FEBS Letters 413 (1997) 174–176 FEBS 19057

Purification, characterization and cloning of a novel variant of the superantigen *Yersinia pseudotuberculosis*-derived mitogen

T. Ramamurthy[1,a], Ken-ichi Yoshino[a], Jun Abe[b], Norikazu Ikeda[a,c], Tae Takeda[a,*]

[a]*Department of Infectious Diseases Research, National Children's Medical Research Center, Taishido 3-35-31, Setagaya-ku, Tokyo-154, Japan*
[b]*Department of Pediatric Ecology, National Children's Medical Research Center, Taishido 3-35-31, Setagaya-ku, Tokyo-154, Japan*
[c]*Institute of Skin Sciences, Club Cosmetics Co. Ltd., Nishi-ku, Osaka-shi, Osaka-550, Japan*

Received 27 June 1997; revised version received 11 July 1997

Abstract The novel superantigen designated as *Yersinia pseudotuberculosis*-derived mitogen typeB (YPMb) was purified from the cell lysate of *Y. pseudotuberculosis* O:6 strain (R-104). Proliferative response of human peripheral blood mononuclear cells to the purified YPMb was detectable at a concentration of as low as 1 pg/ml, that is comparable to the previously documented YPM (YPMa). The Vβ repertoire specificity (3, 9, 13.1 and 13.2) of YPMb was also the same as that of YPMa. A gene (*ypmB*) encoding YPMb was cloned and its nucleotide sequence was determined. The open reading frame (ORF 453 bp) of the *ypmB* encodes a protein with 150 amino acid residues, though the precursor protein of YPMa consists of 151 amino acid residues. There is a nucleotide sequence homology of 88.9% between *ypmB* and *ypmA*. The low homology in the downstream of the structural gene between *ypmB* and *ypmA* and the difference in the GC content in the ORF of *ypmB* and *ypmA* from that of the base usage of *Y. pseudotuberculosis* suggest that the *ypm* gene originates from another organism. The alignment of the amino acid sequences of mature proteins of YPMb and YPMa revealed that there is 83% homology (108 amino acid residues are identical). Between YPMa and YPMb, the central region is less homologous than the N- and C-terminal regions. Based on the functional similarity of two superantigen molecules, the less homology suggested that the central region is less important for the function of both YPM molecules.

© 1997 Federation of European Biochemical Societies.

Key words: Superantigen; YPM; Novel variant; Amino acid sequence; Nucleotide sequence; *Yersinia pseudotuberculosis*

1. Introduction

Yersinia pseudotuberculosis is a Gram-negative enteropathogenic bacillus which is capable to induce not only gastrointestinal symptoms but also a variety of systemic manifestations such as fever, scarlatiniform rash and desquamation. *Y. pseudotuberculosis* is known to elaborate a superantigen *Y. pseudotuberculosis*-derived mitogen (YPM) [1–6] which plays an important role in the pathogenesis of *Y. pseudotuberculosis* infection [7–9]. A superantigen is a highly potent immunostimulatory molecule. Unlike conventional peptide antigen, a superantigen binds directly as an intact protein to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells at a site distinct from the antigen-binding groove. The superantigen/MHC complex then reacts with the T-cell receptors (TCR) through the particular β-chain of variable region (Vβ), rather than with the normal antigen-binding groove on TCR. The result of these interactions is proliferation of a large number of T-cells with consequent release of cytokines. This ability of the superantigen directly accounts for the pathogenic effects and clinical symptoms of the infection [10,11].

In addition to YPM, more than 10 species of bacterial superantigens, such as toxic shock syndrome toxin-1, staphylococcal enterotoxins, streptococcal pyrogenic exotoxins, exfoliative toxins and streptococcal superantigen, are well established [12–14]. Although almost all members are produced by Gram-positive cocci, *Staphylococcus aureus* and *Streptococcus pyogene*, YPM is the only known Gram-negative bacillary superantigen whose primary structure substantially differs from other bacterial superantigens.

