Effect of various glycosidase treatments on the resistance of the HIV-1 envelope to degradation

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Abstract Using a CD4-binding assay to assess the conformation of the human immunodeficiency virus envelope glycoprotein (CHO^+ Env), we studied the effect of treatment with various glycosidases on the stability of Env in denaturing environments and in biological media: cleavage from Env of either high-mannose-type glycans (HMT^- Env) by endoglycosidase H or sialic acid residues ($Sial^-$ Env) by sialidase did not alter Env stability whereas its complete deglycosylation (CHO^- Env) by *N*-glycanase had a large effect. The influence of glycan removal on Env sensitivity to proteases was also studied. Thrombin cleavage within V3 was affected by *N*-glycanase treatment; both HMT^- Env and CHO^- Env displayed an increased sensitivity to other endoproteases. Thus, partial deglycosylation increases Env sensitivity to proteases but only its total deglycosylation alters its stability.

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Key words: Conformation; Denaturation; Envelope; Glycosylation; HIV; Proteolysis

1. Introduction

The mature human immunodeficiency virus type-1 (HIV-1) envelope (Env) is composed of gp120 and gp41, the cleavage products of the gp160 precursor. At the virus surface, gp120 mediates HIV binding to $CD4^+$ cells; gp41 induces the subsequent membrane fusion between HIV and the host cell. Parts of gp120, including its third variable domain (V3), are also involved in the fusion process [1].

HIV Env is heavily glycosylated; Env from various origins presents 15–17 sialylated complex glycans and 6–8 high-mannose-type structures (HMT) [2]. Their positions are conserved among different isolates and they are evenly distributed along the molecule [3]. The current view is that glycosylation is required, during biosynthesis, for Env processing, folding and expression at the cell surface; in contrast, glycans are not necessary for Env function after biosynthesis [4–6].

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Abbreviations: ABB, ammonium bicarbonate buffer; BHK, baby hamster kidney; CD, cluster of differentiation; CHO, chinese hamster ovary; CHO^+, glycosylated molecule; CHO^-, *N*-glycanase-treated molecule; EH, endo-β-*N*-acetylglucosaminidase H; gp160, envelope precursor glycoprotein; gp120, outer membrane envelope glycoprotein; gp41, transmembrane envelope glycoprotein; HIV-1, human immunodeficiency virus type 1; HMT, high-mannose-type structures; HMT^-, endo-β-*N*-acetylglucosaminidase H-treated molecule; HPLC, high-pressure liquid chromatography; NG, *N*-glycanase; PBS, phosphate-buffered saline; PBSC, PBS 2% casein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Sial, sialidase; $Sial^-$, sialidase-treated molecule; V3, third variable domain of the outer membrane envelope glycoprotein

Env is the primary target for protective antibodies and the key component of vaccine trials [7]. Its glycans help HIV to escape the immune response and the use of deglycosylated gp160 as an immunogen may overcome this problem, despite several drawbacks: (i) mutation of some glycan sites alters the Env antigenic conformation but does not uncover epitopes [8]; (ii) as suggested [5,9] to explain that small amounts of SDS alter CD4 binding to deglycosylated, but not to glycosylated, Env [9,10], it has been shown that deglycosylation renders Env susceptible to treatments that alter protein stability [11]. The role that each of the two main glycan structures, i.e. the complex structures and the HMT, plays in the stability of Env under the conditions described previously [11] and in the resistance to proteolysis remained to be studied. Here, we addressed these points by means of endoglycosidase H (EH) treatment to investigate the role of the HMT and desialylation with sialidase (Sial) to study that of sialic acid residues which are responsible for most of the biological properties of the complex glycans [12]. Our criteria to study Env degradation were its CD4-binding ability — a good marker of Env conformation as it requires several domains to form the binding site [1] — and polyacrylamide gel electrophoresis (PAGE) patterns.

