# Human Immunodeficiency Virus Type 1 Activates the Classical Pathway of Complement by Direct C1 Binding through Specific Sites in the Transmembrane Glycoprotein gp41

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## Summary

Human immunodeficiency virus type 1 (HIV-1), in contrast to animal retroviruses such as murine leukemia virus, is not lysed by human complement. Nevertheless, HIV-1 activates complement via the classical pathway independent of antibody, and C3b deposition facilitates infection of complement receptor-bearing cells. Using gel exclusion chromatography on Sephacryl S-1000, purified virions were found to bind $^{125}$I-labeled C1q, but not $^{125}$I-labeled dimeric proenzyme C1s. Virions activated the C1 complex, reconstituted from C1q, proenzyme C1r, and $^{125}$I-labeled proenzyme C1s, to an extent comparable with that obtained with immunoglobulin G–ovalbumin immune complexes. To determine the activating viral component, recombinant viral proteins were used: in the solid phase, soluble gp41 (sgp41) (the outer membrane part of gp41, residues 539–684 of gp160) bound C1q, but not dimeric proenzyme C1s, while gp120 was ineffective. In the fluid phase, sgp41 activated the C1 complex in a dose- and time-dependent manner, more efficiently than aggregated Ig, but less efficiently than immune complexes. To localize the C1 activating site(s) in gp41, synthetic peptides (15-residue oligomers spanning amino acids 531–695 of gp160) were used. Peptides covering positions 591–605 and 601–620 and, to a lesser extent, positions 561–575, had both the ability to bind C1q and to induce C3 deposition. These data provide the first experimental evidence of a direct interaction between the C1 complex and HIV-1, and indicate that C1 binding and activation are mediated by specific sites in gp41.

Retroviruses isolated from avian, feline, murine, and simian sources have been found to be lysed by normal human serum (1–3). Lysis is induced by direct antibody-independent triggering of the classical complement pathway (4). It was previously believed that this mechanism protected the individual from retroviral disease (2). Human retroviral pathogenes (HIV-1 and HTLV-1) have since been identified, and several laboratories have shown that these viruses are not lysed efficiently by human serum (5, 6), although animal sera from felidae or muridae are capable of lysis (7).

As in the case with other retroviruses, cells infected with HIV-1 activate the complement system independent of antibody via the alternative pathway. Subsequent deposition of C3b/C3d resulted in rosetting between HIV-1-infected cells and cells bearing complement receptors (CR) (8). In contrast, isolated HIV-1 (strain IIIB) activated the classical pathway independent of antibody (8). The biological relevance of the latter mechanism was demonstrated through the observation that infection of CR-bearing cells by HIV-1 is enhanced at low multiplicity of infection (9, 10), i.e., under conditions that probably represent the typical in vivo situation during the first contact between HIV-1 and the host. The validity of this concept has been proven by other groups (11–13).

The fact that complement activation by HIV-1 does not result in lysis of the virus could be explained either by a viral component interfering with the complement cascade or by restriction mechanisms similar to those protecting cells of an individual against its own complement system (14, 15), such as decay-accelerating factor (16) and membrane cofactor protein (17); such a factor originating from the host cell may be embedded in the membrane of HIV-1 and protect the retrovirus against the lytic activity of human complement. Among examples of such a protection mechanism in other viruses is glycoprotein C of HSV-1, which binds C3b (18); vaccinia virus has a protein with structural homology to C4b-binding protein (19) and EBV accelerates the decay of the alternative pathway C3 convertase (20, 21).

In an attempt to elucidate the molecular mechanisms in-

J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/12/1417/08 $2.00
Volume 174 December 1991 1417–1424

volved in the early steps of the activation of the classical pathway of complement by HIV-1, we provide the first experimental evidence of a direct interaction between the C1 complex and HIV-1 and show that C1 binding and activation are mediated by specific sites in gp41.

## Materials and Methods

*Reagents and Buffers.* IgG-OVA immune complexes were prepared at equivalence as described previously (22). Heat-aggregated Igs were prepared by heating purified rabbit Ig (15 mg/ml) for 15 min at 63°C. Particulate material was removed by centrifugation and the soluble aggregates were used for C1 activation.

