DIPHTHERIA TOXIN: EVIDENCE FOR PRESENCE OF FRAGMENT B IN CORYNEBACTERIOPHAGE.

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Summary

The protein composition of a virulent β tox^+ corynebacteriophage has been characterized by SDS-polyacrylamide gel electrophoresis. The major protein in the purified phage preparations shows size identity with the B fragment of purified diphtheria toxin. Phage is capable of protecting KB cells from subsequent intoxication with diphtheria toxin which supports the identification of B fragment present in the phage preparations. The concentration of iron in growth media had no effect on the protein composition of the purfied phage.

Introduction

Only strains of Corynebacterium diphtheriae which are infected with tox^+ corynebacteriophages are capable of producing diphtheria toxin (1,2). The recent isolation of phage mutants, the new lysogens of which produce altered toxin proteins, provides strong evidence that the toxin structural gene is part of the phage genome (3,4,5). To date, however, the role that toxin plays in phage development is unknown.

We have examined the protein composition of purified tox^+ corynebacteriophage to determine if any of these proteins share identity with diphtheria toxin or toxin fragments. Our results show that the major protein present in the phage preparations has size identity with one of the major diphtheria toxin fragments.

Materials and Methods

A. Phage growth and purification:

A tox^+ virulent β corynebacteriophage, β^{vir}, (6) was propagated in C_7 (-) tox (-) strain of C. diphtheriae in PGT medium supplemented with 4.0 μg iron/ml. The media employed, the methods for bacterial and phage growth and plaquing techniques were those described by Matsuda and Barksdale (7). The phage preparations were purified as follows: Crude lysates containing approximately

2 X 10^{10} PFU/ml were centrifuged at 12,000 X g (low speed) for 20 minutes to remove bacterial debris. The phage was then centrifuged out of suspension at 105,000 X g (high speed) for 2 hours. Phage was resuspended in sterile 0.1 M sodium phosphate buffer, pH 7.2 and subjected to three more cycles of low and high speed centrifugations and centrifuged to equilibrium in $CsCl_2$. The $CsCl_2$ gradients were fractionated by collecting drop fractions from the bottom of the tubes. All fractions were assayed for infectivity and only the tubes with peak infectivity were used as a source of purified phage. The final preparation of phage was exhaustively dialysed against 0.1 M sodium phosphate buffe pH 7.2.

B. Polyacrylamide gel electrophoresis:

Polyacrylamide gels containing 10% acrylamide and 0.26% methylene bisacrylamide in 0.1 M sodium phosphate buffer, pH 7.2 and 0.1% SDS were prepared according to the method of Weber and Osborn (8). For each gel (17 X 0.8 cm) a sample of approximately 100 µg protein was dissolved in 100 µl of 0.1 M sodium phosphate buffer containing 1% SDS and 10% sucrose. Samples were heated at 100 C for 2 minutes prior to loading. The gels were electrophoresed for 14 hours at 4.5 ma/gel in 0.1 M phosphate buffer, pH 7.2 containing 0.1% SDS. Gels were fixed in 12% TCA for 1 hour, stained at least 6 hours in 0.2% Coomassie brilliant blue and destained by diffusion in a solution of 20% methanol and 10% acetic acid.

Purified diphtheria toxin was split into its two major components (Fragments A and B) according to the trypsinization procedure of Gill and Dinius (9).

C. KB cell protection studies:

Tube cultures of KB cells were grown to confluency in Hanks' MEM medium containing 12% fetal calf serum and 1% glutamine. The medium was poured off and various concentrations of β^{vir} phage, bovine serum albumin, or T_4 coliphage diluted in 10% Hanks' BSS containing 1% calf serum were added to the cells in a fin volume of 0.9 ml. Following 1 hour incubation at 37 C, 0.1 ml of diphtheria toxin was added to the tubes in sufficient concentration to inhibit KB cell protein

