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Glycosylation Is Necessary for the Correct Folding of Human Immunodeficiency Virus gp120 in CD4 Binding

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Conflicting results have been reported regarding the role of carbohydrate on human immunodeficiency virus (HIV) envelope glycoprotein gp120 in CD4 receptor binding. Glycosylated, deglycosylated, and nonglycosylated forms of HIV type 1 (HIV-1) and HIV-2 gp120s were used to examine CD4 receptor-binding activity. Nonglycosylated forms of gp120 generated either by deletion of the signal sequence of HIV-1 gp120 or by synthesis in the presence of tunicamycin failed to bind to CD4. In contrast, highly mannosylated gp120 bound to soluble CD4 molecules well. Enzymatic removal of carbohydrate chains from glycosylated gp120 by endoglycosidase H or an endoglycosidase F/N glycanase mixture had no effect on the ability of gp120 to bind CD4. An experiment which measured the ability of gp120 to bind to CD4 as an assay of the proper conformation of gp120 showed that carbohydrate chains on gp120 are not required for the interaction between gp120 and CD4 but that N-linked glycosylation is essential for generation of the proper conformation of gp120 to provide a CD4-binding site.

The interaction of envelope glycoprotein gp120 of human immunodeficiency viruses (HIV) with its receptor, CD4, represents the initial step in virus infection and accounts for HIV's tropism and cytopathic effect (4, 13, 27). This interaction appears to be completely dependent upon the overall conformation of the envelope glycoprotein (14, 18). gp120 consists of more than 20 potential N-linked glycosylation sites, and the N-linked glycans represent almost 50% of the apparent molecular mass of the protein, which has both high-mannose and complex branched-type carbohydrates (7, 9, 17). Therefore, carbohydrates are likely to be prominent structures on the surface of HIV but their precise biological functions remain elusive. There has been a great deal of interest in the requirements for glycosylation of both CD4 and gp120 in their successful interactions. For CD4, the sites of interaction with gp120 have been clearly defined (1, 2, 22) and there is no requirement for either of the two N-linked carbohydrate side chains (1). For gp120, however, the nature of the site of interaction with CD4 is more complex (3, 14). Although it has been shown that glycosylation is not required for generation of neutralizing antibodies (23), the exact role of the carbohydrate moiety on gp120 in the formation of a complex with CD4 remains unclear. Conflicting results have been reported regarding this issue; some reports claim that nonglycosylated gp120 expressed in bacteria (19, 23) and enzymatically deglycosylated gp120 are unable to bind to the CD4 receptor (6, 17). In contrast, other reports claim that enzymatic removal of carbohydrate from glycosylated gp120 does not significantly reduce the ability to bind to CD4 (7, 8). In view of these apparent discrepancies, as well as the importance of virus-host cell interactions in virus infection, we felt it necessary to re-examine the nature of the interaction between gp120 and CD4. We employed a baculovirus expression system to prepare nonglycosylated and fully glycosylated forms of gp120. We analyzed the abilities of glycosylated, nonglycosylated, and enzymatically deglycosylated forms of gp120 to bind to CD4.

We expressed two forms of HIV type 1 (HIV-1) gp120 in SF9 cells containing or lacking the natural signal sequences which control intracellular localization and glycosylation and found that positively charged amino acids in the natural signal sequence of HIV-1 gp120 were crucial in the expression level, glycosylation, and secretion of glycosylated gp120 (unpublished data). Deletion of the signal sequence of HIV-1 gp120, which represents 30 amino acids at the N terminus, results in the synthesis of large quantities of a nonglycosylated and nonsecreted form with an apparent molecular mass of 53 kDa (p53). Fusion of heterologous signal sequences from honeybee mellitin (29) or interleukin-3 (33) to nonglycosylated gp120 results in high levels of expression and secretion of a glycosylated gp120 molecule (unpublished data). We also expressed HIV-2 gp120 containing its natural signal sequence in *Spodoptera frugiperda* (SF9) cells by using a recombinant baculovirus vector.

