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CD4-affinity purification of recombinant and native HIV gp120 and comparison of the affinity constants for the receptor

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Soluble CD4-immunoglobulin chimeric proteins were covalently attached to CNBr-activated Sepharose. This affinity matrix was used to establish a powerful new method to isolate different species of the HIV external glycoprotein gp120 from cell-free culture supernatants. Recombinant gp120 was expressed in Baculovirus-infected insect cells and isolated from cell-free culture supernatants. The recombinant protein has an apparent molecular mass of 95 kDa whereas the native gp120 purified from a persistently HIV-1 infected H9 cell culture has an apparent molecular mass of 130 kDa. These two gp120 species were shown to be of identical molecular size after complete deglycosylation achieved by endoglycosidase treatment, and they bound to CD4-Hγ1 with the same binding constant, that was reported for native forms of gp120 and CD4. Thus the different glycosylation of gp120 does not influence its affinity to CD4 and the gp120-CD4 complex can be reversibly dissociated.

HIV-1gp120; CD4; Affinity purification

1. INTRODUCTION

The human immunodeficiency virus HIV is the etiological agent of the acquired immunodeficiency syndrome, AIDS [1,2]. The primary immunologic abnormality consists of the progressive depletion and functional impairment of a subset of T-lymphocytes bearing the CD4 surface antigen [3,4]. The specific formation of a high affinity complex between the major virus envelope glycoprotein, gp120, and the non-polymorphic cell surface protein CD4 represents the initial step of the infection and accounts for HIV's cellular tropism and cytotoxicity [5,6]. Isolated gp120 could thus be very helpful for the study of viral infectivity and pathogenesis. Furthermore the development of therapeutic agents that are able to block the specific CD4-gp120 interaction is greatly facilitated by the availability of purified gp120. The primary *env* gene product is a glycosylated 160 kDa protein (gp160) processed by a cellular protease yielding the external gp120 and the transmembrane gp41. The aminoterminal fusogenic domain of gp41 mediates the entry of the virus into the cell [7,8]. Since the gp120-gp41 interaction is non-covalent, soluble gp120 is shed from the surface of the virion as well as infected cells [9].

Gp120 is heavily glycosylated, the amino acid sequence reveals more than 20 potential *N*-glycosylation sites [10]. The N-linked glycans of the secreted gp120 consist of oligomannosidic as well as fucosylated and partially sialyted bi- and triantennary complex-type oligosaccharides [11]. The pattern of oligosaccharides differs between gp120 of HIV and gp130 of a related simian immunodeficiency virus indicating an influence of the amino acid sequence on the processing of the oligosaccharides [12]. Roughly one-half of the apparent molecular mass of gp120 can be attributed to carbohydrates [13]. Truncated forms of the HIV receptors CD4 have been expressed in eukaryotic cells [14]. More recently chimeric proteins consisting of the two aminoterminal domains of CD4 and various carboxy-terminal domains of immunoglobulins were produced with high yield in mouse myeloma cells [15]. Soluble CD4 derivatives containing the extracellular CD4 domains V_1 and V_2 were reported to bind gp120 with the same affinity as cell-surface CD4 [16]. SF9 insect cells infected with a recombinant Baculovirus shed gp120 about 1000 times more efficient than HIV-1 infected H9 cells (L. Dirckx, unpublished observation), however the recombinant gp120 most probably contains only oligosaccharides of the low mannose type [17,18].

This study reports a new potent purification strategy to isolate gp120 from cell-free culture supernatants. The most effective step of this method exploits the high-affinity interaction between gp120 and the CD4-immunoglobulin chimeric molecule, CD4-Hγ3. By applying this method it was possible to isolate both native gp120 from persistently HIV-1 infected H9 cells as well as recombinant gp120 expressed in the Baculovirus-system.

2. MATERIALS AND METHODS

2.1. *Proteins*

Purified chimeric CD4-immunoglobulin molecules CD4-Hγ3 and CD4-Hγ1 were kindly provided by A. Traunecker and K. Karjalainen

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(Basel Institute for Immunology, Switzerland). CNBr-activated Sepharose 4B, lentil lectin-Sepharose 4B, Protein A Sepharose CL-4B and Protein G Sepharose 4 Fast Flow were purchased from Pharmacia.

