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The potential molecular targets of marine sulfated polymannuroguluronate interfering with HIV-1 entry Interaction between SPMG and HIV-1 rgp120 and CD4 molecule

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Abstract

The potential targets of marine sulfated polymannuroguluronate (SPMG) involved in inhibition of HIV-1 entry were investigated by surface plasmon resonance and flow cytometry. Results indicated that binding of SPMG either to soluble oligomeric rgp120 or to complexed rgp120–sCD4 mainly resided in V3 loop region. In addition, SPMG was shown to be less accessible for sCD4 when sCD4 had pre-interacted with rgp120, though SPMG per se multivalently bound to sCD4 with relatively low affinity. While the pre-incubation of SPMG with rgp120 caused a partial blockade of rgp120 binding to sCD4, suggesting that SPMG either shared common binding sites on gp120 with sCD4 or masked the docking sites of gp120 for sCD4. Taken together, V3 domain was demonstrated to be the major site mediating interaction of SPMG with complexed rgp120–sCD4. It seems likely that SPMG binds to both rgp120 and sCD4, but has less accessibility for sCD4 when sCD4 has already bound to rgp120. Nevertheless, addition of SPMG either prior to or after the interaction of rgp120 with sCD4 may suppress rgp120 binding to sCD4. The exact pattern of this trimolecular complex formation at the cell membrane-anchored virus level requires further clarification.

Keywords: Sulfated polymannuroguluronate; rgp120; V3 loop; sCD4; Surface plasmon resonance; Flow cytometry

1. Introduction

Although the HIV-1 entry process is one of the earliest targets examined for therapeutic intervention, it has still not led to any real drugs used clinically. However, the elucidation of the crystal structure of the HIV-1 envelop glycoprotein gp120, together with understanding of co-receptors that facilitate crucial new insights into the mechanisms of HIV-1 entry have afforded significantly new opportunities for HIV-1 drug discovery (Watson et al., 1999; Blair et al., 2000).

Of the potential HIV-1 entry inhibitors, sulfated polysaccharides have since long been most intensively studied. It has been suggested that the polyanonic polysaccharides exert their anti-HIV activity by binding to the viral glycoprotein gp120 or to the cellular CD4 receptor (Baba et al., 1988; Witvrouw et al., 1994; Roderiquez et al., 1995; Nakashima et al., 1995; Nishimura et al., 1998).

Marine sulfated polymannoroguluronate (SPMG), a new kind of sulfated polysaccharide extracted from *brown algae* and fractioned by enzymatic hydrolysis, bears a unique ratio of 1,4-linked β-D-mannuronic acid over α-L-guluronic acid blocks with implanted sulfate groups. Our previous work has demonstrated a significant inhibitory effect of SPMG on the replication of HIV and SIV both in vitro and in vivo. This anti-HIV action has been attributed to the inhibitory effect of SPMG on the process of HIV entry (Xin et al., 2000). Yet the detailed mode of action for understanding the involvement of SPMG in HIV-1 entry at the molecular level has not been elucidated.

In the present study, the influence of SPMG on HIV entry based on the exploration of its interaction with rgp120

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and/or sCD4 was investigated by surface plasmon resonance analysis and flow cytometric assay.

2. Materials and methods

2.1. Materials

SPMG (a new kind of sulfated polysaccharide extracted from *brown algae*, depolymerized by enzymatic hydrolysis, followed by sulfation) with an average molecular weight of 8 kDa, was provided by Marine Drug and Food Institute, Ocean University of China, Qingdao, PR China.

Recombinant gp120 (rgp120, strain MN) was purchased from ViroStat, Portland, ME. Soluble CD4 molecule (Human soluble CD4, sCD4) was provided by R&D System, Inc, Minneapolis, MN. Benzylated CD4-derived synthetic peptide (amino acids 81–92) was purchased from Sigma, St. Louis, MO.

V3 loop (28 amino acid sequence) and V3 conserved motif (6 amino acid sequence) were all synthesized by GL Biochemical Company, Shanghai, PR China. The V3 loop region of 28 amino acids was synthesized, with the following sequence: *(H_2N)-Asn-Tyr-Asn-Lys-Arg-Lys-Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Thr-Lys-Asn-Ile-Ile-Gly-Thr-Ile-Arg-Gln-(COOH).* The conserved V3 motif (six amino acids) was synthesized, with the following sequence: *(H_2N)-Gly-Pro-Gly-Arg-Ala-Phe-(COOH).*

CM5 biosensor chip was purchased from Amersham Pharmacia, Biacore AB, Uppsala, Sweden. Sulfo-NHS-biotin and streptavidin were provided by Sigma. Other chemical agents used were all purchased from Sigma.

