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Sequence Note

Genotypic Characterization and Comparison of Full-Length Envelope Glycoproteins from South African HIV Type 1 Subtype C Primary Isolates That Utilize CCR5 and/or CXCR4

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Abstract

CCR5 has preferentially been used by all circulating HIV-1 subtype C viruses for cell entry. Recently, we reported the highest proportion of CXCR4-utilizing primary isolates among a cohort of 20 South African AIDS patients. This study describes and compares the Env genotypic characteristics from these 20 HIV-1 subtype C (and unique CD recombinant) primary isolates. Fourteen primary isolates utilized CCR5, four (including the CD recombinant) used CXCR4, and two were dual tropic. Extensive analysis and comparison of important structural motifs such as the N-linked glycosylation sites, signal sequences, CD4-binding sites, variable loops, cleavage sites, known neutralizing antibody and small molecule inhibitor binding sites confirmed that other than the expected differences in the V3 loop, no sequence motifs distinguished between R5 and X4 tropism. Further correlation of the *env* genotype to functionally relevant motifs is necessary to elucidate the relationship between biologically and immunologically relevant sites and aid vaccine and novel drug design.

Entry of human immunodeficiency virus type 1 (HIV-1) into host cells is facilitated by its envelope glycoprotein (*env*/gp160). The precursor gp160 is cleaved to yield the surface gp120 and transmembrane gp41 subunits of the envelope, which assemble as trimers on the surface of the HIV-1 virion.[1] Classical entry of HIV-1 into CD4-positive T cells is initiated by the interaction of trimeric gp120 and the host cell surface receptor CD4.[1,2] This interaction causes conformational changes in gp120, which lead to the transient exposure or formation of a coreceptor binding site, principally for CXCR4 or CCR5.[2,3] Post-CD4 and coreceptor binding, the gp120/gp41 association is considerably weakened, leading to exposure of the gp41 fusion peptide. The gp41 trimers enter a fusion-active conformation by folding back on themselves to form parallel coiled-coil trimers (six-helix bundle), and bring the viral and host-cell membranes together to allow for membrane fusion.[4,5] X-ray crystallographic studies, coupled with mutagenic and antigenic analysis, have led to a model in which the coreceptor interacts with V3 and a conserved bridging sheet composed of the V1/V2 stem and an antiparallel, four-stranded structure that includes sequences in the C4 region.[2]

Viral tropism (i.e., macrophage or T cell tropism) is largely linked to coreceptor usage, with CXCR4(X4)-utilizing viruses being T-tropic and syncytium-inducing (SI), and CCR5 (R5)-utilizing viruses being M-tropic and non-syncytium-inducing (NSI).[6–8] Coreceptor usage and switching have been extensively studied among the various HIV-1 subtypes and circulating recombinant forms (CRFs). Studies on subtype B have shown that transmitted HIV-1 viruses are generally NSI, and these R5-using viruses tend to predominate in early and asymptomatic HIV infection.[6,8] In later infection, however, CXCR4-utilizing isolates often emerge and this switch from a primarily R5 to a primarily X4 virus population is closely correlated with increased $CD4^+$ T cell decline and progression to AIDS.[9–11] Some CXCR4-using viruses can also

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utilize CCR5 (R5/X4 or dual tropic). When compared to R5 viruses, X4 viruses show increased cytopathicity *in vitro*,[10] which may account for the link between coreceptor switching and increased pathogenicity of HIV *in vivo*.[9,11]

Interestingly, despite the fact that over 50% of new HIV-1 infections worldwide are caused by subtype C, less than 30 CXCR4-utilizing viruses have been isolated from this subtype worldwide after 25 years of research.[7,12–18] We recently reported the highest proportion of CXCR4-utilizing HIV-1 subtype C primary isolates among 20 South African antiretroviral drug-naive AIDS patients,[19] suggesting the ongoing evolution of *env* and the epidemic in this country. It is therefore imperative to understand the unique biological and immunological properties of these viruses. This study describes the extensive genotypic analysis and comparison of the full-length gp160 of the 20 recently isolated HIV-1 subtype C (and unique CD recombinant) primary isolates that utilize CCR5 and/or CXCR4 for cell entry.

