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Functional Role of the Glycan Cluster of the Human Immunodeficiency Virus Type 1 Transmembrane Glycoprotein (gp41) Ectodomain

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To examine the role of the glycans of human immunodeficiency virus type 1 transmembrane glycoprotein gp41, conserved glycosylation sites within the *env* sequence (Asn-621, Asn-630, and Asn-642) were mutated to Gln. The mutated and control wild-type *env* genes were introduced into recombinant vaccinia virus and used to infect BHK-21 or CD4$^+$ CEM cells. Mutated gp41 appeared as a 35-kDa band in a Western blot (immunoblot), and it comigrated with the deglycosylated form of wild-type gp41. Proteolytic cleavage of the recombinant wild-type and mutant forms of the gp160 envelope glycoprotein precursor was analyzed by pulse-chase experiments and enzyme-linked immunosorbent assay: gp160 synthesis was similar whether cells were infected with control or mutated *env*-expressing recombinant vaccinia virus, but about 10-fold less cleaved gp120 and gp41 was produced by the mutated construct than the control construct. The rates of gp120-gp41 cleavage at each of the two potential sites appeared to be comparable in the two constructs. By using a panel of antibodies specific for gp41 and gp120 epitopes, it was shown that the overall immunoreactivities of control and mutated gp41 proteins were similar but that reactivity to epitopes at the C and N termini of gp120, as present on gp160 produced by the mutated construct, was enhanced. This was no longer observed for cleaved gp120 in supernatants. Both gp120 proteins, from control and mutated *env*, were expressed on the cell surface under a cleaved form and could bind to membrane CD4, as determined by quantitative immunofluorescence assay. In contrast, and despite sufficient expression of *env* products at the cell membrane, gp41 produced by the mutated construct was unable to induce membrane fusion. Therefore, while contradictory results reported in the literature suggest that gp41 individual glycosylation sites are dispensable for the bioactivity and conformation of *env* products, it appears that such is not the case when the whole gp41 glycan cluster is removed.

The mature envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1)—outer membrane gp120 and transmembrane gp41—are proteolytic cleavage products of precursor gp160, and they are responsible for virus binding to CD4$^+$ cells and fusion of virus and cell membranes, respectively (for reviews, see references 13 and 23). Previous results indicate that after biosynthesis, glycans present on mature recombinant or viral gp120 and gp41 are not directly involved in the biological activity of these molecules (9, 12). In contrast, by using an inhibitor of α-glucosidases—1-deoxynojirimycin—which induces abnormal glycosylation, we have recently shown that at least a number of the approximately 30 glycans (16, 26) of gp120 contribute, during biosynthesis, to the folding, conformation (10, 19), and bioactivity (10) of nascent recombinant gp160. This effect was due to the presence on gp120 of 1-deoxynojirimycin-induced abnormal high-mannose-type glycans with high molecular mass and not to that of high-mannose glycans per se, inasmuch as baculovirus-produced gp120 was perfectly able to bind to CD4 (10, 32).

The role of gp41 glycosylation during biosynthesis could not be studied by this approach. While 1-deoxynojirimycin affects a sufficient proportion of the numerous gp120 glycans to induce a detectable effect, its incomplete efficiency for a given glycoprotein (7) makes it inappropriate for examination of the role of the limited number of glycans on gp41 (4, 15): gp41 synthesis in the presence of 1-deoxynojirimycin would result in molecules of which a high proportion were normally glycosylated, and the properties of these species would then mask those of the abnormally glycosylated species.

The ectodomain of gp41 of HIV-1_{LAI} (41) presents five putative glycosylation sites (4), at least three of which are glycosylated (12). A 30-amino-acid-long peptide (residues 615 to 645), glycosylation of which is conserved among HIV-1, HIV-2, and simian immunodeficiency virus (4), appears to have features similar to those of transmembrane glycoproteins of other lentiviruses (15). These sites are located in a region that corresponds to a variable, weak neutralizing epitope, and they are close to the gp41 immunodominant region and to a putative domain involved in the noncovalent binding of gp41 to gp120 (for a review, see reference 15). According to current models (15), they might also be spatially close to the gp41 cleavage site and fusion peptide (14); therefore, the hydrophilicity of the corresponding glycans may contribute to the "hiding" of this hydrophobic region before it is exposed to initiate membrane fusion.

Recently, apparently conflicting results have been obtained by two independent groups which examined the role of gp41 glycans by using single glycosylation site mutation. Dedera et al. (3) "demonstrate(d) that individual conserved potential N-glycosylation sites are dispensable for produc-

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tion of infectious, cytopathic virus." Effectively, removal of individual glycosylation sites from gp41 modified neither the cytopathic effect of an HXB2 clone nor reverse transcriptase activity. Nevertheless, of the three single potential glycosylation sites removed, mutation of Asn-618 had the greatest effect on syncytium formation for CEM cells (fivefold reduction) but not for Sup T1 cells. In the same study, a vaccinia virus (VV) vector harboring the same *env* mutant exhibited a reduced (16-fold with CEM, 9-fold with Sup T1 cells) but still patent ability to induce syncytia and "no significant effects on envelope protein synthesis, conformation, processing . . . or release into the culture medium" were observed.

In contrast, by using the same virus clone and Sup T1 cells, Lee et al. (25) showed that mutation of Asn-642 caused a reduction in HXB2 infectivity (as determined by delayed reverse transcriptase production kinetics but not as a reduced tissue culture infective dose), which contrasted with the absence of an effect of this mutation noted in the previous study. Surprisingly, in this work, removal of the glycosylation site corresponding to Asn-618 increased HXB2 infectivity. Therefore, the extent to which any single glycosylation site affects *env* bioactivity remains uncertain and appears to be highly dependent on the experimental conditions. It therefore remains possible that single gp41 glycosylation sites are dispensable for HIV-1 infectivity. This is not surprising when one considers the literature which shows that, in contrast to viral glycoprotein multiple glycosylation site mutations, single glycosylation site removal usually does not affect viral glycoprotein bioactivity (5, 24, 27, 31).

For these reasons, we mutated the gp41 cluster of the three glycosylation sites that are conserved among HIV-1, HIV-2, and simian immunodeficiency virus (4). Processing and cleavage of the recombinant mutated glycoprotein were examined and compared with those of the wild-type recombinant glycoprotein, both expressed by a VV vector. Conformation, presentation at the cell surface, and biological activity of both gp41 and gp120, as well as their noncovalent association, were evaluated.

MATERIALS AND METHODS

Mutation of the gp160-encoding gene and generation of recombinant VV. The HIV-1_{LAI} *env* sequence used (clone VVTG9-1) (21) was from Transgène SA (Strasbourg, France). A gp160 gene lacking the three conserved carbohydrate addition sites at positions 7663, 7690, and 7721 in gp41 (corresponding to Asn residues located at positions 621, 630, and 642, respectively) was constructed by polymerase chain reaction generation of two *env* fragments incorporating the carbohydrate changes, followed by ligation to reconstruct the full gene.

