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The Role of the First 14 Amino Acids of Mature M1 Protein of *Streptococcus pyogenes* on Fibronectin-Binding Activity and Dimer Formation

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Streptococcus pyogenes is one of the most important human pathogens which express a multi-facet of virulence factors on its cell surface. One of the virulence factors that has been intensively-studied is the M protein that binds several human proteins. M1 protein, a member of the M protein family, was previously found to bind human fibronectin (Fn), an activity that is responsible for bacterial internalization. A structural study showed that this protein consists of four regions: A, B, S, and C. The study was intended to investigate the role of the first 14 amino acid residues located at the non-helical region of M1 protein in binding Fn, and its ability to form a dimer. The DNA fragment encoding for the ABS protein lacking its first 14 amino acids ($ABS\Delta_{14aa}$) was cloned into pET-16b, overexpressed in *Escherichia coli* BL21(DE3), and the protein was purified by affinity chromatography. The purified protein was characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the Fn-binding activtiy was assayed by enzyme linked immunosorbent assay. The result indicated that the M1 lacking its first 14 amino acids retains its dimerization and Fn-binding activities.

Key words: ABS fragment of M1 protein, dimerization, Fn-binding activity, non-helical region, *Streptococcus pyogenes*

Streptococcus pyogenes (Group A Streptococcus, GAS) causes a broad range of diseases, from mild ones, such as pharingitis and superficial skin infection, to more severe and even fatal ones, such as nectrotizing fasciitis, rheumatoid arthritis, and Streptococcal toxic shock-like syndrome. Since mid 1980s, GAS infections have reemerged and caused at least 616 million cases of throat infections, and 111 million cases of skin infections worldwide per year. It has also caused about 500,000 mortalities/year worldwide (Vlaminck *et al.* 2007; Bessen 2009; Guilherme *et al.* 2009). To cause the illness, GAS produces various virulent factors; among which is the M protein which participates in adhesion, colonization, and internalization in the host cells (Vlaminck *et al.* 2007; Courtney *et al.* 2002). The M protein also has a role in streptococcal resistance to the phagocytes system of the host and triggers tissue-factor up-regulation which leads to pro-coagulant activity of cells (Pahlman *et al.* 2007).

At present, there are at least 200 types of GAS based on the amino acid sequences of the hypervariable region (HVR) located at the N-terminus of the M protein (Bessen *et al.* 2009). Among them, M1 and M3 serotypes are known to be the most invasive strains (Courtney *et al.* 2002). The M1 protein of strain 90226, a highly invasive strain, has been studied for its interaction with host cells via the human fibronectin (Fn), a component of human extracellular matrix. A structural study showed that M1 protein has a helical coiled-coil conformation and consists of several different regions, i.e. A, B, S and C. The HVR that accounts for serotype specificity of M1 protein resides in the first 50 residues of the A region of mature protein (McNamara *et al*. 2008). At the amino-terminus of HVR, there is a non-helical region whose length varies depending on the serotype, 11 amino acids in the M6 protein, and 21 amino acids in the M1 protein (Fischetti 1989; McNamara *et al*. 2008).

A previous study demonstrated that strain 90-226 was internalized into the A549 human lung epithelial cells. However, this ability was lost in the M1-deficient mutant which showed that the M1 protein was responsible for the internalization. A purified intact M1 protein was found to bind Fn (Cue *et al*. 1998). A genetic approach was done to localize the Fn-binding domain by creating several truncated M1 proteins. The evidence showed that a truncated M1 protein containing ABSC, ABS, and BSC regions retained Fn-binding ability and dimer formation (Cue *et al*. 1998; Cue *et al*. 2001). Deletion of both A and B regions abolished the ability to bind Fn. Deletion of either A or B region did not eliminate Fn-binding ability completely, although it was considerably reduced. The data strongly indicated that the M1 protein may have two independent Fn-binding domains (Cue *et al*. 2001).

