Biochemical and Biophysical Research Communications 430 (2013) 14–19

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

A deeper analysis of the epitope/paratope of PLY-5, a mouse monoclonal antibody which recognises the conserved undecapeptide tryptophan-rich loop (ECTGLAWEWWR) of bacterial cholesterol-dependent cytolysins

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ARTICLE INFO

Article history:
Received 1 November 2012
Available online 15 November 2012

Keywords:
Epitope
Paratope
PLY-5
Tryptophan-rich loop
Cholesterol-dependent cytolysin

ABSTRACT

A previous study showed that the minimal epitope recognised by the PLY-5 mAb in the conserved undecapeptide Trp-rich loop of bacterial CDCs should consist of WEWWRT (Jacobs et al., 1999) [5]. Now, through immunoscreening of amino acid substitution analogues, it is concluded that the second Trp and the Arg residues are essential in the PLY-5 epitope. The E residue is an auxiliary epitope contributor. Antibody modelling and docking simulations provided support for these findings. For recognition by the antibody, the Trp-rich loop flipped out, mimicking the mechanism of membrane insertion. The displaced second Trp was seen to establish aromatic stacking interactions with aromatic residues of the antibody paratope and the notably extruded guanidium tip of the arginine residue mediated electrostatic interactions with well-exposed carboxylic groups of glutamic residues on the surface of the paratope. Thus, the epitope/paratope interaction is mainly mediated by aromatic and by ionic interactions.

1. Introduction

Bacterial cholesterol-dependent cytolysins (CDCs) are proteins which form pores on cholesterol-bearing membranes [1,2] and are therefore lytic for sheep red blood cells (SRBCs). Most of them have a highly conserved tryptophan-rich undecapeptide (ECTGLAWEWWR) which is essential for membrane insertion and subsequent lytic activity; this is the case of pneumolysin (PLY).

The crystal structure of four CDCs – perfringloysin O (PFO), intermedilysin (ILY), anthrolysin O (ALO) and suilysin (SLY) – has been solved [3]. In the four, the undecapeptide forms a flexible loop that is well exposed to the solvent. The mostly hydrophobic character of this loop confers on it an unfavourable antigenic propensity.

The PLY-5 mAb was raised against native PLY after extensive immunisation [4] and shown to recognise the conserved Trp-rich undecapeptide of some bacterial CDCs. Its minimal epitope should consist of WEWWRT [5].

In spite of some weaknesses and pitfalls still present, homology modelling of antibodies from their sequences, based on the structural data of well-known examples, is one of the methodologies with allows researchers to predict and build three dimensional models of antibodies with very high accuracy [6,7]. The characteristics of the binding site (paratope) predicted by these models may be of help in explaining its reactivity patterns.

From an immunological viewpoint, the above-mentioned features of the Trp-rich loop make of it an interesting subject for of analysis. By immunoscreening of CDCs with amino acid variations in the undecapeptide (Table 1) and by means of amino acid substitution analogues, we describe here new experimental findings. Together with data from Fv modelling and docking simulations, we discuss the nature of the epitope recognised by the PLY-5 mAb and its binding characteristics.

2. Materials and methods

2.1. Purified toxins

Purified pyolysin (PYO) was kindly provided by Prof. Michael Palmer, Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada; and purified vaginolysin (VLY) by Prof. Adam J. Ratner, Departments of Paediatrics and Microbiology and Immunology, Columbia University, New York, USA [8]. Purified recombinant PLY was prepared by us [9].

2.2. PLY-5 mAb

The PLY-5 secreting-mouse hybridoma was grown in BioWhittaker® UltraDOMA™ – PF Serum-free, Protein-free Medium

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http://dx.doi.org/10.1016/j.bbrc.2012.11.012

Table 1
Multiple alignment of the Trp-rich undecapeptide of some known CDCs. Discrepancies with the consensus sequence are in bold.

