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Hypothesis

The second cholera toxin, Zot, and its plasmid-encoded and phage-encoded homologues constitute a group of putative ATPases with an altered purine NTP-binding motif

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It is shown that the second cholera toxin, Zot, ORF3 product of *Pseudomonas* plasmid pKB740, and ORF424 product of bacteriophage Pf1 are a group of closely related proteins containing a modified version of the purine NTP-binding motif, with a drastic substitution of tyrosine for a conserved glycine. They are distantly but reliably related to the product of gene I of filamentous bacteriophages which is a putative ATPase containing the classical NTP-binding motif and is involved in bacteriophage assembly and exit from the bacterial cell. Hydropathy analysis suggests that the Zot and gene I product may have a similar transmembrane topology. It is hypothesized that Zot may possess ATPase activity and modify the membrane structure of its target cells in an ATP-dependent fashion. Genes for Zot and the related protein of pKB740 are likely to have evolved from gene I of a Pf1-like bacteriophage.

Zot protein; Second cholera toxin; NTP-binding motif; ATPase; Filamentous phage gpI

1. INTRODUCTION

It has been shown recently that in addition to the well known cholera toxin, *Vibrio cholerae* encodes a second toxin called Zot (zonula occludens toxin) that increases the permeability of small intestinal mucosa by inducing a modification of the structure of the intercellular tight junction, or zonula occludens [1]. The sequence of the *zot* gene has been determined, and no significant sequence similarity to other proteins has been reported [2].

A database search using the BLAST program [3] with the Non-redundant Database (National Center for Biotechnology Information, NIH) revealed that Zot is closely related to the ORF3 product of pKB740, a 8.3 kilobase *Pseudomonas* plasmid bearing genes for the enzymes of 2-aminobenzoate metabolism [4] and to the ORF424 product of filamentous bacteriophage Pf1, also from *Pseudomonas* [5]. The probability of matching by chance alone for Zot and these proteins was 4.5×10^{-25} and 1.6×10^{-18}, respectively.

The position of ORF424 in the Pf1 genome and the previous mention of distant relationship between most Pf1 ORFs and gene products of filamentous single-stranded DNA bacteriophages [5] led me to suspect that its product might be related to the product of gene I (gpI) of these phages. GpI contains the Walker-type purine NTP-binding pattern and is thought to be an ATPase involved in phage assembly and exit from the bacterial cell [6–8].

2. ALIGNMENT

The alignment of Zot with the two related proteins revealed a sequence resembling the A motif of the NTP-binding pattern, < hydrophobic region > [GA]xx(G)xGK[TS], with the exception that Tyr is substituted for the third Gly. Comparison of the sequences of these proteins with those of gpI using the MACAW program [9] detected three conserved regions (Fig. 1). Region 1 corresponds to the A motif of the NTP-binding pattern that had the classical form in gpI and the modified form in Zot and the related plasmid and phage proteins. Region 2 corresponds to the B motif of the NTP-binding pattern; this motif conformed to the original consensus [6] in all the aligned proteins.

Region 3 is specific for this set of proteins and interestingly shows the highest level of conservation, partic-

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Abbreviations: Zot, zonula occludens toxin; SpV1, *Spiroplasma* virus 1; NTP, nucleoside triphosphate.

ularly between the proteins of the Zot group and gpI proteins of filamentous coliphages (Fig. 1). The signature RxxxWD[ILVF]x[LF]xxx[DN][IL] was found to be unique to these proteins upon search of the entire database even when conservative substitutions of the hydrophobic amino acid residues were allowed.

The multiple alignment of the Zot-related proteins with the phage gpI proteins (Fig. 1) generated by OPTAL program [10] scored 9.9 standard deviations over the random expectation indicating a definite though distant relationship. These findings led me to consider this set of proteins a specific family of putative NTPases, with Zot, and the ORF3 and ORF424 products constituting a distinct subfamily.

3. A MODIFIED NUCLEOTIDE-BINDING MOTIF

I predict that despite the drastic substitution of Tyr for a Gly residue conserved in a wide variety of NTP-utilizing enzymes, Zot, and the products of ORF3 and ORF424 have a functional NTP-binding site and may possess NTPase activity. The most important feature of the A motif is the existence of a hydrophobic β-strand followed by a flexible Gly-rich loop that interacts with the phosphates of the NTP substrate [6,11]. Inspection of the sequences (Fig. 1) and secondary structure prediction (not shown) suggested that these features are probably retained by the counterpart of the A motif in Zot and the related proteins. Moreover, analogous substitutions of Tyr or Phe for Gly have been observed in two proteins that are known to possess ATPase activity and belong to well-defined families of ATPases, namely in the F plasmid partitioning protein SopA and the bacteriophage T4 DNA repair protein UvsX (Fig. 1). Site-directed mutagenesis or other direct analysis of the putative unusual NTP-binding motif in these proteins has not yet been reported. Nevertheless, statistically sig-