The first polymerase chain reaction used the forward primer 5'-CCGGGAGCTCATGAGAGTGAAGGAGAAA-3' and the reverse primer 5'-GGTCATGGCATTCCAAATC TGTTCCAGAGATTTTTGACTCCAACTAGCGTTCCA-3' to generate a 1,900-bp fragment from the 5' end of the gp160 gene flanked by unique restriction sites *Sac*I at the 5' end and *Bsm*I at the 3' end. The reverse primer also incorporated base changes to change the Asn of the most upstream carbohydrate site at position 7663 to Gln and remove a single internal *Bsm*I site by a third base change that did not affect the amino acid sequence.

In the second polymerase chain reaction, the forward primer 5'-ATTTGGAATGCCATGACCTGGATGGAGTGG GACAGAGAAATTAACCAATACAC-3' and the reverse primer 5'-CCGGTCTAGATTATAGCAAAATCCTTTCCA A-3' were used to generate a 720-bp fragment from the 3' end of the gp160 gene flanked by unique restriction sites *Bsm*I at the 5' end and *Xba*I at the 3' end. The forward primer also incorporated base changes to mutate Asn to Gln in the two downstream carbohydrate sites at positions 7690 and 7727.

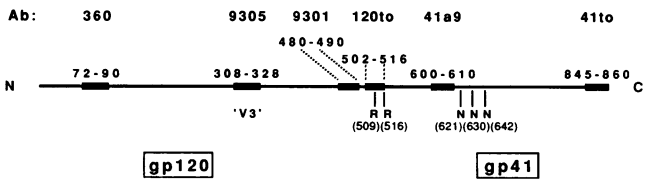

FIG. 1. Schematic drawing of gp160 indicating the epitopes recognized by the different Abs used in this study. Locations of the potential cleavage sites and conserved glycosylation sites are indicated. Numbers refer to amino acid residues.

In both cases, the amplification conditions were 10 ng of template DNA (a full-length *env* clone of HIV-1_{LAI}), 100 ng of each primer, and 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. DNA fragments generated by polymerase chain reaction were gel eluted, digested with *Bsm*I, and ligated for 2 h at room temperature. The ligation reaction was inactivated by heating, and the resulting fragment was recut with *Sac*I and *Xba*I and cloned into pUC118 between the *Sac*I and *Xba*I sites. The structure of the final gene was confirmed by restriction and sequence analyses.

The mutated *env* gene was introduced into plasmid pTG186POLY by allowing homologous recombination with the VV genome. Recombinant VV (rVV) was generated by using the protocol described in reference 20.

Production of recombinant *env* glycoproteins. BHK-21 cells were infected as described in references 10 and 21, with rVV expressing the wild-type (VVTG9-1 [21]; referred to as VV9-1 thereafter) or mutant (VV0) *env* gene. Cells were cultured in Glasgow medium (J. Bio, Les Ullis, France), which contained 1% glutamine, 5% fetal calf serum, and 5% tryptose phosphate, as previously described (10, 21). They were infected with either VV9-1 or VV0 (0.2 PFU per cell) in fetal calf serum-free medium to allow easy further supernatant concentration and analysis. After 2 days, cell pellets were lysed with phosphate-buffered saline (PBS)–2% Triton X-100 (Sigma, St. Louis, Mo.) and supernatants were concentrated fivefold by using a 10-kDa cutoff Centriprep system (10) (Amicon, Danvers, Mass.).

Antibodies. Sheep polyclonal immunoglobulin G antibody (Ab) D7324 (referred to thereafter as Ab120to; Aalto, Dublin, Ireland) recognizes a peptide encompassing the two gp160 potential cleavage sites (amino acids [aa] 502 to 516) at the gp120 C terminus; sheep Ab D7323 (Ab41to; Aalto) recognizes a C-terminal peptide of gp41 (aa 845 to 860) (11) (Fig. 1).

Monoclonal antibody (MAb) mAb360 (a gift from H. Holmes, MRC AIDS Reagent Project, Potters Bar, United Kingdom) is a mouse MAb that recognizes the gp120 N terminus (aa 72 to 90). mAb9305 and mAb9301 (Du Pont de Nemours, Dreieich, Germany) are MAbs directed to aa 318 to 328 (in the V3 loop of gp120) and 480 to 490 (C terminus of gp120), respectively. mAb110-4 (Genetic Systems, Seattle, Wash.) is another anti-V3 loop Ab. mAb41a9 (a gift from F. Traincard, Hybridolab, Institut Pasteur, Paris, France) and mAb41-1 (Genetic Systems) are MAbs directed to the immunodominant region of gp41 (aa 602 to 610) (11; Fig. 1).

Western blot (immunoblot; WB) characterization of recombinant glycoproteins. Two days postinfection with VV9-1 or

VV0, cell lysates were not treated or treated overnight with 200 mU of an endoglycosidase F *N*-glycanase mixture (endo F *N*-glycanase; Boehringer, Mannheim, Germany), as previously described (10, 12), and submitted to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After migration, blotting was performed as previously described (10, 11, 12) and staining was realized with human anti-HIV-1 Ab Ab120to or Ab41to (diluted 1:1,000) or mAb9305 (diluted 1:100). After overnight incubation at 20°C (8), peroxidase-coupled anti-sheep, -mouse, or -human Abs diluted 1:1,000 (Dakopatts, Glostrup, Denmark) were added for 2 h and color was developed by using diaminobenzidine.

HIV-1 viral pellets (Diagnostics Pasteur, Marne-la-Coquette, France), not deglycosylated or deglycosylated as previously described (12), were studied in parallel under identical conditions.

Immunoreactivity and dosage of recombinant glycoproteins by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc, Roskilde, Denmark) were coated with Ab120to or Ab41to (200 ng/100 μl) in 20 mM sodium bicarbonate buffer, pH 8. After 2 h at 20°C, plates were blocked with 5% casein. Increasing amounts of cell lysates or supernatants were added for 3 h at 20°C after dilution in PBS–1% Tween–1% casein, pH 7.4. After two washes, mouse MAbs or a pool of human anti-HIV-1 Abs (diluted 1:100) was added for 3 h at 20°C in SS buffer (supplemented with 10% normal sheep serum) that was added to inhibit nonspecific Ab binding as previously described (29). After two washes, anti-mouse or anti-human Abs coupled to peroxidase (1:1,000 dilution in SS buffer) was added and incubated for 2 h at 20°C. Color was developed with the appropriate chromogen after five washes.

Metabolic labeling and pulse-chase experiments. Fifteen hours after rVV infection of 10×10^6 BHK-21 cells, 3 ml of low-Met-Cys Glasgow medium (J. Bio) was added as previously described (21). After 1 h, 150 μCi of radiolabeled [^{35}S]Met-Cys (Trans Label; ICN, Costa Mesa, Calif.) per ml was added and incubated for 30 min. The supernatant was then removed, cells were washed, and Met-supplemented (100 μg/ml) Glasgow medium was added and incubated for 1, 4, or 18 h. Cells and supernatants were separated by centrifugation immediately after the pulse or after each chase period. Cells were lysed with 25 mM Tris–300 mM NaCl–0.25% SDS–0.5% Triton X-100, pH 7. Recombinant glycoproteins in cell lysates or supernatants were immunoprecipitated by an AIDS patient's serum diluted 1:100 and protein A-Sepharose (Pharmacia, Uppsala, Sweden) as previously described (21). After five washes in 2 ml of buffer, recombinant glycoproteins were eluted by using 10% SDS–5% β-mercaptoethanol and heating (80°C, 5 min). Eluates were analyzed by SDS-PAGE and autofluorography as previously described (21).