2. Materials and methods

2.1. Reagents

Monoclonal antibody 41a9 (Hybridolab, Paris, France) binds the gp41 sequence 605–609. Soluble (s) recombinant $gp160_{Lai}$ from BHK-21 cells [13] was obtained from Transgène S.A. (Strasbourg, France); $gp120_{GB8}$ from CHO cells was obtained from CAMR (Salisbury, UK); sCD4 (a gift from I. Jones, Institute of Virology, Oxford and the MRC ADP, UK) was expressed using the baculovirus expression system.

2.2. Labeling procedures

CD4, gp120 and gp160 (5 μg in 40 μl of phosphate buffer, 150 mM NaCl, pH 7.4 (PBS)) were labeled with 200 μCi of ^{125}INa in a 4 nM iodogen-coated tube for 20 min at 20°C (specific radioactivity: 15 μCi/μg, irrespective of the antigen), as described in [9], and then purified on a PD10 column (Pharmacia, Uppsala, Sweden).

2.3. Env treatments

2.3.1. Enzymatic treatments. Labeled (10^6 cpm/30 μl of PBS final volume) or unlabeled (2 μg/10 μl) glycosylated native (CHO^+) Env was treated overnight at 37°C as described in [14–16] with (i) type X Sial (Boehringer Mannheim, Germany; 20 mU) (Sial-treated molecules are named $Sial^-$ thereafter) or (ii) EH (Boehringer; 6 mU) to cleave HMT (EH-treated molecules: HMT^-) or (iii) *N*-glycanase (NG; Boehringer; 300 mU) to cleave all *N*-glycans (NG-treated molecules: CHO^-). Env (2.10^5 cpm) were treated at 37°C with various endoproteinases (Boehringer): (i) Arg C (2–200 mU/30 μl of 0.1 M ammonium bicarbonate buffer (ABB), pH 8.5, for 6 h); (ii) Glu C (0.04–40 U/30 μl of ABB for 6 h); (iii) pronase (0.02–200 ng/30 μl of PBS for 6 h); (iv) bovine thrombin (1.25–125 U/30 μl of ABB for 18 h). The samples were treated with 5% SDS and 5% β-mercapto-

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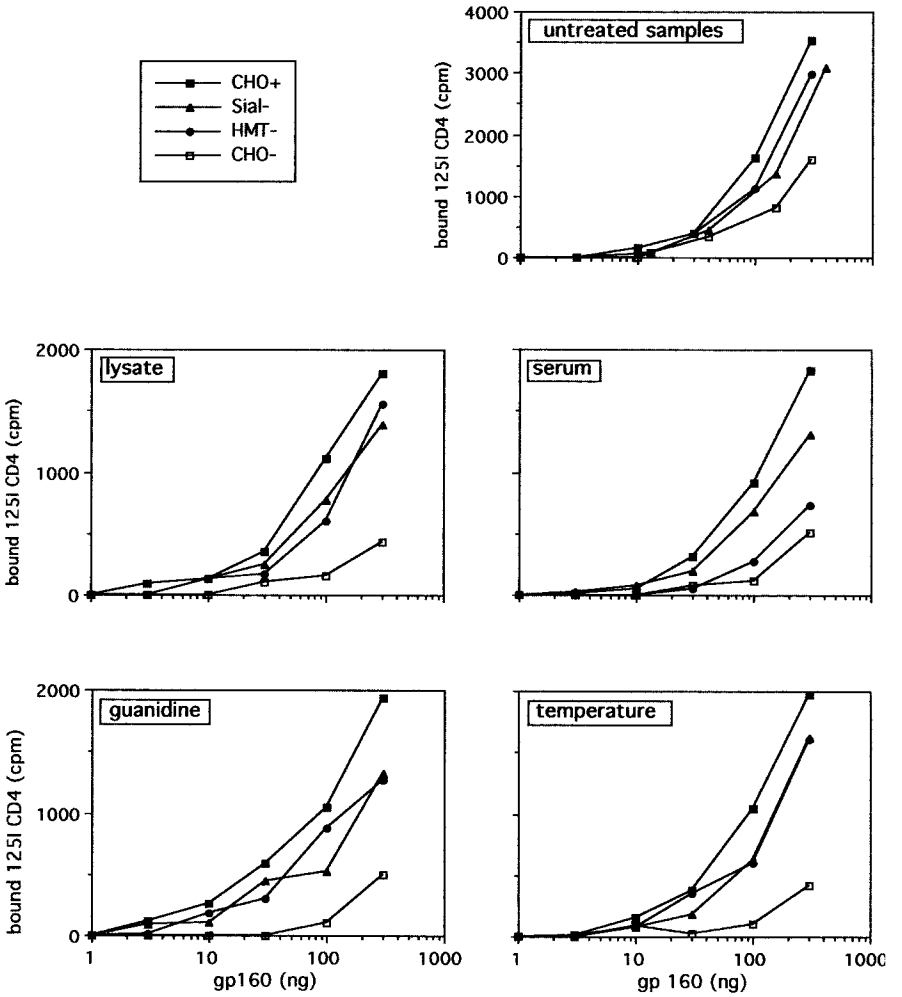