Veronal-buffered saline (VBS)[1] contained 5 mM sodium barbital (pH 7.4), 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, and either 150 mM NaCl (physiological ionic strength) or 75 mM NaCl (half-physiological ionic strength). Recombinant protein p138 from EBV was obtained from Biotest (Dreieich, Germany).

*C1 Subcomponents.* C1q, proenyzme C1r, and proenzyme C1s were isolated from human plasma as described previously (22, 23). The concentrations of purified C1q, C1r, and C1s were determined by using values of E (1%, 1 cm) at 280 nm of 6.8, 12.4, and 14.5, and molecular weights of 459,300, 86,300, and 78,900 (24, 25). C1q and C1s were labeled with $^{125}I$ either by the immobilized lactoperoxidase-glucose oxidase method using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) or with Iodobeads (Pierce Chemical Co., Rockford, IL) as recommended by the manufacturers. Unbound $^{125}I$ was removed by exhaustive dialysis or by centrifugation on a Sephadex G 50 fine column (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously (26).

*Cells and Virions Preparation.* H9 cells chronically infected with HIV-1 (HTLV-IIIB strain) were cultivated with four parts of uninfected H9 cells in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and antibiotics (all from Seralab, Sussex, UK) for 48–72 h. Supernatants were harvested by centrifugation at 400 *g* for 10 min, followed by centrifugation at 800 *g* for 30 min, and subsequent filtration through a 0.2-μm membrane (Millipore Continental Water Systems, Molsheim, France). These supernatants were centrifuged at 100,000 *g* for 90 min to concentrate the virus. Virus was resuspended in 500 μl of VBS at half-physiological ionic strength and loaded under containment conditions onto a disposable 10-ml Sephacryl S-1000 column (Pharmacia Fine Chemicals) equilibrated with the same buffer. Virions were eluted with this buffer and fractions were collected. P24 was measured with a capture ELISA (Coulter Immunology, Hialeah, FL) using recombinant p24 as a standard (27). In parallel, supernatants of uninfected H9 cells were centrifuged, passed through the column, and used as a control.

*Proteins and Peptides from HIV-1.* Recombinant protein gp120 (HTLV-IIIB strain) was obtained from the MRC AIDS Directed Program (Herts, UK). Soluble gp41 (sgp41), the proposed outer membrane part of gp41 (28), was derived from clone BH 10. The restriction sites RsaI and SspI were used to clone this fragment into the plasmid pSB6 and generate the expression of a 18-kD polypeptide (amino acids 539–684 of gp160) in *Escherichia coli*. It was purified to homogeneity by a three-step method (27). Before use for activation studies, the final preparation, in 0.1 M Tris-HCl (pH 9.0) containing 0.2% SDS, 0.3 M urea, 10% glycerol, and 14 mM β-mercaptoethanol, was submitted to several dialysis cycles against the appropriate buffer, using a microdialyzer (Pierce Chemical Co.). Synthetic peptides Env 54–Env 69 (29) (15-residue oligomers with five–amino acid overlap) covering positions 531–695 of gp160 (HIV-1 strain SF-2) were either kindly provided by Dr. J. Denner (Frankfurt, Germany) or obtained from the MRC AIDS Directed Program.

*Gel Exclusion Chromatography.* Gel exclusion chromatography was performed as described (30–32). Briefly, 100-μl samples of virus (1.5 μg of p24/ml) were incubated with either 1 μg of $^{125}I$-labeled C1q (112,000 cpm/μg) or 1 μg of $^{125}I$-labeled dimeric proenzyme C1s (98,000 cpm/μg) for 30 min at room temperature in VBS at half-physiological ionic strength and loaded onto 2-ml disposable Sephacryl S-1000 columns equilibrated with the same buffer. Virus was eluted from the column, and 250-μl fractions were collected. Virus was neutralized by addition of Triton X-100 to 1%. Each fraction was assayed for $^{125}I$ radioactivity and p24.