2.2. *Cell culture*

The preparation of cell-free culture supernatants from recombinant gp120-expressing *Spodoptera frugiperda* insect cells (SF9) has been described elsewhere [19]. Chronically HIV-1 infected H9 cells (ATCC CRL 8543) [20] were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (Amimed), 1 mM sodium pyruvate (Gibco), 2 mM Glutamine (Gibco), 1 × non-essential amino acids (Gibco) and 100 U of penicillin and 100 μg streptomycin per ml (Gibco). The cell density was adjusted to 5×10^5 per ml by dilution with fresh medium three times a week. Virus production was augmented by adding non-infected H9 cells once weekly. Culture supernatant of HIV-1 infected H-9 cells was prepared by sedimenting the cells from 1.61 liters of culture for 10 min at 370 × *g* followed by centrifuging the supernatant for 20 min at 10000 × *g*. The latter supernatant was stored at −20°C and after thawing 1 mM phenylmethylsulfonylfluoride (Calbiochem) and 5 μg/ml Aprotinin (Boehringer Mannheim Biochemicals, FRG) were added as protease inhibitors.

2.3. *Isolation of gp120 from cell culture supernatants*

The following procedure was applied to isolate recombinant gp120 from 50 ml 10-fold concentrated SF9 cell-free culture fluid supernatant as well as native gp120 from a 1.6 liter culture supernatant. A CD4-affinity column was prepared by covalently attaching 3–10 mg purified CD4-Hγ3 to 0.5–1 g CNBr-activated Sepharose 4B using the method suggested by the manufacturer. The gp120-containing supernatants from 500 ml of gp120 expressing SF9 cells or 1.6 liter of HIV-1 infected H9 cells were passed over the column at 4°C with a maximum flow rate of 10 cm/h. Washing of the column with 5 volumes PBS containing 0.5% NP 40 and 5 volumes PBS was followed by subsequent elution of the gp120 from the resin with 3 M KSCN. The eluate was dialysed over night at 4°C against PBS and applied to a 2 ml lentil lectin-Sepharose minicolumn. Conditions for chromatography and washing were the same as described above. Elution was accomplished by an aqueous solution of Methyl-α-D-mannopyranoside (1 M) and KSCN (3 M). Gp120-containing fractions were identified by Western blot analysis performed as described [21]. After combining the peak fractions followed by dialysis as described above, the solution was concentrated 10-fold by immersing the dialysis tubing into solid polyethyleneglycol. To remove residual CD4-Hγ3 the concentrated solution was adsorbed to Protein G Sepharose. The homogeneity of the preparation was analysed by electrophoresis on a 8% sodium dodecyl sulfate polyacrylamide gel [22].

2.4. *Determination of the gp120-CD4 affinity constant*

Highly purified gp120 was radioiodinated with the Bolton Hunter reagent (Amersham) [23] yielding specific activities in the range of 3.7–18.7 μCi/μg. 2–4 ng of CD4-Hγ1 was incubated with increasing amounts of [^{125}I]gp120 for 15 h and 4°C in PBS containing 0.25% gelatin and 0.34% NP 40. Formed complexes were adsorbed with protein A Sepharose and extensively washed with PBS/0.25% gelatin/0.5% NP 40. Bound [^{125}I]gp120 was measured using a γ-counter. Specifically bound [^{125}I]gp120 was determined from the difference in binding in the presence and absence of CD4-Hγ1. Less than 10% of the specifically bound activity was attached to protein A-Sepharose in the absence of CD4-Hγ1. Radioiodinated gp120 bound unspecifically to CD4-Hγ3-Sepharose. Therefore CD4-Hγ3 was used in the binding assays.

2.5. *Analytical Deglycosylation of [^{125}I]gp120*

For complete deglycosylation 18 ng of recombinant [^{125}I]gp120 (100000 cpm) and 3 ng of native [^{125}I]gp120 (60000 cpm) were incubated with 0.2 U endoglycosidase F/N-glycosidase F (Boehringer Mannheim Biochemicals, FRG) in PBS/0.02% SDS/1% Triton X-100 at pH 7.2 and 37°C for 20 h [24].