Fig. 1. Effect of various treatments on the subsequent CD4 binding of differently glycosylated gp160s. After heating at 70°C or incubation in 2 M guanidine, in human serum or in cell lysate — or no treatment (untreated samples) — the different gp160s (CHO^+-, $Sial^-$-, HMT^-- and CHO^- gp160) were absorbed onto 41a9-coated wells. After incubation with ^{125}I-labeled CD4, bound radioactivity was counted.

ethanol at 95°C and the proteins were separated by 10% SDS-PAGE; the pattern was scanned with a PhosphoImager (Bio-Rad, Les Ullis, France). To facilitate scanning and because, despite several gp160 purification steps performed by the manufacturer, contaminants were detected on gels, the sensitivity to proteases was studied using a batch of gp120 that migrated as a single band and was glycosylated like gp160.

2.3.2. Incubation of Env in various environments. Labeled (2.10^5 cpm/30 μl of final volume) or unlabeled (2 μg/30 μl) Env preparations were (i) subjected for 2 h to 2 M guanidine-HCl and then diluted 1:100 in PBS–2% casein (PBSC); (ii) heated for 5 min at 70°C in a water bath; (iii) incubated for 18 h at 37°C in 50% serum (obtained from a healthy donor) or with cell lysate (10^6 BHK-21 cells (ECAAC, Salisbury, UK) cultured in Glasgow medium (Flowbio, Les Ulis, France), 5% fetal calf serum, were lysed by three freeze–thaw cycles [11]). After dilution (at least 1:5), CD4 binding was tested. To avoid interference from albumin, samples incubated with serum were immunopurified with a pool of anti-HIV^+ human sera (1:100 dilution) and Protein A Sepharose (Pharmacia, Uppsala, Sweden) [14] before SDS-PAGE.

2.4. Binding assays

Wells (Maxisorp; Nunc, Roskild, Denmark) were coated with 41a9 antibody (1.5 μg/100 μl of 50 mM ABB, pH 9.6) overnight and blocked with PBSC. gp160 was incubated for 2 h at 20°C. After a wash, ^{125}I-labeled CD4 (2.10^4 cpm) was incubated for 2 h. After three washes, bound radioactivity was counted [11]. The signal in wells from which gp160 was omitted was defined as background. The treatments did not modify gp160 binding to wells as the binding of ^{125}I-labeledgp160 to 41a9-coated wells was unchanged after similar treatment (not shown). When serum or guanidine was used, gp160 bound to the well was quantified by ELISA with a pool of anti-HIV^+ human sera (1:100) and anti-human IgG coupled to peroxidase as reported in [15].

The figures illustrate a representative experiment ($n = 3$).

