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Tryptophan 375 stabilizes the outer-domain core of gp120 for HIV vaccine immunogen design

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ABSTRACT

The outer-domain core of gp120 may serve as a better HIV vaccine immunogen than the full-length gp120 because of its greater stability and immunogenicity. In our previous report, we introduced two disulfide bonds to the outer-domain core of gp120 to fix its conformation into a CD4-bound state, which resulted in a significant increase in its immunogenicity when compared to the wild-type outer-domain core. In this report, to further improve the immunogenicity of the outer-domain core based immunogen, we have introduced a Tryptophan residue at gp120 amino acid sequence position 375 (375S/W). Our data from immunized guinea pigs indeed shows a striking increase in the immune response due to this stabilized core outer-domain. Therefore, we conclude that the addition of 375W to the outer-domain core of gp120 further stabilizes the structure of immunogen and increases the immunogenicity.

1. Introduction

The CD4-binding site (CD4-BS) of gp120 in HIV-1 has been recognized to be the most vulnerable site for antibody targeting and viral neutralization [1–8]. Recently, identification of several potent neutralizing antibodies through the screening of HIV-1 patient sera, such as VRC01 [9] and PGV04 [5], have been known to be elicited by the CD4-BS of gp120. Further analysis of these antibodies has found that the binding sites for some CD4-BS antibodies primarily depend on the outer domain of gp120, which are unlike CD4 binding wherein the binding requires all the three domains: outer domain, inner domain and the bridging sheet [10]. It is known that the outer domain is relatively more stable than the inner domain, and the outer-domain core structure, which does not include the V3 loop, is even more stable [11,12]. An optimized outer domain from a HIV-1 subtype A strain can bind to the CD4-BS antibodies VRC01 and PG04. The outer domain structure in complex with PG04 has been also solved [13]. The outer domain used as an immunogen has been tested for its immunogenicity previously, and it shows a comparable immune response to gp120, but a weaker response than the gp140 envelope trimer. However, it is an un-stabilized wildtype outer domain in which the V3-loop and the glycan site (antibody 2G12) were focused, not the CD4-BS [14]. A membrane anchored-outer domain is recognized by antibody b12 and is also found to have elicited CD4-BS antibodies [15]. All of these previous studies are based on the wildtype outer-domains, but the outcomes have provided the feasibility for developing outer-domain based immunogens.

CD4 binding to gp120 causes a large scale conformational change of gp120 which involves the gp120 core structure and the transitions of inner-domain layers (layer 1, 2 and 3), but also involves the movement of the major loops (V1, V2 and V3) [16–19]. For instance, the V2-loop joins the bridging sheet formation with the C4 region β20-β21 hairpin of the outer-domain, and the V3-loop opens up to contact the coreceptor, CCR5 or CXCR4 [17,18,20]. A tryptophan residue at amino acid position 375 (375W) of gp120 will fill the Phenylaniane-43 (Phe-43) cavity and stabilizes the gp120 into a CD4-bound conformation, but does not completely achieve the CD4-bound state [21–23]. More stabilization changes such as the addition of disulfides, salt-bridges added to the S375W conformation of the gp120 core structure have enhanced the stability and increased the immunogenicity [22,24]. However, in respect to the outer-domain core structure only, we

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do not know whether the 375W can play a role in further improving the stability of the CD4-bound state. Here we have conducted the research into an outer-domain based HIV immunogen design. The antigenic nature of the CD4-BS has been recognized as conformation dependent because it is known to be located at the junction of three domains. Interestingly, the binding sites of some CD4-BS antibodies such as VRC01 or PGV04 are actually located on the outer domain, and the binding is almost independent from the inner domain and the bridging sheet. Since the outer-domain of gp120 is a relatively stable domain, it has become an ideal antibody target for vaccine immunogen design.

In our previous study, a stabilized outer-domain, through the introduction of two-disulfide bonds, was significantly more immunogenic than the wild-type outer domain. To further improve this outer-domain based immunogen, we have introduced a Tryptophan (W) mutation at the position 375, which is known to be the Phe-43 cavity filling residue in the CD4-BS. We expect that the 375W will further stabilize the outer-domain into the CD4-bound conformation in addition to the two-disulfide bond stabilization. Thus, it will serve as a better immunogen to induce the production of CD4-BS neutralizing antibodies.

2. Materials and methods

2.1. Outer domain OD3 mutagenesis

The outer domain mutant 3 (OD3) of HIV-1 gp120 from subtype C strain 1084i essentially was designed based on the outer domain mutant 2 (OD2) structure as described previously [25]. The S375W mutation was introduced by Site-directed mutagenesis using the mutagenesis kit from Agilent. The sequences of the mutations were confirmed by DNA sequencing. The models of the OD1, OD2 and OD3 structures were predicted based on the gp120-VRC01 structure complex (PDB: 3NGB) from an HIV-1 subtype B strain, and conducted using Discovery Studio Client 4.0 (Biovia, San Diego, CA). The protein sequence numbering of OD1, OD2 and OD3 is based on the envelope sequence of the HIV-1 prototypic HXBc2 strain [26].

