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Mechanism of Anti-HIV Activity of Succinylated Human Serum Albumin

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ABSTRACT. In the present study, we described the interaction of succinylated human serum albumin (Suc-HSA), a negatively charged anti-HIV-1 active protein, with HIV-1 gp120 and in detail with the third variable domain of gp120 (V3 loop). To this end, different assay formats were tested in which gp120- and V3-related peptides were presented in various configurations in order to investigate the effect of the conformational structure of the V3 loop on the interaction with negatively charged albumins. When gp120 presented via a lectin was used, it was observed that Suc-HSA bound to native gp120. The binding site appeared to be located at or near the thrombin digestion site (GPGRAF sequence) in the V3 loop of gp120, since the cleavage of the loop resulted in decreased binding of Suc-HSA. In addition, Suc-HSA was able to protect the V3 region of gp120 from cleaving with thrombin. In contrast, significant binding of Suc-HSA to V3 loop or gp120 peptides was not observed when both were presented in a fluid phase system, suggesting the involvement of a monovalent-low affinity binding of Suc-HSA. Using overlapping peptides delineating the whole V3 loop immobilized to CNBr–Sepharose, we noticed that the interaction of the V3 loop with Suc-HSA was predominantly induced by electrostatic interactions between positively charged linearized peptide fragments and Suc-HSA and was positively influenced by the presence of hydrophobic amino in the V3 loop fragments as well. Moreover, the highest affinity site was located at sites near the GPGRAF sequence. These observations add to the evidence, collected earlier, that Suc-HSA interferes at the level of virus entry, independent of interaction with the CD4 receptor. Since the recently discovered chemokine receptors are negatively charged, we can hypothesize that Suc-HSA is able to prevent the positively charged V3 loop from interacting with these types of receptors, thereby inhibiting virus entry. BIOCHEM PHARMACOL **57**;8:889–898, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. HIV-1; gp120 peptides; polyanion; binding studies; V3 loop peptides

In previous work from our laboratories, the binding of NCAs,§ a novel class of proteins with anti-HIV-1 activity, to the env-glycoprotein gp120 of HIV-1 was reported [1, 2]. Binding of these albumins, modified either by succinylation (Suc-HSA) or aconitylation (Aco-HSA), to the positively charged V3 loop of gp120 was observed. It was concluded that the mechanism of NCA anti-HIV-1 activity (inhibition of virus cell binding, fusion and/or assemblage of newly generated viruses [3]) may therefore partially be explained by the binding to this gp120 domain.

The entry of HIV-1 into host cells is a multistep process that, although only partially understood, is mediated largely by the gp120 of HIV-1. The first step in virus adsorption is thought to be the binding of gp120 to the CD4 receptor of the host cell. This interaction results in a conformational change that exposes the V3 loop (amino acids 296–330) in gp120 [4, 5]. Earlier studies hypothesized that the cleavage of the tip of the V3 loop (GPGRAF sequence) by proteases at the host cell surface was one of the triggering events in the fusion of virus and host cell [4, 6–8]. However, more recent studies have reported that in addition to the CD4 receptor, HIV-1 uses the chemokine receptors (CKCR-4 or CC-CKR-5) for subsequent fusion steps [9–12]. It was

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§ *Abbreviations:* NCA, negatively charged albumin; Suc-HSA, succinylated human serum albumin; Aco-HSA, aconitylated human serum albumin; V3 loop, third variable domain of gp120; SI, syncytium inducing; NSI, non-syncytium inducing env-protein, envelope protein; GNL, Galanthus nivalis lectin; NRS, normal rabbit serum; EIA, electroimmunoassay; BAL, British anti-lewisite; and CNBr, ω-chloroacetophenone bromide.

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suggested that the exposed V3 loop was able to interact with either one of these chemokine receptors. This interaction could then trigger the final conformational changes in the env-protein, which enables the fusion peptide of gp41 to insert into the target cell lipid bilayer [13, 14].

In the present study, we investigated the interaction of the NCAs with the circular V3 loop peptide and gp120 env-protein in more detail. To this end, we used different assay formats in which gp120 and the V3 loop peptide were presented in different configurations and tested the binding property of antiviral albumins. Although the role of a 'thrombin like' protease in the infection cycle of HIV-1 is still obscure, we also used thrombin as a model protease to cleave the V3 loop between Arg^{315} and Ala^{316} [4, 7] in order to investigate the influences of conformational changes on the interaction of the NCAs and the (cleaved) V3 loop and gp120, respectively.

MATERIALS AND METHODS

HIV-1 rgp120 Env-protein

To obtain HIV-1 rgp120/gp160, 120×10^6 CV-1 cells (monkey fibroblasts) were infected with 1 plaque-forming unit rVV (recombinant vaccinia virus)/cell for 48 hr (37° and 5% CO_2). rVV is a construct able to express rgp120/160 of HIV variant p160-BAL, an NSI variant of HIV-1.* After infection, the cells were centrifuged for 10 min at 2000 *g* and the pellet was suspended at 4° in 1 mL PBS/0.1% Triton X-100, containing the protease inhibitors PMSF (phenylmethylsulfonyl fluoride) (7 μg/mL) (Sigma Chemical Co.) and pepstatin A (0.3 μg/mL) (Sigma). The CV-1 cell lysate (p160-BAL) was centrifuged for 20 min at 4000 *g*. The env-containing supernatants were purified further using lentil lectin Sepharose 4B (Pharmacia). One mL lentil lectin suspension was incubated with 1.2 mL supernatant and 10.8 mL PBS/0.2% Tween 20 for 4 hr at 4°. The mixture was washed four times with PBS/0.2% Tween 20 and once with PBS (10 min at 4°). The rgp120/160 was eluted from the lentil lectin by three incubations of 30 min at 4° using 1 mL 0.1 M methyl-α-D-mannopyranoside (Sigma Chemical Co.) and concentrated to 0.5 mL using an Amicon centricon 1Q filter (Amicon Division). The fractionation of rgp120/160 was performed using a Sephacryl S-500 column (Pharmacia). Elution took place with PBS/0.1% Tween 20. Purity was confirmed using SDS-PAGE and immunoblotting (data not shown). The highly purified recombinant gp120 (HIV-1 MN-strain) expressed in Chinese hamster ovary cells was kindly provided by Genentech Inc., CA and handled likewise.