VLY	E	**K**	T	G	L	**V**	W	E	**P**	W	R
ILY	**G**	**A**	T	G	L	A	W	E	**P**	W	R
PYO	E	**A**	T	G	L	A	W	**D**	**P**	W	**W**
SLG	E	C	T	G	L	**F**	W	E	W	W	R
CONSENSUS	E	C	T	G	L	A	W	E	W	W	R

(Lonza Group). From the supernatant, equilibrated with 1.0 M ammonium sulphate, the mAb was purified by means of hydrophobic interaction chromatography on a 20-ml HiLoad™ 16/10 Phenyl Sepharose™ HP column (Amersham Biosciences), on an ÄKTAdesign FPLC system (Amersham Biosciences). The mAb was recovered by elution in a linear gradient from 1.0 M to 0 M ammonium sulphate in 50 mM Tris-buffered saline (TBS), pH 7.0.

2.3. Anti-haemolytic activity of PLY-5 and blocking assays with soluble peptides

Haemolysis neutralisation and blocking assays were performed as already described [4,5,9]. Briefly, the haemolytic activity of recombinant PLY preparations was titered immediately before neutralisation tests and they were assayed at two haemolytic units (HU). Similarly, the neutralisation capacity of PLY-5 was also titered and this mAb was assayed at its neutralising minimal amount.

Synthetic peptides, ⩾95% pure, were supplied by PolyPeptide Laboratories France SAS. L-tryptophan, L-tyrosine, L-phenylalanine, L-arginine and L-lysine were from Sigma.

For competition tests, PLY-5 was mixed with different amounts of the competitor in 10 mM phosphate-buffered saline-0.1% sodium azide (PBS-SA), preincubated for 30 min at 37 °C and then with 2 HU of PLY for another 30 min at 37 °C. Finally, SRBCs at 1% were added and incubated again at 37 °C for 30 min.

In the positive neutralisation control samples, the addition of the competitor was omitted. Determinations were conducted in triplicate.

2.4. Immunoblot analyses

One μg/lane of purified recombinant PLY and 2 μg/lane of purified recombinant VLY and PYO were subjected to 12% polyacrylamide SDS–PAGE under reducing conditions and, subsequently, electrotransferred onto nitrocellulose membranes. After blocking overnight at 4 °C in 50 mM TBS-3% bovine serum albumin-0.1% SA (TBS-BSA-SA), pH 7.5, the membranes were then incubated with the PLY-5 mAb (3 μg/sample-lane), in 10 ml of 10 mM PBS-1% BSA-0.1% SA, with gentle rocking for 2 h at room temperature. After several washing steps, blots were incubated with an 1:50,000 dilution of anti-mouse γ chain specific – HRPO-conjugate (Sigma A3673) in PBS-1% BSA-0.1% Tween-20, for 2 h as above, and finally developed with the Immobilon™ western chemiluminescent HRP Substrate (Millipore).

2.5. Modelling of the Fv of PLY-5 and docking simulations

The VH and VL sequences of PLY-5, deposited in GenBank (accession numbers AAM34748.1 and AAM34749.1, respectively) [9], were submitted to the "RosettaAntibody: F_V Homology Modeling Server" (http://antibody.graylab.jhu.edu/) [10].

The 1M3I PDB file (http://www.rcsb.org/pdb/explore/explore.do?structureId=1M3I) [3] contains the structural information from a new crystal form of perfringolysin O. This toxin is made of four domains, of which the C-terminal domain or domain 4 (residues 391–500) includes the Trp-rich undecapeptide. The atom coordinates of its D4 domain were obtained from this pdb and used for docking on the modelled Fv of PLY-5.

Docking simulations were generated by "The RosettaDock server for local protein–protein docking" (http://rosettadock.graylab.jhu.edu/) [10–12]. Simulations were done with the D4 domain, as above stated, in such a way that the total number of residues involved were 221 for the Fv and 110 for the D4 domain. The PyMOL 1.1 software (DeLano Scientific LLC, Palo Alto, CA, USA) was used to position the tip of the D4 domain in front of the binding site of the modelled PLY-5 Fv for docking as well as to generate molecular renditions. Electrostatic potentials were obtained and visualised using the Molegro Molecular Viewer 2.2.0 (Molegro ApS, Aarhus C, Denmark).