3. Results

3.1. CD4-binding ability of differently glycosylated gp160s after incubation under various conditions

After treatment with glycosidases and incubation in various conditions, the CD4 binding of the resulting gp160 was studied using a test in which Env is captured via its gp41 domain, leaving intact the CD4-binding subunit [11].

CHO^+ gp160 and partially ($Sial^-$ gp160 and HMT^- gp160) and fully deglycosylated (CHO^-) gp160 each bound CD4 in a dose-dependent manner (Fig. 1). CHO^- gp160 binding was one-third of that of CHO^+ gp160, and HMT^- gp160 and $Sial^-$ gp160 presented intermediate binding capacities. None of the various treatments substantially modified the binding of CHO^+ gp160, $Sial^-$ gp160 or HMT^- gp160. However, all the treatments reduced CHO^- gp160 binding to CD4 (Fig. 1).

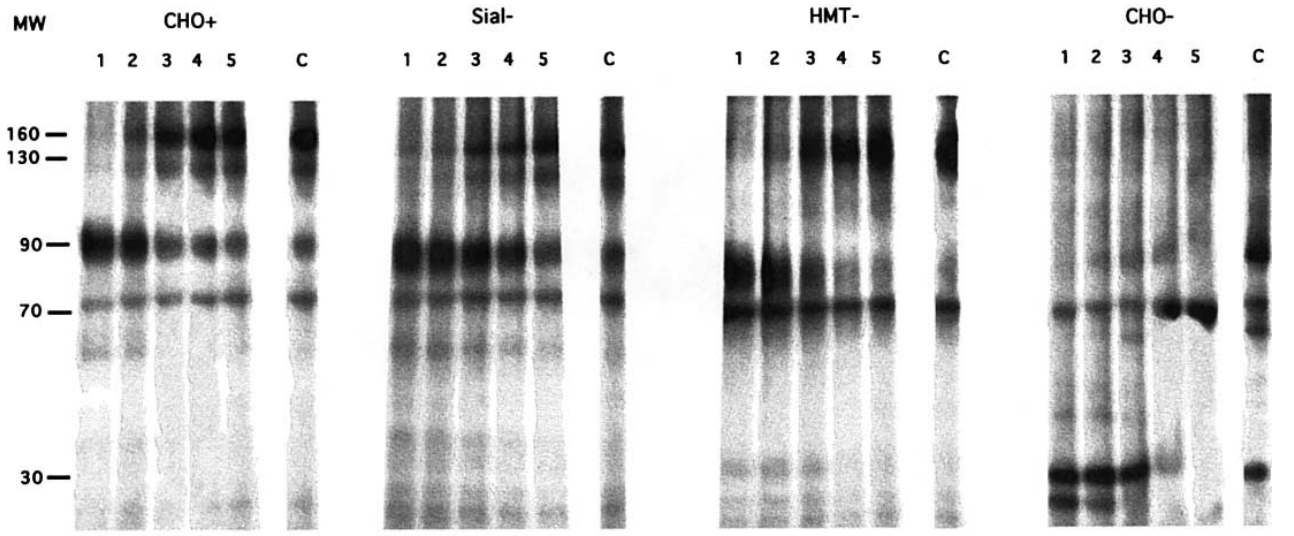

Fig. 2. Sensitivity of differently glycosylated gp160s to thrombin. The different gp160s (CHO^+-, $Sial^-$-, HMT^-- and CHO^- gp160) were treated with thrombin (lane 1, 125 U; lane 2, 42 U; lane 3, 12.5 U; lane 4, 4.2 U; lane 5, 1.25 U; land C, mock treated gp160).

Thus, partial deglycosylation did not alter Env stability under our conditions.

3.2. *Susceptibility of differently glycosylated gp160s to thrombin*

Native Env is cleaved by thrombin only within V3 [17]. Because glycans protect glycoproteins from proteolysis, we studied whether deglycosylation of gp160 allowed cleavage by thrombin at sites other than in V3.

