Signal peptide of HIV envelope protein impacts glycosylation and antigenicity of gp120

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The HIV-1 envelope protein (Env) of early-replicating viruses encodes several distinct transmission signatures. One such signature involves a reduced number of potential N-linked glycosylation sites (PNGs). This transmission signature underscores the importance of posttranslational modifications in the fitness of early-replicating isolates. An additional signature in Env involves the overrepresentation of basic amino acid residues at a specific position in the Env signal peptide (SP). In this report, we investigated the potential impact of this SP signature on gp120 glycosylation and antigenicity. Two recombinant gp120s were constructed, one derived from an isolate that lacks this signature and a second from an early-replicating isolate that includes this signature. Chimeric gp120s were also constructed in which the two SPs were swapped between the isolates. All four gp120s were probed with glycan-, structure- and receptor- specific probes in a surface plasmon resonance binding assay. We found that the SP of Env influences qualitative aspects of Env glycosylation that in turn affect the antigenicity of Env in a major way. The SP impacts the affinity of Env for DC-SIGN, a lectin receptor expressed on dendritic cells that is believed to play a role in mucosal transmission. Additionally, affinity for the monoclonal antibodies 17b and A32, which recognize a CD4-induced, open conformation of Env is also altered. These results demonstrate that natural variation in the SP of HIV Env can significantly impact the antigenicity of mature gp120. Thus, the SP is likely subject to antibody-mediated immune pressure.

gp120 | signal peptide | antigenicity | HIV | vaccine

Transmission of HIV across mucosal tissues involves a complex, yet strikingly inefficient, series of events that are not fully understood (1, 2). It has been shown that among the quasispecies of virus replicating in an infected, sexually transmitting person, very often, only a single isolate establishes infection in the recipient partner (3, 4). Studies suggest that this genetic bottleneck is not stochastic (3, 4). Rather, specific structural features of the HIV-1 envelope protein (Env) appear to provide transmitting isolates with increased transmission fitness (5, 6). The transmitting virus diversifies over time in infected subjects (7) due to random mutations introduced by error prone replication, which are then subjected to immune selective pressure.

The HIV Env is extensively glycosylated, with up to 50% of the mass of gp120 composed of glycans (8). However, the number of potential N-linked glycosylation sites (PNGs) varies significantly between isolates, with a range of 18–33 and a median of 25 (9, 10). Genetic signatures in transmitting HIV isolates have been linked to Env (11, 12). The best-characterized signature involves a reduced number of PNGs in transmitting isolates (12). In addition, shorter variable loops in gp120 have been correlated with this decrease in PNGs (13). Mass spectrometry analysis of the glycan profiles of Env from transmitting viral isolates in acute infection shows increased high-mannose residues compared with the glycan profile of Env from chronic phase isolates, further suggesting a link between Env glycan content and transmission (14) (Fig. 1A). It is currently unknown by what precise mechanisms these alterations in glycosylation influence transmission fitness. One possibility is that altered glycosylation can directly influence the interaction of Env with host cell surface receptors (15), which in turn increase infectivity. Once infection is established, the appearance of new PNGs may shield critical epitopes from neutralizing antibodies that evolve during the course of infection.

A second genetic signature, identified in subtype B isolates, involves an overrepresentation of basic amino acids, particularly histidine, at position 12 of the Env signal peptide (SP) of transmitting viral isolates (Fig. 1 B and C), which is lost in chronic phase viral isolates (11, 16). The presence of a signature in this region of the Env SP of transmitting viral isolates has also been confirmed in simian immunodeficiency virus (SIV) and simian–human immunodeficiency virus (SHIV) transmission studies (17). The SP, which lies at the N terminus of the amino acid sequence of proteins destined for transport to the cell surface or for secretion, directs these nascent peptides to the endoplasmic reticulum (ER) where translation continues, coincident with protein processing (Fig. S1). In the ER, disulfide bonds form and undergo isomerization (18). Of note, the addition of high-mannose glycans begins in the ER (Fig. 1A). The SP is then cleaved before transport of the maturing peptide from the ER to the Golgi where further folding and posttranslational processing, e.g., tyrosine sulfation and complex glycan modification, occur (19). These posttranslational modifications (PTMs), particularly glycosylation, have been shown to greatly influence the structuring of mature proteins as well as protein function (20–22).

