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Clinical resistance to vicriviroc through adaptive V3 loop mutations in HIV-1 subtype D gp120 that alter interactions with the N-terminus and ECL2 of CCR5

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ABSTRACT

The HIV-1 CCR5 co-receptor is a member of the chemokine receptor family of G-protein coupled receptors; for which a number of small molecule antagonists, such as vicriviroc (VCV), have been developed to inhibit HIV-1 R5-tropic replication. In this study, we analyzed an HIV-1 subtype D envelope gene from a clinical trial subject who developed complete resistance to VCV. The HIV-1 resistant envelope has six predominant amino acid changes in the V3 loop, together with one change in the C4 domain of gp120, which are fully responsible for the resistance phenotype. V3 loop mutations Q315E and R321G are essential for resistance to VCV, whereas E328K and G429R in C4 contribute significantly to the infectivity of the resistant variant. Collectively, these amino acid changes influenced the interaction of gp120 with both the N-terminus and ECL2 region of CCR5.

Introduction

HIV-1 infects CD4 positive target cells through one or both of two chemokine receptors—CC motif chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4). The initial interaction between the envelope glycoprotein gp120 homotrimers and CD4 induces a conformational change in gp120 that enables binding to either CCR5 or CXCR4 co-receptors (Wyatt and Sodroski, 1998). In the case of CCR5, the co-receptor binding domain in gp120 is formed largely by the V3 loop and C4 domain in the bridging sheet region (Huang et al., 2007, 2005; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Wyatt et al., 1998; Wyatt and Sodroski, 1998). The coordinated interactions between gp120 and the co-receptor produce additional conformational changes within the gp120/gp41 trimer that leads to triggering of the gp41 fusion peptide and subsequent fusion of virus and cell membranes.

Small molecule CCR5 co-receptor antagonists that block HIV-1 infection bind in a pocket formed by the transmembrane domains of CCR5. The drug-bound co-receptor locks into a conformation which subsequently inhibits R5-tropic HIV-1 entry of host cells (Dragic et al., 2000; Kondru et al., 2008; Maeda et al., 2006; Nishikawa et al., 2005; Seibert et al., 2006; Tsamis et al., 2003). One CCR5 antagonist, maraviroc (MVC), marketed in the US as Selzentry® is approved for use in both naïve and treatment-experienced adult patients (Gulick et al., 2008). Another CCR5 antagonist, vicriviroc (VCV), is currently in late-stage clinical trials (Gulick et al., 2007; Schurmann et al., 2007). Additional understanding of the biology of viral resistance mechanisms associated with this class of inhibitors and identification of signature genotypic changes in the envelope glycoprotein that impart resistance would help further optimize the clinical use of these agents.

Clinical failure with CCR5 co-receptor antagonists has most often correlated with the expansion of pre-existing, low-level X4 using virus (Gulick et al., 2008, 2007; Landovitz et al., 2008; Moore and Kuritzkes, 2009; Pfizer, Inc., 2007; Tsibris et al., 2008), but classical resistance to CCR5 antagonists has also been reported (Gulick et al., 2008, 2007; Pfizer, Inc., 2007; Tsibris et al., 2008). In phase II studies for VCV, 1/29 subjects who failed therapy in the ACTG A5211 study (Gulick et al., 2007; Tsibris et al., 2008) and 4/26 subjects in the P03802 trial (Landovitz et al., 2008) developed R5 variants with phenotypic resistance to VCV. How R5-resistant variants evolve in clinical subjects has not been well characterized. In the single study subject that was characterized, changes in the V3 loop of gp120 were

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sufficient to confer the resistance phenotype to the baseline envelope (Tsibris et al., 2008).

R5-tropic resistant variants selected *in-vitro* were shown to have adapted to use the drug-bound form of CCR5 (Marozsan et al., 2005; Moore and Kuritzkes, 2009; Ogert et al., 2008; Pugach et al., 2007; Tsibris et al., 2008; Westby et al., 2007) and recent findings show that two lab-adapted resistant viruses acquired increased dependence on the N-terminus of CCR5 for viral entry (Berro et al., 2009; Ogert et al., 2009). In this report, we describe the analysis of 7 mutations present in the CCR5 binding site region of gp120 (6 V3 loop mutations and 1 C4 mutation) identified in a clinical subject who developed complete phenotypic resistance to VCV while participating in a phase II trial. These mutations were systematically characterized for their effect on VCV resistance and interactions with CCR5.

Results

Subject 91 (S91) characteristics

S91 from clinical study P03802 (Landovitz et al., 2008) receiving a regimen of zidovudine + lamivudine in combination with 75 mg VCV QD had a baseline viral load of approximately 10^5 RNA c/ml. This subject experienced nearly complete viral suppression having a nadir viral load (97 c/ml) on day 57. This subject continued on treatment and maintained a stable viral load (10^3 c/ml) from week 14 until study discontinuation at week 48. The CD4 cell count for S91 increased from 188/mm^3 at baseline to 351/mm^3 at week 48. As previously reported (Landovitz et al., 2008), VCV plasma C_{min} concentrations exceeding 100 ng/ml were associated with virologic success and S91 had a VCV C_{min} plasma concentration of 95 ng/ml. HIV-1 from S91 was categorized as subtype D and at study discontinuation was reported to be R5-tropic by the Trofile™ assay (Whitcomb et al., 2007). Susceptibility to VCV was determined using the PhenoSense Entry™ assay; end of treatment viral pools exhibited phenotypic resistance to VCV with a maximum percent inhibition (MPI) of 34.5%. MPI values measured in pseudovirus drug susceptibility assays are used as a representative measure of resistance to co-receptor antagonists based on the nature of the allosteric effect these inhibitors have on CCR5 and the ability of HIV-1 to adapt by using the drug-bound form of CCR5 (Buontempo et al., 2009; Moore and Kuritzkes, 2009; Pugach et al., 2007, 2009; Westby et al., 2007).

Sequence analysis of S91 envelope clones

The HIV-1 envelope gene was amplified from samples obtained at baseline, week 24, and study discontinuation at week 48. Envelope sequences were analyzed for specific amino acid changes in the V3 loop and C4 domain which represent the principal CCR5 co-receptor binding regions in gp120 (Huang et al., 2007, 2005; Rizzuto et al., 1998; Wu et al., 1996; Wyatt et al., 1998). 12 clones from each time point were sequenced and the V3 loop amino acids for each clone are shown in Fig. 1A. The sequence of the V3 loop/bridging sheet region of S91 gp120 present in the majority of clones at week 48 are highlighted in the molecular model of the gp120-CD4 complex in Fig. 1B.

Sequence analysis of subtype D viruses in the Los Alamos HIV-1 database and the frequency of amino acids at each of these S91 positions were determined. The subtype D analysis showed the following results: positions 309-F (0%), L (4.4%), I (82.2%); 314-G (92.9%), W(0%); 315-Q (54.1%), E (0.6%); 317-L (48.3%), F (21.1%); 320-D (3.1%); 321-R (14.6%), G (22.8%), K (22.8%); 328-E (0.7%), K (7.6%), Q (65.1%); and 429-G (61.2%), R (3.7%). Of special note, the D (N) amino acid at position 320 in V3 was present in only 43/1367 subtype D envelope sequences in the public HIV-1 database and therefore can be considered a rare insertion mutation.

Analysis of $S91_{Baseline}$ and $S91_{Wk\ 48}$ replication competent HIV-1 produced with pNL4-3/S91 gp160 provirus constructs

Replicating cultures of $S91_{Baseline}$ and $S91_{Wk\ 48}$ HIV-1 were analyzed for susceptibility to VCV in U87-CD4-CCR5 cells. VCV dose–response curves for $S91_{Baseline}$ HIV-1 showed susceptibility to VCV with an IC_{50} of 1.3 nM; whereas, $S91_{Wk\ 48}$ HIV-1 was completely resistant to VCV with a MPI of −130% (Fig. 2A). Resistance was characterized by replication enhancement in the presence of VCV at concentrations ≥100 nM.