3. Results

3.1. Soluble peptide blocking assays on the neutralising capacity of PLY-5

The neutralising capacity of PLY-5 on the haemolytic activity of PLY was challenged by means of blocking assays with 11-mer soluble peptides corresponding to the undecapeptide sequences of VLY, ILY, PYO, SLG (seeligeriolysin O), and with PLY as a consensus sequence of CDCs. Peptides were first probed at 1 mg/ml. Only the SLG and the consensus undecapeptides showed blocking activity on the PLY-5 paratope (Table 2). These blocking peptides were next assayed at progressively doubling dilutions; and, as shown

Table 2
Soluble peptides assayed and their capacity to block the neutralising activity of the PLY-5 Ab in PLY-mediated haemolysis.

Peptide	Blocking capacity
VLY undecapeptide: E**K**TGL**V**WE**P**WR	–
ILY undecapeptide: **GA**TGLAWE**P**WR	–
PYO undecapeptide: E**A**TGLAW**DP**W**W**	–
SLG undecapeptide: ECTGL**F**WEWWR	+
CONSENSUS undecapeptide: ECTGLAWEWWR	+
Designed variants[a]	
Variant of the VLY undecapeptide: EKTGLVWE**W**WR	+
Variant of the ILY undecapeptide: GATGLAWE**W**WR	+
Variant of the PYO undecapeptide: EATGLAWD**W**WW	+(50%)
Variant of the PYO undecapeptide: EATGLAWD**W**W**R**	+(50%)
Shortenings of the consensus undecapeptide	
CTGLAWEWWRT	+
TGLAWEWWRT	+
GLAWEWWRT	+
LAWEWWRT	+
AWEWWRT	+
Alanine-substituted analogues of the consensus heptapeptide AWEWWRT	
A**A**EWWRT	+(75%)
AW**A**WWRT	+(75%)
AWE**A**WRT	–
AWEW**A**RT	+
AWEWW**A**T	–
AWEWWR**A**	+
Conservative-substituted analogues	
AW**D**WWRT	+(75%)
AWE**F**WRT	–
AWE**Y**WRT	–
AWEWW**K**T	–

[a] Substitutions are in bold.

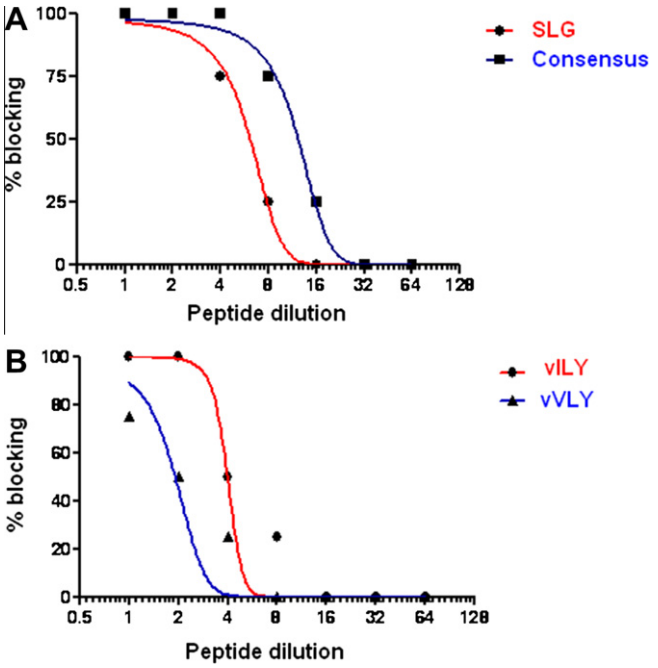

Fig. 1. Competitive neutralisation assays with free soluble SLG and consensus undecapeptides (A), and variant ILY and VLY undecapeptides (B). The figure was generated in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

(Fig. 1A) the neutralisation of PLY by PLY-5 was clearly inhibited by both peptides in a dose-dependent manner.

