Phospholipase Activity in Bacteriophage-Infected *Escherichia coli*

I. Demonstration of a T4 Bacteriophage-Associated phospholipase

E. T. NELSON AND C. S. BULLER

Department of Microbiology, University of Kansas, Lawrence, Kansas 66044

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Phospholipase activity has been found to be associated with T4 phage and T4 ghost particles. The attachment of the phospholipase to the phage persists during purification through cesium chloride gradients and dialysis, indicating that it is firmly bound. The presence of the enzymatic activity on T4 ghosts suggests that it is not normally packaged within the head of the virus. The enzyme has specificity for phosphatidylglycerol and its activity is stimulated by 0.1% Triton X-100 and 20% methanol. It does not have a requirement for Ca^{2+} and is inactivated at temperatures above 60°C. The association of the phospholipase with T4 phage grown in a phospholipase-deficient host and its absence on unsuppressed T4amtA3 suggests that it may be phage gene specific.

The infection of *Escherichia coli* with T4 phage culminates in the endolytic degradation of host peptidoglycan, leading to release of progeny phage. Although the role of phage lysozyme in lysis from within is well established (25), it is likely that membrane changes are required to permit the enzyme to reach its substrate. The membrane changes could either cause, or be a consequence of, the metabolic arrest observed in T4 phage-infected *E. coli* (16). That the metabolic arrest is not a consequence of lysis is clear from the demonstration that *E. coli* infected with lysozyme-deficient T4 have a block in oxygen uptake occurring at about the same time that lysis would normally occur (16).

Hydrolysis of *E. coli* membrane phospholipids after T4 infection has been demonstrated and it was suggested that phospholipase has a role in the lysis from within process (7). Subsequently, a new class of lysis-deficient mutants, designated as t^- , was described (11). Although these mutants could produce lysozyme, they failed to lyse *E. coli* and they did not cause a block in host respiration (13). It was concluded that the t -gene product, although required for lysis, did not directly cause phospholipid hydrolysis, and that the appearance of free fatty acids at the end of the latent period was a consequence of lysis (12). Nevertheless, it has been demonstrated that phospholipid hydrolysis can substantially precede lysis if T4rIf mutants are used (3, 5).

Because of the potential importance of phos-

pholipases in the explanation of lysis from within, and possibly other events such as the phage-induced efflux of cations (19, 20, 23) and ATP (4), we have examined the role of these enzymes in phage infection. In this communication we describe the detection of a phage-associated phospholipase.

MATERIALS AND METHODS

Bacteria and phage. *E. coli* strains B and K12(λ)IYMel and wild-type T4 phage were obtained from L. Astrachan. T4amtA3 phage were obtained from R. Josslin and *E. coli* K12 strains carrying mutations for the detergent-resistant (*dr*) and/or detergent-sensitive (*ds*) phospholipases (9, 17) were obtained from S. Nojima. High-titer phage stocks were prepared from phage lysates either by differential centrifugation or polyethyleneglycol sedimentation (26). *E. coli* K12(λ)IYMel, containing an amber suppressor, was used in the production of suppressed stocks of T4amtA3. Unsuppressed stocks of this phage were prepared by growth in suppressor-negative *E. coli* B, followed by artificial lysis with chloroform to liberate the phage. T4 ghosts were prepared by an osmotic shocking procedure (10) which usually reduced plaque-forming ability by 99.9% or more. Bacteriophage were assayed by the methods described by Adams (1). Protein content of phage and host preparations was assayed by the procedure of Lowry et al. (15).

Media and reagents. Tryptone broth, containing 1% tryptone (Difco) and 0.1 M NaCl, was used as growth medium. Soft and hard agar for plating phage and bacteria contained 0.6 and 1.5% agar (Difco), respectively. All reagents used in phospholipid extraction and thin-layer chromatography were analytical grade. Cesium chloride was purchased from Schwarz/

Mann (N.Y.), and silica gel, Camag-Type DO, without binder, from Arthur H. Thomas (Philadelphia, Pa.). [1,2-¹⁴C]sodium acetate (56.2 mCi/mM) was obtained from New England Nuclear Corp. (Boston, Mass.).

T4 phage and ghost purification. A 5-ml amount of phage or ghost suspension was layered over a step gradient prepared by successive additions of 4.5 ml of 80, 70, 60, and 50% CsCl and 4.0 ml of 40 and 20% CsCl. After centrifugation for 18 h at 20,000 rpm in a Beckman SW-27 rotor, the isolated phage and ghosts were collected and dialyzed for 18 h against several changes of distilled water.

