Studies on Protein Synthesis in Kappa Particles

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Further evidence that kappa endosymbionts of stock 7 *Paramecium biaurelia* [*Caedibacter varicaedens*] are bacteria is given by the discovery of a prokaryotic translational system in these particles. Kappa proteins incorporate ^{35}S in *in vivo* experiments. However, there is no evidence that the translational systems of the host parametia and the symbionts are shared. On the contrary, experiments with symbiont-bearing and symbiont-free strains strongly indicate that all major proteins found in kappa are synthesized in kappa. In this respect, kappa clearly differs from cell organelles. Protein synthesis in kappa is not inhibited by cycloheximide and the aminoglycoside G418, which do inhibit, although not completely, protein synthesis of the host cells. On the other hand, chloramphenicol quantitatively blocks translation in kappa without any obvious effects on the translational system of the *paramecium* cytoplasm.

Stock 7 kappa was isolated and purified using a filter paper column. The purity of kappa preparations thus obtained was significantly improved by adding a preliminary deciliation step.

INTRODUCTION

Kappa and other endosymbionts are Gram-negative bacteria, living in the cytoplasm or nucleus of *Paramecium* and other ciliated protozoa. They obviously profit from their host, acquiring from it all necessary nutrients, and their intracellular site offers protection from the external environment. Adaptation to an intracellular mode of life has led to degenerative changes: most symbionts are slow growing, and have lost their capacity to grow outside their host. The genome size of several endosymbionts appears to be small (Soldo & Godoy, 1973, 1974; Schmidt, 1982), and the genome seems to be present in a rather high number of copies (5–20) per cell. Numerous other studies have shown the closeness of the relationship between symbionts and host. For instance, many symbionts depend for their maintenance on genes in the host genome (Balsley, 1966; Preer *et al.*, 1974*a*). Studies by Beale & Jurand (1966) indicated that the degree of specificity between these genes and the symbionts is very high. Stock 51 kappa contains an antigen which is found in the cytoplasm of kappa-bearing cells, but not in sensitive, symbiont-free strains (Mueller, 1966). Furthermore, strong arguments exist (Preer, 1977) that the association of kappa and *Paramecium* is very ancient, perhaps going back to pre-Cambrian times. If this is true, then Sonneborn's original view that kappa is an intrinsic organelle of *Paramecium* may be more accurate than the view that it is merely a parasite (for reviews see Sonneborn, 1959; Preer *et al.*, 1974*a*; Preer & Preer, 1983).

One of the most important discoveries on cell organelles such as mitochondria and chloroplasts during the past 15 years was the finding that they contain an independent system of protein synthesis. Although a number of mitochondrial proteins are synthesized by the cytoplasm, some are made in the organelles themselves (for reviews see Ashwell & Work, 1970; Poyton & Groot, 1975; Tzagoloff *et al.*, 1979). The results reported here demonstrate that in this

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respect kappa is much more autonomous than cell organelles. It does have an independent system of protein synthesis, but there is no evidence for a coordinate regulation of the kappa and the paramecium translational systems. No proteins synthesized in the cytoplasm could be found in kappa.

METHODS

Cultures. Stock 7 of *Paramecium bicaurelia* with *Caedibacter varicaedens* (Preer & Preer, 1982, 1983), commonly known as 7 kappa, from the Indiana collection of paramecia was used and cultured as described previously (Sonneborn, 1950) in Cerophyl culture medium on *Klebsiella pneumoniae*. Cultures were purified by washing single cells in several changes of the following sterile solution: yeast autolysate, 1.33 g l⁻¹ (Basamin Bush, Bush Co., St Louis, Missouri, USA); 2.5 mM-sodium acetate; Cerophyl, 0.88 g l⁻¹; 1.2 mM-Na₂HPO₄ (Preer *et al.*, 1974b). Purified cultures were kept monoxenically throughout the course of this study.

Isolation of kappa-free cells. Sensitive (kappa-free, 7S) cells were obtained as follows: a kappa-bearing (7K) culture was grown for one week at maximum fission rate (27 °C) with aureomycin (0.04 mg ml⁻¹) present for the first day of incubation. Single cells were isolated, grown up at the low fission rate of one fission per day for three weeks into small cultures and tested for the presence of kappa. Cell lines without kappa particles were selected by examining crushed cells (Preer & Stark, 1953) and were tested further for their 'spin killing' (Sonneborn, 1970) reaction against cells of stock 152 (*P. triaurelia*), which is very sensitive to the presence of 7 kappa. Once established, 7S cultures were also kept monoxenically.