To further validate the above observations, purified recombinant VLY and PYO were probed, by Western-blot, with the PLY-5 mAb; PLY served as positive control. As shown in Fig. 2, PLY was strongly recognised by this antibody; PYO was hardly reactive; and VLY was not reactive at all.

The VLY, ILY and PYO undecapeptides have a proline instead of the second conserved tryptophan of the consensus sequence; and pyolysin has one more tryptophan instead of arginine. Next, the EKTGLVWE**W**WR, GATGLAWE**W**WR, EATGLAWD**W**WW and EATGLAWD**W**W**R** peptides, in which the P of VLY, ILY and PYO, respectively, was replaced by W, and the final W of PYO by R, were similarly probed. The first two peptides were fully blocking in a dose-dependent manner (Fig. 1B), but the last two were partially blocking (50%) when assayed at 1 mg/ml. These results pointed to a very relevant role of the second conserved W in the antigenicity of the Trp-rich undecapeptide; the E and R residues seem to be also involved; and the amino acid residues at the left of the WEWWR stretch do not seem to be determinants of that antigenicity.

Then, the five progressively shorter peptides: CTGLAWEWWRT, TGLAWEWWRT, GLAWEWWRT, LAWEWWRT, and the AWEWWRT were probed. They were all fully blocking, confirming that the amino acid residues to the left of WEWWRT are dispensable for recognition by the PLY-5 mAb.

Next, the contribution of each of the residues of the consensus sequence AWEWWRT to the building-up of the epitope was evaluated by testing the capacity of six alanine-substituted analogues to be recognised by PLY-5 and to block its neutralising activity on PLY. The AWEW**A**RT and AWEWWR**A** heptapeptides were fully blocking, indicating that the third W and the final T residues are dispensable for antibody recognition. In contrast, AWE**A**WRT and AWEWW**A**T were not blockers at all, meaning that the second W and the R residues are essential in the PLY-5 epitope. The A**A**EWWRT and AW**A**WWRT peptides were mostly blocking, and therefore the first W and the E residues should be envisaged as minor epitope contributors. Thus, according to these last observations, within the WEWWRT sequence, the PLY-5 epitope would be mainly configured by the second aromatic W and the charged R amino acid residues.

Some conservative changes were also tested. The E residue was substituted by D. The AW**D**WWRT peptide was 75% blocking as was its respective Ala substitute; thus, the E residue, being a minor epitope contributor, allows a conservative change. In contrast, when the second W was replaced by F or Y, or the R residue by K, the heptapeptides AWE**F**WRT, AWE**Y**WRT and AWEWW**K**T were not blockers, just like their Ala substitutes, evidencing that the second W and the R residues of the conserved tryptophan-rich undecapeptide (ECTGLAWEWWR) are essential for its recognition by the PLY-5 mAb and that they cannot be replaced without loss of its antigenicity.

In view of the preceding results, free tryptophan, tyrosine, phenylalanine, arginine and lysine were also individually tested but none of them were blocking even when assayed at 1 mg/ml.

Thus, according to all these observations using soluble peptides, the PLY-5 epitope seems to respond to an X^1 X^2 W^3 X^4 R^5 X^6 pattern.

3.2. Analysis of the binding site of PLY-5

The PLY-5 Fv generated by the RosettaAntibody: F_V Homology Modeling Server assigned a very low confidence prediction for the CDR-H3 loop, which comprises three amino acid residues (99EAS101), matching length only.

When this Fv modelling is visualise with the PyMOL 1.1 software (Fig. 3A), the paratope, as defined by the CDRs (Table 3), comprises a deep central crater followed by an eccentric canyon.

This crater includes a lateral concavity, delimited by the LQYASFPP residues of CDR-L3. At the bottom of the crater, is the Trp102 residue, from FR-H4.

One of the walls of the canyon is built up by the aromatic rings of Tyr91 (CDR-L3), Phe32 (CDR-L1) and Tyr49 (FR-L2). On the opposite wall, the Glu99 residue of CDR-H3 and behind this the E 6 residue of FR-H1 protrude above the surface as electronegative horns (Fig. 3B), while the R 59 of CDR-H2 gives rise to an electropositive area.

