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## Evolutionary Gamut of *in vivo* Gag Substitutions during Early HIV-1 Subtype C Infection

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### Abstract

Two analyses of HIV-1 subtype C Gag quasispecies were performed in a prospective cohort of 42 acutely and recently infected individuals by SGA on viral RNA/proviral DNA templates. First, *in vivo* Gag substitutions were assessed in relation to the HIV-1C consensus sequence, which revealed that 29.3% of detected amino acid substitutions can be classified as reversions to subtype consensus, 61.3% as forward substitutions from subtype consensus, and 9.3% as polymorphisms not associated with the subtype consensus sequence. Second, the proportion, dynamics, and relationships within individual pools of viral quasispecies were analyzed. Among reverse substitutions, 16.1% were minor, 11.0% transient, 13.6% dominant, and 59.2% fixed. In contrast, 31.6% of forward substitutions were minor, 59.3% transient, 3.8% dominant, and 5.3% fixed. The distinct patterns in the spectrum and dynamics of reverse and forward Gag substitutions suggest that these differences should be considered in HIV-1 evolutionary studies and analyses of viral mutational pathways.

### Introduction

A comprehensive assessment of viral evolution across HIV-1 genes is critical for the successful design of preventive and therapeutic interventions. Host-driven immune pressure involving both innate and adaptive components during the course of HIV infection results in a robust quasi-steady balance between emerging viral variants and virus fitness (Borghans et al., 2007; Crawford et al., 2007; Leslie et al., 2004; Liu et al., 2007; Martinez-Picado et al., 2006; Peyerl et al., 2004b). The first virus-mediated T cell response can rapidly select escape mutations concurrent with the peak decline in acute HIV infection (Goonetilleke et al., 2009). Successful viral escape from immune recognition can maintain viral replication (Feeney et al., 2004; Geels et al., 2006; Oxenius et al., 2004). However, if the escape mutation impairs virus replicative fitness, such escape might be associated with reduced viral load and better disease outcome in humans and rhesus macaques (Chopera et al., 2008;

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Crawford et al., 2007; Friedrich et al., 2004a; Friedrich et al., 2004b; Friedrich et al., 2004c; Goepfert et al., 2008; Martinez-Picado et al., 2006; Migueles et al., 2003; Peyerl et al., 2004a; Peyerl et al., 2004b; Schneidewind et al., 2007). For example, in subjects expressing HLA-B\*57/5801, the virus mutates from Thr to Asn at position 242 in Gag which results in a reduction of viral replication due to the fitness cost of the T242N mutation (Brockman et al., 2007; Chopera et al., 2008; Crawford et al., 2007; Leslie et al., 2004; Martinez-Picado et al., 2006). Because host genetic factors play an important role in disease progression in HIV-infected individuals (Carrington et al., 1999; Fellay et al., 2007; Kaslow et al., 1996; Klein et al., 1998; O'Brien and Nelson, 2004), it is believed that the pathway of viral escape from immune recognition can be predicted by the profile of host MHC class I HLA alleles (Allen et al., 2005; Brumme et al., 2008a; Brumme et al., 2007; Brumme et al., 2008b; Rousseau et al., 2008).

Transmission of the viral escape variant to a new host may result in a rapid reversion to the wild type virus (which corresponds to the HIV-1 subtype consensus sequence in most epidemics), particularly in the absence of selective pressure in the new host due to a different profile of HLA alleles (Allen et al., 2004; Chopera et al., 2008; Crawford et al., 2007; Davenport et al., 2008; Duda et al., 2009; Friedrich et al., 2004a; Leslie et al., 2004; Li et al., 2007; Matthews et al., 2008; Rousseau et al., 2008). Reverse amino acid substitutions to the HIV-1 subtype consensus are critical for maintaining virus within the optimal evolutionary space in the local epidemic. The breadth of immune response plays an important role in disease outcome, and is likely to be associated with the breadth of viral mutational pathways. It is widely agreed that viral escape (or forward) and reverse amino acid substitutions contribute significantly to HIV-1 evolution at both the intra-patient and population levels (Allen et al., 2005; Bhattacharya et al., 2007; Borrow et al., 1997; Brumme et al., 2008a; Friedrich et al., 2004a; Koenig et al., 1995; Leslie et al., 2005; Leslie et al., 2004; Li et al., 2007; Moore et al., 2002; Phillips et al., 1991; Price et al., 1997; Rousseau et al., 2008). While inpatient evolution of HIV does not necessarily translate into evolution of HIV at the population level (Leslie et al., 2004), it is likely that the circulating variants of HIV-1 in the local epidemic represent equilibrium between cumulative immune pressure of the host population and viral fitness of locally transmitting viruses.

The HIV-1 Gag is able to induce potent virus-specific T cell responses that were shown to be associated with control of viral replication, lower viral set point, and better disease prognosis (Betts et al., 2001; Boaz et al., 2002; Edwards et al., 2002; Geldmacher et al., 2007; Kiepiela et al., 2007; Masemola et al., 2004; Ndongala et al., 2009; Novitsky et al., 2003; Novitsky et al., 2006; Ramduth et al., 2005; Rolland et al., 2008; Serwanga et al., 2009; Zuniga et al., 2006), and therefore represents an attractive target for an HIV-1 vaccine design. Better understanding of viral dynamics and the spectrum of *in vivo* Gag substitutions may advance the rational design and development of an HIV-1 vaccine.

