Glycosylation and stability of mature HIV envelope glycoprotein conformation under various conditions

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Abstract The role of the glycans of the mature human immunodeficiency virus (HIV) envelope (gp160) in its stability in various conditions was studied. gp160 conformation was monitored through its subsequent ability to bind [^{125}I]CD4. Treatment of glycosylated (CHO+) gp160 with (i) sodium dodecyl sulfate (SDS) concentrations above 0.01% impaired subsequent CD4 binding while 0.3% SDS abolished it; (ii) β-mercaptoethanol (MSH) concentrations above 0.01% impaired CD4 binding while 0.03% MSH abolished it; (iii) 2 M guanidine-HCl had no effect; (iv) temperatures between 50°C and 80°C altered CD4 binding while, above 80°C, the binding was abolished; (v) CD4 binding was decreased by 50% by 2 freeze-thaw cycles but was not further affected by subsequent (up to 15) cycles; (vi) gp160 incubation in serum or cell lysate had no effect on CD4 binding. Glycanase treated (CHO−) gp160 binding activity was only 3-fold lower than that of CHO+ gp160. Only 2 M guanidine-HCl and heating at 70°C differentially affected the binding of CHO+ and CHO− gp160, the effects being larger for CHO− gp160. CHO− gp160 binding was impaired after incubation in either serum or cell lysate. Thus, glycans stabilize gp160 conformation in some environments. However, CHO− gp160 appears to be resistant to denaturation as compared to other glycoproteins reported in the literature.

Key words: Glycan; HIV-1; Envelope glycoprotein; Conformation; Stability

1. Introduction

N-linked glycans of glycoproteins are important for intracellular routing, biosynthesis, folding, biological activity, antigenicity and protection against proteolytic and physico-chemical degradations [1–5]. Thus, glycosylation is a post-translational modification which affects many characteristics of a glycoprotein.

For many viral envelope (Env) glycoproteins, glycans contribute to various steps of the virus cycle, e.g. folding, virus budding, infectivity and antigenicity changes [1,6,7]. Early in the 90's, the involvement of glycans of the human immunodeficiency virus type-1 (HIV-1) Env in such phenomena was studied [3,6]: glycans are necessary both to create the functional conformation of Env [3,6] and for its efficient intracellular transport [2,4]. In contrast, glycans present on mature Env after biosynthesis are required neither for HIV binding to, nor for infection of, CD4+ cells [8–10] although glycans may help HIV to escape host immune response [11]. The contribution of HIV glycans in the stabilization of the mature Env has been suggested on several occasions [6,8,9,11] but no study has adressed this point. Yet, stabilization of gp160 through its glycans is an important point inasmuch as removal of gp160 glycan padding may improve its immunogenicity.

The mature HIV-1 glycoproteins, outer membrane gp120 and transmembrane gp41, are proteolytic cleavage products of precursor gp160. They are responsible for HIV binding to CD4+ cells and fusion of virus and cell membranes, respectively [12]. gp160 displays a property of the cleaved gp120 subunit, i.e. CD4 binding [12]. It is highly glycosylated: glycans are conserved between isolates, equally distributed on Env and responsible for 50% of its MW [13]. Recombinant and viral gp160 exhibit a similar pattern of large high mannose and complex type glycans [8,9,13]. Possibly, this mass of glycans is a selective advantage for HIV by protecting mature Env against degradations.

gp160 has an elaborate organisation with variable and conserved loops bridged by 10 disulfide bonds [12]. These loops interact each other to form the conformation that binds CD4 [12]. Using CD4 binding ability to study Env conformation, we investigated the role of glycans in the protection of gp160 against degradations induced by various treatments.

2. Materials and methods

2.1. Reagents

Mouse monoclonal (m) antibody (Ab) 41a9 (a gift from F. Traincard, Hybridolab, Paris, F) recognizes gp41 amino acids 605–609. Soluble (s) gp160 was encoded by a gene where gp120-gp41 cleavage sites and the transmembrane domain are missing; it was produced in BHK-21 cells via a vaccinia virus vector and purified [14]; sCD4 (a gift from I.M. Jones, NERC, Oxford, UK) was obtained in the baculovirus expression system.