Well-characterized primary HIV-1 isolates used in this study were available from samples from 20 antiretroviral drug-naive AIDS patients attending a clinic in Johannesburg (between January and June, 2005). Biological and phenotypic analysis revealed that isolates 05ZAFV3, 05ZAFV10, 05ZAFV14, and 05ZAFV26 utilized CXCR4, 05ZAFV15 and 05ZAFV27 were dual tropic, and the remainder utilized CCR5 for cell entry. Proviral DNA for each isolate was extracted from p24 antigen-positive cultures with a High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) according to the manufacturer's instructions. Subtype C-specific *env* primers based on the original primers described by Gao *et al.*[20] were designed by comparison of 52 complete HIV-1 subtype C sequences retrieved from the NLM nucleotide database (http://www.ncbi.nlm.nih.gov). These included *env*A-1 (5′-GGCTTAGGCATTTCCTATGGCAGGAAGAA-3′), *env*N-1 (5′-TTGCCAATCAGGGAAGAAGCCTTGTGT-3′), *env*B-1 (5′-GGAAAGAGCAGAAGACAGTGGCAATGA-3′) and *env*M-1 (5′-TAACCCATCCAGTCCCCCCTTTTCTTTTA-3′). The 20 full-length *env* (gp160) genes were each amplified by nested polymerase chain reaction (PCR) using the High Fidelity Expand^{PLUS} PCR kit (Roche) and outer primers *env*A-1 and *env*N-1 and inner primers *env*B-1 and *env*M-1, according to manufacturer's instructions. PCR products were purified using the High Pure PCR Product Purification kit (Roche), as per the manufacturer's instructions. Primers spanning the full-length *env* gene (~2.5 kb) were used to sequence the PCR products in both directions (population-based sequencing). Amplicons were sequenced with the ABI Big Dye terminator system (v3.1) according to the manufacturer's instructions, on the ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were edited using the Sequencing Analysis V3.3 program (Applied Biosystems), and the complete gp160 sequences were assembled using Sequencher V4.5 (Genecodes, Ann Arbor, MI).

A multiple alignment of the gp160 regions with references from HIV-1 subtypes A to K, CRF01_AE, and CRF02_AG (http://hiv-web.lanl.gov) was generated in Clustal X. Aligned sequences were converted to MEGA V3.0 format and used in phylogenetic and molecular evolutionary analyses. A phylogeny reconstruction of each *env* gene was performed by neighbor-joining using the Kimura two-parameter distance matrix. The stability of the nodes was assessed by bootstrap analysis (1000 replicates) and bootstrap values greater than 70% were considered significant (see Papathanasopoulos *et al.*[18] for further details of phylogenetic analysis). Amino acid sequences for the 20 newly characterized South African envelope glycoproteins were derived and compared to the consensus C and ancestral C sequences available from the Los Alamos website (http://hiv-web.lanl.gov).

Nucleotide sequence analysis showed that the newly characterized full-length *env* genes ranged from 2526 to 2634 bp, with intact open reading frames confirming the presence of functional *env* genes in all 20 isolates. Phylogenetic analysis of the 20 full-length *env* nucleotide sequences together with viruses from the major subtypes revealed that 19 isolates clustered within HIV-1 subtype C with a bootstrap value of 100%, while one isolate (05ZAFV10) showed evidence of recombination (Fig. 1). The 05ZAFV10 sequence was further analyzed using the RIP 2.0 Program (http://hiv-web.lanl.gov), which showed that it is a unique CD recombinant with two recombination breakpoints at approximate nucleotide positions of 160 and 1850. Segments within positions 1–160 and 1850–2559 were identified as subtype C, while the segment between positions 160 and 1850 was subtype D. Subtype D is typically found in West/West Central Africa, while subtype C is more common in Southern Africa (see the subtype distribution map at http://hiv-web.lanl.gov). Thus, this strain could have originated in West Central Africa, or it could represent a recombination event between a West Central African and South African strain. The *env* intrasubtype nucleotide diversity for all 19 newly characterized subtype C isolates varied between 10.2% and 17.1%. Additional *env* analysis from 12 South African CXCR4-utilizing subtype C isolates available in the Los Alamos database showed that the intrasubtype nucleotide diversity ranged up to 16.5%. This is higher than previously reported for South African HIV-1 subtype C CCR5-utilizing isolates,[16] and suggests that the genetic diversity of HIV in South Africa is gradually expanding. Interestingly, the *env* intrasubtype distances from Indian subtype C isolates were found to increase from up to 12% in 1995–1996 to 19% in 2003–2004.[21] The phylogenetic relationship among the subtype C *env* sequences revealed a star-like phylogeny with several lineages, and there did not appear to be any major clustering within the subtype C branch between the R5 and X4 *env* sequences (Fig. 1).

