Characterization and HIV-1 Fusion Inhibitory Properties of Monoclonal Fabs Obtained From a Human Non-immune Phage Library Selected Against Diverse Epitopes of the Ectodomain of HIV-1 gp41

John M. Louis1, Carole A. Bewley2*, Elena Gustchina1, Annie Aniana1 and G. Marius Clore1*

1Laboratory of Chemical Physics
National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 5, Room B1/30I
Bethesda, MD 20892, USA
2Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda MD 20892, USA

Using a human non-immune phage library comprising more than 10⁸ functional human antibody specificities in Fab format, we have been able to select a set of eight monoclonal Fabs targeted against diverse epitopes of the ectodomain of gp41 from HIV-1. The antigens used for panning the antibodies comprised two soluble, disulfide-linked, trimeric polypeptides derived from gp41, NCCG-gp41 and N35CCG-N13. The former comprises an exposed trimeric coiled-coil of the N-helices of gp41 fused in helical phase to the minimal thermostable ectodomain of gp41, while the latter comprises only the trimeric coiled-coil of N-helices. The selected Fabs were probed by Western blot analysis against four antigens: NCCG-gp41, N35CCG-N13, N34CCG (a smaller version of N35CCG-N13), and the minimal thermostable ectodomain core of gp41 in its six-helix bundle conformation (6-HB). Three classes of Fabs were found: class A (two Fabs) interact predominantly with the 6-HB; class B (four Fabs) interact with both the 6-HB and the internal trimeric coiled-coil of N-helices; and class C (two Fabs) interact specifically with the internal trimeric coiled-coil of N-helices. The IC₅₀ values for the Fabs, expressed as bivalent mini-antibodies, ranged from 6 µg/ml to 60 µg/ml in a quantitative vaccinia virus-based reporter gene assay for HIV-1 envelope-mediated cell fusion using the envelope from the HIV-1 Tropic strain LAV. The two most potent fusion inhibitors belonged to class B. This panel of Fabs provides a set of useful probes for studying HIV-1 envelope-mediated cell fusion and may serve as a basis for developing Fab-based anti-HIV-1 therapeutics.

Published by Elsevier Ltd.

Keywords: HIV-1 fusion; Virus; HIV envelope; gp41; fusion inhibitors
HIV-1 Fusion Inhibitory Fabs Directed Against gp41

Figure 1. Antigens used for antibody selection and Western blot analysis. (a) NCCG-gp41 and N35CCG-N13 were used for antibody selection, and NCCG-gp41, N35CCG-N13, N34CCG, and gp41 core were used for Western blot analysis. The three chains of the trimer are depicted as ribbons in red, blue, and green, and the intermolecular disulfide bridges that covalently link the chains in NCCG-gp41, N35CCG-N13 and N34CCG-N13 are shown in gold. The structure of the minimal thermostable ectodomain core of HIV-1 gp41 is taken from Tan et al.,22 and the structures of NCCG-gp41, N35CCG-N13 and N34CCG are modeled on the basis of crystal structures of the HIV-1 gp41 ectodomain core.20,21 (b) SDS-PAGE of non-reduced NCCG-gp41 (lane 1), N35CCG-N13 (lane 2), N34CCG (Lane 3) and gp41 core (lane 4) on 20% homogeneous PhastGel (Amersham Biosciences, Piscataway, NJ). M denotes molecular mass markers (Amersham Biosciences low molecular mass calibration kit). Note that NCCG-gp41 is not expressed with a His tag whereas the other three proteins are.12 Note also that the molecular mass of the gp41 core construct on the denaturing gel reflects the molecular mass of a single chain, whereas the molecular mass of the other three constructs reflect three chains, since they are covalently linked by disulfide bridges. Details of the design, construction, expression, purification and folding of NCCG-gp41, N35CCG-N13 and N34CCG have been described elsewhere.11,12 The presence of a His tag at the N terminus of N35CCG-N13 and N34CCG facilitates purification before and after protein folding.12 To obtain N35CCG-N13 and N3CCG without any possible contamination with the C34 peptide (residues 628-661 of HIV-1 LAV) used for initial folding, the protein/C34 complex was denatured in a final concentration of 7.5 M guanidine hydrochloride, bound to Ni-NTA-agarose and washed excessively in 6 M guanidine hydrochloride, 50 mM Tris-HCl (pH 8). The protein was then eluted from the Ni-NTA column, concentrated, subjected to size-exclusion chromatography on Superdex-75, and folded as described.12 To produce the minimal thermostable ectodomain core construct, an Ndel site was introduced preceding the N34 sequence in the NCCG-gp41 construct.11 This intermediate construct was digested with Ndel and BamHI endonucleases to derive the DNA insert encoding the N34-linker-C28 ectodomain core domain,22 which was then cloned into a pET15b vector (Novagen, Madison, WI). Expression of the His-gp41 core construct was carried out as described for the other three constructs. Cells were lysed by sonication in 6 M guanidine HCl. 50 mM Tris–HCl (pH 8), 1 mM β-mercaptoethanol. Following clarification of the lysate by centrifugation at 16,000 rpm (Sorval SS-34) for 30 min at 18°C, the supernatant was subjected to Ni-NTA-agarose affinity chromatography. The His-gp41 core protein fraction eluted from the column in 0.2 M imidazole was further purified by reverse-phase HPLC chromatography on a POROS 20 HQ column (Applied Biosystems, Foster City, CA). The peak fraction was estimated for protein concentration and folded (~0.15 mg/ml) by extensive dialysis against 50 mM sodium formate (pH 3), concentrated and stored at 4°C.
Table 1. Selection strategies for the target antigens N_{CCG-gp41} and N35_{CCG-N13}