At the N-terminus of the A region, a non-helical region spanning for the first 63 amino acids was identified from a crystallography study, and the A regions of two M1 protein molecules form parallel dimers, starting from residues 13-15 of mature protein (McNamara *et al*. 2008). Based on this result, it is suggested that the first 13-15 amino acids of the A-repeat region do not involve in the dimer formation. The information regarding whether these amino acids are crucial for Fn-binding is also limited. Therefore, the current research was aimed at investigating the role of the first 14 amino acids at the non-helical region of the M1 protein on Fn-binding and dimer formation. In this study, we constructed a truncated M1 protein containing A, B, and S regions but lacking the first 14 amino acid residues, and determined its binding activity towards Fn and its ability to form a dimer. A pET-16b/*Escherichia coli* BL21(DE3) expression system was used to overproduce the truncated ABS protein. The affinity-purified protein was assayed for its Fn-binding activity using enzyme linked immunosorbent assay (ELISA).

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MATERIAL AND METHODS

Bacterial Strains and Plasmids. The *S. pyogenes* strain 90-266 was a courtesy of Prof P Patrick Cleary from the Department of Microbiology, Medical School, University of Minnesota, Minneapolis, USA. The cloning was performed in *E. coli* JM109 using the pGEM-T (Promega, Minneapolis, USA) as a cloning vector. The expression was performed in *E. coli* BL21(DE3) using pET-16b (Novagen Inc., Madison, WI, USA) as an expression vector. All *E. coli* cells were grown in Luria Bertani (LB) agar or broth medium containing 100 μg mL^{-1} of ampicillin as a selectable marker at 37°C with vigorous agitation. *S. pyogenes* was grown in Todd-Hewitt medium supplemented with 2% neopeptone (THNB, Difco Laboratories, Detroit, MI, USA). In the fibronectin (Fn) binding assay sheep anti-human Fn antibody (ICN Pharmaceuticals, Costa Mesa, CA, USA) was used as a primary antibody and the alkaline phosphatase conjugated the rabbit anti-sheep IgG (Sigma Chemical Co., St Louis, MO, USA) was used as a secondary antibody. The substrate, para-nitrophenylphosphate, pNPP (Sigma Chemical Co., St Louis, MO, USA) was prepared in 10% diethanol amine and 0.5 mM $MgCl_2$.

Genetic Manipulation. To construct the DNA encoding for ABS fragment that lacks its first 14 amino acids ($ABS\Delta_{14aa}$), a pair of primers was designed using DNAStar program (DNASTAR, Inc.) based on the nucleotide sequences of *emm*1 gene of *S. pyogenes* M1 serotype (accession number AE004092) deposited in the GenBank (http://ncbi.nih.nlm.gov). The *Nde*I and *Bam*HI restriction sites were incorporated to the 5' end of forward and reverse primers, respectively to facilitate the insertion into pET-16b. The oligonucleotide sequences of the primers for amplification of the DNA encoding $ABS\Delta_{14aa}$ protein were 5'-CATATGGCAAACAATCCCGCAATACAA-3' (pF-*abs)* and 5'-GGATCCGATTTGTTTTTCTTCCTCA-3' (pR-*abs*). The restriction sites *Nde*I and *Bam*HI are underlined. The primers were synthesized by Proligo (Singapore) to amplify the DNA in a region that spans from codon 56 to 237 in *emm*1 gene.

The chromosomal DNA of serotype M1 *S. pyogenes* 90-226 was isolated using the Wizard® Genomic DNA Purification Kit (Promega) and used as a template to amplify the DNA encoding for ABS region. The DNA amplification was done by *Taq* DNA polymerase in 30 cycles of 94ºC, 55ºC and 72ºC for the denaturing, annealing and elongation processes, respectively. The PCR product was ligated overnight with pGEM-T, at 4ºC, and transformed into *E. coli* JM109 using the heat-shock method. The transformants were grown on LB agar supplemented with 100 g mL^{-1} ampicillin, isoprophyl-β-thiogalactopyranoside (IPTG), and bromo-chloro-indolyl-galactopyranoside (X-gal) at 37°C and white ampicillin-resistant transformants were isolated. The plasmids isolated from the white colonies were characterized by polymerase chain reaction (PCR) and restriction analyses. The insert of potential clones was sequenced by an automatic DNA sequencer (Macrogen Inc, Seoul, South Korea). The nucleotide sequence was analyzed using Basic Local Alignment Search Tools (BLAST) (http://ncbi.nih.nlm.gov). The confirmed recombinant pGEM-T plasmid was double digested with *Bam*HI and *Nde*I to excise the insert, which was then ligated with pET-16b linearized by the same enzymes. Transformation and characterization of selected transformants were performed as described above. The confirmed recombinant of pET16b-$ABS\Delta_{14aa}$ was transformed into *E. coli* BL21(DE3) for protein overproduction.

