Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 13-20

INTERACTION BETWEEN THE GENE 5 PROTEIN, GENE 5 PROTEIN/SINGLE STRANDED fd DNA COMPLEX AND GENE 8 PROTEIN OF THE FILAMENTOUS PHAGE fd

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Received September 30, 1987

SUMMARY: An affinity column consisting of gene 8 protein, the major coat protein of fd phage, bound to Sepharose was prepared. Isolated gene 5 protein/single stranded fd DNA complex was found to bind to this column and was eluted with fd phage single stranded fd DNA. pH changes, and 1 M $CaCl_2$ were not effective in eluting the protein from the affinity column. Gene 5 protein/single stranded fd DNA complex from the crude extracts of fd-infected E.coli also bound to the column, as did isolated gene 5 protein; whereas fd single stranded DNA alone did not. These results may be relevant for the illucidation of the molecular events occuring in the early stages of fd phage assembly.

The assembly of filamentous phage fd progeny is characterized by 2 features. First, assembly is non-lytic; the E.coli host cell remains intact, grows and divides during fd infection. Second, the mature phage is assembled while being ejected from the host cell; therefore, no mature phage are found within the host (for review see Ref. 1).

A critical step in fd assembly is the formation of a complex between the g5p and ssDNA. When bound to g5p, ssDNA can no longer serve as a template for DNA replication. This g5p/ssDNA complex consists of 1300 g5p monomers per fd ssDNA molecule (6408 bases) and has been physically and chemically characterized in detail (2,3).

The next step in assembly, probably occurs on the inner membrane of E.coli where the g8p is localized (4). There, the ssDNA is then exchanged from the complex to the g8p. This protein is the major coat protein of the fd phage (2700 copies per phage) and along with 4 other minor coat proteins it encap-

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Abbreviations: g8p, gene 8 protein; g5p, gene 5 protein; ssDNA, single stranded fd DNA; TEN buffer, 10 mm Tris, pH 8.1, 1 mM in EDTA, 50 mM in NaCl; SDS, sodium dodecylsulfate; BSA, Bovine serum albumin; Tris, Tris (hydroxymethyl)-aminomethane; moi, multiplicity of infection.

0006-291X/87 $1.50

sulates the ssDNA in a distinct orientation and mature phage particles are produced.

This research was designed to experimentally determine the mutual affinities of the phage components involved in the early steps of its assembly, i.e., g8p, g5p, ssDNA and g5p/ssDNA complex. For this purpose, g8p was immobilized on a Sepharose gel and the retention of the remaining components was investigated.

MATERIAL AND METHODS

Preparation of crude cell extracts of E.coli: Bacteriophage fd and E.coli strain K37 (supD) are from our collection. Routinely, 50 ml of DYT media was infected with an overnight culture of E.coli to an OD_{540} of 0.4. The cells were grown with shaking at 37°C for 0.5 h before infection with fd phage (moi 10) and then grown for a further 4-6 h before harvesting by centrifugation (7,000 xg, 10 min). The cells were washed with TEN buffer and gently lysed with lysozyme (1 mg/30 ml TEN buffer). The supernatant obtained after centrifugation (32,000 xg, 30 min) is the crude cell extract.

Preparation of g5p, g5p/ssDNA and g8p: g5p was isolated using the method described by Oey and Knippers (5) and will be referred to as purified g5p (6). Doubly-labelled g5p/ssDNA complex was a gift from M. Vaccaro and was prepared as previously described (6) using L-[U-^{14}C]arginine (>300 mCi/mmol) and [methyl-^{3}H]thymidine (>40 mCi/mmol) purchased from Amersham Buchler, F.R.G. g8p was isolated from SDS-dissociated phage by column chromatography (Sepharose 12 column, Pharmacia) (7) and was judged by SDS gel electrophoresis and amino acid analysis to be pure.

g8p was determined to be essentially free of membrane phospholipids by extracting a 0.5 mg aliquot with chloroform and methanol (8) and then measuring the phosphate content of the resulting chloroform phase (9). Using this technique as little as 2.5 ng inorganic phosphate can be detected. In our g8p preparation, no phosphate was measured. Protein was determined by the method of Lowry (10).

Binding of g8p to CNBr-Sepharose: 0.6 g CNBr-Sepharose (Pharmacia) previously washed with 120 ml 1 mM HCl was added to 20 mg g8p (solubilized in 0.1% SDS) dissolved in 0.1 M $NaHCO_3$, pH 8.1, 0.5 M NaCl and gently mixed 2 hrs at room temparature. Any unreacted groups were blocked by washing with 0.2 M glycine, pH 8 for 2 hrs. Unbound protein (less than 1 mg) was then removed by washing with 3 cycles of 0.1 M $NaHCO_3$, pH 8; 0.5 M in NaCl and 0.1 M sodium acetate, pH 4, 0.5 M in NaCl. The gel was then poured into a pasteur pipette, the narrow end of which had been stopped with glass wool and equilibrated with 10 column volumes TEN buffer. After use, the column was first washed with 10 column volumes of 0.1 M Tris/HCl, pH 8.5, 0.5 M in NaCl and then 10 column volumes of 0.1 M sodium acetate, pH 4.5, 0.5 M in NaCl and then re-equilibrated with TEN buffer before further use. The column lost no activity over a 3 month period.