*C1q (C1s) Binding Assay.* C1q and C1s were radiolabeled by the immobilized lactoperoxidase-glucose oxidase method (33). Recombinant proteins (100 ng) were incubated in ELISA plates (Immunoplate, Maxisorb; Nunc, Roskilde, Denmark) in a carbonate-buffered saline (pH 9.6) and left overnight at 4°C. Synthetic peptides (1 μg/well) were dried onto ELISA plates at 30°C overnight. Nonspecific binding was blocked by two incubations for 30 min at room temperature with 100 μl of 1% BSA in VBS at half-physiological ionic strength, followed by one wash with the same buffer. Binding was performed by incubation for 30 min at room temperature with either 50 μl of $^{125}I$-labeled C1q (50,000 cpm/well) or 50 μl of $^{125}I$-labeled dimeric proenzyme C1s (50,000 cpm/well). Plates were washed three times with the same buffer containing 0.05% Tween 20. Bound C1q or C1s was removed by addition of 100 μl of 1 M NaOH/well and measured by counting $^{125}I$ radioactivity.

*C1 Activation Assay.* Proenzyme C1 was reconstituted to a concentration of 1 μM by incubating $^{125}I$-labeled C1s (26,000–30,000 cpm/μg), C1q and C1r (C1q/C1r/C1s molar ratios of 1:2:2), for 5 min at 4°C in the presence of 2.5 mM $CaCl_2$, either in VBS at half-physiological ionic strength (activation by HIV-1) or in 145 mM NaCl, 50 mM triethanolamine-HCl, pH 7.4 (activation by recombinant proteins). 20 μl of viral suspension (p24; 1.5 μg/ml) or of protein solution (0.14–2.0 mg/ml) in the appropriate buffer was incubated with 1–12 μg of reconstituted C1 in a final volume of 40 μl for various periods at 30°C. Activation of radiolabeled C1s in C1 was measured by monitoring the conversion of the proenzyme form to its activated two-chain counterpart by SDS-PAGE (34) under reducing conditions. This was performed either by direct measurement of $^{125}I$ radioactivity on the dried gels or by scanning of autoradiographs.

*C3-Deposition ELISA.* Synthetic peptides (1 μg/well) were dried onto ELISA plates at 30°C overnight. Nonspecific binding was blocked by incubation with 100 μl of 1% BSA in VBS at physiological ionic strength for 1 h at room temperature. After one wash with the same buffer, 50 μl of 5% normal human serum (confirmed as negative for antibodies against HIV-1 by HIV-1 ELISA and HIV-1 Western blot) in VBS was added and incubation was performed for 20 min at 30°C. C3 deposition was detected by a rabbit polyclonal anti-C3d antibody (Dakopatts, Glostrup, Denmark) followed by peroxidase-conjugated anti–rabbit Ig antibody (Dakopatts). Color was developed with 1 mM 2,2'-Azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) (Sigma Chemical Co.) and 0.002% (vol/vol) $H_2O_2$ in citrate buffer (pH 4.3) and optical densities were determined at 412 nm.

## Results

*C1q, but Not C1s, Binds to HIV-1.* Since classical pathway activation by HIV-1 has been proven (8), we wanted to determine which

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[1] *Abbreviation used in this paper:* VBS, veronal-buffered saline.