Polyclonal Anti-HSA

Rabbit polyclonal anti-HSA (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services [CLB], Amsterdam, The Netherlands, nr: RbHu 02P; M1001) was immunoaffinity-purified as follows: HSA (CLB) was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) following the instructions of the manufacturer. Ten mL of antiserum was incubated with HSA beads overnight at room temperature in PBS. Beads were washed with PBS and the bound antibody eluted with 0.2 M glycin–HCL pH 2.5. The eluate (predominantly anti-HSA-specific) was dialyzed against PBS and stored at −20° until use.

Polyclonal Antibody W1/08

Polyclonal antibody W1/08 was obtained from rabbits immunized with V3 peptide (CIHIGRGRAFYAARKI (SI phenotype)). The peptide was coupled to keyhole-limpet hemocyanium) (Calbiochem) before immunization of the rabbits. This antibody, as determined by several immunoassays,† binds predominantly to the tip of the V3 loop peptide and is capable of recognizing the V3 loop in gp120 as well. The binding of the antibody to the V3 domain is sensitive to thrombin digestion (see Results).

Peptides

The V3 loop sequences and nomenclature are shown in Table 1, with amino acid residues being designated by the standard single-letter codes. V3-Q17 (168.10) represents the whole circular V3 peptide and is indicative of a T cell tropic SI isolate. The peptide was purchased from Cambridge Research Biochemicals.

Linear overlapping peptides (ninemers) were synthesized with a Milligen 9050 peptide synthesizer according to the procedures provided by the manufacturer using Fmoc-protected activated amino acids (Millipore Corp.). Peptides were purified by liquid chromatography on Sephadex G-15 in 5% acetic acid (Pharmacia Sweden). Sephadex G-15 fractions were analyzed on a Beckman Ultrosphere reversed-phase C18 column applying a gradient of acetonitrile with 0.1% trifluoroacetic acid. Fractions with the same major compound and high purity were pooled, lyophilized, and analyzed for amino acid composition using acid hydrolysis. The amino acid composition analysis of the peptides used was in agreement with the sequence claimed (data not shown).

Synthesis of Neoglycoproteins

The lactosylated neoglycoprotein (Lact-HSA) was synthesized by reductive amination according to Schwartz *et al.* [15] during an 8-hr incubation. The mannosylated neoglycoprotein (Man-HSA) was synthesized using thiophosgene activation of the *p*-aminophenyl sugar derivative according to Kataoka and Tavassoli [16]. Both neoglycoproteins were purified and characterized as previously described [17].

* Koppelman, MHGM and Huisman H, submitted for publication.

† Laman JD and Huisman H, submitted for publication.

TABLE 1. Sequences and charges at pH 8.0 of the different V3-loop peptides

HIV peptide	Sequence	Charge (pH 8.0)*
V3 (Q17)(SI)	CTRPNNNTRKRIHIGPGRAFYTTGQIIGN.IRQAHC	+4.38
V3 (Q17 n°4)	CTRPNNNTRK	+1.68
V3 (Q17 n°6)	NNNTRKRIHK	+2.86
V3 (Q17 n°7)	NTRKRIHIGK	+2.86
V3 (Q17 n°8)	RKRIHIGPGK	+2.91
V3 (Q17 n°9)	RIHIGPGRAK	+1.91
V3 (Q17 n°10)	HIGPGRAFYK	+0.93
V3 (Q17 n°11)	GPGRAFYTTK	+0.97
V3 (Q17 n°12)	GRAFYTTGQK	−0.03
V3 (Q17 n°14)	YTTGQIIGNK	−0.08
V3 (Q17 n°15)	TGQIIGNIRK	+0.92
V3 (Q17 n°16)	QIIGNIRQAK	+0.91
V3 (Q17 n°17)	KIGNIRQAHC	+0.63

* The net charge was determined using CHARGEPRO (PCGene). Charges were calculated on the complete peptide sequence corrected for the coupling to Sepharose beads.

Synthesis of NCAs

HSA (CLB) and neoglycoproteins were derivatized with succinic anhydride or *cis*-aconitic anhydride and characterized as previously described by Jansen *et al.* [18, 19]. The percentage monomeric conjugate as well as the relative net negative charge of the modified albumins was determined as described previously by Kuipers *et al.* [1].