2.2. Labeling procedures

CD4 (5 μg in 40 μl of PBS NaCl 150 mM pH 7.4 (PBS)) was labeled with 150 μCi of ^{125}INa in a 4 nM iodogen-coated tube for 20 min at 20°C (specific radioactivity: 15 μCi/μg) [8]; 5 μg of gp160 in 100 μl of PBS H_2O_2 ($1/10^6$ final dilution) was labeled with 250 μCi of ^{125}INa and 2.5 μg of lactoperoxidase (specific radioactivity: 25 μCi/μg) [8].

2.3. CD4 binding assay

41a9 mAb (1.5 μg/100 μl 50 mM bicarbonate buffer pH 9.6) was adsorbed onto Maxisorp microtiter plates (Nunc, Roskild, Denmark) overnight at 4°C. All subsequent steps were done at 20°C. The wells were blocked using 2% non fat dry milk in PBS (PBSM) that was used in the following steps. Either a fixed gp160 concentration (for determination of denaturing conditions) or increasing concentrations (for experiments using gp160 treated by *N*-glycanase (CHO−) as in [8,9,15]) were added. In competitive experiments, 100 ng of gp160 were incubated for 2 h in mAb-coated wells. After a wash, CD4 (2–200 nM) and [^{125}I]CD4 ($2 \cdot 10^4$ cpm/well) were coincubated for 2 h. After 3 washes, radioactivity was counted. Background was measured by incubating [^{125}I]CD4 directly with the mAb. To ascertain that neither physical (temperature of freeze/thaw cycles) nor chemical (guanidine-HCl, SDS,

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β-mercaptoethanol (MSH)) treatments modified gp160 binding to 41a9 mAb, glycosylated (CHO+) and CHO− [^{125}I]gp160 ($2 \cdot 10^5$ cpm/assay) were similarly treated. Samples were incubated in mAb-coated wells for 2 h. After 3 washes, radioactivity was counted. Background was determined using wells with no mAb but incubated with [^{125}I]gp160. When serum or cell lysate were used, gp160 bound to wells was determined by ELISA using human anti-HIV-1 sera (1:100) [9].

2.4. gp160 treatments

In each experiment described below, the effect of the pretreatment on gp160-mAb binding and CD4 binding was checked as described above. (i) SDS treatment: gp160 (100 ng/10 μl PBS) was incubated with SDS (0 to 3%) for 10 min at 20°C and then diluted in 100 μl PBSM prior the assay. Similarly, CHO+ gp160 and CHO− gp160 (750 ng) were treated in 17 μl PBS and diluted 10-fold prior the assay. (ii) MSH treatment: gp160 (100 ng/10 μl PBS) was treated with MSH (0 to 0.3%) for 10 min at 20°C and then with iodoacetamide (2.5:1, iodoacetamide/MSH ratio), lyophilized to remove MSH, and diluted in 100 μl of PBSM, 10% fetal calf serum prior the assay. Similarly, CHO+ gp160 and CHO− gp160 (750 ng) were treated in 17 μl PBS and diluted 10 fold prior the assay. (iii) Guanidine-HCl treatment: gp160 (500 ng/5 μl) was treated with 2 M guanidine-HCl for 2 h at 20°C and diluted in 500 μl PBSM prior the assay. Similarly, CHO+ gp160 and CHO− gp160 (2 μg) were treated in 7 μl of PBS and then diluted 100-fold. (iv) Resistance to freeze-thaw cycles: gp160 was submitted to up to 15 freeze-thaw cycles (one cycle consisted of incubation at −80°C for 5 min in an ethanol bath followed by an incubation at +30°C for 5 min) prior the assay. (v) Resistance to temperature: gp160 was heated to definite temperatures (20°C to 90°C) for 5 min in a water bath prior the assay. (vi) Incubation with serum or cell lysate: serum was obtained from a healthy person; cell lysate was obtained by a 3 freeze-thaw cycle treatment of BHK21 cells (10^7 cells/100 μl). gp160 was treated with 15 μl of either serum or cell lysate in PBS (30 μl final volume) overnight at 37°C. Samples were diluted at least 5-fold prior the assay.

