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Human Immunodeficiency Virus Type 1 Coreceptor Switching: V1/V2 Gain-of-Fitness Mutations Compensate for V3 Loss-of-Fitness Mutations†

C. Pastore,[1] R. Nedellec,[1] A. Ramos,[1] S. Pontow,[2] L. Ratner,[2] and D. E. Mosier[1]*

Department of Immunology, The Scripps Research Institute, La Jolla, California 92037,[1] and Molecular Oncology Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110[2]

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Human immunodeficiency virus type 1 (HIV-1) entry into target cells is mediated by the virus envelope binding to CD4 and the conformationally altered envelope subsequently binding to one of two chemokine receptors. HIV-1 envelope glycoprotein (gp120) has five variable loops, of which three (V1/V2 and V3) influence the binding of either CCR5 or CXCR4, the two primary coreceptors for virus entry. Minimal sequence changes in V3 are sufficient for changing coreceptor use from CCR5 to CXCR4 in some HIV-1 isolates, but more commonly additional mutations in V1/V2 are observed during coreceptor switching. We have modeled coreceptor switching by introducing most possible combinations of mutations in the variable loops that distinguish a previously identified group of CCR5- and CXCR4-using viruses. We found that V3 mutations entail high risk, ranging from major loss of entry fitness to lethality. Mutations in or near V1/V2 were able to compensate for the deleterious V3 mutations and may need to precede V3 mutations to permit virus survival. V1/V2 mutations in the absence of V3 mutations often increased the capacity of virus to utilize CCR5 but were unable to confer CXCR4 use. V3 mutations were thus necessary but not sufficient for coreceptor switching, and V1/V2 mutations were necessary for virus survival. HIV-1 envelope sequence evolution from CCR5 to CXCR4 use is constrained by relatively frequent lethal mutations, deep fitness valleys, and requirements to make the right amino acid substitution in the right place at the right time.

Human immunodeficiency virus type 1 (HIV-1) entry into target cells is mediated by sequential interaction of the envelope glycoprotein with CD4 and one of two chemokine receptors, CCR5 or CXCR4 (1, 5, 10, 11). Most primary infections involve transmission of viruses using CCR5 as the preferred coreceptor (8, 25). Evolution of coreceptor use by HIV-1 from CCR5 to CXCR4 is known to be associated with poorer clinical prognosis (3, 8) and can be assumed to be one pathway leading to resistance to CCR5 inhibitors currently in clinical trials (28, 30, 34). Moreover, treatment with CCR5 inhibitors may select for minor populations of viruses with the ability to utilize CXCR4. Understanding the evolution of coreceptor switching in terms of the fitness costs to the virus is thus important. Although the sequence of the V3 variable loop of HIV-1 gp120 envelope is known to contribute to coreceptor use (6, 14, 18, 42, 51), sequence variation in or near the V1/V2 loop is also an important influence on coreceptor choice (13, 22, 23, 31, 39, 47, 48, 52, 53). We have previously characterized coreceptor switch mutants selected by rapid substitution of U87-CD4-CXCR4 cells for U87-CD4-CCR5 cells in vitro (32). Mutations confined to the V3 region were sufficient to alter coreceptor use for some virus envelopes, but other viruses required additional mutations in or adjacent to the V1/V2 region for successful coreceptor switching. These prior studies allowed analysis of the starting virus, an occasional intermediate, and the final successful coreceptor switch mutant. The fitness cost of each mutation in envelope on the pathway to successful coreceptor switching could not be assessed.

In the present study, we have used site-directed mutagenesis to introduce most possible combinations of mutations on the pathway from CCR5 to CXCR4 use. The entry efficiency of these mutated envelopes was assessed in a single cycle infection assay. We also measured the ability of mutated envelopes to mediate entry into cell lines expressing CCR5:CXCR4 chimeric coreceptors (36) to determine if coreceptor switch intermediates engaged specific extracellular domains of CXCR4. This study of the potential intermediates between CCR5 and CXCR4 use allows us to measure the costs or benefits of each combination of envelope mutations on the entry process, including CD4 binding, coreceptor binding, and fusion. The evolution of envelope function was studied for four independent coreceptor switch mutants, two derived from the ADA envelope and two derived from the BaL envelope.

The primary finding in these studies was that coreceptor switching is constrained by the high fitness costs of combined mutations in V3 and that the loss of entry efficiency associated with V3 mutations could be offset by compensatory mutations in or near V1/V2. The V1/V2 region mutations alone tended to improve the entry efficiency via CCR5 but generally did not confer CXCR4 use. A significant fraction of all possible evolutionary pathways from CCR5 to CXCR4 use led to dead ends. These results establish that the order of occurrence of mutations associated with coreceptor switching is critical for survival of intermediates. The coreceptor switch mutant viruses previously identified (32) rarely resulted from the most direct mutational pathway. Instead, the reconstructed evolutionary pathway seemed to oscillate between

* Corresponding author. Mailing address: The Scripps Research Institute, Dept. of Immunology, IMM-7, La Jolla, CA 92037. Phone: (858) 784-9121. Fax: (858) 784-9190. E-mail: dmosier@scripps.edu.

