Original Article

Tailoring the binding properties of SpA Ig binding domains by in vitro molecular evolution

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Abstract: Background: Staphylococcus aureus protein A (SpA) is a bacterial immunoglobulin (Ig)-binding protein (IBP) and has fundamental applications in medical and biological sciences associated with IgG. In addition to its high affinity for IgG Fc, SpA also has a low affinity for the VH3 regions of IgG, IgM and IgA Fab, which may complicate its IgG applications. Methods: To diminish its VH3 binding potential and preserve the Fc binding potential, the amino acids which are involved in the interaction with VH3 at positions 29 and 30 in SpA A domain and at positions 36 and 37 in SpA C domain were randomly mutated respectively, meanwhile, a combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA was constructed. Then, a combination of A L29I30-A V29K30 was generated by in vitro molecular evolution using human IgG as bait. Results: The binding assays demonstrated that comparable to that of the its equivalent (A-A), A L29I30-A V29K30 exhibited affinity for IgG, but diminished affinity for IgM and IgA. Horse-radish peroxidase (HRP) conjugated A L29I30-A V29K30 presented enhanced IgG binding potential and improved effects for antibody detection against HIV core antigen in serum. Further, A L29I30-A V29K30 affinity chromatography recovered purified IgG in amount comparable to that for SpA but recovered no detectable IgM and IgA, thus demonstrating the former’s application advantages. Conclusions: This study demonstrates a successful example of functional protein engineering via in vitro molecular evolution and provides a useful approach to remodel the Ig binding property of SpA for application purposes.

Keywords: NEIBM, IgG, phage-based molecular evolution, antibody detection, antibody purification

Introduction

Bacterial immunoglobulin (Ig)-binding proteins (IBPs) specifically bind to immunoglobulins (Igs) and play important roles in the pathogenicity of bacteria [1]. Protein A of Staphylococcus aureus (SpA) [2], protein L of Finegoldia magna, formerly Peptostreptococcus magnus (PpL) [3], and protein G of groups C, G streptococci (SpG) [4], are three well-defined IBPs. Among them, SpA with a molecular weight of 57 kDa comprises 524 amino acid residues [5]. SpA has a tandem repeat sequence that containing five highly homologous IgG-binding domains, which named (from the N terminus) E, D, A, B and C. Each domain comprises of approximately 58 amino acid residues with a characteristic secondary structure consisting of three up-down α-helices that mediate the binding with IgG Fc region [6]. In addition, SpA also binds to a fraction of Ig molecules other than IgG Fc, for instance, the VH3 heavy chains in Fab region of Igs [7-9]. Crystal structure studies have shown that SpA B domain binding with Fc mainly involves residues in helix I and less involves in helix II [10, 11], whereas D domain interacting with VH3 of Fab involves residues in helix II and helix III [5].

SpA and other IBPs have fundamental applications in medical and biological area, e.g. diagnostic antibody detection, antibody purification, immunoprecipitation assays, and immunoadsorption therapy [12-15]. Recombinant monoclonal antibodies have become important biological pharmaceuticals and are widely used as
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research reagents. SpA-based purification, as a key purification step, is the main part of the raw-material costs [16, 17]. In addition to the specific interaction with the Fc region of IgG during antibody purification, SpA may also bind to the Fab domain of the VH3-clan of IgG, IgM and IgA. Therefore, this VH3 binding potential might disturb the purification of recombinant monoclonal antibodies and lead to an inaccurate specific detection of the IgG antibody responses against pathogens by false detection of the IgM antibodies. Developing SpA-derived IgG binding proteins with preserved IgG Fc binding potential and diminished VH3 binding potential remains an interesting research topic.

Numerous novel combinations of Ig-binding domains of SpA, SpG and PpL that do not exist in natural bacterial IBPs were generated by in vitro molecular evolution. These molecules are named as newly evolved Ig-binding molecules (NEIBM) and exhibit novel Ig-binding properties [18-22]. For example, NEIBM LD5 and LD3 not only could interact with human Ig Fab in the VH3 and Vκ regions via a double-binding method, but also show a high affinity for human IgM [19]. Additionally, the horse-radish peroxidase (HRP)-conjugated anti-M13 antibody and helper phage M13K07 were obtained from Pharmacia Biotech (Uppsala, Sweden). Human IgG (hIgG), human IgM (hIgM) and human IgA (hIgA) were purchased from Sigma (St. Louis, MO, USA).