Localization of gp160 cleavage sites. We utilized an ELISA to assess the rate of gp160 proteolytic cleavage at either of its two potential cleavage sites. This was based on the finding that a minority of gp41 molecules react with Ab120to, a polyclonal antibody which partly recognizes the amino acid sequence located between the two potential cleavage sites, an indication that these molecules are cleaved at Arg-509 (11) instead of Arg-516 (1, 11, 18, 28).

The amount of such gp41 species relative to the total amount of gp41 produced was evaluated here as follows. Two days after rVV infection of BHK-21 cells, virus and membrane pellets obtained after ultracentrifugation (100,000 × *g* for 1 h) of the cleared supernatant were lysed by 2% Triton. Only gp120 and gp41, and no gp160, were detected in the lysate by WB and by ELISA with Ab41to-coated plates and mAb9305 staining (data not shown). Increasing amounts of lysates diluted in PBS–1% Tween–1% casein, were incubated in Ab41to- or Ab120to-coated plates as described above. The gp41 bound to Ab41to or Ab120to was stained as already described, with mAb41a9 and peroxidase-coupled anti-mouse Ab in SS buffer.

Flow cytometry analysis. (i) Binding of gp120 to CD4$^+$ cells. Human lymphoid CD4$^+$ CEM13 cells were cultured in RPMI 1640 (J. Bio)–10% fetal calf serum–1% glutamine–1% penicillin-streptomycin as previously described (9, 10, 12). Increasing amounts of concentrated rVV-infected cell supernatants, which contained soluble gp120, were incubated with 10^6 cells for 2 h at 20°C in 100 μl of PBS–0.5% bovine serum albumin–0.05% NaN_3 as previously described (9, 10, 12). Labeling with a pool of anti-HIV-1 human sera or with mAb9305 diluted 1:100 was performed for 1 h at 20°C. Phycoerythrin-coupled sheep anti-human or rabbit anti-mouse immunoglobulin G Abs diluted 1:100 (Becton Dickinson, San Jose, Calif.) were added for 1 h at 20°C (9, 10, 12). Membrane fluorescence was measured with a FACScan apparatus (Becton-Dickinson).

(ii) Expression of gp120 and gp41 on the surface of rVV-infected cells. At 4, 8, or 24 h postinfection with VV9-1 or VV0 (10 PFU per cell), 10^6 CEM13 cells were incubated for 2 h at 20°C with mAb9305, mAb110-4, mAb41a9, or mAb41-1 diluted 1:100 to detect the presence of recombinant glycoproteins at the cell surface. Staining was performed as described above.

Syncytium formation assay. CEM13 cells (5×10^6) were infected with increasing rVV doses (1, 10, and 100 PFU per cell) in RPMI 1640–1% glutamine–10% fetal calf serum. At 1 day postinfection, cultures were photographed and syncytia or aggregates were scored. The culture was monitored for an additional 2 days to observe the evolution of syncytium development in the different samples.

RESULTS

Apparent molecular weights (MWs) of recombinant *env* HIV-1 glycoproteins. First we used WB to determine the MWs of the recombinant molecules obtained following expression in rVV, and then we examined whether gp41 produced by mutated *env* (gp41CHO$^-$) was glycosylated relative to its wild-type counterpart (gp41CHO$^+$). (i) As expected (11), gp41, gp120, and lesser amounts of gp160 were detectable in VV9-1-infected BHK-21 cell lysates (Fig. 2A), and secreted gp120 was detected in the respective supernatants (Fig. 2B). (ii) When lysates of 10^6 VV9-1- or VV0-infected cells were immunoblotted with Ab41to, similar amounts of gp160 precursor were detected while gp41CHO$^-$ was not (Fig. 2C); the latter was readily detected only by using lysates from 10 times more cells (Fig. 2A).

Under the latter conditions (Fig. 2A), a high-MW (about 140,000) glycoprotein was found in VV0-infected cell lysates. The decrease of its MW to 90,000 after endo F N-glycanase (40) deglycosylation, as previously observed (9, 10, 12), indicated that this protein corresponded to the precursor envelope glycoprotein. No gp120 species was distinguished here, presumably because of superimposition of the 140,000-MW band.

A 35,000-MW band should correspond to gp41CHO$^-$, because it also specifically reacted with Ab41to (data not shown) and its MW agreed with that of both endo F N-glycanase-treated viral gp41 (12) and wild-type recombinant gp41CHO$^+$. Moreover, endo F N-glycanase treatment

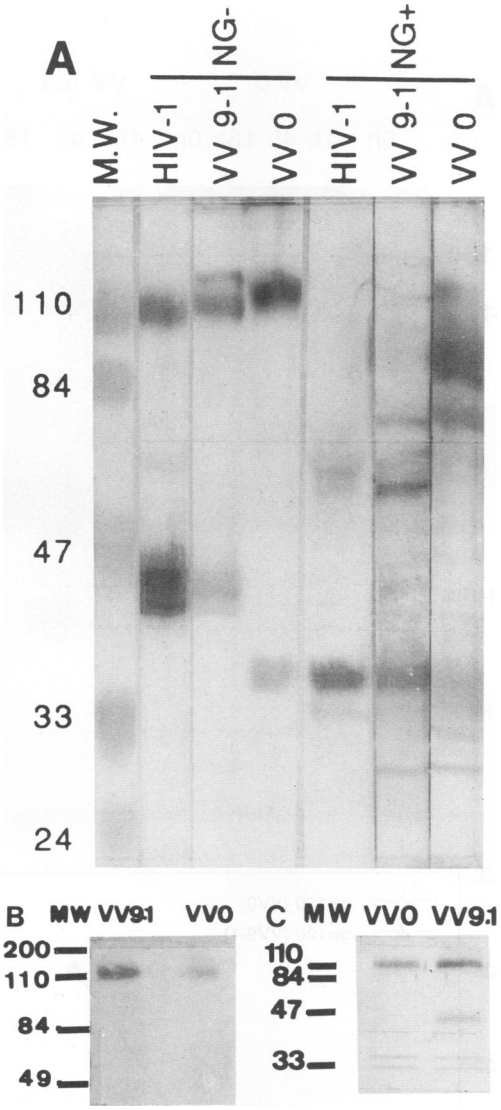

FIG. 2. WB analysis of recombinant glycoproteins. (A) SDS-(10%) PAGE and immunoblotting of a HIV-1 viral preparation and VV9-1- or VV0-infected cell lysates from approximately 10^6 and 10^7 cells, respectively. Preparations were treated (NG+) or not treated (NG−) with endo F N-glycanase. Proteins were detected with a pool of sera from HIV-1-infected patients. (B) Immunoblotting of 20 μl of VV9-1- or VV0-infected cell supernatants (concentrated fivefold) at 3 days postinfection with mAb9305. (C) Immunoblotting of approximately 10^6 VV9-1- and VV0-infected cell lysates with Ab41to. MWs are in thousands.