3.3. Docking simulations

In the crystal form of PFO, the Trp-rich undecapeptide (458)ECTGLAWEWWR(468) conforms two antiparallel β-strands joined by a β-turn. One of these strands is made of ECTGL. The central AWEW tetrad configures the β-turn (Fig. 3C) in such a way that the loop is curled back; on this loop, the free carboxylic acid group of the Glu465 residue produces an electronegative surface spot (Fig. 3D). The following WR residues run backwards parallel to ECTGL; the free guanidium side chain of R 468 confers an electropositive charge to this location.

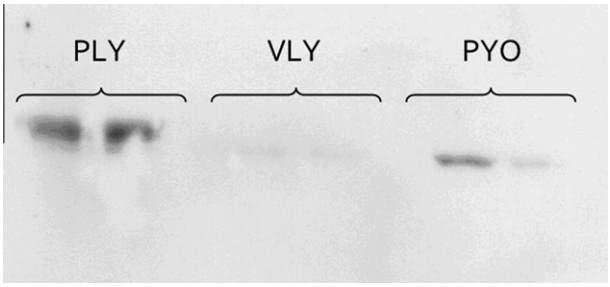

Fig. 2. Western-blots of recombinant PLY, VLY and PYO with the PLY-5 mAb.

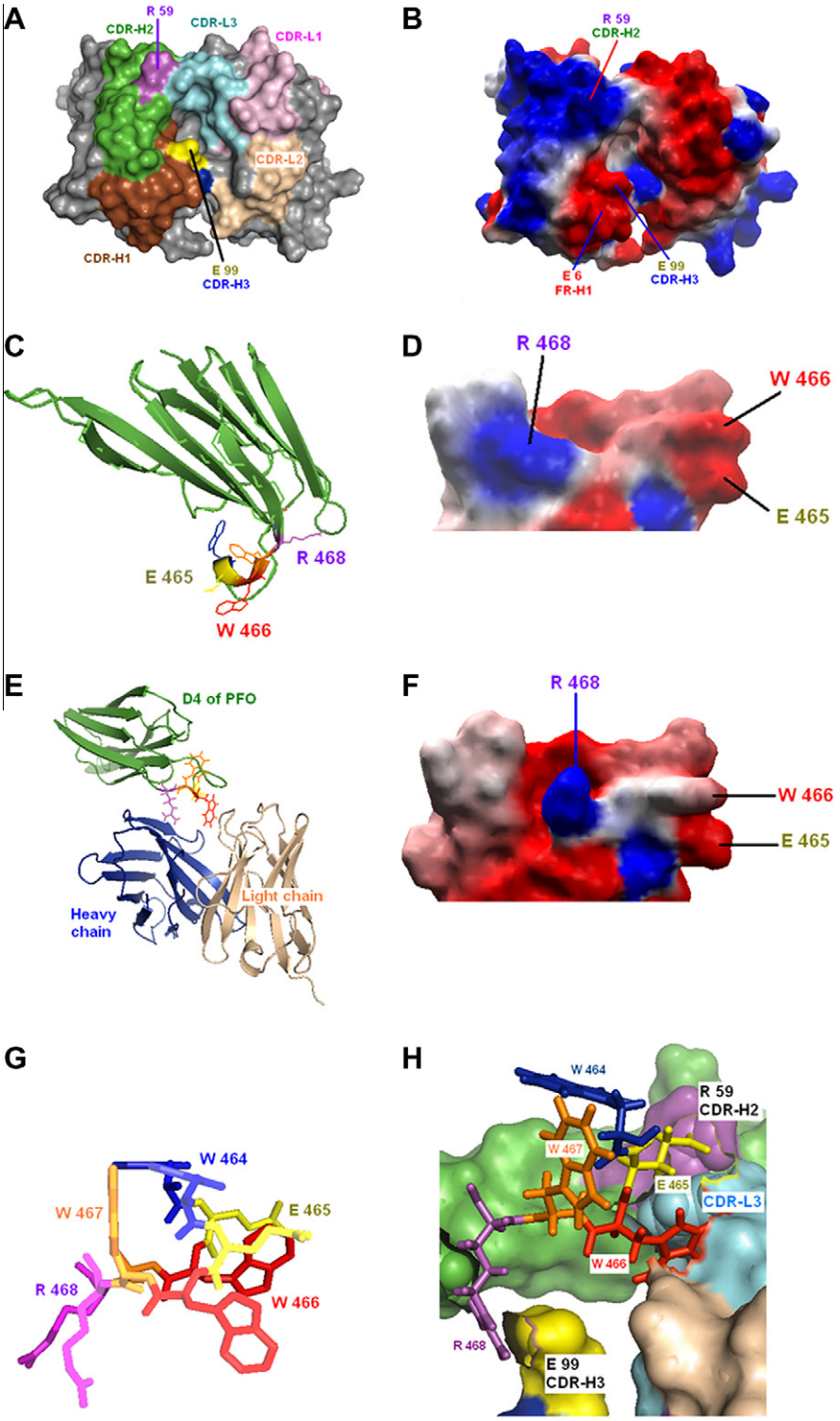

Fig. 3. A: Orthogonal view of the modelled Fv. CDR-H1, brown; CDR-H2, green. The R 59 residue of CDR-H2 is highlighted in magenta. CDR-H3 is in blue. The E 99 residue of CDR-H3 is highlighted in yellow. CDR-L1, pink; CDR-L2, wheat; CDR-L3, cyan. The figure was generated in PyMOL. B: Surface electrostatic map of the modelled Fv. The figure was generated in Molegro. C: The WEWWR residues of the undecapeptide are shown in detail at the tip of the D4 domain of PFO. The figure was generated in PyMOL. D: Close-up view of the surface electrostatic map of the EWWR stretch of the D4 domain of PFO. The figure was generated in Molegro. E: Molecular rendition of the modelled simulation of D4 docking on the Fv of PLY-5. The figure was generated in PyMOL. F: Close-up view of the surface electrostatic map of the EWWR stretch of the D4 domain of PFO as in the docking simulation. The figure was generated in Molegro. G: Superimposition of the WEWWR stretch as in the crystal (at the back, darker colour) and as in the docking simulation (at the