In the present study, the amino acids which are involved in the interaction with VH3 at positions 29 and 30 of SpA A domain and those at positions 36 and 37 of SpA C domain, were randomly mutated, and a combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA was constructed. A NEIBM combination, A_{29G30T}C_{36K37Q}, was obtained by in vitro molecular evolution using human IgG as bait, which preserved the IgG Fc binding potential and diminished the IgM and IgA binding potential.

Materials and methods

Ethical approval

All aspects of the study on the forty anti-HIV-positive human serum samples were approved by the Ethics Committee of Beijing You An Hospital, Capital Medical University, China; the written informed consent was provided by all subjects in the study [23]. And all aspects of the study on the forty anti-HIV-negative human serum samples were approved by the Ethics Committee of Changhai Hospital, Shanghai, China; oral informed consent was provided by all subjects in the study.

Vectors, reagents and serum samples

The phagemid vector pCANTAB5S was constructed in advance in our laboratory. The prokaryotic expression plasmid, pET-32a (+), and E. coli host strains Top10, were purchased from Novagen Company (Darmstadt, Germany), and E. coli TG1 was obtained from Stratagene Company (Cambridge, England). All the primers in this study were synthesized by the Sangon Biotech (Shanghai, China). The horseradish peroxidase (HRP)-conjugated anti-M13 antibody and helper phage M13K07 were obtained from Pharmacia Biotech (Uppsala, Sweden). Human IgG (hIgG), human IgM (hIgM) and human IgA (hIgA) were purchased from Sigma (St. Louis, MO, USA).

Forty anti-HIV-positive human serum samples were collected from the AIDS high-risk cohort at YouAn Hospital in Beijing, China [23]. Forty anti-HIV-negative human serum samples were collected from healthy blood donors at Changhai Hospital, Shanghai, China. Upon receipt, all samples were aliquoted and stored at -80°C. Referring to the related study, the seropositive status of the subjects was confirmed using Western blotting (HIV Blot 2.2 WB, MP Biomedicals Asia Pacific Pte. Ltd., Singapore) and ELISA (Diagnostic Kit for Antibody to HIV (ELISA), Shanghai Kehua Bio-Engineering Co., LTD., China) [23].

Construction of combinatorial phage libraries

The combinatorial phage library displaying randomly-rearranged A domain with randomly mutated amino acids at positions 29 and 30 and C domain with randomly mutated amino acids at positions 36 and 37 of SpA, respectively, was constructed through polymerase chain reaction (PCR)-based random mutagenesis technology using primers listed in Table S1. XbaI restriction sites were introduced at both ends of the fragments of the mutated A and C domain. Then, the combinatorial mutat-
ed phage library was constructed by digesting the PCR products with Xba I and ligating into the phagemid pCANTAB5S on the Xba I site (library 1, Table 1).

Gene fragments with similarly Xba I restriction sites that encode the SpA A and C domains were respectively synthesized by PCR with primers listed in Table S2. Then, the combinatorial phage library displaying various randomly-rearranged A and C domains was built with above mentioned method (library 2, Table 1).

After amplification in E. coli Top10, the recombinant phagemids were transformed into the host bacterial strain TG1. Helper phages M13-K07 (approximately 1.8*10^{11} transformation unit (TU)) were added when the optical density (OD) value at 600 nm was about 0.5, and cultured in the shaker at 230 rpm for 1 h at 37°C. Subsequently, kanamycin (15 μg/ml) was added and the cells kept culturing overnight. After the centrifugation at 5,000 g for 10 min, the supernatant was collected and the phages were harvested by a 0.22 μm filter. Then, the combinatorial phage display libraries displaying the randomly-rearranged A and C domains of SpA were obtained (Table 1). The primer pairs pCANTAB5S-1 (5'-CAACGTGAAAA-AATTATTATTCGC-3') and pCANTAB5S-6 (5'-GTA-AATGAATTTTCTGTATGAGG-3') were applied for the amplification and sequencing analysis of inserted fragments for the positive phages [22].

