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Model for Intracellular Folding of the Human Immunodeficiency Virus Type 1 gp120

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The intracellular folding of the human immunodeficiency virus type 1 gp120 has been assessed by analyzing the ability of the glycoprotein to bind to the viral receptor CD4. Pulse-chase experiments revealed that the glycoprotein was initially produced in a conformation that was unable to bind to CD4 and that the protein attained the appropriate tertiary structure for binding with a half-life of approximately 30 min. The protein appears to fold within the rough endoplasmic reticulum, since blocking of transport to the Golgi apparatus by the oxidative phosphorylation inhibitor carbonyl cyanide *m*-chlorophenylhydrazone did not appear to perturb the folding kinetics of the molecule. The relatively lengthy folding time was not due to modification of the large number of N-linked glycosylation sites on gp120, since inhibition of the first steps in oligosaccharide modification by the inhibitors deoxynojirimycin or deoxymannojirimycin did not impair the CD4-binding activity of the glycoprotein. However, production of the glycoprotein in the presence of tunicamycin and removal of the N-linked sugars by endoglycosidase H treatment both resulted in deglycosylated proteins that were unable to bind to CD4, suggesting in agreement with previous results, that glycosylation contributes to the ability of gp120 to bind to CD4. Interestingly, incomplete endoglycosidase H treatment revealed that a partially glycosylated glycoprotein could bind to the receptor, implying that a subset of glycosylation sites, perhaps some of those conserved in different isolates of human immunodeficiency virus type 1, might be important for binding of the viral glycoprotein to the CD4 receptor.

Infection of cells by human immunodeficiency virus type 1 (HIV-1) is initiated by binding of the viral surface glycoprotein gp120 to the cellular viral receptor CD4 (5, 16, 25). This binding is a high-affinity event that appears to be completely dependent upon the overall conformation of the envelope glycoprotein (22, 26). Initial studies of the gp120 sequence requirements for CD4 interaction demonstrated that spatially distinct regions in the C terminus of the viral glycoprotein were critical for binding to the receptor (7, 18, 22). In addition to the importance of disulfide-induced glycoprotein conformation, the infectivity of HIV appears to be dependent upon the state of glycosylation of gp120. Production of gp120 in bacteria (30) or deglycosylation of native gp120 with endoglycosidase F in the presence of sodium dodecyl sulfate (23) results in proteins unable to bind to the CD4 receptor, while blocking of the initial events in N-linked oligosaccharide side-chain trimming by either castanospermine (32) or deoxynojirimycin (DNM; 14) results in viruses unable to induce syncitium formation or productively infect CD4-positive cells. These results suggest that the overall conformation of gp120 is a consequence of complex intramolecular interactions that embody contributions by both the primary amino acid sequence and posttranslational events, including glycosylation. Unfortunately, little is understood regarding the means by which the newly synthesized viral envelope glycoprotein attains this elaborate native structure.

The intracellular folding of glycoproteins destined for the cellular secretory pathway has been primarily studied by using either conformation-dependent monoclonal antibodies or protein oligomer formation. The great bulk of work in this area has focused on the folding and trimerization of such viral envelope glycoproteins as the vesicular stomatitis virus G protein (20; R. Doms, A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. Rose, J. Cell Biol., in press) and the influenza hemagglutinin (HA) glycoprotein (12, 19, 35). This work has demonstrated that folding and oligomerization occur in the rough endoplasmic reticulum (RER) with a fairly rapid half-life (12). The overall monomeric native conformation of HA appears to be completed before oligomeric assembly, after which the appropriately folded trimer rapidly passes from the RER to the *cis* Golgi apparatus for further oligosaccharide modification (35). Studies in this as well as other glycoprotein systems suggest that the rate of efficiency of RER-to-Golgi transport appears to be dependent upon the attainment of correct tertiary and quaternary structure (13, 20, 34). The role of glycosylation in the folding and transport of secreted glycoproteins has been analyzed in only a few systems, in which blockage of the addition of N-linked sugar residues appears to result in denatured proteins that tend to aggregate or associate with the immunoglobulin-heavy-chain-binding protein BiP and that are inefficiently secreted (3, 6, 13, 19, 34). The role of glycosylation in protein function has been analyzed in a limited fashion in the acetylcholine receptor system, in which it was found that glycosylation appeared to be required for appropriate ligand interaction (29).

Although previous studies have clearly demonstrated the intracellular site of glycoprotein folding as well as its role in cellular transport in a number of antibody- or higher-order-structure-based assays, they have not analyzed any glycoproteins by using an assay that measures the actual function of the molecule. We have previously shown that a recombinant form of the HIV-1 gp120 can be used to study the interactions between the viral glycoprotein and CD4 and that this glycoprotein appears to mimic many of the functions of the virally synthesized molecule (22). In this study we have analyzed the intracellular folding of gp120 by using its binding to CD4 as a functional assay for overall conforma-

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tion. In addition, we have shown that glycosylation of the glycoprotein is critical for its binding to the cellular receptor and that the receptor-binding conformation of the glycoprotein may be, in part, dependent upon the glycosylation of a subset of N-linked sites in gp120.

