A PRECURSOR OF THE NECK APPENDAGE PROTEIN OF *B. SUBTILIS* PHAGE Φ 29

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1. Introduction

The cleavage of precursor proteins to polypeptides of smaller molecular weight is a mechanism involved in the morphogenesis of the head and DNA encapsulation in T4 [1–4]. In this paper we present evidence which indicates the existence of a precursor in the synthesis of the protein which forms the neck appendages of the *Bacillus subtilis* phage Φ29 [5]. Pulse-chase experiments indicate that the neck appendage protein (NP1), of molecular weight 80 000, is synthesized as a precursor (P-NP1) with a molecular weight about 90 000, and tryptic peptide analysis of both proteins (P-NP1 and NP1), show that they contain very similar peptides. These results indicate a precursor–product relationship of proteins P-NP1 and NP1. Infection under nonpermissive conditions with temperature-sensitive mutants in cistron B, the gene coding for the neck appendage protein precursor [6], which have a clear plaque phenotype, gives place to the accumulation of protein P-NP1 in higher amount than that present after normal phage infection. Some evidence for the existence of a precursor of the Φ29 neck appendage protein has been given by Anderson and Reilly [7].

2. Materials and methods

2.1. *Bacteria, phage, medium and ultraviolet irradiation*

B. subtilis 11ONA, an asporogenous mutant of *B. subtilis* 168 try$^-$, was obtained from Dr. F. Moreno. Bacteria were grown in minimal medium and irradiated for 7.5 min with a 15 W germicidal lamp located 50 cm above the sample as indicated [8].

The temperature-sensitive mutants tsB66, tsB73 and tsB74 were from the collection of Talavera et al. [9]. The preparation of phage stocks and the phage assays were as described [9].

2.2. *Labelling of bacteria*

For the pulse-chase experiments the irradiated bacteria were suspended, at 5×10^8 cells/ml, in complete medium containing 0.5 mM amino acids except leucine, which was 0.01 mM. The bacteria were infected with wild type phage at a multiplicity of 20 and incubated at 37°C. A sample of the irradiated bacteria was kept uninfected as control. At 22 min the irradiated bacteria, infected and uninfected, were pulse-labelled with [^{3}H]leucine (40 μCi/ml; 0.8 μM) and two min later, a 100-fold excess of non-radioactive leucine was added. A sample was immediately removed, added to two volumes of frozen salts and the cells were sedimented by centrifugation for 10 min at 10 000 *g* at 4°C. The cells were washed once with two volumes of salts and sedimented as before. The remainder of the pulse-chased culture was incubated for five additional min and the cells were removed and washed as before. A control of wild type-infected cells, pulse-labelled with [^{14}C]leucine (7.5 μCi/ml; 0.023 mM) from 21 to 26 min postinfection and chased with a 100-fold excess of non-radioactive leucine for five min, was also carried out. To follow phage development samples were taken at different times from the infected cultures. The time course of the phage development was normal and the burst size was about 20% of that obtained in non-irradiated bacteria.

For the isolation of radioactive proteins from wild type-infected cells for tryptic peptide analysis, the irradiated cells were resuspended, at 5×10^8 cells/ml, in complete medium containing 0.5 mM amino acids except methionine which was 7.5 μM. The bacteria

were infected with phage Φ29 at a multiplicity of 20 and incubated at 37°C. A volume of the culture was labelled from 33.5 to 35 min with [^{35}S]methionine (65 μCi/ml; 0.35 μM). After the pulse, the cells were removed and washed as indicated before. Another volume of the infected cells was labelled from 15 to 35 min with [^{3}H]methionine (50 μCi/ml; 7.8 μM). Samples were taken at different times from the infected culture to follow phage development. A control of irradiated cells was kept uninfected and labelled with [^{35}S]methionine or [^{3}H]methionine, respectively, as indicated before for the infected cells.

For the analysis of the proteins induced in cells infected with temperature-sensitive mutants in cistron B, the cells were irradiated for 7 min and resuspended in complete medium with 0.5 mM amino acids except methionine which was 0.01 mM. The cells were infected with either wild type phage Φ29 or with ts mutants in cistron B (tsB66, tsB73 and tsB74) at a multiplicity of 20 and incubated at 42°C. The bacteria infected with the ts mutants and uninfected cells were labelled with [^{35}S]methionine (32 μCi/ml; 0.17 μM) from 15 to 30 min post-infection. The wild type infected bacteria were labelled at the same time with [^{3}H]methionine (50 μCi/ml; 7.8 μM). After the pulse, the cells were added to two volumes of frozen salts, sedimented and washed as indicated before. As a control, samples were taken at different times from the infected cultures to follow phage development.