Pseudoviruses generated with chimeric ADA envelopes demonstrate phenotypes similar to replication competent S91 HIV-1

We previously found that replacing the C2-V5 region of gp120 in a VCV-susceptible R5-tropic ADA envelope with the corresponding region derived from a lab-adapted VCV-resistant RU570 HIV-1 virus was sufficient to confer resistance to VCV (Ogert et al., 2009, 2008). Using a similar strategy, we generated chimeric ADA envelopes with the C2-V5 region derived from $S91_{Baseline}$ and $S91_{Wk\ 48}$ clones. $S91_{Baseline}$ pseudovirus was completely susceptible to VCV (IC_{50} 0.5 nM; MPI 100%), whereas $S91_{Wk\ 48}$ pseudovirus was completely resistant to VCV with a MPI of −75% (Fig. 2B). The VCV-enhanced pseudovirus entry phenotype correlates with the replication enhancement of $S91_{Wk\ 48}$ HIV-1 in the presence of VCV.

To determine if the V3 loop alone would confer resistance to VCV; we substituted the ADA V3 loop with the V3 loop from $S91_{Baseline}$ or $S91_{Wk\ 48}$ envelope. HIV-1 pseudoviruses generated with these envelopes were tested for susceptibility to VCV. As shown in Fig. 2C, both ADA and ADA V3 ($S91_{Baseline}$) pseudoviruses were completely susceptible to VCV (IC_{50} 0.2 nM). However, when the V3 loop sequence of ADA was replaced with the V3 loop from $S91_{Wk\ 48}$ envelope (ADA V3 $S91_{Wk\ 48}$), HIV-1 pseudoviruses were completely resistant to VCV with a MPI of −50%.

We next asked if envelope clones amplified at week 24 might also be resistant to VCV. The majority of envelope clones (7/12) at week 24 contain a nearly identical sequence as week 48 without a Q315E mutation in the crown region of the V3 loop (Fig. 1A). To analyze this V3 loop sequence present at week 24, an E315Q back mutation was generated in the $S91_{Wk\ 48}$ envelope. Pseudoviruses produced were characterized by VCV dose–response curves showing a MPI plateau level at 91% (Table 1), thus demonstrating only a minimal reduction in susceptibility to VCV. In order to analyze the other V3 loop sequence found in 5/12 week 24 clones, step-wise forward mutagenesis was used to construct this sequence within the context of the baseline clone. Pseudoviruses produced with this $S91_{Wk\ 24}$ V3 loop containing a G314W mutation (Fig. 1A) demonstrated complete resistant to VCV with a MPI of −25% (Table 1). However, by week 48, only 25% of the envelope clones retained this specific V3 loop sequence (Fig. 1A). Because envelope clones containing the Q315E crown mutation were predominant at week 48, we focused the following studies using this envelope.

Phenotypic resistance to VCV is reproduced in the $S91_{Baseline}$ envelope with a combination of 4 specific amino acid substitutions in the V3 loop

To establish the contribution for each V3 loop mutation on resistance to VCV, forward amino acid substitutions were systematically introduced into the $S91_{Baseline}$ envelope. The $S91_{Baseline}$ envelope with a single amino acid substitution (Q315E, R321G or E328K) in V3 was still completely susceptible to VCV with a MPI at 100% and an IC_{50} similar to $S91_{Baseline}$ pseudovirus. $S91_{Baseline}$ Q315E/L317F, $S91_{Baseline}$ D320N/R321G, and $S91_{Baseline}$ F309L/L317F/D320N envelopes were also completely susceptible to VCV when analyzed in pseudovirus assays (Table 1). However, resistance to VCV was established with specific combinations of ≥3 substitutions in the $S91_{Baseline}$ envelope. The level of resistance to VCV varied based on the plateau level in the

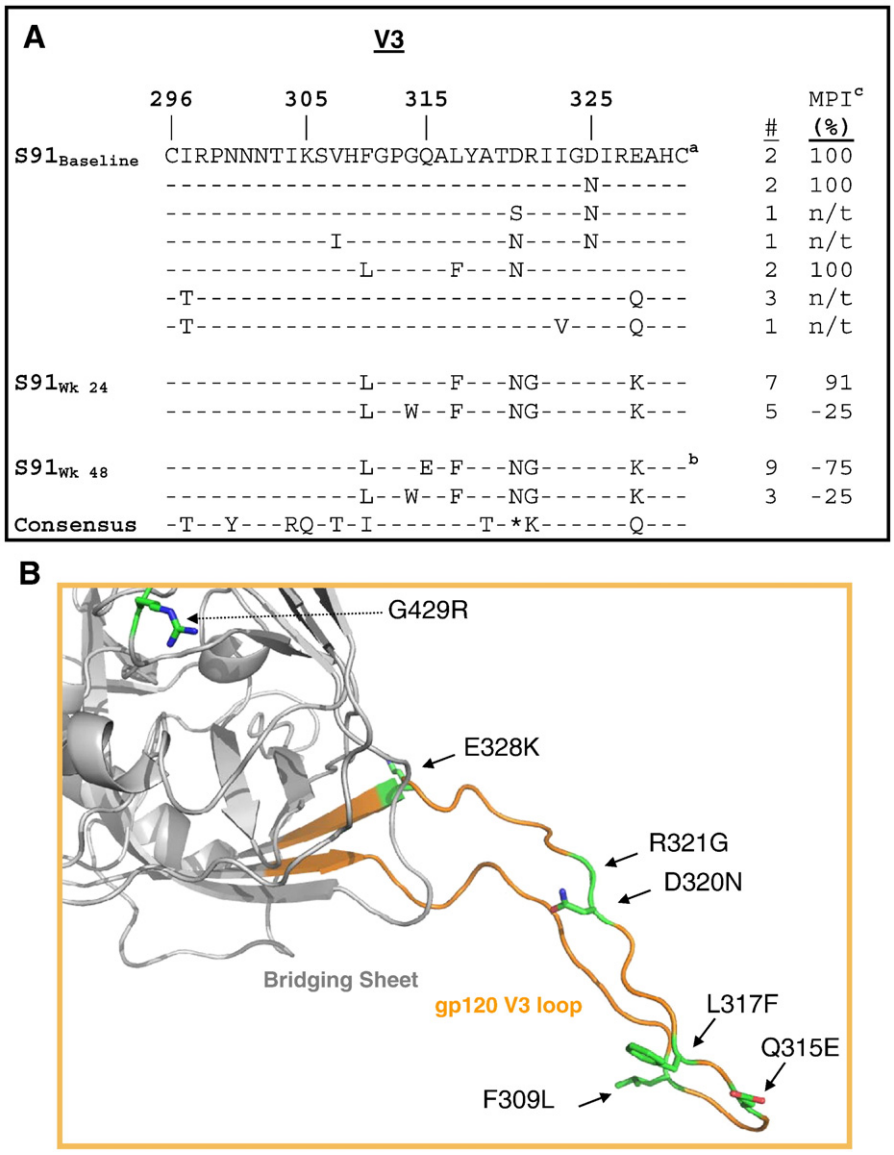

Fig. 1. A) The V3 loop sequence of 12 individual envelope clones amplified from S91 at each time point: baseline, week 24 and week 48. Amino acid numbering is based on the amino acid coordinates for the HXB2 reference strain. The # clones identified with the specific V3 loop sequence are shown. The [a]$S91_{Baseline}$ and [b]$S91_{Wk\ 48}$ clones used in HIV-1 pseudovirus assays for characterizing resistance to VCV. [c]The maximum percent inhibition (MPI) values determined from VCV dose–response curves using HIV-1 pseudoviruses ($n \geq 3$) generated with each of the designated envelopes are shown (n/t = not tested). The consensus sequence determined from analysis of subtype D viruses present in the public database (*—represents no amino acid present). B). Molecular model of Subject 91 gp120/CD4 complex with designated amino acid changes that occurred between baseline and week 48 in the majority of envelope clones sequenced.

MPI for each specific combination tested. Pseudoviruses with the $S91_{Baseline}$ envelope containing Q315E/D320N/R321G, Q315E/L317F/D320N/R321G, or Q315E/D320N/R321G/E328K forward mutations were resistant to VCV with a MPI plateau level of 66%, 83%, and 33%, respectively. The Q315E/D320N/R321G/E328K combination in the $S91_{Baseline}$ envelope produced nearly complete resistance to VCV; however, this combination was unique showing increased virus infectivity compared to either $S91_{Baseline}$ or $S91_{Wk\ 48}$ pseudovirus (data not shown).