MATERIALS AND METHODS

gp120-expressing cell lines. Chinese hamster ovary cell lines producing a secreted form of gp120 were produced as previously described (21). A new plasmid for the expression of a secreted, mature form of gp120 with only nine N-terminal amino acids derived from the herpes simplex virus glycoprotein D gene was constructed (D. Dowbenko and L. Lasky, data not shown). Glycoproteins were labeled with [^{35}S]methionine and [^{35}S]cysteine in methionine-cysteine-free medium as previously described (21, 22). Pulse-chase experiments were performed by labeling the cells for 10 min with 500 μCi of each isotope in methionine-cysteine-free medium, washing them in phosphate-buffered saline, and incubating the pulse-labeled cells for various periods of time in complete medium. At each time point, samples were spun, and the supernatants were saved. The pelleted cells were lysed with 1% Nonidet P-40 and used for coprecipitation with CD4.

CD4-binding assays. CD4-binding assays were performed as previously described (22), with the exception that a highly purified, truncated form of the receptor was used for the binding studies (31). The affinity of this truncated receptor molecule for gp120 has been shown to be identical to that found for the membrane-bound form of the receptor. For all CD4-binding experiments, 1/10 of the supernatant or cell lysate was analyzed by immunoprecipitation with a high-titer rabbit antiserum directed against recombinant gp120, while the remaining 9/10 of the material was analyzed for CD4-binding ability. gp120-CD4 complexes were immunoprecipitated with either the OKT4 or the OKT4a monoclonal antibody (Ortho Pharmaceuticals) and protein A-Sepharose and analyzed on 7.5% polyacrylamide-sodium dodecyl sulfate gels.

Inhibitor studies. For carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) incubations, the cells were pulse-labeled for 10 min as described above. Labeled cells were washed and transferred to glucose-free medium containing 80 μM CCCP (10) for the times indicated below and analyzed for intracellular CD4 binding. For DNM and deoxymannojirimycin (DMM) treatment, cells were labeled for 4 h as described above in the presence of 1 mM of either inhibitor (2, 11). Tunicamycin inhibition was performed at an inhibitor concentration of 15 μg/ml (6). All lysates or supernatants from these inhibitor experiments were then analyzed for CD4 binding as described above.

Glycosidase studies. Deglycosylations were performed on immunoprecipitated gp120-CD4 complexes or gp120 immunoprecipitated with anti-gp120 antiserum to insure complete glycosidase digestion. Pelleted material was washed four times with 0.2% Tween 20–0.2% sodium deoxycholate in phosphate-buffered saline and once with 10 mM Tris hydrochloride, pH7.5–1% Nonidet P-40. For neuraminadase samples, 100 μl of 100 mM sodium acetate, pH 5.2, 2 mM CaCl2, and 0.2 mM EDTA were added, and 100 μl of 100 mM sodium citrate, pH 5.5, was added for the endoglycosidase H samples. Neuraminadase (10 mU; Calbiochem-Behring) or endoglycosidase H (5 mU; Genzyme) was added to the samples, after which they were incubated overnight at 37°C. The pelleted material was washed and analyzed on polyacrylamide gels. Time course experiments showed that a 2-h incubation with 5 mU of endoglycosidase H gave partially digested gp120, and these conditions were used for the partial digestion experiment. In addition, control experiments showed that treatment of recombinant soluble CD4 with either neuraminadase or endoglycosidase H had no effect on its gp120-binding ability.

RESULTS

Kinetics of intracellular folding of recombinant gp120. Previous work with recombinant gp120 utilized a glycoprotein variant that lacked the N-terminal 30 amino acids of the virally encoded glycoprotein and contained 25 amino acids derived from the mature N terminus of the herpes simplex virus type 1 glycoprotein D (21). To make a more natural molecule, a new HIV gp120 gene was constructed that encoded the mature N terminus of the viral glycoprotein in translational phase with the 34 N-terminal amino acids encoded by the glycoprotein D gene, including the 25-amino-acid signal sequence of the protein. Synthesis and secretion of this glycoprotein from mammalian cells resulted in a glycoprotein containing nine N-terminal amino acids from glycoprotein D followed by the HIV gp120 glycoprotein. The absence of any gp41-derived transmembrane anchor sequence from this glycoprotein allowed for its secretion from these cells into the medium. Stable Chinese hamster ovary cell lines expressing this construct were produced as previously described (21), and the affinity of the gp120 isolated from these lines was found to be identical to that previously determined for recombinant gp120 as well as for the native gp120 isolated from virus-infected cells (T. Matthews, personal communication; G. Nakamura and L. Lasky, unpublished data). Both intracellular and secreted forms of gp120 appeared to bind to a soluble form of the CD4 receptor with approximately the same efficiency (5 to 10%) (Fig. 1; 22). This binding appeared to be significant, since as has been previously shown, the complex cannot be immunoprecipitated by the OKT4A monoclonal antibody. In addition, these results show that the intracellular form of gp120 is slightly smaller than the secreted form, suggesting that terminal oligosaccharide modifications occurred to gp120 as it was secreted from these cells.

