained with Coomassie blue.

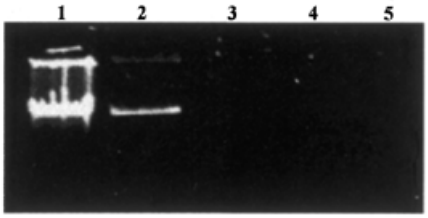

Figure 2. The soluble p16 is functional in the *in vitro* Φ29 DNA packaging assay. Agarose gel stained with ethidium bromide showing in: lane 1, the input DNAp3 in the packaging reaction; lane 2, the DNAp3 packaged in an assay containing all the components; lane 3, in the absence of the soluble p16; lane 4, ATP; or lane 5, pRNA.

To test whether the soluble p16 is functional in DNA translocation, an *in vitro* DNA packaging system was developed based on the one previously described (35). The system is composed of purified Φ29 proheads containing pRNA, the DNAp3 to be packaged (Φ29 DNA with the terminal protein p3 covalently linked to the ends), and the soluble p16 mixed in TMS buffer. After addition of ATP, the mixture is incubated for 30 min at room temperature, and the non-packaged DNA is digested with DNase. The packaged DNA is then extracted from the proheads and visualized in agarose gels with ethidium bromide. Figure 2 (lane 2) shows that the Φ29 genome was packaged inside the proheads only when all the components were present in the reaction, whereas no packaging was detected when protein p16 (lane 3), ATP (line 4) or pRNA (line 5) were absent. The results clearly show that soluble p16 is functional and that the assay is stringent for the *in vitro* DNAp3 packaging reaction.

p16 ATPase activity

Previous studies showed that p16 possesses an *in vitro* prohead and DNAp3-dependent ATPase activity (25), and the prohead dependence could only be uncoupled in an ammonium chloride containing buffer. Unfortunately, under these conditions the protein did not show any DNA packaging activity (26), and thus its behavior could not be compared to other well-studied terminases (39–43). We decided to characterize the ATPase activity of p16 under conditions where the protein is fully competent for DNA packaging. We found that the basal ATPase activity of p16 is very low, but is stimulated 10–20-fold by nucleic acids (Φ29 DNA and RNA) (Table 1). This stimulation is independent of the presence of other proteins (Φ29 connectors or proheads lacking pRNA) or ammonium chloride in the reaction. It is interesting to note that the specific pRNA is twice as efficient at stimulating the p16 ATPase activity as the same amount of the non-specific 5S rRNA. This could be related to the different binding affinity of p16 for the specific and non-specific RNAs, as will be discussed below.

Table 1. p16 ATP hydrolysis activity

Packaging reaction	ATPase activity (nmol ATP/μg p16/min)
p16 basal activity	2 ± 2
p16 + connectors	2 ± 2
p16 + pRNA-free proheads	3.6 ± 3
p16 + pRNA	37 ± 5
p16 + 5S rRNA	17 ± 3
p16 + DNAp3	30 ± 5
p16 + Proheads + pRNA	7.5 ± 3
p16 + pRNA-free proheads + DNAp3	50 ± 8
p16 + proheads + pRNA + DNAp3	50 ± 7

The p16 ATPase activity was assayed under conditions fully competent for the DNAp3 packaging reaction, in TMS buffer without ammonium chloride. Each value represents the average of three independent experiments.

To get an insight into the ATPase activity of the soluble p16 during the DNA translocation process, the ATPase, the DNAp3 and the proheads in the presence or absence of the specific pRNA were mixed under non-saturated conditions in TMS buffer. The reactions were started upon the addition of ATP (0.5 mM ATP containing 50 μCi [γ-^{32}P]ATP), and the ^{32}Pi released during the reaction was quantified. As expected, while pRNA containing proheads were fully active in DNA packaging, RNA-free proheads did not package DNA (not shown), although there was a similar increase in ATP hydrolysis in both cases (Table 1). These results suggest that the presence of prohead and DNAp3 induces the p16 maximum ATPase activity, but the presence of pRNA is needed to couple this activity to the packaging of DNA.