CHO^+ gp160 was treated with thrombin under conditions previously reported to cleave Env only at the crown of V3 [17]. The 160 kDa band visualized by SDS-PAGE was eliminated in a thrombin dose-dependent manner (Fig. 2) concomitantly with the accumulation of a 90 kDa species also found in the control sample, suggesting that a fraction of gp160 was already cleaved in the preparation we used. This 90 kDa form corresponds to the previously described C-terminal cleavage product [17]. The 70 kDa band was not an *env* sequence and it did not correspond to the N-terminal thrombin cleavage product of about 70 kDa [17]: it was found in the controls, it was not affected by either thrombin or glycosidase treatments and it did not react with anti-HIV^+ human sera (not shown).

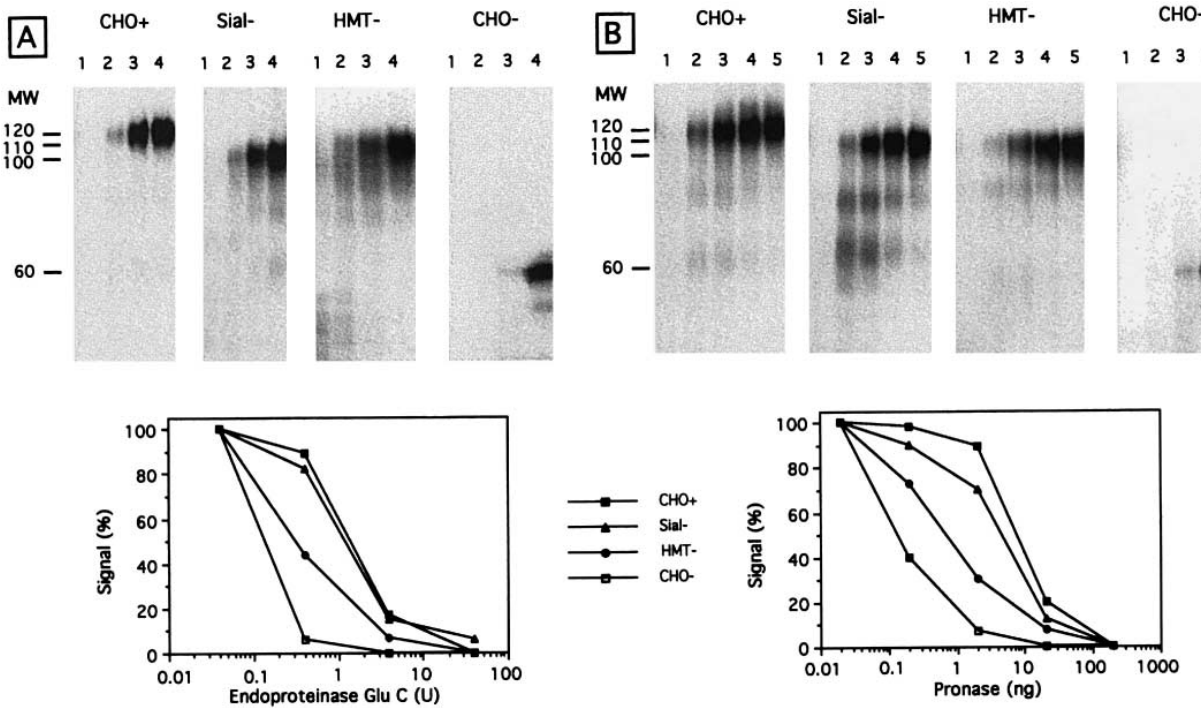

Fig. 3. Sensitivity of differently glycosylated gp120s to proteases. The different gp120s (CHO^+-, $Sial^-$-, HMT^-- and CHO^- gp120) were treated with either Glu C (A: lane 1, 40 U; lane 2, 4 U; lane 3, 0.4 U; lane 4, 0.04 U) or pronase (B: lane 1, 200 ng; lane 2, 20 ng; lane 3, 2 ng; lane 4, 0.2 ng; lane 5, 0.02 ng). The amount of radioactivity corresponding to the original species was quantified with a PhosphoImager scanner. (The lane corresponding to the control situation is the 100% signal.)