Preparation of ssDNA: ssDNA was prepared by phenol/chloroform extraction (11). The concentration was determined by measuring the optical density at 260 nM using the conversion factor 1 OD unit = 43 µg DNA. To obtain [^{3}H]-labelled-ssDNA, cells were grown in 10 ml minimal media (12) supplemented with amino acids and added [methyl-^{3}H]thymidine (200 mCi, sp.Act. 2 Ci/mmol). After centrifugation to remove E.coli cells, phage were precipitated from

the supernatant with polyethylene glycol and the [^{3}H]-labelled-ssDNA was then prepared as described above.

RESULTS

Application of crude cell extracts to g8p affinity column

Crude cell extract (1 mg protein) prepared from E.coli cells infected with fd phage was applied to the g8p-Sepharose column. After extensive washing with TEN buffer, 20 μg fd ssDNA was applied and 1 ml fractions of the effluent were collected. 42.2 μg protein was recovered in the first 1-2 fractions along with 6.4 μg DNA. This corresponds to a DNA to protein (w:w) ratio of 1:6.6 which correlates well with the 1600-1300 g5p monomers/6400 bases reported by several authors (13,14). When these fractions were concentrated and applied to a 20% acrylamide gel containing SDS (Fig. 1A) one major protein band was obtained (lane 4) which co-migrated with purified g5p (6). Both of these protein bands also stained positively after transferral to nitrocellulose sheets and immunoblotting with antibodies raised against purified g5p (Fig. 1B).

Since molecular weight determinations using SDS gel electrophoresis are considered unreliable in the low molecular weight regions, electrophoresis

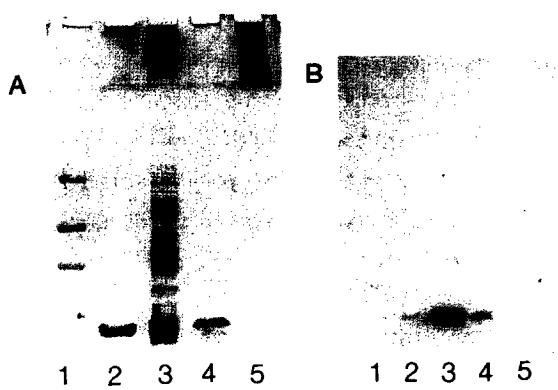

Figure 1. SDS gel electrophoresis and immunoblotting of g5p.
Crude extract (0.5 mg) from infected cells was applied to the g8p-Sepharose column. After application of 20 μg ssDNA, the first 1-2 fractions were collected, pooled, concentrated, made up to 1% in SDS, placed in a boiling water bath for 3 min and applied to 20% polyacrylamide gels (30:0.8; acrylamide:bisacrylamide) containing 1% SDS and prepared in 0.3 M Tris pH 8.9. After electrophoresis (150 V, 2.5 h) the gel was subjected to Western blotting (17) using antibodies raised against g5p, or silver staining (18). Figure 1A is the polyacrylamide gel; figure 1B is the corresponding immunoblot.
In each case, the following proteins were applied: to lane 1, low molecular weight standards from Bio-rad; to lane 2, purified g5p (6); to lane 3, cell lysate from fd-infected E.coli; to lane 4, g5p eluted from the g8p-Sepharose column with ssDNA; to lane 5, protein eluted from the BSA-Sepharose column with ssDNA.