p16–prohead interaction

It has already been described that p16 interacts with Φ29 proheads containing the connector structure (25). In this work we used ELISA tests and sucrose gradient centrifugation assays to characterize such interaction. The ELISA tests show that Φ29 proheads bind to the vinyl plate when this is covered with the ATPase p16, regardless of the presence or absence of pRNA (Fig. 3A). We also used sucrose gradient centrifugation to test this interaction in solution (Fig. 3B and C). These experiments showed that after the centrifugation the ATPase cosedimented with proheads. This interaction, although is independent of the presence of pRNA or ATP in the reaction, showed a strong salt dependence, since the co-sedimentation of p16 with the proheads (with or without pRNA) occured only when the gradients were run in a low ionic strength buffer. Note that p16 appears in two peaks after the centrifugation with the proheads containing pRNA. The electron microscopy analysis of the fractions from the first peak revealed that they contained ribosome-like structures, which disappeared after the EDTA/RNase treatment of the prohead samples (not shown).

p16–connector interaction

The above described results suggest that the interaction of p16 with the Φ29 proheads is not mediated by the pRNA. It has been suggested that this interaction is actually mediated by the connector, although no direct data has been provided (25). To further characterize this point, we studied the interaction in solution of soluble p16 with the isolated connector using immunoprecipitation techniques. Both proteins were incubated

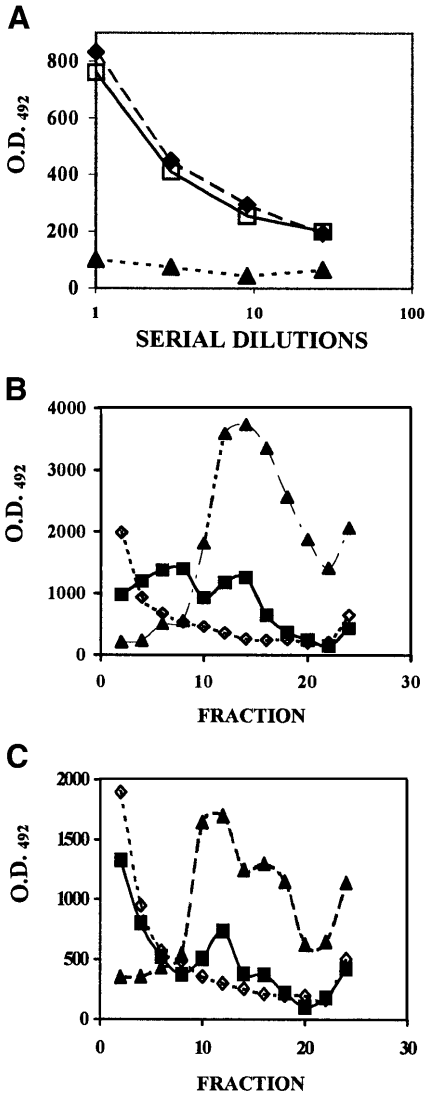

Figure 3. p16 interacts with Φ29 proheads. (**A**) Result of an ELISA test showing the specific retention by a p16 carpet of serial dilutions of Φ29 proheads (5 μg/ml starting concentration) with either pRNA (squares) and without pRNA (diamonds). The Φ29 proheads do not bind to the surface uncoated with p16 (triangles). The retained protein was measured by the absorbance at 492 nm. To check the interaction in solution, Φ29 proheads with (**B**) or without (**C**) pRNA were mixed with p16 in TMS buffer. The mixtures were loaded and centrifuged in linear sucrose density gradients containing TM buffer. The gradients were fractionated and the protein content of each fraction was analyzed by ELISA, as described in Material and Methods. (B) and (C) show that protein p16 (diamonds) remains at the top of the gradient when incubate alone. After incubation with proheads (triangles) with (B) or without (C) pRNA, p16 (squares) is now found associated to these protein structures. The same results were obtained in the presence of ATP (1 mM) in the reactions. Note that the prohead population is heterogeneous and it sediments either as a wide peak (B) or as a peak with a shoulder (C). The direction of the sedimentation is left to the right. O.D.$_{492}$, optical density at 492 nm.