To determine the kinetics of intracellular folding of gp120, the ability of the glycoprotein to bind to CD4 was measured in a pulse-chase experiment. The glycoprotein was almost completely unable to bind to CD4 after the initial pulse-label (Fig. 2), suggesting that it is originally synthesized as a predominantly denatured, highly glycosylated molecule. With time after the pulse-label, the protein gradually attained a native conformation that enabled it to bind to CD4. The half-life of this folding event appears to be approximately 30 min (Fig. 2B), substantially longer than has been found for the attainment of a native, oligomeric structure by the influenza virus HA (~5 min) (12, 35). The initially synthesized glycoprotein underwent posttranslational sugar cleavage (Fig. 2), so that there was a clear decrease in the size of the molecule with time of chase. The faint, higher-molecular-weight band is presumed to be terminally glycosylated material that has yet to be secreted from the cell. Finally, the glycoprotein appears to be secreted from the cell with a half-life of approximately 60 to 90 min.

Intracellular site of folding of gp120. The fairly long half-life of folding and obvious trimming of the sugar residues of gp120 shown in Fig. 2 led us to ask if the glycoprotein folded within the RER before transport to the *cis* Golgi apparatus.

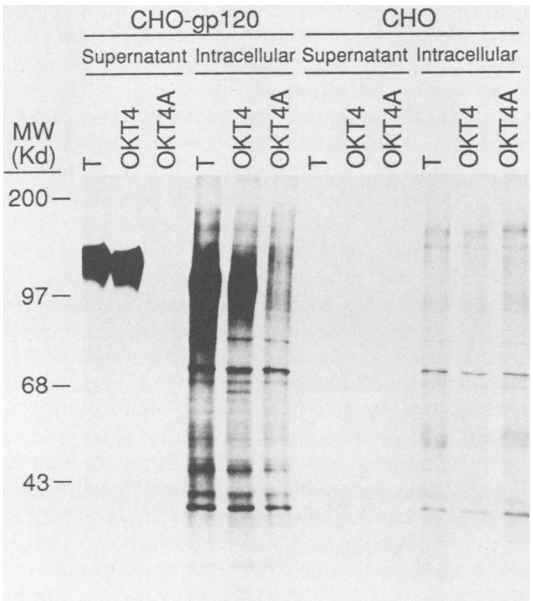

FIG. 1. CD4 binding of secreted and intracellular recombinant gp120. The left side of the figure illustrates CD4 binding of secreted and intracellular gp120 produced in a permanent mammalian cell line, while the right side of the figure illustrates the same binding experiments in a control cell line. One-tenth of the total (T) material was immunoprecipitated with high-titer antiserum directed against recombinant gp120. The remaining 9/10 of the material was incubated with soluble CD4, and the complex was coprecipitated with either OKT4 or OKT4A. The precipitated material was then analyzed on 7.5% polyacrylamide-sodium dodecyl sulfate gels. MW, Molecular size; Kd, kilodaltons.

Previous work has demonstrated that transfer of glycoproteins from the RER to the *cis* Golgi apparatus is an ATP-requiring step (1) and that the oxidative phosphorylation inhibitor CCCP appears to block this transfer (10). Cells were pulse-labeled for 10 min, after which they were incubated in the presence of CCCP in glucose-free medium for the indicated times. Total and CD4-bindable gp120 were analyzed at each time point. gp120 that was prevented from exiting the RER appeared to fold with approximately the same kinetics as the normally transported glycoprotein (Fig. 3). The lack of intracellular transport is illustrated by the inhibition of core oligosaccharide trimming, clear absence of gp120 loss from the cell by secretion (see the 120-min time point in Fig. 2 versus that in Fig. 3), as well as the lack of the higher-molecular-weight, terminally glycosylated gp120 molecule seen in the pulse-chase experiment shown in Fig. 2A.