2.3. *Polyacrylamide gel electrophoresis*

The labelled cells were resuspended in half the original volume of lysis buffer (30 mM Tris–HCl, pH 6.8; 1 mM EDTA; 0.58 mM phenylmethylsulfonylfluoride) and incubated with lysozyme (280 μg/ml) for 2.5 hr at 0°C; the sample was then frozen and thawed three times and treated with pancreatic ribonuclease (10 μg/ml) for 30 min at 0°C. In some cases, to remove residual radioactive amino acid, the protein was precipitated by addition of 10 vol of acetone after heating for 2 min in a bath of boiling water in the presence of 1% (w/v) sodium dodecyl sulfate and 5% (v/v) 2-mercaptoethanol. The precipitated protein was sedimented by centrifugation for 15 min at 10 000 *g*. The acetone was removed and the residue dried under a nitrogen stream.

The sample to be analyzed by electrophoresis was dissolved in 0.2–0.3 ml of a buffer containing 0.0625 M Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol and 6 M urea and heated for 3 min in a bath of boiling water. Electrophoresis was carried out in 10 cm long gels containing 10% acrylamide, 0.25% *N*, *N'*-methylenebisacrylamide and 0.1% sodium dodecylsulfate [8], at a constant voltage op 90 V for about 5.5 hr. The gels were cut in 0.8 mm slices and the radioactivity determined as described [8].

2.4. *Tryptic peptide analysis*

Φ29-Infected cells, labelled with [^{35}S] or [^{3}H] methionine as indicated before, were lysed and subjected in polyacrylamide gel electrophoresis. The gels were cut in 0.8 mm slices and the protein was eluted out of each gel by overnight incubation at 37°C in 0.5 ml of 0.1% sodium dodecyl sulfate and 0.58 mM phenylmethylsulfonylfluoride after freezing and thawing three times. A 25 μl aliquot of each fraction was counted and the peak fractions of the proteins to be analyzed were pooled and lyophilized; the residue was dissolved in 1 ml of water. Electrophoresis of uninfected cells, labelled in the same conditions as the infected cells, showed that the radioactivity present in the position corresponding to the protein peaks to be subjected to tryptic digestion was negligible.

A mixture of ^{35}S – and ^{3}H-labelled proteins was reduced, carboxymethylated and digested with trypsin after removing the sodium dodecyl sulfate as described elsewhere [10]. The tryptic digest was applied to a column (22 × 0.9 cm) of Beckman PA-35 ion exchange resin maintained at 50°C. The column was developed under pressure, at a rate of about 1 ml/min, with a linear gradient of 200 ml of 0.2 M pyridine–acetic acid (pH 3.1) and 200 ml of 2 M pyridine–acetic acid (pH 5.0). Fractions of 2 ml were collected in small vials (4.5 cm × 1.2 cm) containing a disk of glass-fiber (Whatman GF/A, 2.4 cm diameter), dried at 90°C and counted as described [5].

3. Results and discussion

Fig. 1A shows that in cells infected with phage Φ29 and labelled in a pulse from 22 to 24 min, a protein (P-NP1), of molecular weight about 90 000, appears. This protein is essentially absent in wild type-infected cells labelled from 21–26 min post-infection followed by five min of chase. When the infected cells, labelled