Methods of labelling. Paramecia and their kappa symbionts were labelled with ³⁵S by feeding them radioactively labelled bacteria, prepared by growth overnight at 37 °C with vigorous shaking in a low-sulphate medium with Na₂³⁵SO₄. The low-sulphate medium contained: 0.19 M-Tris, 0.07 M-NH₄Cl, 0.05 M-KCl, 0.17 M-HCl, 3.4 mM-Na₂HPO₄, 0.33 mM-MgCl₂·H₂O, 0.38% glucose, 0.06 mM-Na₂SO₄, 7.5 µCi (277.5 kBq) Na₂³⁵SO₄ ml⁻¹. The labelled bacteria were harvested by centrifugation (Sorvall SS34, 4 °C, 12000 g, 10 min), washed twice and then resuspended in 1 ml Dryl's solution (Dryl, 1959), and incubated for 15 min at 30 °C in the presence of lysozyme (1 mg ml⁻¹). Then 1 mg proteinase K was added and the incubation was extended overnight. The mixture was autoclaved and fed to a 200 ml paramecium culture (750–1000 cells ml⁻¹). Cycloheximide, 1–2 mg ml⁻¹, chloramphenicol, 0.4 mg ml⁻¹ (Sigma) and the aminoglycoside G418, 50 µg ml⁻¹ (Scherring Corp., Bloomfield, NJ, USA; Law *et al.*, 1983) were used for inhibition of protein synthesis.

Experiments to label isolated kappa particles *in vitro* were carried out as follows: kappa particles from a 200 ml culture were resuspended in 80 µl mannitol/PMSF (10 µl PMSF stock per ml 0.6 M-mannitol: PMSF stock, 0.1 g in 2 ml ethanol) and incubated for 50 min at 30 °C in a mixture containing [³⁵S]methionine, which has previously been used successfully for *in vitro* labelling of mitochondria (Steinkeler & Mahler, 1980). Incorporation was determined according to Steinkeler & Mahler (1980), using a model LS100C Beckman scintillation counter.

Isolation of kappa particles. A portion (1 g) of a filter paper ashless tablet (Whatman) was resuspended in water, poured into a 20 ml pipette with some glass wool in the tip, and washed with 100 ml H₂O and with 100 ml 0.01 M-sodium phosphate buffer (pH 7). A homogenate of paramecia (in 1 ml Dryl's solution) was carefully laid on the column. The column was washed with about 500 ml 0.01 M-sodium phosphate buffer (pH 7) at a rate of 1 drop s⁻¹. Pure kappa particles were eluted with 0.01 M-sodium phosphate buffer, containing 0.5 M-NaCl (pH 8) in eight fractions of 1.5 ml each. These fractions were centrifuged (Sorvall SS34, 17000 g) and the kappa particles were pooled and stored frozen. This isolation procedure is a modification of the methods used by Preer *et al.* (1966). In some experiments the paramecia were deciliated before homogenization as follows. Cells were filtered through cotton and centrifuged in an IEC-HN-SII oil-testing centrifuge to concentrate the paramecia. The packed cells were then resuspended in a 1:1 mixture of Dryl's solution and Sten's solution (0.5 M-sucrose, 20 mM-Tris, 2 mM-EDTA, 6 mM-NaCl; pH 7.5) (Hansma & Kung, 1975; Adoutte *et al.*, 1980) and incubated for 10 min on ice. CaCl₂/KCl solution was added to give a final concentration of 10 mM and 30 mM, respectively. The cells were left in this solution for 10 min before centrifugation at 1600 r.p.m. (20 °C) in an IEC-HN-SII centrifuge. The pellet was resuspended in 1 ml Dryl's solution, and homogenized with a Potter–Elvehjem homogenizer at 4 °C.

Electrophoresis and autoradiography. SDS-PAGE was done according to Laemmli (1970) on 8 and 10% (w/v) polyacrylamide slab gels, and on 7.5–22.5% or 5–15% (w/v) exponential gradient gels. Gels were stained with Coomassie Brilliant Blue R, then washed with methanol/acetic acid/water (4.5:1:4.5, by vol.) and enhanced for 1 h with Enhance (New England Nuclear). Following this treatment the gel was placed into double-distilled H₂O for 1 h, and dried on Whatman filter paper. Dried gels were put on sheets of Kodak XRP-1 X-ray film and incubated at –70 °C for periods ranging from 3 d to 2 weeks.

Myoglobin, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, conalbumin (Sigma) and β-galactosidase (Worthington) were dissolved in sample buffer [10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.0625 M-Tris/HCl, pH 6.8] at concentrations of 50 µg ml⁻¹ and used as size markers. The positions of these non-radioactive size markers were determined by comparisons of autoradiographs with stained gels.