An alignment of the predicted gp160 amino acid sequences of all isolates was extensively analyzed and compared (Fig. 2). The predicted proteins varied from 842 to 878 amino acids in length, primarily due to insertions/deletions in the variable regions (V1 toV5) of the gp120 and deletions in the gp41 cytoplasmic tail. It has been suggested that an unusually high number of positively charged residues in the signal sequence (first 30 amino acids) of Env affects the rate of cleavage of the signal peptide and is responsible for slower rates of folding, intracellular transport, and secretion.[22] Analysis of the overall positive charges within this region for all subtypes revealed that the HIV-1 subtype C isolates have between two and four positively charged residues in the N-terminus of the signal peptide (results not shown). This was lower than the calculated value of the non-C subtypes, which had between three and six positively charged residues in the same region. This implies that HIV-1 subtype C may have more efficient cleavage of the signal peptide and thus Env

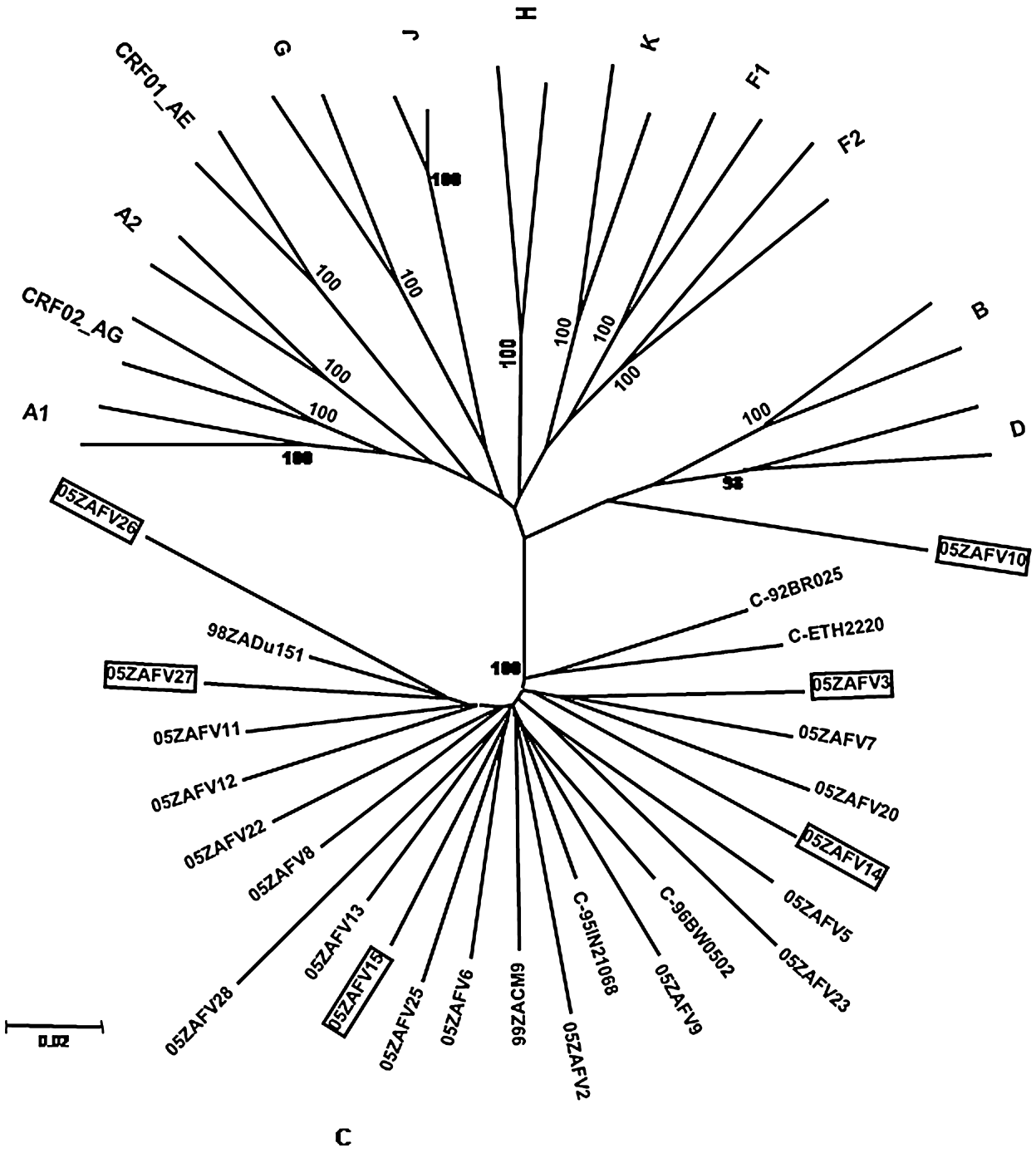

FIG. 1. Phylogenetic relationships of the full-length envelope glycoproteins from the 20 newly characterized South African subtype C (and unique CD recombinant) primary isolates with HIV-1 subtype reference sequences (Los Alamos database; http://hiv-web.lanl.gov). Sequences from the six isolates able to use the CXCR4 coreceptor are boxed. The phylogenetic tree was constructed from nucleotide sequences, using the neighbor-joining method with maximum parsimony bootstrap values to estimate the stability of the nodes. Only bootstrap values of 70% or higher are shown.

