Chapter 4

Lectibody: Design and characterization of a cyanovirin-N – Fc chimera

Abstract

Cyanovirin-N (CVN) is a protein that is a broadly potent inhibitor of many enveloped viruses, including HIV, Ebola, and influenza. It acts to neutralize these viruses by binding to glycoproteins on the viral envelope and preventing viral fusion to the host cell. Although CVN has already been shown to be quite effective against these viruses, we hope to make a variant that has more potential therapeutic value by recruiting activities of the human adaptive immune system. We present here a CVN-Fc chimeric fusion protein. This protein, termed a "lectibody" for its fusion of a lectin (CVN) and the constant region (Fc) of an antibody, is designed to incorporate the viral neutralization properties of CVN with Fc-mediated effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), increased serum half-life, and antibody-dependent cell-mediated phagocytosis (ADCP). Here, we show that a CVN lectibody has similar neutralization activity to wild-type CVN in an anti-HIV assay and that there is significant higher order oligomerization of the protein that is due in some part to domain swapping of CVN. This new class of antiviral protein could act to neutralize free viral particles as well as invoke an immune response surrounding virus-infected cells.

Introduction

Antibodies are a vital component of the mammalian adaptive immune system. They are responsible for neutralizing infectious particles by binding to them and directly inhibiting them as well as by recruiting other components of the immune system, including macrophages, neutrophils, and natural killer (NK) cells, to the site of an infection.[1,2] An antibody consists of two major regions, the variable region (Fab) and the constant region (Fc). The Fab portion of the antibody is highly variable and is specific to the antigen, whereas the relatively conserved Fc portion contains binding sites for Fc receptors (FcRs) and engages the immune effector functions. There are five major isotypes of antibodies: IgM, IgA, IgD, IgE, and IgG; these differ in their heavy chain sequence and oligomerization state and mediate different responses. While all of these isotypes are important in an immune response, IgG is the most abundant antibody type found in humans, has the longest serum half-life, and is involved in most of the major effector functions.[1] For these reasons, the Fc of IgG1 was chosen for this study.

In addition to direct neutralization of potential pathogens via the Fab regions of an antibody, effector functions mediated through Fc binding are vital to a normally functioning immune system. The Fc of IgG1 specifically interacts with FcRn and Fc receptors specific to the γ chain (FcγR), including FcγRI, FcγRII, and FcγRIII.[1,3-5] These receptors act as messengers, linking antibody-mediated responses to cellular responses. The interaction between Fc and FcRn is involved in recycling antibodies, thereby extending their lifetime *in vivo,* and in transporting antibodies across epithelial barriers.[4,6-8] The other FcγRs, when complexed with antigen-bound IgG1, can mediate antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-

mediated phagocytosis (ADCP), and endocytosis.[3-5] In addition to FcR-mediated cellular responses, Fc can also activate the complement pathway, which leads to cell lysis or phagocytosis.[1,9]

Due to their simple protein A-based purification, extended *in vivo* lifetime, and Fc-mediated effector functions, Fc fusion proteins have become increasingly popular.[10-12] In a recent review, Jazayeri and Carroll report that at least six Fc fusion proteins are currently used clinically for several indications, including asthma, psoriasis, and rheumatoid arthritis.[12] In addition, countless other fusions have been made for both pharmaceutical and basic research purposes. While many researchers are interested in the increased lifetime of small, soluble proteins that is conferred by addition of an Fc,[13,14] Fc fusions have also been used to display Fc in a reverse orientation in order to study Fc-mediated effector functions,[15] to investigate protein-protein interactions,[16] and as potential therapeutics for various diseases or conditions.[10-12] One particularly relevant Fc fusion is CD4-Fc.[17,18] Various constructs combining the soluble portion of the HIV receptor CD4 with the Fc domain of an antibody were investigated for inhibition of HIV *in vivo*. Unfortunately, the results of clinical trials on these specific constructs were disappointing, but the constructs were able to induce ADCC of HIV-infected cells in culture and were efficiently transferred across the placenta in non-human primates.[19]