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TABLE 1. Sequence and numbering of mutations in gp120 associated with coreceptor switching

Envelopes and mutant(s)	Sequence
ADA wt, ADA-1, ADA-3	
V2[a] and C2[b]	● ● ● CSFNITTSIRDKVKDYALFYRLDVVPIDNDNTSYRLINCNTSTITQACPKVSFEPIPIHYCTPAGFAILK... ----------D----------T------... 1 2 ---K----------I---N----------... 1 2 3
V3	● CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC wt -----H----R----R-------EK---------- ADA-1 3 4 5 67 ----------R-------K---------- ADA-3 4 5
BaL wt, BaL-1B, BaL-2A	
V1/V2	● ● ● ● ● NCTDLRNATNGNDTNTTSSREMMGGGEMKNCSFKITTNIRGKVQKEYALFYELDIVPIDNNSNNRYRLISC -------T----------K---------- 1 2 D----------K---------- 1 2
V3	● CTRPNNNTRKSIHIGPGRALYTTGEIIGDIRQAHC wt ----------KI----K---------- BaL-1B 34 5 ----------K---------- BaL-2A + K490T 3 4

[a] Filled symbols above NxT(S) sequences indicate N-linked glycosylation sites. wt, wild type.
[b] The V2 sequence is underlined; the remaining sequence is C2.

loss-of-fitness mutations in V3 and compensatory mutations elsewhere to arrive at an envelope sequence with moderate utilization of CXCR4.

MATERIALS AND METHODS

Cell lines. U87-CD4-CCR5 and U87-CD4-CXCR4 cells (2) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 ng of puromycin/ml, and 300 ng of G418/ml. U87.CD4 cells expressing chimeric CCR5:CXCR4 coreceptors (36) were maintained under identical conditions. The control 5555 (N-terminal, EC1, EC2, and EC3 domains) and 4444 cell lines contained constructs similar to those of the chimeric cell lines and differed in CCR5 and CXCR4 expression from the U87-CD4-CCR5 and U87-CD4-CXCR4 cell lines used for initial infectivity studies. The chimeric cell lines are designated according to the origin of the N-terminal, EC1, EC2, and EC3 extracellular domains; i.e., 5545 contains EC2 from CXCR4 and the rest of the protein is CCR5.

Cloning and mutagenesis. The ADA and BaL full-length envelope glycoproteins were amplified by PCR from the virus plasmids pNL4-3-ADA and pR8-BaL (32), respectively, with two sets of primers spanning the 5′ and 3′ sites of the ADA and BaL *env* genes and inserting a 5′ SalI and a 3′ XhoI restriction site for ADA and a 5′ SalI and a 3′ HpaI site for BaL. The ADA SalI-XhoI and the BaL SalI-HpaI fragments of the PCR were cloned into the expression plasmid pSVIII (45, 46).

Each single or combination of mutations was introduced in the cloned envelopes by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA) following the manufacturer's instructions. The sequence of each mutant envelope was confirmed using the four primer pairs previously described (32).

Entry assay. The entry fitness of mutated envelopes was measured in a single-cycle pseudovirus infection assay. Mutant envelope clones inserted into the pSVIII plasmid were cotransfected with env-negative, luciferase-positive (NL4-3-Luc+E-R- [7]) reporter plasmids into 293T cells, and the resulting pseudoviruses were harvested, standardized for p24 content, and used to infect either U87-CD4-CCR5 cells, U87-CD4-CXCR4 cells, or U87-CD4 cells with chimeric coreceptors. The luciferase activity from triplicate wells was measured on a luminometer (EG&G Berthold LB 96V; Perkin Elmer, Gaithersburg, MD) with the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Virus infectivity was compared to the parental R5 virus by calculating the slope of the infectivity curve by plotting p24 input versus light units of luciferase activity after 48 to 72 h of culture. The slope was calculated using linear regression analysis (Prism 4; GraphPad Software, San Diego, CA). Determination of infectivity was repeated in three replicate experiments, and the mean was normalized to the percentage of ADA or BaL wild-type envelope infectivity for CCR5-expressing target cells or the final X4 or R5X4 mutant infectivity for CXCR4-expressing target cells.

RESULTS

Envelope mutations spanning the sequence space between CCR5 and CXCR4 coreceptor use. We chose to examine in detail four coreceptor switch mutants generated by replacing U87-CD4-CCR5 target cells first with U87-CD4-CXCR4 targets and then with MT-2 cells, as described in our previous study (32). ADA-1 and ADA-3 were two independent coreceptor switch mutants derived from the parental ADA virus that typed as X4 only (32) and had incurred seven and five mutations, respectively (Table 1). All possible intermediate mutations leading to ADA-3 ($2^5 = 32$) were generated by site-directed mutagenesis. ADA-1 had five mutations in V3 and two mutations in C2, including a loss of a potential N-linked glycosylation site (PNGS) at position 197 at the base of the V2 loop (Table 1). All possible combinations of V3 mutations were generated, as well as a subset of possible C2 mutations for a total of 53 mutant envelopes. The N197D mutation was found to have a major impact on envelope function, while the A221T mutation in C2 was