3. Results

3.1. Evaluation of the CD4 binding assay

We showed previously [8] that, while CHO+ gp160 binding to CD4 exhibits a $K_{0.5}$ of $1.25 \cdot 10^{-8}$ M, completely deglycosylated gp160 binds with high affinity cell membrane CD4 ($K_{0.5} = 5 \cdot 10^{-8}$ M) [8,9]. Here, we verified that [^{125}I]CD4 binding to gp160 adsorbed onto 41a9 mAb-coated wells fullfilled the criteria of a specific high affinity interaction. We also verified that the assay allowed quantitative measurement of the binding.

MAb 41a9 was used because (i) it recognizes with high affinity ($3 \cdot 10^{-10}$ M; F. Traincard, personal communication) a region that is not involved in CD4 binding and (ii) gp160 deglycosylation does not affect its binding (not shown). Labeled CD4 migrated as a single sharp band in SDS PAGE (not shown). It was bioactive because (i) gp160 binding was inhibited by CD4 and (ii) the affinity ($K_{0.5} = 7 \cdot 10^{-9}$ M) was in line with previous data [8,9] (Fig. 1A). After *N*-glycanase treatment, CHO− gp160 migrated as a 90 kDa band (not shown). [^{125}I]CD4 bound CHO− gp160 in a dose dependent manner but its binding was 3-fold lower than that of CHO+ gp160 (Fig. 1B).

3.2. CD4 binding ability of gp160 submitted to various treatments

We first determined concentrations of various chemical agents that affect protein conformation. CHO+ gp160 was treated with these reagents and its subsequent ability to bind CD4 was studied ($n = 3$) under conditions where these reagents, except those bound to treated Env, were no more present in the assay as, after mAb binding, wash was done. Concentrations above 0.01% of SDS or MSH inhibited CD4 binding while

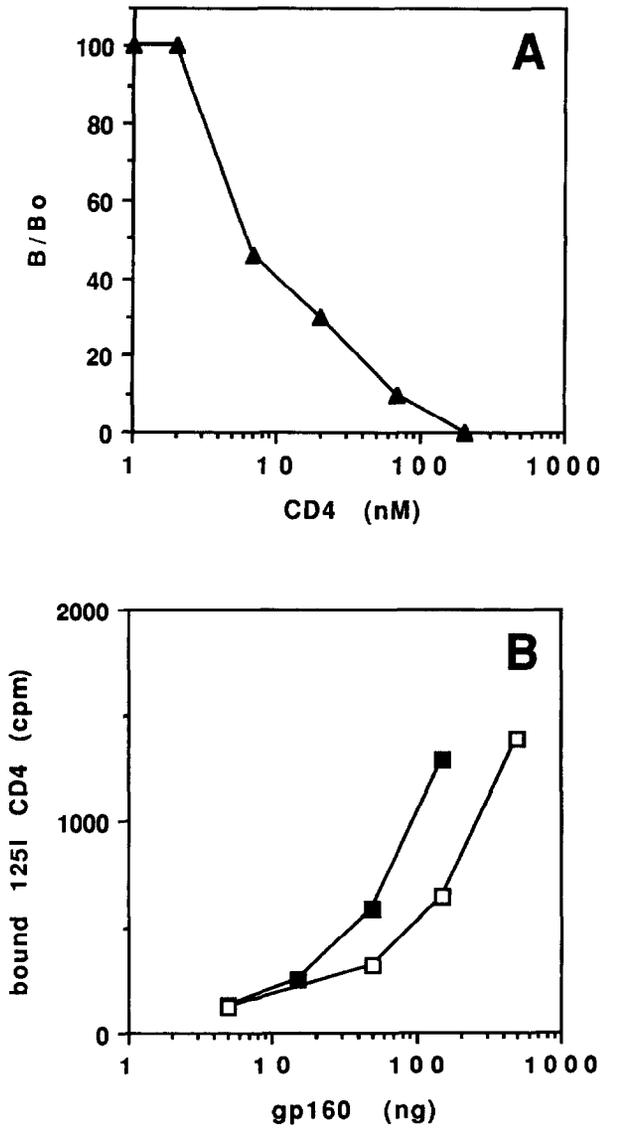

Fig. 1. Evaluation of the gp160-CD4 binding assay. (A) Affinity of native gp160 for [^{125}I]CD4. After binding onto 41a9 mAb-coated wells, gp160 (100 ng) was coincubated with CD4 (2–200 nM) and [^{125}I]CD4 (50 000 cpm). Bound radioactivity was counted. (B) Effect of the deglycosylation of gp160 on CD4 binding. After incubation of either CHO+ (■) or CHO− gp160 (□) (5–300 ng) onto 41a9 mAb-coated wells, [^{125}I]CD4 was added and radioactivity was counted. A representative experiment ($n = 3$) is shown.