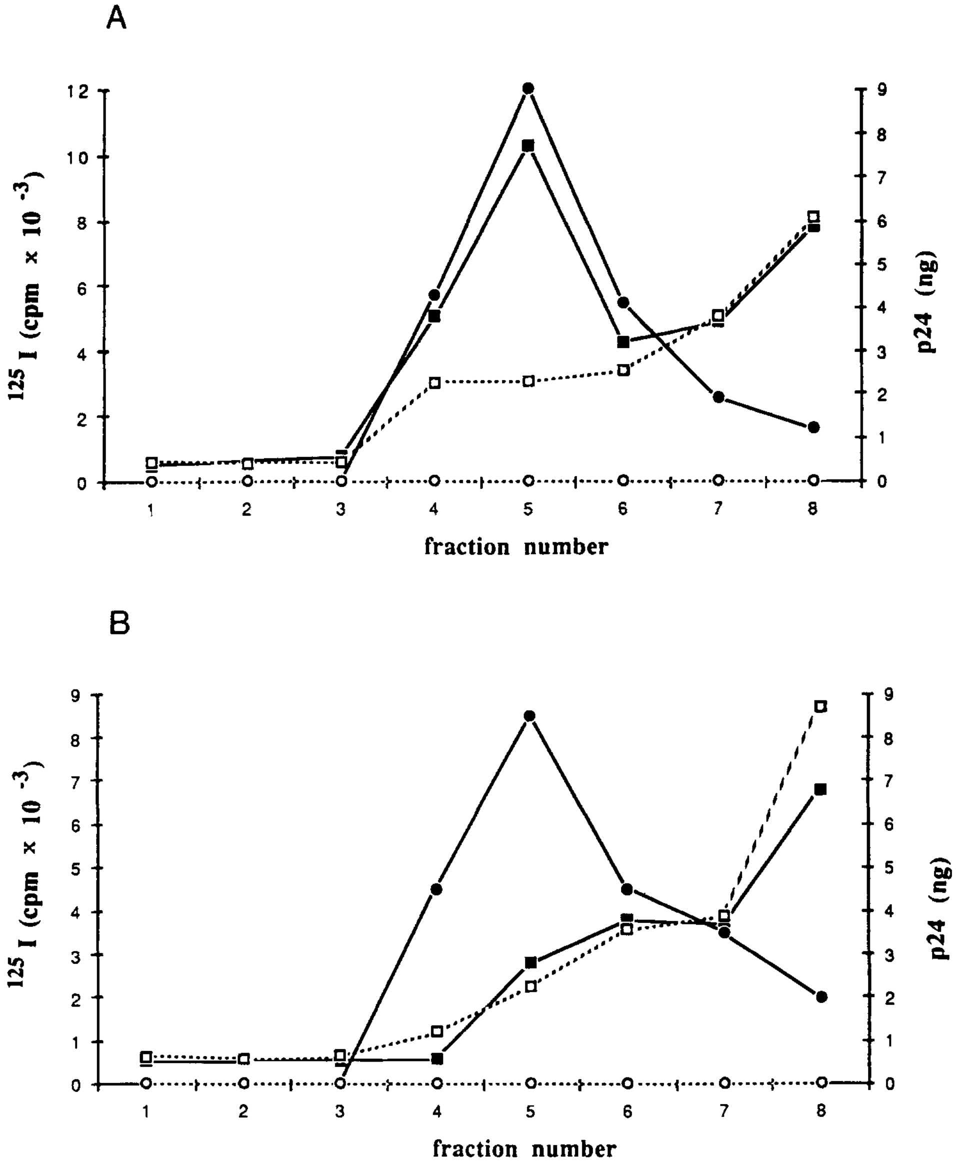

**Figure 1.** C1q (C1s) binding to HIV-1. $^{125}$I-labeled C1q (*A*) or C1s (*B*) were incubated with HIV-1 and then subjected to gel exclusion chromatography on Sephacryl S-1000. (□ and ■) $^{125}$I; (○ and ●) p24; (*closed symbols* and *continuous lines*) samples containing HIV-1; (*open symbols* and *dotted lines*) samples containing control preparation.

C1 subcomponent(s) is (are) involved in the interaction between the C1 complex and HIV-1. To this end, gel exclusion chromatography on Sephacryl S-1000 was used because of its ability to separate virions from proteins. Preliminary experiments indicated that virions eluted between 1.0 and 1.5 ml, as demonstrated by p24 capture ELISA, whereas free C1q and free dimeric proenzyme C1s eluted between 1.75 and 2.5 ml. As shown in Fig. 1 *A*, when virions were preincubated with C1q, part of C1q coeluted with the virions. In control experiments, the elution position of C1q was not modified after incubation with the control preparation (uninfected H9 supernatants prepared in parallel to virions). In contrast, experiments performed with dimeric proenzyme C1s gave no evidence for an interaction between C1s and the virus (Fig. 1 *B*).

*HIV-1 Activates the C1 Complex.* The ability of HIV-1 to activate C1 was tested by incubating the virions with the reconstituted C1 complex for various time periods at 30°C. Activation was measured through conversion of proenzyme C1s into its active two-chain form C1s. As shown in Fig. 2, purified HIV-1 was found to activate the C1 complex in a time-dependent fashion. The activation rate was comparable to that observed with IgG-OVA immune complexes. In contrast, no significant C1 activation was induced by incubation with the control preparation.