3. RESULTS

3.1. *Purification of recombinant and native HIV gp120*

The objective of this work was to establish a method based on CD4-affinity chromatography for the purification of the external HIV glycoprotein gp120 from different sources. Recombinant gp120 was expressed and secreted from Baculovirus-infected insect cells as described elsewhere [19]. Native gp120 was obtained from a persistently HIV-1 infected H9 cell line which was co-cultivated with non-infected H9 cells to stimulate virus production and augment the titer of secreted gp120. To establish an analytical method for working out the isolation procedure, HIV-1 infected H9 cells were metabolically labeled with [^{35}S]cysteine. The first step in the isolation procedure was the adsorption of gp120 from the culture fluid of the labeled cells to a CD4-Hγ3 loaded affinity matrix. The elution of the bound gp120 was found to be almost quantitative using a 3 M aqueous solution of the chaotropic salt KSCN. Rebinding to the CD4-matrix could be demonstrated

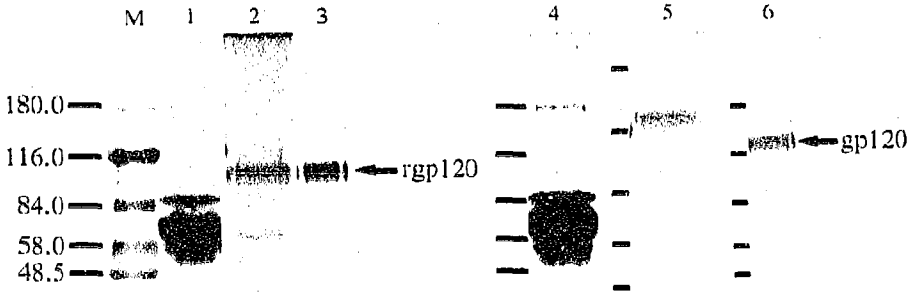


Fig. 1. Analysis of culture fluid polypeptides and purified gp120 by SDS polyacrylamide electrophoresis and Western blot analysis. **1**, Cell culture supernatant from Baculovirus-infected SF9 insect cells secreting soluble recombinant gp120, **2,3** purified recombinant gp120, **4**, cell culture supernatant of HIV-1 infected H9 cells, **5,6** purified native gp120. The polypeptides were visualized either by staining with Coomassie brilliant blue **(1,2,4,5)**, or Western blot analysis **(3,6)**. Molecular masses of marker proteins (M) are indicated in kDa.

after extensively dialysing the eluate against PBS (not shown). The analytical procedure was scaled up to isolate preparative quantities of gp120. 50 ml of a 10-fold concentrated cell free supernatant containing recombinant gp120 and 1.6 liter H9/HIV-1 supernatant as a source for native gp120 were passed over 3 ml CD4-Hγ3-Sepharose (2 mg of ligand/ml of settled bed). Then the column was washed and subsequently eluted with 3 M KSCN as described in section 2. The eluate was dialysed against PBS and passed over 2 ml of lentil lectin-Sepharose. Because gp120 was only partially eluted by 1 M methyl-α-D-mannopyranoside, 3 M KSCN was additionally included in the elution buffer. The eluate was again dialysed and concentrated. Finally CD4-Hγ3 that eventually leaked from the Sepharose was removed by batch adsorption to protein G-Sepharose. The resulting gp120-preparations consisted of one major band of both recombinant and native gp120 as demonstrated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis. The apparent molecular mass was determined as 95 kDa for the recombinant and 130 kDa for the native gp120 (Fig. 1b,e).

Immunoblotting and thus reactivity of both gp120 molecules with pooled HIV-1 specific sera from HIV-infected individuals proved the identity of the isolated proteins (Fig. 1c,f). To demonstrate that the difference in molecular mass was due to different glycosylation patterns described in inect cells and mammalian cells, both species of ^{125}I-labeled gp120 molecules were extensively deglycosylated using endoglycosidase F. The result of the experiment revealed that both molecules were shifted to the same apparent molecular mass of about 60 kDa after enzyme treatment (Fig. 2). The native [^{125}I]gp120 contains minor impurities of about 90, 50 and 40 kDa (Fig. 2, lane 1), whereas only a single additional band of about 40 kD_a is visible after complete deglycosylation (Fig. 2, lane 3). Removal of oligosaccharides may have decreased the size of the glycopeptides to less than 36 kDa or may have yielded hydrophobic polypeptides. Both of which may have been lost in our analysis.