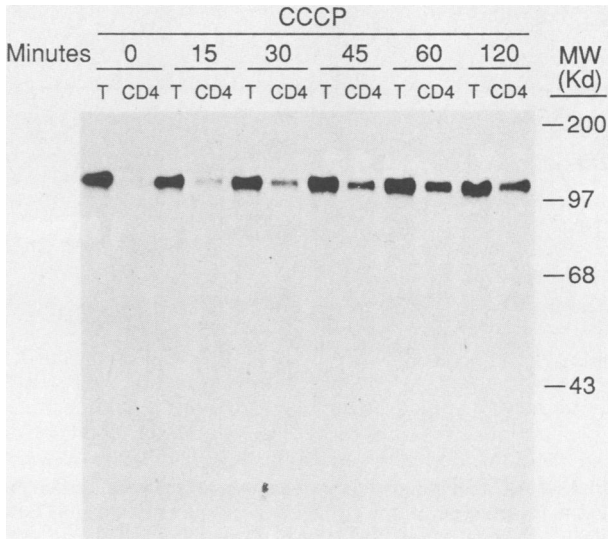

FIG. 3. Pulse-chase analysis of CD4 binding by intracellular gp120 in the presence of CCCP. Cells were pulse-labeled for 10 min with [^{35}S]cysteine and [^{35}S]methionine, washed, and then incubated in glucose-free medium in the presence of CCCP for the indicated times. Cell lysates were analyzed for total and CD4-bindable gp120 as described in the legend to Fig. 2. MW, Molecular size; Kd, Kilodaltons.

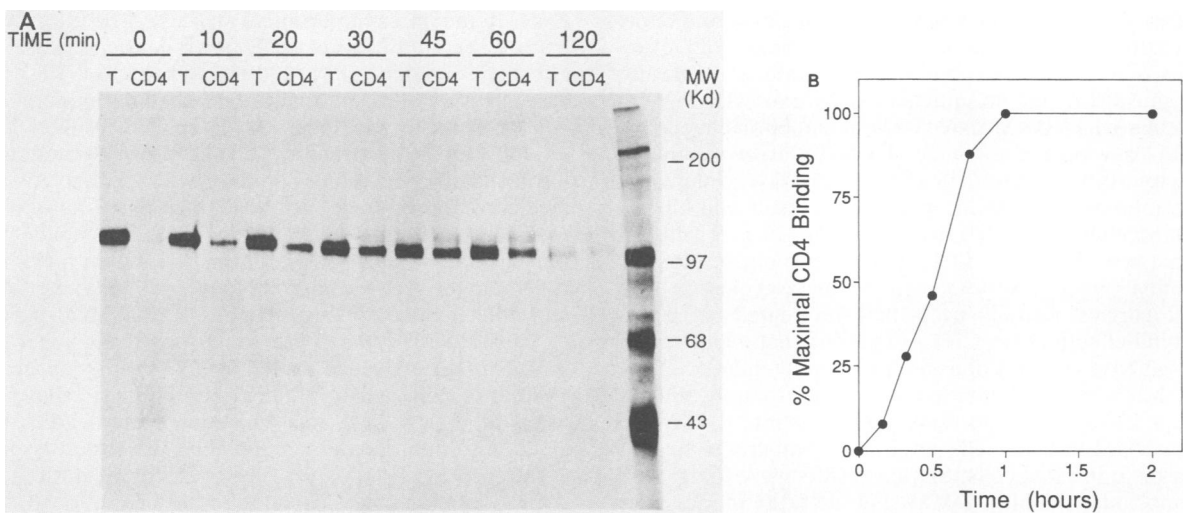

FIG. 2. Pulse-chase analysis of CD4 binding by intracellular gp120. Cells were pulse-labeled for 10 min with [^{35}S]cysteine and [^{35}S]methionine, washed, and chased for the indicated times. Intracellular material was either immunoprecipitated with anti-gp120 antiserum (1/10) (T) or OKT4 after complexing with soluble CD4 (9/10) (CD4). (B) Time course of CD4 binding as determined by densitometric measurement of the gel shown in panel A, with each point being normalized to the maximal binding seen for the 1- and 2-h time points. MW, Molecular size; Kd, Kilodaltons.

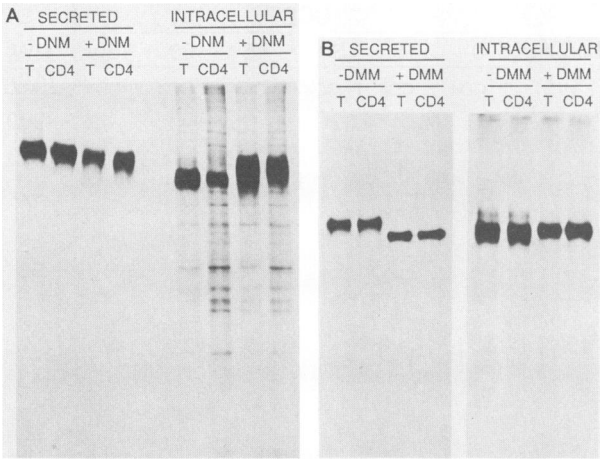

FIG. 4. CD4 binding of intracellular and secreted gp120 produced in the presence of the glycosidase inhibitors DNM and DMM. Cells were labeled with [^{35}S]cysteine and methionine in the presence of 1 mM concentrations of each inhibitor for 4 h, after which the cell supernatants and cell lysates were analyzed for total (T) and CD4-binding (CD4) gp120 as described in Materials and Methods. (A), Intracellular and supernatant material produced in the presence of DNM. (B) Intracellular and supernatant material produced in the presence of DMM.