2.2. Surface plasmon resonance assays (optical biosensor interaction analysis evaluation)

The surface plasmon resonance assays were conducted on a SPR biosensor instrument (BIAcore® X, Amersham Pharmacia). In this assay, a polysaccharide ligand (SPMG) was biotinylated and then immobilized onto the surface of a carboxymethylated dextran sensor chip (CM5) via streptavidin–biotin coupling (Li et al., 2003). SPMG was first biotinylated and the process is described as follows: 4.0 mg of SPMG was dissolved in 250 μl of 0.4 M 1,6-hexanediamine in aqueous glacial acetic acid (10%). Then, 250 μl of 2 M aqueous sodium cyanoborohydride was added and incubated at 37 °C for 8 h. The obtained SPMG-hexanediamine was further separated by a Microcon YM-3 (Millipore) and then followed by incubating with a three-fold molar excess of sulfo-NHS-biotin (Sigma) in 100 mM sodium bicarbonate buffer at pH 8.5 for 1 h at room temperature, and finally to yield biotinylated SPMG. Excess biotin was then removed by centrifugation via Microcon YM-3 (Millipore). Then, the immobilization of biotinylated SPMG onto CM5 sensor chip surface was carried out according to BIApplications Handbook (Biacore AB). Briefly, the biotinylated SPMG was immobilized onto one of the flow cell (FC1) of CM5 sensor chip surface. The immobilization procedure was carried out at 25 °C and at a constant flow rate of 5 μl/min HBS–EP buffer composed of 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v). To assess real-time binding of the interactants, 35 μl of each tested sample, including rgp120 or V3 peptide or V3 conserved motif or sCD4 molecule or benzylated CD4-derived peptide or concomitant existence of any molecules dissolved in HBS–EP, was injected over the sensor chip surface with SPMG immobilized, followed by a 5-min wash with HBS–EP buffer. The sensor chip surface was then regenerated by washing with 60 μl of 2 M NaCl. All binding experiments were carried out at 25 °C with a constant flow rate of 5 μl/min HBS–EP buffer. To correct for non-specific binding and bulk refractive index change, a blank channel without SPMG was used and run simultaneously for each experiment. The sensorgram for all binding interactions were recorded in real time and were analyzed after subtracting the sensorgram from the blank channel (FC2). Then, the changes in mass due to the binding response were recorded as resonance units.

2.3. Preparation of fluorescein isothiocyanate (FITC)-labeled rgp120

Twenty microliters of 7.33 μM rgp120 was mixed with 20 μl of 257.0 μM FITC in aqueous Na_2HPO_4 (0.1 M). The reaction mixture was incubated at 4 °C for 12 h. Excess FITC was removed by centrifugation via Microcon YM-10 (Millipore) according to the manufacturer's instructions, and PBS as a washing reagent.

2.4. Flow cytometry assay

Heparinized peripheral blood samples from healthy volunteers were obtained and evaluated by a whole blood lysing technique. Briefly, 100 μl peripheral blood was exposed to anti-CD3-cychrom mAb, anti-CD8-PE mAb, and rgp120–FITC, together with the simultaneous addition of SPMG at the given concentrations. After incubation for 30 min, the samples were lysed with BD lysing solution and fixed with 4% polyformaldehyde, and then washed three times with PBS buffer, and analyzed by three-color analysis using a Vantage flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Data were acquired with Cellquest II software by using fluorescence triggering in the FL2/FL3 channel to gate on $CD3^+CD8^-$ lymphocyte populations ($CD4^+$ cell). The 10000 $CD4^+$ T lymphocytes were gated, and then the mean fluorescence intensity reflecting rgp120–FITC binding to CD4 receptors on T lymphocytes (FL1 channel) was measured.

3. Results

3.1. Inhibition of rgp120 binding to CD4$^+$ T cells by SPMG with flow cytometric analysis

CD4 is generally considered to be the primary receptor for the human immunodeficiency virus type 1 (HIV-1) envelop glycoprotein 120 (gp120), the interaction of which plays a crucial role in the initiation of the entry process of HIV-1 into CD4$^+$ T cells. Large evidence has highlighted the involvement of sulfated polysaccharides such as heparin or the like in interacting with either gp120 or CD4 receptor and thus resulting in HIV-1 entry inhibition (Baba et al., 1988; Witvrouw et al., 1994; Roderiquez et al., 1995; Nakashima et al., 1995). Whether SPMG interfered with rgp120 binding to CD4$^+$ T cells was investigated by flow cytometry. Results indicated that SPMG at concentrations of 1, 10, and 100 μg/ml resulted in a dramatic inhibition in rgp120 binding to CD4$^+$ T lymphocytes in a concentration-dependent manner, with SPMG at a concentration of 100 μg/ml reaching the maximal effect (Fig. 1).

3.2. Inhibition of rgp120 binding to sCD4 by SPMG with surface plasmon resonance assay

The surface plasmon resonance assay (SPR assay) based on optical biosensor was adapted to provide a means of looking at the impact of SPMG on the interaction of rgp120 with sCD4 molecule as it occurred in real time. Thus, the kinetics of the interaction, in addition to the affinity, could be measured. Alterations in the relationship between SPMG and rgp120 and sCD4 thus could be easily seen as changes in association and dissociation of the interactant molecules.