Surprisingly, types of viral amino acid substitutions have not been defined and used uniformly. While some types of substitutions are well defined (e.g., synonymous, nonsynonymous, fixed), terms for other viral substitutions are used inconsistently, leading to confusion or misunderstanding. For example, “reverse mutation” or “reverse substitution” may mean reversion either to the transmitted virus, or to subtype consensus. Using a detailed classification of terms, this study addressed and characterized the types and frequency of the *in vivo* Gag substitutions in the early stage of HIV-1 subtype C infection in a cohort of 42 subjects with estimated time of seroconversion. The frequency distributions of amino acid substitutions within the individual pools of viral quasispecies were analyzed longitudinally. Two temporal aspects in the evolution of *in vivo* Gag quasispecies were addressed. Amino acid substitutions across Gag were analyzed by their relation to the HIV-1 subtype C consensus, and by fixation and stability.

## Methods

### Study subjects

Longitudinal sets of *gag* sequences were analyzed in subjects enrolled in a primary HIV-1 subtype C infection cohort in Botswana (Novitsky et al., 2009c; Novitsky et al., 2009d; Novitsky et al., 2008) from April 2004 to April 2008. A total of 42 enrolled subjects included 8 detected with acute HIV-1 infection (Fiebig stage II) and 34 detected with “recent” infection (Fiebig stage IV or V). Details on estimating time of seroconversion are provided elsewhere (Novitsky et al., 2009b). A “zero” time corresponded to seroconversion, as a more accurate and measurable time point than estimation the time of HIV infection (Novitsky et al., 2010c). The time of HIV infection can be estimated by adding 14 to 21 days. The median (IQR) time elapsed between estimated time of seroconversion and first available sequence was 44.0 (10.8 to 54.0) days. There were 9 male (2 acute) and 33 female (6 acute) participants. The median age at enrollment was 27 years (IQR 25-32, range 20-56). All subjects were Botswana nationals, and all infections were HIV-1 subtype C (Novitsky et al., 2009a; Novitsky et al., 2009d). As described previously (Novitsky et al., 2009a; Novitsky et al., 2009d; Novitsky et al., 2008), viral load and CD4+ T cell counts were assessed during follow-up. Blood samples were analyzed at median (IRQ) of 5 (4-6) time points per subject. The follow up period was 415 (321-447) days post-seroconversion (p/s). Ten of 42 (24%) subjects initiated ART within the observed period of time due to a drop in CD4+ T cells (time of ART initiation, if any, is shown in Table S1). The study was approved by Institutional Review Boards in Botswana and the US. Written informed consent was obtained from each participant.

### Single-genome amplification and sequencing

Previous evolutionary studies demonstrated that the HIV-1 *gag* and *env* sequences derived from PBMC are comparable to viruses circulating in plasma (Geels et al., 2003; Shankarappa et al., 1999). The similarities between viral sequences originating from alternative templates, viral RNA and proviral DNA, are particularly evident at the early phase of HIV infection (Novitsky V., unpublished data). In this study both viral RNA from plasma and cell-associated proviral DNA were used as templates for amplification of viral sequences. Single genome amplification and direct sequencing was performed as described previously (Novitsky et al., 2009c; Novitsky et al., 2010c). Sequence contigs were assembled by SeqScape v.2.6. Multiple sequence alignment was performed by HIV-align at the Los Alamos HIV Database site (<http://www.hiv.lanl.gov/>) using the hidden Markov model and the codon-alignment option followed by minor manual adjustments in BioEdit (Hall, 1999). The obtained sequences were tested by HYPERMUT v.2.0 (Rose and Korber, 2000) and hypermutated sequences were excluded from analysis. A total of 2,595 *gag* sequences included 827 sequences originating from the viral RNA template, and 1,768 sequences obtained from the proviral DNA template. The median (IQR) of analyzed *gag* sequences was 11.7 (8.8-13.9) per time point, and 61.5 (43.3-76.0) per subject. The accession numbers of the analyzed *gag* sequences are GQ275380–GQ277569, GQ375107–GQ375128, and GQ870874–GQ871183.

### Amino acid frequencies in the pool of viral quasispecies

Only non-synonymous substitutions were analyzed in this study. The frequencies of translated amino acid sequences in Gag were analyzed using MargFreq (Ray, 2008) per time point per subject. The frequency of each amino acid was expressed as a fraction of 1 in the pool of viral quasispecies at a given time point. Frequency changes in the pool of viral quasispecies at each amino acid position over time were tracked according the following criteria. If any change in the proportion of amino acid(s) in the pool of viral quasispecies was observed over time, the amino acid with increased frequency was the subject of

frequency assessment. Sites with a single amino acid present in the pool of the earliest available quasispecies were examined for appearance of any alternative amino acid over the time of follow up, and the new amino acid was subject for evaluation. Examples of this approach were presented elsewhere (e.g., Figure 2 in (Novitsky et al., 2010c)). If two amino acids were present in the pool of the earliest quasispecies, the amino acid that showed an increase in frequency over time was a subject for analysis. Although multiple amino acids can be present in the pool of viral quasispecies, we observed no cases with more than two amino acids at the earliest time points in this study. Therefore, no sites with multiple increasing amino acids were detected.