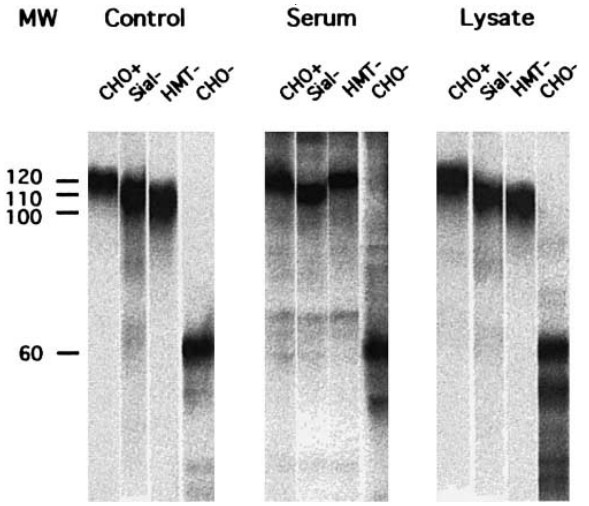

Fig. 4. Effect of biological media on differently glycosylated gp120s. The different gp120s (CHO^+-, $Sial^-$-, HMT^-- and CHO^- gp120) were incubated with either serum or cell lysate. Control: gp120 not preincubated in the media.

Presumably, the expected N-terminal cleavage product was not radiolabeled: indeed, non-denaturing labeling labels only one or few Tyr residues among the many available on a protein. Thrombin treatment of $Sial^-$ gp160 (MW of the original band: 140 kDa) and HMT^- gp160 (130 kDa) resulted in the accumulation of 85 kDa and 80 kDa species, respectively. These MW are consistent with the partial deglycosylation of the C-terminal fragment. The susceptibility of CHO^+ gp160, $Sial^-$ gp160 and HMT^- gp160 to thrombin activity was similar (complete degradation with 40 U). gp160 was completely deglycosylated under our conditions [9] and resulted in a deglycosylated species of about 90 kDa. Thrombin cleavage of CHO^- Env within V3 was expected to give a 55 kDa fragment corresponding to the CHO^- C-terminal fragment. Unexpectedly, CHO^- gp160 was cleaved by 1.25 U of thrombin to give a 70 kDa band; thus, cleavage occurred at a site other than V3. In the presence of high thrombin doses, the 70 kDa band was lost and a 30 kDa fragment appeared.

Thus, partial deglycosylation did not significantly influence Env sensitivity to thrombin, whereas total deglycosylation unmasked new cleavage site(s).

3.3. Susceptibility of differently glycosylated gp120s to various proteases and to incubation in biological media

3.3.1. Incubation with proteases. Because deglycosylation affected Env cleavage by thrombin, we studied whether glycans protect Env against various other proteases.

Sial-, EH- or NG-treatment of gp120 resulted in homogeneous bands of 110, 100 and 60 kDa, respectively (Fig. 3). The dose of Glu C (Fig. 3A) required to degrade completely Env was higher for CHO^+ gp120 and $Sial^-$ gp120 than for HMT^- gp120; CHO^- gp120 was the most sensitive. The gels were scanned: CHO^- gp120 and HMT^- gp120 were degraded at 10-fold and 5-fold lower enzyme concentrations, respectively, than those required for $Sial^-$ gp120 and CHO^+ gp120 digestions. Similarly, treatment of CHO^+ gp120 and $Sial^-$ gp120 with the maximum Arg C (0.2 U) or Lys C (0.12 U) dose which could be used in our test led to a 50% loss of the native band (not shown). These doses completely degraded HMT^-- and CHO^- gp120. Finally, the sensitivity of CHO^+ gp120 and $Sial^-$ gp120 to pronase was similar whereas the sensitivity of gp120 was increased by EH- or NG-treatment (Fig. 3B). Similar results were obtained with our batch of gp160 (not shown).