expression. It is also interesting to note that, on average, within the subtype C signal peptides, X4-utilizing viruses had a slightly higher net positive charge than the R5-using viruses, implying that the R5 envelopes may be more efficiently expressed. There is also an ongoing controversy about the cleavage recognition site for the signal peptidases. The majority of non-C subtypes have a recognition sequence of CSA, followed by CNA and CSV, whereas the consensus subtype C is CNV. The new subtype C sequences include several different motifs, such as CNG, which could have an effect on efficient recognition by the signal peptidases, and thus expression. Nevertheless, this feature poses an interesting question about the pathogenesis of HIV-1 subtype C, and should be evaluated further.

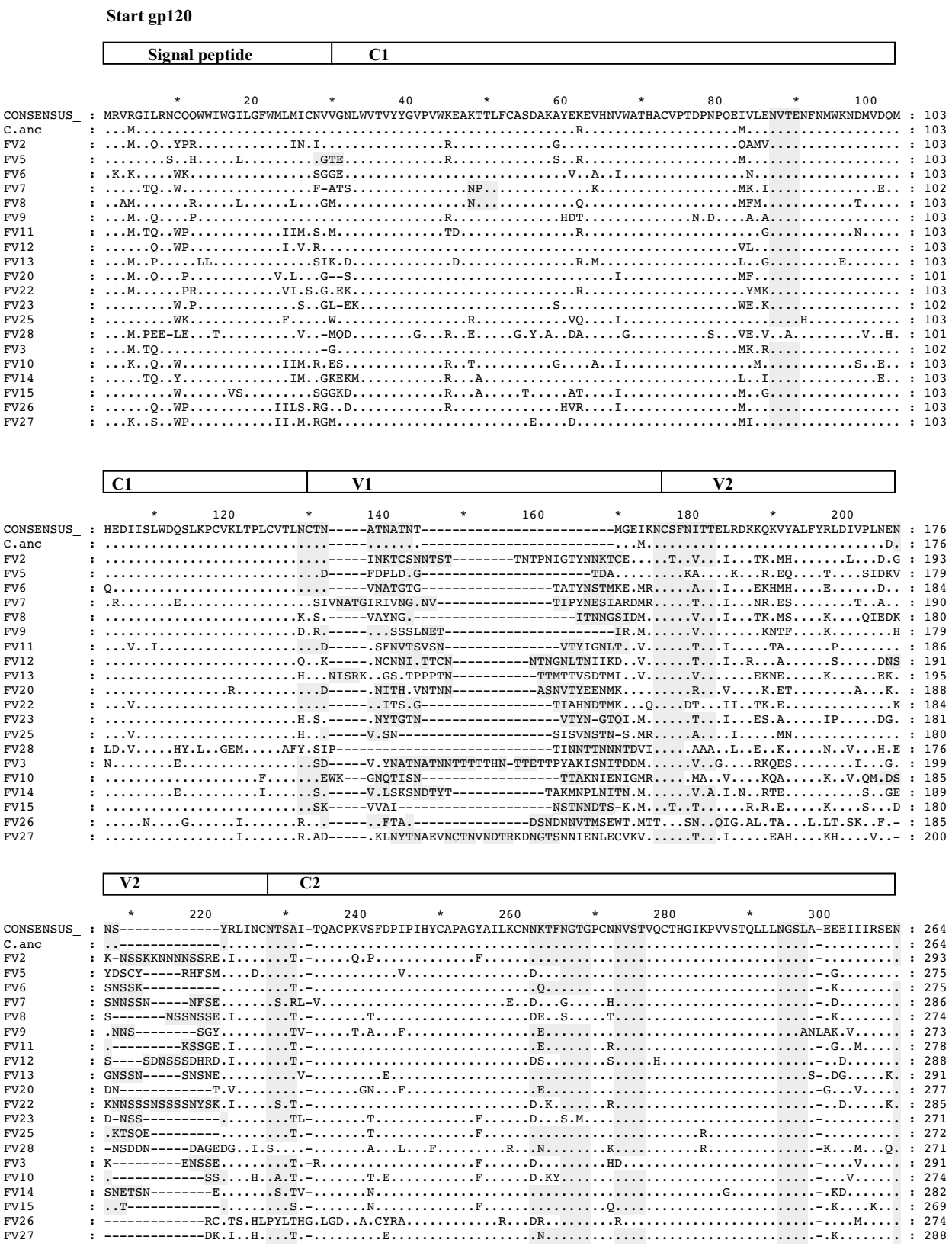