**In vitro molecular evolution of the libraries by hlgG molecule**

Dilute the hlgG to the concentration of 10 μg/ml in 0.1 M NaHCO₃ (pH9.6), then add to 96-well ELISA plates for incubating at 37°C for 3 h. Then, block the plates with 0.01 M phosphate buffered saline (PBS) containing 10% of skimmed milk, 0.1% of Tween 20 and 0.2% of mercurothiolate (blocking buffer) for 3 h. Add the constructed phage-displaying libraries into the plates and incubate at 37°C for 2 h. Wash each well with PBS containing 0.1% of Tween 20 for 10 times to remove the unbound phages. When the OD at 600 nm was about 0.5, 100 μl of the E. coli TG1 was added and the plates were incubated at 37°C for 1 h. Take 10 ml infected TG1 cells to culture in tryptone-yeast extract (2X) plates containing ampicillin (100 μg/ml) and then, calculate the number of colony forming units. After growing with helper phages M13K07 (approximately 1.8*10^{11} TU) overnight, the residual cells were cultured in 2X tryptone-yeast extract medium (8 ml) containing ampicillin (100 μg/ml). After the centrifugation at 5,000 g for 10 min, the supernatant was collected and the phages were harvested by a 0.22 μm filter for the next selection round with the hlgG as well. The selection was performed 3-6 rounds totally.

A total of 22 phage clones that randomly picked from each primary library and each round of the post-selection libraries were cultured with 2X tryptone-yeast extract medium (1 ml) at 37°C overnight. The primers pCANTAB5S-1 and pCANTAB5S-6 were applied for PCR amplification and analysis of the inserted fragments of phages. The empty plasmid pCANTAB5S was used as negative control for the evolution [22].

**Phage ELISA test**

Screening the hlgG binding phages in post-selection libraries by ELISA: The recombinant phagemids in the post-selection library 1 and library 2 were transformed into the E. coli TG1, respectively. Subsequently, select ninety clones from each library randomly and then culture them by shaking at 230 rpm at 37°C overnight. With the helper phages M13K07 (about 1.8*10^{11} TU), monoclonal phages were selected out from the last round from each post-

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**Table 1.** The names, mutational sites and evaluation of the combinatorial phage libraries

<table>
<thead>
<tr>
<th>Phage display libraries</th>
<th>Name</th>
<th>Mutational amino acid sites of SpA A domain</th>
<th>Mutational amino acid sites of SpA C domain</th>
<th>Transformation efficiency (cfu)</th>
<th>Titre (TU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Library 1</td>
<td>A₂₉₉, A₃₀₀, and C₃₆₆, C₃₇₇, site-directed randomly mutational phage display library</td>
<td>29.30</td>
<td>36.37</td>
<td>5.3*10^{6}</td>
<td>1.8*10^{12}</td>
</tr>
<tr>
<td>Library 2</td>
<td>A and C randomly combinatorial phage display library</td>
<td>None</td>
<td>None</td>
<td>3.6*10^{6}</td>
<td>1.6*10^{12}</td>
</tr>
</tbody>
</table>

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selection library and added to plates after coating with 10 μg/ml of hIgG as mentioned above, and the plates were incubated at 37°C for 2 h. After washing with the solution containing 0.25% of Tris, 0.05% of Tween 20, HRP-conjugated anti-M13 phage antibody was applied to detect the bound phages. Then, 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA) and H2O2 were adopted as the substrate for HRP, and the absorbance at 450 nm was measured with an ELISA Reader. Plasmid pCANTABSS-phage was applied as a negative control [22].

Sequence analyses: A total of 10 positive phage clones with the highest OD 450 nm were sequenced with the primers pCANTAB5S-1 and pCANTAB5S-6 by JIE LI Biology Company (Shanghai China). The sequences of target amino acid were deduced on the basis of DNA sequencing result, and the DNASTAR software package was adopt of the multiple sequence alignment analysis.

Prokaryotic expression, purification, and binding analysis of AL29I30-AV29K30 and A-A

Expression and purification of AL29I30-AV29K30 and A-A: With the primer pairs U-AA-BamH and D-AA-Sal, the representative phagemids, including AL29I30-AV29K30 and A-A, were used as templates to amplify DNA fragments by PCR using primers in Table S3 respectively. After the amplification, the target DNA was inserted into the plasmid pET-32a (+) and the result was examined via DNA sequencing. The proteins expression of AL29I30-AV29K30 and A-A was mediated by isopropyl-beta-D-thiogalactopyranoside (1 mmol/L) in E. coli BL21 (DE3). After sonication for 30 min, the proteins were collected and purified by a Ni-NTA column (Amersham Pharmacia Biotech).

