Peptides 30 (2009) 2127–2135

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Peptide sequences identified by phage display are immunodominant functional motifs of Pet and Pic serine proteases secreted by *Escherichia coli* and *Shigella flexneri*

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ARTICLE INFO

Article history:
Received 13 May 2009
Received in revised form 14 September 2009
Accepted 15 September 2009
Available online 20 September 2009

Keywords:
Escherichia coli
Shigella
SPATEs
Phage display
Immunodominant epitopes
Mimotopes

ABSTRACT

Plasmid-encoded toxin (Pet) and protein involved in colonization (Pic), are serine protease autotransporters of *Enterobacteriaceae* (SPATEs) secreted by enteroaggregative *Escherichia coli* (EAEC), which display the GDSGSG sequence or the serine motif. Our research was directed to localize functional sites in both proteins using the phage display method. From a 12mer linear and a 7mer cysteine-constrained (C7C) libraries displayed on the M13 phage pIII protein we selected different mimotopes using IgG purified from sera of children naturally infected with EAEC producing Pet and Pic proteins, and anti-Pet and anti-Pic IgG purified from rabbits immunized with each one of these proteins. Children IgG selected a homologous group of sequences forming the consensus sequence, motif, PQPxK, and the motifs PGxI/LN and CxPDDSSxC were selected by the rabbit anti-Pet and anti-Pic IgGs, respectively. Analysis of the amino terminal region of a panel of SPATEs showed the presence in all of them of sequences matching the PGxI/LN or CxPDDSSxC motifs, and in a three-dimensional model (Modeller 9v2) designed for Pet, both these motifs were found in the globular portion of the protein, close to the protease active site GDSGSG. Antibodies induced in mice by mimotopes carrying the three aforementioned motifs were reactive with Pet, Pic, and with synthetic peptides carrying the immunogenic mimotope sequences TY**PG**YI**N**HSKA and LL**P**Q**P**P**K**LLLP, thus confirming that the peptide moiety of the selected phages induced the antibodies specific for the toxins. The antibodies induced in mice to the PGxI/LN and CxPDDSSxC mimotopes inhibited fodrin proteolysis and macrophage chemotaxis biological activities of Pet. Our results showed that we were able to generate, by a phage display procedure, mimotopes with sequence motifs PGxI/LN and CxPDDSSxC, and to identify them as functional motifs of the Pet, Pic and other SPATEs involved in their biological activities.

1. Introduction

Serine proteases constitute one-third of all reported proteases. Studies on the activity of these enzymes show different effects of serine proteases on eukaryotic cells. According to their structural similarities, serine proteases have been classified into 36 families, which are in turn grouped into 15 clans (http://merops.sanger.ac.uk). The serine protease autotransporters of *Enterobacteriaceae* (SPATEs) which have been defined as virulence factors, use the Type V system of secretion, are integrants of the subfamily of IgA serine protease family from the chymotrypsin-like clan. *Escherichia coli*, *Shigella* sp., *Salmonella* sp. and *Citrobacter* sp. are some of the bacteria which produce these proteases [30,44,46]. Their common characteristics are: (i) the translocation to periplasmic space is associated to a signal sequence in the amino terminal portion and related with the *secA* secretion apparatus; (ii) their enzyme active site is located in the middle of the passenger domain, and formed by the amino acids: histidine (catalytic base) asparagine (electrophilic) and the first serine of the GDSGSG motif (nucleophylic), similar to that described for the chymotrypsin clan [11]; in addition, this active site has been related to the different properties described to the SPATEs [7,20,46]; (iii) the third dominion or β-barrel is located in the C-terminal region of the protein and is

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doi:10.1016/j.peptides.2009.09.019

functionally implicated, together with some factors of the external membrane proteins (YaeT/Omp85), in SPATEs secretion [21].

Plasmid-encoded toxin (Pet) and protein involved in colonization (Pic) identified in enteroaggregative *E. coli* (EAEC) and *Shigella flexneri* are members of the SPATEs [10,19], both with different biological properties. Pet shows enterotoxic and cytotoxic activity, and a fodrin-degrading effect [26,43]. Histological studies of mice intestine samples challenged with EAEC and from rat ileal loops inoculated with Pet, showed a strong inflammatory response [10,32]. It was recently shown that Pet induces a chemotaxis effect on mononuclear cells (preliminary data). Pic has been described as a protein that contributes to intestinal colonization, and induces mucine degradation, serum resistance [18,19], and hemoglobin disruption [27].

Dutta et al. [8], using random mutation assays on the passenger domain of Pet, identified the existence of domains to bind and internalize the protein into eukaryotic cells, and described their association with the bacterial secretion protein. Yen et al. [46], in a bioinformatic study of the SPATEs' structure, described conserved amino acid sequences in the passenger domain of these proteases, which seem to contribute to the structural and functional stability of SPATEs. Despite the large number of studies available on SPATEs, when we started this work, the literature lacked information on both, immunologically active epitopes and structural motifs associated with their biochemical and physiological properties [7,19,43,46].

Peptide selection from random libraries displayed on filamentous phage surface proteins pIII or pVIII, proposed by Smith and other researches from his group [31,35,37,38], is successfully employed for mimotope selection by means of antibodies and their use for localization of immunologically active sites (epitopes) in proteins, carbohydrates, lipids, and as individual epitope-mimicking antigens and immunogens [14,17,29]. In addition, phage display is a powerful high throughput methodology for drug design, discovery of peptide-mimetics of receptor ligands with agonistic and antagonistic activity [1,14], scaffold-based protein engineering [36], discovery of novel substrates [3], development of mimotope-based vaccines [16,23,39], and for research purposes and clinical applications [2].

