Asp 187 and Phe 190 residues in lethal factor are required for the expression of anthrax lethal toxin activity

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Received 13 December 2001; received in revised form 4 March 2002; accepted 25 April 2002

First published online 23 May 2002

Abstract

Anthrax toxin consists of three proteins, protective antigen, lethal factor, and edema factor. Protective antigen translocates lethal factor and edema factor to the cytosol of mammalian cells. The amino-termini of lethal factor and edema factor have several homologous stretches. These regions are presumably involved in binding to protective antigen. In the present study we have determined the role of one such homologous stretch in lethal factor. Residues 187AspLeuLeuPhe190 were replaced by alanine. Asp187Ala and Phe190Ala were found to be non-toxic in combination with protective antigen. Their protective antigen-binding ability was drastically reduced. We propose that Asp187 and Phe190 are crucial for the expression of anthrax lethal toxin activity.

Keywords: Anthrax; Lethal toxin; Lethal factor; Osmotic lysis

1. Introduction

The anthrax toxin is the major virulence factor secreted by Bacillus anthracis. It consists of protective antigen (PA), lethal factor (LF), and edema factor (EF). LF and EF are the catalytic components of the toxin, whereas PA is the receptor-binding component, which mediates the entry of LF and EF to the cytosol of mammalian cells by a mechanism associated with its ability to heptamerize, and form a transmembrane pore [1]. LF and EF bind competitively to the receptor-bound PA. When EF combines with PA, edema toxin is formed, whereas the combination of LF and PA forms lethal toxin [2]. The lethal toxin is considered to be the cause of death in fatal anthrax infections. Mouse peritoneal macrophages and macrophage-like cell lines such as RAW264.7 and J774A.1 are sensitive to anthrax lethal toxin [3]. Continuous protein synthesis and the presence of extracellular calcium is required for the expression of anthrax lethal toxin activity [4,5]. LF is a zinc-dependent metalloprotease [6]. LF cleaves several mitogen-activated protein kinase kinases (MAPKKs) from their N-termini and inactivates the signal transduction pathway [7]. However, cleavage of MAPKKs by LF has not yet been associated with its lethality.

The crystal structure of LF has been determined [8]. The LF molecule consists of four domains. Domain 1 (residues 1–254) is the PA-binding domain. Sequence examination of LF and EF shows that their amino-termini have extensive sequence homology. Since the intracellular actions and enzymatic activities of these two proteins are different, the regions of homology at their amino-termini have been viewed as the sites responsible for high-affinity binding to PA. The residues of one such homologous stretch in LF as well as EF are indispensable for binding to PA [9,10]. The present study was initiated to determine the importance of another homologous region of LF consisting of residues Asp187, Leu188, Leu189 and Phe190.

2. Materials and methods

2.1. Materials

Biochemicals were purchased from USB Chemicals (USA). Bacterial culture medium was purchased from Hi-Media Laboratories (India). Trypsin, PEG1000 and...
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (USA). RAW264.7, a macrophage-like cell line, was obtained from American Type Culture Collection (USA).

2.2. Plasmid construction and mutagenesis

The LF gene was mutagenized using previously constructed plasmid pPGLF1 as template [11]. Long PCR amplification was done using mutagenic oligonucleotides, which annealed on the opposing DNA strands [12]. PCR-amplified products were subjected to DpnI treatment and transformed into Escherichia coli DH5α cells. Transformants were screened by restriction digestion and verified by sequencing. Mutant proteins were purified according to the method previously described [9].

2.3. Cell culture and cytotoxicity assay

Macrophage-like cell line RAW264.7 was maintained in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 25 mM HEPES, 100 U ml⁻¹ penicillin and 200 µg ml⁻¹ streptomycin. LF mutants were added in varying concentrations with PA (1.0 µg ml⁻¹) and incubated for 3 h at 37°C. LF from B. anthracis along with PA was used as a positive control. Cytotoxicity was determined by MTT assay [13].

2.4. Binding of LF mutants to PA in solution

To study the binding of LF to proteolytically nicked PA, the PA molecule was digested with trypsin [14]. Trypsin-nicked PA (1.0 mg ml⁻¹) was incubated with mutant LF proteins (1.0 mg ml⁻¹) in 25 mM Tris, pH 9.0, containing 2.0 mg ml⁻¹ CHAPS (3-[3-cholamidopropyl] dimethyl ammonio]propanesulfonic acid) for 15 min at room temperature. Samples were applied to a non-denaturing 4.5% polyacrylamide gel. Gels were stained in Coomassie brilliant blue, destained and analyzed.

2.5. Direct introduction of LF mutants into the cytosol of RAW264.7 cells

LF was directly introduced into the cytosol of cells by the method of osmotic lysis of pinosomes [3,15]. 50–100 µg ml⁻¹ of LF mutant proteins were taken. B. anthracis LF was taken as a positive control. Cytotoxicity was measured by MTT assay.