Preparation of extracts from T4-infected *E. coli*. *E. coli* grown to log phase in 300 ml of tryptone broth were infected with phage at input multiplicities of 5. After 10 min of incubation at 37°C with shaking, the cells were collected by centrifugation and resuspended in 5 ml of tryptone broth. Incubation at 37°C was continued for 30 min, and the suspensions were then sonically treated for 6 min with a Biosonik III, at 35% of maximum intensity. After centrifugation at $38,000 \times g$ for 1 h, the supernatant fractions were dialyzed against distilled water and then used for phospholipase assays.

Preparation of ¹⁴C-labeled phospholipids. *E. coli* was grown to late log phase in tryptone broth containing 0.2 μ Ci of [1,2-¹⁴C]sodium acetate per ml. After collecting bacteria by centrifugation, phospholipids were extracted with chloroform-methanol (1:2, vol/vol), by the procedure of Kates et al. (14). Based on distribution of radioactivity on thin-layer plates the extracts were comprised of phosphatidylethanolamine (PE), 74 to 78%; phosphatidylglycerol (PG), 16 to 20%; and cardiolipin (CL), 5 to 7%. The free fatty acid (FFA) content in these lipid extracts was less than 1%. Lipid phosphorus was determined by using the procedure of Ames (2).

Phospholipase assay. A portion of labeled phospholipids, containing a known amount of lipid and radioactivity, was added to a screw-cap tube. After evaporating the CHCl₃ solvent in a stream of N₂, 0.5 ml of phage, ghosts, or cell extract, Tris-hydrochloride buffer, pH 7.8, 100 mM final concentration, and methanol at final concentration of 20% were added. The suspensions were incubated with shaking at 37°C, usually for 12 h, after which the reaction was terminated by the addition of 1 ml of CHCl₃. The mixture was vortexed for 2 min, and after centrifugation the CHCl₃ phase was collected by aspiration. The aqueous phase was again extracted by adding 3 ml of CHCl₃-methanol (1:2, vol/vol). After mixing, 1 ml of water and 0.5 ml of CHCl₃ were added to make the system biphasic. The CHCl₃ phase was removed and the aqueous phase, after acidification with H₂SO₄, was immediately extracted with 1.5 ml of petroleum ether. This latter step, although routinely used as a precautionary measure to ensure protonation and extraction of any salts of fatty acids which might be present, did not significantly alter the yields of fatty acids. The organic phases were combined, adjusted to 10.0 ml with CHCl₃, and a 2.0-ml sample was removed for assay of radioactivity, to determine the efficiency of extraction. The efficiency was greater

than 90% in these experiments. The remaining 8.0 ml of the lipid extract was concentrated by evaporation under a stream of N₂ and used for thin-layer chromatography. The extent of FFA release from radioactive phospholipids was used as the index of phospholipase activity.

Fractionation of lipids and assay of radioactivity. Thin-layer chromatography was performed by the methods described by Skipski (24). End plates (5 by 20 cm) were coated with Camag gel type DO, without binder. For separation of phospholipid classes, plates were developed in chloroform-methanol-water (65:25:4, vol/vol). The neutral lipids were separated from phospholipids by developing the plates in a solvent system containing isopropyl ether-acetic acid (96:4, vol/vol). Lipids were detected by exposing the developed plates to iodine vapors. The radioactivity in the individual spots was determined by quantitatively transferring the gel to vials for counting in a Tri-Carb liquid scintillation spectrometer. To assure complete recovery all of the gel in a lane was routinely assayed by this procedure. The scintillation fluid used contained 5 g of 2,5-diphenyloxazole and 0.1 g of dimethyl 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter of toluene. The identity of the individual phospholipids was established as previously described (18). To obtain labeled phospholipids for assays of phospholipase substrate specificity, gels in each spot separated by thin-layer chromatography were scraped from the plate and the lipids were eluted with the developing solvents.