HIV Env SPs show a high degree of sequence diversity (Fig. 1B); however, several conserved structural features have been identified. Much like the SP of all secreted or membrane proteins, the

Significance

Significant heterogeneity exists in the structure and antigenicity of HIV-1 envelope protein (Env) gp120 due, in part, to various posttranslational modifications. Using glycan and antigenic analysis, we show that the signal peptide (SP) of HIV Env can impact the glycan profile of gp120, which in turn impacts antigenicity of the mature protein. Thus, although the SP is not present in the mature gp120 protein, it is likely subject to immune pressure. Moreover, to the extent that glycan content impacts transmission fitness, variation in the SP may influence transmission. These observations potentially help us better understand both the early events in HIV transmission as well as the mechanisms whereby HIV evades humoral immune responses, providing additional information for HIV vaccine design.
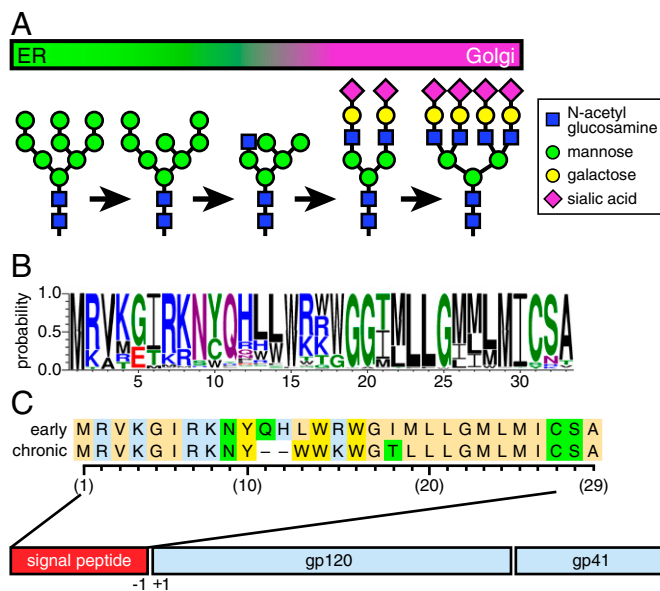
Fig. 1. HIV Env SP role in early HIV Env processing and glycosylation. (A) N-linked glycans are added in the ER to the asparagine in an N-linked glycosylation sequon of nascent peptides as $Glc_3Man_9GlcNAc_2$. The three Glcs are sequentially removed followed by removal of one Man residue. This glycosidase activity is intimately associated with glycoprotein folding assisted by ER resident chaperones and determines whether a protein traffics to the Golgi or is degraded by the proteasome. Once in the Golgi, the glycans are further modified with the addition of GlcNAc, Gal, Fuc, sialic acids, or other complex saccharide linkages. Gal, galactose; Glc, glucose; GlcNAc, N-acetyl glucosamine; Fuc, fucose; Man, mannose. (B) Sequence logo of natural variation found in 2,025 Clade B HIV Env SP sequences from the Los Alamos National Laboratory HIV Database using Weblogo 3. The height of each symbol indicates relative frequency of each amino acid at that position. Amino acid symbols are color coded for hydrophobicity: blue, hydrophilic; green, neutral; black, hydrophobic. (C) SP amino acid sequence alignment of the SP of the early-replicating isolate bearing the SP transmission signature and of the isolate that does not bear the SP transmission signature. Amino acid residues are color coded for side chain chemistry: orange, nonpolar; blue, basic; green, polar; yellow, aromatic; red, acidic.

Env SP includes a positively charged n-region, a hydrophobic core (h-region) and a cleavage domain. These regions strongly influence the rate of processing of Env as it transits through the ER and Golgi (23–26). This is believed to alter Env synthesis including the trafficking of nascent Env to the ER, the rate of SP cleavage inside the ER, and/or the retention time of Env in the ER. Asmal et al. (11) concluded that the SP signature at position 12 provides transmitting HIV isolates with higher overall Env expression, which translates into higher Env incorporation into virions.

There are no studies to date that investigate how natural variation in the SP impacts the overall structure or antigenicity of Env. Identification of a transmission signature located in a region of the SP sequence that is absent in the mature full-length protein (Fig. 1C) suggests a potential role for SP variation in impacting Env structure and function. Thus, SP variation may be subject to immune pressure despite the fact that this domain is absent from the mature protein.