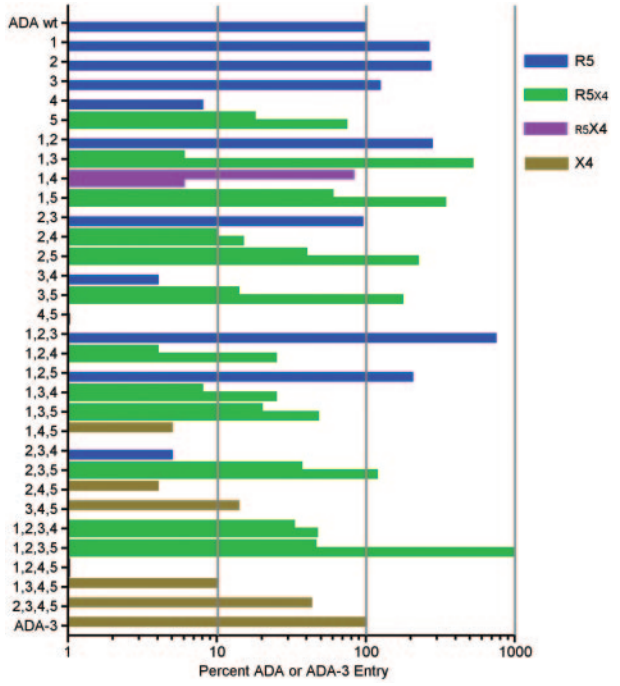

FIG. 1. Entry efficiency of ADA-3 mutant envelopes in a single-cycle infection assay employing either U87-CD4-CCR5 or U87-CD4-CXCR4 target cells. Mutations 1 to 5 correspond to V2 mutations N157K (loss of PNGS), V178I, and D182N and V3 mutations G312R and E320K, respectively (numbering based on HxB2), and are further described in Table 1. Data are normalized to percentage of ADA wild type (wt) for CCR5-expressing target cells or ADA-3 for CXCR4-expressing target cells. Envelopes that mediate entry only via CCR5 are shown in blue, envelopes that mediate entry via both CCR5 and CXCR4 but more efficiently via CCR5 are shown in green, envelopes that mediate entry via both CCR5 and CXCR4 but more efficiently via CXCR4 are shown in purple, and envelopes that mediate entry only via CXCR4 are shown in brown.

found to have little impact (see below), obviating the need to introduce all 128 possible combinations of mutations. For comparison, we analyzed two independent BaL coreceptor switch mutants that had incurred four (BaL-2A) or five (BaL-1B) mutations leading to an R5X4 phenotype (32). The sites of these mutations are shown in Table 1. BaL-2A had a K490T mutation in C5 that has little impact on envelope function. BaL-1B also had two mutations in gp41 that were not reproduced by site-directed mutagenesis. In total, 123 mutant envelopes were generated for these studies.

The function of ADA- or BaL-related envelope mutations was measured in single-cycle infection assays as described in Materials and Methods employing either U87-CD4-CCR5 or U87-CD4-CXCR4 target cells. In addition to measuring infectivity on target cells expressing native CCR5 or CXCR4, we also tested all of the envelope mutations on a panel of U87-CD4 target cells expressing a subset of the CCR5:CXCR4 chimeric coreceptors described by Pontow and Ratner (36).

Sequence evolution from ADA to ADA-3. The five mutations associated with the R5 to X4 switch in ADA-3 were introduced in all possible combinations and tested in single-round infectivity assays. The normalized results are presented in Fig. 1, with percentage of parental (CCR5) or final mutant (CXCR4) entry displayed on a log scale to capture the wide variability in infectivity. Introduction of the V2 mutations, either alone or in combination, improved the entry efficiency on CCR5 targets and (with one exception) did not permit entry via CXCR4. The V3 mutation G312R was highly deleterious but did allow limited use of CXCR4. The common E320K mutation conferred modest entry via CXCR4 and maintained entry via CCR5. Introduction of both V3 mutations resulted in an envelope with less than 1% of wild-type entry efficiency on CCR5 targets and no infectivity for CXCR4 target cells. This suggests that V2 mutations must have occurred prior to the two V3 mutations in the selection of the ADA-3 virus. An intermediate ADA-3p virus was identified during the selection process (32), and the 3p envelope contained V2 mutation 3 (D182N) and V3 mutation 4 (G312R). Both of these mutations involved highly prevalent G-to-A transitions (32), and the fitness cost of mutation 3 in V2 is much less than that of mutation 4 in V3 (Fig. 1), suggesting that the most likely order of mutations was 3 followed by 4. Note that introduction of all the observed mutations except for mutation 3 results in a minimally infectious envelope (Fig. 1), indicating that mutation 3 is critical for survival of the evolving coreceptor switch mutant.

The ability of pseudotyped viruses expressing the ADA-3 series of mutated envelopes to infect CCR5:CXCR4 chimeric coreceptor-expressing target cells was assessed. Infection was scored positive if the mean relative light units was three times the background or greater in both of two replicate experiments. The results are shown in Table 2. No single mutation, including mutation 5 (E320K), supported entry with any coreceptor other than 5555. The expression of CXCR4 on the 4444 target cells is less than that on the U87-CD4-CXCR4 cells used to generate the data in Fig. 1, and this difference appears to impact their sensitivity to infection by envelopes with poor ability to use CXCR4. The combination of mutations 1 (N157K) and 3 (D182N), 4 (G312R), or 5 (E320K) conferred the ability to infect 4444, 4444, or 4555 target cells, respectively. Three envelopes containing the combination of mutations 1 and 5; 2, 4, and 5; or 1, 2, 3, and 5 were able to utilize one or more chimeric coreceptors but not 4444. ADA-3 (all five mutations) and its likely precursors with four mutations showed the broadest use of chimeric coreceptors and improved use of 4444. Use of native CCR5 (5555) by these mutants was observed in these target cell lines, in contrast with the results with U87-CD4-CCR5 cells used in the entry assays (Fig. 1 versus Table 2), and this difference correlated with levels of CCR5 expression. It is notable that we observed eight distinct patterns of chimeric coreceptor use in a series of 32 mutant envelopes. For the ADA-3 series of mutants, no envelope was completely noninfectious on any target cell line. This result did not extend to the ADA-1 or BaL series of mutant envelopes (see below).