binding was abolished with 0.3% and 0.03%, respectively (Fig. 2A,B). Treatment of gp160 with 2 M guanidine-HCl did not significantly modify CD4 binding while concentrations above 3 M interfered with mAb binding (not shown). We studied also the influence of physical denaturations on gp160: 2 freeze-thaw cycles significantly diminished binding but subsequent cycles (up to 15) had little further effect (Fig. 2C and data not shown). CD4 binding was modified by preincubation at temperature over 50°C and binding was abolished above 80°C (Fig. 2D). Incubation with either serum or cell lysate (50%) did not significantly alter gp160-CD4 binding (not shown).

In each experiment, gp160-41a9 mAb binding was assessed using treated [^{125}I]gp160. The conditions reported above did not

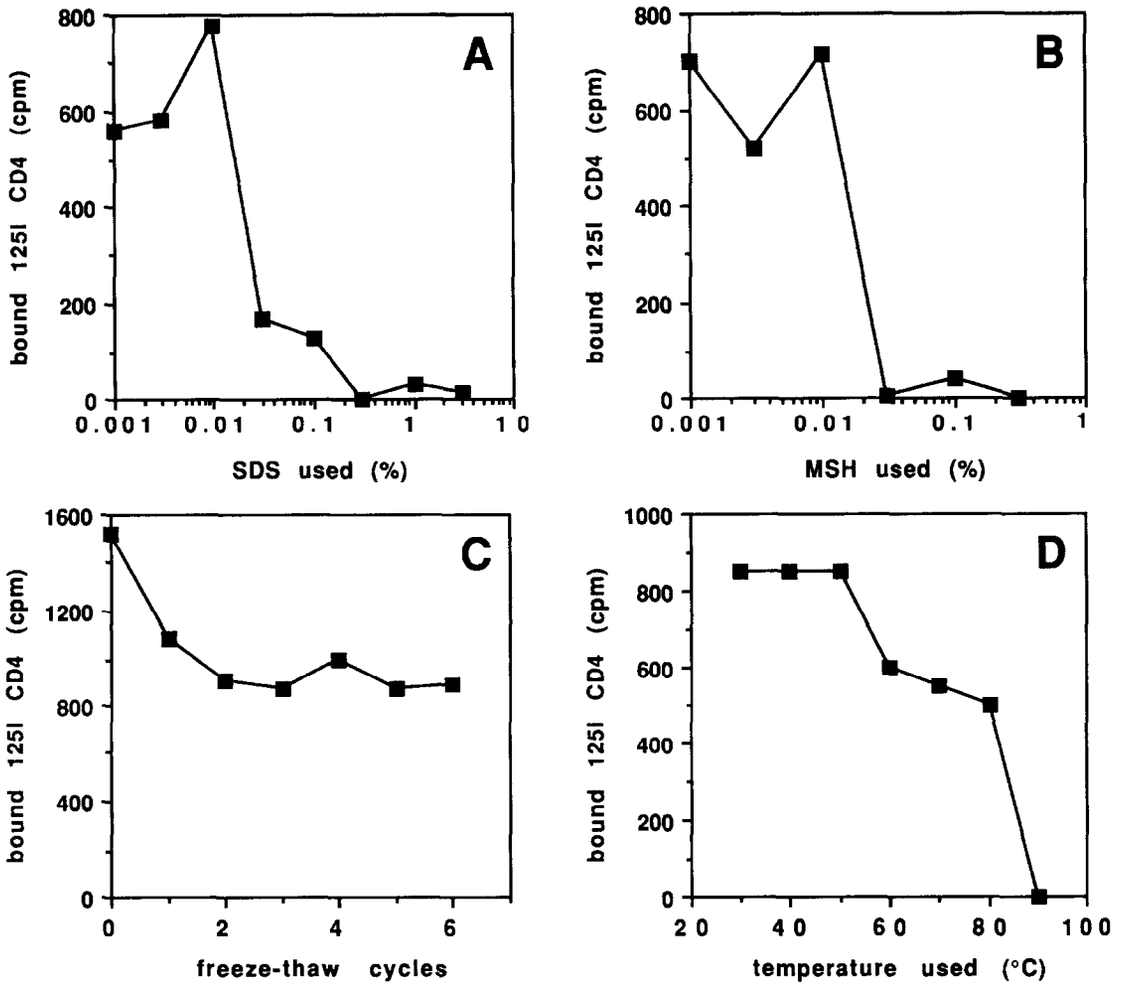

Fig. 2. Effect of physico-chemical treatments of native gp160 on its subsequent ability to bind CD4. gp160 was pretreated with increasing concentrations of either SDS (0.003–3%) (A) or MSH (0.003–0.3%) (B), or subjected to freeze-thaw cycles (C) or preincubated at various temperatures (30–90°C) (D). CD4 binding was then tested as described in Fig. 1. A representative experiment (n = 3) is shown.

significantly modify the binding. In addition, [^{125}I]CD4 binding specificity was assessed in each experiment by incubation with an excess of CD4 (not shown). Similar controls were also carried out in the experiments described below with both CHO+ gp160 and CHO− gp160: no significant difference in the mAb binding step was noted (not shown).

3.3. CD4 binding ability of deglycosylated gp160 submitted to various treatments