Soluble CD4 was immobilized on the CM5 sensor chip surface, and rgp120 (at 50 μg/ml) or SPMG (at 50 μg/ml) or both rgp120 and SPMG (both at a concentration of 50 μg/ml) were injected over the sensor chip surface. Results indicated that rgp120 intensively bound to sCD4 molecule, and this was partially blocked by the concomitant presence of SPMG (Fig. 2). This inhibition may be explained by the fact that SPMG might hide the binding site of sCD4 on rgp120 by steric hindrance, or SPMG may competitively occupy the same binding sites of rgp120 with sCD4, or vice versa. However, the above findings failed to explain whether SPMG bound to rgp120 alone or sCD4 alone or bound to both rgp120 and sCD4 simultaneously.

3.3. Binding of SPMG to rgp120 in monomeric form by SPR assay

With SPMG immobilized on the sensor chip surface, rgp120 at concentrations of 20, 41, 83, 167, or 334 nM was injected over the sensor chip surface. In this situation rgp120 was in its monomeric form rather than its oliogomeric complex state on cell surface. Results indicated that binding of SPMG to rgp120 was increased in a concentration-dependent fashion, revealing a relatively

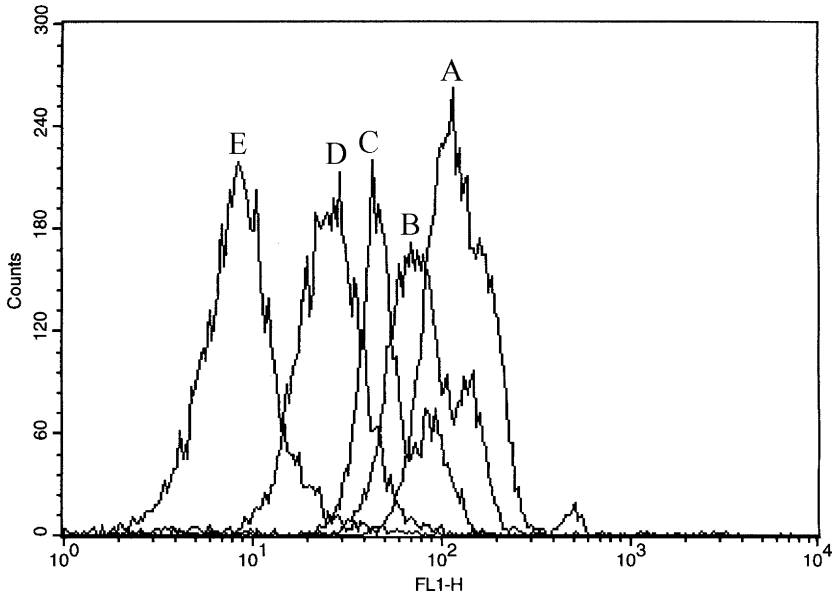

Fig. 1. The inhibitory effect of SPMG on rgp120 binding to CD4$^+$ T cells by flow cytometry. Peripheral blood (100 μl) from healthy volunteers was exposed to anti-CD3 mAb, anti-CD8-PE mAb, and rgp120–FITC, together with simultaneous addition of SPMG at concentrations of 1, 10, and 100 μg/ml, respectively. Data were acquired with Cellquest II software by using fluorescence triggering in the FL2/FL3 channel to gate on CD3$^+$CD8$^-$ lymphocyte populations (CD4$^+$ cell). The 10000 CD4$^+$ T lymphocytes were gated, and then the mean fluorescence intensity reflecting rgp120–FITC binding to CD4 receptor (FL1 channel) on T lymphocytes was measured. Here, A stands for rgp120-treated alone group, whereas E stands for PBS-treated alone group. B stands for SPMG-treated group at a concentration of 1 μg/ml, while C stands for SPMG-treated group at a concentration of 10 μg/ml, and D stands for SPMG-treated group at a concentration of 100 μg/ml.