Sequence evolution from ADA to ADA-1. The ADA-1 mutant incurred seven mutations with five replacements in V3 (Table 1). We made all combinations of mutations in V3 and selected mutations in C2 (Fig. 2). This was possible because many combinations of mutations in V3, including all five mutations, resulted in envelopes that were unable to mediate entry into any target cell line. Mutation 1 in C2 (N197D, loss of PNGS) rescued infectivity of the V3-mutated envelope, but mutation 2 in C2 (A221T) was unable to rescue infectivity.

TABLE 2. Proportional use[a] of chimeric CCR5:CXCR4 chemokine coreceptors by ADA-3 envelope mutants

Mutation or mutation combination	5555	5554	5545	4555	4444
0	100				
1	100				
2	100				
3	100				
4	100				
5	100				
1, 2	100				
1, 3	99.91				0.09
1, 4	98.12				1.88
1, 5	99.23			0.77	
2, 3	100				
2, 4	100				
2, 5	100				
3, 4	100				
3, 5	100				
4, 5	100				
1, 2, 3	100				
1, 2, 4	100				
1, 2, 5	99.32		0.48		0.21
1, 3, 4	96.87		2.24	0.30	0.59
1, 3, 5	99.12		0.29	0.31	0.27
1, 4, 5	86.99				13.01
2, 3, 4	100				
2, 3, 5	100				
2, 4, 5	77.95		16.19	5.86	
3, 4, 5	62.45		17.68	10.26	9.60
1, 2, 3, 4	98.87				1.13
1, 2, 3, 5	99.51			0.49	
1, 2, 4, 5	69.19		11.27	7.46	12.07
1, 3, 4, 5	76.85	3.15	10.22	2.58	7.19
2, 3, 4, 5	75.71		11.34	8.58	4.37
1, 2, 3, 4, 5	74.71	0.53	14.13	4.63	6.00

[a] Numbers represent the percentage of total infection mediated by one chemokine receptor compared to infection mediated by all coreceptors. Note that the level of infectivity of each mutant is shown in Fig. 1 and is not taken into account in calculating the relative use of each parental or chimeric coreceptor.

Moreover, introduction of mutation 2 alone conferred little change in envelope function, and the combination of both C2 mutations had the same result as mutation 1 alone (Fig. 2). We therefore focused on the V3 mutations and the C2 mutation N197D.

Introduction of single mutations in V3 produced infectious envelopes, and mutation 5 (P311R) or 7 (E320K) permitted some level of infectivity via CXCR4. Mutation 5 was known to have occurred first in the evolution of the ADA-1 mutant (32). No single mutation was lethal, and mutation 4 (S306R) exacted the greatest cost in entry efficiency. The combination of mutation 1 (N197D) and any single V3 mutation improved entry efficiency on both CCR5 and CXCR4 target cells compared to single V3 mutations alone. Several combinations of two V3 mutations were lethal, and only the 5, 7 (P311R and E320K) and the 6, 7 (G319E and E320K) mutations produced envelopes with high entry efficiency on both CCR5 and CXCR4 target cells. Mutations 4 and 5 (S306R and P311R) conferred CXCR4 use that was almost as efficient as the final ADA-1 mutant. The addition of C2 mutation 1 (N197D) to two V3 mutations improved entry efficiency and utilization of CXCR4. Mutation 1 rescued the lethal 3 and 5 combination mutations, and the 1, 5, 7 mutant envelope (N197D, P311R, and E320K) was 10-fold more infectious for CXCR4 target cells than the final ADA-1 mutant. This set of mutations would have represented a better solution for coreceptor switching than the observed mutations. However, the evolution of coreceptor switching must have included other more deleterious mutations prior to the introduction of these three mutations. Introduction of C2 mutation 2 (A221T) did not improve the entry efficiency of V3 mutations 5 and 7, but it did alter the use of chimeric coreceptors (Fig. 2B). Introduction of three, four, or all five mutations in V3 resulted in a high percentage of noninfectious envelopes. In particular, all combinations of four V3 mutations were lethal, except for mutation 4, 5, 6, 7 (S306R, P311R, G319E, and E320K), which generated an envelope with modest entry only on native CXCR4-expressing targets. This result suggests that mutation 3 (N301H), which disrupts a highly conserved PNGS in V3, was the most damaging for V3 function. The high proportion of lethal V3 mutations indicates that the compensatory mutation 1 in C2 must have occurred soon after V3 mutation 5 to preserve the evolution of viable envelopes leading to ADA-1. This is directly confirmed by showing that the addition of mutation 1 (N197D) to combinations of four or five V3 mutations rescues envelope function for infection of CXCR4 target cells and several of the chimeric coreceptor lines expressing CXCR4 extracellular domains (Fig. 2A and 2B). Two of these mutant envelopes (combinations 1, 4, 5, 6, 7 and 1, 3, 4, 5, 7) are more efficient at infecting