GNL Capture ELISA

This capture ELISA was adopted from Weiler *et al.* [20] and modified as such. In brief, a 96-well round-bottom ELISA microtitre plate (Falcon 3911 MicroTest III™ Flexible Assay Plate, Becton Dickinson) was coated with 25 μl GNL (25 μg/mL PBS) (Vector Laboratories Inc.) overnight at 4°. Blocking of the remaining sites was performed with 100 μL PBS/0.5% gelatin. After each step the plates were washed with PBS/0.005% Tween 20. Gp120, because of its high amount of sugar chains, can bind efficiently to this lectin. Both gp120 proteins (p160-BAL and MN) were added in appropriate concentrations (PBS/0.05% Tween 20/0.1% gelatin supplemented with 0.1% BSA). Suc-HSA was tested for its binding to the gp120 proteins at three different concentrations (1, 10, and 100 μg/mL in PBS/Tween 20/gelatin supplemented with 0.1% BSA). The binding was determined after a 1-hr incubation with the purified anti-HSA polyclonal antibody (dilution 1:250 in PBS/Tween 20/gelatin). HSA (1, 10, and 100 μg/mL in PBS/Tween 20/gelatin supplemented with 0.1% BSA), NRS, and polyclonal antibody W1/08 (1:50, 1:250, and 1:1250 dilution in PBS/Tween 20/gelatin) were used as negative and positive controls, respectively. The formed immunocomplex was detected by horse anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (CLB PK17E) (1:10.000 dilution). Tetramethylbenzidine (Sigma) was used as a substrate and extinctions were measured at 450 nm using a microplate reader. All incubation steps took place in the dark at 37°. All experiments were performed twice on separate days, in duplicate. Results are presented as the average of the values obtained in one experiment with a variation of less than 5%.

Fluid Phase Assay

cV3 peptide and gp120 (MN) were radioactive-iodinated as described previously [21]. An appropriate amount of labeled peptide or protein was incubated with 25 μL Suc-HSA (1, 10, or 100 μg/mL in PBS/Tween 20/gelatin supplemented with 0.1% BSA) overnight. HSA (1, 10, and 100 μg/mL in PBS/Tween 20/gelatin supplemented with 0.1% BSA), NRS, and polyclonal antibody W1/08 (1:50, 1:250, and 1:1250 dilution in PBS/Tween 20/gelatin) were used as negative and positive controls, respectively. The putative gp120 or cV3–Suc-HSA complexes were trapped by preformed complexes of polyclonal anti-HSA bound to protein A-Sepharose beads. After 2-hr incubation, the complexes bound to Sepharose beads were washed and counted for gamma radiation.

Thrombin Digestion of the V3 Loop

Digestion of the V3 loop in gp120 or cV3 peptide was performed using thrombin (Sigma). The proteins and peptides were incubated with 5 U/mL thrombin at 30° for several hours. The proteolytic cleavage was blocked by the addition of hirudine (Sigma) and phenylmethylsulfonyl fluoride (Sigma). The cleavage of gp120 was checked by SDS-PAGE and silverstaining or autoradiography in case of radiolabeled gp120.

Coupling of V3 Peptides to CNBr-activated Sepharose

Lyophilized peptides (1 mg) were dissolved in coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) and immobilized to 50 mg CNBr-activated Sepharose (Pharmacia) according to Kuipers *et al.* [1]. Cleavage of the V3-Q17 loop

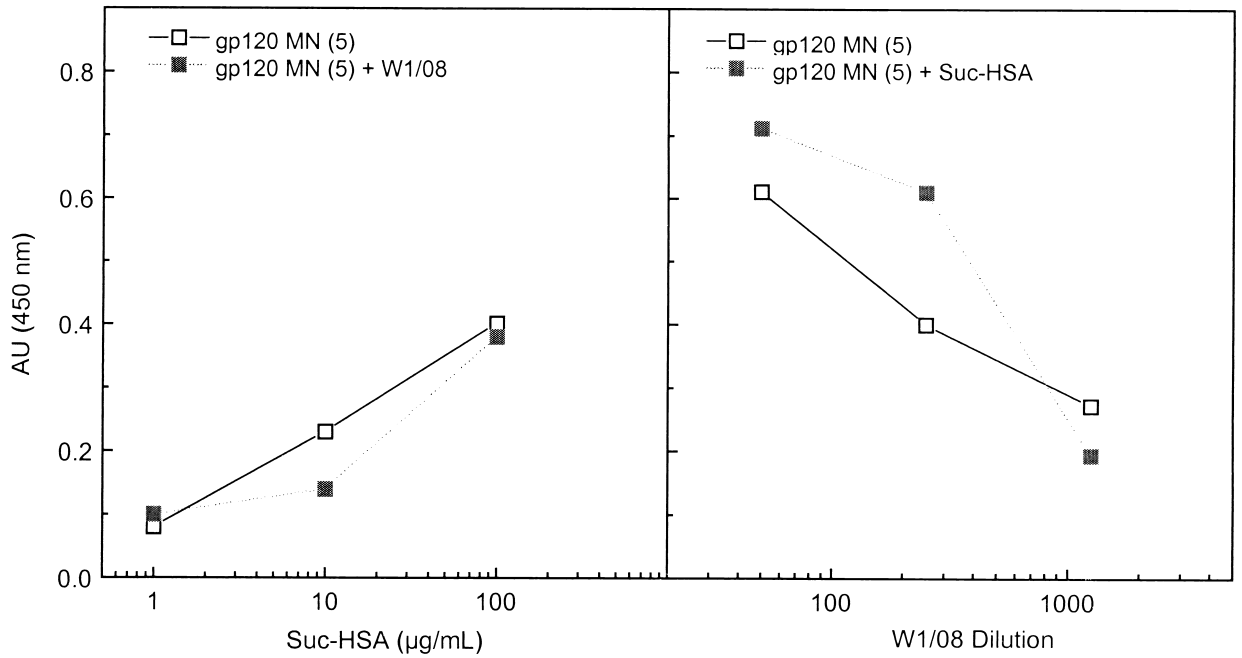

FIG. 1. The binding to MN gp120 presented via GNL (capture EIA). Gp120 was tested at 5 μg/mL. Results are presented as the average of the values obtained in one experiment with variation of less than 5%. Left: The binding of Suc-HSA to gp120. Suc-HSA was tested at concentrations of 1, 10, and 100 μg/mL and identified via purified polyclonal antibody HSA. Preincubation with a 1:50 dilution of W1/08 was used to compete for the binding. Right: The binding of W1/08 to gp120 W1/08 was used in a 1:50, 1:250, and 1:1250 dilution. PK17-horseradish peroxidase was used to measure binding of W1/08. Preincubation with 100 μg Suc-HSA was used to compete for the binding.