To study the receptor-binding capacity of nonglycosylated and glycosylated forms of gp120, we used an assay system based on the ability of a monoclonal antibody against CD4 known as OKT4 to immunoprecipitate the CD4-gp120 complex (14, 18). SF9 cells infected with recombinant baculovirus were labeled with [^{35}S]methionine for 1 h, and membrane and cytosolic fractions of ^{35}S-labeled cells were prepared with 1% Nonidet P-40. One half of the cytosolic fraction was analyzed for total gp120 by immunoprecipitation with rabbit antiserum directed against recombinant gp120 (see Fig. 1 legend). The remaining half of the sample was incubated with soluble CD4, and formation of the gp120-CD4 complex was analyzed by immunoprecipitation of the complex with an excess of OKT4. The intensity of the band that comigrated with gp120 on sodium dodecyl sulfate

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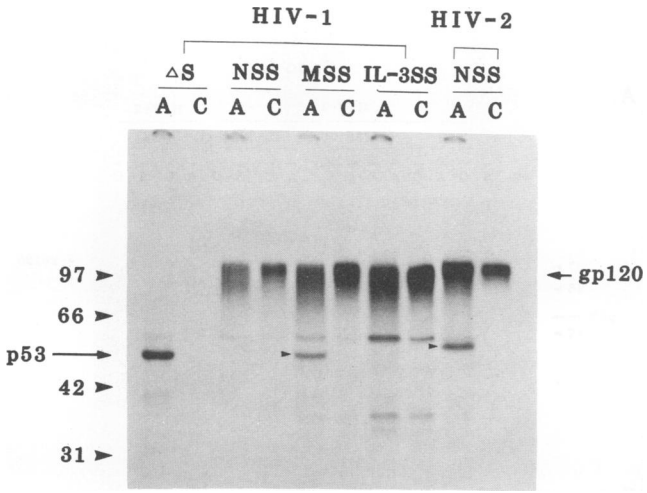

FIG. 1. CD4 binding to recombinant gp120 expressed in SF9 cells. CD4-binding assays were performed by following the procedure of Lasky et al. (14), except that highly purified, soluble CD4 (ABT Inc.) was used. SF9 cells were infected with recombinant baculovirus at a multiplicity of infection of 5 PFU per cell. At 36 h postinfection, the infected SF9 cells were pulse-labeled for 1 h with [^{35}S]methionine (250 μCi/ml) and lysed by addition of 1 ml of extraction buffer (50 mM Tris-HCl [pH 7.5] containing 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). One half of the cell lysate was incubated with 5 μl of rabbit antiserum directed against recombinant gp120 (lanes A). Rabbit anti-gp120 serum was prepared as follows. SF9 cells infected with recombinant baculovirus containing HIV-1 gp120-ΔS or HIV-2 gp120-NSS were harvested at 3 days postinfection, lysed with 1× dissociation buffer (50 mM Tris-HCl [pH 6.8], 5% SDS, 12.5% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), and heated to 100°C for 5 min. Samples containing approximately 2 mg of the expressed gp120 were analyzed by SDS–10% PAGE, and protein bands were visualized by using the KCl method (11). The band containing the nonglycosylated form of gp120 was excised, and protein was electroeluted from the gel with a Bio-Rad electroelutor. Rabbits were immunized twice with 1-month interval by intramuscular injection of 10 μg of purified gp120 by using Freund's complete adjuvant for the primary immunization and Freund's incomplete adjuvant for the secondary immunization. Sera were collected 15 days after the last injection. Each serum sample was tested for antibody titer, and the serum with the highest titer was used. The remaining half of the sample was incubated with 0.4 μg of soluble CD4 and 30 μl (1 μg) of OKT4 (Ortho Diagnostics) (lanes C), and then immune complexes were bound to protein A–Sepharose CL-4B (Pharmacia). The immunoprecipitated material was washed three times with RIPA buffer (50 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) and resuspended in 30 μl of 1× dissociation buffer. Samples were analyzed by SDS–10% PAGE and visualized by fluorography. Recombinant baculoviruses containing the following sequences that express gp120 are indicated at the top of the gel (fluorograms): ΔS, gp120 gene without the natural signal sequence; MSS, gp120 gene containing the mellitin signal sequence; IL-3SS, gp120 gene containing the interleukin-3 signal sequence; NSS, HIV-1 or HIV-2 gp120-encoding gene with the natural signal sequence. The arrowheads between lanes represent nonglycosylated HIV-1 and HIV-2 gp120s. The numbers on the left represent molecular masses (10^3) of markers.