The ability to incorporate extended *in vivo* lifetimes and activation of cell-mediated effector functions is a very compelling reason to engineer Fc fusion proteins. Additionally, these Fc-mediated functions can be modulated through mutations in the Fc region to either increase or abrogate the response, providing more flexibility to the system.[20,21] Various studies have indicated that single point mutations[22,23] or changes in

the Fc-linked carbohydrate composition[9,24,25] can dramatically increase the ADCC response by increasing the affinity for FcγR. Engineered mutations in the Fc have been shown to increase activation of the complement pathway.[26] Alternatively, Lazar *et al*. showed that a point mutation could destroy the ability for an Fc to activate complement-dependent cytotoxicity (CDC) while retaining or enhancing ADCC and other effector functions.[22] Extending the lifetime of Fc fusions has also been extensively studied. Even though Fc-fused proteins often already have longer *in vivo* lifetimes than the unfused molecule, any improvements in the circulatory half-life of a molecule is a possible benefit for potential therapeutics. A 2- to 2.5-fold increase in the half-life of Fc fusions was accomplished by either a single or double point mutation in the Fc.[27,28] The incorporation of one or more of these mutations allows researchers to specifically study the effects of ADCC, complement, and half-life on a particular system.

The protein of interest in this study, cyanovirin-N (CVN), is a small cyanobacterially-derived protein that inhibits infection by various enveloped viruses including HIV,[29] Ebola,[30] and influenza.[31] CVN is a lectin that specifically binds α1-2 linked high-mannose molecules.[32-35] This type of carbohydrate linkage is found in high concentrations on the envelope proteins of these viruses, including gp120 on HIV.[34,36] CVN effectively neutralizes HIV by binding with high affinity and avidity to the glycosylation on gp120 and blocking interactions with the host cell receptor, CD4, and coreceptors.[37]

Here, we report the generation of a CVN-Fc fusion that retains wild-type (WT) CVN-like HIV neutralization activity. We have termed this construct a “lectibody” as it is a fusion between a lectin (CVN) and the Fc of an antibody (Figure 4-1). Similarly to the

CVN_2 dimers discussed in Chapter 2, we hope that we can improve the efficacy of HIV neutralization by dimerizing the CVN through the Fc domain. The CVN lectibody also has the potential for Fc-mediated effector functions as described above. Previous studies have shown that ADCC plays a role in protection against HIV[38] and that ADCC and other FcR-mediated effector functions provide some protection against viruses[39-42] even when associated with non-neutralizing antibodies.[43,44] We also anticipate that this lectibody construct will have a longer half-life *in vivo*. A study on CVN showed that after subcutaneous injection in mice, WT CVN was mostly cleared from the bloodstream after 7 to 24 hours.[45] Since a daily injection to maintain therapeutic levels would most likely not be feasible, a variant with a longer half-life would make a potential therapeutic more practical. This construct would also benefit from a potential pulmonary delivery route, as Fc fusions have been shown to be effectively transported across the pulmonary epithelial barrier in both humans and non-human primates through FcRn-mediated transcytosis.[6-8] Here, we present initial data showing the viability of a CVN-Fc fusion: a lectibody.

Methods

Construct generation. Lectibody constructs were created by subcloning the WT CVN sequence or the CVN_2 L0 sequence described in Chapter 2 including DNA that encodes a five-amino acid linker (GGSGG) between CVN and the Fc of human IgG1 into the baculovirus expression vector pAc-κ-Fc using the XhoI and SpeI restriction sites (Progen Biotechnik). Sequencing on this construct revealed that the Fc portion was missing the last eight residues and included two point mutations. To rectify this, the last eight Fc residues were added during the second cloning step in which the secretion

signal, CVN, and Fc were subcloned using PCR-based techniques into the mammalian expression vector pcDNA3.1 (Invitrogen) or pTT5 (NRC Biotechnology Research Institute), and the mutations were reversed to give the WT Fc sequence. Human-codon optimized CVN sequences were determined using the Custom Gene Synthesis program from IDT (Integrated DNA Technologies, Inc). The optimized gene was assembled via recursive PCR[46] and ligated into the pcDNA3.1 or pTT5 vector already containing the secretion leader sequence and the Fc sequence. Point mutations were introduced into the lectibody constructs using the QuikChange Site-Directed Mutagenesis kit (Stratagene). All constructs were verified through DNA sequencing.