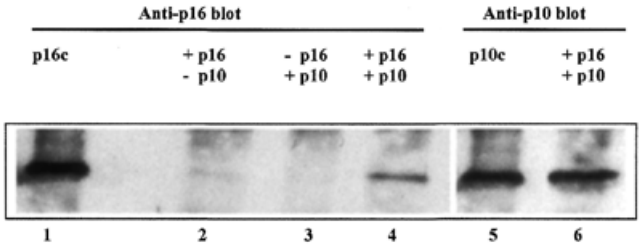

Figure 4. Immunoprecipitation of p16 with Φ29 connectors. The connector protein and the ATPase p16 were incubated together in TMS buffer. The preformed p10 serum-proteinA–Sepharose matrix was used to immunoprecipitate the protein p10 from the reaction. After washing the samples, the presence of p16 in the immmunocomplexes was detected with anti-p16 specific antibodies by western blot analysis (lanes 1–4). The figure shows that no signal of p16 was found in the reactions lacking the connectors (lane 2) or lacking the ATPase p16 (lane 3). However, the protein was found associated to the connector when both proteins were present in the reaction (lane 4). Lane 1, p16 mobility control (p16c). Lanes 5 and 6 show a western blot analysis with anti-p10 specific antibodies of the immunoprecipitated connectors. Lane 5, p10 mobility control (p10c); lane 6, the immunoprecipitated connector under the assayed conditions.

in TMS buffer (10:1 p16:connector molar ratio) and an anti-p10 polyclonal antibody (bound to protein A–Sepharose) was used for the immunoprecipitation of the complexes. The complexes were analyzed by western blot using polyclonal antibody sera against p16 and/or p10. The results show that p16 and the connector interact in solution (Fig. 4). The p16 signal was detected in the immunocomplexes only when it was previously incubated with the connector oligomer (Fig. 4, lane 4) whereas no signal of the protein was detected when the ATPase is incubated with the Sepharose–anti-p10 matrix in the absence of connectors (Fig. 4, lane 2).

The interaction was studied also using an immuno-assay based on the incubation of p16 attached to a plate with serial dilutions of the connector protein. These assays show that whereas native connectors interact with the p16 carpet, they lose this binding affinity after treatment with endoproteinase Glu-C from *S.aureus* (protease V8) (Fig. 5A). The proteinase V8 specifically removes the 13 residues of the N-terminal domain and the last 18 residues of the C-terminal of p10. In fact, the positively charged N-terminal domain is involved in the non-specific interaction of the isolated connector with nucleic acids (34). To check whether this end is involved in the interaction with p16, the connector was preincubated with increasing concentrations of Φ29 DNA or RNA. The results show that the nucleic acids strongly inhibit the binding of the connector to the p16 carpet (Fig. 5B and C). To test the interaction of p16 and nucleic acids we used biotinylated phage DNA and pRNA in ELISA. The absence of binding between the p16 carpet and the biotinylated nucleic acids (not shown) leads to the suggestion that both p16 and nucleic acids compete for the same N-terminal p10 domain. As is discussed below, an electrostatic interaction between p16 and the positively charged N-terminal end of p10 could explain the observed salt dependence of the p16 prohead (connector) interaction (see results above).