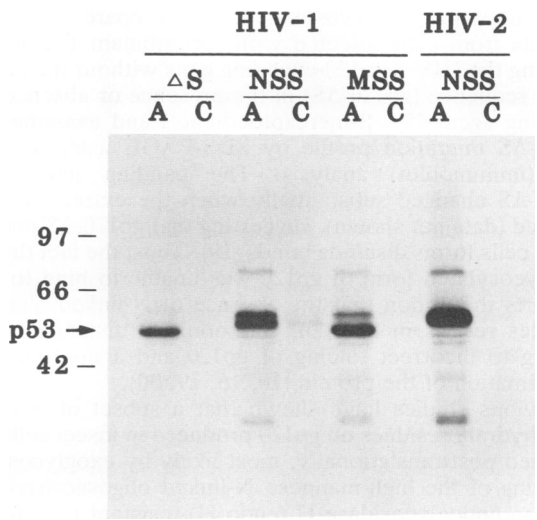

FIG. 2. Effect of tunicamycin treatment on gp120 binding to CD4. SF9 cells were infected with recombinant baculovirus at a multiplicity of infection of 5 PFU per cell. At 20 h postinfection, tunicamycin was added at a concentration of 15 μg/ml, which was maintained throughout the experiment. The cells were labeled with [^{35}S]methionine (250 μCi/ml) for 2 h at 24 h postinfection. An equal portion of intracellular material was incubated with rabbit anti-gp120 serum (lanes A) or with CD4 molecules and then with OKT4 (lanes C). The precipitated material was analyzed by SDS–10% PAGE and visualized by fluorography.

(SDS)-polyacrylamide gel electrophoresis (PAGE) was an indication of the quantity of gp120 bound to CD4.

Figure 1 shows that most of the gp120 with either the natural signal sequence or heterologous signal sequences were glycosylated. However, small portions of these proteins were not glycosylated. We checked the abilities of both glycosylated and nonglycosylated forms of gp120 to bind to CD4 molecules. Only glycosylated forms of gp120 bound to CD4, while both glycosylated and nonglycosylated forms of gp120 were immunoprecipitable with rabbit anti-gp120 serum. In contrast, nonglycosylated HIV-1 gp120 generated by deletion of the signal sequence (gp120-ΔS), nonglycosylated HIV-1 gp120 generated in the presence of the mellitin signal sequence (gp120-MSS), and nonglycosylated HIV-2 gp120 with its own natural signal sequence did not bind to CD4 (Fig. 1). A protein band of approximately 60 kDa detected with OKT4 in cells infected with the recombinant baculovirus containing HIV-1 gp120 with the interleukin-3 signal sequence (gp120-IL-3SS) may represent cellular protein that was nonspecifically immunoprecipitated, because the protein band was also present in all other recombinant virus-infected cell extracts (Fig. 1). Precipitation of glycosylated gp120 by OKT4 was dependent on the addition of exogenous CD4 (data not shown). Our results are consistent with recent reports (5, 21, 31) that although gp120 produced in insect cells has altered carbohydrate structures (high in mannose, no sialic acid, and less processed) compared with the gp120 produced in mammalian cells (15), it exhibits excellent CD4-binding characteristics, as has been reported for gp120 expressed in mammalian cells (6, 14). Conversely, the nonglycosylated gp120 polypeptide backbone is unable to bind to CD4 (19, 23).

To study the requirement for glycosylation of gp120 for binding to CD4 further, we expressed gp120 in the presence of tunicamycin. As can be seen in Fig. 2, gp120 produced in the presence of tunicamycin was nonglycosylated and was similar in size to the nonglycosylated protein expressed when no signal sequences were present. This nonglycosylated protein failed to bind to CD4. These results suggest that glycosylation of gp120 is essential to create a conformational epitope to which CD4 can bind. We examined gp120 folding

with and without glycosylation. We prepared total cell extracts from cells infected with recombinant baculovirus carrying the HIV-1 gp120-encoding gene without the natural signal sequence (gp120-ΔS) in the presence or absence of a reducing agent (5% β-mercaptoethanol) and examined the gp120-ΔS migration profile by SDS-PAGE using Western blot (immunoblot) analysis. The banding pattern for gp120-ΔS changed substantially when the extract was not reduced (data not shown), suggesting that gp120-ΔS made in insect cells forms disulfide bonds (19). Thus, the fact that the nonglycosylated form of gp120 was unable to bind to CD4 supports the notion that the absence of N-linked oligosaccharides results in aberrant formation of disulfide bonds, leading to incorrect folding of gp120 and a nonfunctional conformation of the protein (10, 16, 19, 30).

