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A SIMPLE ONE-STEP METHOD FOR THE PREPARATION OF HIV-1 ENVELOPE GLYCOPROTEIN IMMUNOGENS BASED ON A CD4 MIMIC PEPTIDE

Grégoire Martin[a], **Yide Sun**[b], **Bernadette Heyd**[a], **Olivier Combes**[a], **Jeffrey B. Ulmer**[b], **Anne Descours**[a], **Susan W. Barnett**[b], **Indresh K. Srivastava**[b], and **Loïc Martin**[a,*]

a*CEA, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, Gif sur Yvette, F-91191, France.*

b*Novartis Vaccines and Diagnostics Corporation, Emeryville, California 94608.*

Abstract

To counteract the problems associated with the purification of HIV envelopes, we developed a new purification method exploiting the high affinity of a peptide mimicking CD4 towards the viral glycoprotein. This miniCD4 was used as a ligand in affinity chromatography and allowed the separation in one step of HIV envelope monomer from cell supernatant and the capture of pre-purified trimer. This simple and robust method of purification yielded to active and intact HIV envelopes as proved by the binding of CCR5 HIV co-receptor, CD4 and a panel of well-characterized monoclonal antibodies. The immunogenicity of miniCD4-purified HIV envelope was further assessed in rats. The analysis of the humoral response indicated that elicited antibodies were able to recognize a broad range of HIV envelopes. Finally, this method based on a chemically synthesized peptide may represent a convenient and versatile tool for protein purification compatible far scale-up in both academic and pharmaceutical researches.

Keywords

HIV1; Monomer; Trimer; CD4 mimic; Affinity chromatography; AIDS vaccine

Introduction

Acquired immunodeficiency syndrome (AIDS) continues to be a major health problem throughout the world, with in 2007 approximately 33 million people infected with the human immunodeficiency virus type 1 (HIV-1), 2.5 million new infections, and 2.1 million people died of AIDS-related illnesses (UNAIDS/WHO, 2007). Therefore, there is an urgent need for an effective anti-HIV vaccine. The HIV-1 envelope glycoprotein (Env) is the target of broadly neutralizing antibodies and should be an important component of a successful HIV vaccine (Girard et al., 2006; Phogat and Wyatt, 2007). Env is formed by gp120 and gp41 that result of the cleavage of gp160 precursor (McCune et al., 1988). These two subunits are noncovalently associated in a trimeric structure (Wyatt and Sodroski, 1998). In order to mimic the native form

*Corresponding author. Telephone and Fax numbers: (+33)-169087133, (+33)- 169089071, e-mail: loic.martin@cea.fr, (L. MARTIN); address: CEA, iBiTecS, SIMOPRO, Bât 152, Gif sur Yvette, F-91191, France.

of HIV-1 envelope, gp140 non-covalent trimer lacking gp41 trans-membrane domain has been engineered (Jeffs et al., 2004; Kieny et al., 1988; Sanders et al., 2002; Srivastava et al., 2002, 2003).

Recombinant HIV-1 glycoproteins are produced at high scale in both trimeric and monomeric forms for pre-clinical and clinical researches on HIV vaccine. Several studies have demonstrated that gp140 is particularly attractive as potent immunogen and that oligomers are more effective in inducing strong antibody responses to conformational epitopes compared to monomer (Barnett et al., 2008; Mascola et al., 1996; Srivastava et al,. 2003; Zhang et al., 2007). Indeed, the few monoclonal antibodies (mAbs) that potently neutralize HIV-1 all recognize epitopes exposed on the native Env complex (Burton and Parren, 2000, Fouts et al., 1998; Parren et al., 1998; Sattentau and Moore, 1995). In contrast, nonneutralizing mAbs that do not bind to the native complex have been found following vaccination with gp120 monomer (Burton and Parren, 2000, Parren et al., 1998). Therefore it is important to produce Env oligomers with intact epitopes and functional receptor binding sites for inclusion in a potential HIV-vaccine.

In order to address this point, we have developed a new method of purification of HIV-1 Env suitable for the capture of gp120 or gp140. There are a few pre-existing methods for the purification and the preparation of active Env. Two main approaches can be pinpointed. First, lectin is utilized in affinity chromatography to capture glycoproteins (Cefai et al., 1990; Jeffs et al., 2004; Jones et al., 1995, Srivastava et al., 2002, 2003). Unfortunately, this approach is unspecific to HIV-1 Env and requires several additional steps of purification such as ion exchange and size exclusion chromatography. Second, Env glycoproteins are purified with anti-gp120 or anti-gp140 mAbs (Jeffs et al., 2004; Kalyanaraman et al., 1990; Kwong et al, 1999). This approach presents several difficulties. Antibody-based methods are expensive and necessitate the coupling of anti-Env antibody to a solid support, which may reduce its affinity for Env. Moreover, there are few antibodies suitable for the purification of a large panel of envelope; and the half-life of antibody is relatively limited, particularly when used in acidic conditions.

Our objective was to define a new method of purification of HIV envelope, which was simple, robust, not expensive, suitable for envelope monomer or trimer and above all which preserved HIV envelope antigenicity and its ability to bind both CD4 and chemokine receptor. Therefore, we investigated the usefulness of a peptide mimicking the human receptor CD4 as a ligand in affinity chromatography. MiniCD4 peptides were initially obtained by the transfer of critical elements of CD4/gp120 interface onto a structurally compatible small scaffold (Martin et al., 2003; Vita et al., 1999). Their affinity towards gp120 and gp140 had been improved by combinatorial chemistry and directed amino-acid substitution based on the crystal structure of the complex gp120-miniCD4 (Huang et al., 2005; Stricher et al., 2005, 2008; Van Herrewege et al., 2008). In the affinity chromatography method presented here, we used CD4M48 an optimized version of miniCD4 that presents a sub-nanomolar affinity for SF162 isolate and a CD4-like affinity for clade B envelopes in neutralization assays (Stricher et al., 2008; Van Herrewege et al., 2008). The miniCD4 was grafted to a solid support and evaluated for its ability to purify gp120SF162 and gp120YU2 from mammalian cells supernatant. In parallel, the peptide was assessed for its capacity to capture gp140SF162 trimer. The yield of purification of each HIV-1 Env was measured and antigenic properties of miniCD4-purified Env were evaluated through the binding of CD4, CCR5 co-receptor and a panel of well-characterized neutralizing monoclonal antibodies. Finally, the immunogenicity of miniCD4-purified gp120YU2 was evaluated in a small scale animal trial.

Results

Purification of monomeric HIV envelope by miniCD4

A miniCD4-based affinity chromatography was designed to purify monomeric HIV-1 envelope from supernatant of cell over-expressing gp120SF162. 500 µl of cell supernatant (approximate concentration of gp120SF162: 150 mg/L) was incubated with 500 µl of miniCD4-beads. Bound proteins were then eluted in 1.5 ml of citric acid instantaneously neutralized by citrate buffer (see materials and methods).

The yield of miniCD4-affinity chromatography was evaluated by a CD4 binding assay through the measurement of the concentration of active gp120SF162 in cell supernatant and in the three fractions eluted from miniCD4-beads (Table 1). Typically, cell supernatant or eluted fractions (named E1–E3) were incubated in the presence of immobilized D7324 anti-gp120 mAb. Bound proteins were then detected after three subsequent incubations with (i) soluble CD4 (sCD4), (ii) L120.3 anti-CD4 mAb, (iii) a HRP labeled antibody directed against L120.3 mAb. Concentrations of gp120SF162 before and after miniCD4 capture were evaluated by comparison to several dilutions of gp120SF162 at known concentrations (gp120SF162 purified with the multi-step method). In the whole study, the multi-step method of purification of HIV envelope was use as a method of reference (see materials and methods). This method involving a capture and two cleaning steps had been shown to efficiently purify both HIV envelope monomer and trimer (Srivastava et al., 2002,2003). We carried out 9 independent experiments and obtained 55.8 % as average yield of purification with the miniCD4 affinity chromatography method.

