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pH-Dependent membrane interactions of diphtheria toxin: A genetic approach

R. JOHN COLLIER
Department of Microbiology and Molecular Genetics and Shipley Institute of Medicine, Harvard Medical School, Boston, Massachusetts 02115, USA

Abstract. A genetic approach is described for exploring the mechanism by which diphtheria toxin undergoes pH-dependent membrane insertion and transfer of its enzymic A fragment into the cytoplasm of mammalian cells. The cloned toxin expressed in *Escherichia coli* is secreted to the periplasmic space, where it is processed normally and folds into a native structure. When bacteria synthesizing the toxin are exposed to pH 5, they die rapidly. The toxin undergoes a conformational change that is believed to allow it to be inserted into the bacterial inner membrane and form channels, which proves lethal for the cell. The membrane insertion event mimics the process by which the toxin inserts into the endosomal membrane of mammalian cells, leading to release of the enzymic A fragment into the cytoplasm. The observation of pH-dependent bacterial lethality provides the basis for a positive genetic selection method for mutant forms of the toxin that are altered in ability to undergo membrane insertion or pore formation.

Keywords. Diphtheria toxin; enzymic A; pH-dependent lethality.

Introduction

Diphtheria toxin (DT) is representative of a group of toxic proteins of bacterial or plant origin that act by covalently modifying substrates within the cytoplasm of mammalian cells (Collier, 1975; Pappenheimer, 1980). Members of this group function as an unusual group of enzymes, which undergo a complex series of events, leading to the introduction of an enzymic moiety of the toxin into the cytosol. Although the mechanism of entry has not been described in detail for any of these toxins, DT has been studied intensively in this regard, and a good working model for entry now exists. In this communication a novel genetic approach that may permit us to isolate mutant forms of the toxin that are defective in their membrane interactions has been reported.

Mechanism of DT action

DT (535 residues) kills mammalian cells by inhibiting protein synthesis. Before or soon after the toxin binds to cells it is proteolytically processed into 2 chains, termed fragments A and B. The enzymic moiety of the toxin (fragment A, or DTA; 193 residues) is delivered to the cytosol by a process that begins with receptor-mediated endocytosis. After binding to cell surface receptors, the toxin is conveyed to an intracellular acidic compartment (endosome) where the acidic conditions induce a conformational change in the protein. This exposes hydrophobic regions within fragment B (DTB), which leads to insertion of the toxin into the endosomal membrane. Concurrently or subsequently DTA is translocated across the

Abbreviations used: DT, Diphtheria toxin; EF-2, elongation factor-2.

endosomal membrane into cytosol, where it catalyzes the ADP-ribosylation of elongation factor-2 (EF-2). The factor is thereby inactivated and protein synthesis ceases.

The mechanisms responsible for intoxication by DT are slowly emerging at a highly detailed level. For example, an amino acid residue that is believed to participate in catalysis has been identified (Glu-148) (Carroll and Collier, 1984; Carroll *et al.,* 1985). By contrast, the process of membrane insertion/translocation remains largely unexplored at a detailed level. It is known that DT and certain fragments form ion-conductive channels in artificial lipid bilayers under acidic pH conditions (Donovan *et al.,* 1981; Kagan *et al.,* 1981) and recent reports suggest that similar channels are formed during toxin translocation in mammalian cell membranes (Sandvig and Olsnes, 1988). Although it is clear that central regions of DTB contain highly hydrophobic sequences that almost certainly participate in the insertion/translocation, there are few data regarding the roles of specific residues or regions.

An approach to isolate mutant forms of DT toxin

Here an attempt has been made to isolate mutant forms of DT that are defective in membrane interactions at acidic pH. It has been found that *E. coli* cells in which cloned DT is synthesized and secreted to the periplasm are killed by exposure to acidic conditions (O'Keefee and Collier, 1989). An enzymically attenuated mutant form of the toxin, DT-E148S, has been used in order to comply with the NIH recombinant DNA guidelines. Mutagenesis of the toxin gene followed by genetic selection at acidic pH should yield mutant forms of the toxin that are nonlethal for *E. coli* due to defects in their ability to interact with membranes. A subset of these, at a minimum, would be expected to exhibit defects in ability to intoxicate mammalian cells.

The notion that DT within the periplasmic compartment might prove lethal to the producing cells under acidic conditions derives directly from the finding that DT inserts and forms channels in artificial lipid bilayers at acidic pH. Thus if the toxin were to form a pore in the inner membrane of *E. coli,* this would presumably cause a loss of membrane potential and permit leakage of ions and perhaps other small molecules of the cytoplasm. Lethality for the bacteria would therefore be independent of the ADP-ribosyltransferase function of the toxin; and indeed it is known that bacterial protein synthesis, in contrast to that in mammalian cells, is not inhibited by the toxin.

The pH-dependent lethality of the toxin in *E. coli* may be demonstrated by plating toxin-producing cells on agar media at a variety of pHs (O'Keefe and Collier, 1989). Cells synthesizing DT-E148S showed drastic loss of viability at low pHs, with only 1 in 10^7 cells surviving at pH 5. Cells producing the corresponding fragment A showed no loss of viability at low pH. Production of a toxin fragment, termed F2, which contains all of DTA and part of DTB (including most of the central hydrophobic domain) also showed loss of viability under acidic conditions, but the effect was less pronounced than that with the whole toxin. Cells producing wild-type F2 fragment showed identical loss of viability to that seen with F2-E148S, confirming that the catalytic function of the toxin plays no part in lethality in *E. coli.*

When the physiological consequences of producing DT-E148S or DTA-E148S were examined under acidic conditions, it was found that cells synthesizing the whole toxin lost membrane potential, the capacity to import proline by active transport, and the ability to retain intra-cellular ^{86}Rb (O'Kefee and Collier, 1989). Cells producing only the A chain were unaffected.

Mutagenesis of the toxin gene followed by transformation of *E. coli* and selection of clones that survive at pH 5 provides a potential route to the direct selection of mutant forms of the toxin that are deficient in the ability to insert into membranes and/or form transmembrane channels in a pH-dependent manner. This method, which provides a unique approach to identifying specific amino acids that are functional in membrane insertion or channel formation, should complement biophysical and biochemical that have been applied to such questions to date. The method may be applicable to other toxins, and possibly to mammalian viruses, that undergo pH-dependent steps in entry into the cell, and it could yield information relevant to broader questions concerning the insertion of other classes of proteins into membranes.

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