By adding additional mutations, further variations in both resistance and virus infectivity were observed. F309L in combination with the Q315E/L317F/D320N/R321G mutations produced a substantial increase in resistance to VCV with a MPI at −385% (Table 1), however virus infectivity was reduced >100-fold (data not shown). The addition of L317F to the Q315E/D320N/R321G/E328K combination produced a similar MPI plateau level (Table 1), whereas all 6V3 loop substitutions together with G429R in C4 reproduced a VCV resistance phenotype that matched $S91_{Wk\ 48}$ pseudovirus (Fig. 2B and Table 1).

Subject 91 VCV-resistant virus remains R5-tropic

To determine if any of the V3 mutations acquired in S91 HIV-1 during treatment allowed for CXCR4 usage, we assayed the ability of pseudoviruses generated with baseline, week 48, and baseline envelopes containing different forward substitutions for infection of U87-CD4-CXCR4 cells (Table 1). The HXB2 CXCR4 positive control produced high relative light units (RLU) in U87-CD4-CXCR4 cells (10^6 RLU) that was reduced to background RLU (10^3 RLU) with 1 μM CXCR4 antagonist AMD3100. The RLU results for ADA R5-tropic pseudovirus were also similar to the background RLU. Pseudoviruses generated with $S91_{Baseline}$, $S91_{Wk\ 48}$, and $S91_{Baseline}$ envelopes containing different forward substitutions did not infect U87-CD4-CXCR4 cells, producing RLU results similar to background (10^3 RLU).

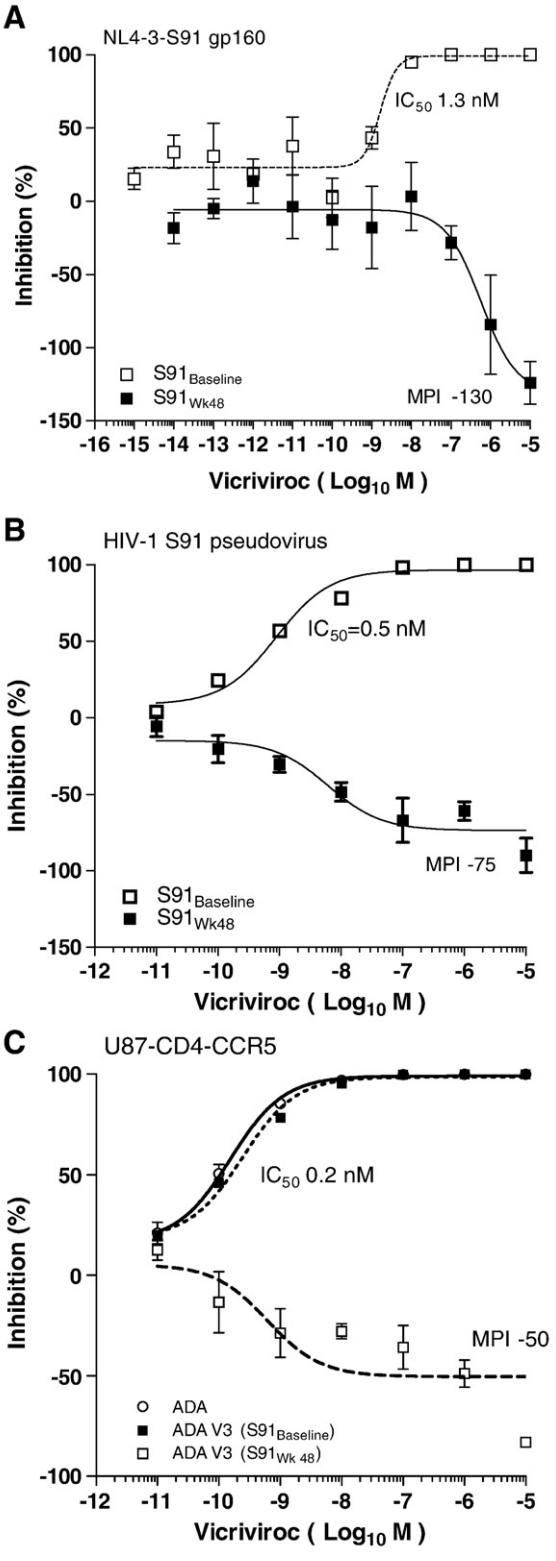

Fig. 2. A) VCV dose–response curves for $S91_{baseline}$ and $S91_{Wk\ 48}$ replication competent HIV-1. Data is average ± SD of triplicates and is representative of 2 independent assays. B) VCV dose–response curves for S91 ADA C2-$V5_{Baseline}$ and S91 ADA C2-$V5_{Wk\ 48}$ envelopes. Data represents the average ± SD of 4 replicates and is representative of 5 independent assays. C). VCV dose–response curves for ○—ADA, ■—ADA V3 ($S91_{Baseline}$), and □—ADA V3 ($S91_{Wk\ 48}$) HIV-1 pseudoviruses. Data is the average ± SD of 4 replicates and represents results from 2 independent assays.

Table 1
Summary of results for single and combined VCV resistance mutations generated in the $S91_{Baseline}$ envelope.

Forward mutations	MPI	EC_{50} (nM)	Tropism[a]
$S91_{Baseline}$ (CIRPNNNTIKSVHFGPGQALYATDRIIGDIREAHC)	100	0.9	R5
Q315E	100	0.2	
Q315E/L317F	100	0.4	
R321G	100	0.4	
D320N/R321G	100	0.8	
E328K	100	1.0	
F309L/L317F/D320N	100	0.8	
Q315E/D320N/R321G	66	0.1	R5
Q315E/L317F/D320N/R321G	83	0.1	R5
Q315E/D320N/R321G/E328K	33		R5
Q315E/L317F/D320N/R321G/E328K	33		R5
F309L/Q315E/L317F/D320N/R321G	−385		R5
F309L/Q315E/L317F/D320N/R321G/E328K	−250		R5
F309L/G314W/L317F/D320N/R321G/E328K[b]	−25		
F309L/L317F/D320N/R321G/E328K/G429R[b,c]	91	5.1	
F309L/Q315E/D320N/R321G/E328K/G429R[d]	73	0.1	
F309L/Q315E/L317F/D320N/E328K/G429R[e]	100	0.1	
F309L/Q315E/L317F/D320N/R321G/E328K/G429R	−60		R5
$S91_{Wk\ 48}$ (CIRPNNNTIKSVH**L**GPG**EAF**YAT**NG**IIGDIR**K**AHC)	−75		R5
ADA	100	0.2	R5
HXB2			X4

Bold data represent 6 V3-loop Mutations Found at Week 48.
[a] Tropism determined in U87-CD4-CCR5 and U87-CD4-CXCR4 cells.
[b] Week 24 V3 loop sequence.
[c] Reverse mutation E315Q in $S91_{Wk\ 48}$ envelope.
[d] Reverse mutation F317L in $S91_{Wk\ 48}$ envelope.
[e] Reverse mutation G321R in $S91_{Wk\ 48}$ envelope.

These results are consistent with the R5 tropism determined for $S91_{Wk\ 48}$ viral pools analyzed using the Trofile™ assay.

S91 VCV-resistant HIV-1 is more sensitive than $S91_{Baseline}$ virus to inhibition with Mabs that specifically recognize either the N-terminus or ECL2 region of CCR5

Monoclonal antibodies (Mabs) 2D7 and CTC5 were produced following immunization with human CCR5 and have been previously shown to specifically recognize either ECL2 or the N-terminus of CCR5, respectively (Lee et al., 1999; Wu et al., 1997). In this study, these Mabs were used to neutralize S91 pseudovirus infections in U87-CD4-CCR5 cells in the presence and absence of VCV. $S91_{Wk\ 48}$ virus was inhibited to a similar degree by CTC5 anti-N-terminus Mab in the presence or absence of VCV, as shown in Fig. 3A. Approximately 75% of the virus infection was neutralized by the CTC5 Mab. In contrast, $S91_{Baseline}$ virus was not susceptible to neutralization by CTC5 Mab.