Given that we now have evidence for SP selection in the context of HIV transmission, we sought to determine whether the natural variation that occurs in SPs impacts Env glycosylation and structure, which in turn could influence Env antigenicity. Here, we provide evidence that naturally occurring variation in SP can impact both Env glycosylation and structure in a way that might potentially impact the efficiency of replication in early-transmitting viruses. Our findings provide evidence that natural variation in the SP of Env can influence a key antigenic feature of gp120 in a major

way. In addition, we demonstrate that antigenic manipulation of Env in the context of a vaccine can be carried out by simple modification of the SP.

Results

HIV Env Signal Peptide Influences the Molecular Mass of gp120. To address the influence of the SP signature on Env characteristics associated with transmission, recombinant gp120 proteins were generated from viral sequences of HIV-positive patients. We chose two gp120s from isolates previously evaluated in the initial Asmal et al. (11) report describing the SP signature associated with transmission. First, a subtype B gp120 derived from a viral sequence isolated from an early-replicating isolate was generated. This gp120 contains a basic amino acid histidine at position 12 of the Env SP and is therefore referred to as "early replicating" (Fig. 1C). A second recombinant gp120 was generated from an isolate from an infected patient that lacked this SP signature. This isolate was termed "chronic" since it did not bear the SP transmission signature (Fig. 1C). Two chimeric gp120s were then generated from these two constructs in which the SPs of the two isolates were swapped, resulting in the early-replicating gp120 with the chronic SP and a chronic gp120 with the early-replicating SP. Using the parental and chimeric gp120 sequences, we sought to determine whether their SPs could influence important antigenic features of the gp120s.

Size exclusion chromatography (SEC) provides an approximation of the Stokes radius of a protein. Although SEC does not provide a precise measure of molecular mass it can identify relative differences in the size of structurally related proteins. Using SEC, we asked whether the early-replicating and chronic gp120s encoding their wild-type SPs along with the two proteins encoding swapped SPs exhibited altered molecular mass. The calculated molecular mass for the early-replicating gp120 was 102 kDa (Fig. 2). Despite containing the identical mature peptide sequence, the early-replicating gp120 with the chronic SP showed a molecular mass of 179 kDa (Fig. 2A). Thus, replacing the early-replicating SP with the chronic SP substantially increased the molecular mass of gp120.
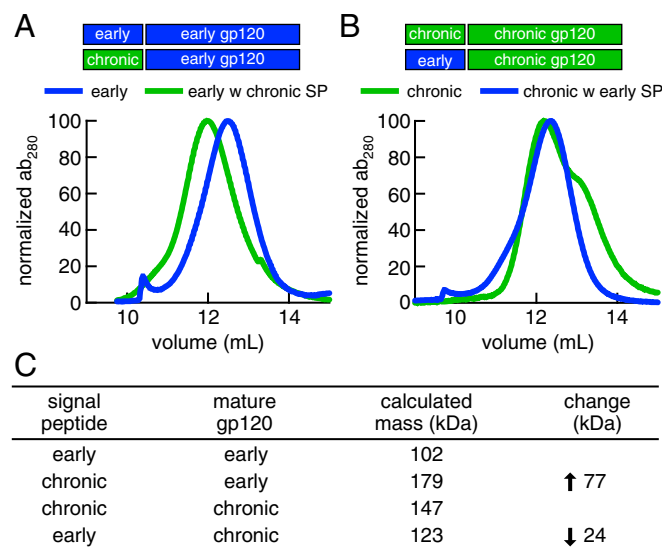

signal peptide	mature gp120	calculated mass (kDa)	change (kDa)
early	early	102	
chronic	early	179	↑ 77
chronic	chronic	147	
early	chronic	123	↓ 24

Fig. 2. HIV Env SP impacts recombinant gp120 molecular mass. Size exclusion chromatography (SEC) chromatograms of the recombinant (A) early-replicating gp120 with its wild-type SP (blue), the chronic SP (green), (B) the chronic gp120 with its wild-type SP (green), and the early-replicating SP (blue) produced in CHO-S cells and purified via Galanthus nivalis lectin column. (C) Tabulated molecular mass and molecular mass changes for all four proteins. w, with.

Conversely, the chronic gp120 with the early-replicating SP showed a decrease in molecular mass compared with the chronic gp120 with its wild-type chronic SP (147 kDa vs. 123 kDa) (Fig. 2B). These SEC data indicate that variations in the SP of Env can have a significant impact on the molecular mass of mature gp120 peptides.