In the present report we describe the identification by phage display of functional motifs of Pet and Pic, which we tentatively assigned to the N-terminal of the amino acid sequence of Pet, Pic and of other SPATE members. Once the three-dimensional model of the Pet molecule was obtained, these motifs were located in the globular region I, near the serine protease active site. Experiments with antibodies raised against the generated mimotopes displaying the motifs, showed that the mimicked protein sites may be implicated in the regulation of Pet biological functions.

2. Materials and methods

2.1. Pet and Pic purification

Pet and Pic toxins were purified from supernatants of the HB101(pCEFN1) and HB101(p56) clones cultured in Luria (LB) medium as described by Villaseca et al. [43]. The EspC protein was provided by Dr. Angel Manjarrez; the protein concentration was determined by the Bradford method [43].

2.2. Serum preparation

Sera prepared from blood samples of 21 children (less than 5 years old) naturally infected with an EAEC (O? is a somatic antigen non determined (ND):H10) strain, and sera of rabbits (New Zealand) inoculated with Pet and Pic toxins, were prepared by standard procedure and analyzed by ELISA to select those showing a highest reactivity with Pet and Pic proteins. Briefly, Pet or Pic (7 μg/ml in 100 μl PBS) was incubated overnight at 4 °C in a well of 96-well microplate, the surface of the well containing the bound protein was blocked with 2% (w/v) BSA–0.5% (w/v) dry milk in 100 μl PBS and incubated with serum 1:100 dilution. Anti-human or anti-rabbit second antibodies conjugated with alkaline phosphatase (Zymed, USA) at 1:1000 dilution and *p*-nitrophenylphosphate (Sigma–Aldrich, USA) were used to detect bound antibody in an ELISA Reader (MR 580; Dynatech) at 405 nm.

2.3. IgG purification

IgG was obtained from sera of patients and rabbits immunized with Pet or Pic that showed highest reactivity with these proteins. Protein G-agarose affinity chromatography was used as previously done [13]. Briefly, 250 μl of Protein G-agarose (Invitrogene, USA) were mixed with 500 μl of each serum, incubated for 20 min at room temperature (RT), mixing gently every 2 min, centrifuged for 30 s at 500 × *g* (Sorvall RC5), the supernatant was discarded and the IgG-Protein G-agarose complex washed five times by 0.1 M glycine, pH 9.0. After the last wash, the complex was dispersed in 750 μl of elution buffer (0.1 M glycine–HCl, pH 2.2) and centrifuged at 500 × *g* for 30 s. The supernatant containing purified IgG fraction was adjusted to pH 7.0 with 2 M Tris base. Protein concentration was determine by Bradford procedure.

2.4. Phage display libraries, biopanning, cloning and characterization of mimotopes

Peptide libraries, 12mer linear, and 7mer cysteine-constrained were purchased from New England BioLabs Inc. (Beverly, MA, USA). In each of them, random peptides (approximately 2.7×10^9 electroporated sequences) are fused to a minor coat protein (pIII) of M13 phage and expressed at its N-terminus separated by Gly-Gly-Gly-Ser spacer. Shortly before use, libraries were amplified to have about 20 copies of each sequence per 10 μl, the amount used in single biopanning experiment.

Biopanning procedure as described in [6,38] with minor adjustments [29] was used. Two wells in a 96-well polystyrene microtiter plate (Immulon 4 flat bottom plates, Dynatech Lab Inc., USA) were coated with IgG fraction affinity purified from each serum. For this, one well was filled with 150 μg/ml IgG, the second one with 75 μg/ml IgG, each in 100 μl PBS, and the plate was kept overnight at 4 °C with gentle rocking. Unbound IgG was removed and wells were washed 6 times with 0.1% (v/v) Tween 20 in PBS (PBS-T) and blocked 1 h at 4 °C with blocking buffer (PBS–1% BSA), followed by five consecutive washing steps using PBS-T. For affinity selection, 10 μl of the library (2×10^{10} plaque-forming units, p.f.u.) were added to 190 μl of PBS-T and the mixture distributed in the two wells (100 μl per well) with immobilized and blocked IgGs; the wells were incubated 1 h at RT while rocking gently to allow phage to bind. The unbound phage was pipetted out and wells were washed 10 times with PBS-T at RT. Bound phage was eluted from each well by stirring with 100 μl of elution buffer (0.1N HCl–glycine, pH 2.2). Eluted phage (designated further as "eluate") from two wells was combined and quickly neutralized by addition of 35 μl of 2 M Tris base. Phage was grown on lag-phase *E. coli* ER2738 (BioLabs) on LB plates and the number of plaques was quantified to estimate the phage titer in p.f.u.; about 3/4th of the eluate was amplified in 30 ml of 2× YT for 4 h. After pelleting the cells by centrifugation, PEG/NaCl (20%/40%) was added to supernatant (4 °C), the precipitated phage centrifuged at 6000 × *g*, 10 min, 4 °C (Sorvall RC), the pellet re-suspended in PBS and clarified by centrifugation at 6000 × *g*, 2 min, 4 °C (Sorvall RC); the supernatant was transferred to a fresh centrifuge tube for repeated phage precipitation with PEG/NaCl as described. The recovered phage was tittered as indicated above, re-suspended in PBS at