RESULTS

Phage-associated phospholipase. To assay for phage-associated phosphatide acylhydrolase activity, T4 phage and ghosts were incubated initially with aqueous suspensions of ¹⁴C-labeled phospholipids extracted from *E. coli* B. After 3 h of incubation at 37°C, the phage-phospholipid mixtures were extracted and lipids were fractionated as described in Materials and Methods. The resulting distribution of radioactivity among the phospholipid classes and FFA is shown in Table 1A. Although T4 ghosts were capable of FFA formation, phage purified by differential centrifugation were inactive. In other experiments (Table 1B), it was found that these phage did contain phospholipase but that it was not activated unless the phage were further purified by CsCl gradient centrifugation and dialysis. In these experiments (Table 1B) a second preparation of phospholipid, containing more label and differing slightly in composition, was used. Subsequently, it was found that dialysis alone was sufficient to activate the phage-associated phospholipase activity, suggesting the presence of a dialyzable inhibitor.

The preceding results (Table 1) were obtained with phage or ghosts incubated with aqueous suspensions of the phospholipids. It was subse-

TABLE 1. *In vitro* phospholipid hydrolysis by T4 phage and T4 ghosts^a

Reaction mixture ^b	FFA		CL		PE		PG	
	Counts/min	%	Counts/min	%	Counts/min	%	Counts/min	%
(A)								
Control ^c	279	0.7	2,591	6.5	30,533	76.6	6,417	16.1
Ghosts ^d	1,395	3.5	2,511	6.3	30,134	75.6	5,780	14.5
Phage ^e	278	0.7	2,671	6.7	30,573	76.7	6,338	15.9
(B)								
Control ^c	287	0.3	4,975	5.2	70,805	73.9	19,423	20.3
Purified phage ^f	5,932	6.2	4,592	4.8	69,944	73.1	15,022	15.7

^a Results are an average of two independent experiments. The difference between the percentage of distribution in the two experiments did not exceed 0.9%, except for PG + purified phage, which was 1.5%.

^b Reaction mixture consisted of aqueous suspensions of radioactive *E. coli* phospholipids and 0.1 mg of phage or ghost protein, as indicated. Reaction volume, 1.0 ml.

^c Controls, no phage or ghosts added.

^d Ghosts, purified by CsCl gradient and dialysis.

^e T4 phage, purified by differential centrifugation.

^f T4 phage, purified by CsCl gradient.

quently found that the phage-associated phospholipase activity was enhanced if the phospholipids were dispersed in Tris-hydrochloride (100 mM, pH 7.8) containing 20% methanol (vol/vol). At concentrations greater than 20% (vol/vol) the presence of methanol became inhibitory. The phospholipase activity was also stimulated by 0.1% Triton X-100, but 1% Triton X-100 and 0.1% sodium dodecyl sulfate were inhibitory. The chloride salts of Ca²⁺, Mg²⁺, Na⁺, K⁺, and Cs⁺ were also tested for ability to stimulate enzyme activity. At concentrations of 2 or 20 mM they either had no effect or were to varying degrees inhibitory. The addition of 10 mM EDTA to assay mixtures resulted in complete inhibition of activity, suggesting the requirement of an unknown cation.

Specificity of phage-associated phospholipase. The preceding experiments (Table 1) suggested that PG was the principal phospholipid being deacylated. To determine if the phage-associated phospholipase exhibited substrate specificity, T4 phage purified by CsCl gradient centrifugation and dialysis were incubated with equivalent amounts of PG, PE, and CL. At various times of incubation samples were removed and the lipids were extracted and fractionated as described in Materials and Methods. Figure 1, indicating the amount of radioactive fatty acids released from each of the phospholipids demonstrates that the enzyme is almost completely specific for PG.

The effect of incubation temperature on phage phospholipase was examined. When assay mixtures were incubated at room temperature, the level of FFA formation was about 50%

FIG. 1. Substrate specificity of phage-associated phospholipase. Reaction mixtures containing 0.187 mg of T4 phage protein, 20% methanol, and Tris-hydrochloride buffer (100 mM, pH 7.8) were incubated at 37°C with 0.2 mM PE, PG, or CL, each containing about 10,000 counts/min of ¹⁴C label in their acyl groups. Final reaction volume was 0.5 ml. At the indicated time intervals samples were removed and lipids were extracted and assayed as described in Materials and Methods. Symbols: ○, PE; ▲, PG; and ●, CL.

of that observed at 37°C, whereas incubation at 50°C resulted in a 10-fold stimulation of activity. Preincubation of phage for 5 min at 60, 80, and 100°C and then subsequent assay for phospholipase activity at 37°C indicated that virtu-

ally complete denaturation occurred at 80 and 100 C. Samples preheated to 60 C retained only 35% of their activity.