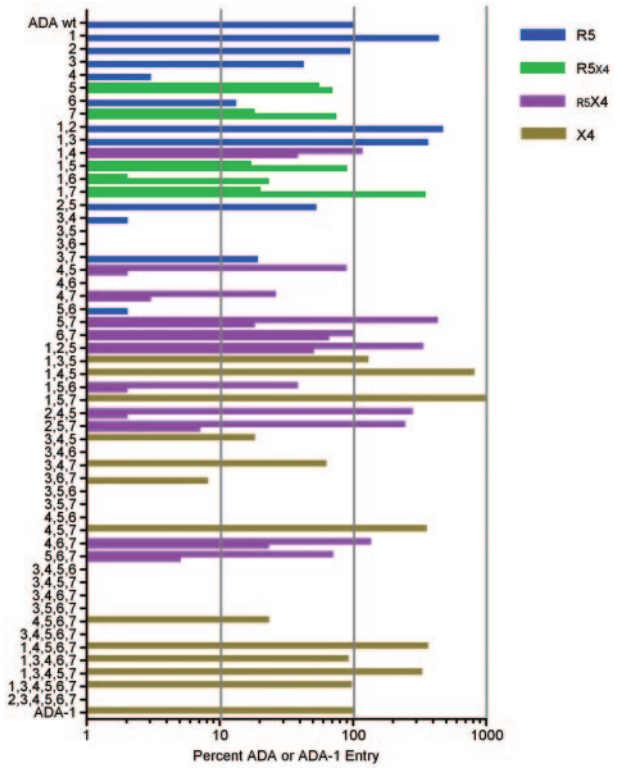

FIG. 2. Entry efficiency of ADA-1 mutant envelopes in a single-cycle infection assay as described in the legend to Fig. 1. Mutations 1 to 7 correspond to C2 mutations N197D (base of V2 loop, PNGS), and A221T and V3 mutations N301H, S306R, P311R, G319E, and E320K, respectively (numbering based on HxB2), and are further described in Table 1. Color coding is described in the legend to Fig. 1. wt, wild type.

CXCR4 target cells than the ADA-1 mutant, suggesting that they were unlikely to have been generated during the evolution of ADA-1 prior to the introduction of the deleterious combination of mutations 3 and 6.

The ability of the ADA-1 series of mutated envelopes to mediate infection of target cells expressing chimeric coreceptors contrasts with the results with the ADA-3 mutant envelopes (Table 3). Use of CCR5 (5555) was abandoned by several of the V3 triple mutants and all of the infectious envelopes containing five or more mutations. The final ADA-1 mutant containing all seven mutations appears to utilize either the N-terminal domain or EC2 of CXCR4 for entry. There were 13 different patterns of chimeric coreceptor use represented among the series of ADA-1 mutant envelopes. These findings suggest considerable flexibility in the ability of the set of envelopes to engage either CCR5 or CXCR4 extracellular domains.

Sequence evolution from BaL to BaL-2A or BaL-1B. We have previously described several coreceptor switch variants with an R5X4 phenotype derived from the BaL envelope (32). We generated combinations of mutations leading to two of these R5X4 isolates, BaL-2A and BaL-1B. BaL-2A had four mutations as indicated in Table 1, and BaL-1B had five mutations, including three in V3. The K490T mutation in C5 in the BaL-2A envelope had little impact on function (Fig. 3) and was not introduced in all possible combinations. Mutation 1 (N130D, loss of PNGS) conferred very modest use of CXCR4 in addition to CCR5. Mutation 2 (E178K) or 3 (E320K) introduced separately had little impact on envelope function. The double mutation 1, 2 improved CCR5 use, whereas the double mutation 1, 3 or 2, 3 allowed modest use of CXCR4 while preserving entry efficiency on CCR5 target cells. All three mutations reproduced the R5X4 phenotype of the original BaL-2A virus and allowed efficient infection of all coreceptor chimeric target cell lines (Table 4). The addition of the C5 region mutation K490T slightly improved CXCR4 entry and slightly decreased CCR5-mediated entry and did not alter the pattern of chimeric coreceptor use. BaL-2A appears to have evolved without encountering major fitness obstacles, in contrast to the other viruses analyzed.

The five mutations leading to the R5X4 BaL-1B virus were examined in all possible combinations. The results are also presented in Fig. 3 and Table 4. All five possible single mutations were well tolerated, with only mutation 3 (A314K in V3) causing a significant reduction in CCR5-mediated entry. However, two of the double mutants (1, 4 [A136T and L315I] and 2, 4 [E178K and L315I]) were unable to infect any target cells. Modest use of CXCR4 was associated with the presence of mutation 5 (E320K). Triple mutants 1, 4, 5 and 2, 4, 5 were noninfectious for U87-CD4-CCR5 cells but did show very low-level entry with the 5555 target cells. Several triple mutants that included mutation 5 (E320K) showed improving use of CXCR4. Mutants 2, 3, 5 and 2, 4, 5 mediated entry via all chimeric coreceptors, but the level of infection was much higher with the 2, 3, 5 combination (E178K, A314K, and E320K), suggesting again that mutation 4 (L315I) impaired entry efficiency. Four of the possible five quadruple mutants showed a more robust R5X4 phenotype and infected most or all of the cell lines expressing chimeric coreceptors (Table 4). The final mutant envelope containing all five BaL-1B mutations reproduced the phenotype of the original BaL-1B virus and infected target cells bearing all chimeric coreceptors. The dualtropic BaL-1B mutant thus showed as broad a usage of the chimeric coreceptors as the X4 ADA-1 mutants. As with the ADA-1 and ADA-3 mutant series, the evolution of BaL-1B was constrained by potential combinations of mutations that were lethal.