To study the role of glycans in the protection of gp160 conformation against denaturations, CHO+ and CHO− gp160 were submitted to the treatments described above. To detect with a reliable efficiency any fragilization of CHO− gp160, we chose the most severe conditions under which CD4 binding was detectable (e.g. 0.01% MSH and SDS, 70°C incubation and 4 freeze-thaw cycles). Guanidine-HCl (2 M), serum (50%) and cell lysate (50%) incubations were performed at the highest concentration usable.

The binding of CHO− gp160 treated with SDS or MSH (Fig. 3A,B) was 4 fold lower than that of CHO+ gp160. This is comparable with results obtained in Fig. 1B: thus, glycan removal did not alter gp160 resistance to SDS and MSH treatments. In contrast, 2 M guanidine-HCl treatment which had no effect on CHO+ gp160-CD4 binding, strongly impaired CHO− gp160-CD4 binding (Fig. 3C). Similarly, incubation at 70°C which only slightly affected CHO+ gp160-CD4 binding, impaired CHO− gp160-CD4 binding (Fig. 4A). CHO+ gp160 and CHO− gp160 were similarly resistant to freeze-thaw cycles (Fig. 4B). Incubation in serum or cell lysate for 24 h at 37°C altered CD4 binding of the sole CHO− gp160 (Fig. 5A,B).

4. Discussion

Glycans are neccesary for HIV Env folding [3,6] while they appear dispensable after biosynthesis to maintain its functional conformation [3,6,8,10]: completely [8] deglycosylated gp120 binds with high affinity CD4 ($K_{0.5} = 5 \cdot 10^{-8}$ M for CHO− gp120 and $1.25 \cdot 10^{-8}$ M for native gp120) [8–10]. However, the presence of 0.025% SDS during the deglycosylation procedure alters subsequent CD4 interaction [16]. Thus, a low SDS concentration, which does not affect native gp120 conformation, has a large effect on the conformation of its deglycosylated counterpart. This suggests that glycan removal alters gp160 stability. To verify this point, we studied the role of the glycans of