At the same time, captured proteins were analyzed by SDS-PAGE, immunoblot, and size exclusion chromatography (SEC) (Fig. 1). Purification of gp120SF162 was achieved with a degree of purity of approximately 60% as shown by SDS-PAGE followed by silver staining (Fig. 1A lane 2). The major contaminant was shown to be a serum protein, identified by N-terminal sequencing as bovine serum albumin precursor (Fig. 1A lane 2). The identity of HIV envelope was confirmed by immunoblot using anti-gp120 D7324 mAb (Fig. 1A lane 3). MiniCD4-purified gp120SF162 was further analyzed on a pre-calibrated Bio-Sil 250 column (Fig. 1B). Consistent with SDS-PAGE analysis, the elution profile shows that only a low amount of aggregate was eluted in death volume and peak A fractions. The elution volume of peak C fractions corresponds to protein with gp120 molecular weight (7.4 ml). Peak B fractions could be composed of gp120 aggregates or by non-envelope proteins found in cell supernatant, which possess a size of 300 KDa (6.5 ml). The fractions constituting peak C were pooled and analyzed by SDS-PAGE (Fig. 1C). Silver staining revealed a purity of gp120SF162 estimated at > 90 % by densitometric scanning.

Binding of miniCD4-purified gp120SF162 to CCR5 receptor

The interaction of CD4 with gp120 allows the subsequent binding of gp120 to a chemokine receptor through an important conformational change. In order to evaluate the activity of gp120SF162 purified from miniCD4 affinity chromatography, HIV envelope was pre-incubated or not with soluble CD4 (sCD4) and then assessed for its binding activity towards CCR5 chemokine receptor. Addition of sCD4 increased the binding of gp120SF162 purified by miniCD4 affinity chromatography to CCR5 receptor (Fig. 2, comparison of lane 1 and lane 3), leading to a binding activity comparable to gp120 purified by the reference method and associated with sCD4 (Fig. 2, lane 3 compared to lane 4).

Immunochemical characterization of miniCD4-purified gp120SF162

The structural integrity of gp120SF162 purified with the miniCD4 affinity chromatography method was further analyzed by surface immunoprobing with a panel of mAbs of defined

epitope specificities, using Surface Plasmon Resonance (SPR) and capture ELISA. Upon binding to sCD4, miniCD4-purified gp120SF162 underwent conformational changes as reflected by an increase binding of X5 and 48D CD4-induced mAbs (CD4i Abs; Fig. 3A and B). MiniCD4-purified gp120SF162 was also recognized by 44752D anti-V3 loop and b12 anti-CD4 binding site mAbs (Fig. 3C and D).

The miniCD4 affinity chromatography was further compared to the reference method. HIV envelope gp120SF162 purified by the two methods were analyzed for their ability to bind a panel of well-defined mAbs. First, the concentration of HIV envelopes was normalized through ELISA assays using D7324 mAb (Fig. 4A). Then, gp120SF162 envelopes purified by miniCD4 (named miniCD4) or by the reference method (named multi) were assessed by using mAbs directed against CD4i epitopes (X5, 48D and 17b), CD4 binding site (b12, F105), carbohydrate moieties (2G12) and V3 loop (44752D). Despite the moderate degree of purity of gp120SF162 purified in one step by miniCD4 (Fig. 1A), a similar antibody-binding was observed for both gp120SF162 glycoproteins (Fig. 4B–H), suggesting that miniCD4 allows the separation of HIV-1 envelope with intact epitopes.

Purification of trimeric gp140 by miniCD4

MiniCD4 affinity chromatography was further assessed for its ability to capture gp140 trimer. 50 μg of gp140SF162ΔV2 purified by the method of reference were diluted in 500 μl of PBS and incubated 1 hour at room temperature with an equal volume of miniCD4-beads. Then, unbound and bound proteins from three independent experiments were collected in a final volume of 500 μl each. All the fractions were further analyzed by spectrometry at 280 nm and by SPR (evaluation of gp140 binding to 48D CD4i mAb). A percentage of 92.4 ± 2.2 % of gp140 captured by miniCD4 was measured by spectrometry, a comparable yield of purification was observed by SPR.

MiniCD4-purified gp140SF162ΔV2 was further analyzed by SDS-PAGE, immunoblot and SEC (Fig. 5). MiniCD4 affinity chromatography allowed the elimination of contaminating proteins of lower molecular mass (Fig. 5A, comparison of lane 1 and lane 2). MiniCD4 affinity chromatography appeared efficient to reinforce the grade of purity of pre-purified gp140, contaminants proteins of 50–70 KDa size should be composed of Bovine Serum Albumin, as previously shown for gp120 (Fig. 5A, line 1). Consistent with the yield of purification described above, a very low proportion of protein with gp140 molecular weight was found in unbound fractions (Fig. 5A, lane 1). As expected, miniCD4-purified gp140SF162ΔV2 migrated with an apparent molecular mass of 140 kDa in reducing and denaturing conditions due to the presence of gp41 ectodomain downstream gp120 core protein.

MiniCD4-purified gp140SF162ΔV2 was further analyzed over pre-calibrated size exclusion HPLC column (Bio-Sil SEC-250, Fig. 5B, panel 2). Based on the volume of elution of gp120 (7.4 ml), gp140 (6.2 ml) and standards (670 kDa = 5.52 ml; 158 kDa = 7.12 ml; 44 kDa = 7.79 ml; 17 kDa = 9.28 ml and 1.35 kDa = 10.55 ml), the calculated molecular masses of gp120SF162 and gp140SF162ΔV2 are 132.3 and 418.3 kDa, respectively. The molecular mass of purified gp140SF162ΔV2 is the expected molecular mass for a trimer (3 × 140 kDa [monomer] = 420 kDa [trimer]), while gp120 one is slightly higher. Consistent with the previous experiments, the SEC profile of unbound fractions indicates the presence of a low proportion of gp140 and contaminating proteins of lower molecular weight (Fig. 5B, panel 1).

Immunochemical characterization of miniCD4-purified gp140SF162ΔV2

The structural integrity of gp140SF162ΔV2 purified with miniCD4 affinity chromatography was further evaluated by SPR using the panel of antibodies described for gp120SF162. MiniCD4-purified gp140 showed an increased affinity for both X5 and 48D CD4i mAbs in

the presence of sCD4 (Fig. 6A and B), and was recognized by b12 and 44752D mAbs (Fig. 6C and D, respectively). Interestingly, gp140SF162ΔV2 k_{off} is reduced for all the antibodies tested compared to gp120SF162 one (see Fig. 3 and Fig. 6), suggesting an increased avidity for gp140 consistent with the trimeric conformation observed by SEC (Fig. 5B).

As described above for gp120SF162, gp140ΔV2SF162 purified by miniCD4 was finally compared to gp140ΔV2SF162 purified by the reference method exclusively. Both gp140ΔV2SF162 concentrations were normalized in ELISA by using 2G12 mAb (Fig. 7A). Afterwards, the two trimers were analyzed for their ability to bind mAbs described above (X5, 48D, 17b, b12, F105, 44752D and MN215 mAbs). We found that SF162 trimer antigenicity was perfectly preserved by the miniCD4 method of purification (Fig. 7B–H). Taken together, these data underline the usefulness of miniCD4 chromatographic method for the purification of functional and intact Env trimer.