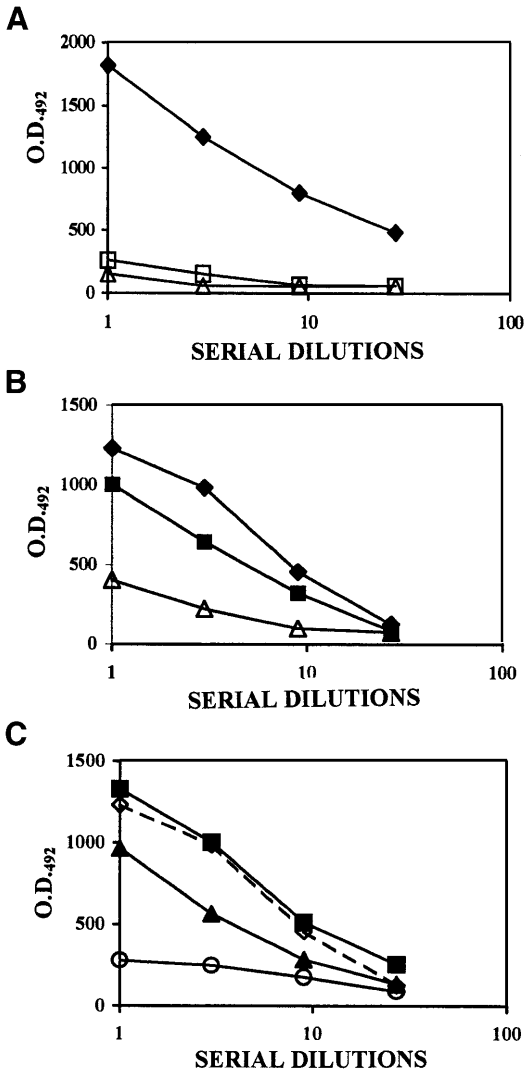

Figure 5. The N-terminal domain of Φ29 connectors is involved in the interaction with p16. (**A**) p16 covered ELISA plates retain serial dilutions of Φ29 connectors (diamonds) but not the connectors pretreated with protease V8 (squares). Control connector binding to a p16-free ELISA plate surface after the BSA blocking step (triangles). (**B**) and (**C**) show that the blocking of the N-terminal end of p10 with increasing concentrations of DNAp3 (B) and pRNA (C) inhibits the connector binding to the p16 carpet. In (B) connector:DNAp3 molar ratio 24:1 (triangles), 13:1 (squares), 1:0 (diamonds). In (C) pRNA:connector molar ratio 27:1 (circles), 10:1 (triangles), 3:1 (diamonds), 0:1 (squares). $O.D._{492}$, optical density measured at 492 nm, as an estimation of the retained connectors.

p16–(p)RNA interaction

As mentioned above, a peculiar feature of the Φ29 DNA packaging system is the requirement of a specific RNA molecule (called pRNA), coded by the phage genome (44,45). It has been reported that the isolated Φ29 connectors, but not p16, are able to bind a labeled pRNA probe in solution (45). Nevertheless, the presence of a RNA binding conserved motif in the p16 sequence (26), and the modulation of the p16 ATPase activity by both (p)RNA and DNA (Table 1) suggest that the protein might interact with these nucleic acids in solution. To further