In addition, endoglycosidase H experiments have shown that intracellular gp120 remains endoglycosidase H sensitive for the first 90 min of the chase, suggesting a long transit time from the RER to the Golgi compartment (data not shown). These results suggest that the gp120 glycoprotein is folded within the RER compartment of the cell and that the molecule presumably must fold before transport to the Golgi apparatus. In addition, the results suggest that this folding process does not require intracellular ATP.

The prolonged folding time for gp120 may have been due to the processing of the large number of N-linked oligosaccharide groups by the glucosidase and mannosidase enzyme systems of the secretory pathway. Previous work with castanosperimine, a glucosidase inhibitor, DNM, another glucosidase inhibitor, and DMM, a mannosidase inhibitor, suggested that these compounds have a profound effect on HIV infectivity, but they did not clearly address the nature of this effect (14, 32). Mammalian cells expressing gp120 were incubated in DNM and DMM at inhibitor concentrations that have been previously shown to effectively block the activity of the glucosidase and mannosidase systems (2, 11). The inhibition by DNM appeared to result in a higher-molecular-weight form of intracellular gp120 (Fig. 4A), a result that was expected since the glycoprotein maintained the terminal glucose residues on its oligosaccharide side chains. Figure 4A also illustrates that the treated gp120 was able to bind effectively to CD4, suggesting that maintenance of these glucose residues does not profoundly influence CD4 binding. Interestingly, inhibition of glucose trimming did not appear to block secretion, since the amount of secreted gp120 in DNM-treated cells appeared comparable to that found in the untreated cells. Similar results were found with the mannosidase inhibitor DMM (Fig. 4B); the intracellular material appeared as a discrete, untrimmed band that appeared able to bind effectively to CD4. Again, this treatment did not seem to inhibit secretion, although the extracellular material produced in DMM-treated cells was of lower molecular weight, presumably due to inhibition of terminal glycosylation on the untrimmed intracellular precursor. These results suggest that the prolonged folding time of intracellular gp120 is not due to trimming of the large number of oligosaccharide side-chain residues.

Role of glycosylation in gp120-CD4 interaction. The studies reported thus far suggested that gp120 is produced as a highly glycosylated, denatured molecule that then folds with relatively slow kinetics to give rise to a correctly folded CD4-binding glycoprotein. Previous work on the virally synthesized glycoprotein suggested that removal of the oligosaccharide side chains with endoglycosidase F in the presence of low levels of sodium dodecyl sulfate without disulfide reduction results in a presumably normally disulfide-linked molecule incapable of binding to CD4 (25). This result suggested that the oligosaccharide side chains of gp120 contribute in some way to its ability to bind to CD4. As can be seen in Fig. 5, production of gp120 in the presence of the inhibitor tunicamycin resulted in a lower-molecular-weight molecule that was incapable of binding efficiently to CD4. The low level of binding to CD4 seen in this experiment may be due to aggregation of nonglycosylated gp120 and is probably nonspecific, since precipitation of the complex with OKT4A, a CD4 monoclonal antibody known to bind to a site at or near the gp120 binding site (Fig. 1; 5, 16), showed the same light band (compare the CD4 immunoprecipitation with the CD4 [OKT4A] precipitation). In addition, treatment of cells producing gp120 with tunicamycin completely blocked secretion of the HIV glycoprotein. These results suggest that the cotranslational addition of oligosaccharide side chains to the newly synthesized envelope glycoprotein is required for appropriate CD4 binding as well as for secretion of the molecule, although secondary toxic effects of tunicamycin on secretion cannot be ruled out.

The next series of experiments was designed to determine the effect of various types of oligosaccharide modification on the ability of gp120 to bind to CD4. Unlike those in previously reported work (23), these glycosidase digests were performed on the gp120-CD4 complex to investigate if the heterodimer is resistant to deglycosylation. The N-linked glycosylation sites in soluble, purified CD4 (31) were of the complex type and almost completely resistant to endoglycosidase H digestion (M. Spellman, personal communication). Removal of the terminal sialic acid residues from the mature secreted form of gp120 with neuraminadase had no effect on the ability of gp120 to bind to CD4 (Fig. 5). This agrees with the finding that the intracellular material, which is not terminally modified with sialic acid residues, appears to bind well to the viral receptor. However, treatment of the intracellular gp120-CD4 complex with endoglycosidase H appeared to profoundly decrease the ability of gp120 to bind to CD4. As will be described below, the faint smudge seen in the CD4-bound, endoglycosidase H-treated intracellular material may be residual, partially glycosylated material that is still able to bind to the receptor. This result suggests, in agreement with those of previous studies using endoglycosidase F on the mature, secreted form of the glycoprotein, that even after appropriate folding and binding to the receptor, the viral glycoprotein must conserve some degree of glycosylation for continued maintenance of receptor association.