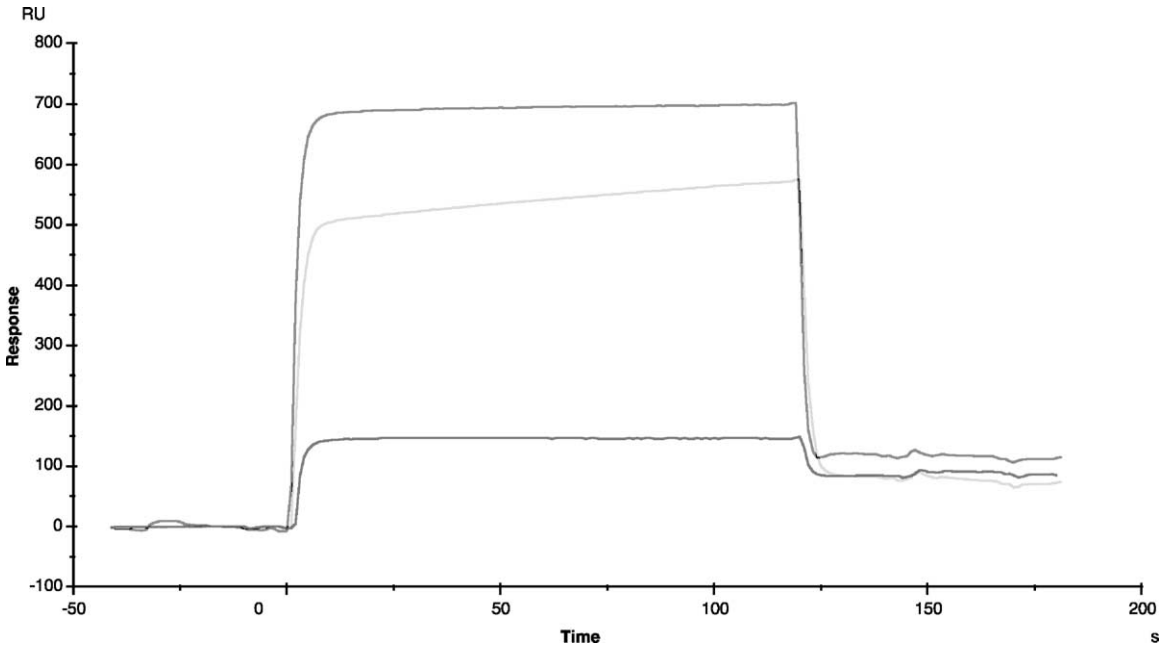

Fig. 2. Interaction of sCD4 with rgp120 or SPMG, or both rgp120 and SPMG, by surface plasmon resonance assay. sCD4 was immobilized onto the CM5 sensor chip surface. SPMG, rgp120 or both rgp120 and SPMG flowed over the sensor chip surface. The sensorgrams from the bottom to the top were SPMG (50 μg/ml), rgp120 (50 μg/ml) + SPMG (50 μg/ml), or rgp120 (50 μg/ml) alone, respectively. *X*-axis stands for the flow time, while *Y*-axis stands for the binding resonance unit. The experiment was carried out at 25 °C with a constant flow rate of 5 μl/min HBS–EP buffer.

high affinity with a K_D equivalent to 3.50E−08M (35 nM, Table 1).

3.4. Binding of SPMG to V3 molecule alone or in its concomitant presence with monomeric rgp120 by SPR assay

It has long been argued that sulfated polysaccharides exert their anti-HIV by a specific interaction with the V3 region of gp120 (Dowd et al., 2001; Stantchev and Broder, 2001). Thus, it was hypothesized that the interaction of SPMG with rgp120 (in monomeric form) as evaluated by SPR might be attributed to the binding of SPMG to V3 loop region of rgp120. With SPMG immobilized on the biosensor chip surface, a synthetic V3 fragment (a 28 amino acid peptide) at concentrations of 3.76, 7.5, 15, 30, and 60 nM was injected over the biosensor surface accordingly. Results showed that SPMG bound to V3 peptide with a K_D equivalent to 1.38E−09M (1.38 nM, Table 1). In addition, a synthetic conserved motif V3 of a six amino acid *(NH_2)-Gly-Pro-Gly-Arg-Ala-Phe-(COOH)* was introduced to the above reaction and resulted in a significant blockade of the binding of SPMG to the V3 loop, whereas the binding of SPMG to rgp120 in a monomeric form was not influenced by the addition of this V3 conserved motif even at concentration of 125-fold higher compared to rgp120 (data not shown).

The difference in avidity between binding of SPMG to rgp120 in monomeric form and that of SPMG to V3 peptide, especially along with the finding that the V3 motif failed to influence SPMG binding to monomeric rgp120 (data not shown), suggested, at least in part, that SPMG might bind to monomeric rgp120 via sites other than the V3 domain. This raises the possibility that SPMG binding sites on monomeric rgp120 might not overlap with those on oligomeric rgp120.

Table 1
The kinetic parameters of the interactions between SPMG and rgp120, or SPMG and V3, or SPMG and sCD4, or SPMG and rgp120–sCD4 complex

Ligand–analyte	k_a (1/M s)	k_d (1/s)	K_A (1/M)	K_D (M)
SPMG–rgp120	8.60E+04	3.51E−03	2.45E+07	3.50E−08
SPMG–V3	2.08E+05	1.70E−03	1.22E+08	1.38E−09
SPMG–sCD4	3.30E+04	5.59E−03	5.91E+06	3.79E−07
SPMG–rgp120–sCD4	2.17E+05	7.23E−03	3.00E+07	3.09E−08

3.5. Binding of SPMG to rgp120 in its oligomeric form by SPR assay

In fact, rgp120 in solution is present in its native monomeric form, which might fail to expose the V3 loop region. Actually, steric structure of the envelop appears important for the exposure of the V3 loop region, with oligomerization as a possible key requirement (Roderiquez et al., 1995; Seddiki et al., 1997; Stanfield et al., 1999). It has become increasingly clear, however, that monomeric rgp120 is often a poor mimic of the membrane-anchored oligomeric counterpart. Yet, it has been confirmed that the soluble oligomerization of rgp120 induced by the presence of 10 mM $CaCl_2$ constitutes a better model than monomeric

