Structure and Properties of the Rapidly Sedimenting Replicating Complex of Staphylococcal Phage K DNA

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SUMMARY

Rapidly sedimenting complexes (RSCs) of replicating phage K DNA, isolated by rate zonal centrifugation in sucrose gradients, contain bacterial membrane lipids and protein. During the first half of the latent period the number of DNA molecules in a RSC increased from 1 to about 27. Digestion by Pronase caused the complexes to dissociate and release virion lengths of DNA which sedimented slowly like free mature DNA. RSCs treated with SDS disintegrated and released tangled DNA molecules, each about one virion length in size, but these structures retained their fast sedimentation characteristic. Chloramphenicol (CM) at 100 μg/ml did not completely inhibit complex formation or DNA replication, indicating that pre-existing host proteins were involved in these processes. CM reduced DNA replication by 50 to 80%. It is concluded that phage K DNA replicates attached to the cytoplasmic membrane of the host.

INTRODUCTION

Phage K is a virulent phage for Staphylococcus aureus and resembles the T-even coliphages in a number of morphological features. It has a contractile tail but, unlike the latter phages, no prominent hinged tail fibres (Rees & Fry, 1981 a). Infection causes host DNA synthesis to cease, and pre-existing DNA is broken down, the products being used in the synthesis of virus nucleic acid. Soon after entry into the host the parental phage DNA passes into a replicative form which sediments rapidly in sucrose gradients. We have previously presented evidence that this rapidly sedimenting complex (RSC) is the main replicative form of phage K DNA (Rees & Fry, 1981 b).

A further intracellular type of K phage DNA, the fast sedimenting form (FSF), sedimenting more quickly than mature type DNA but not as fast as the RSC, has also been found, but this is regarded as being a breakdown product from the RSCs and is not a true intermediate in the replication of the phage DNA (Rees & Fry, 1981 b).

Each RSC complex contains a number of DNA molecules associated with a small discrete mass of electron-dense matter presumed to be material other than DNA (Rees & Fry, 1981 b). The DNAs of other phages such as T2, T7 and λ replicate in association with the cytoplasmic membrane (Serwer, 1974; Siegel & Schaechter, 1973; Hallick & Echols, 1973). We show below that the RSCs of K phage DNA contain lipid components together with protein: the latter is especially important in maintaining the integrity of the complexes.

METHODS

General methods. The phage, its propagating strain, S. aureus NCTC 9318, defined media (DM1 and DM2), measurement of bacterial growth (as A610) and the production of K phage with DNA labelled with either [Me-3H]thymidine or 32P are described in Rees & Fry (1981 a, b). In all experiments, 5 min was allowed for adsorption of the phage to the bacteria. Bacteria with isotopically labelled phospholipids were obtained by growth in DM1 containing [2-3H]glycerol (Amersham International; 200 mCi/mmol, 1 μCi/ml final concn.). Determinations of 3H and 32P in the same sample were made in a two-channel scintillation counter (Nuclear Chicago Isocap, programme 10) using an external standard. Unless shown otherwise, all incubations were at 37 °C.

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Phage DNA associated with cytoplasmic membranes can be detected by co-sedimentation with membraneous material adhering to insoluble crystals of magnesium sarcosinate. Such crystals form a band, the M-band, when centrifuged through a sucrose gradient (Earhart et al., 1968). Bacteria were infected (m.o.i. = 5) with phage K containing $^{32}$P-labelled DNA and incubated in DM1 (5 x $10^8$ cells/ml). Samples (5 ml) were taken, the bacteria collected, washed and resuspended in 0.4 ml of 0.05 M-Tris-HCl, 0.0012 M-EDTA, 0.145 M-NaCl, pH 7.4 (TEN) containing 25% (w/v) sucrose. After 1 h at 4°C with 10 units of lysostaphin, 50 µl of 1 M-MgCl$_2$ was added and the sample layered on to an 8 ml gradient of 15 to 47% (w/v) sucrose. All sucrose solutions were in 0.01 M-Tris-HCl, 0.01 M-magnesium acetate, 0.1 M-KCl pH 7.0. Sarkosyl NL97 (Geigy; 50% (w/v), 40 µl) was added and mixed gently with the sample. The cells lysed almost immediately and crystals of magnesium lauroyl sarcosinate were formed. The M-bands were formed by centrifugation (22 500 g for 20 min at 4°C) in a 6 x 14 ml swinging bucket rotor (MSE Prepspin 50 centrifuge). There was no visible pellet and little or no labelled material in the gradient below the M-band.