FIG. 2. Alignment of the predicted envelope glycoprotein amino acid sequences of 20 South African HIV-1 subtype C primary isolates. The bars above the amino acid sequences indicate the approximate locations of regions of the envelope glycoproteins. V1 to V5 indicate segments of the envelope that show high variability between isolates, and C1 to C5 denote regions that remain relatively constant. The dots indicate identity to the consensus subtype C sequence and dashes indicate insertions/deletions. Potential N-linked glycosylation sites are shaded yellow, and the functionally relevant sites for the gp120–gp41 cleavage site, T-20 resistance, and 2F5, 4E10 neutralizing antibody sites are highlighted in blue, pink, green, and red, respectively.

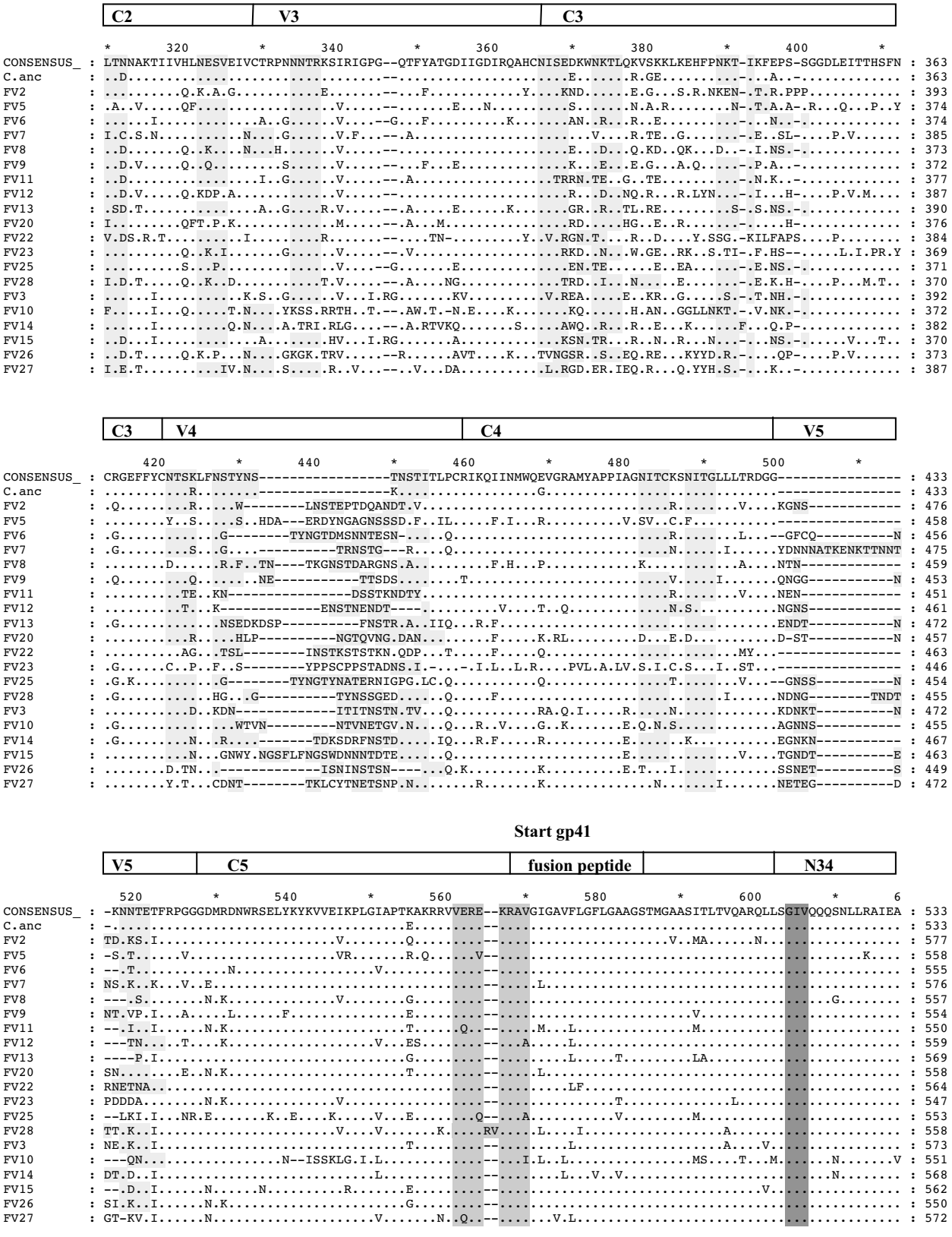

FIG. 2. (*continued*).