RESULTS

Kappa particles were isolated and purified from *Paramecium biaurelia* (stock 7) cultures. *In vitro* labelling with [^{35}S]methionine, using *Klebsiella pneumoniae* samples as a control, was not successful. Although *K. pneumoniae* incorporated the label readily, there was insignificant incorporation of label by kappa, because homogenates from kappa-free paramecia showed a comparable incorporation when subjected to the *in vitro* system. However, kappa particles could be labelled *in vivo*, when radioactive bacteria were fed to a kappa-bearing paramecium culture. Usually, about 35% of the radioactivity originally added was incorporated by paramecium. *In vivo* labelled kappa particles could be purified from the paramecium homogenates (Fig. 1). All the radioactivity eluted in kappa-containing fractions sedimented together with the kappa particles, thus excluding the possibility of soluble contaminants.

Chloramphenicol inhibited cell division of paramecium at concentrations of 0.2 mg ml^{-1} . At concentrations of 0.4 mg ml^{-1} it prevented *in vivo* labelling of kappa, when added 30 min before paramecium was fed with radioactive bacteria. The incorporation of ^{35}S into paramecium proteins was not prevented, however. This was shown by using kappa-free cultures, and by measuring the radioactivity of homogenates from which kappa had been removed quantitatively by centrifugation at $17000 g$ for 10 min.

Cycloheximide and the aminoglycoside G418, when used at concentrations of 1 mg ml^{-1} and $50 \mu\text{g ml}^{-1}$, respectively, significantly reduced the incorporation of ^{35}S into paramecium proteins. However, they did not prevent it completely, and they did not eliminate the *in vivo* labelling of kappa.

Kappa proteins were subjected to electrophoresis on SDS-polyacrylamide gels and subsequent autoradiography. About 40 major bands were found with very distinctive bands of 165, 86, 78, 59, 52, 41, 40, 18 and 14 kDal (Fig. 2). The pattern was essentially identical in experiments with cycloheximide as an inhibitor. Figure 3 shows the autoradiograph of kappa proteins from an

Fig. 1. Chromatographic profile of a radioactive 7K homogenate on a filter paper column. All fractions (1.4 ml) were centrifuged at $17000 g$ (10 min, 4°C), and radioactivity was measured in $5 \mu\text{l}$ of the supernatant. Sediments were dissolved in $100 \mu\text{l}$ sample buffer, and radioactivity was measured in $5 \mu\text{l}$ of this suspension. The start of elution with low ionic strength buffer pH 7 (0.01 M-sodium phosphate) and with high ionic strength buffer pH 8 (0.01 M-sodium phosphate, 0.5 M-NaCl) is indicated by bold arrows. SD, sediment; SN, supernatant. Undigested food bacteria were eluted with low ionic strength buffer, and were usually found in fractions 5–20.

Fig. 2

Fig. 3

Fig. 2. Autoradiograph of highly purified 7 kappa proteins on a 7.5–22.5% exponential gradient SDS-polyacrylamide gel. Kappas were labelled *in vivo* without inhibitors. Cells were deciliated before isolating kappa particles on a filter paper column. The size-marker positions were found by matching autoradiograph and stained gel.

Fig. 3. Autoradiograph of 7 kappa proteins on a 5–15% SDS-polyacrylamide gradient gel. CH, cycloheximide used during *in vivo* labelling at a concentration of 1 mg ml^{-1} ; CAP, chloramphenicol used at a concentration of 0.4 mg ml^{-1} . The CH lane is overexposed in order to show bands present in the CAP lane.

experiment with cycloheximide and chloramphenicol as inhibitors. No bands were visible with a short exposure (2–3 d), when chloramphenicol was used. However, a long (2–3 weeks) exposure revealed some bands in the kappa lane. The possibility that these bands were contaminants from food bacteria was excluded by the following experiments. Food bacteria, after labelling, were treated with lysozyme and proteinase K, and autoclaved. This effectively reduced or eliminated all distinctive bands from the electrophoretic pattern of the bacterial proteins, but had no influence on the electrophoretic pattern of the kappa proteins (Fig. 4). Furthermore, when labelled food bacteria were mixed with a homogenate of unlabelled kappa-bearing paramecia before treatment on the filter paper column, the resulting kappa preparation was completely free from radioactivity.