Preparation of lysates containing rapidly sedimenting complexes. The bacteria were grown in DM1, harvested (when $A_{610}$ was 0.9), suspended in DM2 (5 x $10^8$ bacteria/ml) and infected with phage K (m.o.i. = 5). Samples were taken at specified times and, if required, pulse-labelled with $[^3H]$thymidine (5 µCi/ml) for 30 s. The bacteria were collected and lysed using lysostaphin and 1% (w/v) Triton X-100 or 1% (w/v) Brij 35 (method 2 in Rees & Fry, 1981 b). Unless stated otherwise, samples of the lysate were then sedimented through a 3-5 ml 5 to 20% sucrose gradient over a 1 ml 50% sucrose cushion in a Beckman SW39 rotor at 75 000 g for 50 min at 4°C. The rapidly sedimenting complexes were found near the bottom of the gradient (Rees & Fry, 1981 b). Samples of gradient fractions were taken directly for electron microscopy and mounted on grids by the micro-version of the diffusion procedure of Lang & Mitani (1970), then stained with uranyl acetate and shadowed with Pt-Pd (Rees & Fry, 1981 a).

Infection and treatment of bacteria with labelled membranes. Labelled bacteria were harvested ($A_{610}$ = 0.9), resuspended in DM1 (no $[^3H]$glycerol) and after 10 min, infected with $^{32}$P-labelled K phage (m.o.i. = 2). Samples (5 ml) were taken as required and quickly frozen. After thawing at 4°C, the bacteria were sedimented, resuspended in TEN, and treated with lysostaphin (10 units/ml for 1 h) and either 1% (w/v) Triton X-100 or 1% (w/v) Brij 35. Lysate samples were centrifuged through a 10 ml 5 to 20% sucrose gradient over a 2 ml cushion of 50% sucrose (75 000 g for 50 min at 4°C in MSE Prepspin 6 x 14 ml swinging bucket rotor).

RESULTS

Membrane association of K phage DNA

The rapidly sedimenting complexes (RSC) of replicating K phage DNA contain small prominent masses of electron-dense material (Fig. 1). The qualitative identification of components present in the latter was begun by using the M-band technique and S. aureus in which the phospholipids of the cytoplasmic membrane were prelabelled with $[^3H]$glycerol. The M-bands recovered from both uninfected and infected staphylococci regularly contained 47% of the total membrane label. The specificity of the M-band technique for the detection of only membrane-bound DNA was tested by mixing free phage DNA (obtained from complete virions by phenol extraction) with a suspension of uninfected S. aureus before lysis. About 20% of the added K phage DNA was consistently found in the resultant M-band. This result contrasts with that obtained by Earhart et al. (1968) who, in similar experiments with Bacillus megaterium, found that only 3% of the added phage DNA spontaneously associated with membrane material. However, M-bands prepared from S. aureus infected with labelled K phage contained between 55 and 60% of the parental DNA, i.e. three times the amount which was non-specifically trapped during the formation of M-bands for uninfected bacteria.

While the M-band technique with S. aureus does not give results which were as clear as those with B. megaterium, they indicated that replication of K phage DNA also involved association with the host cytoplasmic membrane. In order to identify the intracellular forms of K DNA which associated with the membrane, the phospholipids of S. aureus were prelabelled with $[^3H]$glycerol, and after a period in unlabelled medium in order to use up labelled intermediates in intracellular pools, the bacteria were infected with labelled K phage. Sucrose gradient analysis of lysates produced by treatment with lysostaphin and either Triton X-100 or Brij 35 showed profiles with three peaks containing parental phage DNA. From their position in the gradient and the radioactivity in the individual peaks, the RSC and FSF material in the Triton extract contained 47% (21% and 26% respectively) of the parental phage DNA, and in the Brij
Rapidly sedimenting complex of phage K DNA

Fig. 1. Electron micrograph of a rapidly sedimenting complex (RSC) of K phage DNA. Note the central core of electron-dense material to which the strands of DNA are attached. Bar marker represents 1 μm.