Interestingly, endoglycosidase H treatment of the secreted, terminally glycosylated molecule demonstrated that some of the N-linked glycosylation sites of gp120 remained in a high-mannose form that was endoglycosidase H sensitive (Fig. 5). Analysis of the sugar residues of secreted purified recombinant gp120 has shown that a fraction of these oligosaccharides are in the high-mannose form (M.

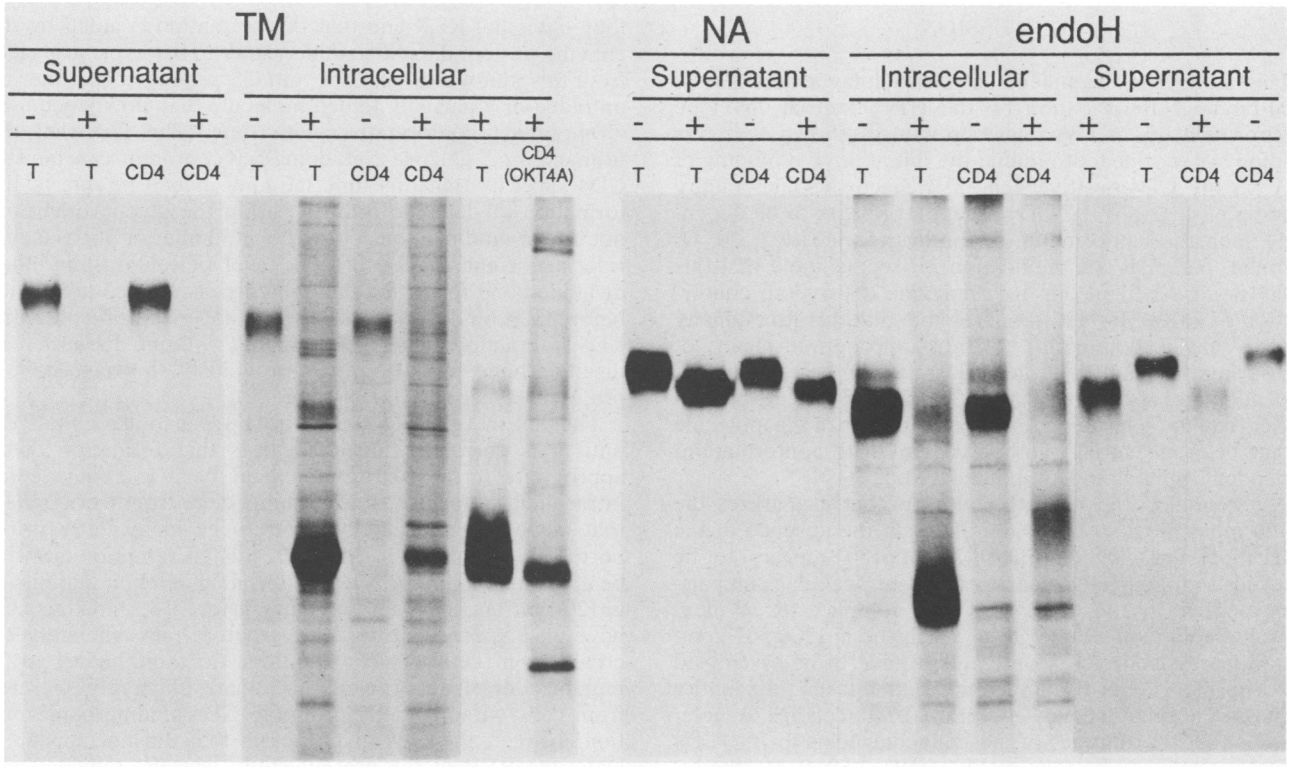

FIG. 5. The effects of glycosylation on CD4 binding of gp120. Cells were labeled in the presence of tunicamycin (TM), after which the intracellular and supernatant materials were analyzed for total (T) and CD4-binding (CD4) gp120. As an additional control, the CD4-gp120 complexes from tunicamycin-treated cells were also analyzed with the nonprecipitating OKT4A monoclonal antibody. The contribution of sialic acid to CD4 binding was analyzed by incubation of the secreted viral glycoprotein-CD4 complexes with neuraminadase (NA) and analysis of total and CD4-bindable gp120. The contribution of core glycosylation to CD4 binding was analyzed by treatment of the intracellular and secreted viral glycoprotein-CD4 complexes with endoglycosidase H (endoH) and analysis of the total and CD4-bindable viral glycoprotein.