front, light colour). The figure was generated in PyMOL. H: Close-up view of the docking. The R 59 residue of CDR-H2 and the E 99 residue of CDR-H3 are in magenta and yellow, respectively, as in A. The figure was generated in PyMOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the third lowest energy score conformer (the one which best fitted experimental observations), according to the simulations of docking of the D4 domain of PFO with the modelled Fv of PLY-5, the tip of the D4 domain lay perpendicular to the binding surface of the PLY-5 Fv (Fig. 3E). The electropositive guanidium tip of R 468 was markedly extruded (Fig. 3F) and was located between

Table 3
CDRs characteristics of PLY-5 according to the Rosetta model.

PLY-5 mAb	Amino acid residues in CDR1-CRD2-CDR3		
	CDR1	CDR2	CDR3
VH11-16-3[a]	(26)G**Y**SITSD**Y**A**W**N(36)	(51)**F**IT**Y**SGSTR**Y**NPSLKS(66)	(99)EAS(101)
VL11-7-9	(24)RASQEISD**F**LS(34)	(50)AASTLES(56)	(89)LQ**Y**AS**F**PPT(97)

Aromatic residues are in bold.
[a] Number of amino acid residues in CDRs.

the electronegative carboxylic ends of Glu99 of CDR-H3 and Glu6 of FR-H1. The aromatic ring of Trp466 was somewhat displaced (Fig. 3G) and lodged in the above-mentioned central crater, between the aromatic rings of Tyr91 and Phe94 of CDR-L3 (Fig. 3H). In addition, the E 465 residue of the undecapeptide made contacts with the R 59 of CDR-H2. In this simulation, neither the CDR-H1, CDR-L1 nor the CDR-L2 residues made any apparent contact with the undecapeptide and neither did the Trp464 and the Trp467 residues of the undecapeptide make contact with the antibody paratope. From all this it can be deduced that the binding of the undecapeptide loop to the PLY-5 antibody is mainly mediated by aromatic and by ionic interactions.