Bacterially expressed constructs were created as described in Chapter 2. Point mutations for bacterially expressed variants were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

Expression and purification. Lectibody constructs were expressed in transiently transfected, suspended HEK293-T or HEK293-6E cells (NRC Biotechnology Research Institute). The cells were transfected with 1 mg of plasmid DNA per liter of culture using a polyethylenimine-mediated transfection protocol (PEI). The secreted protein was harvested from the cell supernatants after 6-8 days and buffer exchanged into 100 mM sodium phosphate buffer pH 7.5, 150 mM NaCl. The protein was purified on a Protein A column, eluted in pH 3.0 elution buffer (Pierce) and immediately neutralized with Tris-base. A second purification step on a Superdex-200 gel filtration column (GE Healthcare) in 25 mM sodium phosphate pH 7.4, 150mM NaCl was used to separate high molecular

weight aggregates from smaller species. Protein was stored as eluted or concentrated in a 10,000 MWCO centrifugal concentrator (Millipore) then kept at 4˚C.

Deglycosylation of lectibody proteins was accomplished using PNGase F (New England Biolabs). The protein was denatured, then PNGase F was added according to the manufacturer's protocol. Complete deglycosylation was achieved after 1-2 hours. After removing the carbohydrates, the apparent molecular weights of the proteins were assessed by SDS-PAGE.

Bacterial expression and purification of non-Fc fusion constructs were performed as described in Chapter 2 of this thesis.

Circular dichroism. Circular dichroism (CD) spectra were obtained on an Aviv 62DS spectrometer with a 1 mm path length cell. Samples were 50 μM protein in 25 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl. Wavelength scans were collected at various temperatures between 200 and 250 nm with a 1 nm step size. A single scan was collected for each variant with an averaging time of 5 sec. Temperature denaturation was monitored at 233 nm from 1˚C to 99˚C. The sample was equilibrated at each temperature for a minimum of 2 minutes before the data was averaged for 30 seconds and recorded. The denaturation curves were not reversible and therefore thermodynamic parameters could not be determined. Instead, the data were fit to a two-state model[47] to estimate the midpoint of thermal denaturation (T_m), an estimate of thermal stability.

Neutralization assays. Neutralization assays were performed as described in Chapter 2 of this thesis.[48] All variants were tested against strain SC422661.8 from clade

B and compared to WT CVN from the same 96-well plate unless otherwise noted. Due to the low concentrations of various constructs, some assays were performed with twice the standard volume of protein to increase the final concentration in the well.

Surface plasmon resonance (SPR). SPR experiments were conducted on a T100 instrument from Biacore. Approximately 30 response units (RUs) of bacterially expressed WT CVN were immobilized on a CM5 chip using standard amine coupling. All assays were conducted in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.0005% v/v Surfactant P20, 1 mM EDTA; Biacore). Various analytes were injected over the surface for 60 seconds at a flow rate of 30 μL/min. The chip was regenerated with two pulses of 50 mM NaOH. Complete regeneration was not achieved after lectibody variants were analyzed and therefore proteins injected later may have exhibited binding to the unregenerated surface and not to the surface itself. We therefore repeated the assay on a new surface and tested the samples in reverse order to confirm the results of the first experiment. The data were analyzed for binding or lack of binding based on the sensorgram.