ELISA test: The ELISA test was conducted as previously described [22]. Briefly, the immunoassay strips (Nunc, Rochester, NY, USA) were coated with the purified AL29I30-AV29K30 and A-A at a concentration of 1 μg per well that diluted by 0.1 M NaHCO3 (pH9.6) and incubated at 37°C for 3 h. After blocking with skimmed milk, 100 μl of serial double-diluting solutions of HRP-AL29I30-AV29K30 and HRP-A-A (1 mg/ml) were added to each well which was then incubated at 37°C for 45 min. The plates were developed by TMB and the absorbance at 450 nm was measured by ELISA Reader.

Detection of anti-HIV antibody: To detect the anti-HIV antibody using HRP-AL29I30-AV29K30 and HRP-A-A, the immunoassay strips were coated with hlgG, hlgM and hlgA respectively (1 μg per well) using 0.1 M NaHCO3 (pH9.6) and were incubated at 37°C for 3 h. After blocking with skimmed milk, 100 μl of serial double-diluting solutions of HRP-AL29I30-AV29K30 and HRP-A-A (1 mg/ml) were added to each well which was then incubated at 37°C for 45 min. The plates were developed by TMB and the absorbance at 450 nm was measured with an ELISA Reader.
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37°C for 45 min. The strips were developed with TMB and the absorbance at 450 nm was measured by ELISA Reader.

Biosensor analyses

The binding properties of $A_{29I30} - A_{V29K30}$ and A-A to hlgG, hlgM, and hlgA were studied by surface plasmon resonance (SPR) using the Bio-Rad XPR36 protein interaction array system. Briefly, hlgG, hlgM and hlgA (diluted in 10 mM sodium acetate, pH4.5) were coupled to a GLC ProteOn sensor chip using amine-coupling chemistry according to the manufacturer’s instructions. The association and dissociation condition were measured with serial 1:5 dilutions of $A_{29I30} - A_{V29K30}$ and A-A with the concentrations of 1.6 nM, 8 nM, 40 nM, 200 nM, 1 µM and 5 µM. The flow rate was set as 100 µl/min using PBST (pH7.4, 0.005% Tween 20) as flow buffer. The sensor-chip surfaces were regenerated by using 10 mM Glycine-HCl (pH2.0). KA (affinity constant) = $k_a$ (association rate constant)/$k_d$ (dissociation rate constant) [21].

Data analysis

All the experiments were repeat no less than three times in each triplicate ($n = 9$) and performed independently. The data were analyzed with one-way ANOVA or Student’s t-test. $P$ value < 0.05 was considered significant.

Results

In vitro molecular evolution of the combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA

To eliminate the VH3 binding activity, the amino acids at positions 29 and 30 of SpA A domain and the amino acids at positions 36 and 37 of SpA C domain, which both interact with VH3 of Igks, were randomly mutated (Figure 1) [22]. A combinatorial phage library displaying the randomly-rearranged mutated A and C domains

Figure 1. Alignment of the amino acid sequences of the five SpA domains and the mutation sites of SpA A domain and SpA C domain. The SpA A domain residues involved in the interaction with Fab are highlighted in green, and the A domain residues mediating Fc binding are highlighted in red with the overlapping residue Gln-32 (highlighted in yellow). The mutation sites of A and C domains are marked by solid triangles.
of SpA (library 1) was constructed and subjected to in vitro molecular evolution with the bait hlgG. As a control, the combinatorial phage library displaying randomly-rearranged A and C domains of SpA without any mutation (library 2) was also constructed and subjected to in vitro molecular evolution as well. The library 1 had \(5.3 \times 10^6\) members, and the titre of the phage library was \(1.8 \times 10^{12}\) TU/ml (Table 1). The library 2 had \(3.6 \times 10^6\) members, and the titre of the phage library was \(1.6 \times 10^{12}\) TU/ml (Table 1). The capacity of the two established libraries satisfied the needs of the subsequent in vitro molecular evolution.