Previous studies have shown that a subset of N-linked carbohydrate residues on gp120 produced in insect cells was modified posttranslationally, most likely by exoglycosidase trimming of the high-mannose N-linked oligosaccharide to yield an endoglycosidase H (endo H)-resistant trimannosyl core (12, 31; unpublished data). Therefore, a series of experiments was performed to determine the effects of various types of oligosaccharide side chains on the ability of gp120 to bind to CD4. By using a modified form of the method of Fennie and Lasky (6), endo H digestion of intracellular gp120 was conducted on gp120-CD4 complexes in the presence of SDS (0.5%). After digestion, the gp120-CD4 complex was dissociated by boiling at 100°C for 3 min. Equal portions of the samples were diluted to a final SDS concentration of 0.02% and then incubated with either rabbit anti-gp120 serum or soluble CD4 followed by OKT4. SDS-PAGE analyses of these molecules were performed before and after endoglycosidase treatment. As shown in Fig. 3A, gp120 without endo H treatment could be precipitated either with rabbit anti-gp120 serum or with soluble CD4 followed by OKT4. However, after treatment of the gp120-CD4 complex with endo H under denaturing conditions in the presence of SDS, two faster migrating polypeptides appeared after precipitation with OKT4. The major component was endo H sensitive, with a molecular mass of 58 kDa, which is consistent with the mass estimated on the basis of the amino acid sequence of the protein backbone (6, 17). The minor component was partially endo H resistant and had a molecular mass of 61 kDa (Fig. 3A). Interestingly, not only the partially deglycosylated form (partially endo H resistant) but also the completely deglycosylated form (fully endo H sensitive) of gp120 exhibited CD4-binding activity similar to that of the nonglycosylated gp120 that reacted only with rabbit antiserum (Fig. 3A). However, it has been demonstrated that secreted gp120 has encountered enzymes involved in oligosaccharide processing during its transport through the exocytic pathway and appears to be partially endo H resistant (31; unpublished data). Therefore, it was important to demonstrate that secreted gp120 after deglycosylation is still capable of binding to CD4. Therefore, the secreted gp120 was treated with endo H or an endo F/N glycanase mixture in the absence of SDS. Enzyme-treated gp120 and untreated gp120 were then analyzed for CD4 binding. As shown in Fig. 3B, after endo H treatment, rabbit anti-gp120 serum recognized the deglycosylated form of gp120 completely but not the partially endo H-resistant forms. In contrast, CD4 recognized heterogeneous glycoproteins which migrated in the 58- to 76-kDa range. The major binding component within this heterogeneous group of glycoproteins appeared to be higher-molecular-mass material (about 61 and 76 kDa) that represents partially deglycosy-