Results

Mammalian expression. All CVN-Fc (lectibody) constructs were expressed and secreted in mammalian cell culture. Yields were typically low for the pcDNA constructs with *Escherichia coli*-optimized CVN sequences (between 100 and 500 μg protein per L of cell culture). For comparison, a similar construct containing only the expression leader sequence and Fc expressed approximately 4 mg/L. To try to resolve this problem, we

made various constructs intended to increase protein expression. We found that changing the vector from pcDNA3.1 to pTT5 did not significantly improve the expression and in multiple trials actually produced a larger fraction of degradation product. We did find, however, that changing the codons of the CVN gene to correspond with optimal human codon usage produced an approximately 10% increase in soluble expression. These yields were sufficient for the assays in this study, but are still much lower than desired.

Glycosylation. After Protein A purification, the initial lectibody construct, CVN-Fc, appeared to migrate much slower on an SDS-PAGE gel than expected (data not shown). Therefore, we deglycosylated the protein to confirm the expected molecular weight. However, upon deglycosylation, it became clear that the protein contained two separate N-linked glycosylation sites instead of only the expected site on the Fc. The NetNGlyc 1.0 Server[49] was used to predict potential N-linked glycosylation sites and found a highly probable site at position 30 of the CVN sequence in addition to the known glycosylation site in the Fc. This potential glycosylation site in CVN is located on the surface of the protein and has the sequence N-T-S, which is consistent with the N-X-(S/T) consensus sequence for N-linked glycosylation (where X is any amino acid except proline).[50] Visual inspection of the NMR and crystal structures indicated that glycosylation of residue 32 may interfere with substrate binding since this residue is near one of the binding sites of CVN. This result was confirmed by HIV neutralization assays, which showed that CVN-Fc had no neutralization activity (data not shown).

To remove the non-native glycosylation site in CVN, we constructed four variants in the bacterially expressed WT background to assess their effect on the structure and

function of CVN. Both N30 and S32 make side chain-backbone hydrogen bonds in the crystal structure, so we constructed two variants for each position, an Ala mutation that deleted the side chain and a polar mutation that may be able to satisfy the hydrogen bond (N30S and S32N). An S32T mutation would have possibly satisfied the hydrogen bond requirement, but it would have also met the glycosylation consensus and therefore would not have destroyed the site.

The four glycosylation deletion variants were assayed for changes in their secondary structure and thermal stability by CD, and their HIV neutralization abilities were compared to WT (Figure 4-2). No significant differences were seen in the CD wavelength scans of the four variants compared to WT CVN, indicating that the secondary structure was not affected by the mutation (Figure 4-2A). We did see slight differences in the midpoint of thermal denaturation (T_m) of the variants, however (Figure 4-2B). WT and the two N30 mutants had T_ms that were within experimental error (48.8˚C to 49.8˚C), whereas S32A and S32N were destabilized by approximately 5˚C and 8˚C, respectively. The results indicate that the physiological temperatures at which the HIV neutralization assays are performed may partially denature the S32N variant, making it less than ideal for our purposes. The neutralization assays showed that the N30A variant was slightly less active than WT, whereas the other three variants were WT-like in their HIV neutralization (Figure 4-2C). All this data together indicated that N30S was the best mutation to incorporate into the lectibody construct. N30S in the background of WT CVN had WT-like HIV neutralization activity, secondary structure, and thermal stability. Additionally, mutation at N30 guarantees the elimination of the N-linked glycosylation,

whereas mutation at position 32 leaves the Asn to which glycosylation would be attached intact, giving rise to a small possibility that glycosylation could still occur.

CVN-Fc N30S. After determining the ideal mutation to remove the non-native glycosylation site from CVN-Fc, we expressed and purified CVN-Fc N30S. This variant, similarly to the WT lectibody, had a significantly higher apparent molecular weight than expected as assayed by gel filtration chromatography, due to higher order oligomers or to aggregation (Figure 4-3). The expected elution volume for dimeric lectibody was approximately 0.60 CV. Although the majority of the protein elutes in the void volume, there was a small peak approximately corresponding to dimeric lectibody. Although Protein A purified protein and fractions containing high molecular weight species showed WT-like HIV neutralization activity, this fraction contained no activity (Figure 4-3B). We therefore sought to solve this unwanted higher order oligomerization problem to obtain monodispersive samples for assaying.