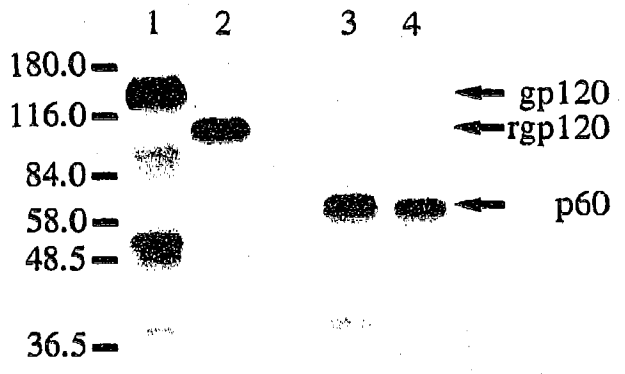
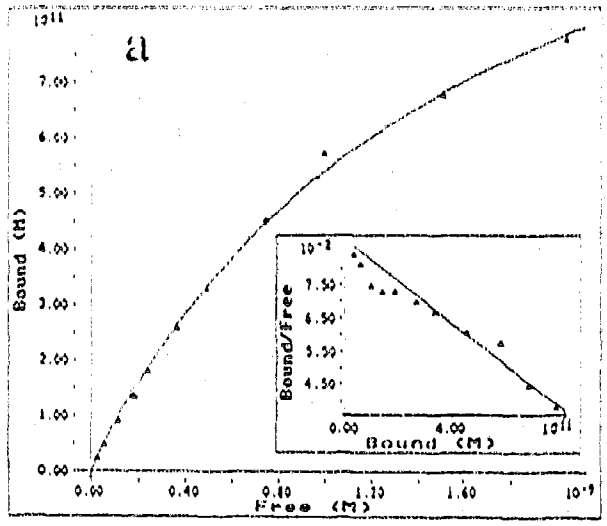


Fig. 2. Complete deglycosylation of recombinant and native HIV-gp120 after endoglycosidase treatment. Proteins were analysed by SDS-PAGE and autoradiography. Lane 1, native [^{125}I]gp120; Lane 2, recombinant [^{125}I]gp120; Lane 3, native [^{125}I]gp120 after complete deglycosylation; Lane 4 recombinant [^{125}I]gp120 after complete deglycosylation.

3.2. *Determination of the gp120-CD4 affinity constants*

Recombinant and native gp120 radio-iodinated using the Bolton Hunter reagent, were proven to be largely in-



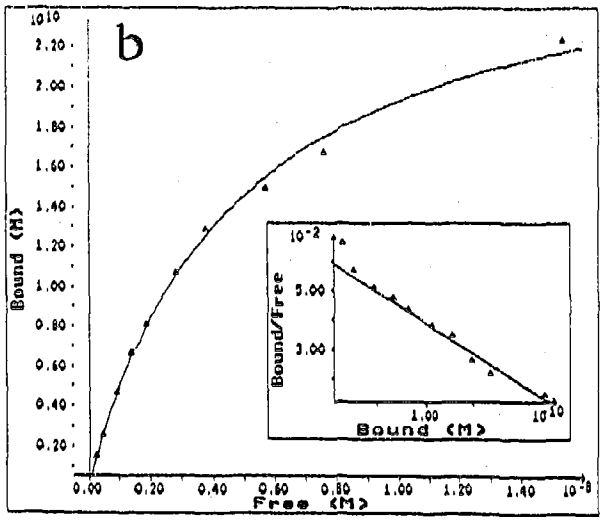


Fig. 3. Saturation binding analysis of native (a) and recombinant (b) [^{125}I]gp120 binding to CD4-Hγl. CD4-Hγl was incubated with increasing amounts of radio-iodinated gp120. CD4-gp120 complexes were quantitatively adsorbed to protein A Sepharose. Specifically bound [^{125}I]gp120 was determined from the difference in binding in the presence and absence of CD4-Hγl and plotted against the total [^{125}I]gp120 concentration. Unspecific binding was corrected by substracting the [^{125}I]gp120 bound to protein A sepharose in the absence of CD4-Hγl. The data were linearized by Scatchard analysis and fitted by unweighted least-squares linear regression analysis using the ENZFITTER programme (R.J. Leatherbarrow, 1987, published by Elsevier-Biosoft). Dissociation constants were calculated as means of two independent experiments.

tact after iodination by specific reaction with an anti HIV-1 serum as well as CD4 (not shown). The affinity constants for the gp120-CD4 interactions were measured as described above by saturation binding analysis with soluble CD4-Hγ1 chimeric molecules and recombinant and native [^{125}I]gp120 as radioligands. Specifically bound [^{125}I]gp120 was determined from the difference in binding in the presence and the absence of CD4 and plotted against the total concentration of ^{125}I-labeled gp120. Scatchard analysis revealed a single homogeneous class of gp120 binding sites on each molecule with apparent dissociation constants (K_d of 2.6 nM for the native and 3.8 nM for the recombinant gp120 molecule (Fig. 3a,b). These data represent the arithmetic means of two independent measurements for both the recombinant and native gp120, respectively. Thus both gp120 molecules bind to CD4 with affinities, that could not be distinguished by our methods.