Mab 2D7 which recognizes CCR5 ECL2 was previously shown to inhibit entry of numerous R5-tropic strains of HIV-1 (Wu et al., 1997). In Fig. 3B, complete inhibition of $S91_{Baseline}$ virus was not achieved with Mab 2D7 at concentrations ≤25 μg/ml. However, $S91_{Wk\ 48}$ virus was completely inhibited by Mab 2D7 at these concentrations (IC_{50} 0.1 nM). As the inset in Fig. 2B shows, when pseudovirus stocks are normalized to p24 and equivalent amounts of virus are assayed, $S91_{Baseline}$ virus is 4-fold more infectious for unbound CCR5 than is $S91_{Wk\ 48}$ virus. This provides a rationale for the reduced sensitivity of the $S91_{Baseline}$ virus with this Mab. In a similar vein, $S91_{Wk\ 48}$ virus infectivity is increased >2-fold with drug-bound CCR5 (inset), thus making it more difficult to inhibit $S91_{Wk\ 48}$ virus infection with MAb 2D7 in the presence of VCV (Fig. 3B).

Subject 91 VCV-resistant virus acquires an increase in dependence on the N-terminus of CCR5

To probe specific interactions between $S91_{Baseline}$ and $S91_{Wk\ 48}$ pseudovirus with CCR5, single point mutations Y3A, Y10A, D11A,

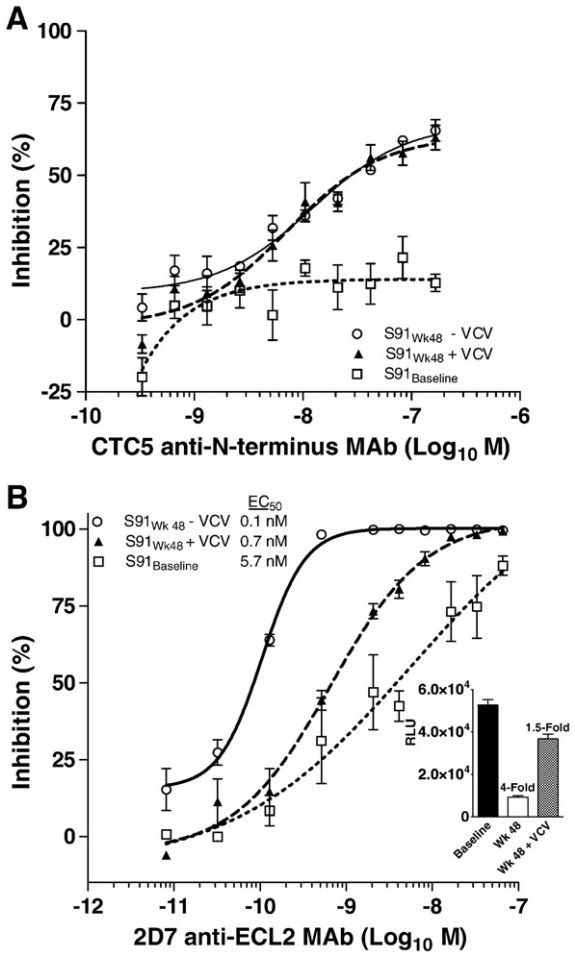

Fig. 3. Antibody neutralization of HIV-1 pseudoviruses with A) CTC5 anti-N-terminus MAb and B) 2D7 anti-ECL2 MAb. ○—$S91_{Wk\ 48}$, ▲—$S91_{Wk\ 48}$ + 10 μM VCV, and □—$S91_{Baseline}$ HIV-1 pseudovirus. Inset: Infectivity of $S91_{Baseline}$ and $S91_{Wk\ 48}$ HIV-1 pseudovirus determined using equivalent levels of HIV-1 pseudovirus from p24 normalized stocks in U87-CD4-CCR5 cells in the absence or presence (+VCV) of vicriviroc. Data represents the average ± SD of 4 replicates and represents results of 3 independent assays.

N13A, Y14A, and Y15A in the N-terminus of CCR5 and an N-terminal (Δ 2–17) CCR5 deletion mutant were constructed and analyzed. The infectivity of $S91_{Baseline}$ and $S91_{Wk\ 48}$ pseudoviruses was assessed in 293T cells transiently expressing CD4 and each CCR5 mutant. The expression levels on the surface of 293T cells for each CCR5 mutant as well as co-expressed CD4 was determined by FACS using anti-CCR5 and anti-CD4 Mabs. The cell surface expression observed for CD4 and each CCR5 mutant was similar to wt CCR5/CD4 expression (data not shown). In addition, we previously reported the effects of each mutation on ADA pseudovirus entry as a control (Ogert et al., 2009).

Fig. 4A shows the amino acids in the N-terminus of CCR5 based on the structural model of the G-protein coupled β2-adrenergic receptor (Cherezov et al., 2007). Each of the single point mutations that were analyzed is designated. As shown in Fig. 4B, $S91_{Baseline}$ pseudovirus infection was reduced by only 25% with either the Y3A or D11A mutations compared to wt CCR5, whereas infection of cells expressing the Y10A, N13A, Y14A, and Y15A mutations was reduced further to approximately 40–50% of wt CCR5 infection, whereas $S91_{Baseline}$ pseudovirus was unable to infect cells expressing the CCR5 Δ 2–17 deletion mutant. In terms of the $S91_{Wk\ 48}$ VCV-resistant pseudovirus, this virus was unable to infect cells expressing CCR5 with Y10A, D11A, Y14A, or Y15A single point mutations either in the presence (data not shown) or absence (Fig. 4C) of VCV. In contrast, infectivity >50% of wt CCR5 infection was observed with cells expressing either the CCR5 Y3A or N13A mutation. Similar to the $S91_{Baseline}$ virus, $S91_{Wk\ 48}$ VCV-resistant pseudovirus was unable to infect cells expressing the CCR5 Δ 2–17 mutant.

Molecular model predicts specific amino acids in the V3 loop of the VCV-resistant envelope that make direct contacts with residues in the N-terminus of CCR5

The potential structural implications of the $S91_{Wk\ 48}$ adaptive mutations in V3 were analyzed in structural models with either $S91_{Baseline}$ or $S91_{Wk\ 48}$ gp120/CD4 complex and the $CCR5^{2-15}$ N-terminus peptide. We used Discovery Studio (Accelrys, San Diego, CA) to construct homology models based on the previously reported structure of the HIV-1 YU-2 gp120-CD4 complex docked with the $CCR5^{2-15}$ N-terminal peptide (Huang et al., 2007) and structural models of CCR5 diagramed in Figs. 4A and 7B were based on the previously published structural model of the G-protein coupled β2-adrenergic receptor (Cherezov et al., 2007).

In the published structural model of the N-terminal peptide, the C4 amino acid $Arg440^{gp120}$ in YU-2 forms an ionic interaction with Asp11 in the N-terminus of CCR5 (Huang et al., 2007). In models constructed with the $S91_{Baseline}$ and $S91_{Wk\ 48}$ envelopes, a similar interaction between $Lys440^{gp120}$ in C4 and Asp 11 in the N-terminus of CCR5 is also predicted (Figs. 5A and B). However, in the $S91_{Wk\ 48}$ envelope the additional D320N/R321G mutations present in the stem region of the returning strand of the V3-loop allow for the formation of an additional H-bond between Asn 320^{gp120} and Asp 11 in the N-terminus (Fig. 5B). This interaction is facilitated by the R321G mutation since Gly 321^{gp120} would relieve conformational constraints imposed by Arg 320^{gp120} present in the $S91_{Baseline}$ envelope. The Gly 321^{gp120} residue in the resistant envelope enables the rotation of $S91_{Wk\ 48}$ Asn 320^{gp120} allowing it to form an H-bond with Asp 11.

The model also predicts that the E328K mutation in the base region of V3 in the VCV-resistant envelope forms a new intra-molecular contact within V3 (Fig. 5B). In the $S91_{Baseline}$ structural model, Glu 328^{gp120} and Asp 325^{gp120} both stabilize the highly conserved Arg 419^{gp120} residue in C4 (Fig. 5A). However, in the $S91_{Wk\ 48}$ envelope, Glu 328^{gp120} is replaced by $Lys328^{gp120}$ which enables contact with $Asp325^{gp120}$ by rotating away from Arg 419^{gp120} in C4. Arg 419^{gp120} is then able to form a new ionic interaction with the negatively charged O-sulfated tyrosine at position 10 in the N-terminus (Fig. 5B). The specific interactions predicted by these models are consistent with results obtained when single alanine substitutions in the N-terminus of CCR5 were made. Both Y10A and D11A abolished S91 VCV-resistant pseudovirus entry, whereas $S91_{Baseline}$ pseudovirus entry was only reduced approximately 50% compared to wt CCR5. Overall, the additional contacts predicted for the D320N and E328K adaptive mutations in conjunction with the interaction of R419 with Y10 support an improved affinity between VCV-resistant gp120 and the N-terminus of CCR5.