was assessed according to Clements *et al.* [7] as follows: thrombin (Sigma) was dissolved in 1 mL 40 mM HEPES, pH 7.0 (activity approximately 100 units/mL). Ten μg V3-Q17 peptide was incubated with 5 units thrombin for 6 hr at 37° in 40 mM HEPES, pH 7.0. The V3 loop digested by thrombin was coupled to Sepharose beads using the procedure described previously.

Binding and Inhibition Studies Using CNBr-activated Sepharose

Binding of various NCAs (^{125}I-labeled [21]) to overlapping peptides of the V3 loop, immobilized to Sepharose beads, were performed as earlier described by Kuipers *et al.* [1]. Results are given as the percentage of added radiolabeled NCA bound to the solid phase peptide. For the inhibition studies, a constant amount of labeled NCA was diluted with various concentrations of non-radiolabeled inhibitor. The assay was further performed as described previously [1].

RESULTS

In earlier studies, we demonstrated that the antiviral mechanism of action of NCAs was likely caused by interaction with the V3 loop of gp120 [1]. In these latter experiments, circular V3 peptides, indicative of an SI or NSI isolate, or a linearized V3 peptide were immobilized to CNBr–Sepharose and tested for their interaction with the NCAs. In the present study, we used different assay formats to investigate in more detail the binding of NCA to the V3 loop in the env-protein of HIV and a variety of V3 peptides.

Binding of Suc-HSA to Immobilized gp120 Using EIA Techniques

The binding of Suc-HSA to the env-protein of HIV gp120 was tested using a capture EIA, in which gp120 was presented via a GNL. In this assay, the MN variant of gp120, which had been expressed and purified from Chinese hamster ovary cells, was incubated with this lectin, and Suc-HSA or antibodies were examined for their binding capacity. Figure 1 is a representative example of the capacity of Suc-HSA to bind to gp120 in a concentration-dependent manner. HSA and NRS were used as negative controls, with neither showing binding. Antibody W1/08 was used as a positive control. This antibody was able to recognize the disulfide-bridged conformation of the V3 loop of gp120. This recognition by W1/08 has been determined using the V3 loop as a peptide as well as the whole gp120 protein, both in a concentration-dependent manner (not shown).

Since we had found that W1/08 bound predominantly to the tip of the V3 loop, we performed a competition experiment with Suc-HSA and W1/08. We preincubated gp120 with either an excess of Suc-HSA (100 μg) or of W1/08 (1:50 dilution) and tested the binding of the counterpart, i.e. W1/08 or Suc-HSA, respectively. As is

demonstrated in Fig. 1, neither compound was able to inhibit the binding of the other compound to gp120. The results with the NSI variant p160-B of gp120, expressed in CV-1 cells, were comparable with the above-mentioned results, suggesting that the binding of NCAs to gp120 is HIV-1 phenotype-independent [22].

Mapping of Bindings Sites of gp120 Using EIA Techniques

INFLUENCES OF V3 LOOP DIGESTION. The digestion of the gp120 of the V3 loop with thrombin was used to investigate the influence of conformational changes in the env-protein and the involvement of the amino acids susceptible to thrombin digestion on binding to NCA. Thrombin was used as a model protease to cleave the V3 loop between Arg^{315} and Ala^{316} [4]. After digestion with thrombin, gp120 was immobilized to a lectin (GNA) on an EIA plate and tested for its binding to Suc-HSA or W1/08. The results are shown in Fig. 2. For both Suc-HSA and W1/08, the binding to the digested gp120 was decreased as compared to the binding to the intact protein (Fig. 2, columns a and b). However, binding could not be reduced to zero. In Fig. 1, we have already demonstrated that preincubation of gp120 with W1/08 did not influence the binding of Suc-HSA to gp120 (Fig. 2, column c). In addition, we show in Fig. 2 that the binding of Suc-HSA to digested gp120 was not influenced when digested gp120 was preincubated with W1/08 (column d). Identical results were found for the binding of W1/08 when digested gp120 was preincubated with Suc-HSA.

PROTECTION EXPERIMENTS. Since we had demonstrated that the binding of W1/08 was sensitive to thrombin digestion, we used this antibody as a read-out in protection experiments with Suc-HSA. As is shown in Fig. 3, preincubation of gp120 with either HSA or Suc-HSA did not influence the binding of W1/08 to gp120 (column a vs b and d). We showed earlier that digestion of gp120 by thrombin, prior to the addition of Suc-HSA, resulted in a decreased affinity of Suc-HSA or W1/08 for gp120 (Fig. 2, columns a and b). Since HSA did not bind to the V3 loop [1], preincubation of gp120 with HSA before thrombin addition resulted in a decreased affinity of W1/08 for gp120 (Fig. 3, column c). In contrast, preincubation of gp120 with Suc-HSA before thrombin addition resulted in an identical binding of W1/08, indicating that the epitope for W1/08 was prevented from cleaving (Fig. 3, columns e vs a). It appears that protection of the digestion of the V3 loop by preincubation with Suc-HSA resulted in preservation of the binding site for anti-V3 antibody W1/08.