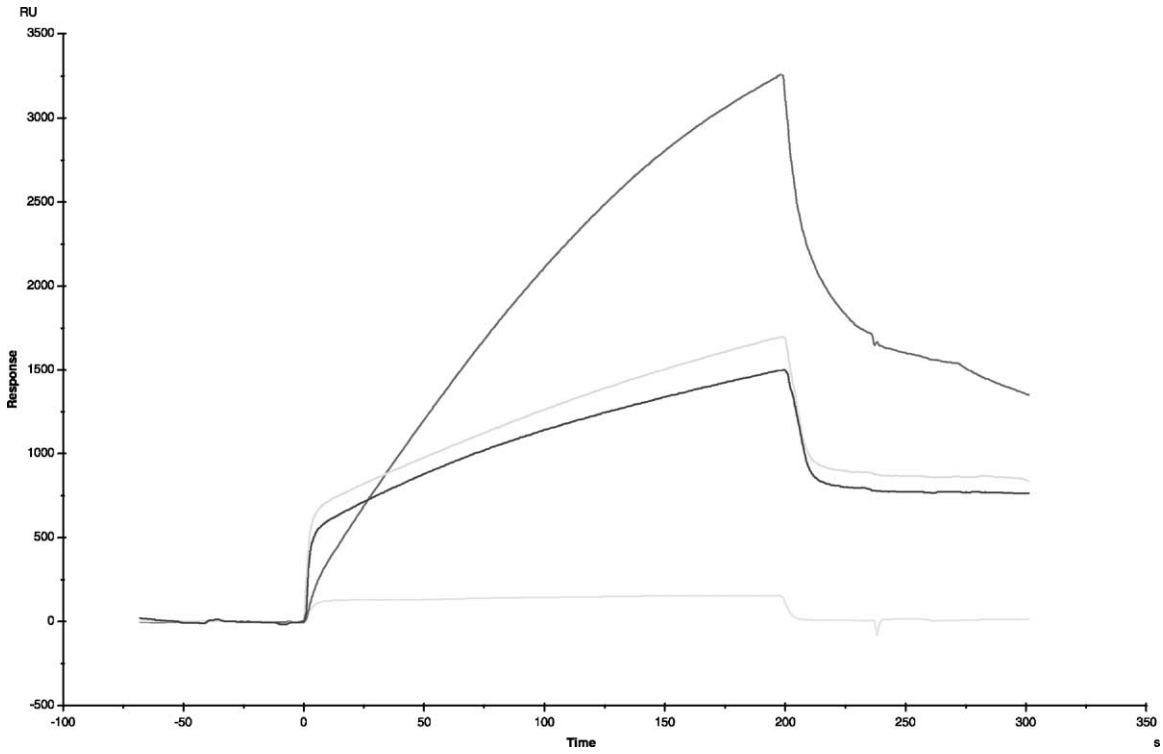

Fig. 3. Effect of V3 conserved motif on the interaction of oligomeric rgp120 with SPMG. The biotinylated SPMG was immobilized onto streptavidin-CM5 sensor chip surface. Conserved motif V3 (10 μM), oligomeric rgp120 (83 nM) with or without conserved motif V3 or monomeric rgp120 were injected over the sensor chip surface, respectively. The sensorgrams from the bottom to the top were V3 conserved motif (100 μM), oligomeric rgp120 (83 nM) and V3 conserved motif (10 μM), oligomeric rgp120 (83 nM) alone, and monomeric rgp120 (83 nM) alone, respectively. *X*-axis stands for the flow time, while *Y*-axis stands for the binding resonance unit. The experiment was carried out at 25 °C with a constant flow rate of 5 μl/min HBS–EP buffer.

molecule for studying gp120 ligand binding properties on the virion surface (Seddiki et al., 1997). Thus, in the present study, we further assessed the binding of SPMG to soluble oligomeric rgp120 induced by 10 mM $CaCl_2$. With SPMG immobilized, soluble oligomeric rgp120 in 10 mM $CaCl_2$ and monomeric rgp120 in buffer solution were injected over the sensor chip surface. Result demonstrated that the binding affinity of SPMG to oligomeric rgp120 was as much as 10-fold lower than that to monomeric rgp120 in buffer solution (data not shown). This binding response triggered by the interaction of oligomeric rgp120 with SPMG was partially blocked by the addition of a six amino acid V3 motif, the same concentration of which failed to neutralize the binding of SPMG to monomeric rgp120 (Fig. 3). These findings might favor the explanation that V3 domain was the major binding domain modulating the interaction of SPMG and oligomeric rgp120.

3.6. Binding of SPMG to sCD4 molecule by SPR assay

An increasing amount of evidence has highlighted the impact of sulfated polysaccharides on the entry of HIV-1 by interacting with CD4 molecule (Roderiquez et al., 1995; Watson et al., 1999; Ugolini et al., 1999). Ample evidence has stated that the positively charged amino acid side chains within the CD4 molecule might serve as an electrostatic docking site for the negatively charged sulfated polysaccharides (Szekely et al., 1998). This prompted us to consider whether it was true for SPMG to bind to the CD4 molecule rather than rgp120. In our experiment, also with SPMG being immobilized, sCD4 molecule at given concentrations was flowed over the biosensor chip surface. Data revealed a marked binding of SPMG to sCD4 molecule in a concentration-dependent manner with a K_D equivalent to 3.79E−07M (379.0 nM), implicating a much lower affinity of SPMG for sCD4 as compared to rgp120 molecule (Table 1). In addition, the stoichiometry revealed that one molecule of SPMG bound to nearly six molecules of sCD4 molecules, indicative of its multivalent binding fashion.