Immunogenicity of miniCD4-purified envelopes

MiniCD4 affinity chromatography was used to purify 1 mg of gp120YU2 from 1 liter of supernatant from cells over-expressing the viral envelope (approximate concentration of gp120YU2 in cell supernatant: 2 mg/L). In one step, gp120YU2 with a high grade of purity was obtained, as shown by SDS-PAGE, immunoblot and SEC analysis (Fig. 8A and B). Afterwards, three Wistar rats were immunized with miniCD4-purified gp120YU2 or miniCD4 peptide (used as control) using an intra-peritoneal boosting regimen. After the second and the third intramuscular boosts with gp120YU2, an important titer of antibody directed against the HIV envelope was observed. Sera from the three rats immunized by either gp120YU2 or miniCD4 were pooled and IgG were purified by affinity chromatography using G protein (Amersham). Then antibodies elicited by the immunogens were evaluated for their ability to recognize a panel of HIV-1 envelope monomers and trimers from diverse genetic sub-types (clades B, C and F). Table 2 presents the dilutions of antibody resulting in half-maximum binding to HIV-1 envelopes. MiniCD4-purified gp120YU2 elicited a high titre of antibody directed against envelopes monomers from B and C clades. However, elicited antibodies were less efficient in recognizing gp140 trimers from B, C and F clades; suggesting a certain proportion of antibody directed against envelope domains which are inaccessible on oligomer surface. This small scale immunogenicity study indicates that miniCD4-purified gp120 is suitable for vaccine candidate preparation. Furthermore, taken that miniCD4-purified envelopes possess an intact CD4 binding site, this method of purification may be particularly appropriate for the preparation of antigen aiming to induce antibodies directed against the CD4 binding site.

Discussion

To address the many problems associated with the purification of HIV envelopes and particularly the separation of intact HIV envelope trimer, we exploited the affinity of miniCD4 for gp120 and gp140 to develop an innovative method suitable for the purification of highly active and immunogenic HIV envelope.

HIV-1 envelope glycoprotein represents a major target for the development of AIDS vaccines. Initial trials of HIV-1 Env-based vaccines showed that soluble recombinant envelope glycoproteins were well-tolerated and elicited neutralizing antibodies to the homologous vaccine strain, but not to heterologous primary virus isolates (Graham and Wright, 1995; Jeffs et al., 2004; Mascola et al., 1996). Furthermore, these vaccines elicited sterilizing immunity against homologous virus and a restricted panel of heterologous virus in non-human primate models (Berman et al., 1990; Girard et al., 1995, 1996; Stott et al., 1998). Those observations had led to numerous envelope-based vaccines evaluated at both preclinical and clinical stages (Graham, 2002; Graham and Mascola 2005; Gallo 2005; Girard et al., 2006; Phogat and Wyatt,

2007). Nevertheless, the achievement of these different vaccine candidates requires important quantities of recombinant envelope monomer and/or trimer with intact epitopes and functional CD4-binding sites. Therefore, it is necessary to have a simple and reproducible method that lets the purification of active Env in good yields.

Pre-existing methods are mainly based on lectin capture or anti-gp120, anti-gp140 mAb affinity chromatography. These methods may require the conjunction of several steps of purification such as ion exchange and SEC and in most cases are specific to one HIV-1 envelope. To avoid a multi-step procedure and the use of anti-Env antibody, we used a miniCD4 peptide presenting a high affinity for gp120 and gp140 (Martin et al., 2003; Stricher et al., 2005, 2008, Van Herrewege et al., 2008; Vita et al., 1999). We showed that miniCD4 allowed the separation in one step of either gp120SF162 or gp120YU2 from cell supernatant. MiniCD4-purified gp120SF162 exhibited a high affinity for CD4, CCR5 and a panel of well-characterized mAbs directed against the CD4 binding site, CD4i epitopes and V3 loop. The affinity of miniCD4-purified gp120SF162 towards those ligands was comparable to the one of gp120SF162 purified with a multi-step procedure (Srivastava et al., 2002, 2003). Taken together, these data suggest that the use of acidic conditions for the elution of gp120SF162 does not affect the antigenic properties of the envelope glycoprotein. Finally, we performed an immunogenicity study indicating that miniCD4 method of purification is suitable for vaccine preparation, as miniCD4-purified gp120YU2 elicited anti-envelope antibodies directed against a broad range of genetic sub-types.

Taken the important need of envelope oligomers presenting intact epitopes for vaccine application, miniCD4 affinity chromatography was also evaluated for its ability to capture Env trimer. In order to achieve better immunogenecity with Env based-vaccines, attention has focused on Env oligomer rather than monomer. The association of envelope subunits in a trimeric conformation on HIV-1 surface shields potential epitopes. Thus, antibodies induced by gp120 monomer react more strongly to monomer as compared to oligomer (Burton and Parren, 2000, Broder et al., 1994; Parren et al., 1998; VanCott et al., 1995). Moreover, it has been well documented that gp120 monomers are limited in their ability to neutralize primary isolates (Mascola et al., 1996; Moore et al., 1995; VanCott et al., 1995; Wrin and Nunberg, 1994). Taken together, those observations had led to the engineering of several gp140 trimers and their evaluation as vaccine candidate (Barnett et al., 2008; Jeffs et al., 2004; Srivastava et al., 2002, 2003, 2008; Zhang et al., 2007). There are relatively few published studies that describe the purification of recombinant gp140 from eukaryotic expression systems. The non-covalent association of the different subunits forming gp140 trimer renders difficult the purification of envelope oligomers with classical method such as lectin capture and antibody-affinity chromatography. Indeed, Jeffs and others had noted the propensity of recombinant gp140/gp160 to form high molecular weight aggregates, particularly when lectin affinity chromatography is used (Binley et al., 2000; Jeffs et al., 2004; Rhodes et al., 1994). In this study, we found that miniCD4 was able to efficiently capture gp140ΔV2SF162 trimer and that miniCD4-purified trimer presented intact epitope as shown by CD4 and mAbs binding. We also showed that the grade of purity of pre-purified gp140ΔV2SF162 can be slightly increased after the miniCD4 purification step. Indeed, we showed that miniCD4-eluted gp140 was separated from lower molecular mass contaminants. Moreover, we did not observe aggregate formation following the miniCD4 affinity purification. However, to address precisely this point, miniCD4 purification should be repeated on cell supernatant of cell over-expressing gp140 rather than pre-purified oligomer.

The advantages of the use of miniCD4 in a purification method are many fold. First, miniCD4 presents a high stability compared to monoclonal antibodies. Indeed, miniCD4 is not affected by pH ranging from 2 to 9, variation of temperature from 20° to 90 °C and presents an important resistance to proteolytic degradation. Thus, miniCD4 beads can be stored at 4°C for a long

period and reused several times without significant loss in their ability to capture Env. Second, because of its well-defined 3D structure and its reduced size (27 amino acids), miniCD4 can be easily and rapidly produced by chemical synthesis and labeled with affinity tag such as biotin moiety. In addition, as miniCD4 derives from a natural mini-protein is refolding is particularly easy (see material and method part). Third, in a recent study, we and others showed that CD4M48 and, more particularly, its derivative M48-U1 were able to neutralize a broad range of primary isolates (Van Herrewege et al., 2008). Those data emphasized the high affinity of miniCD4 for oligomeric envelopes belonging to a broad spectrum of HIV-1 primary strains. Thus, CD4M48 and M48-U1 could be advantageously used to purify subtype C gp140, as well as a large panel of Env from different clades. Fourth, another advantage inherent to miniCD4-purification is the selection of protein with accessible and well-folded CD4-binding site. This region is recognized by a broadly neutralizing antibody, b12 mAb (Binley et al., 2004, Ruprecht et al., 2003) which has been shown to present protective effects in non-human primate challenged with SHIV (simian/human immunodeficiency virus) (Parren et al., 1995, 2001; Ruprecht et al., 2003; Veazey et al., 2003). A recent study has shown that b12 mAb binding site has considerable overlap with both CD4 and miniCD4 ones (Huang et al., 2005; Zhou et al., 2007), suggesting that the selection of envelopes with intact miniCD4 binding site might be particularly attractive in vaccine preparation. Finally, the use of miniCD4 in a purification method represents a new example of applications of mini-protein engineered by the transfer of active site onto small-size scaffolds. Thus, biologically active mini-proteins may become not only useful tools in biology and medical research, but also a convenient step for protein purification in academic and pharmaceutical researches.