We investigated a correlation between the presence of *ypm* gene encoding YPM and the production of a superantigen among 411 strains of *Y. pseudotuberculosis* using the colony hybridization assay with oligonucleotide probe and the proliferative assay on human peripheral blood mononuclear cells (PBMC). Of the 411 strains investigated, 28 strains expressed mitogenic activity, despite failure to hybridize with the specific probe for the *ypm* gene [15]. These facts implied that the possibility of the production of a novel superantigen by *ypm*-negative strains of *Y. pseudotuberculosis*. This finding formed the impetus for the present study in which we report the purification, cloning and nucleotide sequencing of a novel Gram-negative bacillary superantigen designated as YPM type B (YPMb) produced by a *ypm*-negative strain of *Y. pseudotuberculosis*. (In this nomenclature, the previously reported *YPM* and *ypm* gene are renamed *YPMa* and *ypmA* gene, respectively.)

2. Materials and methods

2.1. Bacterial strains and culture conditions

A *ypmA*-negative strain (R-104) of *Y. pseudotuberculosis* belonging to the serotype O:6, isolated from a wild-mouse in Japan was used in the present study, which was kindly donated by Dr. H. Fukushima of Public Health Institute of Shimane Prefecture, Matsue, Japan. The strain was cultured as described previously [1].

2.2. Purification of YPMb

Cell lysate of *Y. pseudotuberculosis* R-104, prepared as described previously [4], was precipitated with 80% ammonium sulfate. After centrifugation at 15000×*g* for 30 min at 4°C, the resulting pellet was resuspended in 20 mM Tris-HCl buffer (pH 7.2), and dialyzed against the same buffer. Dialyzed sample was purified further by high-performance ion-exchange chromatography (HPIEC) and reversed-phase high-performance liquid chromatography (RP-HPLC). The used running condition of HPIEC was described previously [4]. RP-HPLC for purification of YPMb was carried out using a Develosil

*Corresponding author. Fax: (81) 3-3411-7308.
E-mail: tae@nch.go.jp

[1]Present address: National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, P.O. Box 177, Calcutta-700 010, India.

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(97)00909-5

300C4-HG-5 (4.6×150 mm) or a Develosil PhA-HG-5 (4.6×150 mm) previously equilibrated with 30% acetonitrile in 0.1% trifluoroacetic acid (TFA) and run with a linear gradient of acetonitrile (30–40% in 30 min), at a flow rate of 1.0 ml/min. In the entire process of purification, the mitogenic activity of a sample was monitored by measuring the proliferative response of human PBMC.

2.3. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Positive-ion MALDI-TOF-MS was performed using a Voyger RP time-of-flight mass spectrometer (PerSeptive Biosystems, Inc., Framingham, MA). The spectrum was obtained using a linear-mode measurement. A protein solution (≈2 pmol/μl in 50% acetonitrile in 0.1% TFA) mixed with same volume of matrix solution the supernatant of 50% acetonitrile solution saturated with 3,5-dimethoxy-4-hydroxycinnamic acid. An aliquot (1 μl) of the mixture was placed on concave surface of a stainless plate. The ions were generated by irradiating the sample area with the output of a nitrogen laser (337 nm).

2.4. Biological assay

Proliferative response was measured with human PBMC as described previously [16]. Vβ repertoire specificity was analyzed by a semiquantitative PCR method as described previously [1].

2.5. Cloning and sequencing of ypmB gene

The chromosomal DNA from *Y. pseudotuberculosis* R-104 was partially digested with *Sau*3AI and then purified by ultracentrifugation in 10–40% sucrose gradient. DNA fragments with desired size were ligated in *Bam*HI site of a vector pUC18. The ligation mixture was used to transform *Escherichia coli* DH-5α. Transformants were transferred to an Amersham Hybond N^+ membrane and screened with the ^{32}P-labelled DNA probe prepared by PCR using the primer pair as described previously [7]. Positive colonies were picked up and were assayed for mitogenic activity on human PBMC.

The plasmid DNA from a positive clone was further digested with *Hinc*II. The fragment was ligated into the *Sma*I site of pUC18 to obtain a subclone. A subclone with the intact *ypmB* gene, which was confirmed by the mitogen assay, was used for sequencing experiments. Nucleotide sequence of the clone was determined by dideoxynucleotide chain termination methods as modified for use with Sequenase© (US Biochemical, Cleveland, OH).