2.2. Cloning and expression of OD1, OD2 and OD3

The molecular clones of OD1 and OD2 were made during previous work [25], and the OD3 mutant was generated by mutagenesis in the vector pET28b (Novagen). To express the recombinant proteins in *E. coli BL21 (DE3) pLysS cells* (Invitrogen), the pET28b-OD1, OD2 or OD3 plasmids were transformed into the cells and a single colony was grown overnight in LB media supplemented with kanamycin (50 mg/L) at 37 °C until the optical density (OD) reached between 0.4 and 0.6 at 600 nm. The cells were then induced by addition of isopropyl-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and were further incubated for 4 h at 37 °C. The cells were then harvested by centrifugation at 5000*g* for 10 min, washed once with STE buffer (100 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5) and finally resuspended in STE buffer containing 0.5% NP40, lysozyme (100 mg/L) and supplemented with protease inhibitors. After incubation for 30 min at 4 °C, the resuspended cells were disrupted by sonication and the lysate was centrifuged (12,000*g*, 10 min, 4 °C) to separate the insolubilized fraction. Both the supernatant and the insolubilized pellet fraction were tested for the presence of recombinant proteins by running SDS-PAGE followed by Coomassie blue staining. The majority of the expressed protein was detected in the insolubilized fraction of inclusion bodies. The insoluble fraction was further purified by sequentially washing with 1x PBS containing 2 M urea, 0.1% SDS, 1% NP-40 and 1% TritonX-100, and was solubilized in solubilization buffer (50 mM NaCl, 25 mM Tris (pH-8.0), 2 mM EDTA, 8 M Urea and 5 mM DTT). The protein was finally renatured by successive dialysis against refolding buffer (50 mM Tris, pH 8.0, 400 mM L-Arginine, 2 mM EDTA, 10 mM DTT and 10% glycerol) containing 4, 2, 1 and 0 M Urea at 4 °C. Ni-NTA resin beads (Thermo Scientific) were used to further purify the protein. The concentrations of the protein samples were determined by a BCA protein kit (Pierce). The protein samples were finally evaluated by Coomassie blue staining and Western blotting.

2.3. Western blot assay

Purified protein samples were loaded onto a 10% SDS-denaturing gel along with negative (bovine serum albumin (BSA)) or positive (recombinant gp120) controls. The gels were run for 75 min at 60 V. Nitrocellulose membrane transfer followed SDS-PAGE. After 30 min of blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS), nitrocellulose membranes were incubated for one hour at room temperature with primary antibodies. Following six washing steps (five minutes each) with PBS + 0.1% Tween-20, horseradish peroxidase (HRP) conjugated secondary antibodies were incubated with membranes for 30 min at room temperature. Six washes were followed as previously described. The outcome of antigen-antibody binding was detected either using SuperSignal West Dura Extended Duration Substrate kit (Pierce) or using a BioRad ChemiDoc.

2.4. Circular Dichroism (CD)

A desalting column (zeba™ Spin Desalting Columns, 7 K MWCO, 5 mL) was used to remove L-arginine from the protein solution to avoid noisy signals. A Jasco J-815 CD spectrophotometer equipped with Peltier-type thermostat in the cuvette holder and a water circulator was used to collect the data. The acquisition and data analysis software are also from Jasco. Data were recorded using a 0.01 cm pathlength, 1500 μL circular quartz cuvette at 20 °C. The data were exported in XY asci format and analyzed with the program CD-Pro located at the Dichro Web-site from the University of London, for the purpose of estimation of the secondary structure of the protein. The data were converted to molar ellipticity (deg·cm2·dmol-1) from raw machine units (millidegrees) with the same resolution as the acquired data (1 data point per nanometer). The cell path, average molecular weight and protein concentration were provided prior to the fitting, which was carried out using the various models available (CONTINLL and SELCON3 yielded the lowest RMSD values) until a satisfactory fit was obtained. The results were imported into Sigmaplot (SPSS), which was used to plot the data along with the simulated secondary structure spectrum. The three protein samples were measured at the same concentration of 0.35 μg/ml, solubilized in buffer containing 20mmTris-HCl and 2 mm EDTA at pH 8.0, and the recording path-length is 0.01 cm.

2.5. Guinea pig immunizations

The standard 63-Day immunization protocol of Cocalico Biologicals, Inc. (CBI) for guinea pigs was followed. 16 guinea pigs were assigned evenly into 4 groups: inoculated with OD1, OD2, OD3 or adjuvant only (Complete Freund's Adjuvant for initial inoculation, but Incomplete Freund's Adjuvant for boosters). Three booster inoculations by Subcutaneous (s.c.) injections were carried out following the first injection. Blood samples were collected from all guinea pigs on Day 0, prior to immunization as pre-bleed samples; on Day 35 as test bleed samples after 2 boosts on Day 14 and Day 21, respectively; and on Day 63 as exsanguination samples following the third boost on Day 49. Blood samples collected (pre-bleeds, test bleeds and final bleeds) were used in antibody testing, ELISA and neutralization assays. All animal experiments were performed