Protein Overproduction, Purification and Characterization. Overproduction of $ABS\Delta_{14aa}$ protein was performed in an LB liquid medium, with and without the addition of 0.3 mM of IPTG. A single colony of recombinant *E. coli* BL21(DE3)/ pET16b-$ABS\Delta_{14aa}$ was grown in LB medium containing 100 μg mL^{-1} of ampicillin at 37°C overnight. A 1% of overnight culture was added to the fresh LB medium containing 100 μg mL^{-1} of ampicillin, and then incubated at 37°C until it reached the optical density at 600 nm (OD_{600}) of 0.7. A 0.3 mM IPTG was added to the bacterial culture and incubation was continued for another 18 h. at 37ºC. Cells were harvested using centrifugation at 8 000 g at 4°C for 10 min. The cell pellets were washed with phosphate buffer saline (PBS) and centrifuged in the same condition. The cell pellets were then resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication (4 MHz, 1 min. pulses with 1 min. break for each cycle) until the solution became clear. Total intracellular proteins were separated from cell debris by centrifugation at 32 000 g at 4°C for 15 min. The supernatant was collected and the total proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

$ABS\Delta_{14aa}$ protein was produced as a fusion protein with His-tag at its N-terminus and was purified using nickel-NTA column (Novagen, Madison, WI, USA) based on the gravity. The total proteins were loaded into a prepared column at a flow rate of 5 mL h^{-1}. The $ABS\Delta_{14aa}$ protein was eluted using buffer (1.0 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). To remove the imidazole from the solution, the eluted $ABS\Delta_{14aa}$ protein was dialysed in 1:2 000 PBS overnight and the purified protein was analyzed by 12% SDS-PAGE.

Binding Assay. The binding assay was performed in two set of experiments which were with various amounts of of $ABS\Delta_{14aa}$ protein and binding assay with various concentrations of human fibronectin, Fn. The microtiter plate of Nunc-Immuno™ Plate Maxisorp™ Surface (Nalge Nunc Inc., Rochester, NY, USA) was used to immobilize the $ABS\Delta_{14aa}$ protein. Wells of microtiter plate were coated with different amounts of $ABS\Delta_{14aa}$ protein (400, 800, 1000, 1200 and 1500 ng), and immobilization was done at 37°C for 18 hours. Unbound proteins were washed three times with PBST (PBS containing 0.05% Tween 20, and 1mM

$MgCl_2$). The bound protein was blocked with 5% skim milk in PBS for 1.5 h at 37°C. After blocking, the wells were washed three times with PBST and once with the Tris-Buffered Saline (TBS). An amount of 320 ng of human Fn (Boehringer Mannheim, Indianapolis, IN, USA) were added and the plate was incubated for 1.5 h at 37°C. To remove the unbound Fn, the washing steps were done as described above. The sheep anti-human Fn antibody was diluted 1: 7 500 in PBS and added 50 µL into each well and the plate was incubated for 1.5 h at 37°C. The alkaline phosphatase conjugated the rabbit anti-sheep IgG diluted 1:10 000 in TBS was added into all wells and incubated at 37°C for 1 h and the wells were washed as described above. To develop the color, 1 mg mL^{-1} substrate para-nitrophenylphosphate, pNPP was added and incubated at 37°C for 1 h. The absorbance was measured at 405 nm by ELISA-*Reader* (Bio-Rad). The binding assay with various amount of Fn was done by using 80, 160, 320 and 640 ng of Fn and 1 000 ng of ABS_{13aa} protein. The rest of procedure was the same as above. All experiments were done in duplicate and the negative control was included to rule out the non-specific binding by skipping one step of the assay at a time, i.e. the addition of human Fn, anti-human Fn, or rabbit anti-sheep IgG.

RESULTS

ABS Protein Lacking its First 14 Amino Acids Has Been Generated. The characterization of the recombinant pET-16b from selected transformant is presented in Fig 1 and 2. The PCR analysis shows that both primers used in the cloning process gave a PCR product which is close to the theoretical size of the of $ABS\Delta_{14aa}$ coding region, 546 bp (Fig 1a). The restriction analysis result is shown in Fig 1b which demonstrates that a single digestion of recombinant pET-16b using *Bam*HI or *Nde*I resulted in a DNA fragment of 6300 bp which is the proper size of recombinant pET16b-$ABS\Delta_{14aa}$. The homology studies using Basic Local Alignment Search Tools (BLAST) (http://ncbi.nih.nlm.gov) showed that the nucleotide sequences of insert has 99% identity to those of *emm*1 of *S. pyogenes* (accession number Ae004092). The characterization results indicated that the DNA insert of recomibinat pET16b is the right ABS coding region of *emm*1 of *S. pyogenes*.