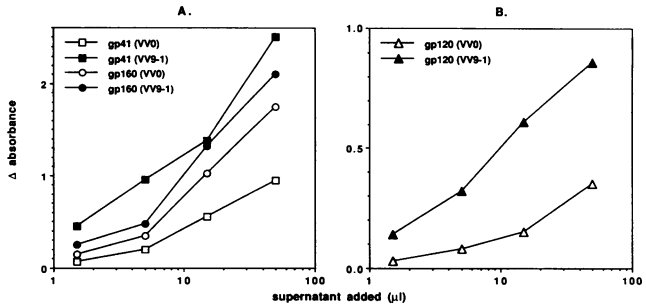

FIG. 3. Determination of glycoproteins in rVV-infected cells and supernatants by ELISA. (A) Comparison of cell-associated gp160 and gp41. Ab41to-coated wells were incubated with increasing volumes of VV9-1- or VV0-infected cell lysates. mAb41a9 was used to detect both captured gp160 and gp41, and mAb9305 was used to detect gp160 only. (B) Comparison of amounts of gp120 in supernatants. Ab120to-coated wells were incubated with increasing volumes of VV9-1- or VV0-infected cell supernatants. The captured glycoprotein was detected with mAb9305. Control optical densities determined with noninfected BHK-21 cell lysates or supernatants were approximately 0.02. All assays were performed three to five times with reproducible results.

of gp41CHO$^-$ did not result in modification of its MW, an indication that no N-linked glycans were present on the molecule.

Relative amounts of gp120 and gp41 in rVV-infected cells and supernatants. We verified that Ab41to, Ab120to, mAb41a9, mAb41-1, mAb9305, and mAb110-4 recognized VV0- or VV9-1-synthesized glycoproteins to the same extent. Results of quantitative dot blot assays (data not shown) performed as previously described (11), under conditions that abolish conformation-dependent antibody binding, were consistent with those of the ELISA described here, which was performed under liquid-phase conditions that allow conformation-dependent binding. One may then conclude that the accessibility of the epitopes recognized by these Abs was not affected by mutations.

The respective amounts of recombinant gp41 and gp120 that were recovered in cells and supernatants after rVV infection were then assessed by capture ELISA. (i) First, plates were coated with Ab41to (Ab directed against the gp41 C terminus). When the captured molecules were then labeled with mAb9305 (anti-V3 loop MAb), which permitted detection of gp160, there were approximately similar amounts of gp160 in lysates of cells infected with either the control or the mutant construct (Fig. 3A). In contrast, when the captured molecules were labeled with mAb41a9 (anti-gp41 MAb), 7 to 10 times more of the VV0-infected cell pellet was necessary to obtain the same signal as with VV9-1 (Fig. 3A). Although under the conditions used here, detection of precursor gp160, which is present in similar amounts in cells infected with both rVVs, may potentially interfere with dosage of cleaved cell-stored gp41, it nonetheless appears that approximately 10 times less gp41CHO$^-$ than gp41CHO$^+$ was present in the corresponding cell lysates. (ii) Similarly, when Ab120to (directed against the gp120 C terminus) was used for capture, along with labeling with mAb9305, 10 times more supernatant from VV0-infected cells was necessary to obtain the same signal as with the VV9-1-infected cell supernatant (Fig. 3B). One may then assume that the amount of gp120 in the VV0-infected cell supernatant was also about 10-fold lower than in the VV9-1-infected cell supernatant.

These results should be taken together with the WB data of Fig. 2, in which the staining intensity of the *env* precursor observed when lysates of 10^7 VV0-infected cells were analyzed was approximately 10-fold stronger than that noted for 10^6 VV9-1-infected cells, whereas under the same conditions gp41CHO$^+$ staining was similar to that of gp41CHO$^-$.

Thus, it appears that the amount of cleaved gp41 and gp120 produced by VV0-infected cells was approximately 10-fold lower than that in VV9-1 infected cells, while the amounts of gp160 produced in the two cases were approximately similar.

Cleavage of gp160 and kinetics of gp120 release in cell

supernatants. (i) gp160 cleavage. Approximately similar amounts of radioactivity were incorporated into gp160 obtained from VV9-1- or VV0-infected BHK-21 cells, but the proteolytic cleavage kinetics of the two species differed dramatically, as indicated by the respective amounts of gp120 noted after a pulse-chase experiment (Fig. 4A). After a 1-h chase, significant amounts of radiolabeled gp120 and lesser amounts of gp41 (the weak labeling of which was due to the few Met and Cys amino acids present on the molecule [4]) were detected in VV9-1-infected cells, and this persisted at least for the 18-h chase. Far less and transient (only 1 h post pulse) cell-associated gp120 was seen in VV0-infected cells. A longer exposure time, however, showed a weak gp120 band after a 4-h chase (data not shown). These gp120 species migrated faster in SDS-PAGE (similar to the gp120 species observed at time zero and at 1 h in VV9-1-infected cells), owing to a different glycosylation pattern, as determined by endoglycosidase H sensitivity (40). The VV9-1 gp120 species with increased electrophoretic mobility correspond to species the high mannose chains of which have been trimmed by glycosidases but not replaced by higher-MW complex type chains by glycosyltransferases (Fig. 4A, VV9-1, 1 h, where both species coexist). This is in line with acquisition of endoglycosidase H resistance (data not shown) and with the results previously obtained with other expression systems for HIV-1 and HIV-2 envelope glycoproteins and discussed (2, 42).

These results confirm the quantitative ELISA data (Fig. 3B) regarding respective amounts of cleaved gp120 and gp41 produced by the rVV constructs. Also, the apparently reduced MW of gp160 obtained after VV0 infection was consistent with the WB data of Fig. 2, and part of this MW difference may be accounted for by lack of gp41 glycosylation.

(ii) Quantitation of glycoproteins produced. The quantitative ELISA was used in parallel to determine gp120 release kinetics in the supernatant of rVV-infected cells, taking as a reference the plot obtained with a soluble gp120 batch (a gift of the Medical Research Council AIDS Reagent Program) with a known concentration. This showed that after 3 days, 10^6 VV9-1-infected cells secreted approximately 800 ng of gp120, compared with 100 ng after VV0 infection (Fig. 4B).

In addition, a semiquantitative dot blot assay (11) allowed us to evaluate the amount of both cleaved and uncleaved cell-associated gp41, with mAb41a9 and recombinant gp160 VVTG-1163 (11, 21) as a reference. This approach was taken because gp160 from VVTG-1163 does not express the epitope recognized by Ab41to (12a), which makes it impossible to use in a capture ELISA. We thus determined that at 2 days post rVV infection, there were approximately 1 μg and 200 ng of VV9-1- and VV0-produced gp41 and/or gp160, respectively, per 10^7 cells.

Rate of gp160 cleavage at each of its potential sites. We have recently shown that, normally, about 10 to 20% of both recombinant and viral gp41 molecules are recognized by Ab120to in an ELISA and an immunoprecipitation assay. This indicates that these species express at least part of the epitope recognized by the Ab and, thus, that they should be cleaved at Arg-509 instead of Arg-516 for the rest (approximately 80 to 90%) of the gp41 species (11). Here we investigated by ELISA whether reduced cleavage of VV0-produced gp160 is related to different rates at either of these sites and whether cleavage at one site is suppressed or enhanced relative to the wild-type product.