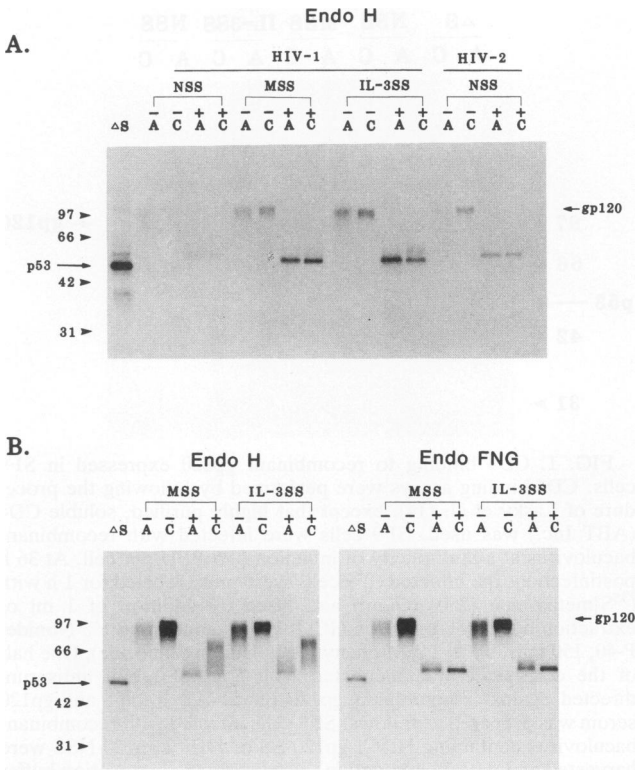

FIG. 3. Effect of deglycosylation of gp120 on binding of CD4. For the intracellular CD4-binding assay (A), pulse-labeled cell lysates (as described in the legend to Fig. 1) were incubated with 1 μg of soluble CD4 at 4°C for 2 h, 60 μl of OKT4 was added, and incubation was continued at 4°C overnight. Immune complexes were bound to protein A–Sepharose CL-4B and washed three times with RIPA buffer (described in the legend to Fig. 1). The immunoprecipitates were resuspended by boiling in 100 μl of 1% SDS–50 mM Tris-HCl (pH 6.8) for 3 min. The Sepharose CL-4B beads were spun down, and the supernatants were divided into two equal aliquots. One aliquot received an equivalent volume of digestion buffer (150 mM Na citrate [pH 5.3], 2 mM phenylmethylsulfonyl fluoride) containing 3 mU of endo H (Boehringer Mannheim), while the other served as a control. Digestion was performed at 37°C for at least 16 h, after which the samples with (+) and without (−) endo H were further divided into two equal aliquots. Each aliquot was brought to 500 μl by addition of 450 μl of extraction buffer. One sample was incubated with 5 μl of rabbit anti-gp120 serum (lanes A), while another was incubated with 0.4 μg of soluble CD4 followed by 30 μl of OKT4 (lanes C) at 4°C overnight. Complexes were bound to protein A-Sepharose and analyzed by SDS–10% PAGE. For the extracellular CD4-binding assay (B), recombinant baculovirus-infected SF9 cells were labeled with [^{35}S]methionine and chased for 2 h. Culture medium was clarified and adjusted to 50 mM sodium phosphate (pH 5.0)–1 mM phenylmethylsulfonyl fluoride. One half of the medium (1 ml) was incubated without enzyme (−), while the remaining half was incubated with either 10 mU of endo H (+) or 2 U of endo F/N glycanase (Boehringer Mannheim) (+) at 37°C for 16 h, after which the supernatants were divided into two equal aliquots. One aliquot was incubated with 5 μl of rabbit anti-gp120 serum (lanes A), and the other half was incubated with 0.4 μg of soluble CD4 and then with 30 μl of OKT4 (lanes C) at 4°C overnight. Immunoprecipitates were analyzed on SDS–10% PAGE as described above. For the comparison, radiolabeled gp120-ΔS immunoprecipitated with rabbit anti-gp120 serum was used as a marker. Endo FNG, endo F/N glycanase.

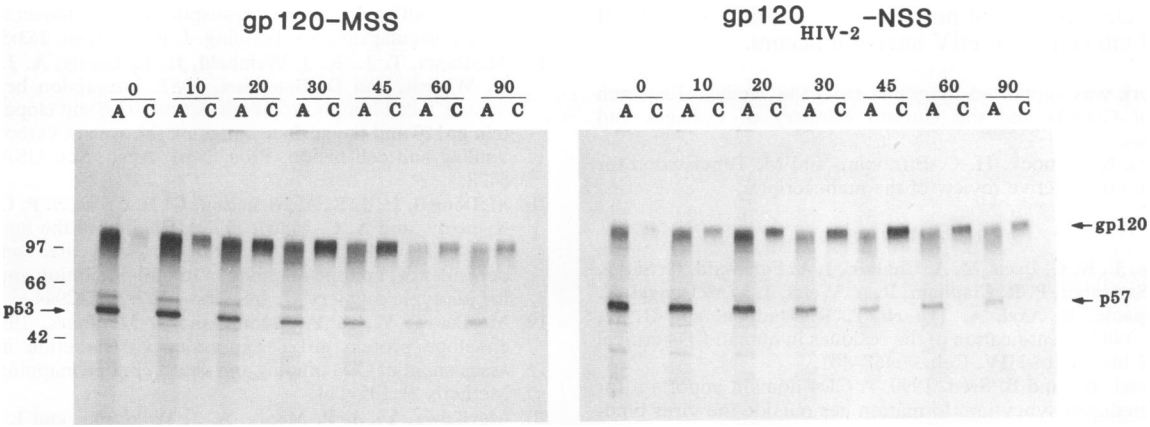

FIG. 4. Time course of intracellular folding of gp120. Infected SF9 cells were pulse-labeled for 10 min with [^{35}S]methionine at 36 h postinfection and chased for the indicated times (minutes). Equal portions of cell lysates were incubated with either rabbit anti-gp120 serum (lanes A) or OKT4 after incubation with soluble CD4 (lanes C). The precipitated material was then analyzed by SDS–10% PAGE and visualized by fluorography.