HIV-1 gp120 mutations at residues predicted to interact with the N-terminus of CCR5 disrupt virus infectivity

To probe the contacts in gp120 predicted in the molecular models, single alanine substitutions at positions 325, 327, 419 and 440 in VCV-resistant gp120 were generated. Pseudoviruses produced with these envelopes were analyzed together with $S91_{Wk\ 48}$, $S91_{Wk\ 48}$ N320D, $S91_{Wk\ 48}$ G321R, and $S91_{Wk\ 48}$ K328E pseudoviruses in U87-CD4-CCR5 cells using p24 normalized pseudovirus stocks. Pseudovirus infections in the absence (Fig. 6, left panel) or presence of 10 μM VCV (Fig. 6, right panel) were compared to VCV-resistant $S91_{Wk\ 48}$ pseudovirus. Pseudoviruses generated with the $S91_{Wk\ 48}$ envelope containing either the D325A or R327A mutation were completely inactive in U87-CD4-CCR5 cells; whereas the K440A mutation reduced pseudovirus infectivity by 10-fold and both N320D and K328E mutations reduced infectivity 5-fold. The G321R mutation reduced virus infectivity by <2-

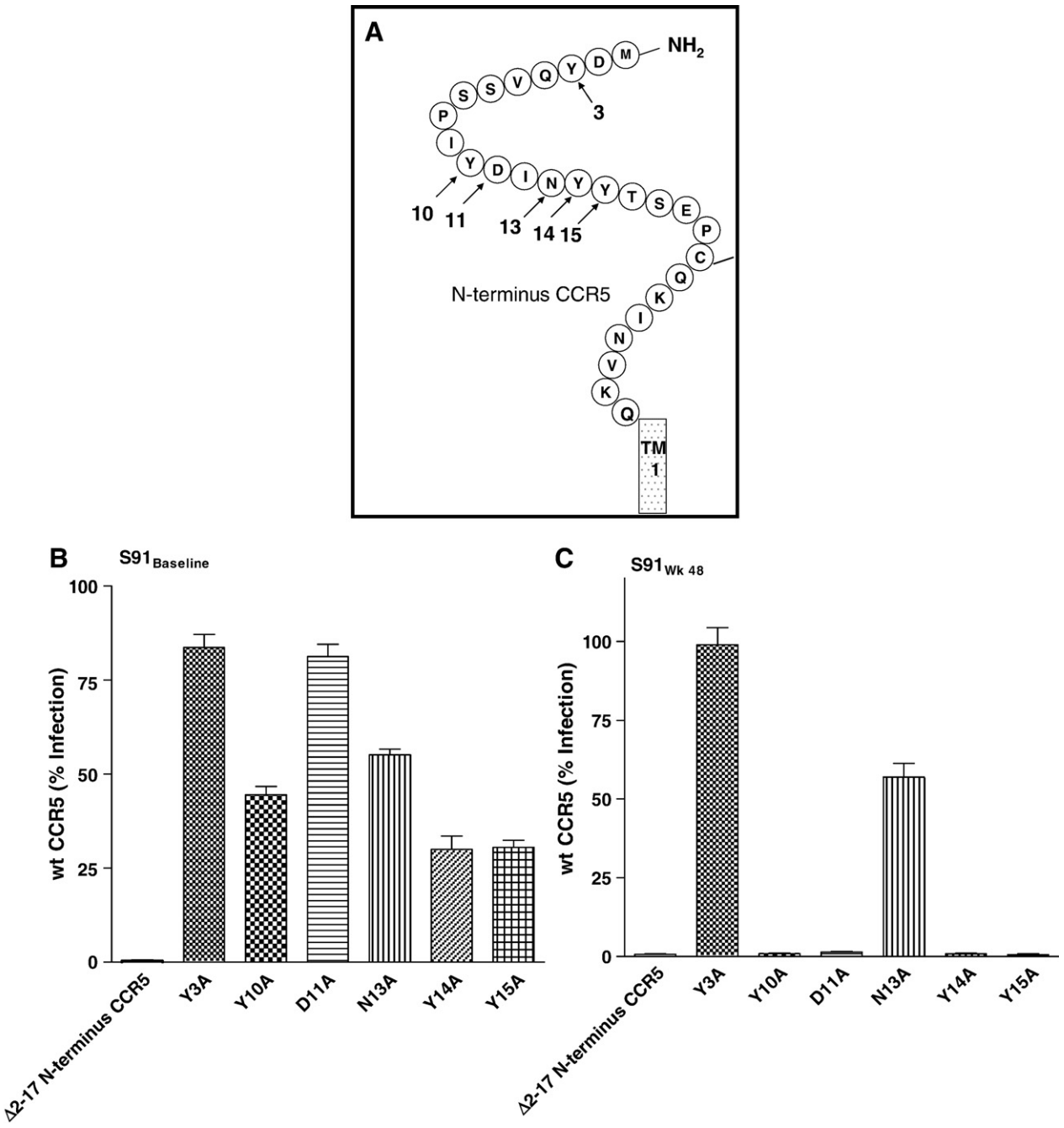

Fig. 4. The effect of single alanine substitutions in the N-terminus of CCR5 on HIV-1 entry. A) Single alanine substitutions Y3A, Y10A, D11A, N13A, Y14A, and Y15A in the N-terminus of CCR5 are depicted in a molecular diagram based on the previously published structural model of the G-protein coupled β2-adrenergic receptor (Cherezov et al., 2007). The results of each single alanine substitution and a Δ 2–17 N-terminal amino acid mutant on B) $S91_{Baseline}$ and C) $S91_{Wk\ 48}$ HIV-1 pseudovirus entry expressed as a percentage of wt CCR5 infection are the average ± SD of ≥5 independent assays.

fold for CCR5 and also restored complete susceptibility to VCV (Table 1). In the presence of 10 μM VCV, both N320D and K440A mutations reduced infectivity 10-fold, whereas the K328E mutation reduced infectivity 3-fold with drug-bound CCR5. The R419A mutation had the greatest impact on pseudovirus infections reducing infectivity >50-fold with unbound CCR5 and 10-fold for the drug-bound co-receptor. The amino acid at position 419 in the resistant envelope is predicted to form a new contact with the negatively charged sulfotyrosine at position 10 in the N-terminus of CCR5. In agreement with this prediction, making a conservative R419K mutation only reduced infection 2-fold with CCR5, and virus entry via drug-bound CCR5 was unaffected.

In summary, single mutations at residues predicted to be associated with binding of gp120 to the N-terminus of CCR5 predicted by our model primarily affected the overall fitness of envelope on virus entry. The N320D and K328E back mutations, as well as R327A, R419A, and K440A alanine substitutions compromised virus infectivity with both the drug-free and drug-bound conformations of CCR5. Together these results support the validity of the gp120 contacts predicted in the structural homology model to interact with residues in the N-terminus of CCR5.