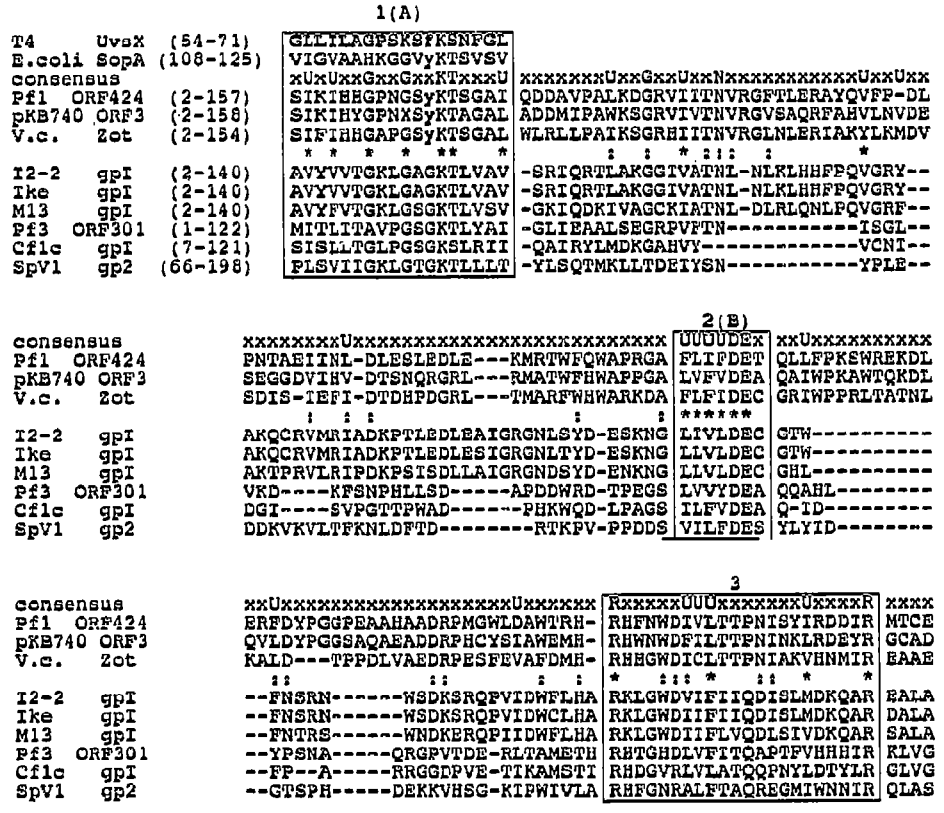

Fig. 1. Amino acid sequence alignment of the proteins of the Zot group with putative NTPases encoded by gene I of filamentous bacteriophages. The alignment was generated by OPTAL program [10]. Motifs delineated by MACAW program are boxed. The 'consensus' line includes amino acid residues that are conserved in at least eight of the nine aligned sequences. U indicates a hydrophobic residue (I,L,V,M,F,Y,W). Asterisks denote residues that are conserved (identical or similar) in the sequences of the Zot-related proteins and in the six bacteriophage sequences, colons denote residues that are conserved specifically in the Zot-related proteins and in the three coliphage sequences. The position of the aligned regions in the respective proteins is indicated in parentheses. The Tyr (Phe) residue substituting for the conserved Gly in the A motif is shown in lower case and in bold typing. The sequence of ORF3 of pKB740 contained an obvious frameshift separating the N-terminal 10 residues from the rest of the protein. Reconstruction of the sequence has led to an uncertainty in one position within the A motif (designated by an X). Another frameshift was found in this sequence downstream from the region shown in the figure. For UvsX and SopA only short segments spanning the A motif are shown to emphasize the substitution of Tyr(Phe) for Gly. I2-2, IKe and M13 are coliphages, Cf1c is a *Xanthomonas campestris* bacteriophage, Pf1 and Pf3 are *Pseudomonas* bacteriophages, and SpV1 is *Spiroplasma* virus 1R8A2B; V.c., *Vibrio cholerae*. The sequences were from: [2] (Zot), [5] (Pf1), [14] (pKB740), [17] (Cf1c), and [18] (SpV1). The references for the sequences of gpI proteins of coliphages and Pf3 can be found in [6].