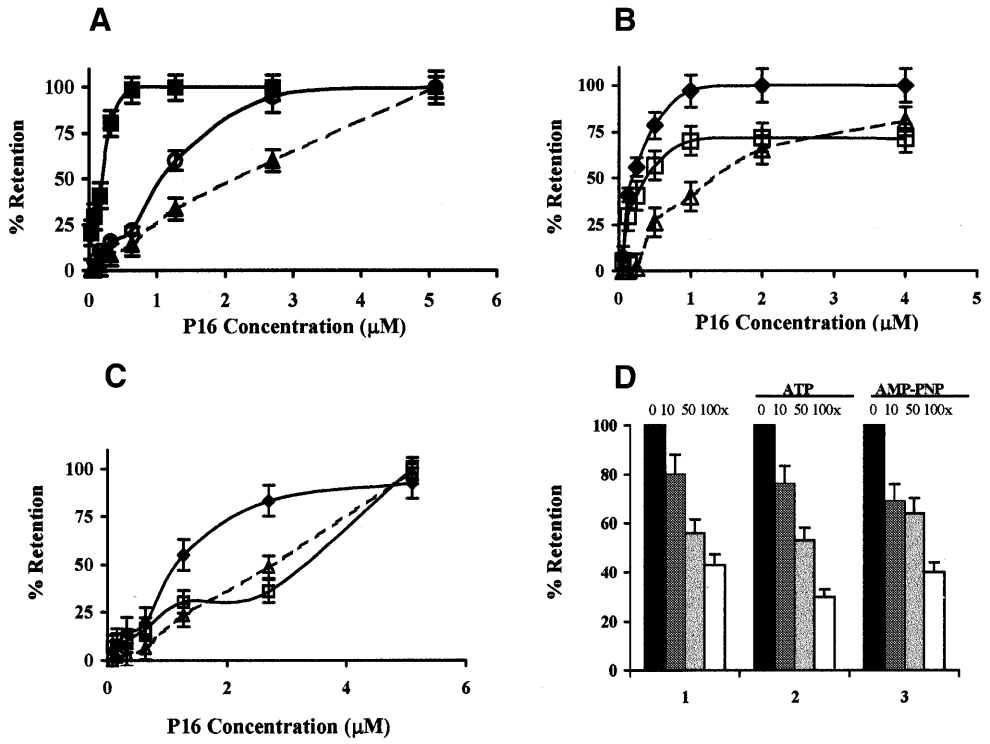

Figure 6. Interaction of the soluble p16 with RNA. Increasing concentrations of p16 (μM) were incubated with a constant amount of radiolabeled RNA probes (0.015 pmol) in a high salt buffer (300 mM NaCl) containing either ATP or AMP-PNP. The samples were filtered through a nitrocellulose filter, washed and the proportion of the retained RNA probe was determined in a phosphorimager. (**A**) p16 binds the pRNA probe with a $K_d = 1.5 \times 10^{-7}$ (squares) an affinity 10-fold higher than that for the non-specific RNA_{240}, $K_d \approx 10^{-6}$ (circles). The binding affinity for the pRNA probe strongly decreases if the pRNA is previously denatured (triangles). (**B** and **C**) Effect of 1 mM ATP and 1 mM AMP-PNP over the p16–RNA binding. (B) p16-specific pRNA interaction in the presence of AMP-PNP (triangles), ATP (squares) or without nucleotide (diamonds). (C) p16–non-specific RNA_{240} interaction in the presence of AMP-PNP (triangles), ATP (squares) or without nucleotide (diamonds). (**D**) Effect of Φ29 DNA on the p16–pRNA binding. The p16 (0.2 μM in 1 and 2, 1 μM in 3) pRNA interaction was competed with increasing amounts of Φ29 DNA (1) [pRNA:DNA molar ratio 1:0 (0×), 1:10 (10×), 1:50 (50×), 1:100 (100×)]. Similar values were obtained in the presence of ATP (2) or AMP-PNP (3). Each value in every assay was the average of at least three independent experiments.

characterize these events we studied the interaction of the purified and soluble p16 with RNA.

An *in vitro* labeled [α-^{32}P]GTP transcript-specific pRNA and an unrelated RNA probe, containing double-stranded regions and of a similar size (240 nt) RNA_{240}, were incubated with increasing amounts of p16 in TMS buffer containing a high salt concentration (300 mM) and 1 mM DTT. The samples were filtered through nitrocellulose filters and the retained label was measured in a phosphorimager to calculate the affinity constant between the interacting species (Fig. 6). The results show that p16 bound RNA in solution. The affinity of p16 towards the specific pRNA probe ($K_d = 1.5 \times 10^{-7}$ M) was around seven times higher than that of the non-specific RNA_{240} probe ($K_d = 8 \times 10^{-7}$ to 10^{-6} M). The incubation of heat-denatured pRNA with the ATPase yielded values similar to those of the non-specific RNA (Fig. 6), suggesting that this interaction depends on the secondary (and/or tertiary) structure of pRNA in solution. It is interesting to note that the affinity of p16 towards the native pRNA probe was very similar to that of the isolated connector for the probe under the same incubation conditions ($K_d = 1.6 \times 10^{-7}$ M; not shown).

We also analyzed the effect of different nucleotides (ATP and AMP-PNP) on the interaction of p16 with the RNA probes. Figure 6B shows that the incubation of pRNA with p16 in the presence of 1 mM AMP-PNP (a non-hydrolysable analog of ATP), decreased 10-fold the affinity of the ATPase towards pRNA, similar to the affinity of the protein for the unrelated RNA_{240} probe in the absence of nucleotide. However, if the non-hydrolysable AMP-PNP was replaced by the same amount of ATP, a small decrease of the total amount of retained pRNA was observed but the protein maintained the standard affinity constant for the nucleic acid. These results suggest a dynamic regulation of the p16–RNA interaction by the sequential binding and hydrolysis of ATP. A similar behavior is observed in the interaction of p16 with the non-specific RNA_{240} in the presence of nucleotides (Fig. 6C). In this case, because of the difficulty in increasing the p16 concentration >5 μM the final decrease of the binding affinity could not be determined.

We also tested the competition by Φ29 DNA on the binding of p16 to the pRNA probe. Increasing amounts of Φ29 DNA (either with the terminal protein p3, or without it) were added to a mixture of p16 and pRNA under non-saturated conditions. The results are shown in Figure 6D. Increasing concentrations of Φ29 DNA decreased the amount of pRNA retained by the p16 in the nitrocellulose membrane, suggesting that this nucleic acid competed with the pRNA probe in the binding to p16. In fact, our results showed that soluble p16 interacted with

the Φ29 genome in solution (not shown), consistent with previously described results (27). It is interesting to note that the competition did not increase in the presence of nucleotides (AMP-PNP) as it would be expected if we take into account that the affinity for the pRNA was reduced 10-fold under these conditions. These results suggest that the effect of nucleotide binding could regulate not only the dynamic binding to the RNA but also the binding to the DNA. This behavior may be important to understand the dynamic function of the DNA packaging machinery.