*Recombinant sgp41, but Not Recombinant gp120, Binds C1q.* To identify the viral component involved in the interaction with the C1q subcomponent of C1, recombinant sgp41 and gp120 were used in a solid phase assay. As shown in Fig. 3, sgp41 bound radiolabeled C1q to an extent comparable to that observed with immune complexes. In contrast, no significant binding was observed with gp120. Parallel experiments performed with dimeric proenzyme C1s gave no evidence for an interaction between C1s and either sgp41 or gp120 (data not shown).

*Recombinant sgp41 Activates the C1 Complex.* With a view to test the ability of sgp41 and gp120 to induce C1 activation, the reconstituted complex was incubated with increasing amounts of these proteins for 20 min at 30°C. Under these conditions, sgp41 was found to induce activation of the C1 complex in a dose-

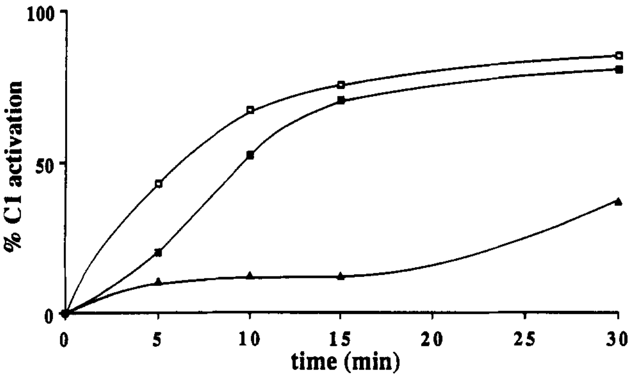

**Figure 2.** Kinetics of C1 activation by HIV-1 and IgG-OVA immune complexes. The reconstituted C1 complex (1 μg) was incubated for varying periods at 30°C with (□) Ig-OVA immune complexes (1 μg), (■) HIV-1 (30 ng of p24), and (▲) control preparation.

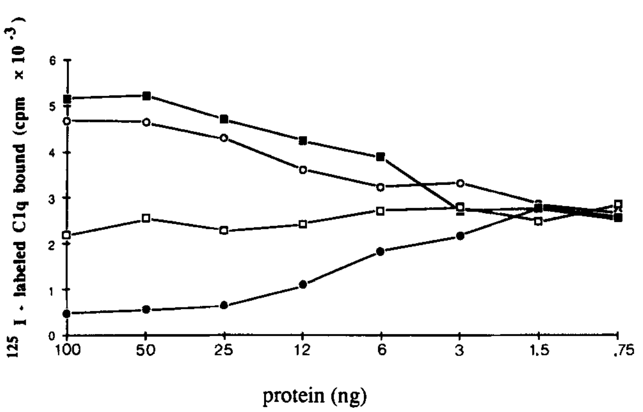

**Figure 3.** C1q binding to IgG-OVA immune complexes and recombinant proteins from HIV-1. Serial dilutions of IgG-OVA immune complexes, recombinant proteins, and control protein (BSA) were bound to ELISA plates, and subsequent binding of $^{125}$I-labeled C1q was measured. (■) IgG-OVA immune complexes, (○) sgp41, (●) gp120, (□) BSA.

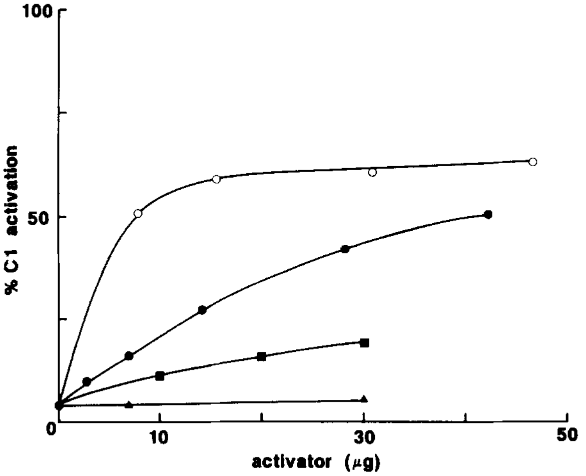

**Figure 4.** C1 activation in the presence of varying amounts of IgG-OVA immune complexes, aggregated Ig, and recombinant proteins from HIV-1. C1 activation was measured after incubation of the reconstituted complex (12 μg) for 20 min at 30°C (○) IgG-OVA complexes; (●) sgp41; (■) heat-aggregated Ig; (▲) gp120.