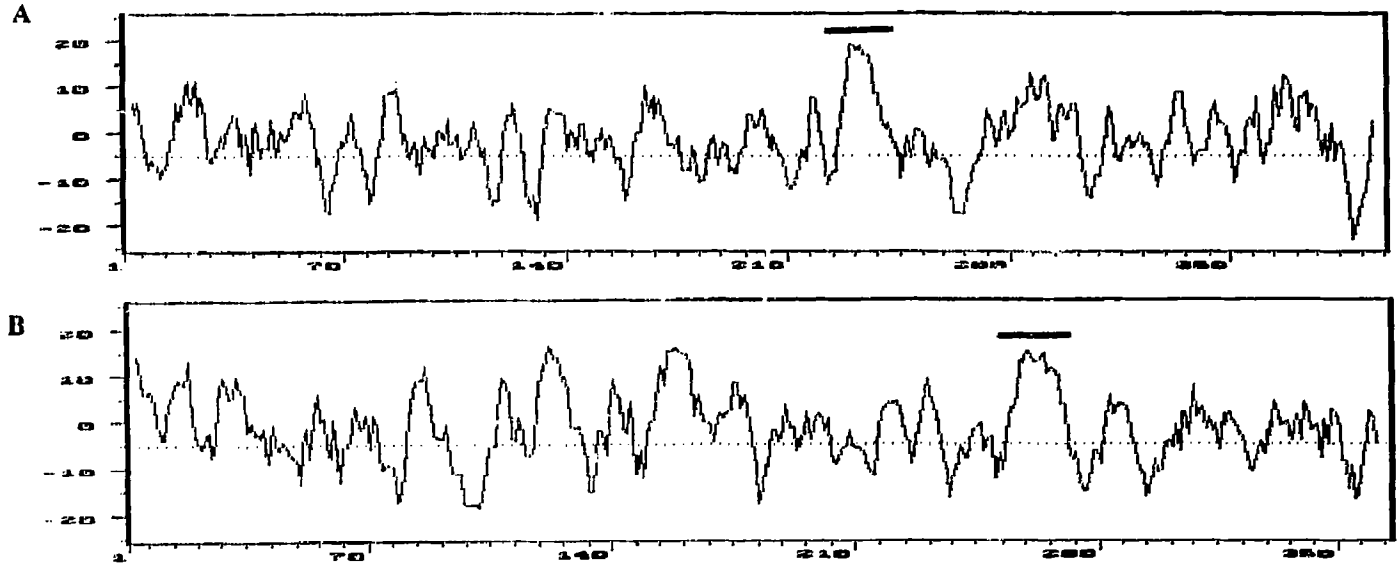

Fig. 2. Comparison of the hydropathicity plots for Zot (A) and gpI of bacteriophage I2-2 (B). The hydropathicity was calculated using the method of Kyte and Doolittle with the window size of seven [19]. The abscissa shows amino acid residue numbers and the ordinate shows hydropathicity units. Hydrophobic regions are above and hydrophilic regions are below the dotted midpoint line. Bars denote transmembrane segments predicted according to [20]. The computation was performed using the program SOAP in the PC/GENE package [21].

nificant alignments with the homologues containing the classical motif leave little doubt about the authenticity of the Tyr and Phe-containing motif ([6,12], and unpublished observations).

4. ZOT IS A MEMBRANE PROTEIN

GpI of filamentous bacteriophages is an integral membrane protein [7]. Comparison of the hydropathy plots for Zot and gpI revealed considerable similarity, with one membrane-spanning segment located downstream from the putative NTPase domain being predicted for each protein (Fig. 2). Thus it is likely that Zot is a membrane protein too, and that Zot and gpI have similar transmembrane topology.

In addition to its participation in phage assembly, gpI is involved in phage exit from the cell through induction of so-called contact zones between the inner and outer membranes [7]. When overexpressed, gpI kills the bacterial cell through rapid loss of the membrane potential [13]. It is tempting to draw an analogy between these properties of gpI and the induction of modifications of cellular membranes by Zot and to speculate that the latter effect may be dependent on the ATPase activity of Zot.

5. EVOLUTIONARY IMPLICATIONS

Finally, these observations led to interesting evolutionary implications. Our finding that the ORF3 product is related to gpI, along with the previously noted similarity between ORF5 product and gpII [14], makes it tempting to speculate that pKB740 could evolve from a filamentous bacteriophage, with the other phage genes having been replaced by the genes encoding 2-aminobenzoate metabolizing enzymes. Origin from gene I of a Pf1-like bacteriophage is likely for *zot* gene too. This and not the opposite direction of evolution is preferred since, on the one hand, Zot and ORF3 are closely related to gpI of Pf1 but only distantly related to gpI of other bacteriophages, and on the other hand, it appears most likely that all filamentous bacteriophages have a common origin. The *zot* gene, together with the adjacent *ctx* operon encoding the two subunits of the classical cholera toxin, belongs to a site-specific transposable element [15]. It is interesting to speculate that this element could have evolved from a filamentous bacteriophage via a plasmid intermediate resembling pKB740. This seems particularly plausible as pKB740 as well as the phage SpV1 are able to integrate into the bacterial chromosome [15,16].

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