The $ABS\Delta_{14aa}$ protein was expressed as a protein fusion with decahistidine with 22 amino acids extra at the N-terminus. The theoretical size of decahistidine-ABS_{14aa} protein is 24 kDa. However, from SDS-PAGE analysis its apparent size was higher, i.e. 28.20 kDa. The SDS-PAGE analysis demonstrated that $ABS\Delta_{14aa}$ protein was produced almost equally in terms of its quantity in both induced and non-induced conditions (Fig 2A, lane 2 and 3). However, in induced condition, the protein was produced slightly more compared to that in uninduced ones. This is possible due to the leaky expression that is frequently occured in the pET expression system (Robert *et al.* 1999). The actual reason of this problem has been unknown. It may be due to the few quantity of lactose in the medium (Trudy *et al*. 1998).

Table 1. Fn-binding activity and dimer formation of truncated M1 proteins + = positive; ND = not determined

Truncated M1 proteins	Amino acid positions	Dimerization	Fn-binding	References
Pep ABSC	42-382	+	+	Cue *et al.* 1998; Cue *et al.* 2001
Pep ABS	42-231	+	+	Cue *et al.* 2001
Pep BSC	132-382	+	+	Cue *et al.* 2001
Pep AB	42-132	+	NA	McNamara *et al*. 2008
ABSΔ	56-237	+	+	This study

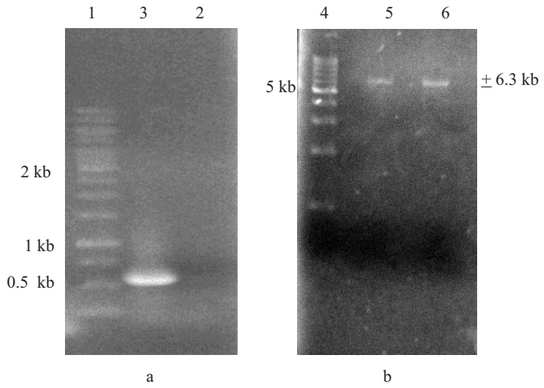

Fig 1 Characterization of recombinant pET16b-$ABS\Delta_{14aa}$. a, PCR product of 577 bp was produced when the pET16b-$ABS\Delta_{14aa}$ was used as a template; b. The restriction analysis. 1 and 4, DNA marker; 2, PCR product; 3, negative control; 5, pET16b-$ABS\Delta_{14aa}$ digested with *Bam*HI; 6, pET16b-$ABS\Delta_{14aa}$ digested with *Nde*I.

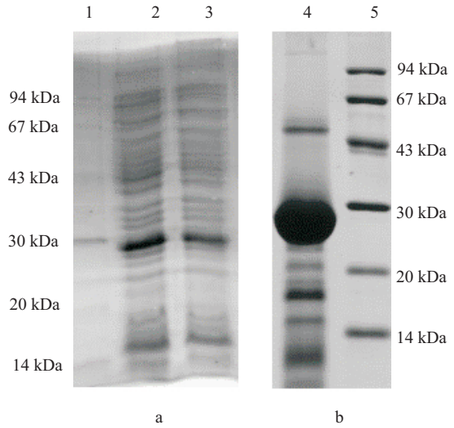

Fig 2 SDS-PAGE analysis of $ABS\Delta_{14aa}$ protein overproduction and purification. a, Total intracellular proteins of *E. coli* pET16b-$ABS\Delta_{14aa}$: 2, under IPTG induction; 3, without IPTG induction. B, Affinity purified $ABS\Delta_{14aa}$ protein; 4, purified $ABS\Delta_{14aa}$ protein; 1 and 5, protein marker.