<table>
<thead>
<tr>
<th>Antigen</th>
<th>N_{CCG-gp41}</th>
<th>N_{35_{CCG-N13}}</th>
<th>N35_{CCG-N13}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking of phage library</td>
<td>BSA</td>
<td>BSA</td>
<td>BSA + N_{CCG-gp41}</td>
</tr>
<tr>
<td>Hits</td>
<td>23</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Clones analyzed</td>
<td>368</td>
<td>368</td>
<td>368</td>
</tr>
<tr>
<td>Total number of unique Fabs</td>
<td>17</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Specificity in ELISAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fabs specific for N_{CCG-gp41}</td>
<td>11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fabs specific for N35_{CCG-N13}</td>
<td>–</td>
<td>–</td>
<td>1*</td>
</tr>
<tr>
<td>Fabs binding to both antigens</td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* BSA, bovine serum albumin.

** The rationale behind the selection strategy using the N35_{CCG-N13} antigen with concomitant blocking of the phage library with N_{CCG-gp41} is that it permits one to select for an epitope around the C-terminal end of the N35 segment that is not accessible in N_{CCG-gp41} owing to steric obstruction from the C-HR helix of the ectodomain core.

** The Fabs were also probed in ELISAs against a series of control antigens, none of which showed any signal above background. The control antigens employed were BSA, ubiquitin, N1-CD33-His<sub>6</sub> and apotransferrin.

** The two Fabs with the largest signal above background on ELISA were chosen for further study: #1014 and #1019.

** Corresponds to Fab #1492.

** Four of the six Fabs were chosen for further study: #1010, #1018, #1020 and #1022.

** Corresponds to Fab #1034.

546–558), thereby duplicating the first 13 residues of N35_{CCG}. N35_{CCG-N13} also forms a disulfide-linked trimeric coiled-coil and presents only a single target; namely, the stably folded N-HR trimeric coiled-coil. The two antigens were immobilized at a concentration of 60 μg/ml in 50 mM sodium formate (pH 3.5) by passive adsorption onto a microtiter plate. The selection strategy is summarized in Table 1. After panning, the enriched pool of Fab genes was isolated and inserted into an Escherichia coli vector expressing functional periplasmic bivalent so-called mini-antibodies. Each mini-antibody comprises a small homodimerization domain (dHLX), a Myc tag, and a His<sub>6</sub> tag at the C terminus of the heavy chain (Fab-DHLX-MH). After transformation of E. coli TG1<sup>r</sup> (strain TG1 depleted for the F-pilus) with the ligated expression vectors, individual colonies were picked randomly and grown in microtiter plates. After induction of antibody expression with 1 mM isopropyl-thiogalactopyranoside (IPTG) overnight at 22°C, the cultures were lysed, and the crude extracts were tested against immobilized antigen in an ELISA format for the presence of binding antibody fragments. All clones with a signal at least fivefold over background in the ELISA were considered as hits and the sequence of their V<sub>H</sub> CDR3 region was determined. Colonies containing antibodies with unique HCDR3 sequences were chosen for subsequent purification. Of these, the two antibodies derived from the panning against N35_{CCG-N13} and the six antibodies with the strongest signal in ELISA of the 17 antibodies obtained from the panning against N_{CCG-gp41} were chosen for further study.