TABLE 3. Proportional use[a] of chimeric CCR5:CXCR4 chemokine coreceptors by ADA-1 envelope mutants

Mutation or mutation combination	5555	5554	5545	5455	4555	4444
0	100					
1	100					
2	100					
3	100					
4	100					
5	98.16		1.84			
6	100					
7	100					
1, 2	100					
1, 3	100					
1, 4	90.46	1.60	3.48		1.92	2.54
1, 5	99.77				0.23	
1, 6	100					
1, 7	100					
2, 4	91.39		0.82		3.51	4.29
3, 4	100					
3, 5						
3, 6	100					
3, 7	100					
4, 5	100					
4, 6						
5, 6	100					
5, 7	55.22	2.23	24.57		6.80	11.19
6, 7	98.14		0.92		0.94	
1, 2, 5	90.85	0.32	2.70		5.05	1.09
1, 3, 5			61.22		8.30	30.48
1, 4, 5	16.25	5.19	40.09		17.64	20.84
1, 5, 6	98.80				1.20	
1, 5, 7	28.97	3.31	36.67	1.56	16.78	12.70
2, 4, 5	29.74	3.16	26.99		17.62	22.48
2, 5, 7	36.34	3.24	23.21	0.55	17.70	18.96
3, 4, 5			100			
3, 4, 6						
3, 4, 7			22.21			77.79
3, 5, 6						
3, 6, 7	100					
4, 5, 6						
4, 5, 7		4.98	38.03		27.10	29.89
4, 6, 7	85.09		7.94		0.98	5.99
5, 6, 7	57.91		38.03		4.06	
3, 4, 5, 6						
3, 4, 5, 7						
3, 4, 6, 7						
3, 5, 6, 7						
4, 5, 6, 7						100
3, 4, 5, 6, 7						
1, 3, 4, 5, 6		4.46	41.19	0.88	19.80	33.67
1, 3, 4, 6, 7		3.71	37.90		25.33	33.05
1, 4, 5, 6, 7		2.20	39.53		29.45	28.82
1, 3, 4, 5, 6, 7			39.74			60.26
2, 3, 4, 5, 6, 7						
1, 2, 3, 4, 5, 6, 7			48.16		42.53	9.31

[a] Numbers represent the percentage of total infection mediated by one chemokine receptor compared to infection mediated by all coreceptors. Mutations are numbered as described in Table 1.

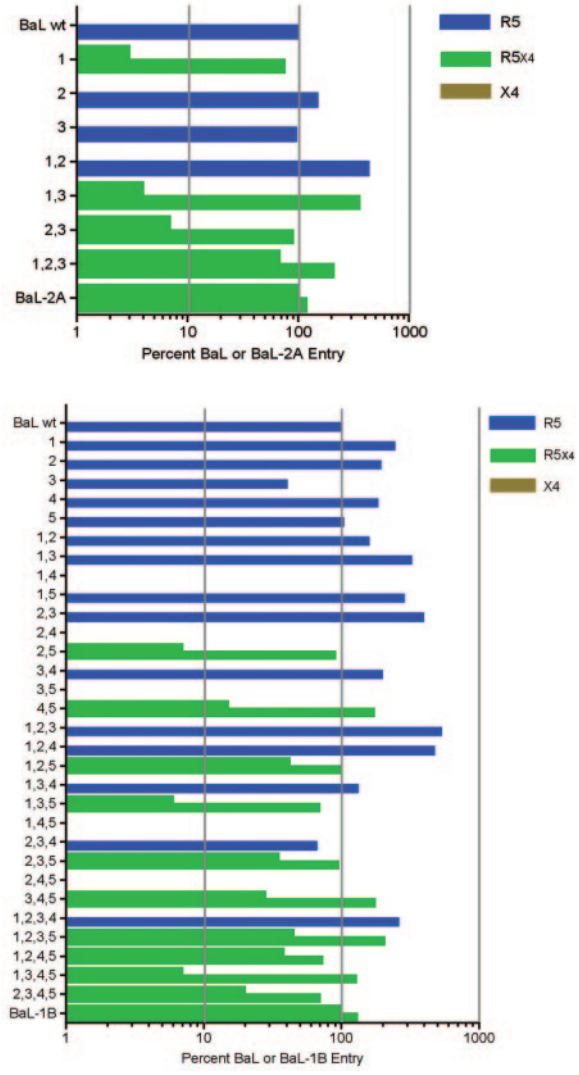

FIG. 3. Entry efficiency of BaL-derived mutant envelopes in a single-cycle infection assay as described in the legend to Fig. 1. Mutations 1 to 4 in BaL-2A and 1 to 5 in BaL-1B are described in Table 1. Mutations 1 to 4 in BaL-2A correspond to N130D (loss of PNGS), E178K, E320K, and K490T (numbering based on HxB2). Mutations 1 to 5 in BaL-1B correspond to A136T, E178K, A314K, L315I, and E320K. Color coding is described in the legend to Fig. 1. wt, wild type.

DISCUSSION

The results of these extensive analyses of envelope mutations associated with coreceptor switching provide more insight into the long delay between primary infection and the emergence of CXCR4-utilizing HIV-1 (40, 41). In addition to the previously identified obstacles to coreceptor switching (20, 32, 35, 49), the mutations involved in coreceptor switching appear to carry a greater risk than previously appreciated. Up to 25% (ADA-1-related mutants) of potential coreceptor switch intermediates were noninfectious for either CCR5- or CXCR4-expressing target cells (Fig. 2). Lethal combinations of mutations were concentrated in the V3 region and often included substitutions that are uncommon in the HIV sequence database. Substitutions in the conserved GPGR sequence at the tip of the V3 loop in the ADA-1 and ADA-3 series are rare and may impact the predicted β-turn at that site and coreceptor engagement (16, 44). Loss of the V3 N-linked glycosylation site by mutation 4 (N301H) in the ADA-1 series is known to be associated with changes in coreceptor binding (35). Although the E320K change in V3 is commonly associated with coreceptor switching (15, 19), that mutation in association with other V3 substitutions was often lethal.

