Characterization of CD4-Independent HIV-1 Envelope as Potential Immunogens

348

Lifei Yang[1], Bradley Cleveland[1], Andrea P.O. Jordan[2], Patricia Polacino[3], James A. Hoxie[2], and Shiu-Lok Hu[1,3]

[1]Department of Pharmaceutics, University of Washington, Seattle, WA, US
[2]Department of Medicine, University of Pennsylvania, Philadelphia, PA, US
[3]Washington National Primate Research Center, University of Washington, Seattle, WA, US

Background: We reported earlier that removal of a highly conserved N-linked glycan N197 (N7) on the primary HIV-1 isolate 89.6 improved the immunogenicity of the envelope (Env) protein. Deletion of this glycan made the 89.6 Env more sensitive to CD4-binding site (CD4bs)-specific neutralizing antibodies (Abs) and less dependent on the CD4 receptor to mediate cell fusion[1]. However, it is not clear if the improvement in immunogenicity of 89.6 mutant Env is related to the N7 glycan site mutation per se or the acquisition of a partial CD4-independent (CD4i) phenotype. The goal of the present study is to examine this issue by generating and characterizing Env that acquired CD4i phenotype.

Methods: We generated fully CD4i viruses derived from the WT and N7 mutant of SHIV 89.6 using a biological selection protocol. Mutant *env* genes from virus isolates of CD4i 89.6WT (i89.6WT) and N7 (i89.6N7) were PCR amplified and cloned into pCI-neo expression vector. Infectious molecular clones (IMCs) containing the CD4i *envs* were generated by cloning the *env* genes back into parental SHIV backbone. CD4-independence was confirmed by the replication of CD4i SHIV viruses and the ability of CD4i Envs to mediate cell fusion in CD4-negative cells. The receptor/coreceptor usage of CD4i Envs were examined in cell fusion assay using a luciferase reporter system.
In order to study the immunogenic properties of CD4i Envs as potential immunogens, we generated recombinant vaccinia viruses (RVV) expressing CD4i Envs, which were used as the priming immunogen in poxvirus prime- protein boost immunization regimens. The expression, glycosylation and processing of CD4i Envs by RVV were tested. Antigenicity of CD4i Envs expressed on the surface of RVV infected cells was analyzed using flow cytometry assay. Neutralization sensitivity of CD4i SHIV viruses was tested against a panel of mAbs on TZM-bl cells. The biological function of CD4i Envs expressed by RVV was tested by cell fusion assay.

Results

Derivation of CD4-independent HIV-1 isolates of 89.6

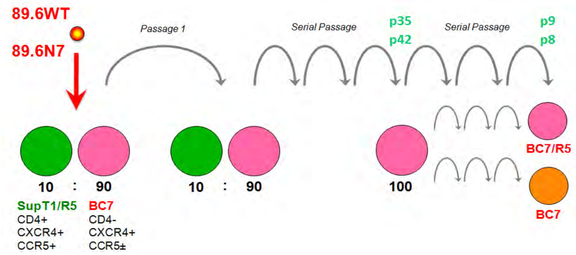


FIG 1: Schematic diagram of the biological selection protocol used for derivation of CD4i HIV-1 isolates of 89.6 WT and N7. CD4i SHIV i89.6 WT and N7 were derived in CD4+ SupT1 T cells and a CD4-negative variant of this line, termed BC7, both transduced with a lentiviral vector to stably express human CCR5 (e.g., SupT1/R5 and BC7/R5). Parental SHIV 89.6 WT and N7 were serially passaged in a 1:10 mixture of SupT1/R5 and BC7/R5 cells respectively, and monitored during each passage for expression of p27gag. When infection of >50% of the cell mixture was documented, virus was passaged in CD4-negative BC7 or BC7/R5 cells alone. Full-length *env* genes were amplified from proviral DNA of the infected cells by PCR. The resulting products representing CD4i *env* genes were cloned into pCI-neo expression vector. An infectious molecular clone (IMC) containing the CD4i *env* was generated by cloning the *env* gene back into parental SHIV backbone. Finally, we isolated two i89.6 WT *env* clones (A2 and B1) and one i89.6N7 *env* clone (D4t), which were derived, respectively, from parental 89.6WT and N7 *envs*. Expression plasmids and IMC expressing i89.6 WT and N7 Envs were generated.