### Types of amino acid substitutions

By analogy with drug-resistance mutations, we define a mutational pathway as a series of sequential changes in the pool of viral quasispecies at a single or at multiple amino acid positions over time. Parameters quantified in this study include sequential frequency of observed amino acids over time in relation to the estimated time of seroconversion. At each position in amino acid alignment, the observed amino acid substitutions were classified based on their relation to subtype C consensus sequence, and fraction in the pool of viral quasispecies.

In analysis of relationships between the observed *in vivo* Gag substitutions and the subtype consensus, an amino acid substitution from the consensus amino acid to the non-consensus was treated as a *forward substitution* (a potential escape mutation that requires experimental confirmation of immune escape). In this study, an amino acid substitution from a non-consensus amino acid to the consensus-matched amino acid was considered a *reverse substitution*, or reversion (note: the alternative interpretation of reverse substitution is related to the transmitted virus, and was not used in this study). Viral substitutions that did not involve the most common HIV-1 subtype C amino acid were treated as non-subtype-consensus-related polymorphisms. Testing of immune responses was out of the scope of the study, resulting in a lack of evidence that observed viral substitutions are directly linked to immune responses.

Based on the frequency in the pool of viral quasispecies, we categorized amino acid substitutions as *minor*, *dominant*, *fixed*, and *transient*. A substitution was considered *minor* if its fraction was less than or equal to 50% in the pool of viral quasispecies during follow-up. A substitution was treated as *dominant* if its fraction reached more than 50% in the pool of viral quasispecies but did not reach complete substitution of the original amino acid. A substitution was *fixed* if it reached 100% in the pool of viral quasispecies during the follow-up period. A substitution was considered *transient* if its frequency subsequently decreased below 1.0 for fixed substitutions, or below 0.5 for dominant substitutions, or if minor substitution disappeared over the observation period. Once amino acid substitutions in Gag reached the state of dominance or fixation, they rarely disappeared but rather shared their frequency in the pool of viral quasispecies with alternative amino acid(s) by balancing and frequency fluctuation over time. Thus, in this study transient substitutions are predominantly those that were minor and disappeared from the pool of viral quasispecies.

### MHC class I HLA typing

High resolution HLA typing was performed for all study subjects as described previously (Novitsky et al., 2009c). Briefly, the AlleleSEQR HLA Sequencing-Based Typing kit (Celera, Alameda, CA) was used according to the manufacturer's instruction. Contig assembling and assignment of HLA alleles was implemented by Assign SBT ver. 3.5.1.42 (Conexio Genomics, Applecross, Australia). All ambiguous positions were resolved by re-

sequencing. Polymorphisms outside the targeted exons that could not resolve heterozygote combinations were interpreted as two-digit HLA typing results.

### CTL epitopes analysis

The Los Alamos National Laboratory HIV immunology database (<http://www.hiv.lanl.gov/content/immunology>) was screened for known human CTL epitopes identified in the context of HIV-1 subtype C infection and the MHC class I HLA alleles restriction. For each subject, the location of the observed amino acid substitutions in Gag was matched with the retrieved epitopes restricted by the corresponding class I HLA alleles. The number of matched amino acid substitutions within known CTL epitopes in the context of their class I HLA restriction was used for analysis the proportion of Gag substitutions within CTL epitopes.

### Statistical analysis

Data are summarized with medians (IQR range for 25% and 75%). Frequencies of amino acid substitutions by type over time are presented.

## Results

### Intra-patient heterogeneity of HIV-1C Gag substitutions

Dynamics of Gag amino acid substitutions was analyzed in a cohort of 42 HIV-1 subtype C infected individuals with estimated time of seroconversion, which allowed us to synchronize the time of observed substitutions among patients. The actual distribution of HIV-1 subtype C Gag substitutions is presented in supplementary Table S1. In the cumulative analysis of all types of Gag substitutions, the median (IQR) number of reverse substitutions was 7 (4-15.5), forward substitutions was 18 (15.3-25), and polymorphisms was 2 (1-4) per subject. The number of Gag substitutions per subject was considerably less for a subset of dominant and fixed substitutions: median (IQR) number of reverse amino acid substitutions was 5 (1.3-8.8); forward substitutions was 1 (0-3), and polymorphisms was 0 (0-1). The reverse dominant and fixed amino acid substitutions in HIV-1C Gag were found in 37 of 42 (88%) subjects, while forward dominant and fixed substitutions were detected in 27 of 42 (64%) subjects.

To address distribution of amino acid substitutions in HIV-1 subtype C Gag, the identified non-synonymous substitutions were plotted according to their location across Gag in each subject. The substitution map presented on Figure 1 shows distribution of three types of Gag substitutions – reverse (green up-triangles), forward (red down-triangles), and polymorphisms not associated with the subtype consensus sequence (gray circles) – that were accumulated over the follow-up period (up to 500 days p/s). The map on Figure 1 includes all minor, transient, dominant, and fixed substitutions in HIV-1C Gag. It is evident from this map that 1) all types of substitutions are scattered across Gag; 2) the number and type of Gag substitutions differ substantially between subjects, and 3) forward (red) substitutions dominate the map (Fig. 1). However, excluding of minor and transient substitutions revealed in a changed shape of the Gag substitution map (Fig. 2). The reverse substitutions (green) are prevailing on the map of dominant and fixed Gag substitutions, while forward substitutions are less common. The location and number of dominant and fixed substitutions differs considerably between subjects. Substitutions at Gag codon position 242 represented the most common pattern observed in 12 out of 42 subjects including 5 reverse, 5 forward, and 2 polymorphic substitutions, as described elsewhere (Novitsky et al., 2010c).