K191A mutation in ECL2 disrupts VCV-resistant virus entry

To probe for specific contacts within the extra-cellular loop regions of CCR5, single point mutations in both ECL2 and ECL3 were analyzed. Single amino acid substitutions E172A, L174A, and C178A in ECL2

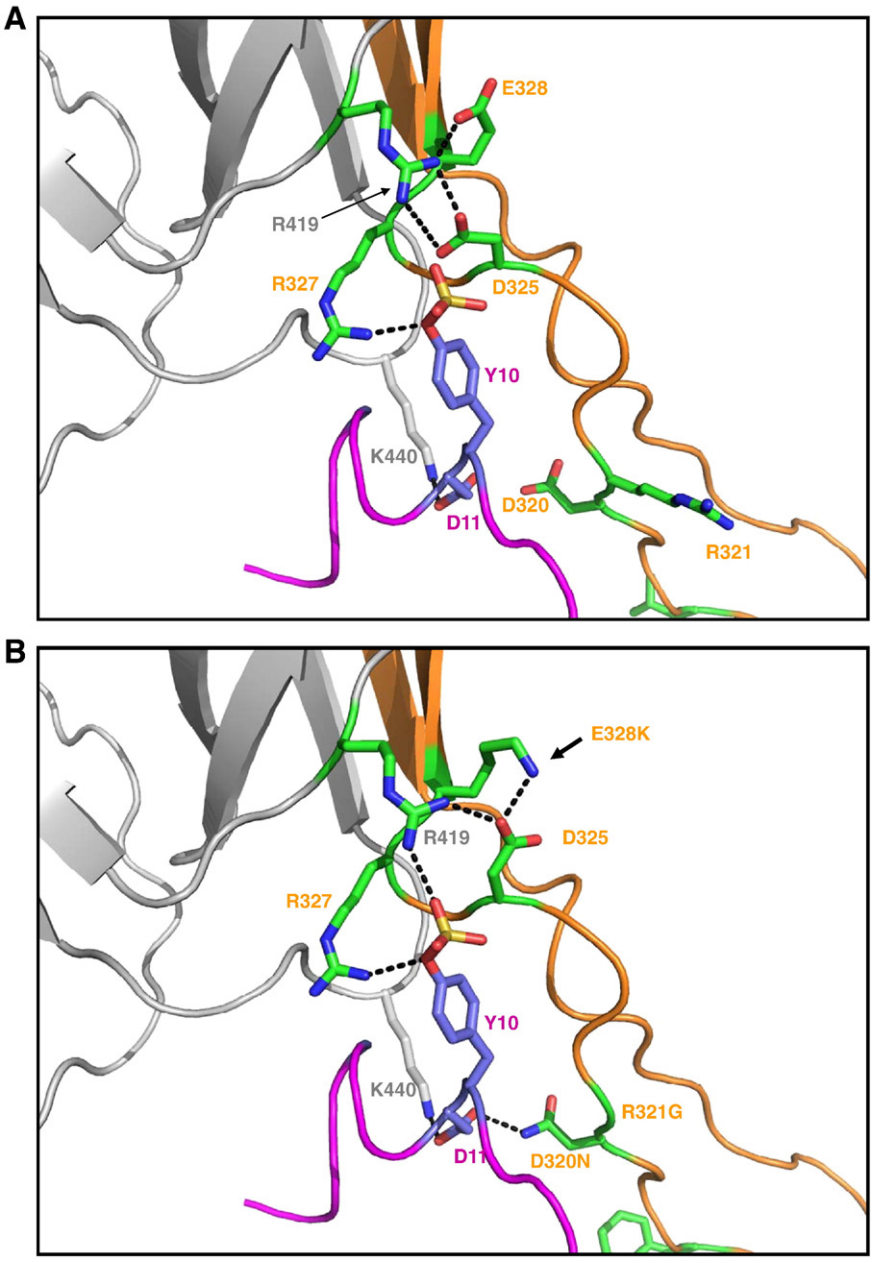

Fig. 5. Molecular model of A) S91 Baseline and B) S91 VCV-resistant gp120 in complex with CD4 and CCR5$^{2-15}$ N-terminus peptide based on coordinates previously reported for a YU2/CD4 complex docked to the CCR5$^{2-15}$ N-terminus peptide (Huang et al., 2007).

were generated. In addition, based on an assumption that the Q315E crown mutation could potentially interact with CCR5 through formation of a salt bridge with a positively charged amino acid, single alanine substitutions at each basic amino acid residue present in ECL2 (R168A, K171A, H175A, H181A, and K191A), ECL3 (R274A), and transmembrane domain 5 (K197A) of CCR5 were also analyzed.

Fig. 7A highlights the amino acid residues in the ECL2 region of CCR5 that were changed to alanine. The ELC2 region shown in this diagram is derived from a structural model of CCR5 based on the G-protein coupled β2-adrenergic receptor (Cherezov et al., 2007). The effect of each single alanine substitution on S91$_{\text{Baseline}}$ virus entry expressed as a percentage of wt CCR5 activity is represented in Fig. 7B. CCR5 C178A and H181A were the only ECL2 mutations that reduced virus infectivity to ≤50% of wt CCR5 infectivity. The C178 residue was previously shown to link ECL1 to ECL2 (Blanpain et al., 1999) and likely disrupts the tertiary structure of ECL2. In ECL3, R274A had no affect on entry of S91$_{\text{Baseline}}$ virus.

The effects of each single alanine substitution on VCV-resistant S91$_{\text{Wk 48}}$ virus entry are depicted in Fig. 7C. The C178A mutation had a similar effect on virus entry as was observed with S91$_{\text{Baseline}}$ virus (≤25% wt CCR5 infection); whereas, the K191A mutation located at the junction of ECL2 and the TM-5 α-helix of CCR5, reduced infectivity of VCV-resistant S91$_{\text{Wk 48}}$ virus to <50% of wt CCR5 infection for the unbound receptor (Fig. 7C, left panel). What is most remarkable is that the K191A substitution in ECL2 completely disrupted VCV-resistant virus entry with drug-bound CCR5 (Fig. 7C, right panel). Also, we observed no effect on ADA virus entry for the K191A mutation in CCR5 (data not shown).

Discussion

The characterization of HIV-1 in S91 demonstrated that multiple mutations in the V3 loop were needed to acquire resistance to VCV. While a minimum of 3 amino acid changes in the S91$_{\text{Baseline}}$ envelope

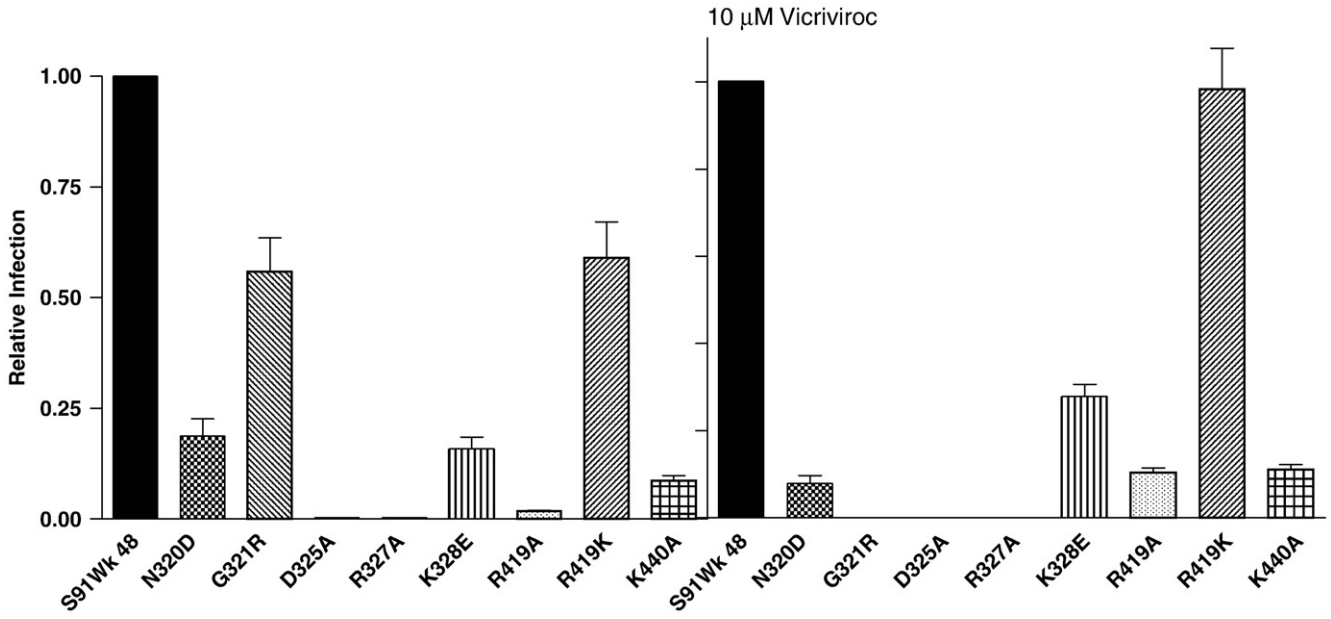

Fig. 6. Infectivity of HIV-1 pseudoviruses expressed as the relative infection of $S91_{Wk\ 48}$ pseudovirus. Single mutations were generated in the $S91_{Wk\ 48}$ envelope and virus infectivity is expressed relative to $S91_{Wk\ 48}$ virus in the absence (left panel) or presence of VCV (right panel). Data is the average ± SD of 4 replicates and results are representative of 3 independent assays.