2.6. PCR for the detection of ypmB gene

PCR for the detection of *ypmB* gene was performed using the primer pair 5′-TTTCTGTCATTACTGACATTA-3′ (sense) and 5′-CCTCTTTCCATCCATCTCTTA-3′ (anti-sense). The nucleotide sequences of sense and anti-sense primers are located at positions 13–33 and 260–280 in *ypmB* gene, respectively.

3. Results and discussion

3.1. Purification of YPMb

The cell lysate of *Y. pseudotuberculosis* R-104 was partially purified by ammonium sulfate precipitation and HPIEC, as described in Section 2.1. The fractions exhibited mitogenic activity were pooled and further purified by RP-HPLC with a Develosil 300C4-HG-5 column. A peak fraction indicated by an arrow in Fig. 1A showed potent mitogenic activity. This fraction was subjected to RP-HPLC with Develosil PhA-HG-5 column to give a pure mitogen and a fraction which showed the mitogenic activity was eluted as one of minor peaks (Fig. 1B). The fraction with marked mitogenicity was subjected to rechromatography to give a sharp and single peak as shown in Fig. 1C, indicating purification to high homogeneity.

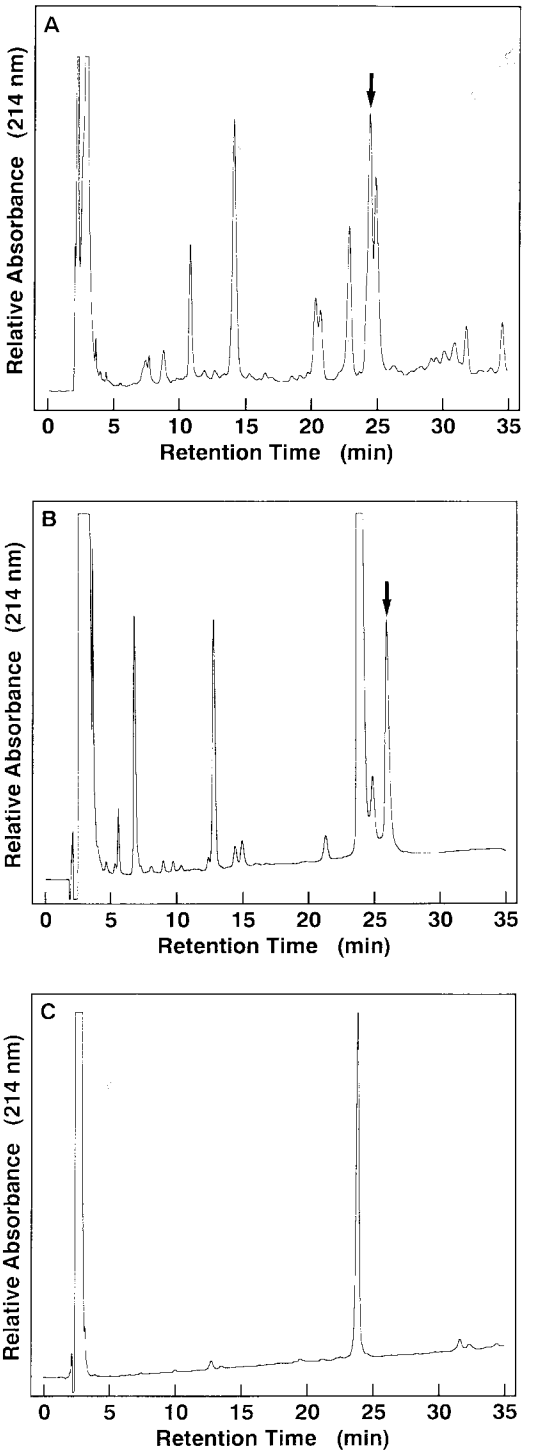

Fig. 1. Purification of YPMb. A: RP-HPLC profile of the crude YPMb obtained by HPIEC with a TSKgel DEAE-5PW column. B: RP-HPLC profile of the mitogenic peak fraction indicated by an arrow in (A). An arrow indicates the peak fraction exhibiting potent mitogenic activity. C: Rechromatography of the purified preparation of YPMb.