Overall, the analysis of viral amino acid substitutions by their relation to the subtype consensus sequence demonstrated the wide range and heterogeneity in the number and location of reverse and forward Gag substitutions among HIV-1 subtype C infected individuals.

### Differential dynamics of Gag substitutions

Temporal trends in kinetics of reverse and forward Gag substitutions in primary HIV-1 subtype C were analyzed over five 100-day intervals up to 500 days p/s (Fig. 3). Forward Gag substitutions were predominant among minor (Fig. 3A) and transient (Fig. 3B) substitutions, except even distribution of minor reverse and minor forward substitutions during the period 0 to 100 days p/s. The relationships between reverse and forward transient substitutions in Gag were remarkably constant over time (Fig. 3B). In contrast, the reverse substitutions outnumbered forward substitutions among dominant (Fig. 3C) and fixed (Fig. 3D) Gag substitutions during the period up to 300 days p/s. However a larger proportion of forward substitutions was evident during 301-400 days p/s with about equal number of forward and reverse dominant and fixed substitutions over the period 401-500 days p/s. This observation suggests a shift in the ratio of reverse/forward amino acid substitution in HIV-1 subtype C Gag around 300 days p/s. The Fisher exact test supported that the frequencies of reverse substitutions within total substitutions were changing over time (p-value for minor, transient, dominant, and fixed substitutions was equal 0.001, 0.36, <0.001, and <0.001, respectively). The observed shift in the ratio of reverse/forward substitutions after 300 days p/s seems to indicate fewer reversions that occur after the first year of HIV-1 infection.

Changes in the spectrum of reverse and forward Gag substitutions were analyzed over five 100-day intervals (Fig. 4). A gradual shift over time was evident in the range of both reverse (Fig. 4A) and forward (Fig. 4B) substitutions. Among reverse Gag substitutions, gradual decrease of dominant and fixed substitutions was accompanied by increased proportion of minor and transient substitutions, which was particularly noticeable after 300 days p/s. Interestingly that among Gag forward substitutions, the fraction of complete substitutions was substantial (10 of 28) at the early stage of HIV-1 subtype C infection (Fig. 4B). However, the proportion of dominant and fixed amino acid substitutions among Gag forward substitutions diminished dramatically at later time points, e.g., after 100 days p/s. As a result, the majority of Gag forward substitutions after 100 days p/s were minor and transient.

The proportion of individuals with detectable reverse or forward amino acid substitutions in Gag was analyzed over the same five 100-day intervals (Figure 5). A gradual decrease from about 80% to 40% of individuals with detectable reverse substitutions was evident over time. In contrast, the proportion of individuals with detectable forward substitutions was the highest during the 201-300 days p/s interval.

Time to fixation of amino acid substitutions in Gag was analyzed for different types of substitutions. The median (IQR) to fixation was 146 (100-185) days p/s for reverse substitutions, 268 (102-380) days p/s for forward substitutions, and 243 (190-440) days p/s for polymorphisms. The difference between time to fixation was statistically significant between reverse and forward substitutions ( $p < 0.001$ ), and between reverse substitutions and polymorphisms ( $p < 0.001$ ), but was similar between forward substitutions and polymorphisms not associated with subtype consensus sequence.

### Relationships between types of amino acid substitutions in Gag

The quantitative relationships between different types of amino acid substitutions were analyzed. The proportions of total reverse substitutions, forward substitutions, and

polymorphisms not directly associated with the subtype consensus in Gag were 30.8%, 59.6%, and 9.7%, respectively (Fig. 6A). The majority of reverse substitutions reached dominance (13.5%) and fixation (60.2%; Fig. 6B). In contrast, forward amino acid substitutions were predominantly minor (31.3%) or transient (58.5%), while only few reached dominance (4.1%) or fixation (6.1%) in the pool of viral quasispecies during the first year of HIV-1 subtype C infection (Fig. 6B). A large fraction of viral polymorphisms not associated with the subtype consensus sequence was represented by minor (21.1%) or transient (55.0%) substitutions, and only about a quarter of polymorphisms reaching dominance (15.6%) or fixation (8.3%) within a year following seroconversion (Fig. 6B). Overall, 68.9% of Gag amino acid substitutions did not exceed the level of 50% or were lost during the observation period (Fig. 6C). Of those, 36.9% were represented by minor amino acid substitutions, and 63.1% by transient substitutions. About one third of Gag substitutions (31.1%) reached dominance or fixation. Among these substitutions, 26.1% remained dominant, while 73.9% reached fixation (Fig. 6C).

### Amino acid substitutions in Gag within known CTL epitopes

To estimate the fraction of amino acid substitutions that are likely to be associated with immune pressure we quantified substitutions within known CTL epitopes restricted by individual MHC class I HLA alleles in each subject. A total of 462 of 1,243 (37.2%) observed amino acid substitutions in Gag were observed with known CTL epitopes restricted by individual class I HLA alleles. The restriction of involved epitopes was approximately even between HLA-A (31.2%), HLA-B (37.4%), and HLA-C (31.4%) loci. The proportion of different types of amino acid substitutions found within known CTL epitopes was similar to the overall distribution of substitutions in Gag: 29%, 63%, and 9% for reversions, forward substitutions, and polymorphisms, respectively. Interestingly, 48% of reverse substitutions and 39% of forward substitutions were found within known CTL epitopes restricted by more than one class I HLA allele. For example, a gradual substitution from Lys to Arg at position 26 in p17 was observed within known overlapping CTL epitope **KIRLRPGGK** restricted by HLA-A\*03:01 and epitope **RPGGKKRYM** restricted by HLA-Cw\*06:02 in recently infected subject OG-2604 expressing both HLA-A\*03:01:01 and HLA-Cw\*06:02. The median (IQR) of observed Gag substitutions within known CTL epitopes per patient was 9 (6; 14) ranging from 1 to 46.