Binding of Suc-HSA to gp120 Using Fluid Phase Format

The next format we looked at was the binding of Suc-HSA to soluble env-protein and env-related peptides radiolabeled with iodine-125. To this end, Suc-HSA was incubated with radiolabeled antigens, and the putative complex was trapped by means of immunoaffinity-purified antibodies to HSA bound to protein A-Sepharose beads. It was observed that there was no complex formation even at elevated amounts of radioactive protein/peptides. As apparent, W1/08 was able to bound to iodinated gp120 and cV3 peptides in a concentration-dependent manner (results not shown).

From the present experiments, it became evident that the interaction of Suc-HSA with gp120 was predominantly located at the V3 loop at sites situated near the thrombin digestion site. In addition, it also seemed clear that this interaction was assay-dependent, probably due to a higher density of protein molecules involved. The assay formats in which gp120 or V3 were presented via a solid phase format (CNBr-activated Sepharose, as shown previously [1], or

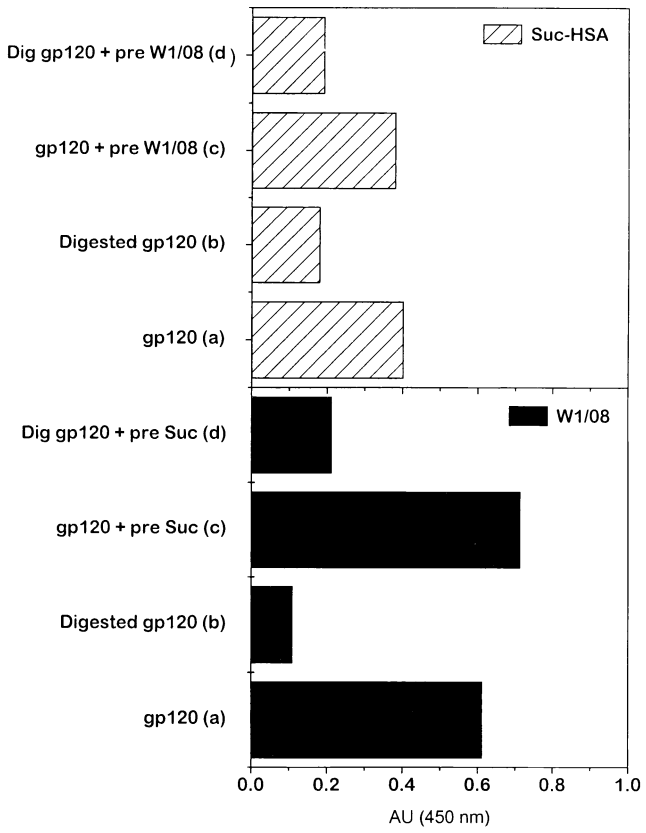

FIG. 2. The binding to MN gp120 and digested gp120 presented via GNL (capture EIA). Five μg/mL gp120 was coated on EIA plates. Upper panel: The binding of Suc-HSA to gp120 and digested gp120 in relation to the binding of W1/08 to these proteins and the competition for the binding to gp120 after pretreatment of gp120 with W1/08. Lower panel: The binding of W1/08 to gp120 and digested gp120 in relation to the binding of Suc-HSA to these proteins and the competition for the binding to gp120 after pretreatment of gp120 with Suc-HSA. Suc-HSA and HSA were used at 100 μg/mL and W1/08 was used at a 1:50 dilution. Results are presented as absorption values at 450 nm, corrected for non-specific binding of HSA and/or NRS, and as the average of the values obtained in one experiment with a variation of less than 5%.

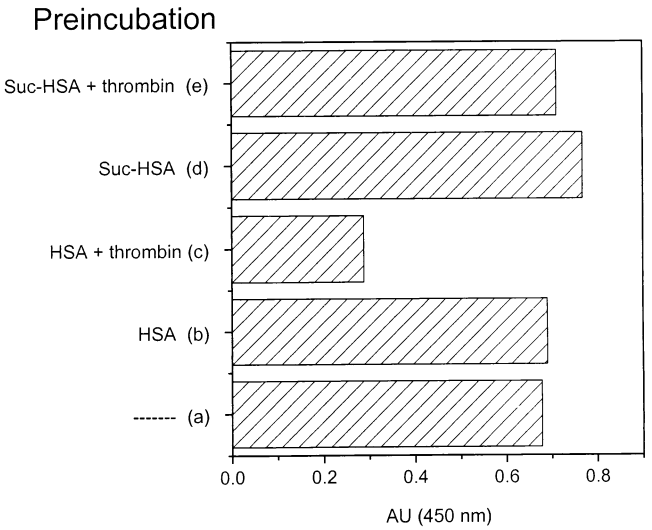

FIG. 3. The binding of W1/08 to MN gp120 and digested gp120 presented via GNL (capture EIA). Five μg/mL gp120 was coated on EIA plates as described above. Preincubation with the specified compounds was performed prior to the addition of W1/08, the latter used at a 1:50 dilution. Suc-HSA and HSA were used at 100 μg/mL. Results are presented as absorption values at 450 nm and as the average of the values obtained in one experiment with a variation of less than 5%.