3.7. Interaction of rgp120–sCD4 complex with SPMG by SPR assay

The above findings prompted us to hypothesize that SPMG could bind to both rgp120 and sCD4. To clarify this assumption, with SPMG immobilized on the sensor chip surface, the concomitant existence of rgp120 and sCD4 was flowed over the desired surface. The SPR biosensor experiment showed that SPMG significantly bound to the rgp120–sCD4 complex with a K_D equivalent to 3.09E−08M (Table 1), the affinity of which was much lower than the affinity of rgp120 for sCD4 (K_D=4.0E−09M). This

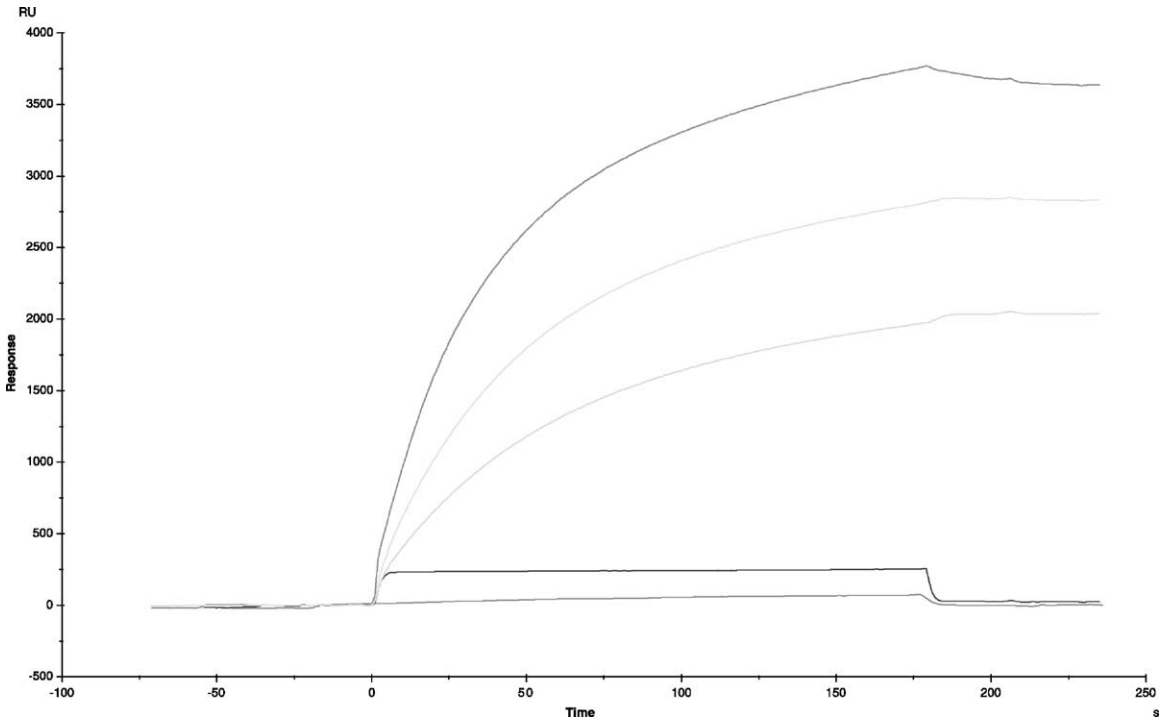

Fig. 4. Effect of benzylated CD4-derived peptide or V3 conserved motif on the interaction of rgp120–sCD4 with SPMG. The biotinylated SPMG was immobilized onto the streptavidin-CM5 sensor chip surface. Different samples were injected over the SPMG-immobilized sensor chip surface. The sensorgrams from the bottom to the top were benzylated CD4-derived peptide alone (16 μM), V3 conserved motif alone (100 μM), rgp120–sCD4 (83 nM) together with the V3 conserved motif, rgp120–sCD4 (83 nM) together with the benzylated CD4-derived peptide domain, and rgp120–sCD4 (83 nM) alone, respectively. *X*-axis stands for the flow time, while *Y*-axis stands for the binding resonance unit. The experiment was carried out at 25 °C with a constant flow rate of 5 μl/min HBS–EP buffer.

observation raised another concept that rgp120 was more often than sCD4 in an accessible state to bind to SPMG. It seems reasonable to assume that SPMG binding sites on sCD4 may be partially occluded by the interaction of rgp120 with sCD4 due to conformational change or steric hindrance. In addition, a benzylated CD4-derived synthetic peptide (amino acid 81–92; CDR3-like domain), which has been demonstrated to block interaction of CD4 with gp120 via V3 region (Batinic and Robey, 1992), was added to the complex of SPMG and rgp120–sCD4. Benzylated CD4 peptide (amino acids 81–92), at a given concentration, partially abolished the binding of SPMG to rgp120–sCD4 complex (Fig. 4). Thus, this partial neutralization benefited from the fact that benzylated CD4 competitively bound to the exposed V3 and thus suppressed the association of rgp120 with SPMG, which was in agreement with our findings that the conserved V3 motif counteracted the interaction of this trimolecular complex to the same degree (Fig. 4).