## Discussion

The study assessed the dynamic spectrum and inter-subject heterogeneity of the *in vivo* Gag substitutions during primary HIV-1 subtype C infection. Longitudinal analysis of viral evolution from seroconversion to up to 500 days p/s revealed the dynamic nature of the *in vivo* mutational pathways in HIV-1C Gag, and their complexity at selected amino acid residues. A high fraction of identified Gag substitutions, i.e., forward substitutions, were transient or minor. In contrast, the majority of Gag reverse substitutions were found to be fixed. A substantial inter-patient heterogeneity was evident in the patterns of appearance, dominance, and fixation of identified amino acid substitutions in HIV-1C Gag.

A considerable fraction of Gag reverse substitutions, 73.7%, reached dominance and/or fixation during the first year of HIV-1 subtype C infection, supporting the hypothesis that reverse substitutions in Gag are driven by viral fitness cost, e.g., ability of reverse substitutions to restore viral fitness. In contrast, only a small fraction of Gag forward substitutions, 10.2%, reached dominance or fixation. More than half of the detected forward substitutions, 58.5%, were transient. Given a relatively short period of detection of some transient substitutions even with frequent sampling, this is likely a conservative estimate. We propose that at least three mechanisms may account for a large fraction of transient forward substitutions in HIV-1 subtype C Gag. First, transient substitutions may represent



mutational noise, although a relatively large overall number of transient substitutions might have a negative impact by diverting immune responses and exhausting the immune system. Second, assuming random appearance, transient substitutions are generated stochastically, they can dramatically reduce viral fitness, and therefore can be eliminated quickly from the pool of viral quasispecies. Third, hypothetically, transient substitutions might induce potent immune responses within a short period of time, and therefore become eliminated by immune pressure. If the last assumption is true, then certain transient substitutions could represent an attractive target for HIV vaccine design due to their potent immunogenicity. According to this scenario, a vaccine antigen with transient amino acid substitutions might rapidly induce a long lasting immune response of high magnitude that cannot be achieved in natural infection due to fast elimination of such viral variant by immune pressure. A high magnitude of the induced immune response might cause cross-reactivity toward viral variants without transient substitution, and if so, should have a protective effect. Presence of both fixed and transient viral substitutions indicates that the overall immune responses to HIV-1 Gag epitopes are substantial, but also provides evidence for a fast appearance of viral variants.

The reverse substitutions play an important role on the population level (Matthews et al., 2008) through their ability to restore the subtype consensus sequence. Upon virus transmission between HLA-distinct hosts and apparent shift in the specificities of HLA restriction, at least some forward substitutions that were generated in previous host revert to the subtype consensus. Thus, being amplified on a population level, the reverse substitutions maintain the subtype consensus sequence of virus in epidemic. Despite the increase in overall viral diversity, the stability of HIV-1C Gag consensus sequence in the epidemic over time is striking (Novitsky et al., 2010a; Novitsky et al., 2010b). A relatively low proportion of amino acid substitutions within known CTL epitopes might indicate that only fraction of CTL epitopes have been mapped in HIV-1C infection, and warrants further studies.

Utilizing the HIV-1 subtype consensus sequence might be biased to the extent of representative sampling in the epidemic, which is one of the study limitations. However our previous analyses demonstrated an extreme similarity between the HIV-1C consensus sequences originating from different geographic areas or collected at different time points (Novitsky et al., 2002; Novitsky et al., 2010b). Another study limitation is that some early reverse and forward substitutions could be missed due to the time of sampling. Samples with the first available sequence in Fiebig stage V might be particularly affected. Therefore, the current study had little to no power to address the earliest amino acid substitutions in Gag.