Purified bivalent Fabs were probed by Western blot analysis against four antigens: N_{CCG-gp41}, N35_{CCG-N13}, N34_{CCG} and the gp41 thermostable ectodomain core. The results are summarized in Figure 2(a) and (b). Three classes of specificity were defined: class A interacts predominantly with 6-HB; class B (#1010, #1018, #1020 and #1022) interacts with both 6-HB and trimeric N-HR; and class C (#1034 and #1492) interacts specifically with the trimeric N-HR. Note that identical specificities on Western blots were obtained for the monovalent forms of these Fabs (comprising a M2 FLAG tag and a StrpII tag at the C terminus of the heavy chain). Classes A and B were derived from the panning against N_{CCG-gp41}, while class C was obtained from selection against N35_{CCG-N13}. In the 6-HB gp41 core, a portion of the surface of the N-helices is exposed between the C-helices. Thus, the three classes of specificity define three broad epitopes: class A interacts predominantly with the exposed surface on the C-helices of the 6-HB; class B interacts with a surface comprising the exposed regions of both the N-HR and C-HR helices on the 6-HB; and class C interacts only with the trimeric coiled-coil of the N-HR. For the two class C Fabs, the specificities can be broken down further, since #1034 interacts with both N35_{CCG-N13} and N_{CCG-gp41} approximately equally, while #1492, for which N_{CCG-gp41} was used to block the phage library during the selection procedure, reacts strongly with N_{CCG-N13} but very weakly with N_{CCG-gp41} (not visible on the Western blot shown in Figure 2(a), and only barely detectable upon higher exposure (Figure 2(b)). This implies that #1492 must recognize an epitope close to the C-terminal end of the N35_{CCG} segment that is minimally accessible for antibody binding in N_{CCG-gp41}.

The ability of the bivalent Fabs to inhibit fusion was tested in a quantitative vaccinia virus-based reporter gene assay for HIV-1 envelope-mediated cell fusion using envelope from the T tropic strain LAV and soluble CD4. Examples of fusion inhibition curves are shown in Figure 3 and the IC<sub>50</sub> values are summarized in Table 2. The most potent antibodies belong to class B, with IC<sub>50</sub> ranging from 6 μg/ml to 40 μg/ml, with two mini-antibodies, #1010 and #1018, having IC<sub>50</sub> values of...
6–7 μg/ml. The two class C antibodies have intermediate IC_{50} values in the range 15–25 μg/ml. The weakest fusion inhibitors are from class A with IC_{50} ranging from 35 μg/ml to 60 μg/ml. We also tested monovalent versions of three of the Fabs in the fusion assay: the IC_{50} values for the monovalent forms of #1010 and #1034 were approximately an order of magnitude higher than for the bivalent forms, while the IC_{50} for monovalent #1034 was about twofold higher. Thus, increasing the valency...
Figure 3. Inhibition of HIV-1 LAV Env-mediated cell fusion by representatives of Fabs from class A (#1014, black), class B (#1018, red), and class C (#1492, blue). The circles represent the experimental data; the vertical bars represent the standard deviations of the experimental data; and the continuous lines represent the fits to the experimental data using the simple activity relationship:
\[
\% \text{ fusion} = 100 / (1 + [\text{Fab}]/\text{IC}_{50}).
\]
Inhibition of Env-mediated cell fusion was carried out as described previously, using a modification of the vaccinia virus-based reporter gene assay employing soluble CD4.9-13,24 B-SC-1 cells were used for both target and effector cells. Target cells were co-infected with recombinant vaccinia viruses vCB21R-LacZ (encoding β-galactosidase) and vCBYF1-fusin (encoding the chemokine coreceptor CCR5).25 and effector cells with vCB4134 (encoding Env from HIV-1 LAV) and vPI1T7gene1 (encoding phage T7 polymerase) at a multiplicity of infection of about 2.5. Following infection for 1.5 h at 37°C, cells were incubated for 18 h at 32°C to allow vaccinia virus-mediated expression of recombinant proteins. For inhibition studies of Env-mediated cell fusion, Fabs were added to an appropriate volume of Dulbecco’s modified Eagle’s medium (DMEM), 2.5% (v/v) fetal calf serum and 10 mM phosphate buffer (pH 7.5) to yield identical buffer compositions (100 µl), followed by addition of 10^5 effector cells in 50 µl of medium and 10^5 target cells in 50 µl of medium per well. Recombinant soluble CD4 (Progenics Pharmaceuticals, Tarrytown, NY) was added to the medium of the target cells at a concentration of 800 nM to yield a final concentration of 200 nM soluble CD4 per well. Following incubation for 2.5 h at 37°C, the assay plates were frozen overnight. The β-galactosidase activity of cell lysates was determined by measurement of the absorbance at 570 nm (Molecular Devices 96-well spectrophotometer) upon addition of chlorophenol-red-β-D-galactopyranoside (Roche, Nutley, NJ).

Table 2. Inhibition of HIV-1 LAV Env-mediated cell fusion by monoclonal bivalent Fabs derived from the HuCAL Gold non-immune phage library of human antibody genes

<table>
<thead>
<tr>
<th>Fab</th>
<th>Class</th>
<th>IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1014</td>
<td>A</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>1019</td>
<td>A</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>1010</td>
<td>B</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>1018</td>
<td>B</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>1020</td>
<td>B</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>1022</td>
<td>B</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>1034</td>
<td>C</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>1492</td>
<td>C</td>
<td>25 ± 2</td>
</tr>
</tbody>
</table>

Acknowledgements

This work was supported by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health and by the Intramural Program of the NIH, National Institute of Allergy and Infectious Diseases.
References


Edited by M. F. Summers

(Received 3 August 2005; received in revised form 14 September 2005; accepted 15 September 2005)

Available online 30 September 2005