3. Results

3.1. Cell culture and cytotoxicity assay

LF mutant proteins were assayed for their functional activity by the cytotoxicity assay. It was observed that D187A, F190A and the combination mutant (D187A L188A L189A F190A) were totally non-toxic even at very high concentrations of 10.0 µg ml⁻¹. F190A was partially toxic at 10.0 µg ml⁻¹. L188A and L189A were fully toxic at normal concentrations (Fig. 1). The EC₅₀ of the non-toxic mutants was found to be >10.0 µg ml⁻¹.

3.2. Binding of LF mutants to PA in solution

Trypsin-nicked PA has the ability to bind LF. LF mutant proteins were incubated with trypsin-nicked PA. PA-LF binding was analyzed on 4.5% polyacrylamide gel. It was found that D187A, F190A and the combination mutant were unable to bind to PA. The PA-binding ability of L188A and L189A was comparable to the native protein (Fig. 2).

![Fig. 1. Cytotoxicity profile of LF mutants. RAW264.7 cells were incubated with PA (1.0 µg ml⁻¹) in combination with varying concentrations of LF mutant proteins for 3 h at 37°C. At the end of the experiment cytotoxicity was determined by MTT assay. The absorption obtained at 540 nm (1.202) with untreated cells was considered 100% viability.](image-url)
3.3. Direct introduction of LF mutants into the cytosol of RAW264.7 cells

The non-toxic mutants were directly introduced into the cytosol of RAW264.7 cells. It was observed that 100 µg ml\(^{-1}\) of all the mutant proteins was fully capable of causing macrophage cytolysis in 3.5 h. The amount of protein required for cytolysis was comparable to the native protein (Fig. 3).

4. Discussion

Binding of LF and EF to PA is an essential step for establishing an anthrax infection and for the resulting pathological events. The intent of this research was to mutagenize the residues 187DLLF190 of the PA-binding domain of LF to determine which residues are involved in interaction with PA. The residues D187, L188, L189, and F190 were changed to alanine individually and in combination in which all the four residues were replaced with alanine.

The mutant proteins were purified and analyzed for their functional activity by the cytotoxicity assay. The cytotoxicity profile of the mutant proteins showed great variation. D187A and the combination mutant were totally non-toxic even at very high concentrations. F190A was partially toxic at very high concentrations, whereas L188A and L189A were fully toxic at normal concentrations. Since the residues D187, L188, L189, and F190 are present in the PA-binding domain of LF, all the mutant proteins were studied for their ability to bind to PA in solution. PA-binding ability of D187A, F190A and the combination mutant was severely impaired. This inability to bind to PA explains the lack of cytotoxic activity even at very high concentrations. The PA-binding ability of L188A and L189A was not affected suggesting that these residues probably do not interact directly with PA and if they participate less directly in PA binding, this function is tolerant of alanine substitution. Similar results were obtained when PA-binding studies were conducted on the cell surface except that in the case of F190A, partial binding was observed (data not shown). Our results suggest that the requirement of Asp at position 187 and Phe at position 190 is highly specific. The side chains of Asp and Phe are probably involved in electrostatic and hydrophobic interactions with PA residues. Alanine substitution at these residues disrupts these interactions leading to the loss of PA binding.

These binding-defective mutants cannot bind PA and hence cannot be internalized into the cytosol by receptor-mediated endocytosis to catalyze their functions. However, if only the binding step is defective, on bypassing this step, these LF mutants should be able to interact with...
their cytosolic substrate, MAPKK2, and cause macrophage cytolysis [3,7]. In vitro and in vivo assays were performed to determine whether the mutant proteins were able to catalyze these activities. It was observed that D187A, F190A and the combination mutant were fully capable of interacting with MAPKK2 and cleaving it from its N-termini (data not shown). Upon direct introduction of these mutants into the RAW264.7 cytosol, cytolysis was observed. These results demonstrate that the mutants generated in this study retain all the functional properties of LF except binding to PA. Therefore, they are expected to retain most of the immunogenic epitopes of the native protein.

The present-day human anthrax vaccine consists predominantly of PA [16]. Recently it has been demonstrated that the immunization with an LF-based DNA vaccine provides complete protection against *B. anthracis* spore challenge [17]. LF-based DNA vaccine appears to be much more immunogenic and produces a longer-lasting immune response than PA-based DNA vaccine [17]. Therefore, LF antibodies can play a very prominent role in providing protection against anthrax. The non-toxic mutants generated in this study can serve as potential vaccine candidates in combination with PA to provide a more efficacious vaccine against anthrax. These results also provide the possible option of inducing protection against anthrax by using LF-based peptides as short as the PA-binding stretch on LF. These peptides can be used therapeutically to neutralize anthrax toxin in infected individuals.

References