We hypothesized that the low pH elution from the Protein A column may cause some partial denaturation of the CVN portion of the lectibody. We therefore assessed the secondary structure, potential changes in oligomerization, and HIV neutralization of WT bacterially-expressed CVN at various pHs (data not shown). These experiments showed no significant differences between protein in pH 7.4 buffer and protein in buffers down to pH 2.0, including the actual Protein A elution buffer (Pierce). We therefore conclude that WT CVN does not show a pH dependence for the general secondary structure, HIV neutralization, or oligomerization.

Another possibility for the higher order oligomers formed by the lectibodies was that CVN, a carbohydrate binding protein, was binding the glycosylation on Fc and therefore causing large complexes of protein specifically bound to other lectibodies. To test this hypothesis, we expressed the lectibody with an additional mutation (N181A, equivalent to position 297 in a full length heavy chain) that eliminates the native Fc glycosylation site. This variant (CVN-Fc noglycos) behaved similarly to CVN-Fc N30S, and most of the protein eluted near the void volume of the gel filtration column, indicating it was almost completely composed of higher order oligomers. There was no apparent molecular weight shift upon deglycosylating this sample, indicating that we did, indeed remove all of the N-linked glycosylation sites. Although almost entirely oligomerized, CVN-Fc noglycos that was eluted from the Protein A column had approximately WT-like activity (as compared to bacterially expressed WT CVN) in the HIV neutralization assay, indicating that glycosylation is not necessary for the proper folding of the protein or for the activity, as expected. We also tested whether glycosylated Fc could bind WT CVN in an SPR assay (Figure 4-4). We saw no evidence of binding to immobilized CVN and therefore concluded that CVN does not bind the glycosylation on Fc. Interestingly, as seen in Figure 4-4, we saw significant amounts of binding of CVN-Fc N30S and CVN-Fc noglycos to the WT CVN surface. Because we know that the Fc is not responsible for the binding, we deduce that the CVN component of the lectibody is aggregating on the surface. WT CVN, on the other hand, shows no evidence of binding the CVN surface. This evidence suggests that the lectibody, although it contains some active and therefore properly folded protein, probably contains some misfolded protein, which has a tendency to aggregate. Additionally, the lectibody could have alternate

domain-swapping properties for the CVN component, leading to intermolecular domain swapping, with either WT CVN or another lectibody protein.

Domain-swapping variant lectibodies. To assess whether domain swapping of CVN is contributing to the formation of higher order oligomers, we created and assayed two new constructs, CVN_2 L0-Fc and N30S/P51G-Fc. In Chapter 2 of this thesis, I describe the dimeric variants of CVN that we created to test the effects of oligomerization on the efficacy of HIV neutralization. We hypothesize that by covalently linking the termini of two copies of CVN we are stabilizing the domain-swapped dimeric form of CVN, which in the context of WT is only metastable.[51] If this hypothesis is true, the CVN_2 L0 variant described in Chapter 2 should be stably domain-swapped and should not interact with other molecules to form intermolecularly domain-swapped complexes. While the CVN2 L0-Fc variant showed a significantly lower proportion of high molecular weight species, this protein was not active against HIV. When we added the N30S mutation to this construct, the majority of protein was shifted to high molecular weight and it remained inactive in the HIV neutralization assay. The second domain-swapping variant appears to hold more promise. In this case, the P51G mutation was added to CVN-Fc N30S. P51G has been shown to shift the equilibrium toward monomeric protein and destabilize the domain-swapped form.[51] N30S/P51G-Fc expressed much more readily in the mammalian expression system, although when it was neutralized after the Protein A column, a significant amount of protein precipitated and was lost. The remaining protein, when separated on a gel filtration column, produced a broad peak around 0.44 CV that contained N30S/P51G-Fc as assayed by SDS-PAGE and

showed WT-like activity in the HIV neutralization assay (Figure 4-5). A second large peak at 0.58 CV was attributed to contamination by BSA from the expression process. This peak contained no anti-HIV activity. Although the N30S/P51G-Fc did not elute at the expected volume, it is not forming the very high order oligomers of previous constructs. This indicates that domain swapping is a concern in the lectibody constructs and is an issue that must be overcome.