DISCUSSION

The packaging of DNA to quasi-crystalline densities into preformed protein shells (proheads) is a sequential and fast process that involves the relative movement of different components of nucleoprotein complexes, driven by the ATP binding and hydrolysis (reviewed in 3–5). The study of this process in the Φ29 system has been limited by the fact that one essential component, the p16 ATPase, aggregates in inclusion bodies when over-expressed in *E.coli* (24), as indeed is the case of other terminases (5).

In this paper we show that the co-expression of p16 with the chaperonins GroEL/ES and the induction at 30°C favors the production of p16 in soluble and active form. When induced at 30°C, the production rate of the enzyme is slower than that at 37°C, and the lower p16 concentration, in addition to the expression of GroEL/ES, facilitates the correct folding of the protein, and leads to the solubilization of a significant amount of the p16 protein. Although we cannot rule out that some soluble p16 could be mis-folded, the purified and soluble p16 preparation is functional and essential in the Φ29 DNA *in vitro* packaging system, and it enables the relationship of p16 with the rest of the DNA packaging machinery components to be studied under functional conditions.

Protein p16 contains both 'A-type' and 'B-type' ATP binding consensus sequences and it has been reported to possess *in vitro* ATPase activity (25,26). We decided to study the ATPase activity of the soluble p16 under conditions fully competent for DNA packaging. Our results show that the protein p16 is a nucleic acid-dependent ATPase. The basal ATPase activity of this protein is very low and is not stimulated by connectors or proheads. However, the ATPase activity of p16 protein is strongly stimulated (10–20-fold) by nucleic acids, either the specific Φ29 DNA, pRNA or the non-specific 5S rRNA. This stimulation is completely independent of the presence in the reaction of connector, proheads or ammonium chloride. These results show that interaction between p16 and the prohead is not needed to activate the ATPase activity of the protein, in contrast with previously reported results (25,26). In fact, while proheads without pRNA do not induce p16 ATPase activity, pRNA containing proheads moderately activate the ATPase, indicating that even the low amount of pRNA present in these proheads (as compared to that present in the free pRNA assays) is able to trigger the ATPase activity. There is a possibility that a fraction of mis-folded, soluble p16 may become active due to a nucleic acid mediated re-folding, but our data favor the hypothesis that the bulk of the protein shows an intrinsic nucleic acid-dependent ATPase activity.

The maximum yield of ATP hydrolysis by p16 is obtained when all the components needed for DNA packaging are present in the reaction. Under these conditions, the ATPase activity is stimulated 25-fold while the DNA is packaged into the proheads. Surprisingly, the same stimulation is observed even if the DNA is not packaged (i.e. when pRNA is eliminated from the system). This suggests that Φ29 DNA in the presence of proheads activates the maximum ATPase activity of p16, while the binding of pRNA to the connector is essential to couple this energy to the actual packaging of DNA. Our results thus indicate that p16 presents a non-specific nucleic acid-dependent ATPase activity not necessary coupled to DNA packaging (non-pac ATPase). This ATP hydrolysis behavior is quite similar to that described for the large subunits of the T3, T4, T7, λ and P2 phage terminases (39–43). Despite the lack of sequence homology, all the known phage packaging ATPases present a nucleic acid dependence that could reflect a common mechanism for the packaging of DNA.

The pivotal role of the ATPase p16 for the transport of DNA indicates that it must transiently interact with the prohead during the DNA packaging process. There is no direct data about the location of p16 in the packaging machinery, although Simpson *et al.* (11) have recently suggested its presence in the pRNA domain of the proheads. Our results show that the soluble p16 interacts with Φ29 proheads in an ATP independent way, and this interaction does not involves the presence of pRNA. The interaction between p16 and the proheads was only detected under low ionic strength, which suggests that the electrostatic forces could play an important role in such interaction.