Spellman, unpublished results), while previous work suggests that the virally produced glycoprotein also contains high-mannose oligosaccharides (27). In addition, the endoglycosidase H-treated glycoprotein was able to bind to CD4, although the relative binding affinity seemed to be somewhat lower (Fig. 5). This result suggested that partial removal of oligosaccharide side chains by endoglycosidase H might result in intracellular glycoproteins that maintained CD4-binding ability. Partial endoglycosidase H treatment of the intracellular gp120-CD4 complex resulted in a lower-molecular-weight, heterogeneous immunoprecipitable glycoprotein smear that migrated from approximately 60 to 76 kilodaltons (Fig. 6). Interestingly, a fraction of the higher-molecular-weight material within this smear appeared to maintain its CD4-binding characteristics. While the binding fraction was somewhat heterogeneous, it appeared to represent the highest-molecular-weight area of the partially deglycosylated material and had a modal size of approximately 76 kilodaltons. It is also clear from this figure that material below this region was incapable of binding to CD4, suggesting that a certain minimal number of oligosaccharide side chains must be preserved to maintain receptor binding. From the amino acid content of the viral protein, completely deglycosylated gp120 is approximately 59,500 molecular weight. Assuming an average oligosaccharide molecular weight of 2,500 to 3,000 (17), these results suggest that an average of six or seven oligosaccharide side chains must be conserved on the envelope glycoprotein in order to maintain its CD4-binding ability.

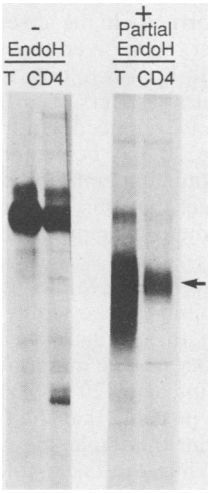

FIG. 6. CD4 binding of partially deglycosylated gp120. Intracellular viral glycoprotein was partially deglycosylated by treatment of a precipitated gp120-CD4 complex with 5 mU of endoglycosidase H for 2 h. The total (T) and CD4-binding (CD4) glycoproteins were analyzed as described above. The left panel shows binding of nontreated intracellular material. Soluble, purified CD4 was found to be predominantly endoglycosidase H resistant (M. Spellman, personal communication).

DISCUSSION

This study has examined the intracellular acquisition of CD4-binding conformation by the HIV external envelope glycoprotein gp120. We have previously shown that this binding assay appears to mimic the initial in vivo binding of HIV to the CD4-positive cell (22). Since the high-affinity binding of gp120 to the CD4 receptor appears to be dependent upon the conformation of both molecules (22, 26; D. Littman, personal communication), it seems likely that this assay is an excellent one for analyzing the overall conformation of gp120. In fact, it is probable that this functionally based assay is potentially the most appropriate means of measuring the conformation of this glycoprotein, since it is dependent upon the overall structural integrity of the protein rather than upon just a regional configuration of the molecule as has been measured in other systems with conformation-dependent monoclonal antibodies (35).

A potential criticism of this work is that it analyzes the gp120 molecule as a truncated entity in the absence of the C-terminal gp41 glycoprotein (21). gp120 appears to be cleaved from a gp160 precursor in an intracellular compartment, either in the RER or Golgi complex (R. Willey, personal communication), after which the gp120-gp41 complex apparently travels to the cell surface to be assembled into virions. The most compelling argument for the identity between the recombinant and virally produced gp120 molecules is that the affinities of these glycoproteins for the CD4 receptor appear to be remarkably similar (22; T. Matthews, unpublished results). In fact, soluble recombinant gp120 can be used to block HIV infectivity in tissue culture (J. Groopman, unpublished results). It is highly unlikely that an inappropriately folded recombinant gp120 will have an affinity as elevated as the correctly folded viral protein, since conformation appears to be critical for CD4 binding. In addition, monoclonal-antibody-blocking studies using CD4-specific antibodies suggest that the recombinant gp120-CD4 interaction is blocked by the same CD4 monoclonal antibodies that hinder viral infectivity (22; G. Nakamura and L. Lasky, unpublished results). These results strongly suggest that gp120 can fold correctly in the absence of the C-terminal gp41 region. However, even given these data, it is unclear if the relatively long half-life for folding found here is actually due to unique structural aspects of gp120 or due to the fact that the glycoprotein is produced in a truncated form. Thus, the folding time of gp120 may be profoundly affected by the inclusion of the C-terminal membrane-bound gp41 glycoprotein. We therefore feel that this system provides a suitable model for the attainment of appropriate tertiary conformation by gp120.