Fig. 2. Detection of co-sedimentation of parental K phage 32P-labelled DNA (○) and 3H-labelled cytoplasmic membrane (●) from infected S. aureus. Lysates of the infected bacteria were prepared using lysostaphin and either Triton X-100 (a) or Brij 35 (b), and then sedimented in a 5 to 20% sucrose gradient (for details, see Methods). ‘RSC’ and ‘FSF’ refer respectively to rapidly sedimenting complex and fast sedimenting form of K phage DNA. Mature-type DNA is at the top of the gradient.

extract 43% (21% and 22% respectively) of the parental DNA. In the Triton extract, the tritium label, indicating the phospholipids in the membrane, was all floating at the top of the gradient (Fig. 2a). In the gradient of the Brij extract, while much of the 3H label was similarly at the top, there were, in addition, two distinct peaks of membrane isotope co-sedimenting precisely with the FSF and RSC peaks of parental phage DNA (Fig. 2b). The amount of membrane label in these two peaks was relatively small (14 and 7% respectively), but this was to be expected since presumably only a small proportion of the total membrane was involved in binding the phage DNA. The lack of co-sedimentation of DNA and membrane labels in the gradient of the Triton extract is in agreement with the known effects of this detergent: it brings about the degradation and solubilization of bacterial cytoplasmic membranes whereas Brij 35 is much less disruptive (Siegel & Schaechter, 1973). The association of K phage DNA with membrane material does not occur spontaneously. When free K phage DNA was mixed with protoplasts derived from uninfected bacteria prior to lysis by Brij or Triton, there was no alteration in the sedimentation of the DNA. It all sedimented as a single sharp peak in the mature DNA position, near the top of the gradient.

Susceptibility of the RSCs to Pronase

Even after removal of phospholipids, the replication complexes of K phage DNA still sedimented rapidly (Fig. 2a), and this led us to determine the importance of protein in
maintaining the RSC structure. A lysostaphin–Triton lysate of pulse-labelled infected bacteria was incubated with and without self-digested Pronase (final concn. 2 mg/ml, 4 h at 37 °C; Young & Sinsheimer, 1967). Incubation alone had no effect on the labelled RSCs which sedimented as usual to the cushion interface at the bottom of the sucrose gradient. Digestion with Pronase destroyed the RSCs and the released labelled DNA formed a broad peak at the top of the gradient in the position expected for mature lengths of phage DNA.

**Origin of protein in the RSC and FSF of K phage DNA**

Chloramphenicol (CM) was used to investigate whether, after infection, protein synthesis was necessary for the formation of RSC (and of FSF) of K phage DNA. In control experiments, 8 μg CM/ml completely inhibited the growth of *S. aureus* 9318. CM (final concn. 100 μg/ml) was added 2 min before infection by 32P-labelled K phage. In the absence of CM, the sedimentation profile of a lysostaphin–Triton extract made at 10 min post-infection again showed three peaks containing parental phage DNA. The presence of CM did not completely inhibit the formation of RSCs and the FSF, but the radioactivity in the corresponding peaks was reduced to 63% and 56% of that in the control. The FSF in the CM-treated bacteria sedimented more slowly (peak at fraction 19 compared to fraction 15 in the control). Exposing the bacteria to CM for a longer period prior to infection and the use of CM at 400 μg/ml did not cause any further reduction in the 32P in the FSF and RSC positions.

To study whether CM (100 μg/ml) affected the synthesis of new DNA in the RSCs, samples of an infected culture were taken and pulsed with [3H]thymidine at 0, 5 and 10 min post-infection. The label was incorporated into RSCs, but the amount was two to five times lower than in the absence of CM. Clearly CM had some inhibitory effect both on the formation of the fast sedimenting forms of intracellular phage DNA and also on DNA replication in the RSCs. However, the inhibition was only partial, suggesting that protein synthesis post-infection was not essential for phage DNA replication, and we therefore conclude that only host enzymes were required.

**The size of RSCs at various times in the latent period and the effect of SDS**

The experiments described above show that RSCs of K phage DNA contain protein and bacterial membrane lipids. Since SDS solubilizes membranes (Wallach & Winzler, 1974) and causes the disaggregation of multimeric proteins (Waehneldt, 1975), the effect of this anionic detergent on RSCs was studied. In lysates from cells prepared at 0, 5 and 10 min after infection, the untreated RSC preparations each produced a single major peak (Fig. 3a, b, c). The peak maximum moved progressively nearer to the bottom of the gradient (in fractions 18, 13 and 10 respectively) as the time of sampling post-infection increased. This suggested that the RSCs were becoming larger during the latent period. After treatment with 3% (w/v) SDS for 1 h, the lysate profiles still showed a single major peak of rapidly sedimenting material (Fig. 3d, e, f). In the 0 min preparation, the peak sedimented in a position slightly lighter (by three fractions) than the corresponding untreated RSCs. At 5 and 10 min, the profiles were similar to the 0 min sample, although there was a small trend towards a heavier position in the gradient (peak maxima at fractions 21, 20 and 18 in the 0, 5 and 10 min samples respectively). Since the untreated RSCs in the 5 and 10 min lysates became progressively heavier and took up markedly different positions in the gradients, these results were interpreted to mean that SDS altered the structure of the RSCs and released DNA structures which sedimented in a position similar to the one isolated at 0 min. This was confirmed when samples of the peak fractions from each gradient were examined in the electron microscope. The untreated RSC at 0 min (Fig. 4a) was a DNA molecule about 1 phage equivalent in length and was associated with a small mass of other material. By 5 min, the complex had increased to a tangled structure with several pieces of associated material from which the DNA strands appeared to radiate (Fig. 4b). From measurements of enlarged photographs, these structures were estimated to contain about five phage equivalents of DNA. The RSCs isolated at 10 min contained about 27 phage equivalents.
Rapidly sedimenting complex of phage K DNA