These dangerous mutations in V3 could be rescued if they were preceded by mutations in or near the V1/V2 region. This is most clearly illustrated in the ADA-1-related mutants, but it was also observed for ADA-3 and BaL1-B mutants. The N197D mutation in the ADA-1 series of envelopes ablated an

TABLE 4. Proportional use[a] of chimeric CCR5:CXCR4 chemokine coreceptors by BaL-2A or BaL-1B envelope mutants

Mutant and mutation or mutation combination	5555	5554	5455	5545	4555	4444
BaL-2A						
0	100					
1	100					
2	100					
3	100					
1, 2	100					
1, 3	100					
2, 3	100					
1, 2, 3	85.46	0.32	4.62	0.07	5.53	4.00
BaL-1B						
1	100					
2	100					
3	100					
4	100					
5	100					
1, 2	100					
1, 3	100					
1, 4						
1, 5	100					
2, 3	100					
2, 4						
2, 5	99.58				0.32	0.10
3, 4	100					
3, 5	100					
4, 5	99.95				0.05	
1, 2, 3	100					
1, 2, 4	100					
1, 2, 5	99.93	0.37	0.15		0.46	0.08
1, 3, 4	100					
1, 3, 5	99.70		0.14		0.16	
1, 4, 5	100					
2, 3, 4	100					
2, 3, 5	94.99	0.09	0.20	0.06	3.31	1.35
2, 4, 5	97.79	0.04	0.10	0.04	1.39	0.65
3, 4, 5	98.79		0.44		0.34	0.43
1, 2, 3, 4	100					
1, 2, 3, 5	95.10	0.15	1.45		1.84	1.46
1, 2, 4, 5	87.07		0.54			12.46
1, 3, 4, 5	99.25	0.05	0.24		0.23	0.23
2, 3, 4, 5	91.41	0.19	2.24	0.22	3.67	2.27
1, 2, 3, 4, 5	89.16	0.31	2.63	0.23	3.90	3.77

[a] Numbers represent the percentage of total infection mediated by one chemokine receptor compared to infection mediated by all coreceptors. Note that the level of infectivity of each mutant is shown in Fig. 3 and is not taken into account in calculating the relative use of each parental or chimeric coreceptor. Sequence designations are given in Table 1.

N-linked glycosylation site at the base of the V2 loop and restored infectivity to several mutated V3 regions, including one containing all five V3 mutations observed in the ADA-1 coreceptor switch mutant (Fig. 2). This site has previously been demonstrated to impact CD4-independent HIV-1 infection (24) and sensitivity to gp120-targeted entry inhibitors (26), and it has been inferred from these findings that loss of the N-linked glycan allows greater flexibility of the V2 loop (54). Analogous results involving the loss of an N-linked glycosylation site in V1 during evolution to CXCR4 use (T-cell line adaptation) have been reported already (4). Our results imply that changes in the positioning of the V2 loop allow complementation of nonfunctional V3 sequences, either through improved CD4 binding or better engagement of CCR5. The combination of N197D with the five V3 mutations in ADA-1 (mutant 1, 3, 4, 5, 6, 7 in Fig. 2) permitted infection of target cell lines expressing only EC2 of CXCR4 but not target cells expressing only the N-terminal domain of CXCR4, a more restricted engagement of CXCR4 than that seen with the final ADA-1 mutant containing all seven mutations and several potential precursors containing four of the final five V3 mutations (Table 3). These results imply that a single amino acid change in V3 combined with the more flexible V2 loop associated with the N197D mutation permits differential use of the extracellular domains of CXCR4. The ADA-1 mutant with the best use of CXCR4 was the 1, 5, 7 envelope (N197D, P311R, and E320K), and this envelope was also most promiscuous in its use of all CCR5:CXCR4 chimeric coreceptors. This envelope represents the optimal solution to the coreceptor switch problem, and the other four mutations found in the ADA-1 virus would appear to be deleterious. The loss of the V3 glycosylation site (mutation 3, N301H) is clearly a high-risk mutation, since several of the lethal combinations of V3 mutations include N301H. Mutation of the equivalent site in SF162 V3 has been shown to reduce use of CCR5 and increase sensitivity to neutralizing antibody (29). However, the ADA envelope containing only the N301H mutation showed only a modest reduction in CCR5-mediated entry (Fig. 2A), so the impact of mutations is cumulative and context dependent. The E320K mutation in the ADA V3 loop has previously been shown to be important for determining coreceptor use (17).

The BaL-1B series of envelope mutations also included several lethal combinations, many of which included the L315I mutation in V3 (mutation 4). This substitution is not uncommon in the V3 sequence database, and it improved CCR5-mediated entry when introduced as a single mutation (Fig. 3B). Nonetheless, this mutation was lethal in combination with V2 mutation 1 (A136T) or 2 (E178K). A functional envelope thus must contain the right combination of mutations in both V1/V2 and V3.