4. DISCUSSION

This study diseribes a new potent approach to isolate gp120 of HIV-1 from the supernatants of cell cultures. The most effective step in the isolation procedure exploits the high affinity of gp120 to its receptor, CD4. CD4 was applied in the form of soluble CD4-IgG chimeric molecules, which could be produced in mg amounts by mouse myeloma cells transfected with the recombinant CD4-IgG gene [15]. This approach is for several reasons superior to previously described methods which are based on anti HIV antibodies specific for gp120 [25,26]. The CD4 affinity matrix represents a homogeneous reagent with reproducible binding capacity, and the binding to gp120 is essential for the replication of the virus and therefore conserved in all variants. Furthermore receptor molecules that may bleed from the affinity matrix can be easily removed from the preparation by Protein G Sepharose. Several methods for elution have been examined with native ^{35}S-labeled gp120 as a ligand. Among elution with high or low pH and chaotropic salts, the elution with 3 M KSCN was shown to be most effective. Gp120 that was eluted by this method could re-bind to CD4 after dialysis. Since the CD4 matrix could be re-used after elution of gp120 repeatedly, structural changes induced by KSCN in the interacting domains of CD4 and gp120 seem to be reversible. In a second step the properties of the carbohydrate moiety were employed to further purify the glycoprotein. However, the classical competitor methyl-α-D-mannopyranoside could elute only marginal proportions of native and recombinant gp120 from *Lens culinaris*-lectin Sepharose. Therefore a mixture of the carbohydrate eluant and chaotropic salt was applied. Recombinant gp120 can also be eluted from lentil lectin-Sepharose by a mixture of methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside (H. Döbeli, personal communication). Thus, the glucose residues of unprocessed recombinant gp120 may be involved in the strong binding of this form of gp120 to lentil lectin. In a final step of the purification procedure CD4-Hγ3 was removed from the eluate by adsorption to Protein G. This detectable impurity may result from the dissociation of the iso-urea linkage formed between the CNBr-activated Sepharose and the CD4 chimeric molecule, or from leakage of non-covalently bound CD4-Hγ3. Western blotting of the purified molecules with an anti HIV-1 positive serum identified a protein with an apparent molecular weight of 95 kDa for the recombinant gp120 and a markedly larger 130 kDa protein for the negative gp120 molecule. Extensive removal of N-linked glycans from both molecules by endoglycosidase treatment provided direct evidence that the difference in the molecular mass is mainly caused by known differences in the glycosylation in insect and mammalian cells. Whereas the N-linked oligosaccharides in human cells are of the high mannose-, hybrid- and of the complex branched-type [11] glycosylation in insect cells is restricted to the addition of high mannose-type oligosaccharides with additional trimming of terminal mannosyl residues [17,18]. It has to be emphasized that irrespective of this difference in glycosylation, the purification strategy succeeded in isolating both molecules through the CD4-gp120 affinity interaction. Since denaturing concentrations of KSCN were applied to dissociate the receptor-ligand complex and for the elution of gp120 from lentil lectin-Sepharose it was important to examine the reversibility of the binding reaction. Thus, saturation binding assays were carried out to determine the affinity constants for the CD4 interaction of both recombinant and native gp120. Scatchard analysis revealed a single homogeneous class of binding sites for each molecule with dissociation constants of 3.8 nM for the recombinant molecule and 2.6 nM for the native gp120. The data obtained for the binding of CD4-IgG to gp120 in solution are in agreement with the affinity constants other investigators have determined for gp120 binding to CD4 on whole cells ($K_d = 4.0$ nM) [8]. These results are important for two reasons: the chimeric CD4-IgG molecule has the same gp120 binding affinity as the native CD4 at the cell surface and gp120 expressed in the Baculovirus system binds with the same affinity to CD4 as the native gp120 obtained from chronically HIV-infected human T-cell line. Obviously the different mode of glycosylation of gp120 produced in insect cells does not significantly change its binding to CD4. Thus the recombinant gp120 and CD4-IgG molecules will be suited to establish a cell-free system for screening inhibitors of the CD4-gp120 interaction.

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