Effect of increasing enzyme concentration.

Figure 2 shows the effect of increasing phage protein concentration on the deacylation of PG. As expected for enzyme catalyzed reactions, the rate of FFA formation was enhanced by increasing protein concentration.

Phospholipase activity of T4 phage grown in *E. coli* phospholipase mutants. *E. coli* K12 has two known acyl hydrolases, one *dr* and the other *ds* (9, 17). The *dr* phospholipase can utilize PE, PG and CL as substrates and probably is the phospholipase A of *E. coli* which has been partially purified and characterized (22). The *ds* enzyme is specific for PG, has no calcium requirement, is thermolabile at 65 C, and is inactivated by 20% methanol. The PG specificity of the phage-associated phospholipase suggests that it might be of host origin, i.e., the *ds* enzyme. To examine this possibility, T4

phage grown in *E. coli dr⁻ds⁻* were assayed for phospholipase and Table 2 indicates that these phage retain the ability to deacylate PG. This, together with differing responses to detergent and methanol, implies that the phage-associated enzyme is not the host *ds* enzyme. Additionally, extracts, prepared as described in Materials and Methods, were prepared from T4-infected *dr⁺ds⁺*, *dr⁻ds⁺*, *dr⁺ds⁻*, and *dr⁻ds⁻* hosts and were compared with those from corresponding, uninfected hosts for ability to deacylate PG. In all cases, phage infection enhanced the activity, but with these crude extracts the results were variable, ranging from 1.05- to 5.4-fold stimulation. A stimulation in activity of extracts from infected cells would be expected if the enzyme is of phage origin.

Assay for phospholipase activity of unsuppressed T4amtA3. The preceding experiments suggest that phage-associated phospholipase may be phage-gene specific. More convincing evidence of this was provided by experiments with T4amtA3. This mutant, described by Joslin (13), does not lyse *E. coli*, nor does it block host respiration at the normal time when grown on hosts not containing amber suppressors. Suppressed and unsuppressed stocks of T4amtA3 were prepared as described in Materials and Methods. After purification and dialysis, these phage preparations were assayed for ability to deacylate PG. Table 3 indicates that T4amtA3, when grown in suppressor-negative hosts, is devoid of phospholipase activity. The ratio of titer to protein for T4amtA3 grown in suppressor-positive hosts was very nearly identical to that for phage grown in restrictive hosts. Wild-type T4, when grown in restrictive hosts, contain phage-associated phospholipase with activity similar to that of T4 grown in suppressor-positive hosts (data not shown). This indicates that the presence or absence of the enzyme on the phage does not depend upon the genotype of the *E. coli* host.

FIG. 2. Phospholipase activity as a function of phage protein concentration. Reaction mixture, 0.5 ml final volume, contained 20% methanol, Tris-hydrochloride buffer (100 mM, pH 7.8), 80 μ M PG, and phage protein as indicated. Phospholipase activity was assayed as described in Materials and Methods.

TABLE 2. Phospholipase activity of T4 phage grown in phospholipase-deficient hosts

Assay mixture ^a	FFA release from PG	
	Counts/min	%
Control ^b	7	0.2
T4 (not dialyzed)	400	13.3
T4 (dialyzed)	1,367	45.6

^a Assay mixture consisted of 0.1 mg of phage protein incubated with 40 μ M PG in 20% methanol and 100 mM Tris-hydrochloride (pH 7.8), for 18 h at 37 C. Final reaction volume, 0.5 ml.

^b Control, no added phage.

DISCUSSION

T4 phage, purified by differential centrifugation, contain a latent phospholipase activity. The enzyme in such preparations can be activated either by further purification of the phage in CsCl gradients, followed by dialysis, or by dialysis alone. The dialysis procedure presumably removed an inhibitor. We do not know where the phospholipase is located on the phage particle. Its association with T4 ghosts implies that it is not normally contained in the head of the phage and released with the DNA. That it is tightly bound to the virus particle is indicated by the retention of the activity during CsCl gradient purification. We have no evidence that it is an integral part of the phage particle and it is possible that it may simply adhere to the phage, as does lysozyme.

The assay for phospholipase activity used throughout was based on measurements of FFA formation, and at this time we have not determined whether the other products of the reaction include lysophosphatidyl-glycerol. In some assays we have observed that more than 50% of the added PG radioactivity could be recovered as FFA, suggesting that both fatty acyl groups can be released.