The characterization of purified $ABS\Delta_{14aa}$ is presented in Figure 2B which shows a thick protein band of 28.20 kDa with some impurities mostly of low molecular weight proteins. There was also a high molecular weight protein of about 50 kDa present in the purified fraction (Fig 2B, lane 4) which was probably a dimer of the $ABS\Delta_{14aa}$ protein. The result obtained was consistent with the previous report of Cue *et al*. (2001) who found that ABS fragment of M1 protein as a dimeric form. The amino acid sequences of the $ABS\Delta_{14aa}$ protein deduced from its nucleotide sequences showed one amino acid that is different from that of the M1 protein used for primer design i.e. amino acid at position 68 of mature protein is His in 90-226 instead of Tyr.

$ABS\Delta_{14aa}$ Protein Binds Specifically to Human Fibronectin. The binding actvity assay result can be seen in the Fig 3 which shows that the optimal binding was obtained when 1 000 of ABS_{14aa} protein and 320 ng of human Fn were used. Apparently, $ABS\Delta_{14aa}$ less than 1 000 ng was inadequate for optimal binding. The immobilization of ABS_{14aa} protein limits the movement of the protein to adjust its proper orientation, thus the interaction with the human Fn also decreased. On the other hand, using more than 1 000 ng of $ABS\Delta_{14aa}$ protein was ineffective since the binding capacity of the microplate is limited (Selby 1999). The interaction of $ABS\Delta_{14aa}$ protein to Fn was indeed due to specific binding because negative control results (binding assay in the absence of human Fn) gave much less absorbance value compared to that in the presence of human Fn (Fig 3a and 3b). Based on the binding assay result it can be concluded that the $ABS\Delta_{14aa}$ protein binds the human Fn.

DISCUSSION

The M1 protein is an important adhesin and invasin of GAS. Its binding to various human extracellular proteins facilitates the attachment of streptococcal cells to human cells, followed by its internalization into host cells where the

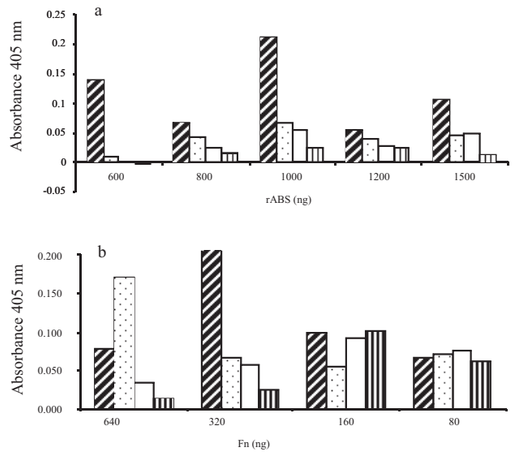

Fig 3 Binding assay of $ABS\Delta_{14aa}$ with a various amount of a, $ABS\Delta_{14aa}$ protein; b, human fibronectin, Fn incubated at 37°C using 1:7 500 of anti human Fn antibody, 1:10 000 conjugate and 1 mg/mL^{-1} substrate, para nitrophenylphoshpate (pNPP). ▨, binding of $ABS\Delta_{14aa}$ to Fn, negative controls; ⁘ without human Fn; □, without primary antibody; ▥, without secondary antibody.

bacteria avoid antibiotics and humoral immune responses. Although the M1 protein has been studied intensively, a deeper understanding is required to find out the binding activity of this protein. This work was intended to study the role of the first 14 amino acids in the HVR of the M1 protein in binding the human Fn and in forming a dimer.

Based on BLAST analysis, the amino acid sequences of the $ABS\Delta_{14aa}$ protein deduced from its nucleotide sequences show 95% to 100% identity to those of fifteen M1 proteins deposited in the GenBank (www.ncbi.nlm.nih.gov). Nine M1 proteins have identical amino acid sequences to the ABS_{14aa} protein. However, three M1 proteins show 99% identity with one amino difference each at H13→Y, N15→T and D17→S, one M1 protein demonstrates 98% identity having two amino acids difference at E138→D and A155→R, and one has 97% identity with four amino acid substitutions, E14→K, deletion of N15, K16→Q and D17→G. There is one M1 protein which shows quite high sequence variability with 95% identity the ABS_{14aa} protein containing eight amino acid substitutions, P4→T, A5→T, I6→V, H13→N, D17→N, E27→N, T140→A and A155→R. These data indicate that the amino acid sequence of M1 protein spanning from residues 56 - 237 are highly conserved, although there are some variations in some M1 proteins. Most of the amino acid substitutions are conservative, for instance E→D, E→K, I→V, H→N, D→N and E→N and these variations are not expected to affect the M1 protein function. Previous result suggested that there are four regions that are highly conserved in Fn-binding of M proteins, M1 and M3, designated as regions a, b, c and d (Cue *et al*. 2001). These regions have been suggested to be Fn-binding domains, although no experimental evidence to provide the notion. The amino acid sequence variations in the fifteen M1 proteins mentioned above are not located in putative Fn-binding domains, suggesting that the Fn-binding activity is evolutionary conserved.