4. Discussion

According to the results of a membrane bound-peptide scan [5], the minimal epitope recognised by PLY-5 on the Trp-rich undecapeptide of PLY should consist of WEWWRT. A W492A mutant of listeriolysin O (LLO) was recognised by PLY-5 but the W491A mutant was not [13]. In accordance with this, and by means of substitution analogues, we now show that the second Trp and the Arg residues are both essential for PLY-5 recognition of soluble peptides related to the Trp-rich undecapeptide and that this recognition responds to an X^1 X^2 W^3 X^4 R^5 X^6 amino acid residue sequence. In bacterial CDCs, X^2 is a positively charged amino acid residue, either E or D, which contributes to the binding interaction with PLY-5 in an auxiliary role.

The essential participation of tryptophan residues in the configuration of B-cell epitopes has already been reported [14,15] but, so far, to our knowledge, there are no descriptions of the characteristics of the antibodies recognising this kind of epitope.

The CDRs of PLY-5 are not especially rich in tyrosine, which is highly abundant in natural antigen-binding sites [16,17], but, as a whole, according to the model described here, the paratope of the PLY-5 mAb seems to be composed of a number of aromatic amino acid residues (Phe, Trp and Tyr) [18,19]. The CDR-H3 is remarkably short, with only three amino acids (EAS); in the modelled Fv, this E residue gives rise to an electronegative surface spot.

The epitope recognised by the PLY-5 mAb resides on the same flexible loop that is involved in membrane binding [1]; to do that, this loop flips out. The docking simulation described here mimics this displacement; this type of conformational change could actually be induced by the antibody to optimise the binding interaction.

Hydrophobic stacking interactions between aromatic side chains of antigens and of antibody CDRs, and van der Waals contacts, are thought to play a paramount role in the antibody recognition of protein antigens. The contribution of aromatic residues to the combining sites of antibodies which recognise aromatic rings has also been documented [20–22]. In our docking model, the aromatic ring of W466 is seen as stacked between those of Tyr91 and Phe94 of CDR-L3.

In the crystal form of PFO, the AWEW tetrad configures a β-turn, whereas the following WRT residues are part of a reverse β-strand. β-turns are well recognised antigenic sites [23].

The model of the Fv of the PLY-5 mAb described here shows a central crater continued on one side by a canyon. According to the docking simulation reported here, the main contacts of the Trp-rich undecapeptide of PFO with the binding site of PLY-5 would be made through its EW-R residues. With respect to the antibody, only residues of its CDR-H2, CDR-H3 and CDR-L3 around that central crater are involved in epitope recognition.

Altogether, our experimental observations indicate that the second Trp residue in the β-turn and the R residue in the following β-strand of the Trp-rich undecapeptide are pivotal in the conformation of the epitope and for the recognition of CDCs by the PLY-5 mAb.

Acknowledgments

This work was partially supported by Proyecto FISS-09-PS09/01911, Ministerio de Ciencia e Innovación, Spain, and by funds (SCT-12-120) from the Universidad de Oviedo. The authors declare no conflict of interest.