dependent manner, less efficiently than IgG-OVA immune complexes, but more efficiently than heat-aggregated Ig (Fig. 4). In contrast to sgp41, gp120 had no significant effect on C1 activation within the range tested (Fig. 4). Considering that recombinant proteins obtained from bacterial expression systems are occasionally contaminated by LPS, a known activator of the C1 complex (35), it appeared necessary to verify that the activating effect observed with sgp41 was not due to trace amounts of LPS. To this end, control experiments were performed in the presence of varying concentrations of polymixin B, an antibiotic that binds to LPS and thereby abrogates its C1-activating ability (35). As shown in Table 1, polymixin B only had a slight inhibitory effect on C1 activation by sgp41, comparable to that observed in the case of IgG-OVA immune complexes. This effect likely reflected an inhibition of the intrinsic C1 activation mechanism, probably due to C1 dissociation, as spontaneous C1 activation (in the absence of activator) was significantly slowed down by polymixin B, as illustrated in Table 1. This hypothesis was further supported by kinetic experiments (data not shown). In contrast, activation of C1 by p138, a recombinant protein from EBV, was abolished in the presence of 1 mg/ml polymixin B, indicating that this preparation was likely contaminated by LPS.

Kinetic experiments performed in the presence of polymixin B indicated that sgp41 and IgG-OVA immune complexes both induced a marked increase in the rate of C1 activation, resulting in

**Table 1.** *Effect of Polymixin B on C1 Activation*

| Polymixin B | C1 activation<br>C1 + buffer | <br>C1 + sgp41 | <br>C1 + immune complexes | <br>C1 + p138 |
|---|---|---|---|---|
| *mg/ml* | | | % | |
| 0 | 5.4 | 71.8 | 72.0 | 68.0 |
| 0.5 | 4.4 | 70.6 | 69.6 | 19.4 |
| 1.0 | 3.6 | 54.4 | 61.5 | 3.4 |

C1 activation was measured after incubation of the reconstituted complex for 20 min at 30°C with different activators (sgp41, 42 μg; IgG-OVA complexes, 40 μg; p138, 42 μg), in the presence of varying concentrations of polymixin B.

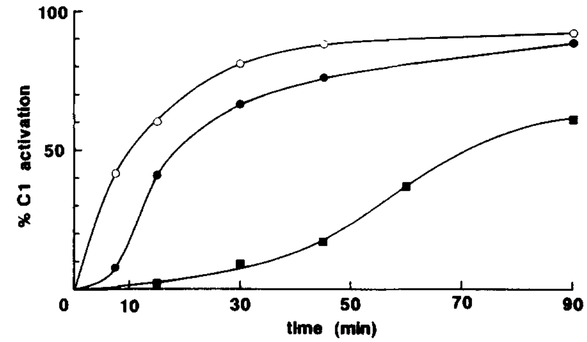

**Figure 5.** Kinetics of C1 activation by IgG-OVA immune complexes and recombinant sgp41. The reconstituted C1 complex (12 μg) was incubated for varying periods at 30°C in the presence of 1 mg/ml polymixin B. (■) C1 alone; (○) C1 + IgG-OVA complexes (28 μg); (●) C1 + sgp41 (28 μg).