Thus, the partial deglycosylation of Env altered its resistance to proteases.

3.3.2. Incubation in serum or in cell lysate. These data suggest that the altered CD4 binding of CHO^- gp160 after incubation in serum or cell lysate may have been due to proteolysis. This was tested (Fig. 4): incubation in these media did not substantially alter the gel pattern of gp120 other than the appearance of minor bands of low MW following CHO^- gp120 incubation in cell lysate. Unexpectedly, but reproducibly, HMT^- gp120 incubated with serum migrated as a 120 kDa band.

Thus, incubation in biological media did not result in a substantial proteolysis of deglycosylated gp120.

4. Discussion

Partial deglycosylation by either EH or a neuraminidase of broad specificity [16] diminished by about 20% and 10%, respectively, the MW of gp120 and gp160; this is in agreement with the nature of the glycans attached to recombinant Env from CHO [2,15] or BHK-21 cells [9].

As reported for various proteins [18–21], heating or incubation in guanidine altered the conformation of CHO^- Env. In contrast, partial deglycosylation did not affect Env stability; as complex glycans are evenly distributed along the protein [3], it is likely that a limited, homogeneously distributed, padding by complex structures is sufficient to preserve Env characteristics. The decreased CD4 binding of CHO^- gp160 after incubation in cell lysate or serum was not due to proteolysis as the incubation in these media did not result in substantial cleavage.

As enhanced sensitivity to proteases after deglycosylation has been described [22–24], we studied the effect of partial deglycosylation on the sensitivity of Env to thrombin [17]. Thrombin-like proteases cleave Env at the crown of V3, a domain that presents a single complex glycan attached 10 amino acid residues upstream from its apex [3]. It has been proposed that this cleavage might be necessary for the changes in conformation required for membrane fusion [1]. Native and partially deglycosylated gp160 digested by thrombin gave SDS-PAGE patterns consistent with cleavage within V3. In strong contrast, CHO^- Env was cleaved by a small amount of thrombin and a fragment that does not result from a cleavage within V3 appeared: indeed, its MW is consistent either with that of the C-terminal fragment of gp160 after thrombin cleavage at Arg^{192} or with that of a N-terminal fragment of Env cleaved at one of the 28 potential sites in the gp41 domain. Furthermore, high thrombin doses generated a short fragment that resulted either from the cleavage of CHO^- Env at sites that are not within V3 or from the further rapid digestion — at some of its 37 potential cleavage sites some of which are likely to be unmasked by deglycosylation — of the C-terminal fragment generated by the cleavage within V3. Thus, glycans restrict thrombin cleavage to V3 in vitro. Similarly, the glycans of the coagulation factor V molecule maintain a conformation required for its functional cleavage by thrombin at a single site [25].

EH, but not neuraminidase, treatment increased Env sensitivity to proteases. Thus, either the HMT per se have a specific role in the protection of Env against proteolysis or the removal of 30% of the glycans is sufficient to uncover proteolytic sites and to increase Env sensitivity to proteolysis, this latter consequence being more pronounced when all glycans are cleaved by NG. The fact that EH treatment exposes several proteolytic Glu C sites is consistent with the observation that 14/26 Glu residues of $gp120_{GB8}$ and 5/6 high mannose glycans at conserved positions on Env produced in CHO cells [3] are located in the C-terminal fragment.

In conclusion, limited glycosylation is sufficient to maintain the biochemical properties of Env whereas the presence of both HMT and complex glycans is necessary for good protection against proteolysis. Thus, in addition to modifying HIV susceptibility to antibody neutralization, changes in HIV glycosylation alter the protection of the viral envelope against degradation.

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