EIA formats) appeared to be a prerequisite for interaction. In order to investigate the interaction with the V3 loop in more detail, using small peptides delineating the whole V3 loop immobilized on a solid phase, we chose the CNBr–Sepharose format. This procedure was selected because earlier attempts to coat small peptides to ELISA plates appeared to be very inefficient.

Binding to Immobilized V3 Peptides

V3 loop and overlapping peptide sequences of V3 immobilized to CNBr-activated Sepharose are listed in Table 1. The net charges of the peptide sequences at pH 8.0, as determined using the CHARGEPRO program from PC-GENE (IntelliGenetics), are also presented in this table. Most of the peptide fragments used in the binding studies were positively charged. The fragments are numbered in consequence, starting with fragment number 4, which represents the 9 amino acid sequence at positions 296 to 304 in the V3 loop, with the other fragments (numbers 5 [aa 300–308] until 17 [aa 322–330]) delineating the 9 amino acids after a consequent shift of 2 amino acids in the loop. An extra lysine in the fragments was included to conjugate the peptides to the Sepharose beads.

The binding of NCAs to the whole V3 loop and the linearilized V3 sequence was performed as described in an earlier study [1], and these data were used as a reference for the present binding studies. The binding affinities of the various fragments of the V3 loop were tested with these modified albumins, using a constant amount of NCA and a variable amount of peptide–Sepharose suspension. The results for fully succinylated HSA (Suc_{60}-HSA) are shown in Fig. 4. It was found that Suc-HSA was able to bind to fragment numbers 6, 7, 8 and, to a much lesser extent, to numbers 9 and 10. These numbers represent peptide fragments all situated at the tip of the V3 loop.

Suc-HSA and Aco-HSA, the conjugates with the most potent anti-HIV-1 activities of the collection of conjugates tested in these experiments, demonstrated a binding affinity for the V3 fragments of the same magnitude. Binding of NCAs to control peptides, as well as binding of unmodified HSA to all the tested peptides, was negative. The negatively charged glycoproteins (Suc_{53}-$Lact_7$-HSA, Aco_{53}-$Lact_7$-HSA, Man_{14}-HSA, Suc_{46}-Man_{14}-HSA, and Aco_{46}-Man_{14}-HSA) showed a comparable binding pattern, although the binding affinity was lower as compared to the parent compounds Suc-HSA and Aco-HSA. This could be due to charge differences between the completely succinylated or aconitylated albumins (Suc_{60}-HSA or Aco_{60}-HSA) and the partially succinylated or aconitylated compounds (e.g. Suc_{53}-$Lact_7$-HSA), as mentioned in earlier studies [1].

Inhibition of Binding to Peptides Immobilized to Sepharose

Inhibition studies using a constant amount of V3 fragment number 8 or 10 of radiolabeled Suc-HSA or Aco-HSA and a variable amount of unlabeled Suc-HSA or Aco-HSA were performed to determine if the binding to the V3 peptides could be blocked using other negatively charged compounds. Results of these experiments are shown in Fig. 5. Binding to both peptide fragments was inhibited by various negatively charged compounds. The extent of inhibition was comparable for both NCAs. These studies were also performed with the other neoglycoalbumins. Generally, Aco_{60}-HSA was the strongest inhibitor, and the relative inhibition action decreased in the order Aco_{46}-

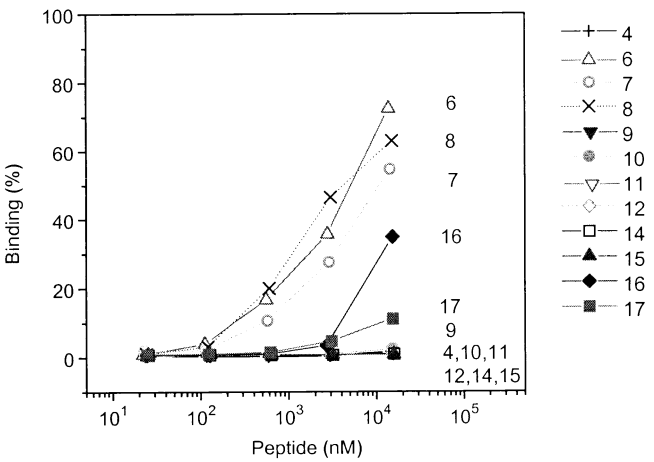

FIG. 4. Binding of Suc-HSA to peptide fragments of the V3 loop immobilized to Sepharose. Results reflect the percentage of added compound bound by the peptide fragments (SEM less than 10%). Amino acid sequences of the fragments are listed in Table 1. Fragment numbers 4(+), 6 (Δ), 7 (○), 8 (x), 9 (▼), 10 (●), 11 (∇), 12 (◇), 14 (□), 15 (▲), 16 (◆), and 17 (■) were used as peptide fragments.