To check the randomness of nucleotides in the mutation sites in A and C domain of SpA from library 1, eighty clones from the original library 1 were randomly selected and primers pCANTAB5S-1 and pCANTAB5S-6 was adopted for the PCR amplification of the inserted fragments of phages [22]. The results are as follows: there were three phage clones displaying three domains, twenty phage clones displaying two domains, forty-six phage clones displaying one domain and eleven phage clones with no inserted fragment. Among them, twenty phage clones displaying two domains were chosen for sequencing analysis. The rearrangements of A and C domain of SpA in the twenty phage clones were as follows: three A-A combinations, two A\(^n\)-A combinations (R refers to reverse complementary sequence), two A-A\(^r\), two C-C\(^n\) combinations, two C\(^n\)-C\(^r\) combinations, two A-C combinations, two A-C\(^n\) combinations, two A\(^r\)-C combinations, one C\(^n\)-C combination, one C-A\(^n\) combination and one C\(^r\)-A\(^r\) combination. Hence, the twenty phage clones included 22 A domains and 18 C domains. In the A domain, the sequence analyses indicating that the A:T:C:G ratio in the NNS mutations of the amino acids at both positions 29 and 30 was 11:10:11:12 for the first base N, 10:13:11:10 for the second base N, and the C:G ratio was 21:23 in the third base S. In the C domain, the A:T:C:G ratio in the NNS mutations of the amino acids at both positions 36 and 37 was 9:8:9:10 for the first base N, 7:8:11:10 for the second base N, and the C:G ratio for the NNS mutation was 19:17 in the third base S. In summary, the variety and randomness of the library 1 satisfied the needs of the subsequent in vitro molecular evolution.

As observed in the previous studies [18, 22], the distribution of the inserted fragment sizes showed remarkable change throughout the in vitro evolution of both libraries in this study (Figure 2), which indicated an effective evolution. As a result, the ratio of phage clones displaying two domains was less than 30% in the original library, and increased to 100% during four rounds of selection of both libraries.

**Analysis of IgG binding activity of phage clones in the post-selection populations**

Ninety monoclonal phages from each of the two post-selection libraries were prepared respectively to evaluate the binding activity with hlgG via ELISA. As shown in Figure 3, phage clones exhibited different binding activities, and ten phage clones with the highest hlgG binding from each of the two post-selection libraries were chosen for sequencing analysis. All ten phage clones from the library 1 that displayed randomly-rearranged mutated A and C domains showed the same NEIBM combination.
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The Ig binding properties of NEIBM AL29I30-AV29K30 and A-A were expressed and purified as fusion proteins using pET-32a (+) expression vector to compare their Ig binding activities (Figure 4). ELISA analysis showed that A_{L29I30}A_{V29K30} and A-A exhibited comparable binding activities with hlgG. In contrast to the remarkable binding activities of A-A with hlgM and hlgA, A_{L29I30}A_{V29K30} presented no hlgM and hlgA binding activities (Figure 5). Consistent with these results, the SPR data (Table 2) also demonstrated that A_{L29I30}A_{V29K30} showed hlgG binding potential which was comparable to that of A-A but its hlgM and hlgA binding potential was diminished.

**Improved hlgG binding potential and anti-HIV detection effect of HRP-A_{L29I30}A_{V29K30}**

The conjugates of HRP-A_{L29I30}A_{V29K30} and HRP-A-A were produced, and their binding activities with hlgG, hlgM, and hlgA were compared. To our surprise, HRP-A_{L29I30}A_{V29K30} exhibited significantly enhanced binding activities with hlgG compared with those of HRP-A-A (Figure 6). Then, we compared the detection effects of the HRP-A_{L29I30}A_{V29K30} and HRP-A-A in a panel comprising of forty anti-HIV-positive human serum samples from HIV patients and forty anti-HIV-negative serum samples from healthy blood donors, respectively. As shown in Figure 7, both HRP-A_{L29I30}A_{V29K30} and HRP-A-A showed the same detection effects for negative serum samples, whereas the HRP-A_{L29I30}A_{V29K30}-based assay presented significantly improved detection effects for positive serum samples compared with HRP-A-A (P < 0.001).

**A_{L29I30}A_{V29K30} affinity chromatography recovered pure IgG from human serum**

To investigate whether A_{L29I30}A_{V29K30} has an application advantage in IgG purification, the purification efficiency of affinity chromatography columns made from A_{L29I30}A_{V29K30} or SpA was compared. As shown in Figure 8, the A_{L29I30}A_{V29K30} affinity column recovered the compara-
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Figure 5. Binding activities of \(A_{29I30}A_{29K30}\) to hIgG, hIgM and hIgA compared to A-A according to ELISA analysis. The plates were coated with purified \(A_{29I30}A_{29K30}\) and A-A, and 1:2 serial dilutions of biotin-labeled hIgG, hIgM and hIgA were incubated in the wells. The reactive complexes were detected using horseradish peroxidase (HRP)-conjugated streptavidin.