If it is assumed that the long folding time for gp120 does in fact represent that found for the virally produced molecule, then it is interesting to speculate on the reasons for this relatively lengthy process. The importance of disulfide bond formation in the binding of gp120 to CD4 has been previously shown (26), and it is possible that the formation of the nine disulfides found within this molecule may contribute to its slow folding time. While the faster-folding HA must fabricate a higher-order trimeric structure, there are only three intramolecular disulfides that must be formed in this glycoprotein, suggesting that the difference in folding times may be influenced by the complexity of the disulfide interactions found in gp120 versus HA. The mechanism of this complex folding process, of course, remains a mystery. Previous work suggested that disulfide bond formation in other cellular glycoproteins appears to occur within protein domains as they enter the RER and that this formation is aided by the enzyme protein disulfide isomerase (9). However, it is clear from this study that gp120 is initially produced as either an unfolded or a partially folded molecule that slowly acquires CD4-binding conformation with time. The fact that the primary (i.e., glucose-containing) glycoprotein can bind to CD4 suggests that this time lag may indeed be due to the formation of disulfide bridges within the glycoprotein and not due to modifications of the large number of oligosaccharide side chains. Of course, a level of folding finer than disulfide bond formation undoubtedly also has to take place before CD4 binding occurs, since mutagenesis of a potential CD4 interaction region, presumably without disruption of disulfide bond formation, was previously shown to have a profound effect on CD4 binding (22).

The fact that the gp120 does not appear to leave the RER until it is correctly folded suggests that formation of an appropriate tertiary structure might be a prerequisite for intracellular transport. It is certainly clear from work on HA that misfolded molecules appear to be inefficiently transported in cells (12, 19). However, this presumption may not be applicable to gp120, since N-terminal deletion mutants of gp120 that are incapable of binding to CD4, and thus that have an inappropriate tertiary structure, are efficiently secreted from cells (7). In addition, the nonbinding gp120 mutants described previously (22) are efficiently secreted from cells without the appropriate CD4-binding conformation. In fact, the only glycoprotein that did not appear to leave the RER in the present study was the tunicamycin-treated molecule, a result that may be interpreted as misfolding or aggregation, the need for oligosaccharide addition as a signal for transport, or tunicamycin-induced cytotoxicity (3, 6, 12). Thus, the possibility that misfolded proteins are incapable of exiting the RER would appear not to apply entirely to the HIV gp120 molecule. However, since the gp120 analyzed here appears to bind to CD4 as a monomer, while the influenza virus HA functions in a trimeric form, the difference in results may be due more to dissimilarities in the species of molecules that have been investigated. In addition, the relative degree of misfolding may have profound effects on the ability of a glycoprotein to exit the RER. It thus must be concluded that misfolding per se is not sufficient to block the intracellular transport of a given glycoprotein.

The roles of glycosylation in glycoprotein function have yet to be clearly elucidated. Oligosaccharides appear to play a role in protein transport, solubility, conformation, and inhibition of antibody neutralization (3, 6, 12, 24). An additional potential role for the sialic acid region of oligosaccharides is to prevent neutralizing-antibody binding to the surface glycoproteins of the caprine arthritis-encephalitis lentivirus (15). The data reported here agree with those of previous experiments on the mature, viral form of the HIV glycoprotein (23) and suggest that glycosylation of gp120 is critical for CD4 binding. One trivial explanation for these results would be that complete deglycosylation of gp120 results in insoluble, aggregated molecules. The question of whether glycosylation is a matter of maintenance of protein solubility or, more interestingly, induction of conformation still remains unanswered. Recent work on assembly of human chorionic gonadotropin suggests that certain N-linked oligosaccharides appear to play a role in the assembly of the alpha and beta chains of this heterodimer (24), while work with the acetylcholine receptor suggests that ligand binding is dependent upon appropriate glycosylation (29). These results suggest that glycosylation may have an impor-

tant effect on protein conformation. While previous work has demonstrated that mutagenesis of N-linked glycosylation sites in the central conserved domain of gp120 does not have a profound effect on CD4 binding (33), it remains to be seen whether mutation of glycosylation sites near the previously characterized C-terminal CD4-binding domain of gp120 (18, 22) has an effect on CD4 interaction.

Another interesting aspect of glycosylation stems from two recent reports that demonstrate that inhibition of the removal of the terminal glucose residues from oligosaccharide side chains by either castanospermine or DNM results in noninfectious virus (14, 30). In agreement with previous work, we have found that DNM produces a gp120 with glucose-containing oligosaccharides that is still able to bind to CD4, although it is not clear if the affinity of this interaction is as high as that previously found for the terminally glycosylated protein (22). The fact that this glycoprotein binds to the receptor but, when incorporated into the virus, results in noninfectious virions, suggests that functions that occur after viral binding might be disrupted. These may include virus fusion, penetration, or unpackaging. From the results demonstrating inhibition of fusion in DNM-treated virus-infected cells, it seems likely that inclusion of these glucose residues on either gp120 or gp41 has a profound negative effect on the virus-cell fusion event. This suggests that fusion may be far more complicated than mere activation of a fusogenic domain by virus-receptor binding and may involve contributions by oligosaccharide groups on both gp120 and gp41.

The data reported here suggest that gp120 might provide an interesting model for the investigation of the roles of both disulfide bond-induced as well as oligosaccharide-induced intracellular folding. Characterization of the requirements for attainment of appropriate gp120 tertiary conformation will not only clarify intracellular pathways of glycoprotein folding, it may also lead to a clearer picture of how the critical primary event of virus-cell interaction in HIV infection occurs.

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