Materials and methods

All Fmoc (fluoren-9-ylmethoxycarbonyl)-protected amino acids were from Nova Biochem (via VWR international, Fontenay-sous-Bois, France). Fmoc PAL-PEG-PS resin was from Applied Biosystems (via Applera Corporation, Norwalk, CT, U.S.A.). All the other reagents and solvents used in the synthesis and purification of CD4M48 were from Fulka (St Quentin Fallavier, France) or SDS (Solvants Documentation Sytheses, Peypin, France). PBS and Biotin and unspecified chemical products were from Sigma Chemical Co. (St Louis, MO). The matrix used to bind the biotinylated miniCD4 was a Streptavidin Sepharose™ high performance from GE Healthcare Ltd (Uppsala, Sweden), the labeled miniCD4 was grafted to the beads at 1 μM final concentration.

Peptide synthesis

The miniCD4 CD4M48 was chemically synthesized on an Applied Biosystems Synthesizer (model 433) by solid-phase method using fluorenylmethyloxycarbonyl-protected amino acids and N-hydroxybenzotriazole/dicyclohexylcarbodiimide coupling strategy (Drakopoulou et al., 1996). The three disulfide bonds were formed with the peptides dissolved at 0.1mg/ml in 50 mM phosphate buffer (pH 7.8) in the presence of sequential addition of 5 mM reduced gluthatione followed by 0.5mM oxidized glutathione. Synthetic peptide was purified by reverse-phase HPLC, and its identity was verified by amino acid analysis and electrospray mass spectrometry. The biotin moiety was specifically introduced at Lys^{11} by using *N*-α-Fmoc-N^{ε}-1-(ivDde-3-methylbutyl)-L-Lysine (where ivDde is 4,4-dimethyl-2,6-dioxocyclohex-1-ylidine) during peptide synthesis and subsequent coupling of Fmoc-8-amino-3,6-dioxaoctanoic acid (linker arm) and biotinamidohexanoic acid *N*-hydroxysuccinimide ester, after ivDde was removed by four treatments of 3 min with 2% hydrazine.

CD4M48 amino acid sequence is the following:

TpaNLHFCQLRC **(K-Biot)** SLGLLGRCAdPTFCACV

Lysine amino-acid was derivatized with a Biotin **(K- Biot)** moiety; dP was for *(D)*-proline and Tpa for thiopropionyl.

Envelope glycoproteins preparation

The CHO cell line DG-44 was used for generation of stable cell lines expressing either gp120SF162 (monomer) or o-gp140SF162ΔV2 (trimer with a partial deletion in the second variable loop V2) (Srivastava et al., 2002, 2003). The gene cassette used for the derivation of the stable cell lines contained the protein-encoding region of the envelope protein fused in frame to the human tissue plasminogen activator (t-PA) signal sequence. The signal peptide of t-PA has been previously described to increase the secretion of recombinant protein in the cell supernatant (Chapman et al., 1991).

Concerning gp120YU2, the HIV-1 envelope glycoprotein was produced in *Spodotera frugiperda* cells infected with *Autographa californica* baculovirus AcSLP10 (Mechulam et al., 2005). As previously described for the expression of HXB2, the natural signal peptide sequence of gp120YU2 was replaced by a new signal sequence isolated from the ecdysteroid glycosyltransferase (EGT) gene of AcSLP10 baculovirus in order to increase the secretion of the glycoprotein in cell supernatant (Misse et al., 1998).

MiniCD4 affinity chromatography

Cell supernatant from cells over-expressing gp120SF162 was concentrated 20-fold through a 100-kDa-pore-size membrane filter and stored at −80°C in the presence of 1 mM EDTA and 1 mM EGTA. Prior the purification process, the culture medium was filtrated over a 0.45 μm filter, and protease inhibitors were added. Afterward, 500 μl of culture medium were applied to 500 μl of miniCD4 beads (CD4M48 final concentration being approximately 1 μM) and incubated with gentle rocking overnight at 4°C. After 5 washes with 500 μl of PBS, proteins bound to the miniCD4 beads were recovered in 3 fractions of 250 μl of 25 mM Citric acid pH 3.6 each. Finally, eluted fractions were neutralized with 250 μl of 400 mM Citrate pH 6.8.

For scale-up growth, 1 liter of cell supernatant from cell infected with baculovirus producing gp120YU2 was divided in 200 ml fractions. Each fraction was then incubated with 10 ml of miniCD4 beads over night in rolling bottles at 4°C. As it was the case for the small scale analysis, the miniCD4 concentration in the mix was about 1 μM. Finally, bound material was eluted in 2.5 ml of acidic solution and neutralized with 2.5 ml of citrate solution as described above.

Multi-step method of purification used as control

HIV-1 envelope glycoprotein purified with the miniCD4 affinity chromatography was compared to Env obtained *via* another method of purification (Srivastava et al., 2002; 2003). Briefly, this multi-step procedure of purification is composed as followed, CHO cell supernatant was loaded onto a DEAE (GE Healthcare Ltd) column equilibrated with buffer (20 mM Tris, 100 mM NaCl, pH 8.0). Under these conditions, gp120SF162 and o-gp140SF162ΔV2 did not bind to the column, but contaminating proteins were retained on the column. The DEAE flowthrough was adjusted to 10 mM Phosphate and pH 6.8 and loaded on to a ceramic hydroxyapatite (CHAP, Biorad, Hercules, CA) column equilibrated with buffer (10 mM Na_2HPO_4, 100 mM NaCl, pH 6.8). Envs were recovered in the flowthrough, and the pH was adjusted to 7.4 with 2 M Tris (pH 8.8) and loaded on to a protein-A-agarose column to remove immunoglobulin contamination. The protein A-agarose flowthrough was loaded onto a *Galanthus Nivalis*-agarose (GNA) (Vector Laboratories, Burlingame, CA) column equilibrated with 20 mM Tris-100 mM NaCl (pH 7.4). Bound Envs were eluted with 500 mM methyl mannose pyranoside (Sigma Chemical Co.) in equilibrated buffer. All the fractions containing the HIV-1 envelope glycoproteins were pooled and fractioned on Superose-6 and

Superdex-200 (GE Healthcare Ltd) tandem columns equilibrated with 10 mM Na-Citrate and 500 mM NaCl. Fractions containing Envs were pooled, concentrated using a Stir cell (Millipore, Inc., Bedford, MA), and stored at −80° until used. HIV-1 envelope glycoproteins purified with this approach were used through this study as control to quantify gp120SF162 and gp120YU2 obtained with the miniCD4 affinity chromatography. Envs obtained with this multi-step purification procedure were also termed 'pre-purified Env' and used to characterize the miniCD4-based method of purification.