Table 2. Surface plasmon resonance analysis of the interactions between \(A_{29I30}A_{29K30}\) or A-A and IgG, IgM or IgA

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Constant</th>
<th>(A_{29I30}A_{29K30})</th>
<th>A-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>(k_a)  (M(^{-1})s(^{-1}))</td>
<td>1.00(*10^5)</td>
<td>1.53(*10^5)</td>
</tr>
<tr>
<td></td>
<td>(k_d)  (s(^{-1}))</td>
<td>2.86(*10^4)</td>
<td>3.61(*10^4)</td>
</tr>
<tr>
<td></td>
<td>(K_A)  (M(^{-1}))</td>
<td>3.50(*10^8)</td>
<td>4.24(*10^8)</td>
</tr>
<tr>
<td>IgM</td>
<td>(k_a)  (M(^{-1})s(^{-3}))</td>
<td>6.56(*10^3)</td>
<td>1.71(*10^3)</td>
</tr>
<tr>
<td></td>
<td>(k_d)  (s(^{-1}))</td>
<td>5.91(*10^5)</td>
<td>1.99(*10^5)</td>
</tr>
<tr>
<td></td>
<td>(K_A)  (M(^{-1}))</td>
<td>1.11(*10^2)</td>
<td>8.59(*10^2)</td>
</tr>
<tr>
<td>IgA</td>
<td>(k_a)  (M(^{-1})s(^{-3}))</td>
<td>10.8</td>
<td>1.08(*10^4)</td>
</tr>
<tr>
<td></td>
<td>(k_d)  (s(^{-1}))</td>
<td>1.51</td>
<td>2.34(*10^4)</td>
</tr>
<tr>
<td></td>
<td>(K_A)  (M(^{-1}))</td>
<td>7.15</td>
<td>4.62(*10^7)</td>
</tr>
</tbody>
</table>

The amount of IgG antibodies from human serum as that recovered by SpA affinity chromatography. Additionally, no IgM and IgA antibodies were recovered by the \(A_{29I30}A_{29K30}\) affinity column, whereas they were recovered by SpA affinity chromatography. The data indicated that \(A_{29I30}A_{29K30}\) had the obvious advantage in IgG purification.

Discussion

SpA, which is a natural IgG binding protein, especially for IgG, has fundamental applications in IgG antibody diagnostic detection, antibody purification, immunoprecipitation assays and immunoabsorption therapy. Crystal studies have shown that each SpA binding domain has two different binding interfaces: one is the Fc binding interface, which is located in helix I and helix II and interacts with one of Fc sites in two IgG heavy chains; the other one is the VH3 Fab binding interface, which is located in helix II and helix III and interacts with the VH3 chain in one Fab of IgG, IgM and IgA [10, 11]. Meanwhile, SpA contains a tandem repeat of five highly homologous Ig-binding domains and thus presents a number of binding avidity models by using some of the five Fc binding interfaces and the five VH3 binding interfaces. Among these potential binding models, the two-site Fc binding mode (Fc-Fc, two Fc binding interfaces from two IgG binding domains of SpA simultaneously bind with two Fc binding sites on one IgG molecule) is the key binding model for IgG and is utilized for the purification and specific detection of IgG. The VH3 binding interfaces may produce a low affinity for Fab of IgG, IgM and IgA, and may thus complicate the applications that are specific for IgG. For example, when a natural SpA chromatography was used for the purification of recombinant IgG consisting of VH3, besides the Fc-Fc binding model, it provided a number of other binding models for a single IgG molecule, such as VH3-Fc, VH3-Fc-Fc, VH3-Fc-Fc-VH3, VH3-VH3 and VH3-VH3-Fc. Namely, these models could recover IgG with different conformations along with that recovered by the Fc-Fc binding model, to generate heterologous IgG conformations, and thus to complicate IgG purification. Based on this consideration, the residues involved in VH3 binding, at positions 29 and 30 of SpA A domain and at positions 36 and 37 of the C domain, were chosen to randomly mutate to diminish the VH3 binding potential and preserve the Fc binding potential.

In theory, proper combinations of the two SpA binding domains are necessary to produce the Fc-Fc binding avidity, and all four types of combinations, A-A, A-C, C-C and C-A, may produce...
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Figure 6. Detection of the binding activities of HRP-AL29I30-AV29K30 and HRP-A-A with hlgG, hlgM and hlgA. The plates were coated with hlgG, hlgM and hlgA. 1:2 serial dilutions of AL29I30-AV29K30 and HRP-A-A were incubated in each well. Binding was detected by the addition of TMB.