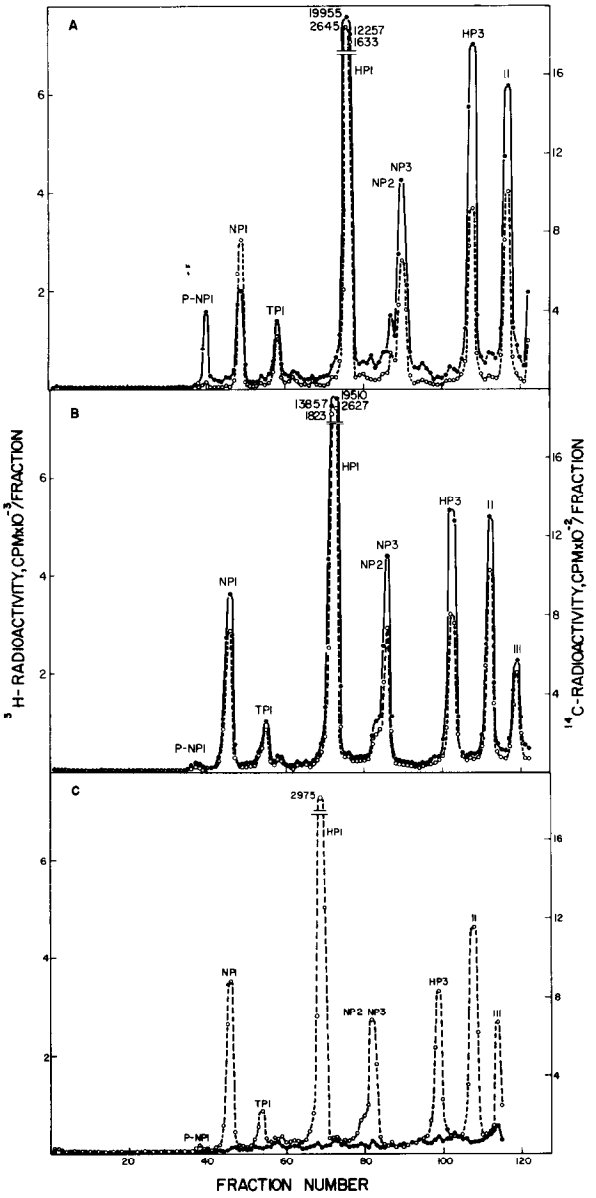

Fig. 1. Polyacrylamide gel electrophoresis of the proteins labelled in a pulse-chase experiment in *B. subtilis* infected with phage ϕ29. *B. subtilis* 110NA cells, irradiated for 7.5 min as indicated in Materials and methods, were infected with phage ϕ29 and labelled with [^{3}H]leucine in a 2 min pulse from 22–24 min after infection, followed by a 5 min chase. Uninfected cells were labelled under the same conditions. Wild-type infected cells were also labelled with [^{14}C]leucine in a 5 min pulse (from 21–26 min post-infection) and 5 min chase. Electrophoresis was carried out as described in Materials and methods. The anode is to the right of the fig. A: Co-electrophoresis of the proteins induced in wild type-infected cells labelled in a 2 min pulse (—●—) and a 5 min pulse and a 5 min chase (- - - ○ - - -); B: Co-electrophoresis of the proteins induced in wild-type-infected cells labelled in a 2 min pulse followed by a 5 min chase (—●—) and a 5 min pulse and a 5 min chase (- - - ● - - -); C: Coelectrophoresis of the proteins from uninfected cells labelled in a 2 min pulse (—●—) and wild type-infected cells labelled in a 5 min pulse and a 5 min chase (- - - ○ - - -). The pattern obtained in uninfected cells labelled in a 2 min pulse followed by a 5 min chase is identical to that shown in C.

for two min, were chased with an excess of non-radioactive leucine for five min, most of the radioactivity present in the 90 000 molecular weight protein dissappeared with a parallel increase in the amount of protein NP1 (fig. 1B). The 90 000 mol. wt. polypeptide is not present in uninfected cells (fig. 1C). The above result suggest a precursor–product relationship of proteins P-NP1 and NP1. A new polypeptide of molecular weight 10 000 which could result by the cleavage of P-NP1 to NP1 was not found after electrophoresis in gels containing 12.5% acrylamide in the presence of sodium dodecyl sulfate and urea, where proteins of molecular weight as small as 4000 do not run out of the gel [8]. It is possible that the fragment overlaps with other phage induced proteins or that it is degraded to low molecular weight products that run out of the gel.

To show a structural relationship of NP1 and P-NP1 by another way, the methionine-containing tryptic peptides of both proteins were analyzed. Fig. 2 shows that the tryptic peptides of both proteins are very similar, indicating a close relationship between them.

An analysis of the proteins synthesized after infection of *B. subtilis* su$^-$ with mutant susB47 indicated that proteins P-NP1 and NP1 were not synthesized [6]. Instead, a protein fragment of lower molecular weight (75 000) appeared. Tryptic peptide analysis showed that this fragment has peptides similar to those of P-NP1 and NP1, indicating that cistron B codes for the precursor of the phage appendage protein (NP1) [6]. Pulse-chase experiments in susB47-infected *B. subtilis* su$^-$ indicated that the 75 000 mol. wt. fragment is not synthesized as a higher molecular weight

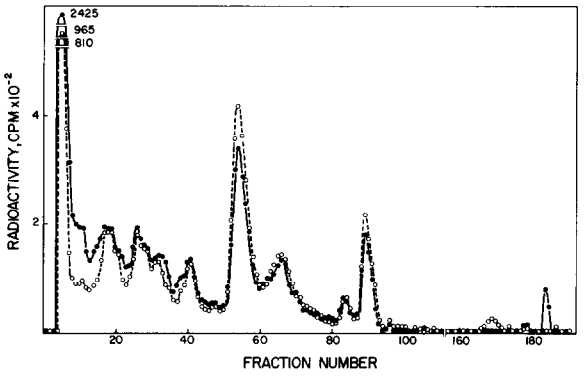

Fig. 2. Tryptic peptide analysis of proteins P-NP1 and NP1. ^{35}S-labelled P-NP1 and ^{3}H-labbelled NP1, synthesized in wild type-infected *B. subtilis*, were isolated as described in Materials and methods. A mixture of P-NP1 (15 000 cpm) and NP1 (26 000 cpm) was reduced, carboxymethylated, treated with trypsin and chromatographed through a column of Beckman PA-35 ion exchange resin as indicated. The recovery was about 80%. (- - - ○ - - -) ^{35}S-P-NP1; (—●—) ^{3}H-NP1.