Fig. 3. Sedimentation of RSCs at various times during the latent period and their sensitivity to SDS. Samples of an infected culture were taken at (a, d) 0, (b, e) 5 and (c, f) 10 min post-infection, pulse-labelled with [3H]thymidine, and lysates prepared. Part of each lysate was incubated at 37 °C for 1 h without (a, b, c) or with (d, e, f) 3% (w/v) SDS and then centrifuged through a 4.5 ml linear 5 to 50% sucrose gradient with no cushion. Other details are described in Methods.

of DNA and had numerous pieces of associated material (Fig. 4c). On the other hand, the SDS-treated complexes, irrespective of the time of sampling, were all similar and appeared as small tangles of apparently linear DNA approximately one phage equivalent in length (Fig. 5). The electron micrographs were therefore in agreement with the interpretation of the observed sedimentation profiles in Fig. 3.
DISCUSSION

The replication of K phage DNA takes place in a rapidly sedimenting complex of DNA molecules and other compounds (Rees & Fry, 1981 b). The amount of DNA in an RSC steadily increased during the first half of the latent period (Fig. 4) and this supports the conclusion that RSCs are primary replication centres. Moreover, this is evidence against the suggestion that these structures are artefacts and formed by aggregation during the extraction procedure.
Rapidly sedimenting complex of phage K DNA

Fig. 5. Electron micrographs of RSCs of K phage DNA isolated at 0, 5 and 10 min post-infection and treated with SDS. Material was taken from peak fractions of the sucrose gradients shown in Fig. 3. Bar markers represent 1 μm.

because, if this were so, the aggregates would be expected to be of similar size irrespective of the time of sampling in the latent period.

Phospholipids derived from the host cytoplasmic membrane co-sediment with RSCs (and with FSF), indicating that phage K DNA replicates attached to the membrane. Removal of these lipids by Triton did not greatly affect the sedimentation characteristics of the RSCs and they are not therefore the compounds primarily responsible for holding the growing mass of replicating DNA molecules together. This is achieved by the protein present in the complex since proteolytic digestion caused complete disintegration of the RSCs. Moreover, as a result of this treatment, the DNA sedimented as a broad band in the lighter part of the gradient, suggesting that the released DNA was not homogeneous and may have contained replicating molecules of different sizes.

Although other phages are known to replicate their DNA in fast sedimenting complexes, the overall structure of these complexes is not necessarily the same and their integrity is not always dependent on protein. Thus, the sedimentation of T7 and T4 complexes is not affected by Pronase (Serwer, 1974; Paetkau et al., 1977; Kemper & Janz, 1976). The K phage RSCs are evidently more like those from infections with phages φX174, λ and SPO1 which are also susceptible to this protease (Knippers & Sinsheimer, 1968; Hallick & Echols, 1973; Levner & Cozzarelli, 1972).

At least some of the proteins present in the phage RSCs are host proteins, since CM cannot prevent their formation. The origin and identity of these proteins is now being explored using host organisms with prelabelled proteins. Since CM did affect the amount of DNA label incorporated into the RSCs (and FSFs), concurrent protein synthesis may not be required for the formation of these DNA complexes but is necessary for an increase in their number and hence the apparent size of the RSC peak in the gradient profile.

SDS produced an interesting effect on the RSCs (Fig. 4 and 5). These were broken up into smaller structures each containing a tangled DNA molecule about one phage genome in length,
but these units still sedimented rapidly in sucrose gradients and much faster than DNA molecules isolated from mature virions. This may be due to their compact nature as well as protein still attached to them. Such structures could represent the basic replication units which are normally held together in an RSC by protein–protein interactions or by (inner leaf) phospholipids of the cytoplasmic membrane.

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REFERENCES


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