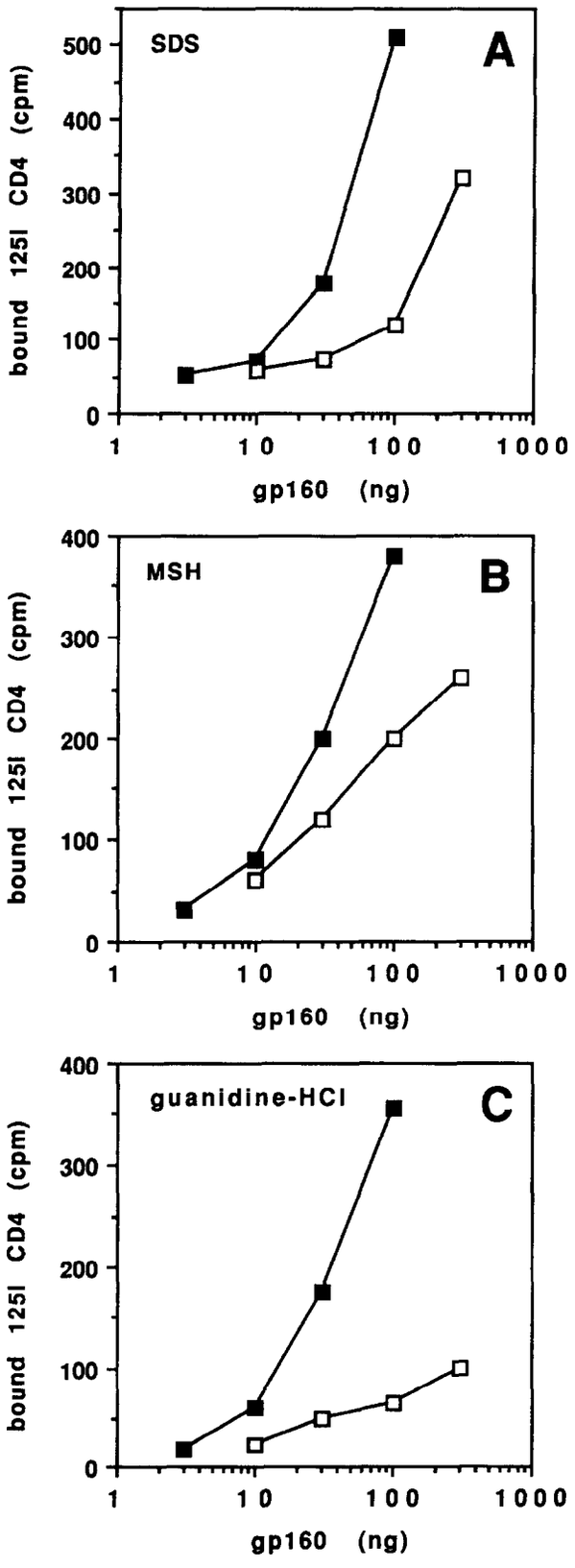

Fig. 3. CD4 binding ability of deglycosylated gp160 submitted to chemical treatments. CHO+ (■) and CHO− (□) gp160 were treated with 0.01% SDS (A), 0.01% MSH (B), 2 M guanidine-HCl (C). CD4 binding was then tested as described in Fig. 1. A representative experiment ($n = 3$) is shown.

recombinant gp160 in its protection against various physicochemical denaturation agents and in its stability in serum or cell lysate, through its subsequent CD4 binding ability. This approach to detect conformation changes was prefered to assays based on antibodies the reactivity of which can be altered by the removal of glycans in the absence of protein conformation changes.

First, we designed an assay in which [^{125}I]CD4 binds gp160 adsorbed onto anti-gp160 mAb coated wells: the affinity was $7 \cdot 10^{-9}$ M and deglycosylated gp160 bound CD4 with an ability reduced by only 3-fold, consistent with previous results [8,12].

Treatment of gp160 with 0.01% SDS was followed by a substantial decrease in subsequent CD4 binding and binding was abolished at 0.3%. This is in line with denaturation by 0.2% SDS of β-lactoperoxidase and ribonuclease [17]. However, many proteins, such as glucose oxidase or papain, remain active in the presence of 1% SDS [18]. Thus, gp160 appears to be rather sensitive to SDS denaturation.

gp160 bound CD4 following treatment with up to 0.01% MSH but activity was abolished at 0.03% MSH (gp160/MSH molar ratio, 1:60,000). The disulfide bond/MSH molar ratio usually used to reduce denatured protein is about 1/100 [19] but reduction of disulfide bonds of native molecules depends on their accessibility: an IgG/MSH molar ratio of 1:1,000 only reduces the disulfide bond between heavy and light chains of native IgG but all intra- and inter-disulfide bonds of denatured IgG [20,21]. Thus, disulfide bonds appear poorly sensitive to MSH on native gp160. Of note, treatment with 0.01% MSH or SDS increased subsequent gp160-CD4 binding, possibly through conformation changes which may promote gp160-CD4 interaction.