It has been previously suggested that the interaction of p16 and the prohead could be mediated by the connector (25). Our results indicate that p16 interacts with native Φ29 connectors in solution and there are several lines of evidence that suggest that the N-terminal domain of protein p10 (the protein that builds up the 12-fold oligomeric connector) is the binding region of p16. On the one hand, the ATPase p16 does not interact with the connector lacking the N-terminal region after treatment with the protease V8. Furthermore our results show that both DNA and RNA strongly compete with the p16 for the binding to the connector, which also suggests that the N-terminal end of p10 could be located near the ATPase binding point site, as the p10 N-terminus is involved in binding of nucleic acids (34). In fact, this domain forms a positively charged ring around the connector quaternary structure, being the only region with a charge accumulation on the outside of the connector (11). Electrostatic interactions between these positive amino acids with some of the negative clusters of p16 could explain the salt dependence of the p16–connector interaction. It has already been suggested that electrostatic forces actually play an important role in the interaction between the phages T3 and λ large terminases subunits and their respective connectors (46,47).

It is interesting to note that when the connector is assembled into the prohead the N-terminal end of p10, located in the narrower domain of the connector, is available outside the prohead (7). Our data suggest that the pRNA ring and the p16 ATPase would be close together around this connector region. The neighborhood of p16 and the pRNA is consistent with the possibility of their interaction during the packaging reaction. This hypothesis is supported by the fact that p16 has an RNA recognition motif (26,48) and pRNA strongly stimulates the p16 ATPase activity. In fact, our data show that p16 interacts

with RNA in solution, in contrast with some previously published results (45). The affinity of p16 for a specific pRNA probe is seven times higher than the affinity for a non-specific RNA probe of similar size. When the pRNA is denatured, the affinity of p16 for the nucleic acid strongly decreases, suggesting that the interaction depends upon the secondary or tertiary structure of the pRNA. It has been reported that the structure of the pRNA in solution is also critical for the interaction with the connector assembled into the prohead (49).

An interesting feature of the interaction between p16 and pRNA is that it is regulated by the binding and hydrolysis of nucleotides. Our results show that when p16 was incubated with pRNA in the presence of AMP-PNP, the nucleotide-bound conformation of the protein had reduced ability to interact with pRNA (a 10-fold decrease of the binding affinity constant was observed). However, when AMP-PNP was replaced by the hydrolysable ATP, the protein restores its binding affinity for the nucleic acid. Due to the fact that pRNA strongly stimulated the ATPase activity of p16, it is possible that after hydrolysis p16 adopts a nucleotide-free conformation that would interact better with the pRNA. On the other hand, the competition of DNA with pRNA for p16 binding was not increased in the presence of ATP or AMP-PNP. Taking into account that the binding affinity for the pRNA decreased 10-fold after AMP-PNP binding, this result implies that the p16–nucleotide-bound conformation affects not only the interaction with RNA but also with the DNA.

Conformational changes induced upon ATP interaction were reported for the phage T3 terminase large subunit (47,50), although the p16 case is the first description for a phage ATPase that relates a putative conformational change with the nucleic acid interaction. This dynamic interaction between p16 and the pRNA (and probably DNA) modulated by ATP binding and hydrolysis, could explain the structural transitions involved in the DNA translocation process.

Due to the intrinsic symmetry of DNA, its translocation involves repeated movements, or repeated symmetry operations. The three-dimensional reconstruction of the Φ29 proheads during the packaging reaction show that the DNA is threaded through the inner hole of the translocating machine (11). If the translocase was free to rotate the DNA would simply translate, like a screw passing through a rotating nut. This relative movement would not produce any twist in the DNA molecule preventing supercoilings that complicate its packaging. The biochemical characterization of p16, and its similarities with other well-defined phage terminases, support a model for DNA translocation in which the ATPase, bound and organized around the narrower domain of the connector, acts to produce the power stroke that pumps the DNA in a linear way into the prohead (A.Guasch, J.Pous, B.Ibarra, F.X.Gomis-Ruth, J.M.Valpuesta, N.Sousa, J.L.Carrascosa and M.Coll, manuscript submitted; 4). The pumping force might come from an ordered conformational change induced in the ATPase by the binding of ATP and its nucleic acid dependent hydrolysis. Due to the symmetry mismatch between the 12-fold connector and the 5-fold prohead, the connector might be free to rotate relative to the prohead structure (51). In this model the connector could rotate in a passive way, driven by the electrostatic forces generated between the DNA negative sugar–phosphate backbone and the positive charges distributed along the inner channel of the connector (A.Guasch, J.Pous, B.Ibarra, F.X.Gomis-Ruth, J.M.Valpuesta, N.Sousa, J.L.Carrascosa and M.Coll, manuscript submitted). This rotation might couple the phosphate-backbone DNA structure with the next ATPase to be fired.

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