Acquisition of CD4i phenotype is accompanied by sequence changes in the receptor/coreceptor binding sites

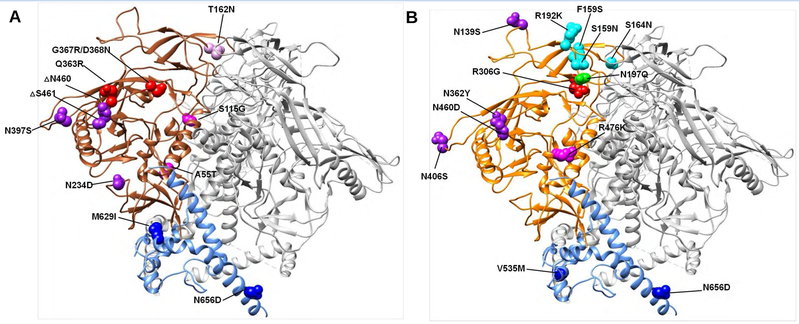


FIG 2: Locations of mutations in CD4i i89.6WT and N7 Envs. 89.6 sequence was threaded onto the SOSIP trimer structure (PDB code 4TVP and 4NCO) to build 89.6 structure model using UCSF Chimera. Mutations in CD4i i89.6 Envs comparing to parental 89.6 Envs were highlighted on this model, and numbering is based on HxB2 sequence. Some mutations were not mapped on this model due to the lack of structure information in some regions. (A) Locations of the common mutations in CD4i i89.6 WT (A2 and B1) Envs. A2 and B1 share common mutations in gp120 (in brown, including C1, V2, C2, C3, V4 and V5) and gp41 (in light blue). Briefly, four potential N-linked glycosylation sites are mutated (in purple), one mutaion in V2 (in plum), three mutations are located at the CD4bs (in red) which affect the binding and neutralization activity of CD4bs Abs, and their cytoplasmic tails are truncated (not shown). Two common mutations are located at the inner domain (in pink). A2 and B1 also have their own unique mutations which are not shown on this model. (B) Locations of mutations in CD4i i89.6 N7-D4t Env. i89.6 N7-D4t Env does not share many common mutations with i89.6WT Envs (A2 and B1), except for a potential N-lined glycosylation site mutation in V5, a similar gp41 tail truncation, and one mutations (in blue) in gp41 (in light blue) which may stabilize the gp41 interface. In the gp120 (in orange) of i89.6 N7-D4t Env, four potential N-linked glycosylation sites are mutated (in purple), one mutation in the V3 tip (in red) disrupting the CXCR4 binding site, one mutation in C5 (in magenta), N197 mutation (in green), and all the other mutations are located in V1/V2 loop (in cyan).

CD4i SHIV i89.6 viruses replicate well in CD4-negative cells

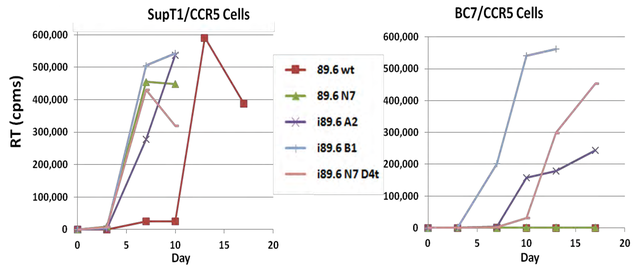


FIG 3: Viral replication in CD4-positive (SupT1/CCR5) and CD4-negative (BC7/CCR5) cell lines for parental SHIV 89.6 WT and N7 viruses or CD4i SHIV i89.6 WT and N7 viruses. Infectious viral stock of each CD4i SHIV virus was generated via co-transfection of SHIV IMC 5' and 3' hemigenomes into 293T cells and propagated in SupT1/R5 cells. For the viral replication assays, SupT1/R5 and BC7/R5 cells were infected with equal amount of SHIV virus normalized by p27gag amount. The replication of each virus in CD4-positive (SupT1/CCR5) and CD4-negative (BC7/CCR5) cell lines was monitored by the reverse transcriptase (RT) activity (counts per minute [cpm]) in the cell culture supernatant. Results show that SHIV i89.6 WT and N7 viruses replicated well in both CD4-positive and CD4-negative cells, but parental SHIV 89.6 WT and N7 viruses can only replicated in CD4-positive cells.