In summary, this study addressed the breadth and relationships of *in vivo* Gag substitutions in primary HIV-1 subtype C infection using a direct mapping of Gag amino acid substitutions along the time line of HIV-1 infection. The study contributes to better understanding of early events in HIV infection and might assist in facilitating the development of preventive strategies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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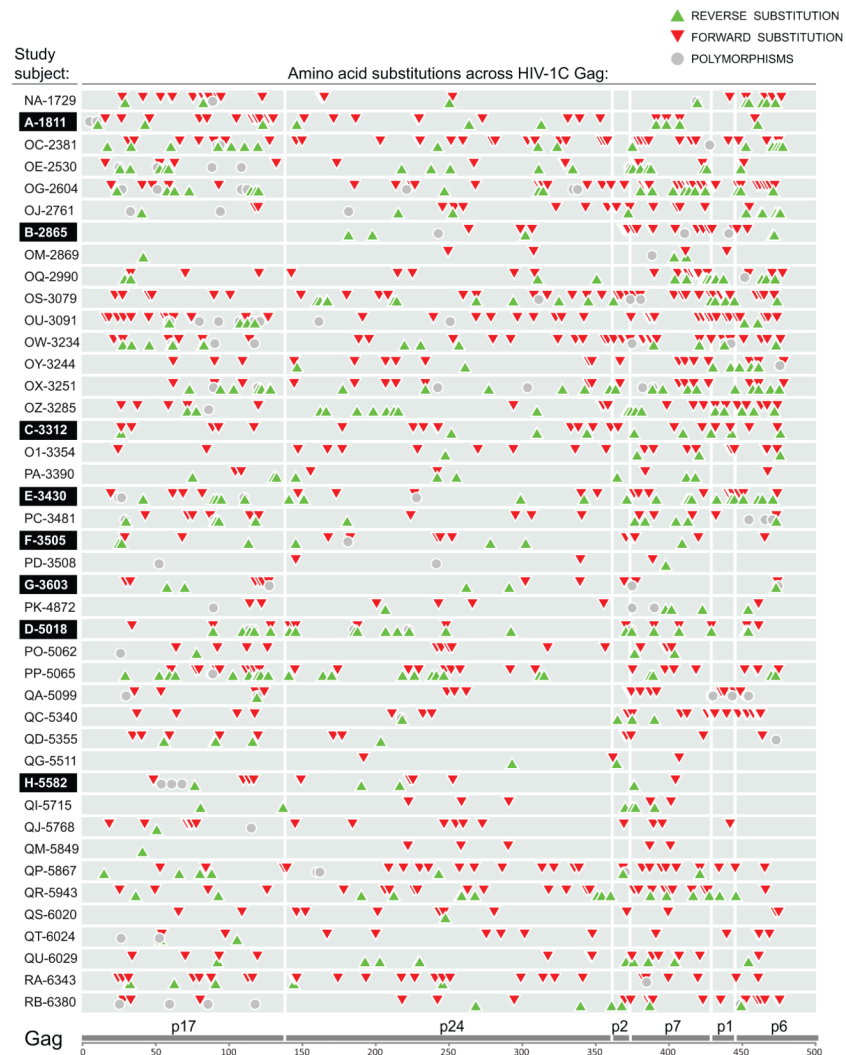
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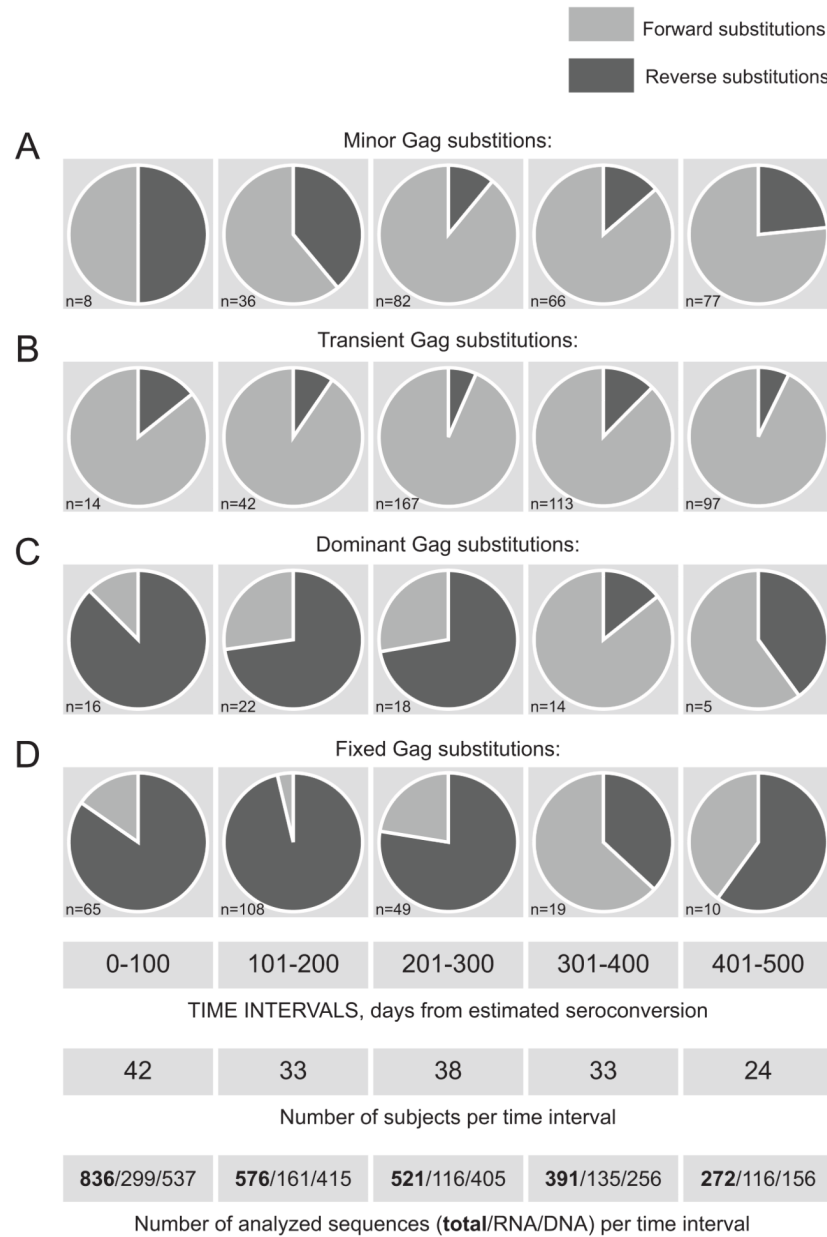


**Figure 1.** Profile and location of total amino acid substitutions across Gag in a cohort of 42 HIV-1 subtype C-infected individuals. The cumulative non-synonymous substitutions identified by SGA during the time period from seroconversion to up to 500 days p/s are presented. The study subjects' code is shown at the left. Eight acutely infected subjects are highlighted. Three types of Gag substitutions are outlined as follows: green up triangle shows amino acid substitutions toward the HIV-1 subtype C consensus sequence – reverse substitutions, red down triangle denotes amino acid substitutions from the subtype C consensus – forward substitutions, and gray circle delineates viral amino acid polymorphisms not associated with the subtype C consensus sequence. Gray bars on the background denote HIV-1 Gag p17, p24, p2/p7/p1/p6 for each subject.