lated gp120 molecules. In contrast, when the secreted gp120 was treated with endo F/N glycanase, which cleaves all glycan moieties from the molecule (7, 28), gp120 migrated as a 58-kDa protein and still displayed CD4-binding activity (Fig. 3B). These results demonstrate that in contrast to previous reports (6, 17), deglycosylation of intracellular and extracellular recombinant gp120 with endo H or endo F/N glycanase under denaturing conditions in the presence of SDS or nondenaturing conditions does not abrogate the ability to bind to CD4. In addition, these results suggest that N-linked oligosaccharides on the gp120 molecule are necessary for correct folding to generate the CD4-binding site but the carbohydrate moiety itself on gp120 is not required to maintain the conformation of the CD4-binding domain once it has formed (7, 8, 30).

To substantiate further the idea that folding of gp120 plays an important role in binding to CD4, we analyzed the intracellular folding of gp120 expressed by recombinant baculovirus. We expressed HIV-1 gp120 containing the mellitin signal sequence and HIV-2 gp120 with its own signal sequence and analyzed their abilities to bind to CD4 as a functional assay for conformation during pulse-chase experiments (6). Recombinant baculovirus-infected SF9 cells were pulse-labeled for 10 min with [^{35}S]methionine and lysed at different times after chasing. One half of each lysate was immunoprecipitated with rabbit anti-gp120 serum, while the remaining half was incubated with excess soluble CD4, followed by immunoprecipitation with OKT4. As described in the legend to Fig. 1, the antiserum we used was raised directly against the denatured, nonglycosylated recombinant gp120-ΔS protein; it reacted efficiently with both the nonglycosylated and glycosylated forms of gp120 but reacted poorly with native, fully glycosylated gp120 (25, 30). Significant amounts of the nonglycosylated and glycosylated forms of gp120 were immunoprecipitated with the antiserum early in the chase period, but this gradually decreased with time (Fig. 4). In contrast, newly synthesized gp120 was unable to bind to CD4 (6) although it was fully glycosylated. Its ability to bind to CD4 gradually increased with time. This result indicates that gp120 is first synthesized as a highly glycosylated form which is not folded into the mature conformation and cannot be recognized by CD4 (6). With increasing time, the protein folds properly to form a CD4-binding domain (Fig. 4). This suggests that the carbohydrate chain plays a role in conferring conformational features on the mature glycoprotein that are not present in the nonglycosylated protein.

The role of the carbohydrate on gp120 of HIV and other virus surface proteins is not firmly established. The lack of tertiary structure information on HIV gp120 makes it difficult to predict the effects of N-linked oligosaccharides on the interaction of gp120 with CD4. Recent information indicates that N-linked oligosaccharides of different viral glycoproteins play a significant role in determining properties of viral glycoproteins which are dependent on folding to form the proper conformation (16, 24, 26, 30, 32). Our results agree with previously published data (7, 8) that indicate that enzymatic removal of glycans in the presence or absence of an ionic detergent (SDS) does not significantly affect the conformation of gp120; deglycosylated gp120 still retains the same structural and biological properties as the native glycoprotein and can bind to CD4. This indicates that carbohydrate side chains per se are unlikely to be involved in direct contact with CD4 (as in the sugar-lectin interaction) and oligosaccharide chains can be altered without loss of CD4-binding ability, as reported here and by others (3, 5, 6, 20, 21). Rather, an N-linked carbohydrate, as others have suggested (6, 20), may act as molecular "padding," required for gp120 to form the proper conformation for CD4 binding. These results suggest that the conformation of gp120 is crucial for CD4 binding. Recent studies on the effect of glycosylation on the reactivity of vesicular stomatitis virus G protein with monoclonal antibodies directed to conformational epitopes have provided evidence that the carbohydrate chains do not influence the antigenic specificity of vesicular stomatitis virus G protein. This supports the notion that N-linked glycosylation plays a key role in promoting the formation of correct disulfide bonds which determine epitope-specific conformation of vesicular stomatitis virus glycoprotein (10).

On the basis of these findings (3, 5, 6–8, 17, 19, 20, 21), our data are compatible with the hypothesis that N-linked glycosylation of HIV gp120 plays a pivotal role in folding of the protein (7). Further characterization of the requirements for correct gp120 tertiary conformation not only will clarify the intracellular pathway of glycoprotein folding but may also

lead to a clear picture of how the critical primary event of virus-cell interaction in HIV infection occurs.

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