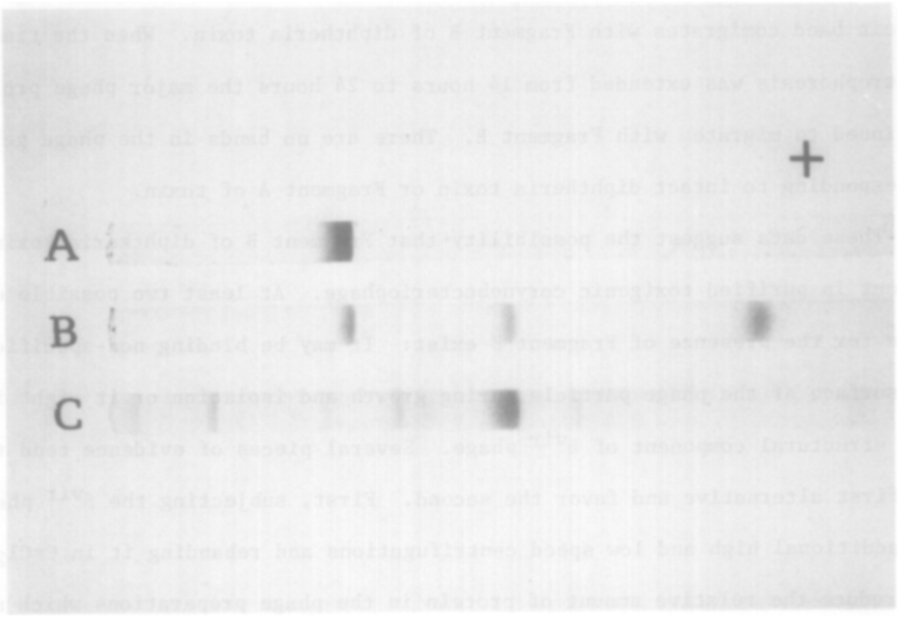

Figure 1. SDS-polyacrylamide gels of diphtheria toxin and phage. Gel A; intact diphtheria toxin. Gel B; trypsinized diphtheria toxin. Fragment A is band closest to cathode, Fragment B is the middle band. Gel C; β^{vir} corynebacteriophage (propagated in high iron medium).

synthesis by 90-95% relative to control cells that received only diluent. After 3 hours further incubation, 0.1 ml of a C^{14} amino acid mixture (New England Nuclear; L-amino acid-C^{14} mixture) was added to each tube at a final specific activity of 0.1 μC/tube. After 2 more hours of incubation the cells were washed two times with phosphate buffered saline and 0.01% trypsin added. The trypsinized cells were vortexed and TCA added to a final concentration of 5%. The precipitates were collected on Millipore filters, washed thoroughly with 5% TCA and 95% ethanol, dried and counted in a liquid scintillation counter.

Results and Discussion

As shown in Figure 1, polyacrylamide gel electrophoresis of SDS treated purified β^{vir} corynebacteriophage reveals multiple protein bands. The major

protein band comigrates with Fragment B of diphtheria toxin. When the time of electrophoresis was extended from 14 hours to 24 hours the major phage protein continued to migrate with Fragment B. There are no bands in the phage gels corresponding to intact diphtheria toxin or Fragment A of toxin.

These data suggest the possibility that Fragment B of diphtheria toxin is present in purified toxigenic corynebacteriophage. At least two possible explanations for the presence of Fragment B exist: It may be binding non-specifically to the surface of the phage particle during growth and isolation or it might indeed be a structural component of β^{vir} phage. Several pieces of evidence tend to rule out the first alternative and favor the second. First, subjecting the β^{vir} phage to two additional high and low speed centrifugations and rebanding it in $CsCl_2$ did not reduce the relative amount of protein in the phage preparations which migrated in SDS-polyacrylamide gels with Fragment B of diphtheria toxin. Second, increasing the final yield of toxin by approximately 50% by propagating phage in cells grown in low iron medium did not result in an increase in the amount of phage protein comigrating with Fragment B. Gels of bacteriophage grown in deferred PGT, in which maximum toxin production occurs, resulted in identical protein band profiles as those seen in our "high iron" preparations. Therefore, irrespective of the iron concentration of the medium, the major protein component of β^{vir} phage preparations comigrates with Fragment B of diphtheria toxin. Furthermore, when β^{vir} phage is extracted with 6 M guanidine hydrochloride, a procedure shown to yield the major protein component of T_4 coliphage (10), the major extractable protein also shows size identity with Fragment B of diphtheria toxin (manuscript in preparation). However, these observations do not conclusively rule out the possibility that Fragment B of diphtheria toxin is merely co-purifying with the β^{vir} phage.