The SDS-PAGE analysis of purified $ABS\Delta_{14aa}$ protein indicated that the proteinexpressed has a dimeric form which is showed by the presence of a higher molecular weight protein (50 kDa) on SDS-PAGE. This result is consistent with previous finding (Cue *et al*. 1998; Cue *et al*. 2001). Several truncated M1 proteins was shown to form dimer using SDS-PAGE and Western blot analysis using Fn and the work indicated that the minimal protein fragment that retained dimer formation was ABS that spans from residues 42-382. Our finding demonstrated for the first time that the ABS protein lacking its first 14 amino acids in the HVR retained its ability to form dimer. The first 14 amino acids are located in the hypervaribile non-helical region of M1 protein and the hypervariable region of M proteins is implicated as a antiphagocytic determinant by binding to C4-binding protein (C4BP), a regulator of complement system (Bergga *et al*. 2001). By not involving in the dimer formation, this HVR is free to bind C4-BP in order for the M protein to function as an antiphagocytic determinant.

Since the $ABS\Delta_{14aa}$ protein was produced as a protein fusion, the structure of the protein of the protein was

analyzed for formation of coiled-coil conformation by paircoil2 program (http://groups.csail.mit.edu/cb/paircoil2/paircoil2.html) to rule out the possibility of structural alteration. The paircoil2 program was run to the whole M1, ABS and ABSΔ_{14aa} proteins. The results convincingly show that there is no significant change in the coiled-coil conformation in ABS$_{14aa}$ protein (data not shown). The formation of coiled-coil conformation is crucial for dimerization which is a requirement for Fn-binding activity. This finding is in agreement with our current result showing that without its first 14 amino acid, the M1 protein is still capable to form a dimer.

Based on the evidence obtained in the current work, it was strongly demonstrated that the ABSΔ_{14aa} protein retained the Fn-binding ability. This present study is in agreement with earlier evidence which demonstrated that a minimal fragment covering A, B and S regions N-terminal A and B regions were accounted for the Fn-binding activity (Cue *et al*. 1998; Cue *et al*. 2001). This current study shows for the first time that deletion of the first 14 amino acids in HVR did not affect the ability of the M1 protein to bind Fn. A stable dimerization is responsible for the binding activity of the M1 protein and human Fn. An ordered dimerization of M1 protein is necessary for building a stable human Fn binding, *in vitro*. The M1 protein contains hydrophobic residues responsible for the formation of a dimeric structure (Fischetti 1989), and under the current experiment, the ABSΔ_{14aa} protein retains its ability for dimerization. This finding is consistent with the result of the previous experiment using crystallography, that region of M1 protein located after amino acid residues in position 53-58 in region A formed a paralel dimeric structure (McNamara *et al*. 2008). M protein has been demonstrated to be a major virulence factor and a multifunctional surface protein. This current study implicated that the amino terminus of M1 protein especially the first 14 amino acids are not involved in the dimer formation and its function is dedicated to provide *S. pyogenes* an ability to resist phagocytosis by binding to C4-BP. Hence, this region is also not important for Fn-binding and this capacity is provided by the rest of A, B and S regions. It means that there is collaboration of different regions in M1 protein to bind two human proteins, C4BP and Fn. Fn-binding of *S. pyogenes* 90-226 cells has been implicated as a prerequisite for internalization into human cell (Cue *et al*. 2001). The HVR focuses in binding to C4BP providing ability of *S. pyogenes* cells to colonize human blood by resisting phagocytic attack, while the remaining regions in ABS protein function in Fn interaction to provide intracellular residence. The result of this work is important to understand how M1 protein as a virulent factor of *S. pyogenes* interacts with each other to function as an invasin through dimerization and binds human extracellular matrix protein, the Fn.

The present study indicated, for the first time, that M1 protein of *S. pyogenes* strain 90-226 lacking its 14 amino acids in the non-helical hypervariable region retains its capability to form dimer and Fn-binding.

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