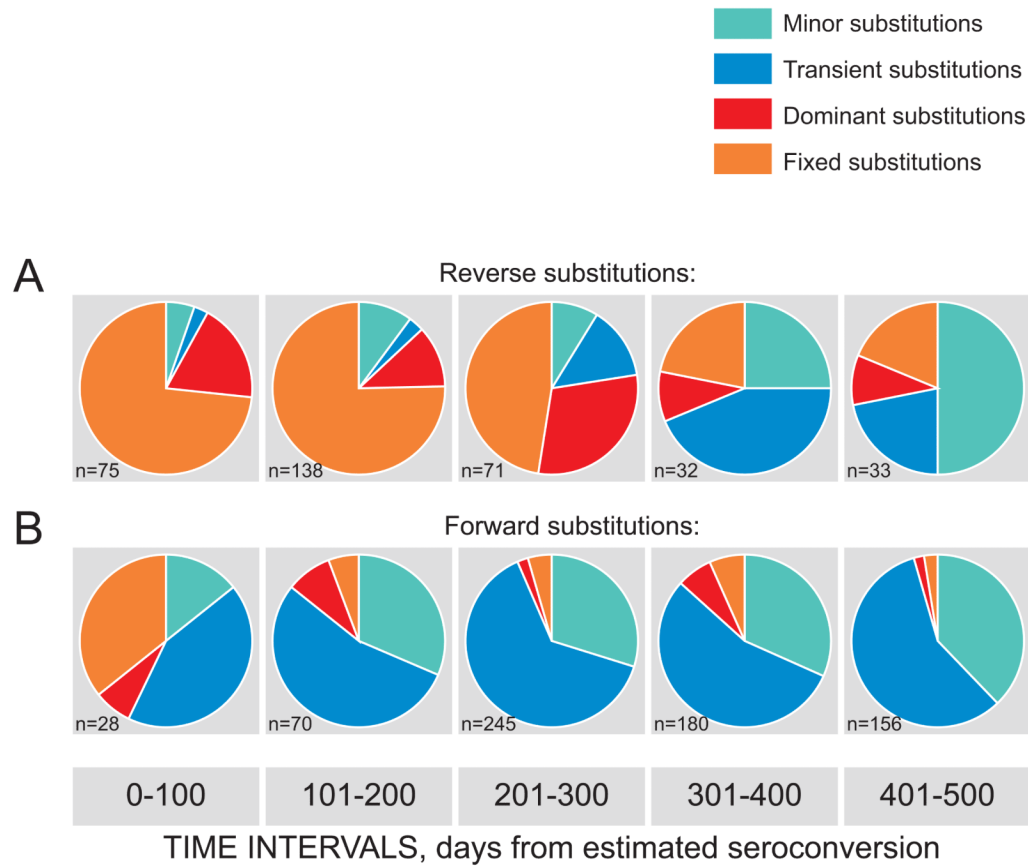


**Figure 2.** Profile and location of dominant and fixed amino acid substitutions across HIV-1C Gag. See legend to Figure 1. The dashed line highlights 12 amino acid substitutions observed at Gag codon position 242.

