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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

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Using a human non-immune phage library comprising more than 10^9 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, N_{CCG}-gp41 and $N35_{CCG}$-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: N_{CCG}-gp41, $N35_{CCG}$-N13, $N34_{CCG}$ (a smaller version of $N35_{CCG}$-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 IC_{50} 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 T 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.

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Keywords: HIV-1 fusion; Virus; HIV envelope; gp41; fusion inhibitors

Human immunodeficiency virus (HIV) envelope (Env)-mediated cell fusion represents the first step of HIV infection and therefore constitutes a potential target for therapeutic intervention.[1–3] Env comprises two glycoproteins, gp120 and gp41. The fusion process involves a set of complex events triggered by the binding of gp120 to two cell-surface receptors, CD4 and a chemokine coreceptor. This initiates a series of conformational changes in both gp120 and gp41 that leads to insertion of the gp41 N-terminal fusion peptide into the target cell membrane, and conversion of the gp41 ectodomain from an extended pre-hairpin intermediate state into a fusogenic six-helix bundle (6-HB), which drives apposition of the viral and target cell membranes and ultimately cell fusion.[3] In the pre-hairpin intermediate state of gp41,[4–6] the internal helical trimeric coiled-coil formed by the N-terminal heptad repeat (N-HR, residues 542–591 of HIV-1 Env) and the C-terminal heptad repeat (C-HR, residues 628–661) do not interact with one another and therefore each can be targeted by various classes of gp41-directed fusion inhibitors.[7–13] Peptides derived from the C-HR bind to the

Abbreviations used: HIV, human immunodeficiency virus; Env, envelope; 6-HB, six-helix bundle; mAb, monoclonal antibody.

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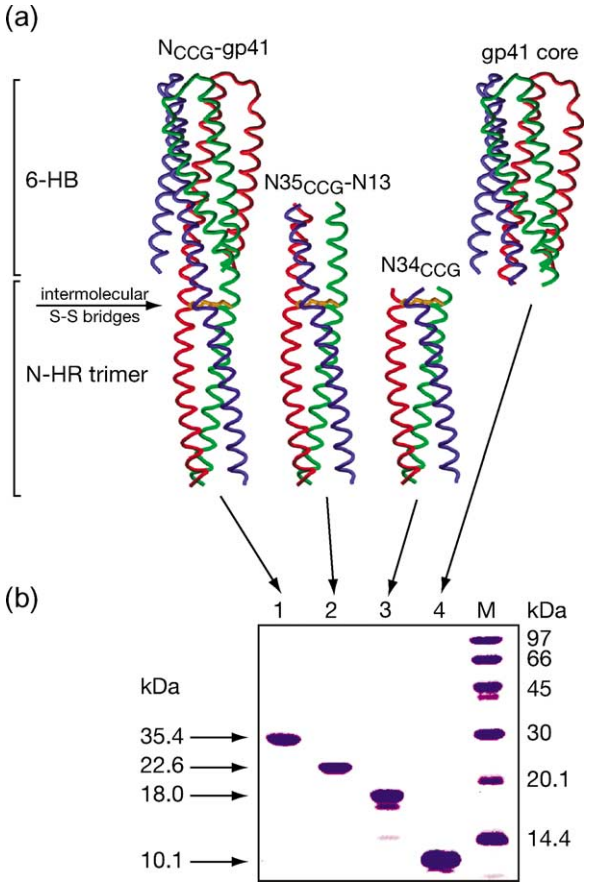

Figure 1. Antigens used for antibody selection and Western blot analysis. (a) N_{CCG}-gp41 and $N35_{CCG}$-N13 were used for antibody selection, and N_{CCG}-gp41, $N35_{CCG}$-N13, $N34_{CCG}$ and gp41 core were used for Western blot analysis. The three chains of the trimer are depicted as ribbons in red, blue and green, and the intermolecular disulfide bridges that covalently link the chains in N_{CCG}-gp41, $N35_{CCG}$-N13 and $N34_{CCG}$-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 N_{CCG}-gp41, $N35_{CCG}$-N13 and $N34_{CCG}$ are modeled[11,12] on the basis of crystal strutures of the HIV-1 gp41 ectodomain core.[20,21] (b) SDS-PAGE of non-reduced N_{CCG}-gp41 (lane 1), $N35_{CCG}$-N13 (lane 2), $N34_{CCG}$ (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 N_{CCG}-gp41 is not expressed with a His tag,[11] 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 N_{CCG}-gp41, $N35_{CCG}$-N13 and $N34_{CCG}$ have been described elsewhere.[11,12] The presence of a His_6 tag at the N terminus of $N35_{CCG}$-N13 and $N34_{CCG}$ facilitates purification before and after protein folding.[12] To obtain $N35_{CCG}$-N13 and $N3_{CCG}$ 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 NdeI site was introduced preceding the N34 sequence in the N_{CCG}-gp41 construct.[11] This intermediate construct was digested with NdeI 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_6-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_6-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.