HIV Env Signal Peptide Influences gp120 Glycosylation. The SEC results described above suggested that variations in the SP of Env may influence the maturation process of gp120. Of the PTMs of the Env, glycosylation has been shown to account for a significant fraction of the molecular mass of the protein (8). Therefore, we sought to determine whether the changes in the gp120 molecular mass that we observed were due, at least in part, to altered glycan processing.

High-mannose carbohydrates are the initial glycans added to nascent peptides (Fig. 1A) and are characteristic of immature glycoproteins. They are also associated with mature proteins that transit through the ER and Golgi in an accelerated way. Conversely, complex carbohydrates exhibit a higher molecular mass than high-mannose glycans (2.4 kDa vs. 1.2 kDa) and are not formed until later in the processing pathway. With an average of 25 PNGs on each gp120, changing all 25 from high mannose-occupied PNGs to all complex glycan-occupied PNGs would result in a ~30-kDa shift in molecular mass.

To quantitate the relative amount of high-mannose glycans on the gp120s, we employed the *Narcissus pseudonarcissus* lectin, which is specific for α-linked mannose (Man) residues, (27) in a surface plasmon resonance (SPR) assay. The early-replicating and chronic Env proteins, along with the two SP chimeras, were immobilized to the surface of a biosensor chip. *N. pseudonarcissus* lectin was passed over each of the four chip surfaces and the total accumulation of lectin bound to each surface was measured over 300 s.

This demonstrated significant Man content on the early-replicating gp120 with the wild-type SP [178.0 response units (RU)] (Fig. 3A, *Left*). Man content was shown to be decreased when we probed the early-replicating gp120 with the chronic SP (130.2 RU) (Fig. 3A, *Left*). The inverse was found when looking at the two chronic gp120s; swapping the SP of the chronic gp120 to that of the early-replicating SP caused an increase in Man content (159.5 RU vs. 225.0 RU) (Fig. 3A, *Right*). These results indicate that the SP alone can significantly influence the Man content of gp120. More specifically, the early-replicating SP, which may favor more rapid transit through the ER and Golgi compartments, increases the relative amount of high mannose carbohydrate present on the two gp120s we evaluated.

We then probed each of the four gp120s with a lectin derived from *Ricinus communis* that preferentially binds complex oligosaccharides ending in galactose (Gal) (28). The early-replicating gp120 showed little detectable reactivity to *R. communis* lectin (17.8 RU) (Fig. 3B, *Left*). In contrast, the same gp120 encoding the chronic SP reacted strongly (494.4 RU) to *R. communis* lectin. Thus, the chronic SP substantially increased the amount of complex oligosaccharides ending in Gal in the early-replicating gp120.

Consistent with this observation, the chronic gp120 encoding its wild-type SP also reacted strongly with *R. communis* lectin (558.8 RU), while the same protein encoding the early-replicating SP showed little or no reactivity with *R. communis* lectin (12.6 RU) (Fig. 3B, *Right*).

Taken together, these results indicate that the chronic SP promotes the addition of complex glycans bearing terminal Gal.

Overall, the manner in which the two SPs differentially impacted the presentation of mannose and complex carbohydrate was consistent insofar as the early-replicating SP appeared to favor the presentation of high mannose on either the early-replicating or the chronic protein, while the chronic SP favored at least one form of complex carbohydrate.

HIV gp120 monoclonal antibodies (mAbs) specific in whole or in part to glycan patches on gp120 have been isolated from HIV-
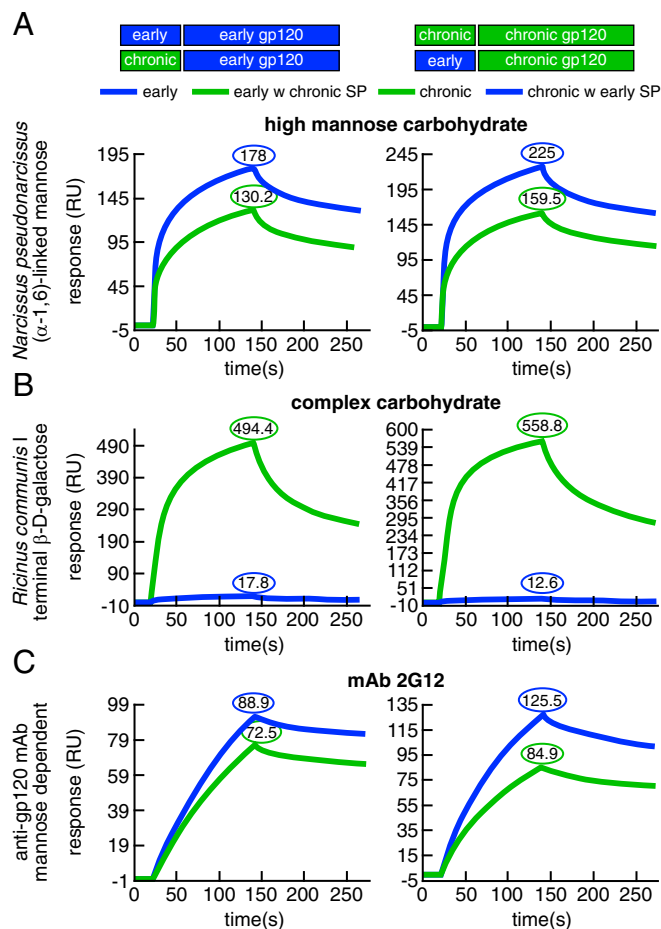