References

[1] G. Polekhina, S.C. Feil, J. Tang, et al., Comparative three-dimensional structure of cholesterol-dependent cytolysins, in: J.E. Alouf, M.R. Popoff (Eds.), The Comprehensive Sourcebook of Bacterial Protein Toxins, 3rd edition., Academic Press, Oxford, England, 2006, pp. 659–670.
[2] E.M. Hotze, R.K. Tweten, Membrane assembly of the cholesterol-dependent cytolysin pore complex, Biochim. Biophys. Acta 2012 (1818) 1028–1038.
[3] <http://www.rcsb.org/pdb/home/home.do>
[4] J.R. de los Toyos, F.J. Méndez, J.F. Aparicio, et al., Functional analysis of pneumolysin by use of monoclonal antibodies, Infect. Immunol. 64 (1996) 480–484.
[5] T. Jacobs, M.D. Cima-Cabal, A. Darji A, et al., The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding, FEBS Lett. 459 (1999) 463–466.
[6] A. Tramontano, The role of molecular modelling in biomedical research, FEBS Lett. 580 (2006) 2928–2934.
[7] J.C. Almagro, M.P. Beavers, F. Hernandez-Guzman, et al., Antibody modeling assessment, Proteins 79 (2011) 3050–3066, http://dx.doi.org/10.1002/prot.23130.
[8] S.E. Gelber, J.L. Aguilar, K.L. Lewis, A.J. Ratner, Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*, J. Bacteriol. 190 (2008) 3896–3903.
[9] B. Suarez-Alvarez, M.delM. Garcia-Suarez, F.J. Mendez, J.R. de los Toyos, Characterisation of mouse monoclonal antibodies for pneumolysin: fine epitope mapping and V gene usage, Immunol. Lett. 88 (2003) 227–239.
[10] A. Sircar, E. Kim, J.J. Gray, RosettaAntibody: antibody variable region homology modeling server, Nucleic Acids Res. 37 (Web Server Issue) (2009) W474–W479.
[11] S. Lyskov, J.J. Gray, The RosettaDock server for local protein–protein docking, Nucleic Acids Res. 36 (Web Server Issue) (2008) W233–W238.
[12] K.W. Kaufmann, G.H. Lemmon, S.L. Deluca, et al., Practically useful: what the Rosetta protein modeling suite can do for you, Biochemistry 49 (2010) 2987–2998, http://dx.doi.org/10.1021/bi902153g.
[13] E. Carrasco-Marín, F. Madrazo-Toca, J.R. de los Toyos, et al., The innate immunity role of cathepsin-D is linked to Trp-491 and Trp-492 residues of listeriolysin O, Mol. Microbiol. 72 (2009) 668–682.
[14] N. Kamal, S. Chowdhury, T. Madan, et al., Tryptophan residue is essential for immunoreactivity of a diagnostically relevant peptide epitope of *A. fumigatus*, Mol. Cell. Biochem. 275 (2005) 223–231.
[15] L.H. Lee, M.F. Tam, H. Chou, et al., Lys, pro and trp are critical core amino acid residues recognized by FUM20, a monoclonal antibody against serine protease pan-fungal allergens, Int. Arch. Allergy Immunol. 143 (2007) 194–200.
[16] I.S. Mian, A.R. Bradwell, A.J. Olson, Structure, function and properties of antibody binding sites, J. Mol. Biol. 217 (1991) 133–151.

[17] S. Koide, S.S. Sidhu, The importance of being tyrosine: lessons in molecular recognition from minimalist synthetic binding proteins, ACS Chem. Biol. 4 (2009) 325–334.
[18] E.J. Sundberg, R.A. Mariuzza, Molecular recognition in antibody-antigen complexes, Adv. Protein Chem. 61 (2002) 119–160.
[19] T. Ramaraj, T. Angel, E.A. Dratz, et al., Antigen-antibody interface properties: Composition, residue interactions, and features of 53 non-redundant structures, Biochim. Biophys. Acta 2012 (1824) 520–532.
[20] J. Anglister, M.W. Bond, T. Frey, et al., Contribution of tryptophan residues to the combining site of a monoclonal anti dinitrophenyl spin-label antibody, Biochemistry 2 (1987) 6058–6064.
[21] R. Robert, M.P. Lefranc, A. Ghochikyan, et al., Restricted V gene usage and VH/VL pairing of mouse humoral response against the N-terminal immunodominant epitope of the amyloid β peptide, Mol. Immunol. 48 (2010) 59–72.
[22] V. Arzhanik, D. Svistunova, O. Koliasnikov, A.M. Egorov, Interaction of antibodies with aromatic ligands: the role of π-stacking, J. Bioinform. Comput. Biol. 8 (2010) 471–483.
[23] J.L. Pellequer, E. Westhof, M.H. Van Regenmortel, Correlation between the location of antigenic sites and the prediction of turns in proteins, Immunol. Lett. 36 (1993) 83–99.