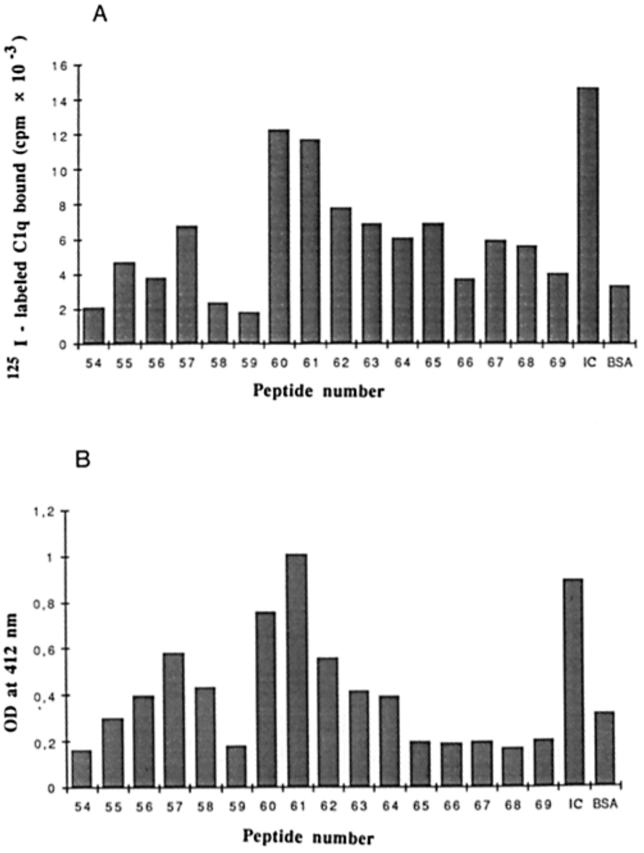

**Figure 6.** Localization of the C1 activating sequence in gp41. Synthetic peptides (1 $\mu$g per well) were dried onto ELISA plates. Subsequent binding of $^{125}$I-labeled C1q (*A*) and C3 deposition (*B*) were measured. Peptides are numbered according to reference 29. IC, BSA, control measurements obtained with IgG-OVA immune complexes (1 $\mu$g), and BSA (1 $\mu$g).

seven- and ninefold enhancements, respectively, after 30 min at 30°C (Fig. 5).

*Localization of the C1 Binding Site(s) in gp41.* To localize the C1 binding site(s) within gp41, synthetic peptides spanning the outer membrane part of gp41 (amino acid residues 531–695 of gp160) were tested in a C1q binding assay and a C3 deposition ELISA. As shown in Fig. 6 *A*, peptides 60 and 61 (amino acid residues 591–605 and 601–620) and, to a lesser extent, peptide 57 (amino acids 561–575) had the ability to bind $^{125}$I-labeled C1q. Similar results were obtained with the C3 deposition ELISA (Fig. 6 *B*).

## Discussion

This paper analyzes the molecular basis of the antibody-independent activation of the classical pathway of complement by HIV-1 and provides the first experimental evidence of: (*a*) a direct interaction between the virus and the C1 complex and (*b*) the ability of the virus to activate the reconstituted C1 complex. These experiments are probably representing the typical in vivo situation during the first encounter between HIV-1 and the host. In this first phase of infection, HIV-1 could be targeted through antibody-independent complement activation to complement receptor–positive cells such as monocytes/macrophages (10). The earlier suggestion that HIV-1 activates the classical pathway (8) is clearly supported by the direct binding of C1q and activation of the C1 complex. These results are further confirmed by the experimental data obtained with recombinant proteins and synthetic peptides. The fact that the alternative pathway was shown to be involved, as stated in a recent report (12), remains unclear. We do not want to rule out the possibility that the alternative pathway may also be involved in the mechanism described here, since C3 deposition via the classical pathway may lead to subsequent activation of the alternative pathway.

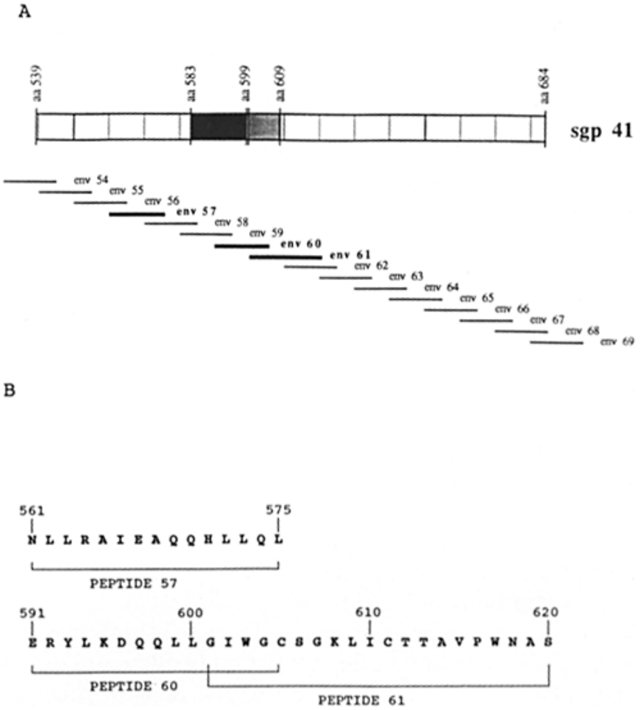

**Figure 7.** (*A*) Location of synthetic peptides from gp41. (*Black box*) Putative HIV immunosuppressive sequence (amino acids 583–599), (*shaded box*) immunodominant epitope (amino acids 600–609). (*B*) Amino acid sequences of the C1 activating peptides. Amino acids are numbered according to reference 63.