exposed N-HR trimeric coiled-coil,[7–9] while engineered constructs that present the internal trimeric coiled-coil of the N-HR in a stable manner target the C-HR of the pre-hairpin intermediate of gp41.[11,12] We showed recently that a polyclonal antibody raised against a construct that exposed the N-HR in a stable trimeric form was able to inhibit HIV-1 Env-mediated cell fusion.[12] Here, we have made use of two gp41-derived constructs to select a set of monoclonal mini-antibodies from a non-immune human phage library comprising more than 10^9 functional human specifies. These mini-antibodies bind to diverse epitopes on gp41 and inhibit, to varying degrees, HIV-1 Env-mediated cell fusion.

Recombinant antibodies were generated from the HuCAL GOLD (Antibodies by Design, Munich) collection of human antibody genes,[14,15] using three rounds of selection (panning) on two immobilized gp41-derived constructs, N_{CCG}-gp41[11] and $N35_{CCG}$-N13[12] (Figure 1), as described.[16,17] N_{CCG}-gp41 is a chimeric protein comprising $N35_{CCG}$ (residues 546–580 of gp41 HIV-1 Env with Leu576, Gln577 and Ala578 substituted by Cys, Cys and Gly, respectively) fused onto the minimal thermostable ectodomain core of gp41.[11] Thus, each chain of N_{CCG}-gp41 comprises $N35_{CCG}$-N34-(L6)-C28, where N34 and C28 represent portions of the N-HR and C-HR regions of gp41 (residues 546–579 and 628–655, respectively) and L6 is a six-residue linker (SGGRGG). Three chains of N_{CCG}-gp41 are linked covalently *via* three intermolecular disulfide bridges to form a stable trimer. Thus, N_{CCG}-gp41 presents two targets: a stably folded N-HR trimeric coiled-coil and the 6-HB of the fusogenic form of gp41. $N35_{CCG}$-N13 is a 48-residue polypeptide that was subcloned from N_{CCG}-gp41 and comprises $N35_{CCG}$ immediately followed by N13 (residues

Table 1. Selection strategies for the target antigens N_{CCG}-gp41 and $N35_{CCG}$-N13

	Antigen		
	N_{CCG}-gp41	$N35_{CCG}$-N13	$N35_{CCG}$-N13
Blocking of phage library	BSA[a]	BSA	BSA + N_{CCG}-gp41[b]
Hits	23	1	7
Clones analyzed	368	368	368
Total number of unique Fabs	17	1	1
Specificity in ELISAs[c]			
Fabs specific for N_{CCG}-gp41	11[d]	–	–
Fabs specific for $N35_{CCG}$-N13	–	–	1[e]
Fabs binding to both antigens	6[f]	1[g]	–

[a] BSA, bovine serum albumin.
[b] 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.
[c] 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_6 and apotransferrin.
[d] The two Fabs with the largest signal above background on ELISA were chosen for further study: #1014 and #1019.
[e] Corresponds to Fab #1492.
[f] Four of the six Fabs were chosen for further study: #1010, #1018, #1020 and #1022.
[g] Corresponds to Fab #1034.

546–558), thereby duplicating the first 13 residues of $N35_{CCG}$.[12] $N35_{CCG}$-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.[18] Each mini-antibody comprises a small homodimerization domain (dHLX), a Myc tag, and a His_6 tag at the C terminus of the heavy chain (Fab-DHLX-MH). After transformation of *E. coli* TG1F$^-$ (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-β-D-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 of at least fivefold over background in the ELISA were considered as hits and the sequence of their V_H 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 observed: class A (#1014 and #1019) 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).[19] Classes A and B were derived from the panning against N_{CCG}-g41, 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.[20–23] 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.[11–13,24] Examples of fusion inhibition curves are shown in Figure 3 and the IC_{50} values are summarized in Table 2. The most potent antibodies belong to class B, with IC_{50} ranging from 6 μg/ml to 40 μg/ml, with two mini-antibodies, #1010 and #1018, having IC_{50} values of