On the other hand, when a kappa-free (7S) culture was labelled, homogenized and mixed with a kappa-bearing non-labelled culture before purification on the filter paper column, the resulting kappa preparation showed contaminating bands, totally unlike bacterial bands (Fig. 5). This provided further proof against bacterial contamination.

Figure 1 indicates that cytoplasmic contaminants were sedimented with kappa, because the supernatant was free from radioactivity, and a careful microscopic study of purified kappa preparations suggested cilia as possible cytoplasmic contaminants. This was confirmed in a

Fig. 4

Fig. 5

Fig. 4. Autoradiograph of *K. pneumoniae* and 7 kappa proteins on a 10% SDS-polyacrylamide gel. BAC, bacterial proteins; A, autoclaved; B, treated with lysozyme, proteinase K and autoclaved. 7 Kappa, kappa proteins; C, from labelling experiment with bacteria, treatment as in B; D, from labelling experiment with autoclaved bacteria, as in A. The positions of the molecular size markers (kDal) are indicated by the pointers.

Fig. 5. Autoradiograph showing cytoplasmic contaminants (arrowed) on an 8% SDS-polyacrylamide gel. A, extended autoradiograph of kappa proteins, labelled without inhibitors. B, autoradiograph of kappa proteins, isolated from a mixture of a non-radioactive 7K and a radioactive 7S culture.

repetition of both experiments (Fig. 6a, b) in which the contaminating bands disappeared if the labelled paramecia were deciliated before the isolation of kappa.

DISCUSSION

The purpose of this study was to see whether any proteins synthesized in the cytoplasm of paramecium move into kappa. Experiments where labelling was done in the presence of chloramphenicol should have shown many labelled cytoplasmic proteins in the cytoplasmic fraction, since chloramphenicol does not affect the protein synthesis on 80S cytoplasmic ribosomes. The kappa fraction should have shown no symbiont proteins except those that were synthesized outside the symbiont.

The experiments confirmed the expectations regarding labelled cytoplasmic proteins. However, comparable autoradiography of kappa proteins revealed no labelled bands. On the other hand, when the autoradiography of the same gel was extended, some bands became visible. Since these bands did not resemble the general pattern of kappa proteins, they could not be explained as being due to incomplete inhibition by chloramphenicol. They could have been proteins that were synthesized in the cytoplasm, and had then moved into kappa. Control

Fig. 6. (a) SDS-polyacrylamide gel (7.5–22.5% exponential gradient) stained with Coomassie Brilliant Blue R. A, kappa proteins from a mixture as in Fig. 5, but with a deciliation step before mixing the homogenates. B and C, two samples of kappa proteins, labelled *in vivo* without inhibitors. For details see text. (b) Autoradiograph of the gel shown in Fig. 6a. Note the absence of any contaminating bands in lane A.

experiments excluded contamination of the kappa preparation by proteins from food bacteria. Contaminating cytoplasmic proteins were found when kappa was isolated from a mixture of labelled kappa-free cells with non-labelled kappa-bearing cells. The symbiont-free cells still had the maintenance gene K for kappa. The K gene product was labelled in the 7S cytoplasm, and kappa could have absorbed the gene product when both homogenates were mixed. However, one would have expected only one contaminating band if this were true.

The discovery of small numbers of cilia in purified kappa preparations established a possible source for cytoplasmic contamination that was easily testable. It was shown that, indeed, cytoplasmic contamination bands disappeared if the labelled symbiont-free culture was first deciliated, and the cilia were separated from the cell bodies before mixing the homogenates and isolating kappa. Kappa preparations obtained in this way were free from ciliary contaminants, as confirmed by light microscopy. When the experiment using chloramphenicol as an inhibitor was repeated, and the cells were deciliated before homogenization, again no bands were seen, even after extended autoradiography.

These results show that no major proteins found in kappa were made outside kappa in the host cytoplasm. Although kappa has previously been shown to be a bacterial endosymbiont (Preer *et al.*, 1974a), these experiments demonstrate for the first time the existence of a prokaryotic translational system in kappa. This translational system seems to be non-functional in isolated kappa preparations, and even in crude cell homogenates, a finding which might be unique for 7 kappa. Isolated 7 kappa particles do not respire (J. R. Preer, Jr, unpublished), whereas stock 51 kappa symbionts do (Kung, 1970). Chloramphenicol was shown to be a potent inhibitor of

protein synthesis in kappa. Cycloheximide and the aminoglycoside G418 influenced cytoplasmic protein synthesis, but did not eliminate it. They did not inhibit protein synthesis in kappa.

The filter paper column method yielded kappa preparations of extremely high purity when combined with a deciliation step before the homogenization of kappa-bearing cells.

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