In agreement with our previous data, after capture with Ab41to and subsequent detection with mAb41a9, the amount

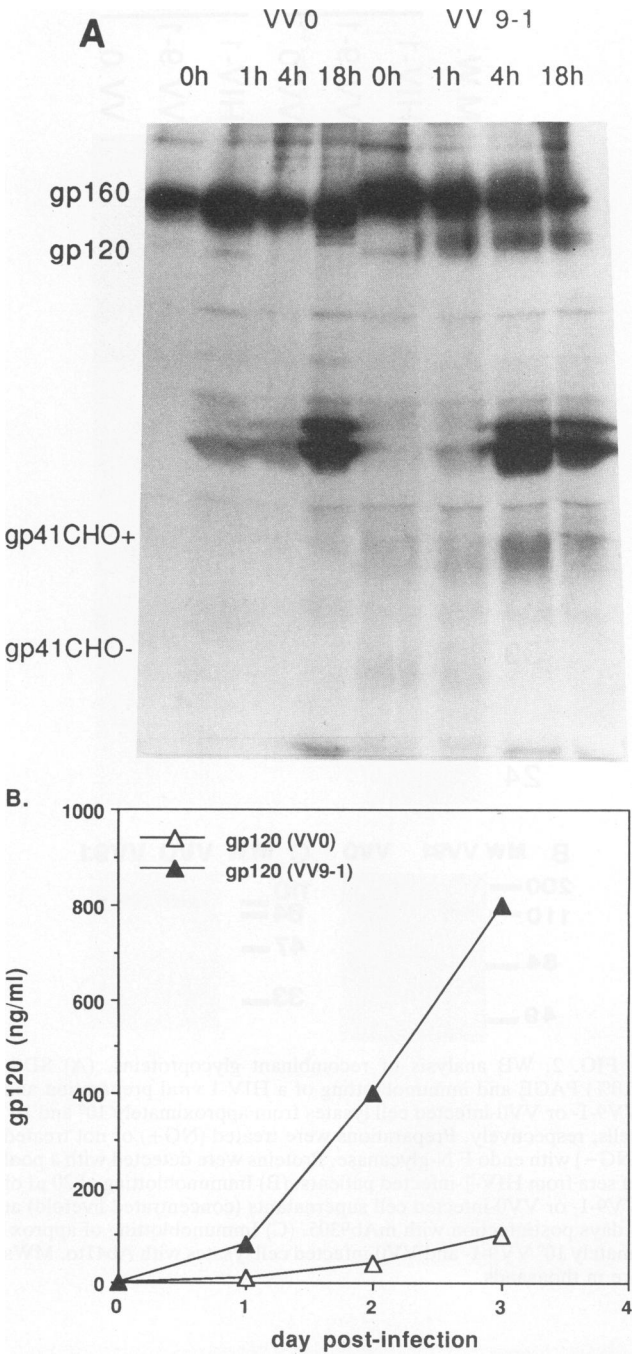

FIG. 4. Glycoprotein cleavage and kinetics of production. (A) Recombinant gp160 cleavage kinetics. Cells (3×10^6) were pulse-labeled (150 μCi/ml) at 18 h after infection with 0.2 PFU of VV0 or VV9-1 per cell. After 30 min, the cells were chased for the lengths of time indicated. After cell pellet lysis, glycoproteins were immunopurified and analyzed by SDS–8% PAGE. MWs are in thousands. (B) Kinetics of gp120 appearance in cell supernatants. Cells were infected with VV0 or VV9-1 as for panel A. At 1, 2, or 3 days postinfection, the amounts of gp120 in the supernatants were quantified by ELISA.

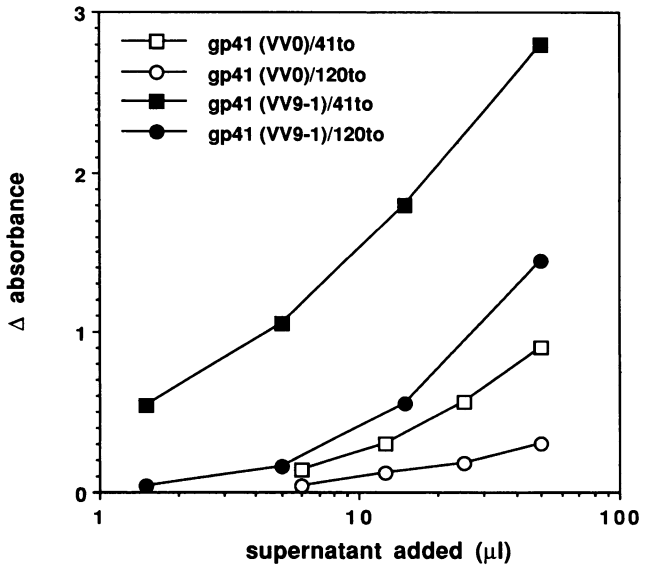

FIG. 5. Determination of gp160 proteolytic cleavage rate at each potential site. Ab120to- or Ab41to-coated wells were incubated with increasing volumes of pellet lysates obtained after ultracentrifugation of VV9-1- or VV0-infected cell supernatants. The captured glycoprotein was detected with mAb41a9. For example, gp41(VV0)/41to stands for the VV0-produced gp41 species captured by Ab41to.

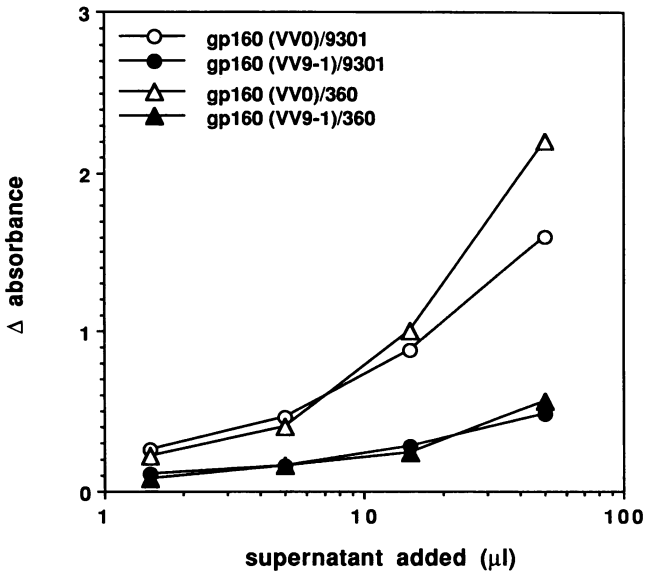

FIG. 6. Comparison of mAb9301 or mAb360 binding to recombinant glycoproteins as determined by ELISA. Ab41to-coated wells were incubated with increasing volumes of VV9-1- or VV0-infected cell lysates. The captured glycoprotein was detected with mAb360 or mAb9301. For example, gp160(VV0)/9301 stands for the VV0-produced gp160 species detected by mAb9301.

of gp41CHO$^-$ detected in cell lysates was approximately 10 times lower than that of gp41CHO$^+$ (Fig. 5). No significant amount of precursor gp160 was detected with mAb9305 in membrane and virus pellets of the cleared supernatants. When the molecules were captured with Ab120to, approximately 10 to 20% of gp41CHO$^+$, as well as gp41CHO$^-$, bound to Ab41to, as previously described (11) (Fig. 5).

Thus, the relative percentages of molecules cleaved at each potential site were similar for the two constructs.