Fig. 3. HIV Env SP influences gp120 glycosylation profile. Four recombinant gp120s were evaluated for glycan composition in a surface plasmon resonance (SPR) assay probing with (A) *N. pseudonarcissus* lectin specific for α-linked mannose residues and (B) *R. communis* I lectin specific for oligosaccharides ending in galactose. (C) The four gp120 were also probed with the glycan-dependent mAb 2G12. (*Left*) Sensograms of the early-replicating gp120 with wild-type SP (blue) and with the chronic SP (green), and (*Right*) sensograms of the chronic gp120 with its wild-type SP (green) and with the early-replicating SP (blue). Peak response units (RU) are presented and circled above each curve. w, with.

infected individuals (29–31). A number of these mAbs show broad and potent neutralizing activity (29, 31, 32). 2G12 is the prototypical glycan-dependent mAb (30, 33). It recognizes a discontinuous dimannose epitope located in the C3 region and the base of the V3 loop in an area often referred to as the silent face of gp120 (33). When probed with mAb 2G12, the early-replicating gp120 encoding the chronic SP showed decreased reactivity compared with the same gp120 encoding the early-replicating SP (72.5 RU vs. 88.9 RU) (Fig. 3C, *Left*). The chronic gp120 with the early-replicating SP showed increased binding to 2G12 compared with the chronic gp120 with its wild-type chronic SP (125.5 RU vs. 84.9 RU) (Fig. 3C, *Right*).

Taken together, these results demonstrate that the reactivity of glycan-specific antibodies to gp120 can be influenced by the SP. Regardless of mature gp120 sequence, the SP of the early-replicating Env biased the mature protein to a high-Man, low-complex carbohydrate profile. Conversely, the SP of the chronic gp120 biased the gp120 to an increased complex carbohydrate profile. Because Man is roughly half the molecular mass of an average complex glycan (~1.2 vs. ~2.4 kDa), and carbohydrate comprises about half the mass of a gp120 surface protein, these
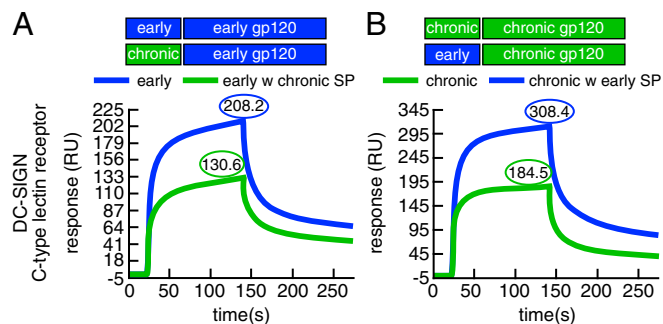
Fig. 4. HIV Env SP influences DC-SIGN reactivity to gp120. (*A*) The early-replicating gp120 with its wild-type SP (blue) and with the chronic SP (green), and (*B*) the chronic gp120 with its wild-type SP (green) and the chronic gp120 with the early-replicating SP (blue) were evaluated for reactivity to soluble tetrameric DC-SIGN, C-type lectin receptor expressed on dendritic cells, in an SPR assay. Peak response units (RU) are presented and circled above each curve. w, with.

alterations in glycan processing are likely playing a major role in the molecular mass shift observed for these peptides shown in Fig. 2.