4. Discussion

The successful elucidation of the crystal structure of gp120–CD4-17b complex provided a possible explanation for the understanding of the mechanism of the gp120–CD4 binding induced HIV-1 entry process. In fact, binding of gp120 to CD4 caused a conformational change within gp120, resulting in the exposure of the V3 loop to bind to a chemokine receptor, another host cell receptor co-localized with CD4. This co-receptor binding to V3 loop induces further conformational change and thus leads to uncover gp41, which participates in the fusion between virus and cells (Blair et al., 2000; Kwong et al., 1998, 2000). The exposed location of gp120 glycoprotein on the virus, which is required for receptor binding, renders the protein potentially vulnerable to either neutralizing antibodies or some inhibitors.

Previous studies have demonstrated that SPMG exerts its anti-HIV-1 activity by interfering with the viral entry process. Thus, the identification of the potential protein targets for SPMG with regards to the inhibition of HIV-1 entry was investigated by using surface plasmon resonance determination and flow cytometric analysis. The results showed that the binding of SPMG either to oligomeric rgp120 or to rgp120–sCD4 complex were mainly dependent on the V3 loop region, whereas SPMG bound to rgp120 in its monomeric form via other sites rather than via the V3 domain.

It has been postulated that the disruption of ionic interactions between charged regions of the viral gp120 and the cell membrane accounts for the anti-HIV action of sulfated polysaccharides. The V3 loop of gp120, an exposed

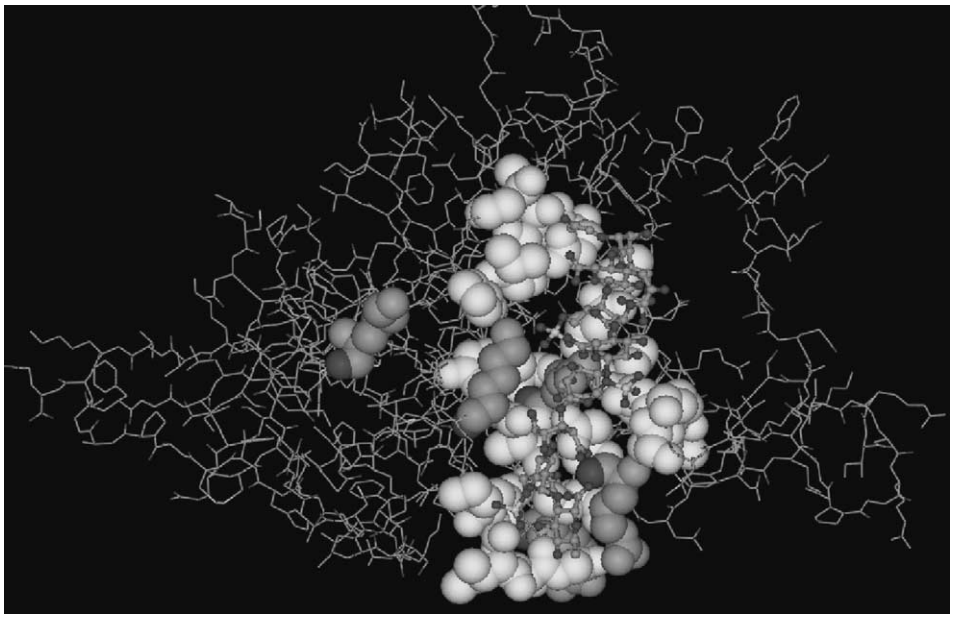

Fig. 5. Computer docking modeling of the V3 loop of gp120 with the octasaccharide unit of SPMG backbone. The binding was mainly due to the electrostatic force.

region on virus, with highly positively charged density, is targeted by negatively charged polysaccharides (Witvrouw et al., 1994; Stanfield et al., 1999). This notion, at least in part, gave us a better insight into the mechanisms underlying the impact of SPMG on HIV-1 entry. Actually, the surface plasmon resonance analysis revealed binding of SPMG to V3 peptide of 28 amino acids with high affinity ($K_D = 1.38$ nM) in a multivalent manner, characterized by one molecule of SPMG binding to three to four molecules of the V3 loop region. This finding was further supported by our computer docking data that the octasaccharide backbone of SPMG just covered the V3 loop, with tetra- or pentasaccharides most tightly bound to residues of the V3 loop (Fig. 5). Cumulative evidence has highlighted that exposure of V3 region requires appropriate conformational change of envelope gp120, with oligomerization being most critical.