Immunoblot analysis

Proteins were fractionated by SDS-PAGE and electro-blotted on PVDF membrane. Transferred proteins were detected by binding of anti-gp120 D7324 mAb (Aalto Bio Reagents, Dublin, Ireland) followed by a horseradish peroxidase conjugated anti-IgG sheep Ab. Immuno-reactive protein bands were detected by autoradiography (Kodak, Sigma) using an enhanced chemiluminescence assay (ECL, Amersham Biosciences, Piscataway, NJ).

CD4 binding assay by ELISA

The capability of purified envelope protein to bind CD4, was determined by using a sandwich ELISA assay (Stricher et al., 2005). Briefly, the 96-wells plates (Maxisorb; Nunc, Rochester, NY) were coated overnight at 4°C with D7324 mAb (50 ng per well); wells were saturated with PBS/5 % BSA buffer, wash three times, then different dilutions of purified gp120SF162 or gp120YU2 were added, followed by an incubation with 62 pg of sCD4 (Progenic, Tarrytown, NY). For detection, anti-CD4 mAb L120.3 (Centralized facilities for AIDS reagents, National Institute for Biological Standards and control (NIBSC), South Mimms, Potters Bar, Herts., U.K.) was added, followed by an incubation with goat-antimouse peroxidase-conjugated antibody (Jackson Immunoresearch, West Grove, PA) and substrate (3,3',5,5'-tetramethylbenzidine; Sigma). The OD was measured at 450 nm and the concentration of purified gp120 was determined by comparison with standard curves derived from a known concentration of recombinant gp120SF162 purified with the multi-step method described above.

Immunological characterization of miniCD4-purified gp120

The binding of well-characterized HIV-Env specific mAbs was performed by a capture ELISA. Typically, the 96-wells plates were coated overnight at 4°C with lectin (concanavalin A, Sigma, 500 ng per well); wells were saturated with PBS/5 % BSA buffer, wash three times, and different dilutions of purified gp120SF162 were added. The capture proteins were revealed by incubation with 50 ng of anti-Env mAbs (Centralized facilities for AIDS reagents, NIBSC) 1 hour at room temperature. Several monoclonal antibodies were tested, directed against the CD4-binding site (b12 and F105), CD4-induced epitopes (17b, X5 and 48D), V3 loop (MN215 and 44752D), the carbohydrates moieties (2G12) and the C-terminal part of gp120 (D7324). For CD4-induced epitopes, gp120SF162 was pre-incubated with an equimolar concentration of miniCD4 before the incubation with immobilized-lectin. To finish, plates were washed, and specifically bound antibodies were detected using peroxidase-conjugated antibody as described earlier.

Size exclusion chromatography (SEC)

Purified gp120SF162, gp120YU2 and o-gp140 SF162ΔV2 were separated over pre-calibrated size exclusion HPLC column (Bio-Sil SEC-250, Biorad) by using ÄKTA purifier apparatus (Amersham Bioscience). Peak elution was monitored either at 280 nm or through Tryptophan fluorescence (λ excitation= 280 nm and λ emission= 340 nm, values expressed in mV: mvolts). Tryptophan fluorescence was measured with a spectrofluorometer FL-detector L-2480 (VWR

international). Eluted proteins were calibrated by comparison with the SEC profile of molecular weight standards of 670, 158, 44, 17 and 1.35 kDa (Biorad).

Surface plasmon resonance (SPR) biosensor analysis

Experiments were conducted at 25° C with 20 µl/min flow rate in HBS (10mM HEPES-buffer saline, 3 mM EDTA, 0.05% Biacore surfactant, pH 7.4) with a Biacore 3000 instrument (GE Healthcare, Biacore AB, Uppsala, Sweden). All the antibodies tested were immobilized at ~1,500 RU by the amine coupling kit (NHS/EDC) provided by the manufacturer. We used the following panel of anti-HIV-1 Env mAbs: D7324, X5, 48D, b12 and 44752D. All the sensorgrams were corrected by subtracting the signal from reference flow cell.

Cell surface CCR5 chemokine receptor binding assays

Adherent CHO-K1 cells expressing CCR5 (Samson et al., 1996) were used to analyze envelope conformational change by flow cytometry FACS (Becton Dickinson, San Jose, CA). Typically, 100 ng of gp120SF162 or gp120YU2 (120 kDa) were pre-incubated or not with 36 ng of sCD4 (44kDa) or 2.5 ng of miniCD4 (3kDa) in 200 µl of PBS and then added to 50 µl of HAMF12-FCS 10% medium containing 2.10^5 CCR5 cells. After 1 h of incubation at room temperature, cells were washed 3 times with PBS/BSA 5 % (pH 7.4). Envelope binding to CCR5 co-receptor was further detected by a phycoerythrin-tagged antibody directed against D7324 antibody.

Immunogenicity studies

Young Wistar rats were immunized by intra-peritoneal path (i.p.) with 50 µg of miniCD4-purified gp120YU2 or 5 µg of miniCD4 CD4M48 emulsified in an equal volume of Alum adjuvant on day 0. Booster inoculations were given i.p. on day 28, 56 and 84. 2 milliliters of serum were collected at day 42, 70 and 98. Antibody titers were assayed using a sandwich ELISA as follows: plates were coated with Concanavalin A (500 ng per well) over night at 4° C, saturated with PBS/BSA 5%, pretreated 1h with gp120YU2 and then treated 1 h with collected sera. Bound antibodies were revealed with goat-anti-rat peroxidase-conjugated antibody.

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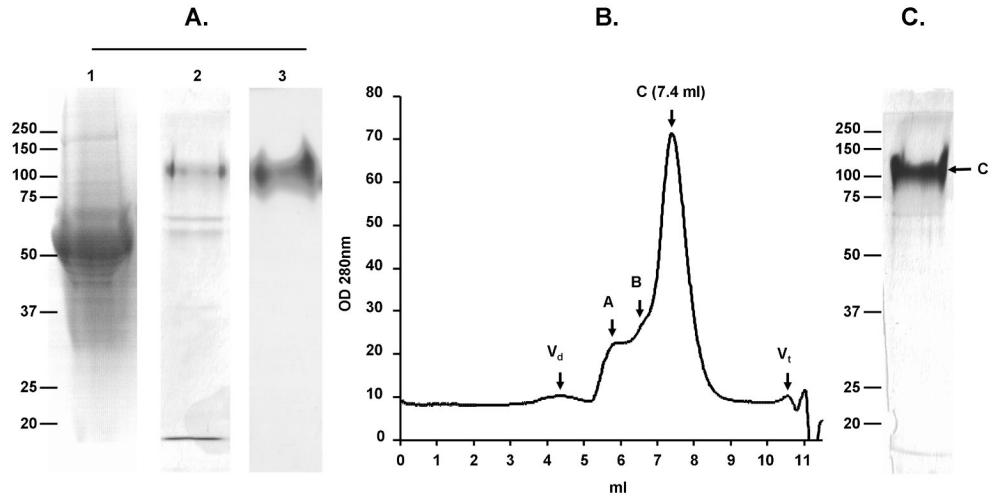

Fig. 1. Purification of gp120SF162 by the miniCD4 method

Analysis of miniCD4-purified gp120SF162 by SDS-PAGE (A). Lanes l and 2 silver staining: lane 1—flowthrough after miniCD4 beads incubation; lane 2—pool of miniCD4 elutions. Lane 3 immunoblot: identification of gp120SF162 among the eluted protein by anti-gp120 D7324 mAb. Size exclusion-HPLC profile of miniCD4-purified gp120SF162 (B). Fractions composing peak C were pooled and analyzed by SDS-PAGE followed by a silver staining (C).