Approximately half the molecular mass of the HIV-1 Env is attributed to N-linked glycans.[23] The typical envelope glycoprotein has approximately 24 N-linked glycosylation sites in gp120, as well as three or four sites in gp41. Previous studies have suggested that these N-linked glycans form a moving shield to mask gp160 epitopes that would otherwise be exposed to neutralizing antibodies.[23] Prosite (http://www.expasy.org/prosite) was used to predict potential N-linked

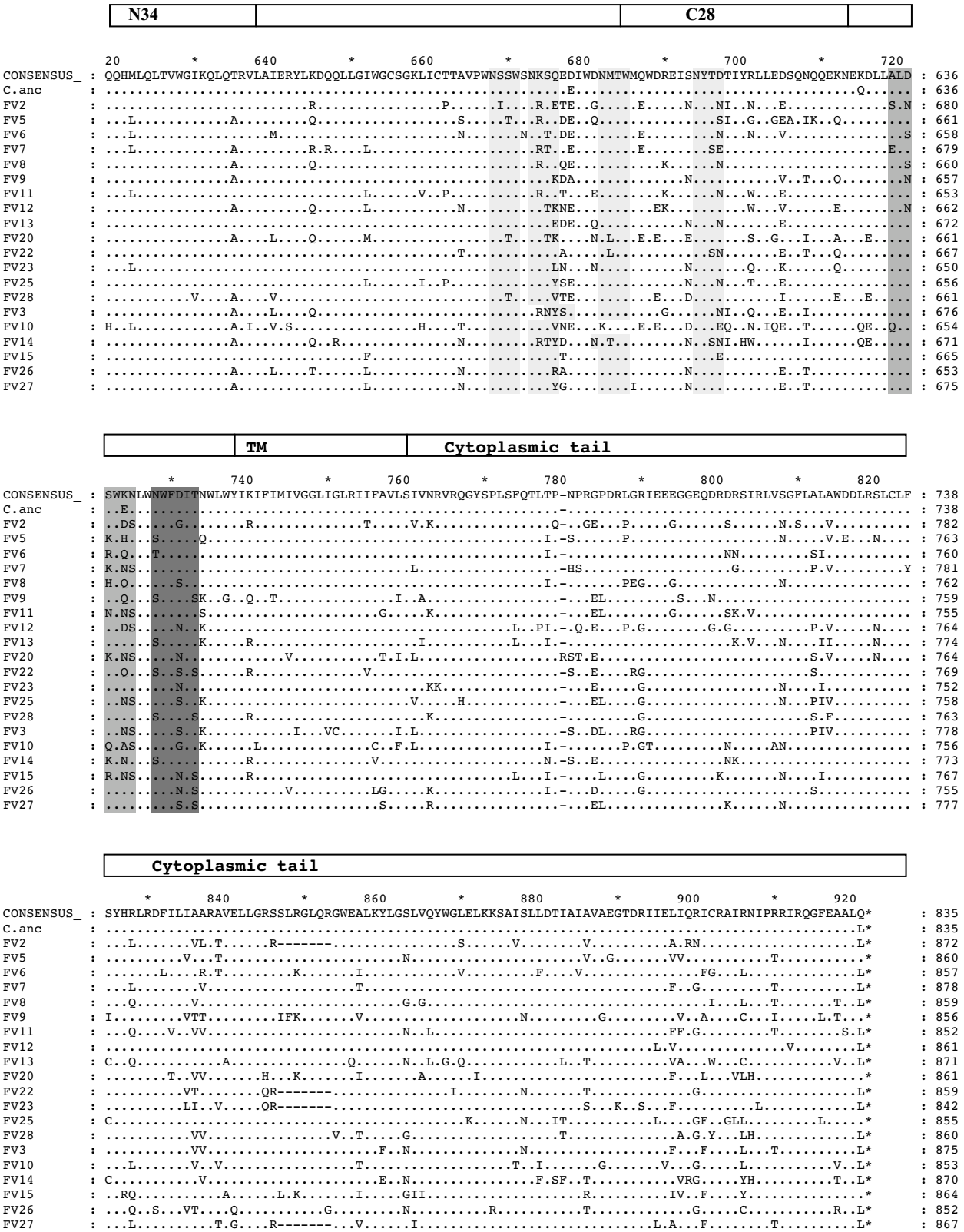

FIG. 2. (*continued*).