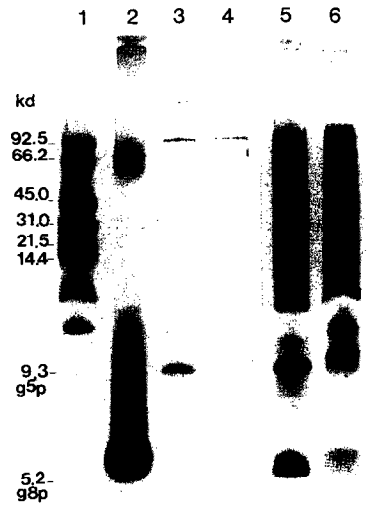

Figure 2. Molecular weight determination using SDS polyacrylamide gels, 8 M in urea.
Protein eluted from the g8p-Sepharose column were prepared as in Figure 1 and applied to a 12.5% polyacrylamide gel, 8 M in urea (14). Lane 1 contains a low molecular weight standard mixture (Phosphorylase B, 92.5 kd; bovine serum albumin; 66.2 kd; ovalbumin, 45.0 kd; carbonic anhydrase, 31.0 kd; trypsin inhibition, 21.5 kd; lysozyme, 14.4 kd). To lane 2, fd phage (containing mostly g8p, 5.2 kd) was applied. To lane 3, the protein obtained by eluting the g8p-Sepharose column with ssDNA (in other words g5p) was applied. Lane 4 contains the fractions obtained when non-infected E.coli cells were applied to the affinity column and eluted with ssDNA. Lane 5 contains crude extract from infected E.coli cells and lane 6 contains the crude extract from lane 5 that has passed through the affinity column without binding.

on 12.5% acrylamide gels containing 8 M urea was performed (Figure 2) (15). The molecular weight thus determined for g5p (Figure 2, lane 3) is 9,300 daltons which corresponds well with the published molecular weight of 9,700 (2) for this protein. When cell extracts from non-infected E.coli cells were treated in the same manner, no silver staining bands were observed on SDS-polyacrylamide gels (Figure 2, lane 4).

Characterization of the column

To test whether the observed binding and recovery of g5p is due to an unspecific interaction with the Sepharose, a column was prepared without bound g8p. When 50 μg g5p/ssDNA complex or crude cell extract was applied to this column (no g8p bound), no binding or retardation of the complex was observed.

To rule out the possibility that the observed results are due to some unspecific hydrophobic interactions, BSA was coupled to Sepharose. When crude extract (1 mg) from infected E.coli cells was applied to this BSA-Sepharose column, 350 μg protein remained bound after washing. After application of 20 μg DNA, 48 μg protein was eluted. This protein was concentrated and placed on a 20% SDS polyacrylamide gel (Fig. 1A). One protein entered the gel

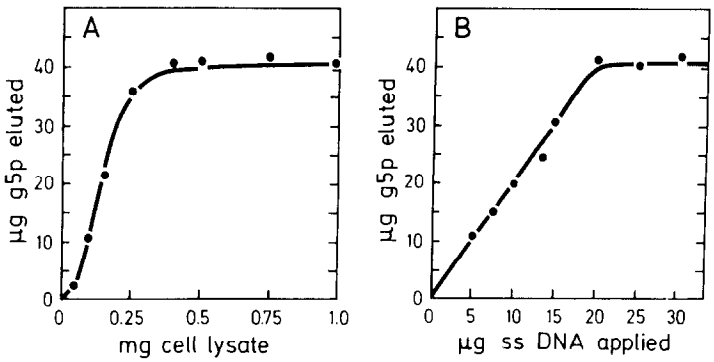

Figure 3. Characterization of the g8p-Sepharose column. Lysates from fd-infected E.coli cells were applied to the g8p-Sepharose column. After elution with ssDNA, fractions absorbing at 280 nm were pooled, dialyzed, lyophilized and protein content was measured (9). Figure 3A diagrams the µg g5p eluted after application of 20 µg ssDNA when the amount of cell lysate applied to the column is varied. Figure 3B shows the results obtained by varying the amount of ssDNA used to elute the g5p from the column. The values shown are average of 3-5 experiments. The standard error is ± 10%.

(others remained in the stacking gel) corresponding to a molecular weight of 92,000. This protein did not cross-react with antibodies raised against g5p (Fig. 1B). Therefore, the observed binding of the g5p/ssDNA complex to the g8p-Sepharose column is due to the interaction between the complex and the bound g8p.

When the amount of crude cell extract from fd-infected cells applied to the column was varied the results diagrammed in Fig. 3A were obtained. There was an increase in the amount of g5p eluted with 20 µg ssDNA when the total amount of protein applied increased from 0 to 0.375 mg. Application of more protein did not lead to increased binding. Thefore with 0.375 mg crude extract, the binding capacity of the column for the g5p-component of the complex was attained. As shown in Fig. 3B when the amount of crude extract applied was held constant (0.5 mg) and the amount of ssDNA used to elute g5p was increased from 0 to 20 µg, there was a corresponding increase in the amount of g5p eluted until 42 µg of protein was recovered. The application of more than 20 µg ssDNA did not lead to an increase in the amount of protein eluted.

Interaction of ssDNA and g5p with g8p-Sepharose column

fd ssDNA when applied to the column alone did not interact with the g8p-Sepharose gel, but appeared immediately in the column effluent. In contrast, purified g5p (0.1 mg) bound to the g8p-Sepharose in a fashion similar to the g5p/ssDNA complex. After washing the column with TEN buffer, 31.5 µg g5p was recovered in the effluent after application of 20 µg fd DNA.