HIV Env Signal Peptide Influences gp120:DC-SIGN Interaction. C-type lectin receptors are thought to facilitate mucosal transmission of HIV (34, 35). Among these receptors, DC-SIGN is perhaps the most extensively characterized (36–38). The carbohydrate-recognition domain of DC-SIGN reacts with high affinity to the high-mannose residues that decorate gp120 (39–41). Considering the capacity of the SP to influence the glycan content of gp120, we sought to determine whether SP variation could also influence gp120 interactions with DC-SIGN.

Using tetrameric recombinant soluble DC-SIGN as a probe, we determined that the early-replicating gp120 with its wild-type SP showed higher reactivity to DC-SIGN than the early-replicating gp120 encoding the chronic SP (208.2 RU vs. 130.6 RU) (Fig. 4*A*). When the wild-type SP of the chronic gp120 was replaced with the early-replicating SP, DC-SIGN reactivity nearly doubled (184.5 RU vs. 308.4 RU) (Fig. 4*B*).

These results are consistent with the results presented above, indicating that the early SP increases the relative proportion of Man presented on gp120 and suggest that the sequence variation encoded in the SP can alter interactions with lectin receptors, which may be relevant in the context of mucosal transmission.

HIV Env Signal Peptide Influences gp120 Antigenicity. Altered glycosylation profiles of Env can greatly influence structural and antigenic features of gp120 (42). A prominent feature of HIV gp120s involves the induction of buried epitopes following CD4 ligation (CD4i epitopes). Such changes are conserved in both HIV-1 and HIV-2 and have a significant impact on antigenicity and susceptibility of viruses to neutralizing antibodies (43, 44). To understand whether the changes in gp120 described above can influence protein antigenicity, we measured reactivity with two CD4i-specific gp120 mAbs, 17b and A32 (45–48).

As a control for this measure, and to establish that our SPR–based assay was able to detect CD4i epitopes that are impacted by glycosylation, we employed a wild-type subtype A gp120, 92UG037, and the same gp120 with a PNG disrupting mutation, N144Q. These proteins were probed with mAbs 17b and A32. In the absence of CD4, mAb 17b reacted well to the wild-type 92UG037 gp120 (22.9 RU) (Fig. 5*A*, *Left*). This reactivity was completely abrogated in the N144Q PNG mutant gp120 (0.0 RU) (Fig. 5*A*, *Left*). The same trend was observed when probing these two gp120s with the mAb A32, in that the wild-type gp120 had significant mAb A32 reactivity, while the N144Q PNG mutant had greatly reduced mAb A32 reactivity (54.6 RU vs.

10.7 RU) (Fig. 5*A*, *Right*). These data confirm that altered glycosylation influences the ability of gp120 to adopt the CD4i open conformation recognized by mAbs 17b and A32.

We next determined whether sequence variation in Env SPs could influence the antigenicity of gp120. To test this hypothesis, we probed our four recombinant gp120s for reactivity with mAbs 17b and A32 in the absence of CD4. The reactivity to mAb 17b was reduced by more than half when the SP of the early-replicating gp120 was swapped from its wild-type SP to that of the chronic SP (14.9 RU vs. 5.4 RU) (Fig. 5*B*, *Left*). Additionally, the same trend of reduced reactivity of the early-replicating gp120 with the chronic SP was observed when the two proteins were probed with mAb A32 (46.5 RU vs. 28.1 RU) (Fig. 5*B*, *Right*).