Discussion

We have successfully created a chimeric CVN-Fc variant that shows WT-like anti-HIV activity. We have shown that a non-native glycosylation site is present in CVN and glycosylated in mammalian tissue culture and that that site must be removed for efficient viral neutralization activity. In addition, we have shown that the lectibody constructs are prone to formation of higher order oligomers, which can in part be prevented by using a variant that stabilizes the monomeric state of CVN over the domain-swapped dimer.[51] Although more work is required to create completely monodispersed lectibody, we have shown that it is possible to modulate the oligomerization through simple mutation. Additional obstacles to overcome include solving a potential misfolding problem that allows the lectibodies to bind to WT CVN, as evidenced by the Biacore experiments.

Unlike in the case of the dimeric CVN molecules (CVN_2s) described in Chapter 2 of this thesis, we do not see a significant increase in the anti-HIV activity of the lectibody as compared to WT CVN attributable to the dimerization. This may be due to the fact that we have yet to isolate pure dimeric lectibody and the samples may be significantly

contaminated by partially or fully unfolded, nonfunctional protein. Additionally, some of the carbohydrate binding sites on CVN could be sterically inhibited by the high order oligomerization that we are seeing. By generating a variant that is monodispersed and dimeric, we hope to resolve these issues. It is also possible that the conformation of the Fc does not allow the CVNs to interact in a way that they are able to in the pure CVN_2 samples, perhaps not allowing the proper domain-swapping interactions.

Our success in generating a functional lectibody is only partially complete. In order to fully realize the goals of this project, we must assay the Fc effector function and *in vivo* half-life. Although we expect the lectibody to exhibit all the potential functions of the Fc, we hope to assay both antibody-dependent cell-mediated cytotoxicity (ADCC) and complement functions in the near future to verify.

Acknowledgements

I would like to thank Jost Vielmetter and Michael Anaya of the Protein Expression Center at Caltech for expression and initial purification of all the Fc fusion proteins. I would also like to acknowledge Priyanthi Peiris for running the HIV neutralization assays that are presented in this work. Josh Klein supplied the initial Fc sequence as well as helpful advice at all stages of this project. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: SVPB8 (Drs. David Montefiori and Feng Gao); $pSG3^{\Delta env}$ (Drs. John C. Kappes and Xiaoyun Wu); Tzm-Bl cells (Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme, Inc.)

References

1. Janeway, C., Travers, P., Walport, M. & Sholmchik, M. J. (2005). *Immunobiology: the immune system in health and disease*. 6th edit, Garland Science Publishing, New York, NY.

2. Huber, M. & Trkola, A. (2007). Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med* **262**, 5-25.

3. Daeron, M. (1997). Fc receptor biology. *Annu Rev Immunol* **15**, 203-34.

4. Raghavan, M. & Bjorkman, P. J. (1996). Fc receptors and their interactions with immunoglobulins. *Annu Rev Cell Dev Biol* **12**, 181-220.

5. Gessner, J. E., Heiken, H., Tamm, A. & Schmidt, R. E. (1998). The IgG Fc receptor family. *Ann Hematol* **76**, 231-48.

6. Bitonti, A. J., Dumont, J. A., Low, S. C., Peters, R. T., Kropp, K. E., Palombella, V. J., Stattel, J. M., Lu, Y., Tan, C. A., Song, J. J., Garcia, A. M., Simister, N. E., Spiekermann, G. M., Lencer, W. I. & Blumberg, R. S. (2004). Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway. *Proc Natl Acad Sci U S A* **101**, 9763-8.