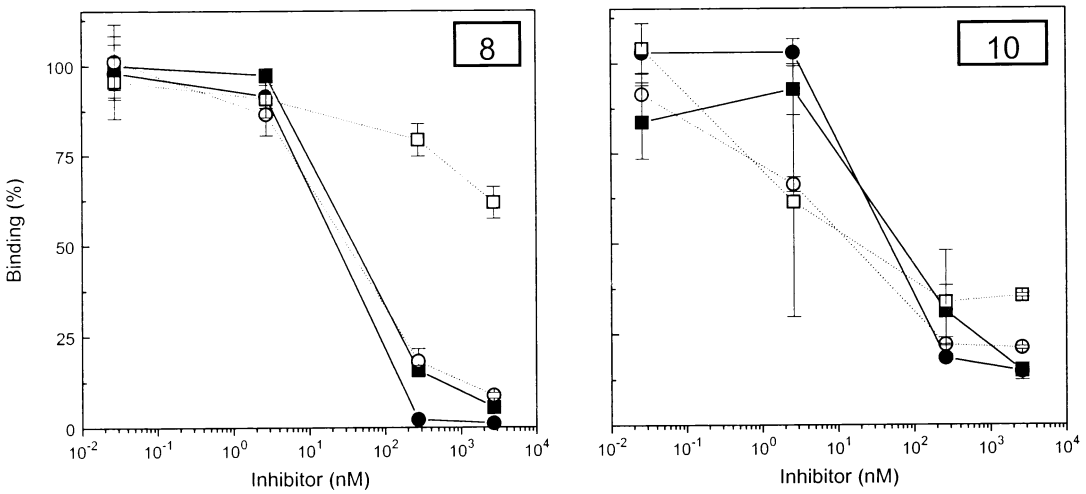

FIG. 5. Inhibition by NCAs of the binding of ^{125}I-radiolabeled NCAs to the V3-peptide fragment numbers 8 (left) and 10 (right) immobilized to Sepharose. V3-peptides were incubated with Suc-HSA (closed figures) or Aco-HSA (open figures) in the presence of serial dilution of inhibitors (±SEM). Left: V3 no. 8: Inhibition of ^{125}I-Suc-HSA binding by unlabeled Suc-HSA (■) or unlabeled Aco-HSA (□). Inhibition of ^{125}I-Aco-HSA binding by unlabeled Suc-HSA (●) or unlabeled Aco-HSA (○). Right: V3 no. 10: Inhibition of ^{125}I-Suc-HSA binding by unlabeled Suc-HSA (■) or unlabeled Aco-HSA (□). Inhibition of ^{125}I-Aco-HSA binding by unlabeled Suc-HSA (●) or unlabeled Aco-HSA (○).

Man_{14}-HSA> Aco_{53}-$Lact_7$-HSA> Suc_{53}-$Lact_7$-HSA> Suc_{46}-Man_{14}-HSA> Suc_{60}-HSA> Man_{14}-HSA.

Binding Studies with the Digested V3 Loop Peptide

The V3 peptide was digested by thrombin as described above. After immobilization of the cleaved V3 peptide to Sepharose beads, the immobilization of both variants of the V3 loop was confirmed by V3-specific polyclonal antibodies with discriminating binding capacities to the V3 loop (Fig. 2). These peptides were then tested for their binding affinity to the NCAs. In Fig. 6, the binding capacities of Suc-HSA and Aco-HSA to the V3 loop were compared to the binding capacities to the digested V3 loop. For both negatively charged albumins, it was observed that digestion of the V3 loop dramatically decreased binding affinities, which is in agreement with previous experiments (Figs. 1 and 2).

DISCUSSION

In this study, we addressed the binding mechanism of negatively charged, antivirally active, albumins to the HIV-1 env-protein. We used different assay formats to test the influences of presentation and conformation of the gp120 or V3 loop on the previously observed interaction with succinylated or aconitylated HSA.

When using HIV-1 env-proteins immobilized via a lectin on EIA plates, irrespective of the phenotype (SI vs NSI), we observed that Suc-HSA was able to bind to native gp120 and that the epitope for binding was located at or near the thrombin digestion site in the V3 loop of gp120. In

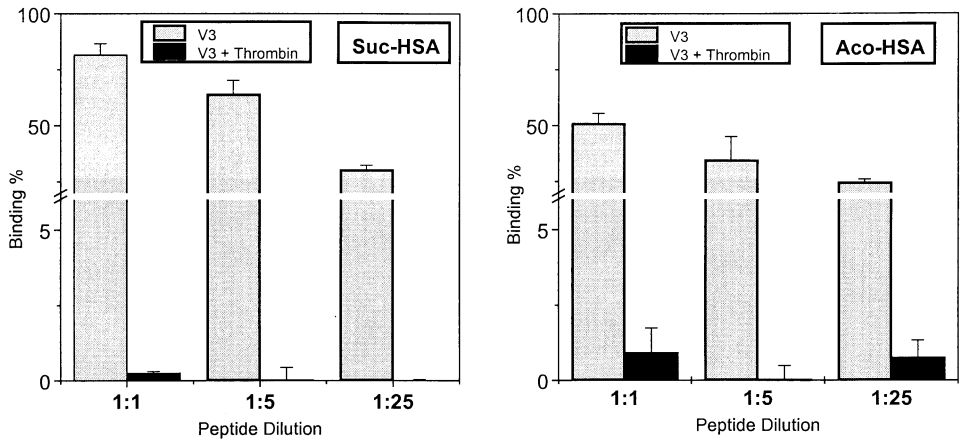

FIG. 6. Binding of Suc-HSA (left) and Aco-HSA (right) to V3-Q17 peptide. V3 peptide was treated without (▭) or with thrombin (■) before immobilization to Sepharose beads (±SEM).

addition, we found that the binding site for Suc-HSA on the V3 loop was not identical to the epitope determined earlier for a control anti-V3 antibody (polyclonal antibody W1/08) (see footnote on page 6), since both components could bind to gp120 independently of each other. We also noticed, using this assay format, that Suc-HSA was able to prevent the proteolytic digestion of the V3 loop by thrombin when preincubated with gp120.