Significantly, oligomerization showed a greater dependence on exposure of V3 region than the monomeric state of gp120 (Sattentau and Moore, 1995; Sullivan et al., 1995; Ugolini et al., 1999). In fact, there exists a high degree of conservation of the *Gly-Pro-Gly-Arg/Gln* motif on the top of the V3 loop, the conserved structure being in association with its biological functions, although the V3 domain is a disulfide-linked loop of approximately 40 amino acids with a high degree of sequence diversity among different viral isolates (Stanfield et al., 1999). Therefore, in our experimental condition, a synthetic conserved V3 motif with the six amino acid sequencing *(NH_2)-Gly-Pro-Gly-Arg-Ala-Phe-(COOH)* was thus introduced in order to obtain further insight. The observation that V3 conserved motif partially suppressed the binding of SPMG to the oligomeric form of rgp120 induced by 10 mM $CaCl_2$, in line with the finding that the V3 motif markedly blocked V3 loop binding to SPMG, suggested that the oligomeric rgp120 favored the interaction of SPMG with the exposed V3 loop domain (Seddiki et al., 1997). Additionally, the blockade of the association of SPMG with rgp120–sCD4 complex by the conserved V3 motif further emphasized the importance of V3 loop region in modulating the interaction of SPMG with the rgp120–sCD4 complex. Moreover, the partial suppression on the interaction between SPMG and rgp120–sCD4 by benzylated CD4-derived peptide added another support to the foregoing conclusion, since this benzylated CD4-derived synthetic peptide caused a micro-precipitation in the V3 domain resulting in suppression of sulfated polysaccharide binding to rgp120 (Batinic and Robey, 1992). Taken together, these experiments favor the conclusion that the V3 loop region is the most important binding site for SPMG on an oligomeric rgp120 molecule.

Another key finding of the present work was the observation that the synthetic conserved V3 motif failed to inhibit SPMG binding to monomeric rgp120. This prompted us to consider that V3 loop might not be the desired binding site in regulating the association of SPMG with monomeric rgp120, especially when 10-fold lower avidity of SPMG for monomeric rgp120 in comparison to that of SPMG for V3 loop was taken into consideration. Alternatively, there might exist another binding site of SPMG on monomeric rgp120. In fact, efforts to map the binding sites with regard to sulfated polysaccharides on HIV-1 envelope revealed that not only the V3 loop but also the C4 region were considered to be the binding sites of sulfated polysaccharides for the enveloped gp120. Both V3 and C4 domains are positively charged regions of gp120, which is compatible with an ionic interaction between these domains

and negatively charged sulfated molecules. Portions of the V3 and C4 domains may be juxtaposed to each other in the native molecule, and therefore these regions together may constitute binding sites of positively charged sulfated polysaccharides (Callahan et al., 1991; Batinic and Robey, 1992; Moore et al., 1993). The binding of gp120 to sulfated polysaccharides varies, depending on both the type and degree of sulfation of polysaccharides and gp120 charge (Ugolini et al., 1999). All these interpretations favored our expectation that more than one binding site of SPMG on gp120 existed. Importantly, the exposure or masking of these binding sites was modulated by the molecular state of gp120 either in monomeric or oligomeric form. Therefore, we proposed that monomeric form of gp120 might prefer to open the desired binding regions rather than V3 domain for SPMG. Detailed investigations are required to further elucidate the binding sites of SPMG on rgp120 in its monomeric form.

The interaction between SPMG and sCD4 in the process of HIV-1 entry was further explored by the surface plasmon resonance analysis, which revealed that SPMG multivalently bound to sCD4 molecules with a relatively low affinity. Actually, the positively charged amino acid side chains within the sCD4 molecule were demonstrated to serve as an electrostatic docking site for the negatively charged sulfated polysaccharides (Szekely et al., 1998). Interestingly, pre-incubation of rgp120 with SPMG triggered partial suppression of rgp120 binding to sCD4. This might imply that SPMG competitively occupied the same binding sites on rgp120 as sCD4. That means that SPMG may not only bind to the V3 domain but also to other regions of rgp120. Also, we could not exclude the possibility that SPMG binding to rgp120 might mask the docking sites of rgp120 for sCD4.

Another interesting finding was that the binding affinity of SPMG for complexed rgp120–sCD4 ($K_D = 30.9$ nM) was nearly the same to that of SPMG for rgp120 alone ($K_D = 35.0$ nM), which, however, was about 10-fold lower than that of rgp120 for CD4 ($K_D = 4.0$ nM). This assumes that SPMG was less accessible for sCD4 when the sCD4 molecule was firstly captured by rgp120, while this capture yet allowed SPMG to bind to rgp120. This notion was strongly supported by the evidence that the high affinity binding of CD4 to gp120, usually in the order of 4.0 nM (Dowd et al., 2001), makes CD4 fall into a pocket of gp120 (Ugolini et al., 1999; Kwong et al., 2000), which might block the access of SPMG to sCD4 molecules.

It seems likely that SPMG bound to rgp120 and sCD4 simultaneously, but showed less accessibility for sCD4 than that for rgp120 when sCD4 was pre-combined with rgp120. Nevertheless, either addition of SPMG prior to or after the interaction of rgp120 with sCD4 may both suppress the multivalent binding of rgp120 to sCD4 receptor, endowing SPMG with both preventive and therapeutic potential. However, the exact pattern of this ternary molecular complex at the virion-bound cellular level requires our further clarification.

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