Acquisition of the CD4i phenotype is accompanied by changes of the receptor/coreceptor usage

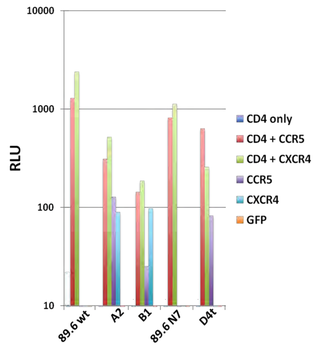


FIG 4: Summary of the cell-cell fusion activity mediated by CD4i Envs expressed by pCI-neo expressing vector. The ability of CD4i Envs to mediate cell-cell fusion was evaluated by using a luciferase-based gene reporter assay. Briefly, quail QT6 cells were transfected with plasmids containing HIV *env* genes and infected with a vaccinia virus expressing T7 RNA polymerase. These cells were mixed with quail QT6 cells transiently expressing human CXCR4 or CCR5 with or without human CD4 and the luciferase gene under the control of the T7 promoter. Fusion was quantified by relative light units (RLU). A vector containing only GFP was used as a negative control.
Results show that i89.6 WT Envs (A2 and B1) use both CCR5 and CXCR4 to mediate cell fusion in CD4-negative cells, but i89.6 N7 Env (D4t) only use CCR5 not CXCR4 to mediate cell fusion in CD4-negative cells which is most likely due to the mutation (R306G) in V3 tip disrupting its CXCR4 binding site.

CD4i Envs can be expressed, glycosylated and properly cleaved in recombinant vaccinia virus expression system

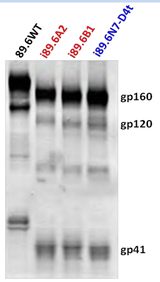


FIG 5: Western blot analysis of CD4i 89.6 Envs expressed by recombinant vaccinia viruses. RVV expressing these CD4i Envs were generated because the first arm of our prime-boost immunization regimen consists of priming with recombinant vaccinia virus. Crude recombinant vaccinia virus infected cell lysates were loaded on SDS-PAGE and probed with anti-HIV human polyclonal antibodies. All the CD4i Envs are expressed mainly in gp160 form and partially cleaved into gp120 and gp41. Consistent with the gp41 truncation and the loss of several potential N-linked glycans in gp120, the molecular weights of CD4i Env gp160, gp120 and gp41 are smaller compared to the counterparts of 89.6WT Env proteins.

Cell surface expressed CD4i Envs preserve epitopes recognized by most broadly neutralizing Abs

Env \ Ab	CD4bs				CD4i	V3			V1/V2		2G12	gp41 MPER		
	b12	VRC01	CD4-IgG	VRC03	17b	447-52D	PGT121	PGT126	PG9	PG16		2F5	4E10	10E8
v-NY	-	-	-	-	-	-	-	-	-	-	-	-	-	-
89.6wt	+	+	+	+	+	+	+	+	-	-	+	+	+	+
89.6N7	+	+	+	+	+	+	+	+	-	-	+	+	+	+
i89.6A2	-	+	+	-	+	+	+	+	-	-	+	+	+	+
i89.6B1	-	+	+	-	+	+	+	+	-	-	+	+	+	+
i89.6N7-D4t	+	+	+	+	+	+	+	+	+	-	+	+	+	+