Immunoreactivity of recombinant gp160, gp120, and gp41. Keeping in mind that the avidities of binding of Ab120to, Ab41to, mAb9305, and mAb41a9 to VV9-1- and VV0-produced glycoproteins were similar and, thus, that the corresponding epitopes were exposed to similar extents on both, we further evaluated the immunoreactivity of two additional MAbs by ELISA: mAb360 (directed against the gp120 N terminus) and mAb9301 (directed against the gp120 C terminus 10 aa upstream of the peptide recognized by Ab120to). Capture Abs were either Ab41to (which allowed for detection of only precursor gp160) or Ab120to (a condition under which both gp160 and gp120 were detectable). Both mAb360 and mAb9301 displayed increased reactivity to cell-associated mutated VV0 glycoproteins, by more than 10-fold relative to VV9-1 (Fig. 6), whereas they recognized gp120 present in supernatants of cells infected with either construct after capture by Ab120to to the same limited extent (data not shown).

This indicates that these gp120 N- and C-terminal epitopes are particularly well exposed when expressed on mutated uncleaved precursor gp160 but that the reactivities of the mutant and the wild type were similar once cleavage of gp120 had occurred.

Binding of recombinant gp120 to CD4$^+$ cells. The capacity of gp120 produced by the mutated and control constructs to bind to membrane CD4 was evaluated by immunofluorescence analysis. Various amounts of concentrated rVV-infected cell supernatants were incubated with CD4$^+$ CEM13 cells. Bound gp120 was then labeled with mAb9305. gp120 from the reference batch was used as a control to quantify bioactive rVV-produced gp120 (Fig. 7).

Taking into account the soluble gp120 ELISA quantitation described in Fig. 4B, the same quantity of VV9-1, VV0, and

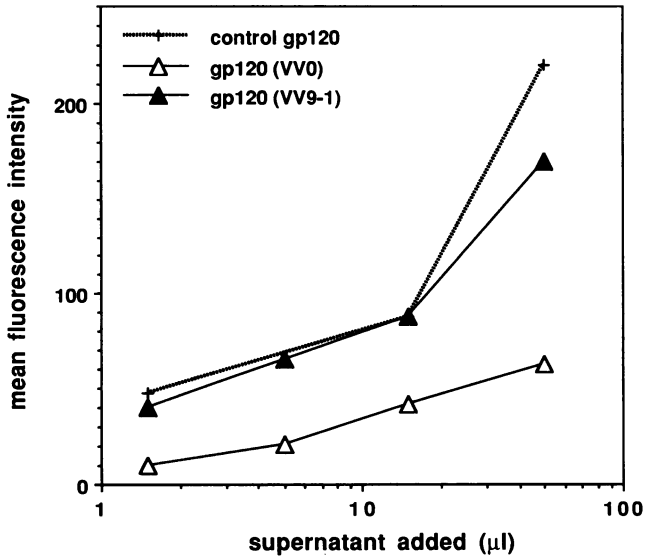

FIG. 7. Binding of recombinant secreted gp120 to the membrane of CD4$^+$ CEM13 cells as determined by flow cytometry analysis after labeling with mAb9305. Cells (5×10^5) were incubated with increasing volumes of fivefold-concentrated VV9-1- or VV0-infected cell supernatants. Results are expressed as mean fluorescence intensities. The reference batch (control gp120) contained 2 μg of gp120 per ml.

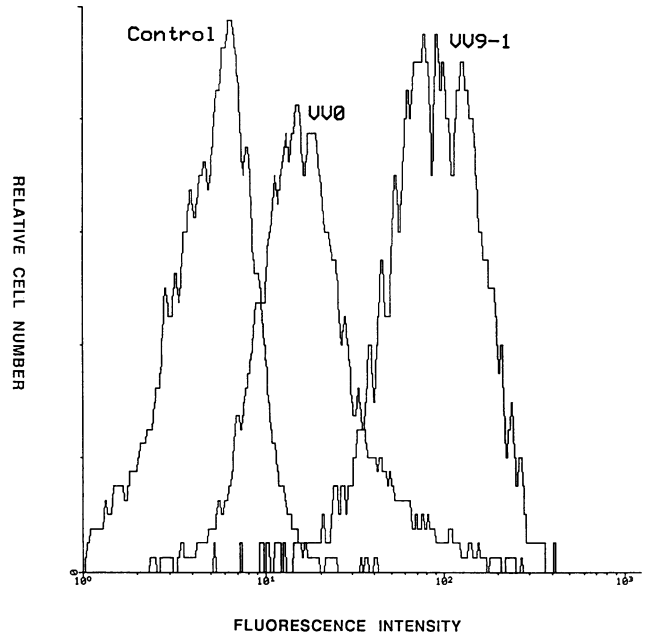

FIG. 8. Evaluation of gp120 expression on the surface of rVV-infected CEM13 cells. Cells (10^6) were infected with VV9-1 or VV0 at 10 PFU per cell. At 24 h postinfection, cells were analyzed by flow cytometry after mAb9305 labeling. Uninfected cells served as a control. Results are expressed as cell numbers relative to fluorescence intensity.

gp120 from the reference batch yielded the same labeling signal, indicating that the three recombinant gp120 proteins had the same bioactivity inasmuch as they bound to membrane CD4 to the same extent, even though 10-fold more VV0 than VV9-1 supernatant was necessary. Comparable results were obtained when human Abs were used for labeling (data not shown).

Expression of gp120 on the surface of rVV-infected cells. At 2 days post rVV infection, CEM13 cells were incubated with mAb9305 (or with mAb110-4; data not shown) to detect gp120 present on the cell surface. Both VV9-1 and VV0-infected cells expressed gp120, but the latter in a lesser amount (Fig. 8). Reduced labeling observed after VV0 infection, compared with VV9-1 infection, was consistent with an approximately 10-fold reduction of gp120 cell surface expression. Because no gp120 was detected in the supernatant under these conditions, one may exclude labeling due to gp120 capture by membrane CD4. In agreement with what has been reported (39), neither of the anti-gp41 MAbs used (mAb41a9 and mAb41-1) allowed us to detect cell surface recombinant gp41. The same results were obtained with rVV-infected BHK-21 cells (data not shown).

Syncytium formation of rVV-infected $CD4^+$ lymphoid cells. To determine whether $gp41CHO^-$ could mediate membrane fusion of rVV-infected and noninfected cells following gp120 binding to CD4, CEM13 cells were infected with different concentrations—spanning 3 orders of magnitude—of VV0 or VV9-1. All doses of VV9-1 resulted in formation of cell aggregates, detectable at 4 to 5 h postinfection, which indicated proper binding capacity of gp120 on the surface of infected cells with membrane CD4 on adjacent cells. These aggregates developed into gp41-mediated syncytia within a few additional hours. In contrast, while aggregates were also observed after VV0 infection, no fusion occurred here, even at the highest rVV concentration used (Fig. 9), and no further syncytia were detected when the culture period was prolonged by 3 days (data not shown). There was, however, a remote possibility that a threshold level of envelope glycoprotein per cell was actually required for fusion and that this could not be attained with VV0, even with 100 PFU per cell. That such was not the case is implied by the experiment described in Fig. 10, in which the kinetics of gp120 expression on the membrane and the ability to mediate syncytia of rVV-infected cells (100 PFU per cell) were compared. At 8 h after infection, VV9-1-infected cells already mediated syncytia at a time when membrane gp120 detection was limited, whereas by 24 h still no syncytia were noted with VV0-infected cells, despite a greater amount of *env* product expression at the cell membrane.