Figure 7. Comparison of the detection effects of HRP-AL29I30-AV29K30 and HRP-A-A in anti-HIV ELISA. The strips were coated with HIV core antigen recombinant protein. One hundred-microliter of 10-fold dilutions of the forty anti-HIV-positive human serum samples and forty anti-HIV-negative human serum samples were separately added. One hundred-microliter of 1000-fold dilutions of HRP-AL29I30-AV29K30 and HRP-A-A were added, and the plates were incubated. The strips were developed upon the addition of TMB and detected at 450 nm on an ELISA Reader.


Fc-Fc binding avidity. To our surprise, in our study, only the A-A combinations (AL29I30-AV29K30 and A-A) were selected by in vitro molecular evolution of two phage libraries, which indicated that the A-A combinations possess some advantages in generating the perfect Fc-Fc binding avidity compared to other combinations. Interestingly, only the mutated A-A combination, AL29I30-AV29K30, was selected from the phage library displaying the randomly-rearranged mutated A and C domain, which should contain the A-A combination, indicating that the mutations at positions 29 and 30 of AL29I30-AV29K30 should favor Fc binding. In the present study, our strategy of in vitro molecular evolution only guaranteed the selection of phage clones with the strongest Fc binding potential but did not guarantee the selection of phage clones with eliminated VH3 binding potential. Hence, the elimination of VH3 binding potential of AL29I30-AV29K30 is not the result of in vitro molecular evolution but is an incidental consequence of the mutations that favor IgG binding. A fine-resolution map of the sequence function landscape of computational designed IgG binding protein FcB6 revealed that substitution of any of the core residues involving Fc binding are usually depleted and that most of the substitutions of any of the other residues are allowed or not allowed, part of them are depleted, and only few are favored [25]. In this...
study, considering the randomly mutated residues at positions 29 and 30 are adjacent to the residues at positions 28, 31, and 32, which are involved in Fc binding, the substitutions at positions 29 or 30 are likely not allowed, or depleted, and few are favored. The combined substitutions at both positions should have less chance to generate the mutants that favor Fc binding. However, on the other hand, the simultaneous substitutions at positions 29 and 30 could possibly compensate for their individual negative effects, and increased the chance to generate the mutants which benefit Fc binding. In this study, only one mutant, $A_{29I30}^{L29I30}$, which has combined substitutions at positions 29 and 30 in both A domain, was obligatorily selected and favor Fc binding. This finding should be helpful for designing mutations of targeted amino acids of functional domains or proteins to achieve successful protein engineering via in vitro molecular evolution.

Unexpectedly, HRP-labeled $A_{29I30}^{L29I30}$ showed obviously improved IgG binding activity compared to HRP-labeled A-A (Figure 6), whereas the binding assays clearly demonstrated that $A_{29I30}^{L29I30}$ and A-A showed comparable IgG binding activities (Figure 5). Our explanation for these results is as follows. The SpA binding domains adopt different residues to interact with Fc and VH3, respectively. The residues involved in Fc binding are primarily located in helix I, and less involves in helix II [10, 11], whereas the residues involved in VH3 binding are located in helix II and helix III [5]. The substitutions of residues at positions 29 and 30 in $A_{29I30}^{L29I30}$, which are involved in VH3 binding and located in the second and third turns of helix II, respectively, possibly induce conformation adjustment, and the neighboring residues at positions 28, 31 and 32 in helix II, which are involved in Fc binding, could be affected. Interestingly, the residue at position 29 in wild type A domain is G, which is conservative in all five domains of SpA and has a strong propensity for breaking the α-helical structure. In contrast, all the substituted amino acids in $A_{29I30}^{L29I30}$ V, K, L and I, have a strong or medium propensity for forming α-helical structure. These substitutions may contribute to the stability of helix II and therefore benefit the Fc binding of neighboring residues at positions 28, 31 and 32 in helix II. The reinforced helix II in $A_{29I30}^{L29I30}$ could also confer more resistance to the HRP labelling than wild-type SpA and may thus contribute to the improved IgG binding. This result revealed that in vitro molecular evolution may have more substantial effects on protein property than those that we designed only according to their binding property. This finding, together with our previous finding [22], might have a significant impact on protein engineering via in vitro molecular evolution to improve binding activity and application potential.