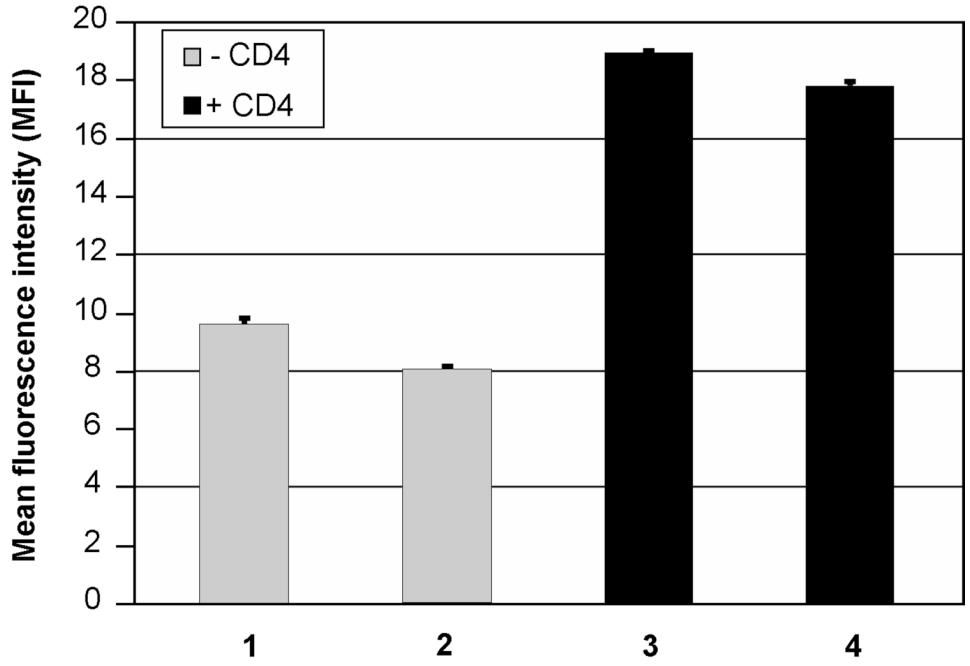

Fig. 2. Binding of miniCD4-purified gp120SF162 to CCR5 chemokine receptor

Over-expressing CCR5 cells were incubated with 100 ng of gp120SF162 without (lanes 1 and 2) or with 36 ng of sCD4 (lanes 3, 4). Bound envelope glycoprotein was detected with D7324 anti-gp120 mAb. Cells were analyzed for envelope binding by FACS flow cytometry. Lane 1—miniCD4-purified gp120SF162; lane 2—gp120SF162 purified by the reference method; lane 3—miniCD4-purified gp120SF162+ sCD4; lane 4—gp120SF162 purified by the reference method + sCD4.

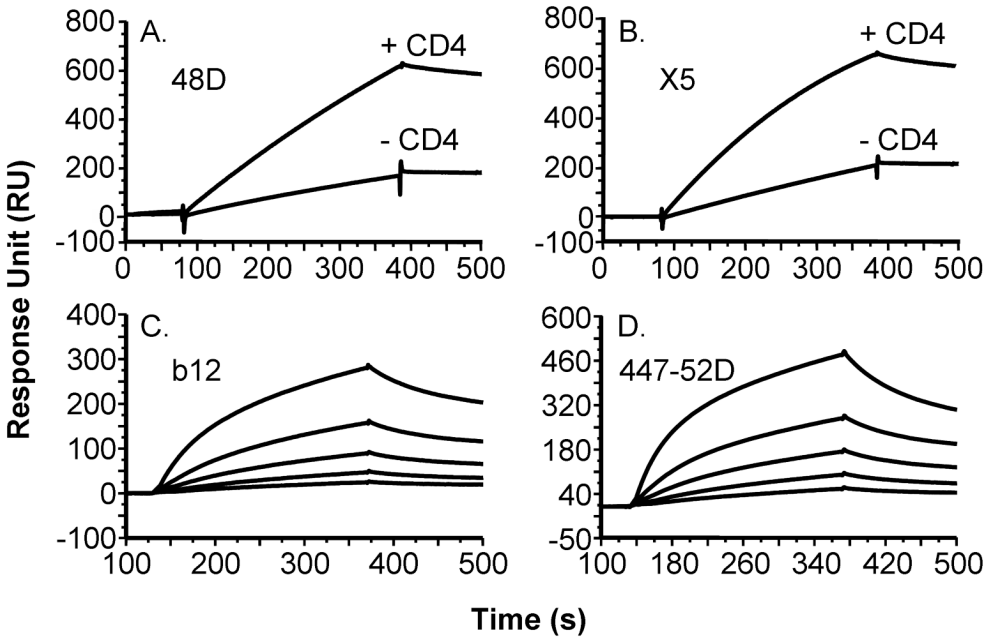

Fig. 3. Binding sensorgrams of miniCD4-purified gp120SF162 to anti-gp120 mAbs

The binding of miniCD4-purified gp120SF162 on 48D (A) or X5 (B) antibody, in the presence or not of 100 nM sCD4 (soluble CD4) was analyzed by surface plasmon resonance (SPR). Binding sensorgrams of miniCD4-purified gp120SF162 diluted at 50 nM, 25 nM, 12.5 nM, 6.25 nM and 3.125 nM to b12 (C) or 44752D (D) mAbs.

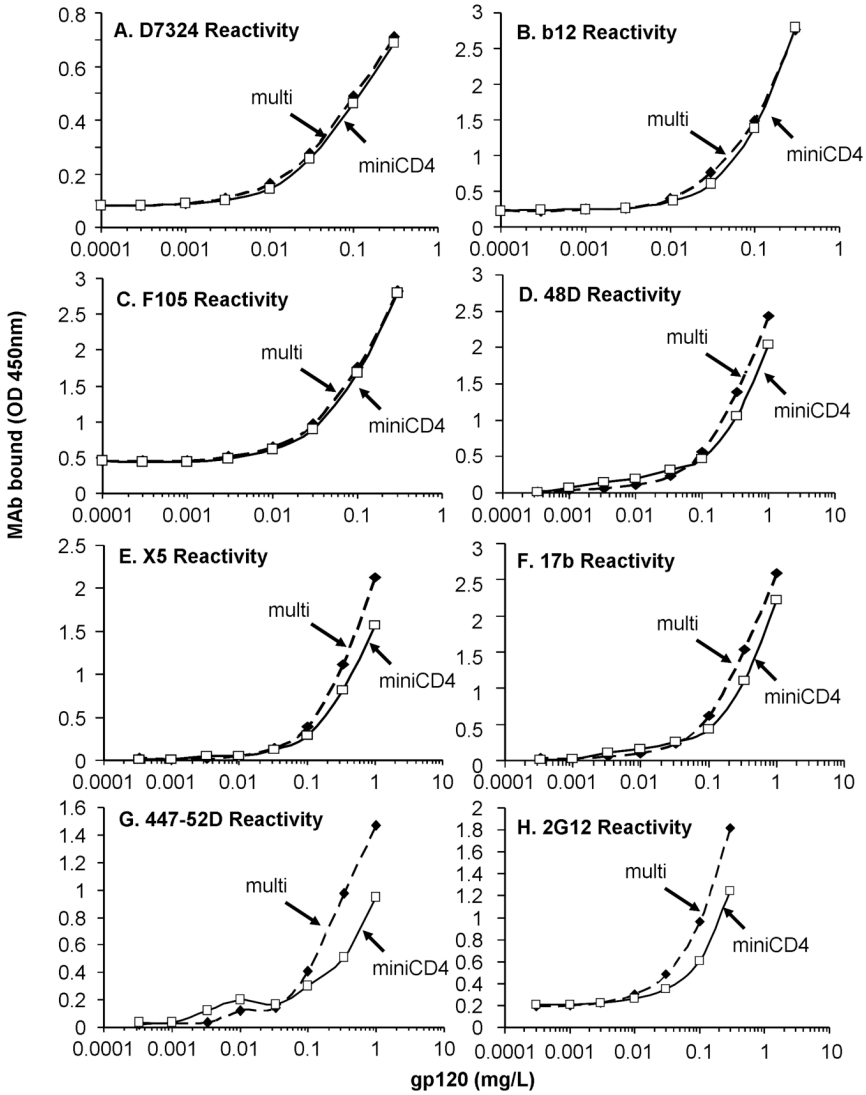

Fig. 4. Comparison of the binding to a panel of well-characterized mAbs of miniCD4-purified gp120SF162 and gp120SF162 purified with a multi-step method