3.2. Chemical and biological properties of YPMb

Automated Edman degradation of the purified YPMb provided the N-terminal sequence of 39 amino acid residues as A–D–Y–D–N–T–L–N–S–I–P–S–L–R–I–P–N–I–E–T–Y–T–G–X–I–Q–G–K–G–E–V–X–I–R–G–N–K–E–G–K–X–R– (X denotes a cycle in which no identifiable residue was detected). Out of the determined 39 residues, three amino acid residues differed between YPMa and YPMb. The molecular weight of the purified YPM was measured to be 14629 by MALDI-TOF-MS, which is comparable to that of the previously documented YPMa (14527).

Proliferative response of PBMC to the purified YPMb was detected at a concentration as low as 1 pg/ml. The minimum

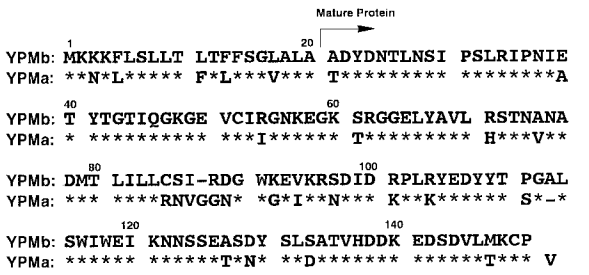

Fig. 2. Comparison of amino acid sequences of precursor proteins of YPMb and YPMa. Homologous residues are shown in asterisks. A dash indicates a gap of one amino acid residue.

effective concentration was comparable to that of the known YPMa. The purified YPMb was found to stimulate human T-cells bearing Vβ3, 9, 13.1 and 13.2 selectively. This Vβ repertoire specificity was identical to that of YPMa.

3.3. Cloning and nucleotide sequence determination of *ypmB*

We cloned a gene (*ypmB*) encoding YPMb and analyzed its nucleotide sequence as described in Section 2.5. The nucleotide sequence data obtained in the present study appears in the DDBJ, EMBL and GenBank nucleotide sequence data bases with the accession number of D88144. The open reading frame (ORF) of the *ypmB* gene (453 bp) encodes a protein with 150 amino acid residues, though the precursor protein of YPMa consists of 151 amino acid residues. The N-terminal 39 amino acid residues determined by automated Edman degradation with the purified YPMb completely match the respective amino acid residues deduced from the nucleotide sequence. The determined N-terminal sequence of the purified protein and the specificity of prokaryote signal peptidase suggest that N-terminal 20 amino acid residues are consisted of a signal sequence. Molecular weight of the mature protein of YPMb with 130 residues was calculated to be 14 596 from the nucleotide sequence. This value is nearly identical to the observed mass value (14 628) of the purified YPMb measured by MALDI-TOF-MS. The alignment of the amino acid sequences of mature proteins of YPMb and YPMa revealed that there is 83% homology (108 amino acid residues are identical) (Fig. 2).

Of the 411 strains of *Y. pseudotuberculosis* investigated, 28 strains did not hybridize with the specific probe for the *ypmA*, despite expression of mitogenic activity [15]. It was demonstrated that high possibility of the presence of *ypmB* in all the *ypmA*-negative and mitogen-positive strains by the PCR test using the specific primer pair for *ypmB*, as described in Section 2.6. This represents that YPMb is responsible for the rest species of superantigen molecule produced by *Y. pseudotuberculosis*.

We concluded that YPMb is a variant of YPMa based on the similarity in amino acid sequence and the biological properties. More than 10 species of Gram-positive coccal superantigen were known. However, so far, YPMa has been the only known Gram-negative bacillary superantigen. In addition to YPMa, the second Gram-negative bacillary superantigen YPMb has been identified in the present study.

Between amino acid sequences of YPMa and YPMb, the central region is less homologous than the N- and C-terminal regions (Fig. 2). As these two superantigen molecules possess the same function, the low homology suggests that the central region is not important for the function of both YPM molecules. In our previous study, it has been identified through competition studies by using synthetic peptides that a region of YPMa encompassing amino acid residues 1 through 23 is involved in the induction of human PBMC proliferation [16]. This is in agreement with the higher homology of the N-terminal portion.

Analysis of the nucleotide sequence homology between *ypmB* and *ypmA* showed that there are 88.9% similarity in ORF, 87.6% in upstream of ORF and 52.1% in downstream of ORF. Although the GC content of the total DNA from a strain of *Y. pseudotuberculosis* is 46.5% [17], these of the ORF of *ypmB* and *ypmA* are 34.4% and 35.3%, respectively. The low homology in the downstream of ORF and the difference in the GC content suggest that the *ypm* gene originates from other organism, implying that the presence of other bacterial superantigen produced by other Gram-negative bacillus [18,19].