In contrast to the deleterious mutations in V3, mutations introduced in or near the V1/V2 region generally improved envelope entry efficiency on CCR5 target cells. The combination of all three V2 region substitutions found in the ADA-3 coreceptor switch variant improved CCR5 use eightfold compared to the ADA parental envelope (Fig. 1). Likewise, the combination of the two C2 mutations in the ADA-1 series improved CCR5 use by sixfold (Fig. 2), and the V1/V2 mutations in the BaL-1B series improved CCR5 use by two- to threefold (Fig. 3). None of these mutations was permissive for CXCR4 use. These results demonstrate that mutations in or near V1/V2 can compensate, directly or indirectly, for potentially deleterious mutations in V3. The mutations in V3 are necessary for coreceptor switching but are insufficient to produce infectious intermediates in the evolution of CXCR4 use. This cooperation between V1/V2 and V3 mutations in determining coreceptor choice confirms earlier studies showing that both regions are important (13, 22, 23, 31, 39, 47, 48, 52, 53) and helps explain a long-forgotten controversy over which region was responsible for the syncytium-inducing phenotype (13). There are other important implications of these results. It may be difficult to generate programs for predicting coreceptor use based solely on V3 sequences (19, 37) if V1/V2 sequences impact V3 function. The ability of V1/V2 mutations to improve entry via CCR5 implies that the original envelope was not optimized for CCR5 use or that mutations that improve CD4 binding have a secondary impact on the efficiency of CCR5 use. Evolution of envelope function under selective pressure from neutralizing antibodies (38, 50) or high levels of chemokines (27) may select for less than optimal engagement of CCR5.

The analysis of infection mediated by the envelope mutants via CCR5:CXCR4 chimeric coreceptors yielded some expected and some unexpected results. As the number of mutations in each envelope increased, there was an expansion in the number of different chimeric coreceptors that were permissive for infection. This growing promiscuity in coreceptor engagement as the virus envelope evolves towards the X4 phenotype could be anticipated from the results of Pontow and Ratner (36). What is surprising is the variety of patterns of chimeric coreceptor use displayed by R5X4 intermediates (Tables 2 to 4). It is difficult to determine which domain of the chimeric coreceptor was critical for virus entry for ADA-3 mutants, because all retained some ability to utilize intact CCR5, but 12 out of 32 mutants could mediate infection via both CCR5 and CXCR4 (4444), and 3 of these 12 could only utilize CCR5 or CXCR4. One primary isolate, BR93019, had this phenotype in the prior study (36), so these results cannot be dismissed as the result of rare mutations that never emerge in patients. For the more complex ADA-1 series of mutations, there were 18 distinct combinations of chimeric coreceptor usage, and the interpretation of extracellular domain engagement was possible because many of the late-stage mutants lost the ability to mediate entry via intact CCR5. The EC2 domain of CXCR4 appeared to be critical for coreceptor function, since many of the infectious mutants with five to seven mutations had higher levels of infection on the 5545 target cell line than the target cells expressing intact CXCR4 (Table 3). Mutations in the V3 region of ADA appeared to influence the use of CXCR4 EC2. ADA-1 V3 mutations 4, 5, and 7 (S306R, P311R, and E320K) mediated equivalent infection of 5545 and 4444 target cells and could not infect 5555 target cells. V3 mutations 4, 6, 7 (S306R, G319E, and E320K) and 5, 6, 7 (P311R, G319E, and E320K) could infect 5555 target cells, but only the latter mutant showed robust infection of 5545 target cells. This result suggests that mutation 5 (P311R) at the tip of the V3 loop was important for engagement of EC2 of CXCR4, although this impact was context dependent. Hu et al. (16) reported that disruption of the V3 crown blocked utilization of EC2 and EC3 of CCR5 but not the N terminus, and the V3 crown appears to determine binding to cell surface CCR5 but not N-terminal

sulfopeptides (9). Our results are thus consistent with these previous findings and further suggest that changes at the crown of the V3 loop may be permissive for CXCR4 EC2 engagement and nonpermissive for CCR5 EC2 engagement.

Although we have sequentially introduced mutations associated with coreceptor switching into envelope, there is no assurance that coreceptor switching in vivo or in our previous experiments is driven solely by mutation. Recombination between HIV-1 genomes is estimated to exceed the rate of mutation (21, 43), and it is thus quite possible that recombination between two envelopes with distinct sets of viable mutations could contribute to the generation of coreceptor switch variants. We did observe some sequence heterogeneity during the selection of coreceptor switch variants (C. Pastore and D. E. Mosier, unpublished observations), but one of the sequences usually encoded the parental R5 envelope. It may be important to note that many mutations introduced into the V1/V2 region directly or indirectly improved CCR5-mediated entry, so they would be subject to positive selection under ordinary conditions but not when CCR5-expressing target cells were being replaced with CXCR4-expressing target cells (32). In vitro selection by target cell replacement would not be expected to mimic the fitness constraints and selective pressures on envelope evolution in patients, and it is possible that a wider array of mutations can be tolerated in vitro than in vivo.

It has recently been appreciated that fitness costs limit the ability of HIV-1 to mutate sequences encoding epitopes targeted by cytotoxic T lymphocytes (12, 33). Our data would suggest that entry fitness costs similarly limit the coreceptor switching process. Almost 25% of potential coreceptor switch intermediates had entry fitness reduced greater than 99%. Of the remaining 75% of possible intermediates, many were highly sensitive to coreceptor inhibitors or neutralizing antibodies (C. Pastore and D. E. Mosier, unpublished). Recognizing the combined challenge of maintaining competitive fitness while generating a distinct combination of envelope mutations that can be selected for improved CXCR4 use makes it easier to understand why coreceptor switching in patients usually takes years rather than days.

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