Guanidine-HCl (2 M) treatment had no significant effect on CD4 binding but this condition is sufficient to alter many proteins [22], e.g. ribonuclease [23], erythropoetin [24], galactose oxydase [25] and yeast invertase [26]. The inactivation of the latter enzymes is reversed by dilution of the sample: thus, 2 M guanidine-HCl may alter gp160 conformation in a reversible way that can be achieved by dilution during the assay.

Temperatures below 50°C did not alter CD4 binding but binding was abolished above 80°C. Resistance of proteins to heat denaturation varies: galactose oxidase activity decreases after 30 min at 60°C [25] while erythropoietin remains active after 30 min at 70°C [27]. However, erythropoietin is denatured at around 55°C, as assessed by circular dichroism analysis [24] and the activity of the heat-treated molecule is due to renaturation. Thus, Env structure may be reversibly altered by temperatures below 70°C and may progressively lose its ability to refold at higher temperatures.

CD4 binding decreased by 50% following 2 freeze-thaw cycles and subsequent cycles had little effect. Freeze-thaw cycles denature many proteins [28] but it is not a general phenomenon: invertase activity resists to 40 freeze-thaw cycles [26] and glucose oxidase to 100 cycles [25]. The mechanism of freeze-thaw denaturation is imputed to the formation of ice within the molecule. This induces an increase in the concentration of substances originally in solution in the environment of the molecule. Freezing injury can thus be compared with osmotic salt injury [29].

We examined whether glycans stabilize gp160 conformation. We treated CHO+ and CHO− gp160 with the conditions determined above. Only guanidine-HCl and heating affected differ-

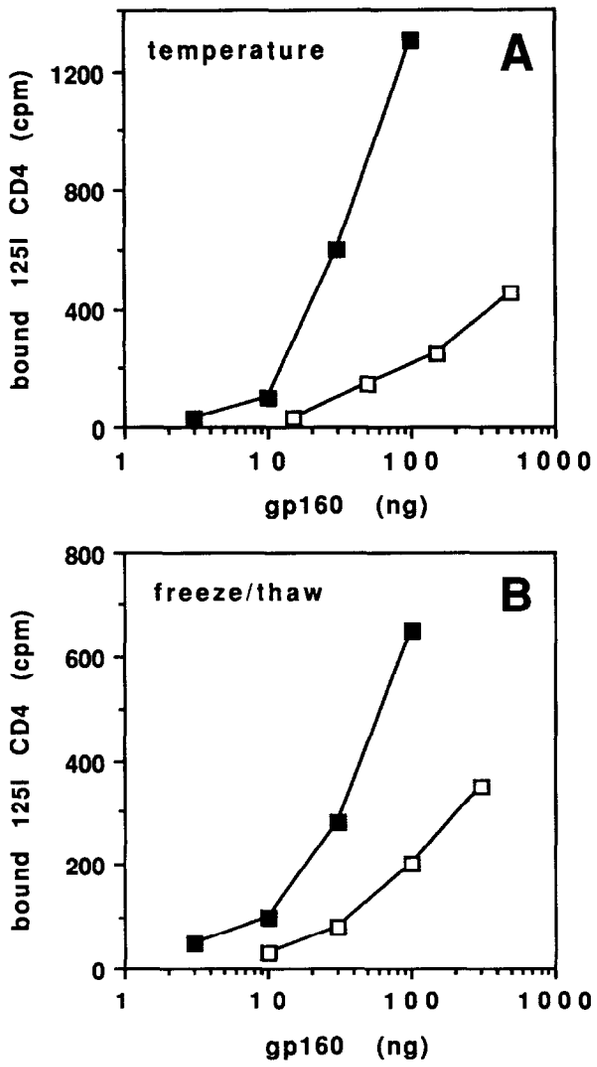

Fig. 4. CD4 binding ability of deglycosylated gp160 submitted to physical treatments. CHO+ (■) and CHO− (□) gp160 were submitted to heating at 70°C (A) or to 4 freeze-thaw cycles (B). CD4 binding was then tested as described in Fig. 1. A representative experiment ($n = 3$) is shown.