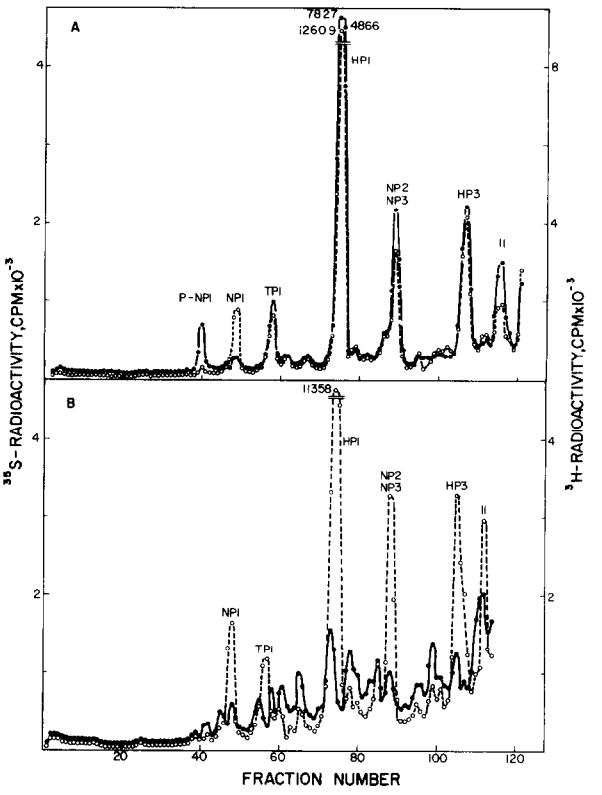

Fig. 3. Polyacrylamide gel electrophoresis of the proteins labelled in tsB73-infected *B. subtilis*, at 42°C. *B. subtilis* 110NA cells, irradiated for 7 min and incubated at 42°C, were infected with mutant tsB73 and labelled with [^{35}S]-methionine from 15–30 min post-infection. Uninfected cells were labelled under the same conditions. Wild type-infected cells were labelled with [^{3}H]methionine at the same time. Electrophoresis was carried out as described in Materials and methods. The anode is to the right of the fig. A: Co-electrophoresis of the proteins induced in tsB73-infected cells (—●—) and wild type-infected cells (- - - ○ - - -); B: Co-electrophoresis of the proteins from uninfected cells (—●—) and wild type-infected cells (- - - ○ - - -).

precursor (Ana Camacho, unpublished results). To see whether or not infection of *B. subtilis* with ts mutants in cistron B is blocked in the conversion of precursor to product, the proteins synthesized at 42°C in cells infected with three ts mutants in cistron B were labelled in a 15–30 min pulse and analyzed by polyacrylamide gel electrophoresis. As shown in fig. 3A, a higher amount of precursor of protein NP1 (P-NP1) is present in tsB73-infected cells than in wild-type infected cells labelled at the same time. The proteins labelled under the same conditions in irradiated, uninfected cells are shown in fig. 3B. A similar result was obtained after infection with mutants tsB66 and tsB74. A control experiment carried out under the same conditions as those described in fig. 3, except for the temperature which was 30°C, showed that infection with mutants tsB73 under permissive conditions produces a normal conversion of P-NP1 to NP1 (results not shown).

An important question is whether the precursor of protein NP1 is cleaved before or after its assembly into the phage particles. An analysis of the proteins present in the particles produced in *B. subtilis* infected with ts mutants in cistron B under restrictive conditions, may aid to answer this question. Also, the study of whether or not P-NP1 is cleaved to NP1 in mutants which are unable to assemble phage structural proteins may clarify this problem.

Bacteriophage No. 11 of *Klebsiella* induces the synthesis of a polysaccharide depolymerase activity which is present in the spikes of the phage particles [11]. These spikes seem similar to the neck appendages of phage Φ29. The induction of such an activity after Φ29 infection and its possible relationship to P-NP1 and/or NP1 are being studied. It is worth noting that

infection of *B. subtilis* with ts mutants in cistron B gives a clear plaque. The same is true in the case of mutant susB47. Anderson and Reilly have found that infection of *B. subtilis* with clear-plaque mutants of phage Φ29 also produce an accumulation of the appendage precursor protein, P (J) [7]. It is possible that these clear-plaque mutants are also mutants in the cistron which codes for the precursor of the appendage protein (J).

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