Acknowledgements: We thank Dr. H. Fukushima of Public Health Institute of Shimane Prefecture (Matsue, Japan) for kindly providing us *Y. pseudotuberculosis* strains. This work was supported in part by Kanagawa Academy of Science and Technology Research Grant (#951065), a Grant-in-Aid for Encouragement of Young Scientists (#08770207) from the Ministry of Education, Science and Culture of Japan, and by Grants-in-Aid from the Ministry of Health and Welfare of Japan, and Japan Health Sciences Foundation.

References

[1] Abe, J., Takeda, T., Watanabe, Y., Nakao, H., Kobayashi, N., Leung, D.Y.M. and Kohsaka, T. (1993) J. Immunol. 151, 4183–4188.
[2] Uchiyama, T., Miyoshi-Akiyama, T., Kato, H., Fujimaki, W., Imanishi, K. and Yan, X.-J. (1993) J. Immunol. 151, 4407–4413.
[3] Miyoshi-Akiyama, T., Imanishi, K. and Uchiyama, T. (1993) Infect. Immun. 61, 3922–3927.
[4] Yoshino, K., Abe, J., Murata, H., Takao, T., Kohsaka, T., Shimonishi, Y. and Takeda, T. (1994) FEBS Lett. 356, 141–144.
[5] Miyoshi-Akiyama, T., Abe, A., Kato, H., Kawahara, K., Narimatsu, H. and Uchiyama, T. (1995) J. Immunol. 154, 5228–5234.
[6] Ito, Y., Abe, J., Yoshino, K., Takeda, T. and Kohsaka, T. (1995) J. Immunol. 154, 5896–5906.
[7] Yoshino, K., Ramamurthy, T., Nair, G.B., Fukushima, H., Ohtomo, Y., Takeda, N., Kaneko, S. and Takeda, T. (1995) J. Clin. Microbiol. 33, 3356–3358.
[8] Miyoshi-Akiyama, T., Fujimaki, W., Yan, X.-J., Yagi, J., Imanishi, K., Kato, H., Tomonari, K. and Uchiyama, T. (1997) Microbiol. Immunol. 41, 345–352.
[9] Abe, J., Onimaru, M., Matsumoto, S., Noma, S., Baba, K., Ito, Y., Kohsaka, T. and Takeda, T. (1997) J. Clin. Invest. 99, 1823–1830.
[10] Herman, A., Kappler, J.W., Marrack, P. and Pullen, A.M. (1991) Annu. Rev. Immunol. 9, 745–772.
[11] Scherer, M.T., Ignatowicz, L., Winslow, G.M., Kappler, J.M. and Marrack, P. (1993) Annu. Rev. Cell Biol. 9, 101–128.
[12] Marrack, P. and Kappler, J. (1990) Science 248, 705–711.
[13] Reda, K.B., Kapur, V., Mollick, J.A., Lamphear, J.G., Musser, J.M. and Rich, R.R. (1994) Infect. Immun. 62, 1867–1874.
[14] Ren, K., Bannan, J.D., Pancholi, V., Cheung, A.L., Robbins, J.C., Fischetti, V.A. and Zabriskie, J.B. (1994) J. Exp. Med. 180, 1657–1683.
[15] K. Yoshino, T. Ramamurthy, and T. Takeda, in: Proc. of the 32nd US–Japan Joint Conference on Cholera and Related Diarrheal Diseases, 1996, pp. 78–83.
[16] Yoshino, K., Takao, T., Ishibashi, M., Samejima, Y., Shimonishi, Y. and Takeda, T. (1996) FEBS Lett. 390, 196–198.
[17] H. Bercover, and H.H. Mollaret, in: N.R. Krieg (Ed.), Genus *Yersinia*, Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984, pp. 498–506.
[18] Stuart, P.M. and Woodward, J.G. (1992) J. Immunol. 148, 225–232.
[19] Stuart, P.M., Munn, R.K., DeMoll, E. and Woodward, J.G. (1995) Human Immunol. 43, 269–275.