We then probed the chronic gp120 with its wild-type SP versus the early-replicating SP with mAbs 17b and A32. We observed low reactivity of mAb 17b with the chronic gp120 with its wild-type SP (11.0 RU) (Fig. 5*C*, *Left*). However, reactivity to mAb 17b increased fourfold when the SP of the chronic gp120 was swapped to that of the early-replicating SP (46.1 RU) (Fig. 5*C*,
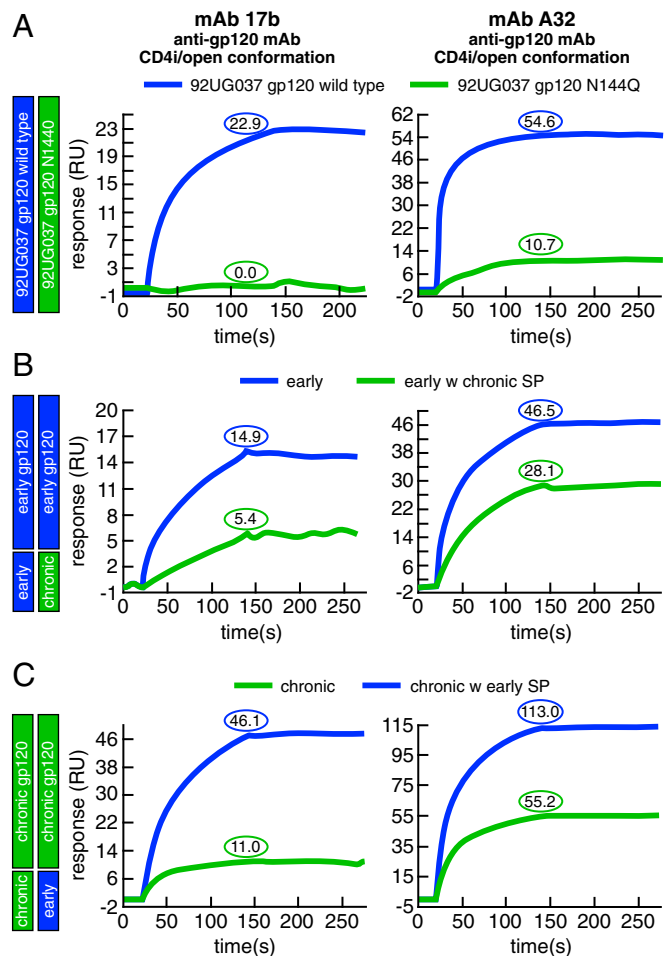

Fig. 5. HIV Env signal peptide influences gp120 structure and antigenicity. (*A*) Subtype A gp120 92UG037 (blue) and the PNG mutant 92UG037 N144Q (green) were probed for reactivity with the CD4i mAbs 17b (*Left*) and A32 (*Right*) in an SPR assay to determine the impact of glycosylation on gp120 antigenicity. (*B*) Early-replicating gp120 with wild-type SP (blue) and early-replicating gp120 with the chronic SP (green) were probed with mAbs 17b (*Left*) and A32 (*Right*) in an SPR assay. (*C*) Chronic gp120 with chronic SP (green) and chronic gp120 with the early-replicating SP (blue) were also probed with the mAbs 17b (*Left*) and A32 (*Right*). Peak response units (RU) are presented and circled above each curve. w, with.

Left). When the two chronic gp120s were probed with mAb A32, reactivity of the mAb to the chronic gp120 with the early-replicating SP was increased twofold compared with the chronic gp120 with its wild-type SP (55.2 RU vs. 113.0 RU) (Fig. 5C, *Right*).

Taken together, these results show that the SP of Env can influence the reactivity of CD4i mAbs to mature gp120s. These data suggest that the glycosylation differences induced by natural SP variation can greatly influence the antigenicity of gp120.

Discussion

The HIV-1 Env is heavily glycosylated (8, 49), and alterations to key PNGs can influence its structure, function, and susceptibility to neutralizing antibodies (22, 33, 50). Evidence of a connection between Env glycosylation and HIV transmission has also been described (5). Previous studies have demonstrated that early-replicating Envs tend to encode fewer PNGs and shorter variable regions in which many PNGs are located (12, 13). Additionally, the glycans that decorate the PNGs of early-replicating Env contain greater high-mannose content compared with Envs isolated from chronic replicating viruses (14). Recently, a second signature of transmission has been described that involves the Env SP of early-replicating viral isolates (11, 16). This SP transmission signature has also been described in SIV and SHIV transmission studies (17). The identification of this signature suggests that the SP may influence the transmission bottleneck that is established around the time of transmission. Understanding the effect of natural variation in the SP coding sequence on the Env protein structure and antigenicity is important insofar as Env is the major target for neutralizing antibodies. In this report, we show that the SP of the HIV Env protein can impact both the structure and antigenicity of gp120.

The identification of a transmission signature located in the SP of Env by Asmal et al. (11) implies that the SP is a target of selective pressure during the early events of transmission. Previous studies in which the SP of HIV-1 Envs have been altered, or swapped with SPs from unrelated proteins have been shown to alter the rates of Env processing (23–25, 51). The conclusions from those studies confirm the importance of certain key features of SPs, including the charged n-region. In the present study, we used the natural variation found in subtype B Envs from two independent patient isolates that incorporate the presence or not of the SP transmission signature. We found that the SP can significantly influence the glycan profile and antigenicity of gp120. This suggests that the observed high degree of sequence variation in SPs can influence Env structure and antigenicity in a manner that has not previously been recognized.