(Q315E/D320N/R321G) produced partial resistance, 4 amino acid changes (Q315E/D320N/R321G/E328K) or more were required for nearly complete phenotypic resistance. Although the Q315E/D320N/R321G/E328K combination produced a phenotype with nearly complete resistance, this combination was not present in any of the envelope clones amplified from S91 HIV-1. Because the F309L/L317F/D320N combination was common to all week 24 and week 48 clones, it is likely the 2 clones at baseline with this combination (Fig. 1A) represent the founder sequence from which the fully resistant clones developed. It is interesting that the additional Q315E and R321G mutations produced a highly resistant virus but infectivity was particularly low with the drug-free receptor. The addition of E328K and G429R resulted in a virus that was completely resistant to VCV and also replicated efficiently using the drug-free receptor.

The S91 VCV-resistant HIV-1 is a subtype D virus and is a new subtype for which resistance to VCV has not been previously characterized. A subtype C virus was previously characterized and mutations in the V3 loop alone were sufficient to confer resistance to VCV (Tsibris et al., 2008). While the pattern of amino acid mutations in the two subtypes is quite different, both resistant subtypes have 3 amino acid changes in the stem region of the returning strand of the V3 loop. Resistance to VCV in this subtype D HIV-1 results in part from an increased reliance on the N-terminus of CCR5 during virus entry. This finding is similar to previous findings for two lab-adapted HIV-1 resistant viruses (Berro et al., 2009; Ogert et al., 2009). Previously, we demonstrated that a lab-adapted RU570 VCV-resistant virus forms two new H-bonds with the N-terminus of CCR5 through a K305R mutation in V3 and P437S mutation in C4 (Ogert et al., 2009). It is interesting that the S91 VCV-resistant virus is also predicted to make two new contacts with the N-terminus of CCR5, but as the result of 3 different V3 loop mutations: D320N, R321G, and E328K. If other combinations of V3 loop changes can also impart structural changes that enhance binding to the N-terminus of CCR5, this helps explain why a signature pattern of resistance mutations has yet to be identified for VCV or other small molecule CCR5 antagonists. Structural modeling and mutagenesis studies of additional HIV-1 isolates from subjects resistant to CCR5 antagonists will help determine the importance of enhanced recognition of the N-terminus of CCR5 for clinical resistance.

We also show that resistance to VCV in S91 is dependent on an improved recognition of the ECL2 region of CCR5 as demonstrated using MAb 2D7 which specifically recognizes an epitope in the N-terminal region of ECL2 in CCR5. Previous results have shown that binding of VCV to CCR5 had essentially no effect on binding of Mab 2D7 to CCR5 (Ji et al., 2007) nor did several other small molecule CCR5 antagonists (Maeda et al., 2008). $S91_{Wk\ 48}$ virus is more susceptible to inhibition with Mab 2D7 in the absence of VCV than it is in the presence of a saturating concentration of VCV. The increased infectivity observed for the $S91_{Wk\ 48}$ resistant virus with drug-bound CCR5 is most likely mediated by an improved recognition of an altered CCR5 configuration induced by VCV. This, in turn, would make it more difficult to inhibit the $S91_{Wk\ 48}$ resistant virus with MAb 2D7 when the receptor is in a drug-bound state. Also, our ECL2 mutagenesis studies show that the K191A mutation in ECL2 ablates $S91_{Wk\ 48}$ virus entry only when the receptor is in the drug-bound state. Preliminary homology models predict a potential for the formation of a salt bridge between E315 in the V3 crown of $S91_{Wk\ 48}$ gp120 and K191 located at the junction of ECL2 and the fifth transmembrane domain of CCR5. Additional mutagenesis/structural modeling studies are ongoing in an attempt to confirm this hypothesis and the determination of additional interactions with other specific residues in the extra-cellular regions of CCR5 that may be required for $S91_{Wk\ 48}$ entry.

Berro et al. (2009) propose that 4 V3-loop amino acid mutations in a lab-adapted VCV-resistant HIV-1 CC101.19 impair interactions between the V3-crown and CCR5 ECL2, while interactions with the N-terminus of CCR5 are enhanced. The enhanced binding of HIV-1 CC101.19c17 envelope to the N-terminus is suggested to compensate for a loss in the interaction between the V3 tip and ECL2 in CCR5 (Berro et al., 2009). This result is consistent with studies showing that deletions in the V3-loop of dual-tropic, HIV-1 R3A (Laakso et al., 2007; Nolan et al., 2009; Nolan et al., 2008) and HIV-2 VCP (Lin et al., 2007) impart complete resistance to co-receptor antagonists suggested to be a result of a loss of interaction between the V3 loop and CCR5 ECL2 (Agrawal-Gamse et al., 2009; Laakso et al., 2007; Nolan et al., 2009; Nolan et al., 2008). However, more recent findings have shown that adaptive mutations that allowed efficient replication of HIV-1 R3A V3 (9, 9) produce an enhanced interaction with CD4 (Agrawal-Gamse et