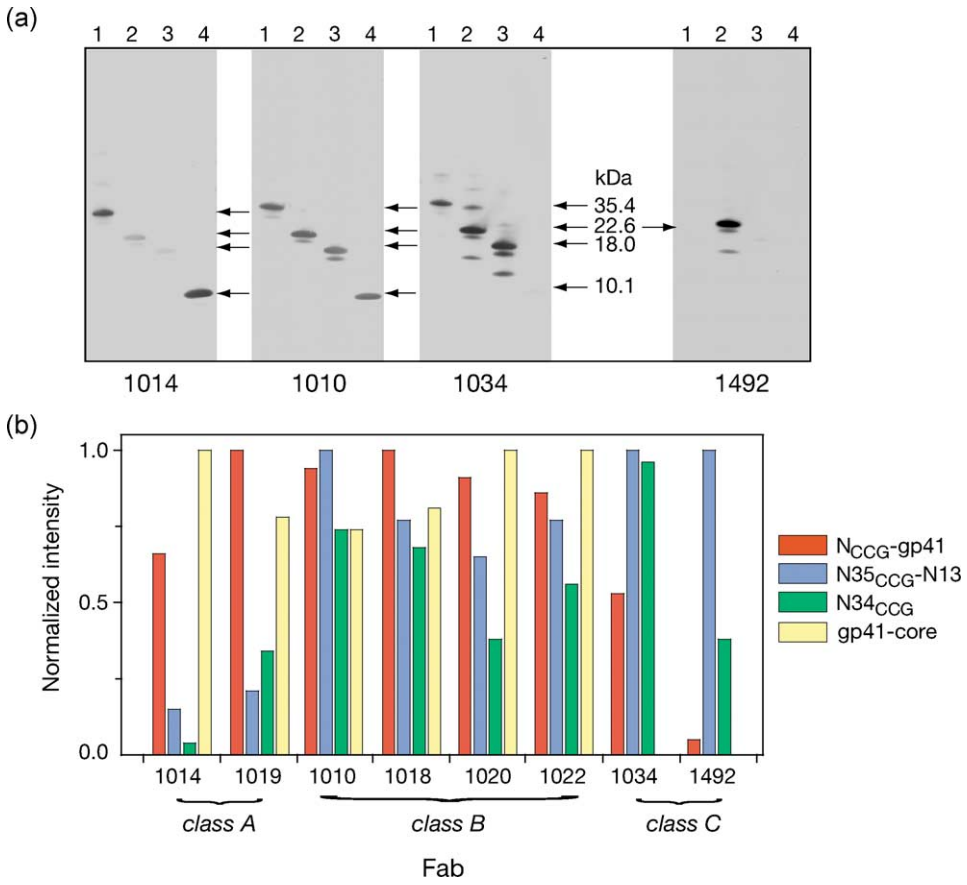

Figure 2. Specificities of Fabs selected against HIV-1 gp41-derived constructs. (a) Western blot analysis of non-reduced $N35_{CCG}$-gp41 (lane 1), $N35_{CCG}$-N13 (Lane 2), $N34_{CCG}$ (lane 3) and gp41 core (lane 4) electrophoresed and immunoblotted with bivalent Fabs #1014, #1010, #1034 and #1492. #1014 is representative of class A, #1010 of class B, and #1034 and #1492 of class C. Note that #1492 reacts much more strongly with $N35_{CCG}$-N13 than with either $N34_{CCG}$ or N_{CCG}-gp41, whereas #1034 reacts similarly with $N35_{CCG}$-N13, $N34_{CCG}$ and N_{CCG}-gp41. See Blue molecular mass markers (Invitrogen, Carlsbad, CA) were used both for calibration and monitoring the efficiency of transfer of proteins onto the membrane. (b) Summary of Western blot data for all bivalent Fabs tested. For each Fab, the intensities of the N_{CCG}-gp41 (red), $N35_{CCG}$-gp41 (blue), $N34_{CCG}$ (green) and gp41 core (yellow) bands are normalized relative to the most intense band in that particular immunoblot. The blot probed with #1492 in (b) represents a duplicate that was developed for a longer period of time than that used for the blot shown in (a) to enable measurement of N_{CCG}-gp41 and $N34_{CCG}$ band intensities relative to that for $N35_{CCG}$-N13. For Fab expression, *E. coli* $TG1F^-$ cultures (250 ml) containing the chosen antibody genes were grown at 30 °C to an $A_{600\ nm}$ of 0.5, and antibody expression was induced by adding IPTG to a final concentration of 0.5 mM. After further incubation for at least 14 h at 30 °C, the cells were harvested, lysed, and the soluble crude extract was subjected to one-step Ni-NTA agarose purification. After elution from the column, the purified antibodies were changed from elution buffer to PBS (pH 7.4), and the concentration was determined by $UV_{280\ nm}$ measurement. Fab purity and activity was tested subsequently by staining with Coomassie brilliant blue following SDS-PAGE and by ELISA, respectively. For Western blot analysis, the four proteins (500 ng/lane) in their non-reduced form were electrophoresed and transferred onto a membrane as described.[12] All subsequent steps were carried out at room temperature. The blot was incubated in TBST (Tris-buffered saline (TBS) containing 0.05% (v/v) Tween-20) and 10% (w/v) bovine serum albumin (BSA) for 1 h. Following three washes in TBST for 5 min each, the blot was incubated for 1 h in TBST containing 1% BSA and 5 μg/ml of antibody. Following three washes in TBST, the blot was incubated for 1 h in TBST containing 1% BSA and 0.3 μg of goat anti-human anti-Fab antibody-alkaline phosphatase conjugate (Jackson ImmunoResearch). The blot was again washed three times in TBST and subsequently developed in Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma, one tablet of FAST BCIP/NBT dissolved in 20 ml of TBST, 1% BSA). Upon acquiring the desired signal, the enzymatic reaction was terminated by rinsing the blot extensively in deionized water. Signal intensities were quantified using the NIH image software (V 1.6.1; rsb.info.nih.gov/nih.image). Under these conditions, band intensity is linearly proportional to protein concentration up to 1 μg/lane of $N35_{CCG}$-N13 and up to 0.6 μg/lane of N_{CCG}-gp41 probed with #1492 and #1019, respectively.