Uchida et al. (4) have recently isolated a mutant β corynebacteriophage (β_{197}), the lysogens of which produce a protein with an altered Fragment A but a normal Fragment B. This protein when pre-incubated with HeLa cells inhibited the action of diphtheria toxin on these cells indicating that Fragment B is that

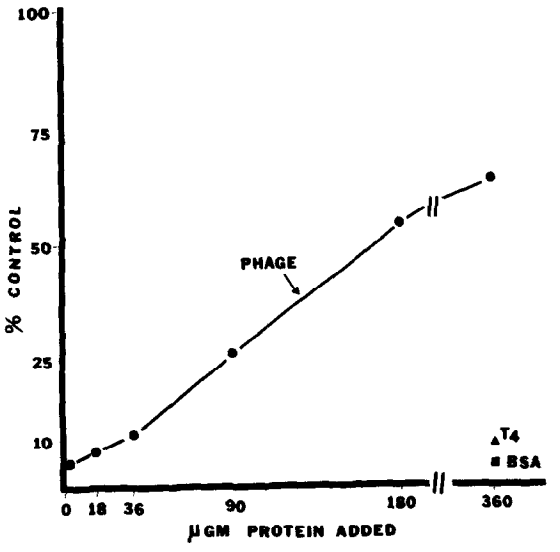

Figure 2. Ability of β^{vir} corynebacteriophage to protect KB cells from subsequent diphtheria intoxication. Experimental procedures explained in Materials and Methods. T_4; T_4 coliphage, BSA; bovine serum albumin. Phage; β^{vir} corynebacteriophage propagated in high iron medium.

portion of the toxin molecule that specifically binds to the susceptible cell membrane (4).

Since the major protein in the β^{vir} phage preparations shares size identity with Fragment B of toxin we tested for the ability of these phage to protect cultured KB cells from diphtheria intoxication. Figure 2 shows the results obtained when increasing concentrations of intact, purified β^{vir} phage were preincubated with KB cells prior to the addition of toxin. Toxin was added at a concentration sufficient to inhibit protein synthesis by approximately 95% (represented by the point touching the ordinate; these cultures received no added "protective protein"). It is clear that with increasing concentrations of β^{vir} phage there is increasing protection of KB cells from diphtheria intoxication. When intact β^{vir} phage was added to the cells at a concentration of 360 μg/culture subsequent intoxication was inhibited by 63%. T_4 coliphage or bovine serum

albumin (BSA) added to the cells at a concentration of 360 µg/culture did not protect the KB cells indicating that protection is not due to non-specific binding of protein to the cells. In some experiments the phage dilutions were poured off and the cells washed several times with buffer prior to the addition of toxin. This procedure did not decrease the degree of cell protection indicating that protection is a function of phage - cell interaction.

The hypothesized presence of Fragment B in β^{vir} corynebacteriophage raises the possibility that diphtheria toxin which reportedly is synthesized as a single polypeptide (11, 12) is cleaved within the cell during phage development. Such cleavages have been shown to be involved in processing enterovirus proteins (13, 14). The role such cleavage mechanisms play in β^{vir} corynebacteriophage development remains to be elucidated.

References

1. Freeman, V. J., J. Bact. 61, 675 (1951).
2. Groman, N. B., J. Bact. 66, 184 (1953).
3. Uchida, T., Gill, D. M. and Pappenheimer, A. M. Jr., Nature New Biol. 233, 8, (1971).
4. Uchida, T., Pappenheimer, A. M. Jr., and Harper, A. A., Science 175, 901 (1972).
5. Matsuda, M., Kanei, C. and Yoneda, M., Biochem. and Biophys. Res. Comm. 46, 43, (1972).
6. Holmes, R. K. and Barksdale, L. J. Virol. 3, 586 (1969).
7. Matsuda, M. and Barksdale, L., J. Bact. 93, 722 (1967).
8. Weber, K. and Osborn, M., J. Biol. Chem. 244, 4406 (1969).
9. Gill, D. M. and Dinius, L., J. Biol. Chem. 246, 1485 (1971).
10. Forrest, G. L. and Cummings, D. J., J. of Virol. 5, 398 (1970).
11. Gill, D. M. and Pappenheimer, A. M. Jr., J. Biol. Chem. 246, 1492 (1971).
12. Collier, R. J. and Kandel, J., J. Biol. Chem. 246, 1496 (1971).
13. Kiehn, E. D. and Holland, J. J., J. of Virol. 5, 358 (1970).
14. Jacobson, M. F. and Baltimore, D., J. Mol. Biol. 33, 369 (1968).