Tab 1: Summary of binding profiles of a panel of mAbs to cell surface expressed CD4i 89.6 Envs. 293T cells were infected with RVV expressing CD4i i89.6 WT and N7 Envs or parental 89.6 WT and N7 Envs at multiplicity of infection (MOI) of 3. The binding reactivity of a panel of HIV-1 mAbs to CD4i Envs expressed on 293T cell surface were tested by flow cytometry. +, indicate there is binding; -, indicate there is no binding. i89.6 WT and N7 Envs preserve epitopes of most broadly neutralizing Abs tested. Cell surface expressed CD4i Envs preserved epitopes of VRC01, CD4-IgG, 17b, 447-52D, 2G12, PGT121, PGT126, 2F5, 4E10 and 10E8. i89.6WT Envs (A2 and B1) don't maintain epitopes of b12 and VRC03, which is due to mutations at the CD4bs. It is quite interesting that i89.6N7-D4t Env can be recognized by conformation dependent Ab PG9 but not PG16, which may be due to mutations in the V1/V2 loop.

Acknowledgements

This work was supported by the Bill and Melinda Gates Foundation award OPP1033102. The content is solely the responsibility of the authors and does not necessarily represent the official views of the BMGF. Thank Kelly K. Lee for helpful comments and suggestions. Special thanks to the CROI Scientific Program Committee for awarding me a Young Investigator Scholarship.

Acquisition of the CD4i phenotype alters the CD4bs on cell surface expressed CD4i Envs

Fold increase in the binding intensity of CD4bs Abs to cell surface expressed CD4i Envs									
Env / Ab	i89.6A2: 89.6WT			i89.6B1: 89.6WT			i89.6N7-D4t: 89.6N7		
	Avg	SD	p	Avg	SD	p	Avg	SD	p
VRC01	0.77	0.09	0.05	0.58	0.05	0.0039	3.55	0.44	0.01
b12	0.07	0.03	0.0004	0.15	0.04	0.0008	1.54	0.18	0.03
CD4-IgG	0.74	0.14	0.08	0.52	0.14	0.03	1.67	0.25	0.04

Tab 2: Altered binding reactivity of CD4bs Abs to cell surface expressed CD4i Envs. 293T cells were infected with RVV expressing CD4i i89.6 WT and N7 Envs or parental 89.6 WT and N7 Envs at 3 MOI. The binding reactivity of CD4bs Abs (VRC01, b12 and CD4-IgG) to cell surface Envs were tested by flow cytometry. The binding intensity (MFI) of CD4bs Ab to each Env was normalized to its corresponding binding intensity of 2G12, and the fold increase in MFI of CD4bs Ab to CD4i Env was compared to parental 89.6WT or N7 Env. Results show that i89.6WT (A2 and B1) exhibit decreased binding ability to CD4bs Abs relative to parental 89.6WT because of mutations in their CD4bs, while i89.6N7-D4t shows increased binding ability to CD4bs Abs relative to parental 89.6N7, indicating the CD4bs on i89.6N7-D4t Env is more exposed. Data shown here were mean value of three independent experiments, including SD and *p* values. Highlight color indicates that there is statistic difference between i89.6 and parental 89.6WT or N7.

Acquisition of the CD4i phenotype alters neutralization sensitivity of CD4i viruses

Virus \ Ab	IC50 (ug/ml)														
	CD4bs				V3			CD4i		2G12	PG9	PG16	gp41 MPER		pooled patient plasma
	IgG1b12	VRC01	CD4-IgG	sCD4	447-52D	PGT121	PGT126	17b without sCD4	17b with sCD4				2F5	4E10	
89.6WT	0.039	1.44	0.024	0.34	0.056	0.019	0.0075	1.85	1.53	0.095	>20	>20	0.12	0.19	5701
89.6N7	0.0083	0.13	0.017	0.066	0.014	0.024	0.0079	0.27	0.42	0.090	>20	>20	0.17	0.35	19501
i89.6 A2	>20	2.78	0.34	>20	0.0066	0.021	0.017	0.28	0.27	0.31	>20	>20	0.052	0.15	1473
i89.6 B1	>20	1.77	0.57	>20	0.0069	0.052	0.042	0.76	0.65	1.00	>20	>20	0.20	0.66	977
i89.6N7-D4t	0.0077	0.11	0.0098	0.049	0.34	>20	>20	0.39	1.13	>20	>20	>20	0.086	0.41	10817

i89.6A2/B1: ↓ CD4bs epitopes; ↑ V3 (linear); ↑ CD4-induced epitope; ↓ 2G12; - <0.01, 0.01-0.1, 0.1-1
i89.6N7-D4t: ↑ CD4bs epitopes; ↓ V3 (linear & quat.); ↑ CD4-induced epitope; ↓ 2G12; - 1-20, >20