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[25] of three of the Fabs in the fusion assay: the IC_{50} values for the monovalent forms of #1010 and 1018 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

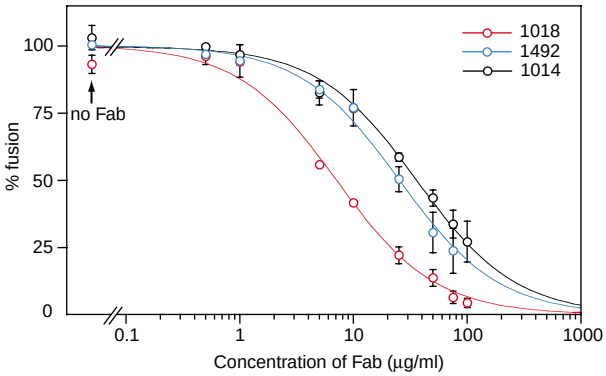

Figure 3. Inhibition of HIV-1 LAV Env-mediated cell fusion by representatives of Fabs from class A (#1014, black), class B (#1018, red), and class C (#1492, blue). The circles represent the experimental data; the vertical bars represent the standard deviations of the experimental data; and the continuous lines represent the fits to the experimental data using the simple activity relationship % fusion = $100/(1+[\text{Fab}]/IC_{50})$. 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.[9,11–13,24] 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 CXCR4),[33] and effector cells with vCB41[34] (encoding Env from HIV-1 Lav) and vP11T7gene1 (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 from 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

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

of the Fabs contributes significantly to their avidity in the context of the fusion assay.

It is of interest to compare the epitope locations and fusion inhibitory activity of the bivalent Fabs described here, with those of previously described, broadly neutralizing monoclonal antibodies (mAbs) directed against gp41. Two classes of the latter involving two distinct epitopes have been described.[25] The first epitope, known as cluster I, is located in the long loop connecting the N-HR and C-HR regions of native gp41 and spans a ten residue sequence containing two cysteine residues. While this epitope is highly immunogenic, only one human mAb from this class, known as clone 3, is neutralizing.[25–28] The second epitope, which is poorly immunogenic, comprises the membrane proximal region (residues 662–667) just C-terminal to the C-HR.[25] Representative, broadly neutralizing human mAbs from this class include 2F5, 4E10 and Z13.[25,29–31] Neither of these two epitopes is present in the antigen constructs employed here (i.e. there is absolutely no overlap between the epitopes targeted by the bivalent Fabs reported here and the previously reported gp41 epitopes for broadly neutralizing anitibodies). The viral neutralizing activity of the broadly neutralizing anti-gp41 mAbs are comparable and broadly similar to 2G12, a broadly neutralizing mAb directed against gp120.[29] In the quantitative vaccinia virus-based reporter gene assay for HIV-1 Env-mediated cell fusion assay employed here, the IC_{50} values for 2F5 and 2G12 are in the range 0.5–1.5 μg/ml (C.A.B., unpublished results),[12] which is approximately an order of magnitude more potent than the two best bivalent Fabs reported here (#1010 and #1018) which have IC_{50} values of 6–7 μg/ml. By way of contrast, the IC_{50} for the mouse MAb NC-1, which was derived from antibodies raised against the ectodomain core of gp41,[32] is 220(±30) μg/ml in the same assay, consistent with previous data indicating that NC-1 was an ineffective fusion inhibitor.

In conclusion, we have been successful in obtaining a series of monoclonal mini-antibodies from a human non-immune phage library that target diverse epitopes on the gp41 ectodomain, and shown that some of these are reasonably potent inhibitors of Env-mediated cell fusion. This set of mini-antibodies should provide useful probes for further analyzing the mechanistic details of Env-mediated cell fusion, and may form the basis for deriving therapeutically useful antibody-based HIV-1 fusion inhibitors.

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