Our results with the human retrovirus are in agreement with previous studies indicating direct triggering of the classical pathway by animal retroviruses (4). In the case of MuLV (the best investigated example), direct attachment of the C1 complex to the viral surface was also demonstrated (36). However, in contrast to HIV-1, both C1q and C1s were shown to bind to the viral surface (36).

To define the viral component responsible for the interaction with the C1 complex, we used recombinant sgp41 (representing the proposed outer membrane part of gp41 [28]) and recombinant gp120. Two different assays (C1q binding and C1 activation) gave similar results. Sgp41 was shown to interact with C1q and to induce C1 activation, whereas gp120 was ineffective in both tests. The rather weak affinity observed in the solid phase binding assays may be explained by the facts that sgp41 is probably monomeric and that the C1q binding affinity is enhanced by multivalent interactions (37). On the other hand, the oligomeric state of gp41 on intact virions (38, 39) probably favors a multivalent binding of C1q and thereby induces rapid activation of the C1 complex. This hypothesis is further supported by the observation that exhaustive removal of SDS from the sgp41 preparation both induces aggregation of the protein and enhances its activation potential (data not shown). The observed binding of C1q to sgp41 is reminiscent of previous studies on MuLV, where the C1 binding component was identified as p15e, the transmembrane protein of MuLV (40).

The peptide studies demonstrated two potential C1 binding sites in gp41. The major site (amino acids 591–620) (Fig. 7 *B*) includes both the immunodominant (41) and the putative immunosuppressive regions (42) of gp41; both regions are highly conserved among most retroviruses (43). The second site (amino acids 561–575) was less efficient in both C1q binding and C3 deposition assays. This site is probably part of the region involved in the interaction between gp120 and gp41 (44–46). It should be stressed that none of these sequences contain the ExKxK motif, previously defined as the binding site for C1q on the C$\gamma$2 domain of IgG (47).

Interestingly, there is recent evidence that the epitope 591–620, which contains the major C1 binding and activating sequence, is exposed after sCD4 binding to gp120 (48). This change of the steric configuration of the CD4-gp120-gp41 complex could facilitate the interaction between gp41 and the C1 complex. The mechanisms described here probably represent the molecular basis for the complement-dependent enhancement of HIV-1 infection (9–13), which presupposes complement activation independent of antibody. Different of this strictly complement-dependent enhancement is the proposal of Robinson and Mitchell (49), who showed that, in addition to an exclusively antibody-dependent enhancement (Fc receptor-mediated antibody-dependent enhancement) (50–53), a mechanism exists in which complement facilitates the antibody-dependent enhancement of infection (complement-mediated antibody-dependent enhancement) (11, 13, 54–59). Besides its enhancing effect on HIV-1 infection, the addition of complement also reduced or abrogated the HIV-1-neutralizing activity of antibodies (54, 60). Interestingly, Robinson et al. (61, 62) mapped complement-mediated antibody-dependent enhancement to a synthetic peptide (amino acid residues 586–620), which in our view contains the C1 activating domain. The common feature between our and Robinson's concepts is the role of human complement. Clearly, we stress the importance of complement in the preimmune phase.

This report provides experimental evidence of a direct interaction between the C1 complex and HIV-1, and indicates that C1 binding and activation are mediated by specific sites in gp41. We suspect that this fact is of major importance in the early phase of the infection by HIV-1.

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The technical help of Gabriele Kienast is gratefully acknowledged. We thank the MRC AIDS directed program (Herts, UK) and J. Denner (Erlangen, Germany) for providing reagents, and Q. Sattentau and J. Moore (London, UK) for communication of a manuscript under submission.

This work was supported by the Ludwig-Boltzmann-Gesellschaft, the state of Tyrol, the Austrian FWF, and the European Community.

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*Received for publication 16 July 1991.*

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