7. Dumont, J. A., Bitonti, A. J., Clark, D., Evans, S., Pickford, M. & Newman, S. P. (2005). Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway. *J Aerosol Med* **18**, 294-303.

8. Low, S. C., Nunes, S. L., Bitonti, A. J. & Dumont, J. A. (2005). Oral and pulmonary delivery of FSH-Fc fusion proteins via neonatal Fc receptor-mediated transcytosis. *Hum Reprod* **20**, 1805-13.

9. Jefferis, R., Lund, J. & Pound, J. D. (1998). IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol Rev* **163**, 59-76.

10. Ashkenazi, A., Capon, D. J. & Ward, R. H. (1993). Immunoadhesins. *Int Rev Immunol* **10**, 219-27.

11. Chamow, S. M. & Ashkenazi, A. (1996). Immunoadhesins: principles and applications. *Trends Biotechnol* **14**, 52-60.

12. Jazayeri, J. A. & Carroll, G. J. (2008). Fc-based cytokines : prospects for engineering superior therapeutics. *BioDrugs* **22**, 11-26.

13. Dwyer, M. A., Huang, A. J., Pan, C. Q. & Lazarus, R. A. (1999). Expression and characterization of a DNase I-Fc fusion enzyme. *J Biol Chem* **274**, 9738-43.

14. Shapiro, R. I., Wen, D., Levesque, M., Hronowski, X., Gill, A., Garber, E. A., Galdes, A., Strauch, K. L. & Taylor, F. R. (2003). Expression of Sonic hedgehog-Fc fusion protein in Pichia pastoris. Identification and control of post-translational, chemical, and proteolytic modifications. *Protein Expr Purif* **29**, 272-83.

15. Stabila, P. F., Wong, S. C., Kaplan, F. A. & Tao, W. (1998). Cell surface expression of a human IgG Fc chimera activates macrophages through Fc receptors. *Nat Biotechnol* **16**, 1357-60.

16. Gurbaxani, B. M. & Morrison, S. L. (2006). Development of new models for the analysis of Fc-FcRn interactions. *Mol Immunol* **43**, 1379-89.

17. Capon, D. J., Chamow, S. M., Mordenti, J., Marsters, S. A., Gregory, T., Mitsuya, H., Byrn, R. A., Lucas, C., Wurm, F. M., Groopman, J. E. & et al. (1989). Designing CD4 immunoadhesins for AIDS therapy. *Nature* **337**, 525-31.

18. Langner, K. D., Niedrig, M., Fultz, P., Anderson, D., Reiner, G., Repke, H., Gelderblom, H., Seed, B., Hilfenhaus, J. & Zettlmeissl, G. (1993). Antiviral effects of different CD4-immunoglobulin constructs against HIV-1 and SIV: immunological characterization, pharmacokinetic data and in vivo experiments. *Arch Virol* **130**, 157-70.

19. Byrn, R. A., Mordenti, J., Lucas, C., Smith, D., Marsters, S. A., Johnson, J. S., Cossum, P., Chamow, S. M., Wurm, F. M., Gregory, T. & et al. (1990). Biological properties of a CD4 immunoadhesin. *Nature* **344**, 667-70.

20. Presta, L. G. (2005). Selection, design, and engineering of therapeutic antibodies. *J Allergy Clin Immunol* **116**, 731-6; quiz 737.

21. Presta, L. G. (2008). Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* **20**, 460-70.

22. Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S. C., Vielmetter, J., Carmichael, D. F., Hayes, R. J. & Dahiyat, B. I. (2006). Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* **103**, 4005-10.

23. Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A. & Presta, L. G. (2001). High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem* **276**, 6591-604.

24. Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H. & Presta, L. G. (2002). Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* **277**, 26733-40.

25. Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N. & Shitara, K. (2003). The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* **278**, 3466-73.

26. Idusogie, E. E., Wong, P. Y., Presta, L. G., Gazzano-Santoro, H., Totpal, K., Ultsch, M. & Mulkerrin, M. G. (2001). Engineered antibodies with increased activity to recruit complement. *J Immunol* **166**, 2571-5.