These data argue for the total inability of $gp41CHO^-$ to mediate membrane fusion.

DISCUSSION

The aim of this study was to examine whether the cluster of N-linked glycans corresponding to the three conserved glycosylation sites of the ectodomain of HIV-1 transmembrane gp41 (4, 15) were involved in its biological property, i.e., essentially mediation of membrane fusion. Because gp41 and gp120 are cleavage products of a common precursor and because it has recently been shown that distant parts of gp120 contribute to molecule folding (30, 36), the possible effects of the absence of these glycans on gp160 proteolytic cleavage and on gp120 characteristics were also investigated. Intracellular routing or oligomerization (6, 34, 38), which are currently under investigation, were out of the scope of this study.

The three conserved glycosylation sites of the *env* sequence were mutated, expressed by using rVV, and used to infect either $CD4^-$ BHK-21 or $CD4^+$ CEM cells to produce a mutated envelope glycoprotein.

First, we showed by WB analysis that the mutations deleted glycans from the gp41 molecules produced. The MW of mutated recombinant $gp41CHO^-$ was 35,000, which in agreement with a previous report (12), corresponded to that of viral gp41 or its wild-type recombinant ($gp41CHO^+$) counterpart after complete deglycosylation by endo F N-glycanase, an enzyme that cleaves all N-linked glycans (40). This reduced MW may be accounted for by the absence of three approximately 2,000-MW glycans (22). That the MW did not change after N-glycanase treatment indicates that $gp41CHO^-$ was not glycosylated at all. It is therefore likely that the three conserved potential glycosylation sites removed (Asn-621, Asn-630, and Asn-642) are the only ones that are actually glycosylated on $gp41CHO^+$. This stands in contrast to the results of Dedera et al. (3) and Lee et al. (25), who suggested that Asn-616 may be glycosylated. However, it is possible that the lack of glycosylation at this site in the VV0 construct is due to impairment of gp41's normal conformation by the mutation, resulting in steric hindrance and inaccessibility of this region to glycosylation enzymes (22).

The rate of synthesis of mutant gp160 was comparable to that of control gp160 in pulse-chase experiments performed after BHK-21 cell infection with either VV9-1 or VV0. Lack of gp41 glycosylation strongly affected the gp160 cleavage rate, which appeared to decrease by approximately 90% as determined by quantitative ELISA. In pulse-chase experiments performed with VV9-1-infected cells, large amounts of gp120 were noted after a 1-h chase and even though it was

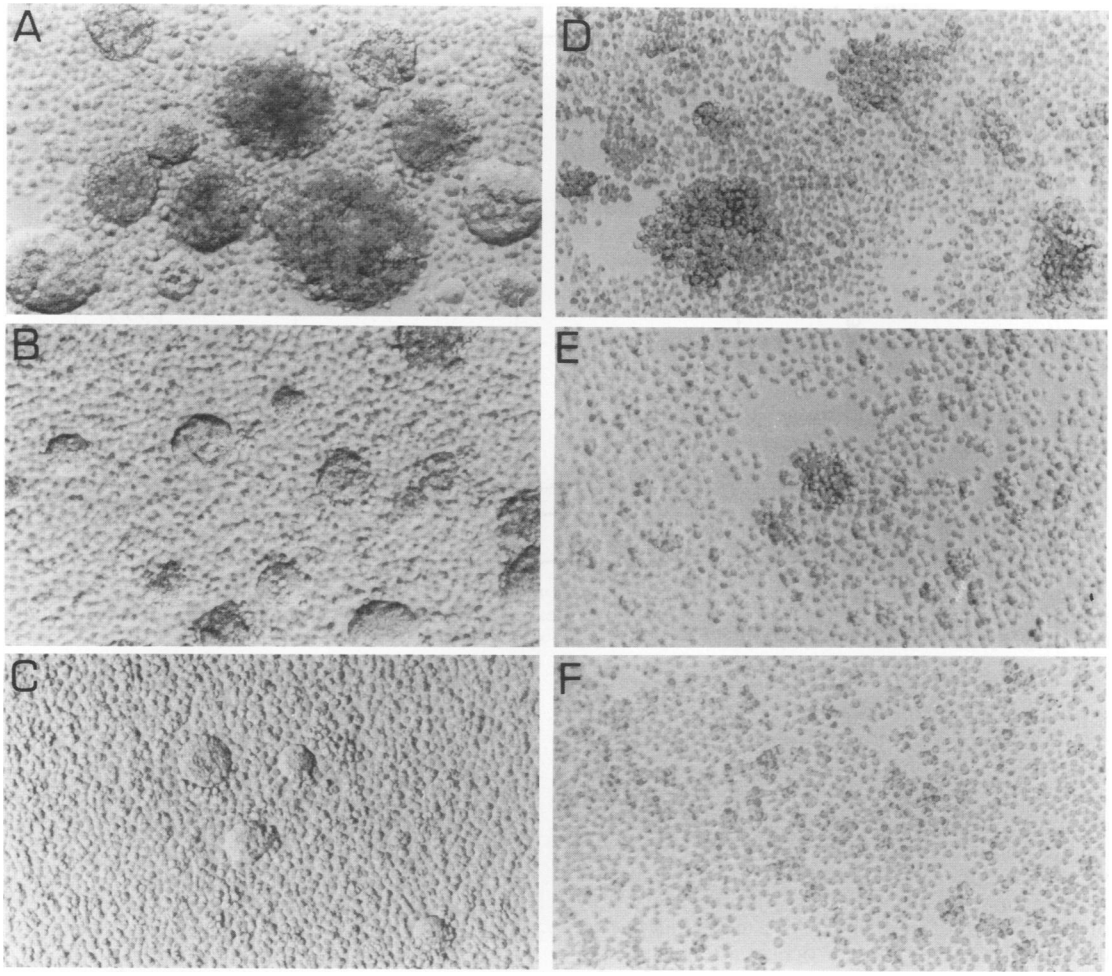

FIG. 9. Ability of VV9-1 and VV0 to mediate syncytium formation. $CD4^+$ CEM13 cells (10^6) were infected with VV9-1 (A, B, and C) or VV0 (D and E) at 100 (A and D), 10 (B and E), or 1 (C) PFU per cell. At 1 day postinfection, plates were photographed. Uninfected cells are shown in panel F.

secreted at high levels in culture supernatants, as determined by ELISA, it was still detected in cells after 18 h. Thus, the remaining radiolabeled gp160 pool continued to be cleaved for a long time after synthesis. In contrast, some gp120 with immature glycan species resulting in a reduced apparent MW appeared in VV0-infected cells during the first hour of the chase, and it was not detectable later. This presumably resulted from shedding of the molecules (6), as determined by a weak level of detection in the supernatant by ELISA. One may then assume that only a small proportion of VV0 gp160 was able to be cleaved normally; transported to the cell surface, as determined by flow cytometry; and progressively shed (6, 38). In contrast with what was noted with VV9-1, lack of cleavage of the remaining gp160 species might be due to the fact that they were directed to a cell compartment where proteolytic activity was not present, a possibility which is under investigation. This has already been described for gp160 after amino acid point mutations (44).