The PG specificity of the phage-associated phospholipase distinguishes it from the *dr* phospholipase of *E. coli* K12 (17). It differs from the PG-specific *ds* phospholipase in that it is activated by methanol and Triton X-100, whereas the *ds* enzyme is inactivated (17). Additionally, the presence of the PG-specific enzyme on phage grown in *E. coli dr⁻ds⁻* indicates that it is distinct from either host *dr* or *ds* phospholipase. Nojima et al. (17) reported that *E. coli* K12 *dr⁻ds⁻* contains less than 1% of the detectable phospholipase of the parent *dr⁺ds⁺*. Extracts prepared from our cultures of this mutant, when incubated with PG in the presence of 20% methanol and 100 mM Tris-hydrochloride (pH 7.8), demonstrated nearly 50% of the level of phospholipase found in *dr⁺ds⁺* extracts. The extracts from the mutant, however, did not hydrolyze PE, indicating that the observed PG deacylation was not due to a "leaky" *dr* mutation (data not shown). These results indicated that *E. coli* may contain additional uncharacterized phospholipases. However, the absence of the PG-specific phospholipase in unsuppressed T4amtA3 particles seems to eliminate the possibility that the phage-associated enzyme is the uncharacterized host phospholipase. Although these observations suggest that the phage-associated phospholipase is T4 gene specific, it is nevertheless possible that the *t*-gene does not

TABLE 3. Assay for T4amtA3-associated phospholipase^a

Assay	FFA produced ^b (nmol)
Suppressed T4amtA3	
33 μ g	10.4
100 μ g	25.6
Unsuppressed T4amtA3	
33 μ g	4.2
100 μ g	5.0
Control ^c	6.5

^a Suppressed and unsuppressed T4amtA3 were prepared as described in Materials and Methods. Assay mixture consisted of phage protein, at indicated concentrations, incubated with 80 μ M PG in 20% methanol and 100 mM Tris-hydrochloride (pH 7.8). Final reaction volume, 0.5 ml.

^b FFA production, after 16 h of incubation with shaking at 37 C, was determined as described in Materials and Methods.

^c Control, no phage added.

code for phospholipase, but rather for another product which can activate a latent phospholipase which is associated with the phage particle. We have no experimental data which distinguishes between these alternatives.

Attempts to directly demonstrate an increase in activity of a PG-specific phospholipase in unlysed, infected cells were not successful. As reported by Cronan and Wulff (7), T4 phage infection results in phospholipid deacylation and the hydrolysis seems to be a consequence of activation of the *dr* phospholipase, which acts on PE, PG, and CL. Since PG comprises only 15 to 20% of the total phospholipid, the effect of the phage-associated enzyme with specificity for PG was masked.

We have no experimental evidence directly concerned with the biological role of the phage-associated phospholipase. It apparently is not required for attachment and DNA injection, since unsuppressed phospholipase-free T4amtA3 can infect *E. coli* K12(λ)IYMel. Phospholipase may, however, have a role in the lysis from within process, as suggested by Cronan and Wulff (7). Subsequently, Josslin (12) reported that FFA formation could occur in cells infected with endolysin-defective T4. Under such conditions lysis from within was not observed, but metabolic arrest of the host occurred at the normal time. Suppressor-negative cells infected with T4amtA3 mutants did not show either lysis from within or blocked respiration, and FFA formation was not observed. He concluded that phospholipid hydrolysis was not

directly involved in the lytic process, but rather that it was a consequence of cytoplasmic membrane degradation which occurred as a function of the *t*-gene product. Bennett et al. (3), however, by using mutants not able to utilize FFA liberated by phospholipase, established that FFA formation can occur prior to lysis. It has been shown that a variety of conditions can activate host phospholipase (6, 21), and even the products of phospholipid hydrolysis (FFA and lysophospholipids), by acting as detergents may have such a role. We believe it is likely that phage phospholipase is required to activate host phospholipase, which in turn degrades the inner membrane, resulting in blocked respiration and escape of endolysin. This would explain why unsuppressed *T4amtA3* mutants do not block respiration or cause lysis, and why they do not cause FFA formation. If this interpretation is correct the phospholipase-deficient *E. coli* cells should either not be lysed from within by T4, or should show extensive lysis inhibition. In one-step growth experiments with T4 and *E. coli* K12 *dr⁻ds⁻*, it was observed that lysis could occur, but only after very extended periods of lysis inhibition (our unpublished results).

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