Various concentrations of miniCD4-purified gp120SF162 (open square) and gp120SF162 purified with a multi-step method (reference method, filled square) were pre-incubated in the presence or not of an equimolar concentration of soluble CD4 (addition of sCD4 for panels D, E and F) on immobilized lectin (Concanavalin A) and incubated with several mAbs i.e. D7324 (A); b12 (B); F105 (C); 48D (D); X5 (E); 17b (F); 44752D (G); 2G12 (H). Anti-gp120 mAbs binding was finally detected with peroxidase-labeled secondary antibodies. Figure 4 shows the representative antibody-binding signal of 4 independent experiments.

Legends: “multi”: gp120SF162 purified with the multi-step procedure, “miniCD4”: gp120SF162 purified by miniCD4 affinity chromatography.

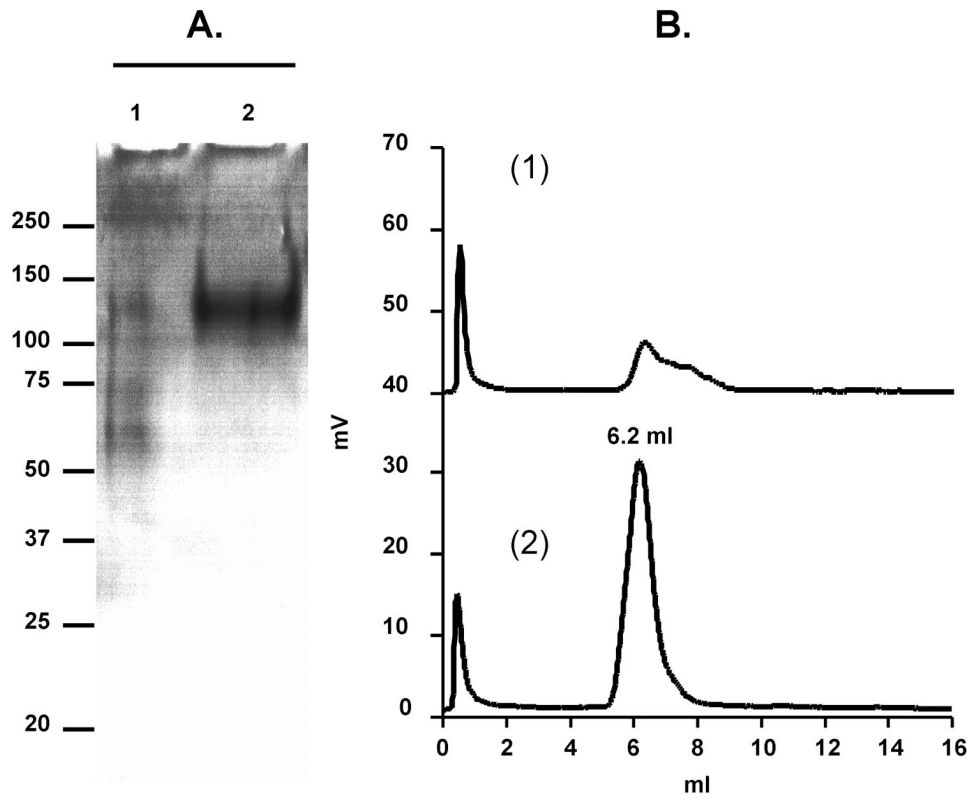

Fig. 5. Purification of gp140SF162ΔV2 by the miniCD4 method

SF162 envelope trimer purified by the multi-step method was applied to miniCD4 beads. Analysis of miniCD4-purified gp140SF162ΔV2 by SDS-PAGE (A: lane 1—flow through after miniCD4 beads incubation; lane 2—pool of miniCD4 elutions). Size exclusion-HPLC profile of miniCD4-purified o-gp140SF162ΔV2 (B; panel 1—flow through after miniCD4 beads incubation; panel 2—pool of miniCD4 elutions).

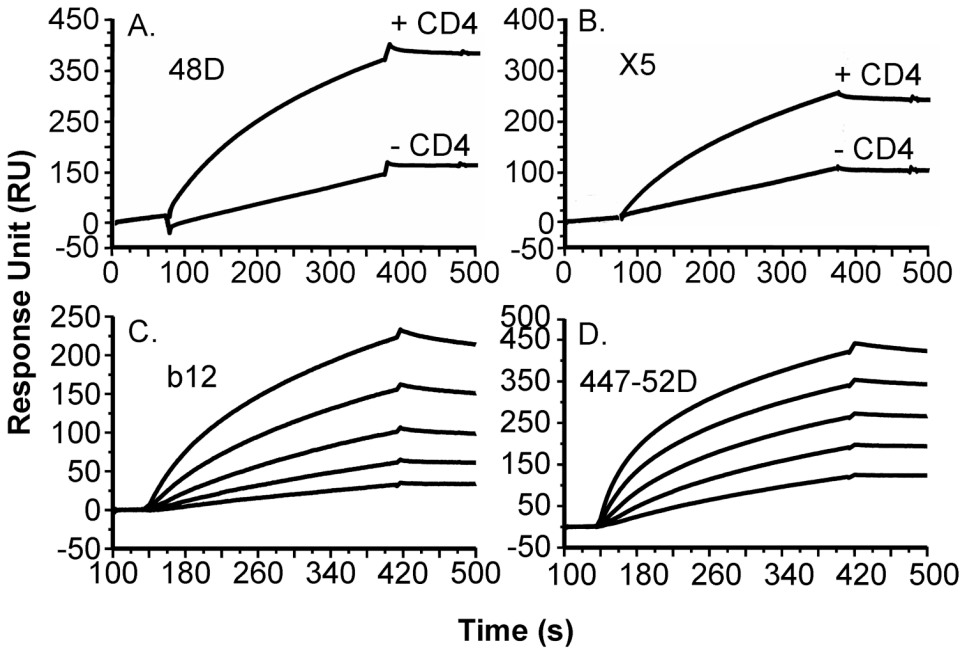

Fig. 6. Binding sensorgrams of miniCD4-purified gp140SF162ΔV2 to anti-gp120 mAbs

The binding of miniCD4-purified gp140SF162ΔV2 on 48D (A) or X5 (B) antibody, in the presence or not of 100 nM sCD4 was analyzed by SPR. Binding sensorgrams of miniCD4-purified gp140SF162ΔV2 diluted at 50 nM, 25 nM, 12.5 nM, 6.25 nM and 3.125 nM to b12 (C) or 44752D (D) mAbs.