Interaction of radioactively labelled g5p/ssDNA complex and ssDNA with the g8p affinity column

In order to quantitatively evaluate the interaction observed between the g5p/ssDNA present in crude extracts of infected E.coli and the g8p-Sepharose column, radioactively labelled g5p/ssDNA was isolated from infected E.coli cells grown in a medium containing [^{14}C]-arginine and [^{3}H]-thymidine. The resulting complex containing 100 μg protein and labelled with 135,000 dpm [^{14}C] in its protein component and 105,500 dpm [^{3}H] in its ssDNA component was applied to the g8p-Sepharose column. Approximately half of the radioactivity applied (67,000 dpm [^{14}C] [49.6%]) and 52,000 dpm [^{3}H] 49.8% was immediately washed through the column with TEN buffer. Further washing with TEN buffer in the pH range 7.2 to 8.7 or with the addition of 0.1% Triton X-100 resulted in no further release of radioactivity. After application of 10 μg fd ssDNA, 29% of the remaining [^{14}C] counts (19,800 dpm [^{14}C]) and 18% of the remaining [^{3}H] counts (9,800 dpm [^{3}H]) were measured in the effluent. Application of a second 10 μg fd ssDNA resulted in the elution of an additional 25% of the [^{14}C] label (17,000 dpm) and 6% of the [^{3}H] label (3,200 dpm). The column was then washed with Tris, pH 8.5, and acetate, pH 4, as described in the materials and methods section. No radioactivity was recovered in these 2 washings. From these experiments it is clear that purified g5p/ssDNA complex binds to the column and is eluted with ssDNA; more ssDNA remains bound as compared to g5p. This suggests that on the column an ssDNA exchange is taking place. The original labelled ssDNA from the g5p/ssDNA complex remained bound to the g8p while the resulting unbound g5p was free to complex with the added unlabelled ssDNA. To test this hypothesis [^{3}H]-labelled fd ssDNA (11,700 dpm) was used to elute the column and unlabelled crude extract was the source of the g5p/ssDNA complex applied to the column. In this case, all of the recovered radioactivity (5140 dpm or 43.9% of that applied) eluted in the g5p-containing fractions. Therefore, it appears that ssDNA is effectively exchanged from the original g5p/ssDNA complex to the g8p-Sepharose column, while addition of more ssDNA leads to formation of new g5p/ssDNA complex.

Effect of $CaCl_2$ on g5p/ssDNA binding to g8p-Sepharose gel

Using circular dichroism (16) it has been established that 0.1 M $CaCl_2$ leads to total dissociation of g5p/ssDNA complex in solution. Therefore, an experiment was performed to determine the effect of this salt on the binding of the g5p/ssDNA complex to g8p-Sepharose. After application of cell lysate (0.5 mg) to the affinity column and extensive washing with TEN buffer, the column was eluted with a 10 ml linear $CaCl_2$ gradient (0 to 1 M) in TEN buffer. No protein was obtained using this procedure and no bands were

seen after silver staining of SDS polyacrylamide gels (results not shown). After application of 20 μg fd ssDNA, protein was eluted which co-migrated with g5p on SDS polyacrylamide gels.

DISCUSSION

Our results show that a complex is formed between the g5p/ssDNA complex and g8p-Sepharose. Recognition of g8p occurs via protein-protein interactions because g5p alone was found to bind to the g8p in a manner similar to the g5p/ssDNA complex. fd ssDNA did not bind to the g8p-Sepharose. The only previous indication for the interaction of fd ssDNA with g8p was published in 1966 (19). However, the "co-aggregation" of g8p and fd ssDNA through CsCl gradients described in this paper was only obtained after treatment with 8 M urea and/or prolonged dialysis (12 h minimum). Since neutral pH was found to optimize this "co-aggregation" the DNA binding experiments described herein were redone at pH 7.0. Again, no interaction between the fd ssDNA applied and the g8p-Sepharose was observed.

The complex formed between g5p/ssDNA and g8p is more stable in the presence of $CaCl_2$ than the g5p/ssDNA complex alone. 1 M $CaCl_2$ did not lead to dissociation of the g8p-Sepharose bound g5p/ssDNA complex whereas 0.1 M $CaCl_2$ results in total dissociation of g5p/ssDNA complex in solution (16). This result suggests that the immobilized ternary complex g8p-g5p-ssDNA is much more stable (at least in the presence of $CaCl_2$) than the binary complex g5p-ssDNA in solution.

Our experiments with [^{14}C]-g5p/[^{3}H]-ssDNA show that more ssDNA remains bound to the g8p-Sepharose column than g5p and that the g5p is eluted with newly applied ssDNA. In conclusion, these results describe the *in vitro* binding of g5p and g5p/ssDNA complex to an immobilized g8p. The addition of ssDNA triggers the release of bound g8p and the regeneration of the g5p/ssDNA complex.

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