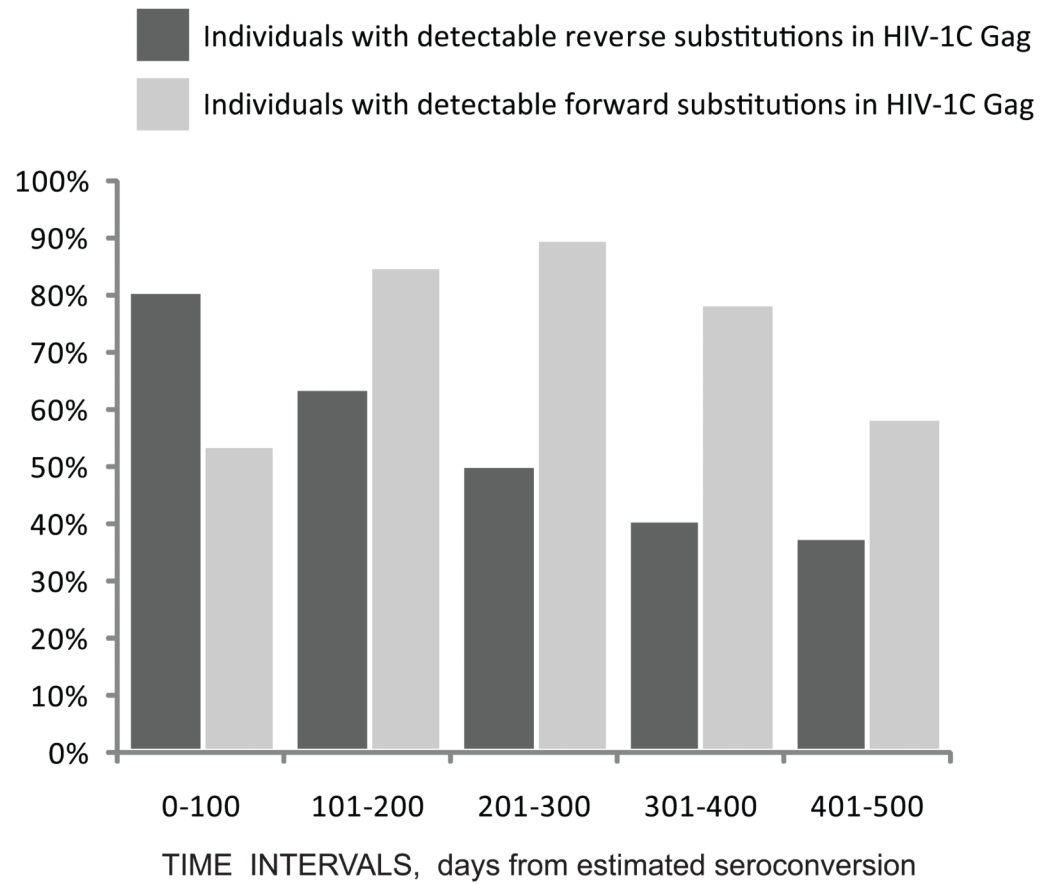




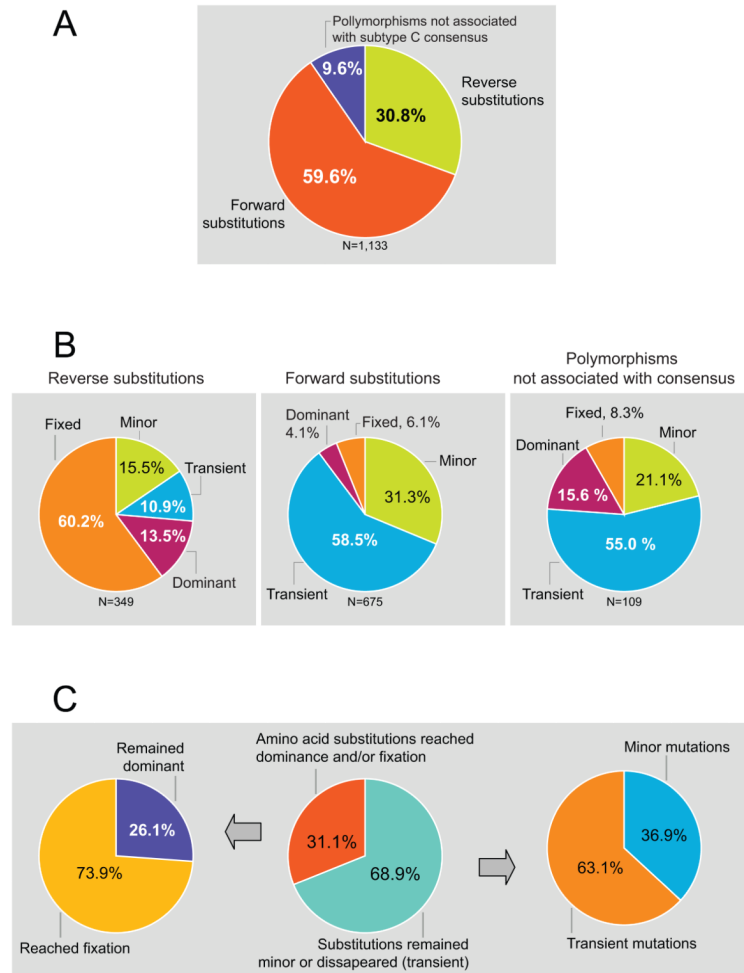
**Figure 3.** Relationships between reverse and forward substitutions in HIV-1C Gag over time. The number of identified amino acid substitutions is shown in the left bottom corner of each graph. Analyzed time intervals in days from estimated seroconversion, the number of subjects, and the number of analyzed sequences per time interval including total, RNA, and DNA are shown at the bottom. A: Minor Gag substitutions (did not reach dominance or fixation, and did not disappear over the follow up time). B: Transient Gag substitutions. C: Dominant Gag substitutions (did not reach fixation over the follow up time). D: Fixed substitutions.



**Figure 4.** Relationships between different types of reverse and forward Gag substitutions over time. The number of identified substitutions is shown in the left bottom corner of each graph. Analyzed time intervals in days from estimated seroconversion are shown at the bottom. A: Reverse substitutions. B: Forward substitutions.



**Figure 5.** Proportion of individuals with detectable amino acid substitutions in Gag over time. Individuals with detectable reverse substitutions are shown by dark bars. Individuals with detectable forward substitutions are shown by light bars. Analyzed time intervals in days from estimated seroconversion are shown at the bottom.



**Figure 6.** Relationships between different types of Gag amino acid substitutions. **A:** Overall relationships between reverse substitutions, forward substitutions, and viral polymorphisms not directly associate with the subtype C consensus sequence. **B:** Relationships within reverse substitutions, forward substitutions, and viral polymorphisms not directly associate with the subtype C consensus sequence. **C:** Breakdown of viral substitutions in Gag that either reached or did not reach dominance and/or fixation.