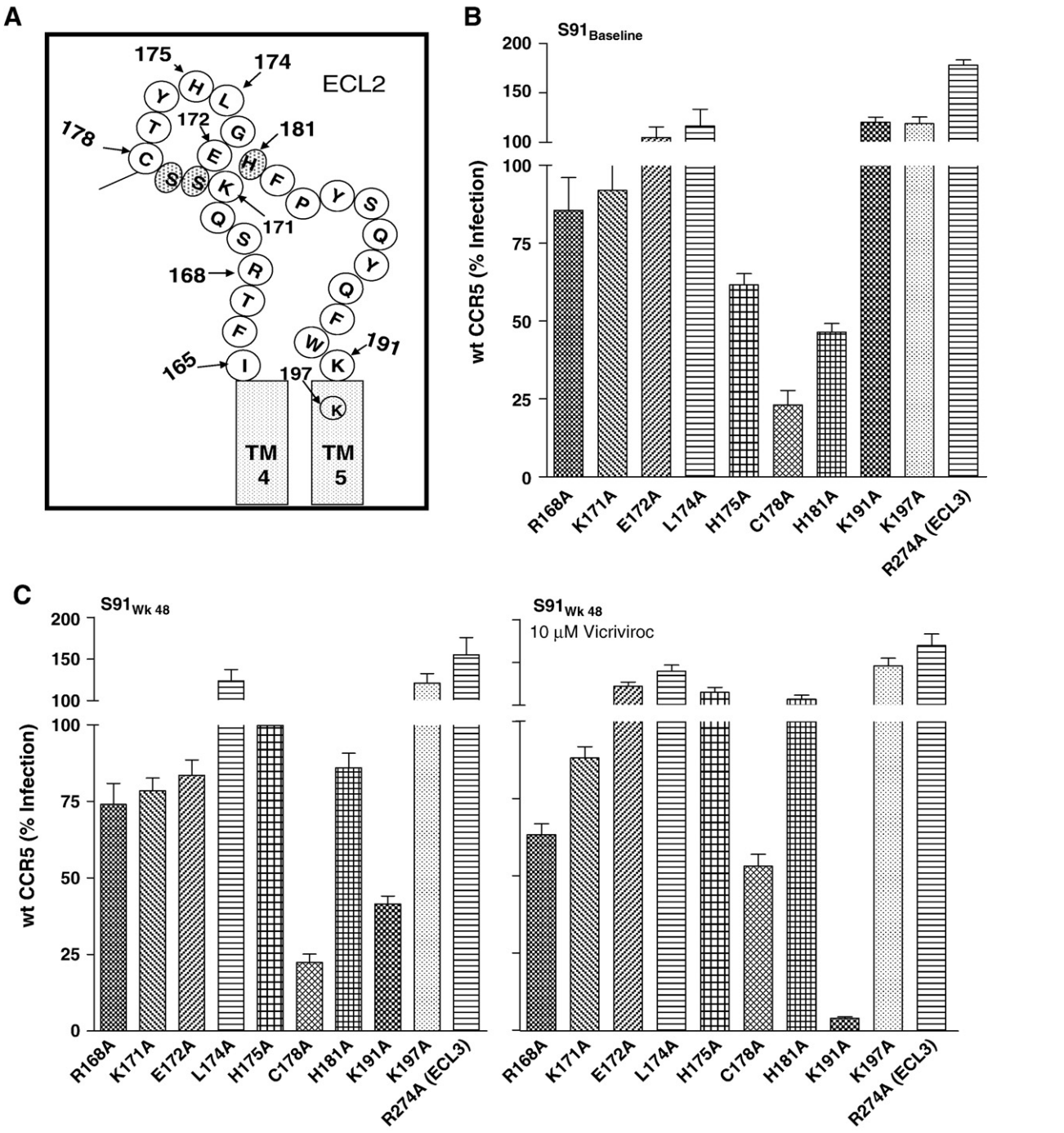

Fig. 7. The effect of single alanine substitutions in ECL2 and ECL3 of CCR5 on HIV-1 pseudovirus entry. A) Single alanine substitutions R168A, K171A, E172A, L174A, H175A, C178A, H181A, K191A in CCR5 ECL2 and K197A in transmembrane domain 5 are depicted in a molecular diagram of ECL2 based on the previously published structural model for the G-protein coupled β2-adrenergic receptor (Cherezov et al., 2007). The R274A mutation generated in ECL3 was also analyzed. The results of each single alanine substitution in CCR5 on B) $S91_{Baseline}$ and C) $S91_{Wk\ 48}$ HIV-1 pseudovirus entry in the absence (C, left panel) and presence of VCV (C, right panel) are expressed as a percentage of wt CCR5 infection. Data is the average ± SD of 12 replicates per mutation and represents ≥2 independent assays.

al., 2009). Our results are more consistent with a model in which S91 HIV-1 developed complete resistance to vicriviroc as a result of mutations that modified interactions between gp120 with residues in both the N-terminus and ECL2 regions of CCR5.

The observation that multiple V3 mutations, were shown to influence binding of gp120 to both the N-terminus and ECL2 regions of CCR5, and were necessary to acquire resistance to VCV suggests a high genetic barrier may exist for developing resistance to VCV. This would also help explain the infrequent development of classical resistance to this agent observed in clinical trials. In addition, it seems plausible that the humoral immune system might have contributed to selection of the $S91_{Wk\ 48}$ virus containing the Q315E mutation. This virus emerged between week 24 and week 48 as the resistant virus containing mutation G314W at week 24 was reduced. However, to support this observation neutralization studies using S91 serum are needed. In addition to these findings, none of the combined mutations in the baseline envelope appeared to confer usage of CXCR4 supporting the clinical observation that the emergence of X4 virus in clinical trials resulted from a pre-existing, minor population of X4 virus undetected at baseline.

Overall our studies suggest that the $S91_{Wk\ 48}$ envelope adapted to allow utilization of the drug-bound receptor by more efficiently using both the N-terminus and ECL2 region of CCR5 during virus entry. Further work with the $S91_{Wk\ 48}$ envelope may increase our understanding of the structural conformation of the drug-bound co-receptor and provide insights into the timing of specific interactions with the structural rearrangements that occur in the envelope trimer during HIV-1 entry.

Materials and methods

Clinical study P03802

The clinical study design, study procedures, and statistical analysis for study P03802 have been previously described (Landovitz et al., 2008). Trofile™ and PhenoSense Entry™ assays were performed by Monogram Biosciences.

Generation of Subject 91 ADA chimeric envelopes

pSV7d-ADA gp160 and pNL4-3E$^-$Luc$^+$ were obtained from Dr. John Moore, Weill Medical College of Cornell University, New York, NY. Envelope sequences for Subject 91 (S91) baseline (clone 28) and week 48 (clone 15) were obtained from Monogram Biosciences. Synthetic DNA clones encoding the entire gp160 in pUC57 were generated by Bio Basic Inc. S91 chimeric ADA envelope clones were constructed by ligation of pSV7d-ADA gp160 digested with Bgl II and EcoN I and the corresponding fragments excised from S91 gp160.

Generation of pNL4-3/S91 baseline gp160 and pNL4-3/S91 week 48 gp160 provirus constructs

The pDOLHIVenv plasmid and pNL4-3 AD8 provirus construct were obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). A shuttle vector, pST-Blue-1/NL4-3 containing the EcoRI to XhoI fragment from pDOLHIVenv was modified by placing a unique PacI site immediately downstream of the gp160 coding sequence. The S91 gp160 coding sequences excised using BbsI and PacI were sub-cloned into the pST-Blue-1/NL4-3 PacI. EcoRI–XhoI fragments excised from the corresponding shuttle vector were cloned directly into the pNL4-3-AD8 provirus construct to produce infectious $S91_{Baseline}$ and $S91_{Wk\ 48}$ HIV-1. NL4-3/$S91_{Baseline}$ and NL4-3/$S91_{Wk\ 48}$ virus stocks were generated in 293T cells using ProFection® calcium phosphate transfection system (Promega).

VCV susceptibility assays with $S91_{Baseline}$ and $S91_{Wk\ 48}$ replication competent HIV-1

U87 astroglioma cells expressing CCR5 or CXCR4 and the U87-CD4 parental cell line were obtained from Dr. Dan Littman, New York University. 10-fold serial dilutions of a VCV stock (final concentration 10^{-5}–10^{-15} M) were added to triplicate wells (5000 U87-CD4-CCR5 cells/well) and plates incubated at 37 °C for 1 h. Supernatants were aspirated and replaced with HIV-1 and inhibitor and plates incubated at 37 °C for 3 h. Virus was washed from wells 2 times with PBS and cells cultured in media with VCV for 72 h at 37 °C. The extra-cellular p24 was measured using Alliance HIV-1 p24 ELISA (PerkinElmer). VCV dose–response data was expressed as the % inhibition of p24 production compared to control wells without VCV.

Site-directed mutagenesis (SDM) of Subject 91 chimeric envelope

SDM of amino acids in S91 chimeric ADA envelopes was performed using QuikChange™ SDM kit (Stratagene) and sequence changes verified by DNA sequence analysis. The following amino acid mutations were generated in $S91_{Baseline}$ (F309L, G314W, Q315E, L317F, D320N, R321G, E328K, G429R, R419A, R419K, and K440A) and $S91_{Wk\ 48}$ (E315Q, F317L, N320D, G321R, K328E, R327A, R419A, R419K, and K440A) envelopes. Amino acid changes are numbered according to HXB2 reference strain coordinates using the Los Alamos National Laboratory HIV sequence locator tool: (http://www.hiv.lanl.gov/content/sequence/LOCATE/locate.html).

CCR5 mutations

The pcDNA3.1 CCR5 expression plasmid was obtained from Dr. Dan Littman, New York University. The generation of mutations in the N-terminus of CCR5 and in ECL2 (R168A, K171A, E172A, L174A, and C178A) and co-expression of these CCR5 mutants with CD4 in 293T cells were previously described (Ogert et al., 2009). Similar results were observed when CCR5 mutants were expressed in the U87-CD4 parental cell line. Therefore, CCR5 mutations R274A in ECL3 and H175A, H181A, K191A, K197A in ECL2 were analyzed in U87-CD4-parental cells using FuGene® transfection reagent. U87-CD4 cells 24 h post transfection were seeded into 96-well luminometer plates (Perkin Elmer) at 5000 cells/well and plates were incubated overnight at 37 °C. HIV-1 pseudovirus infections of transfected U87-CD4 cells was performed as described below.

Generation and characterization of HIV-1 pseudoviruses

HIV-1 pseudoviruses were produced in 293T cells by calcium phosphate transfection of pNL4-3E$^-$Luc$^+$ and HIV-1 envelope expression vectors and pseudovirus assays were performed as described previously (Ogert et al., 2009, 2008). For neutralization of HIV-1 pseudovirus infection with CTC5 anti-N-terminus CCR5 MAb (R&D Systems Inc., Minneapolis, MN; Catalog # MAB1802) and 2D7 anti-ECL2 CCR5 MAb (BD Pharmingen™, San Jose, CA; Catalog # 555991), serial 2-fold dilutions of Ab were added 1 h prior to the addition of HIV-1 pseudovirus. % inhibition was calculated as follows: 100 − (average normalized RLU for HIV-1 pseudovirus plus drug / average normalized RLU for HIV-1 pseudovirus from control wells without drug) × 100.

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