Tab 3: Summary of neutralization profiles of CD4i SHIV i89.6 viruses and parental SHIV 89.6 viruses. Darker shading indicates increasing sensitivity to the mAbs tested, according to the key at the lower right. Gray shading indicates that 50% neutralization was not reached at the highest concentration tested (20 ug/ml). IC50s in the table are mean value of at least two independent experiments performed in duplicate. SHIV i89.6WT viruses show decreased neutralization sensitivity to CD4bs Abs and 2G12, but increased neutralization sensitivity to V3 linear epitope targeting Ab (447-52D) and CD4-induced Ab (17b) compared to parental SHIV 89.6WT virus. SHIV i89.6N7 virus shows increased neutralization sensitivity to CD4bs Abs and CD4-induced Ab (17b) compared to SHIV89.6WT but no statistic difference compared to SHIV89.6N7. SHIV i89.6N7 virus shows decreased neutralization sensitivity to V3 linear and quaternary epitope targeting Abs as well as 2G12. All SHIV i89.6WT and N7 viruses show similar neutralization sensitivity to V1/V2 targeting Abs (PG9, PG16) and MPER targeting Abs compared to parental SHIV 89.6WT and N7 viruses.

CD4i Envs mediate cell fusion in CD4-negative CCR5- and/or CXCR4-expressing cells

Env \ Cell lines	TZM-bl	NP2-CD4/R5	BC7-R5	BC7	NP2-R5
	CD4+ CCR5+ CXCR4+	CD4+ CCR5+ CXCR4-	CD4- CCR5+ hi CXCR4+	CD4- CCR5+ low CXCR4+	CD4- CCR5+ CXCR4-
MOCK	-	-	-	-	-
v-NY	-	-	-	-	-
89.6WT	+	+	+	-	-
89.6N7	+	+	+	-	-
i89.6 A2	+	+	+	+	-
i89.6 B1	+	+	+	+	-
i89.6N7-D4t	+	+	+	-	+

Tab 4: Summary of the cell-cell fusion activity of CD4i i89.6 Envs expressed by recombinant vaccinia viruses. Briefly, cells expressing different coreceptors with or without CD4 receptor were infected with RVV at 1 MOI for 1 hour, 18 hours post infection syncitia were counted under microscopy. The expression of receptor/coreceptor in each cell line was listed. v-NY vaccinia virus was used as a negative control. i89.6 WT Envs mediate cell fusion in CD4-negative CCR5-/CXCR4- expressing cells, but not in CD4-negative CCR5- expressing cells, which maybe due to the amount of CCR5 expressed on the cell surface. Consistent with results in FIG4, i89.6 N7 Env only use CCR5 not CXCR4 to mediate cell fusion in CD4-negative cells. Interestingly, 89.6WT and N7 Env can mediate cell fusion in CD4-negative CCR5hi and CXCR4- expressing cells, which maybe due to the high amount of CCR5 on the cell surface.

Conclusions

- Acquisition of the CD4-independence is accompanied by sequence changes in the receptor/coreceptor binding sites
- Acquisition of the CD4i phenotype is accompanied by changes of the receptor/coreceptor usage
- Acquisition of the CD4i phenotype alters the antigenic properties of the CD4i Env

Future Directions

- Does the ability of the Env to engage the CD4 receptor or coreceptor affect its immunogenicity?
- What are the determinants necessary and sufficient for the CD4i phenotype and how do they impact its immunogenicity?
- What is the structural/biophysical basis of the CD4i phenotype?

References

1) Li, Y., et al., *Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses.* Journal of virology, 2008. **82**(2): p.638-651.