However, gp160 synthesized by the mutated construct did not accumulate in cells, as indicated by the equivalent amounts of intracellular gp160 detected by ELISA in VV9-1- and VV0-infected cell lysates, which agrees with results that suggest that uncleaved gp160 is cleared in cells (43). Some other posttranslational modifications, such as, in particular, changes in glycan processing (22), may occur on uncleaved mutated gp160, as indicated by its decreased MW observed in the WB and pulse-chase experiments. This was further suggested by the increased endoglycosidase H sensitivity of mutated gp160 species relative to their wild-type counterparts (data not shown).

Lack of gp41 glycosylation apparently did not modify the accessibility of the gp120 and gp41 central and C-terminal regions to Abs such as mAb9305, mAb110-4, and Ab120to with respect to gp120 and mAb41a9, mAb41-1, and Ab41to for gp41 when the corresponding epitopes were expressed on intracellular uncleaved gp160. Surprisingly, MAbs directed to an N-terminal gp120 epitope (mAb360, which recognizes the sequence of aa 72 to 90) or to a C-terminal epitope of gp120 (mAb9301), a few residues upstream of that recognized by polyclonal Ab120to, bound to mutated recombinant gp160 to a much greater extent than to its control counterpart, indicating greater accessibility of the corresponding regions of gp120 under these conditions. Because of their distance from the mutated glycosylation sites, this suggests that distant regions of gp160 act in concert to allow normal

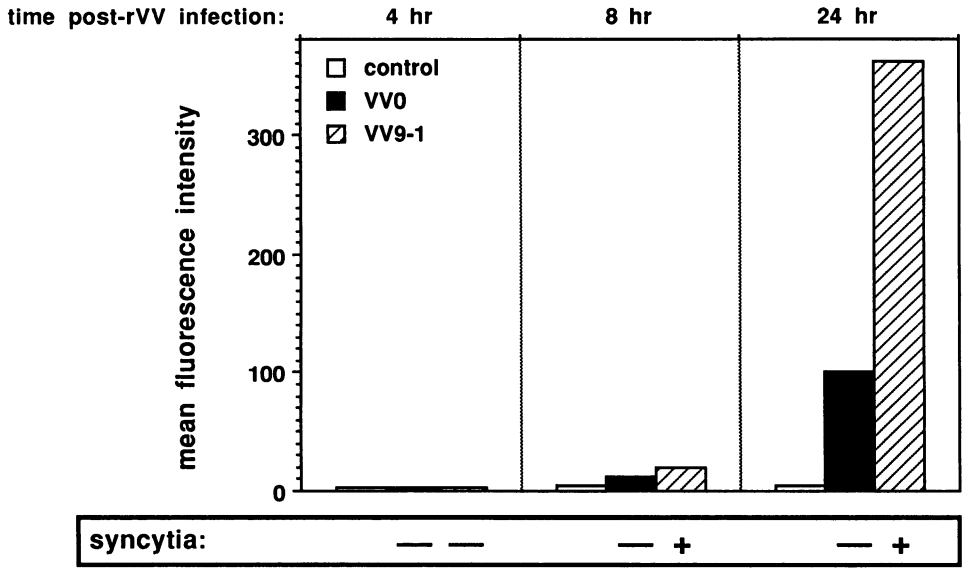

FIG. 10. Comparative evaluation of the kinetics of gp120 expression on the membrane of VV0- and VV9-1-infected cells, as determined by fluorescence-activated cell sorter analysis with mAb9305 and expressed as mean fluorescence intensities, and of the abilities of VV0 and VV9-1 to mediate syncytium formation (+, syncytia observed; −, no syncytia).

conformation of the molecule, which is in line with recent data (30, 36) indicating that distinct regions combine for proper conformation of gp120. Such a mechanism might also explain the observed impaired precursor cleavage due to lack of accessibility to proteases and/or inappropriate conformation of the relevant region (28). However, both types of bioactive gp120 species produced after cleavage and released in the supernatant exhibited similar, very low avidities for mAb360 and mAb9301. As a consequence, it seems that some reshaping might occur after cleavage, resulting in gp120 molecules that are correctly folded for binding to membrane CD4.

We have recently shown that gp160 cleavage occurs predominantly but not exclusively at Arg-516, as reported by others (1, 11, 18, 28), and that approximately 10 to 20% of cleaved subunits result from cleavage at Arg-509 (11). Here, we evaluated the cleavage rate at either of these sites to examine whether the reduced cleavage of mutated gp160 could be accounted for by impairment of cleavage at the preferential site (Arg-516). In fact, although overall cleavage was greatly reduced, cleavage of mutated gp160 occurred at both sites in the same proportion as for wild-type gp160. Transport of the resulting subunits to the cell surface was not abolished, and VV0-produced gp120 could be detected both at the membrane and in the supernatant of VV0-infected cells, albeit at reduced levels (approximately 10%) relative to VV9-1-infected cells.

Our findings on the influence of lack of gp41 glycosylation on gp160 cleavage are in line with findings that abnormal glycosylation resulting from the use of glucosidase inhibitors is associated with human T-cell leukemia virus type 1 (35), as well as with HIV-1 (33; reviewed in reference 37), impaired *env*-encoded glycoprotein cleavage.

We also investigated the ability of mutated *env*-encoded glycoproteins to mediate fusion of rVV-infected and uninfected $CD4^+$ cells, since membrane glycoprotein gp120 of VV9-1-infected cells is already known to bind to membrane CD4 of adjacent cells and to initiate gp41-triggered membrane fusion (21). Relative to this control, VV0-infected cells displayed a greater than 3 log unit-reduced ability to induce syncytia. This effect was much in excess of what would be expected from gp160 cleavage impairment, a known requisite for membrane fusion (21, 28). VV0 *env* products expressed on the cell surface are actually cleaved: no significant amount of gp160 was detected by ELISA and WB in membrane and virus pellet lysates after ultracentrifugation of the cleared supernatant from VV9-1- or VV0-infected cells, which is in line with the absence of precursor gp160 at the cell membrane and in HIV-1 particles (8, 11, 34). Moreover, expression of bioactive gp120 on the surface of these cells was reduced by no more than 10-fold, a sufficient amount to allow for its interaction with $CD4^+$ cells. One may then reasonably hypothesize that lack of syncytium formation capacity is due to the absence of gp41 glycans and that the resulting reduced hydrophilicity may modify the correct presentation of the hydrophobic fusion peptide (14).

These results are in contrast with those of Dedera et al. (3) and Lee et al. (25), who showed that single N-glycosylation sites are dispensable for production of infectious virus and that precursor cleavage and gp41 fusion ability were not significantly affected, while we found that these properties were dramatically affected. Such a discrepancy could signify that single glycosylation site removals per se are not sufficient to abolish gp41 bioactivity, while mutation of clustered glycosylation sites and the resulting lack of gp41 glycans can diminish cleavage of gp160 and completely suppress the fusogenic properties of gp41. This effect might be explained by possible complementation of one site by the others, resulting in only limited consequences of single-site removal. The same type of mechanism has also been suggested for HIV-1 and HIV-2 gp120, in which single glycosylation site removal showed only limited consequences relative to multiple-site removal (5, 24, 31).

In conclusion, our results confirm and extend previous findings that while glycans present on mature gp41 and gp120 after biosynthesis are not of paramount importance for the biological properties of the proteins (9, 12), N-glycosylation

during biosynthesis of both gp120 and gp41 is essential for properly folded bioactive molecules (10, 17, 33).

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