In contrast to the specific interactions of Suc-HSA and env-related peptides in the solid phase assays, we did not observe any binding of Suc-HSA to V3 loop peptides or gp120 when presented in a fluid phase system. Obviously, the conformation of the peptides and gp120 and Suc-HSA in fluid phase is such that their interaction is not possible. It is possible that binding of Suc-HSA to peptides occurs only when one of the components is immobilized and, as a consequence, the local density of the molecules involved is much greater. However, experiments in which Suc-HSA was coupled to CNBr–Sepharose did not result in interaction with any peptide or gp120 (unpublished observations). It seems reasonable to assume that a certain density of peptide molecules is necessary for interaction. This observation also points to an electrostatic interaction between Suc-HSA and the V3 loop. Apparently, interaction between NCA and V3 or gp120 requires a sufficiently high net positive charge for interaction to occur. It is clear that this can be achieved at local high concentrations of peptide molecules. We hypothesize that the linear presentation of amino acids results in higher affinities than a more globular presentation, since the amino acids necessary for interaction are within better reach.

This lack of interaction of Suc-HSA with peptides or gp120 in fluid phase is comparable with the phenomenon of monovalent antigen binding in the case of monovalent Fab fragments. It is known that in fluid phase these interactions (Fab–Ag) are sometimes too weak to detect. However, these Fab fragments do interact with their target molecules (Ag) when presented via a solid phase. Consequently, the density of the target molecules is most important for this monovalent interaction. Likewise, we can hypothesize that a monovalent binding is involved in the interaction of Suc-HSA and HIV proteins.

In order to identify the binding site of Suc-HSA to the V3 loop more precisely, we used the solid phase assay. We immobilized overlapping peptides to CNBr–Sepharose and observed that the interaction between V3 peptides and the NCAs (Suc-HSA, Aco-HSA, and the other negatively charged glycoproteins) was not solely dependent on electrostatic attraction: the actual amino acid composition is also important [1]. This is in line with the earlier conclusion of Meshcheryakova *et al.* [23], who also suggested that both charge and a certain amino acid composition were required for interaction between polyanions and HIV peptides. It was proposed that, ideally, lysine and arginine combinations in a particular sequence should be present for a proper interaction. Fragment numbers 6, 7, and 8 contain these specific amino acid combinations, and our results show that the binding of the NCAs to these fragments was indeed stronger as compared to the other fragments (Fig. 4). It is likely, therefore, that electrostatic attraction is the first prerequisite for the interaction of these peptides and the NCAs, and that this element is probably associated with the presence of arginine and lysine combinations (positively charged amino acids). In addition to positively charged amino acids, the presence of hydrophobic amino acids in the fragments, particularly that of isoleucine, seems to be required. This explains the absence of interaction of the NCAs with the positively charged fragment numbers 4 and 11, which do not contain this particular amino acid. It was surprising, however, that no strong interaction was found with fragments 9, 10, 11, and 12, which contain the thrombin cleavage site of arginine and alanine. In other experiments, we showed that after digestion of the bond between these amino acids with thrombin the interaction with Suc-HSA was greatly diminished. Therefore, we expected that the interaction of Suc-HSA was located at this site of the V3 loop. However, these mapping studies indicate that the highest affinity site is located near the tip of the V3 loop and that the affinity of Suc-HSA for this part is influenced by changes in the conformation of the tip of the loop.

The negatively charged glycoproteins (Suc_{53}-$Lact_7$-HSA, Aco_{53}-$Lact_7$-HSA, and Aco_{46}-Man_{14}-HSA) showed a comparable binding pattern. The affinity of these glycoproteins for all peptide fragments tested was much lower compared with the affinity of Suc-HSA and Aco-HSA for these fragments. This could be due to charge differences between the completely succinylated or aconitylated albumins (Suc_{60}-HSA or Aco_{60}-HSA) and the partially succinylated or aconitylated compounds (e.g. Suc_{53}-$Lact_7$-HSA), and correlates well with the *in vitro* antiviral activities reported previously [1].

From the experiments presented in this study, we may conclude that Suc-HSA is able to bind to linear amino acid sequences in env-related peptides. This phenomenon can best be studied using solid phase assay systems. In addition, these amino acid sequences must contain enough positively charged amino acids, and the presence of isoleucine is also required. The interaction with the V3 loop, therefore, is located on the left near the GPGRAF sequence, and conformational changes of this sequence do have consequences for interaction with Suc-HSA.

These observations add to the evidence collected earlier that Suc-HSA interferes at the level of virus entry, without interaction with the CD4 receptor [19, 22]. As discovered recently, HIV-1 uses a second receptor for entry into host cells [13, 14, 24]. The interactions of gp120 with the chemokine receptors CXCR-4 on T cells and CC-CKR-5 on macrophages were found to be the triggering events in the fusion process [9–11]. Although we reported earlier on the interaction of the NCAs with components in the membrane of the host cell [25], it seems unlikely that interaction of Suc-HSA with either of these chemokine receptors could explain the antiviral mechanism of this

negatively charged albumin, since the chemokine receptors are negatively charged themselves [24, 26]. It seems more likely that Suc-HSA is able to prevent the V3 loop from interacting with these types of receptors, thereby inhibiting virus entry. Cocchi *et al.* [12] have already suggested that the V3 domain is a critical component of the chemokine-mediated blockade of infection. Trkola *et al.* [27] demonstrated same via the inhibition of the chemokine–gp120 interaction using certain V3-directed monoclonal antibodies. In addition, we showed earlier that Suc-HSA was able to inhibit clinical isolates of both SI and NSI variants with a comparable activity [22]. Although an interaction of Suc-HSA, with comparable affinity, with either chemokine is possible, it is our feeling that an interaction of Suc-HSA with the virus or with other more non-specific cellular components is a more probable explanation for its antiviral activity.

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