glycosylation sites in the 20 newly characterized Env sequences, as well as the consensus C and ancestral C sequences (Fig. 2). Overall, there was no drastic alteration in the number of potential glycosylation sites, and the number and positioning of sites appeared to be mostly conserved between the R5 and X4 isolates. All the CCR5-utilizing sequences contained an N-linked glycosylation motif within the V3 loop (positions 334–337), consistent with recent find-

ings highlighting the close association of this site with CCR5 usage.[24] Furthermore, the X4 isolates were more likely to have an extra glycosylation site within the V4 loop. Further investigations could reveal whether they mask any immunologically relevant epitopes. Two N-linked glycans (positions 295 and 332 of $gp120_{HxB2}$, corresponding to positions 328 and 366 in the current sequences) are postulated to form the core epitope for the neutralizing antibody 2G12, which is maintained conformationally by other N-linked glycans (at positions 339, 386, and 392 of $gp120_{HxB2}$, corresponding to positions 373, 421, and 427 in the current sequences).[25] The high mannose glycan of residue 328 was absent in 14 of the 20 isolates, suggesting that they are resistant to neutralization by the monoclonal antibody 2G12 (Fig. 2). Thus, the presence of N328 in three of the five subtype C CXCR4-utilizing envelope glycoproteins, 05ZAFV10 and two CCR5-utilzing isolates, implies these isolates would be susceptible to neutralization by 2G12. *In vitro* phenotypic neutralizing antibody assays would confirm this hypothesis.

Analysis of the residues involved in forming the CD4 binding site (residues D403, E405, W467, V470, and G471 to P478)[3] revealed that most were conserved, although some differences were noted. This was not considered significant, however, since various combinations of the different amino acids can be found in the CD4 binding site of other HIV-1 isolates in the Los Alamos database (http://www.hiv.lanl.gov).

A high frequency of mutations associated with resistance to the BMS-378806 (BMS-806) entry inhibitor, which prevents binding of gp120 to CD4, was found among previously characterized HIV-1 subtype C primary isolates.[26] Amino acids conferring resistance to BMS-806 include W112, T257, S375, F382, and M426 within the Phe 43 cavity of gp120, and D113, V120, K121, K429, and A433 ($HIV\text{-}1_{HXB2}$ numbering), which correspond to amino acid positions W111, T289, S410, F417, M466, D112, V119, K120, E469, and A473 in the 20 newly characterized sequences, respectively. These amino acids are all well conserved within our isolates, with the exception of K429 (E469), where the lysine is present in only 3 of 20 viruses. BMS-806 against a K429L mutant was shown to have an IC_{50} approximately 7-fold higher than the wild-type virus in a previous study.[27] This suggests that our isolates may not be as sensitive to BMS-806 as viruses with a lysine residue in that position, and phenotypic studies to determine sensitivity of the primary viral isolates to BMS-806 inhibition would be valuable if human clinical trials with this compound continue.

The variable loops, particularly the V1/V2 and V3, have been directly implicated in coreceptor usage.[18] The overall positive charges for V1, V2, V3, V4, and V5 were determined and compared. While there were no significant differences between the X4 and R5 viruses within the V1, V2, V4, and V5 loops, the V3 loop of the R5 isolates was more conserved, compared to that of the X4 isolates, and displayed features previously associated with coreceptor usage.[18] The tetrapeptide crown sequence in the V3 loop of all R5 subtype C isolates was GPGQ with a net charge of +1 to +4.5. A neutral serine residue was present at position 11 (corresponding to position 339), and amino acids with a negatively charged side chain (either D or E) were present at position 25 (corresponding to position 355) of the V3 loop, except for 05ZAFV28. In contrast, four of the six CXCR4-using isolates exhibited changes in the V3 tip tetramer motif and two of these isolates each had two amino acid insertions within this motif, which, during coreceptor binding, is postulated to reach down and interact with the second extracellular loop of the coreceptor.[28] The overall positive charges for the X4 isolates range from +4.5 to +8.5. The X4 phenotype has been associated with the presence of at least one basic amino acid substitution at position 11 or 25. Interestingly, all of the CXCR4-using isolates, except for 05ZAFV3, had a positively charged residue at position 11 of the V3 loop and all, except 05ZAFV10, had either a neutral or positively charged residue at position 25. The conserved residues that have been previously implicated in CCR5 coreceptor binding include amino acids N301, K117, K121, T123, I420, K421, Q422, P438, and G441 ($HIV\text{-}1_{HXB2}$ numbering).[29] Analysis of these residues among the 20 new sequences revealed they were mostly conserved among the R5 and X4 sequences (corresponding to positions N334, K116, K120, T122, I460, K461, Q462, P478, and G481). Retention of these sites within CXCR4-utilizing viruses poses numerous questions and the relevance of maintaining these sites in the context of viral coreceptor evolution should be investigated further.