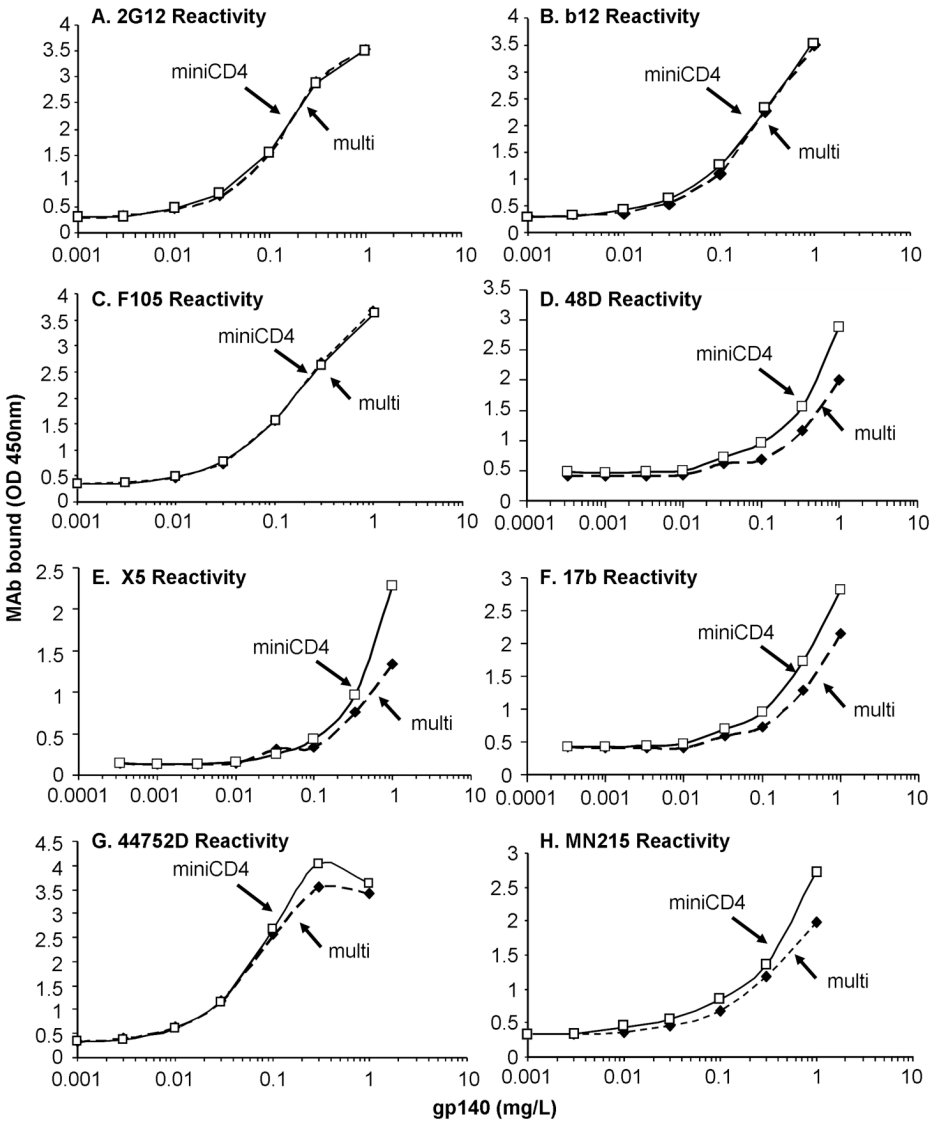

Fig. 7. Immunochemical characterization gp140SF162ΔV2 purified with the multi-step method and submitted (open square) or not (filled square) to miniCD4 capture

Various concentrations of gp140SF162ΔV2 purified with the multi-step method and submitted (open square) or not (filled square) to miniCD4 capture were pre-incubated on immobilized lectin (Concanavalin A) and incubated with a panel of mAbs i.e. 2G12 (A); b12 (B); F105 (C); 48D (D); X5 (E); 17b (F); 44752D (G); MN215 (H). Anti-gp120 mAbs binding was finally detected with peroxidase-labeled secondary antibodies. For X5, 48D and 17b binding gp140SF162ΔV2 was pre-incubated in the presence of an equimolar concentration of sCD4. Figure 7 shows the representative antibody-binding signal of 4 independent experiments. Legends: “multi”: gp140ΔV2SF162 purified by the multi-step procedure, “miniCD4”: gp140ΔV2SF162 purified by miniCD4 affinity chromatography.

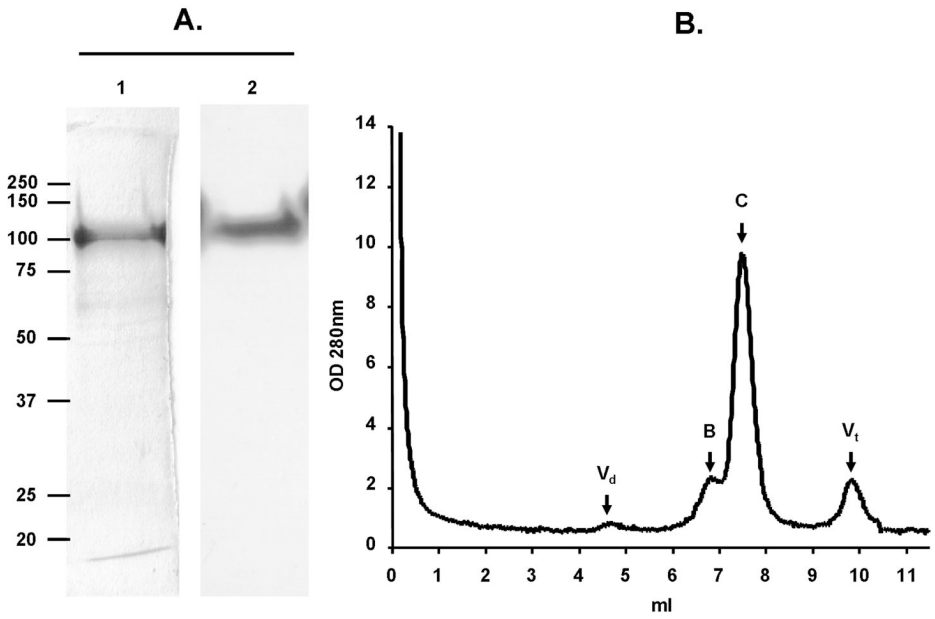

Fig. 8. Purification of gp120YU2 by the miniCD4 method

Analysis of miniCD4-purified gp120YU2 by SDS-PAGE (A: lane 1, silver staining: pool of proteins eluted from miniCD4-beads; lane 2, immunoblot: identification of gp120YU2 among the eluted protein by anti-gp120 D7324 mAb) and size exclusion-HPLC profile of miniCD4-purified gp120YU2 (B).

Table 1

yield of purification of miniCD4 affinity chromatography

Step	Volume (ml)	gp120SF162 (μg)	% of recovery	Purity (%)
Culture supernatant	0.5	72.25 +/− 12.5		
MiniCD4 eluate	1.5	40.3 +/− 4.12	55.8	60

Number of independent experiments: n= 9

Table 2

Dilution of purified antibody resulting in half-maximum binding to several Envelope glycoproteins

Antisera	Antigens	
	miniCD4-purified gp120YU2	**miniCD4**
gp120CN54 (C)	3000	<100
gp120HxB2 (B)	1000	<100
gp120LAI (B)	600	<100
gp120SF162 (B)	1550	<100
gp120SF2 (B)	1400	<100
gp120W61D (B)	2000	<100
gp120YU2 (B)	2000	<100
gp140Br29 (F)	250	<100
gp140Bx08 (B)	450	<100
gp140ZM96 (C)	225	<100