Our findings implicate the SP of Env as a potential regulator of PTMs that influence functional characteristics of gp120 associated with transmission. We found that by swapping the SP of Envs in a way that either inserted or removed the SP transmission signature, the molecular mass of the resulting gp120 was significantly altered, predominantly by a modification of the carbohydrate content of gp120. Thus, these data establish a clear connection between natural variations found in the Env SP and the gp120 carbohydrate profile. It is noteworthy that a domain that does not appear in the mature protein can alter the glycan shield, which plays a key role in immune evasion.

Env has been shown to bind to and interact with multiple host surface proteins and receptors, and affinities for these receptors can change during the course of infection and disease progression (52). One such receptor, believed to be relevant to HIV transmission is the C-type lectin receptor DC-SIGN, which is expressed on the surface of dendritic cells that survey mucosal tissues (36). After antigen capture, these DC-SIGN⁺ dendritic cells migrate to secondary lymphoid tissues and present the captured antigen to T cells (53). DC-SIGN–captured HIV has been shown to promote transinfection of CD4⁺ T cells through its interaction with viral Env (36). This capture and presentation of virus particles to target cells is believed to play a role in mucosal transmission of HIV. We show that the interaction dynamics between DC-SIGN and Env can be altered by variations in the Env SP. This result raises the possibility that one of the selective forces associated with variation in the SP may involve modulation of affinity for DC-SIGN and other C-type lectin receptors involved in transmission.

Consistent with the effects of glycans on Env structure (42), we find that variations in Env SP can substantially influence the antigenicity of gp120. We probed the two native gp120s along with the two SP hybrids with the conformation-specific mAbs 17b and A32 that preferentially recognize gp120s that adopt a CD4i open conformation. We determined that SPs do impact the reactivity of Env with both mAb 17b and A32. The two gp120s with the early-replicating SP reacted more efficiently with these mAbs compared with the two gp120s that lacked the early-replicating SP signature. The capacity of the early SP to promote greater access to CD4i epitopes is likely related to an overrepresentation of high-mannose glycans. In contrast, there are at least two potential explanations for the reduced access to CD4i epitopes mediated by the chronic SP. First, the increased bulk associated with complex carbohydrates may favor a more constrained conformation of the gp120. Second, a less likely explanation is that this increased bulk may sterically occlude CD4i epitopes. Of note, Envs that react with mAbs that recognize the CD4i conformation are associated with greater susceptibility to antibody neutralization (54).

It is important to note that the identification of a SP transmission signature at position 12 was achieved without any predisposition toward identifying sequences that would alter PTMs. Because we do not yet understand the rules by which SP sequences can influence PTMs, it is possible that other SP residues also mediate such effects. Additional studies are required to define these rules insofar as SP sequences hold the potential to influence transmission fitness and susceptibility to antibody neutralization.

Finally, these findings support the concept that SP variations can be utilized in the production of recombinant proteins to optimize the glycan profile and antigenicity of vaccine immunogens. Much work has been done toward producing optimized vaccine antigens that will hopefully elicit broadly neutralizing antibodies (55). The results presented herein provide an additional tool that may be useful in that effort.

Materials and Methods

Two HIV-1 clone sequences from HIV-1–positive patients deposited into the HIV Database at Los Alamos National Library were used in this study: an early-replicating isolate bearing the SP transmission signature termed early replicating (GenBank accession no. EU289198), and a non-SP transmission signature bearing an isolate termed chronic (GenBank accession no. EU575870). Both full-length gp120 DNA sequences were synthesized for expression in mammalian cells (ATUM). The constructs were designed such that swapping of the wild-type SP with an alternative SP could be achieved by single digest and insertion of the complimentary SP without alterations to the mature gp120 protein sequence. Using this approach, two additional recombinant gp120 constructs were generated: early-replicating gp120 with chronic SP and chronic gp120 with early-replicating SP. All recombinant gp120s were transiently transfected into CHO-S Freestyle cells (Invitrogen), purified as previously described (56), and further detailed in *SI Materials and Methods*. Surface plasmon resonance analysis was performed on a Biacore 3000 instrument (GE Life Sciences) using CM5 sensor chips and the data were evaluated with BIAevaluation 4.1 software (GE Life Sciences) as previously described (56) and further detailed in *SI Materials and Methods*.

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