The NEIBM, $A_{29I30}^{L29I30}$ with preserved IgG binding potential and diminished IgM and IgA binding potential, demonstrated some application advantages. In contrast to natural SpA, the $A_{29I30}^{L29I30}$ affinity column recovered pure IgG without the contamination of IgM and IgA from human serum. This provides a novel affinity chromatographic medium with a simple IgG binding mode which could favor the purification of IgG antibodies. How $A_{29I30}^{L29I30}$ acts in the purification of recombinant IgG drug production remains an interesting question. With enhanced IgG binding potential, the HRP-$A_{29I30}^{L29I30}$-based ELISA exhibited a much better detection effect for anti-HIV core antigen than HRP-A-A-based ELISA, which implied the former’s application advantage in the detection of specific IgG antibodies, for the diagnosis of infections by pathogenic organisms. Moreover, $A_{29I30}^{L29I30}$ may contribute to the improvement of detection of specific IgM antibody responses against various pathogens by absorbing IgG from serum without any loss of IgM, which is usually absorbed by natural SpA, and eliminating competitive antigen binding between IgG and IgM.

In this study, a new NEIBM, $A_{29I30}^{L29I30}$ with preserved IgG binding potential and diminished IgM and IgA binding potential was obtained through in vitro phage-based molecular evolution, and it showed substantial application advantages in IgG purification and detection. This study demonstrates a successful example of functional protein engineering via in vitro molecular evolution and provides a useful approach to remodel the Ig binding property of SpA for application purposes.

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Disclosure of conflict of interest

None.

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References


Tailoring the binding properties of SpA Ig binding domains

**Table S1.** Primers for the amplification of DNA fragments encoding the mutants of A and C domains of SpA

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA1-AX-1</td>
<td>Forward amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*GCTGACAAACATTTCAAC</td>
</tr>
<tr>
<td>DA1-A1-1</td>
<td>Reverse amplifying primer</td>
<td>ATCTTTTAAGCTTTGGAATSNNSNN*ATTGCGTTGTTTCCTGGT</td>
</tr>
<tr>
<td>UA2-A1-2</td>
<td>Forward amplifying primer</td>
<td>ATCCAAAGCTAAAAGACCCAGAAAGTCTAAAGT</td>
</tr>
<tr>
<td>DA2-AX-2</td>
<td>Reverse amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*TTCGGTGCTTTGAGATTCC</td>
</tr>
<tr>
<td>UC1-CX-1</td>
<td>Forward amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*GCTGACAAACATTTCAAC</td>
</tr>
<tr>
<td>DC1-C1-1</td>
<td>Reverse amplifying primer</td>
<td>TCTTTTGCTCAGCTGAGGGSNNSNN*TTTAAGGCTTTGAGATGA</td>
</tr>
<tr>
<td>UC2-C1-2</td>
<td>Forward amplifying primer</td>
<td>CTTTCAGTGACAAAGAAATTAGCAGAAAGCT</td>
</tr>
<tr>
<td>DC2-CX-2</td>
<td>Reverse amplifying primer</td>
<td>TCTCTCAGACGCCGTACCTGCTCTAGA*TTCGGTGCTTTGAGATTCC</td>
</tr>
</tbody>
</table>

Note: *The restriction sites are underlined, Xba I cutting site is “TCTAGA”.*

**Table S2.** Primers for the amplification of DNA fragments encoding the A and C domains of SpA

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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</tr>
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<td>UA1-AX-1</td>
<td>Forward amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*GCTGACAAACATTTCAAC</td>
</tr>
<tr>
<td>DA2-AX-2</td>
<td>Reverse amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*TTCGGTGCTTTGAGATTCC</td>
</tr>
<tr>
<td>UC1-CX-1</td>
<td>Forward amplifying primer</td>
<td>TCGTCAGACGCCGTACCTGCTCTAGA*GCTGACAAACATTTCAAC</td>
</tr>
<tr>
<td>DC2-CX-2</td>
<td>Reverse amplifying primer</td>
<td>TCTCTCAGACGCCGTACCTGCTCTAGA*TTCGGTGCTTTGAGATTCC</td>
</tr>
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</table>

Note: *The restriction sites are underlined, Xba I cutting site is “TCTAGA”.*

**Table S3.** Primers for the DNA sequences amplification of A_{129K30} \rightarrow A_{129K30} and A-A

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-AA-BamH</td>
<td>Forward amplifying primer</td>
<td>CGCTCGGGATCC*GCCAGCCGGCTCCT</td>
</tr>
<tr>
<td>D-AA-Sal</td>
<td>Reverse amplifying primer</td>
<td>GTGGGCGTCGAC*CTAAGGCTCAGGCT</td>
</tr>
</tbody>
</table>

Note: *The restriction sites are underlined, BamH I cutting site is “GGATCC”, Sal I cutting site is “GTCGAC”.*