All isolates, except 05ZAFV28, had the consensus basic tetrapeptide motif –R/K–X–R/K–R at the gp120/gp41 cleavage site. Proteolytic activation of the HIV-1 gp160 precursors occurs at the carboxyl side of the consensus motif in this region.[30] This region of the precursor is the main determinant for cleavage, which is catalyzed by a cellular subtilisin-like protein convertase located in the Golgi complex. It is thus expected that the gp160 precursor of all isolates would be efficiently cleaved into the gp120 and gp41 subunits. In the case of 05ZAFV28, cleavage may be affected due to the two amino acid insertions at position 565, which abolishes the consensus cleavage site. HIV-1 does, however, contain a secondary cleavage site, corresponding to position 555, with the sequence KAKRR.[31] This sequence is conserved in 05ZAFV28, and thus cleavage of the envelope may occur here.

The gp41 sequences are highly conserved relative to those of gp120, with fewer potential glycosylation sites and no notable differences between the SI and NSI isolates. It was also noted that four of the 20 new isolates (three CCR5-using and one CXCR4-using) contained five to seven amino acid deletions in their gp41 cytoplasmic tail. The tryptophans in the membrane proximal external region of gp41, which are essentially required for membrane fusion,[32] are all highly conserved (W723, W727, W729, W735, and W737), with the exception of G737 in isolate 05ZAFV9.

The involvement of gp41 in the entry of HIV-1 into target cells presents potential targets for novel antiviral drugs. The first in this class of FDA-approved entry inhibitors, enfuvirtide (T-20), exerts its antiviral activity by interacting with the viral sequence within HR1 and inhibiting its association with the native HR2, thereby preventing gp41-mediated fusion.[33] Sequence analysis of the T-20 resistance domain (Fig. 2, 604–606, GIV) revealed that all primary HIV-1 isolates had the T-20-sensitive genotype. Thus, there is no evidence for baseline resistance to T-20 among any of these isolates, and no difference is expected in sensitivity to the drug between CXCR4- and CCR5-utilizing isolates.

Antibodies that are able to broadly cross-neutralize most HIV isolates/subtypes are of great interest, particularly in vaccine design. Neutralizing antibodies of interest include

2G12 (see above) and IgG1b12 in gp120 and 4E10 and 2F5 in the membrane proximal external region of gp41.[34–36] Resolution of the crystal structure of IgG1b12 showed that this neutralizing antibody interacts with gp120 by the insertion of a tryptophan residue (W100) of IgG1b12 into the same binding pocket of gp120 that is used by Phe 43 of CD4.[34] The critical contact residues of gp120 important for this interaction, which correspond to S400, D403, I406, Y419, and V470 (Fig. 2; S365, D368, I371, Y384, and V420 in HIV-1_{HXB2}), are highly conserved, with minor exceptions. Thus all these isolates should show varying susceptibility to IgG1b12 neutralization. The core of the 4E10 epitope (highlighted in Fig. 2) comprises the NWFDIT residues[35]; however, 4E10 can neutralize isolates that differ from this sequence in positions 1, 4, and 6.[36] In all of our isolates, the core epitope is conserved, and it is expected that these viruses will be susceptible to neutralization by 4E10. All sequences showed variation from the 2F5 binding motif, ELDKWA.[35] It has been shown that only mutations in the D, K, or W residues of this epitope will produce neutralization escape.[35] Among our isolates, the K residue is the most poorly conserved, and it is expected that most of these isolates will not be sensitive to neutralization by 2F5. However, isolates 05ZAFV5, 05ZAFV7, 05ZAFV20, and 05ZAFV14 have retained the DKW motif, and should be susceptible to 2F5 neutralization. Overall, there are no notable differences between the X4 and R5 viruses in terms of the known neutralizing antibody epitopes. However, *in vitro* phenotypic resistance or susceptibility of these primary isolates to these antibodies should be evaluated further, in order to give a clearer indication of the impact of sequence variation in these epitopes on neutralization.

Nucleotide sequencing of the HIV-1 subtype C *env* and analysis of the predicted envelope glycoprotein are important because of the information provided on the vital biological properties associated with this protein. As more functionally relevant sites are identified and correlated with genotypes, we can begin to answer questions concerning, for example, the relationship between immunologically relevant sites and the impact of viral diversity on these sites, which have important implications for both vaccine and drug development studies.

Sequence Data

The 20 *env* sequences were submitted to GenBank using Sequin V5.35 (http://www.ncbi.nlm.nih.gov) and are available under accession numbers DQ382361–DQ382380.

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