entially CD4 binding of CHO+ gp160 and CHO− gp160. Thus, gp160 glycans have a protective role against some denaturing agents. The stabilizing role of glycans depends on the glycoprotein [5,30]: both glycosylated and non glycosylated ribonucleases are equally sensitive to guanidine-HCl and refold equally well [23] while CHO− yeast invertase progressively looses its activity following freeze-thaw cycles whereas its CHO+ counterpart does not. The activity of its two forms is similarly affected by guanidine-HCl but its removal allows recovery of the activity of CHO+ but not of CHO− invertase [26], suggesting that glycans influence refolding. Similarly, native erythropoietin unfolds at 70°C [24] but its activity is restored once the temperature decreases, while its CHO− counterpart is irreversibly affected [27]. Thus, glycans of HIV Env may either stabilize its structure or be involved in refolding after denaturation. The latter is more likely as CHO− gp160 activity was affected by treatments which may be reversed here.

Finally, glycans play a role in the stabilization of gp160 in media that may contain proteases, e.g. serum or cell lysate, inasmuch as they only alter CD4 binding by CHO− gp160. Similar protection is described for many glycoproteins [5,30–32] and may be due to removal of bulky glycans exposing proteolytic sites either by inducing conformation changes of the tertiary structure or by unmasking cleavage sites.

In conclusion, CHO− gp160 appears to be rather resistant to many denaturing agents. The use of abnormally glycosylated gp160 as immunogen was considered as a means to improve the anti-HIV immune response as glycosylation may mask neutralizing epitopes. Because deglycosylation decreases gp160 stability, especially in serum, the characterization of abnormally glycosylated forms with higher stability, and thus with improved plasma half-life, should be investigated to help vaccine design.

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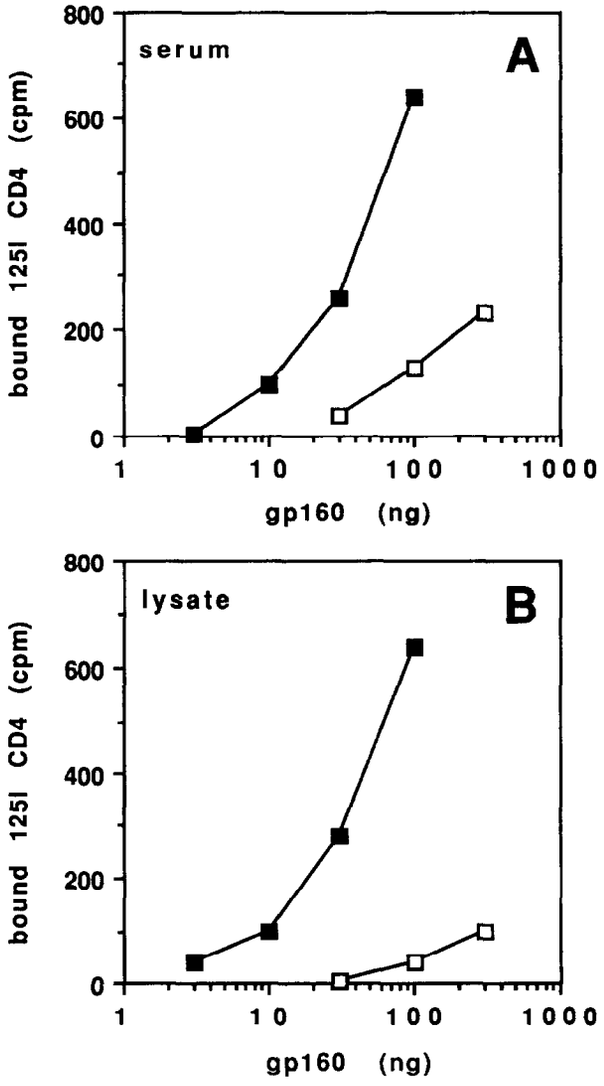

Fig. 5. CD4 binding ability of deglycosylated gp160 incubated with serum or cell lysate. CHO+ (■) and CHO− (□) gp160 were incubated with 50% human serum (A) or cell lysate ($1.5 \cdot 10^6$ cells) (B). CD4 binding was then tested as described in Fig. 1. A representative experiment ($n = 3$) is shown.

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