27. Hinton, P. R., Johlfs, M. G., Xiong, J. M., Hanestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vasquez, M. & Tsurushita, N. (2004). Engineered human IgG antibodies with longer serum half-lives in primates. *J Biol Chem* **279**, 6213-6.

28. Hinton, P. R., Xiong, J. M., Johlfs, M. G., Tang, M. T., Keller, S. & Tsurushita, N. (2006). An engineered human IgG1 antibody with longer serum half-life. *J Immunol* **176**, 346-56.

29. Boyd, M. R., Gustafson, K. R., McMahon, J. B., Shoemaker, R. H., O'Keefe, B. R., Mori, T., Gulakowski, R. J., Wu, L., Rivera, M. I., Laurencot, C. M., Currens, M. J., Cardellina, J. H., 2nd, Buckheit, R. W., Jr., Nara, P. L., Pannell, L. K., Sowder, R. C., 2nd & Henderson, L. E. (1997). Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. *Antimicrob Agents Chemother* **41**, 1521-30.

30. Barrientos, L. G., O'Keefe, B. R., Bray, M., Sanchez, A., Gronenborn, A. M. & Boyd, M. R. (2003). Cyanovirin-N binds to the viral surface glycoprotein, GP1,2 and inhibits infectivity of Ebola virus. *Antiviral Res* **58**, 47-56.

31. O'Keefe, B. R., Smee, D. F., Turpin, J. A., Saucedo, C. J., Gustafson, K. R., Mori, T., Blakeslee, D., Buckheit, R. & Boyd, M. R. (2003). Potent anti-influenza activity of cyanovirin-N and interactions with viral hemagglutinin. *Antimicrob Agents Chemother* **47**, 2518-25.

32. Bewley, C. A. (2001). Solution structure of a cyanovirin-N:Man alpha 1-2Man alpha complex: structural basis for high-affinity carbohydrate-mediated binding to gp120. *Structure* **9**, 931-40.

33. Botos, I., O'Keefe, B. R., Shenoy, S. R., Cartner, L. K., Ratner, D. M., Seeberger, P. H., Boyd, M. R. & Wlodawer, A. (2002). Structures of the complexes of a potent anti-HIV protein cyanovirin-N and high mannose oligosaccharides. *J Biol Chem* **277**, 34336-42.

34. Bolmstedt, A. J., O'Keefe, B. R., Shenoy, S. R., McMahon, J. B. & Boyd, M. R. (2001). Cyanovirin-N defines a new class of antiviral agent targeting N-linked,

high-mannose glycans in an oligosaccharide-specific manner. *Mol Pharmacol* **59**, 949-54.

35. O'Keefe, B. R., Shenoy, S. R., Xie, D., Zhang, W., Muschik, J. M., Currens, M. J., Chaiken, I. & Boyd, M. R. (2000). Analysis of the interaction between the HIV-inactivating protein cyanovirin-N and soluble forms of the envelope glycoproteins gp120 and gp41. *Mol Pharmacol* **58**, 982-92.

36. Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* **265**, 10373-82.

37. Dey, B., Lerner, D. L., Lusso, P., Boyd, M. R., Elder, J. H. & Berger, E. A. (2000). Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. *J Virol* **74**, 4562-9.

38. Hessell, A. J., Hangartner, L., Hunter, M., Havenith, C. E., Beurskens, F. J., Bakker, J. M., Lanigan, C. M., Landucci, G., Forthal, D. N., Parren, P. W., Marx, P. A. & Burton, D. R. (2007). Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* **449**, 101-4.

39. Florese, R. H., Van Rompay, K. K., Aldrich, K., Forthal, D. N., Landucci, G., Mahalanabis, M., Haigwood, N., Venzon, D., Kalyanaraman, V. S., Marthas, M. L. & Robert-Guroff, M. (2006). Evaluation of passively transferred, nonneutralizing antibody-dependent cellular cytotoxicity-mediating IgG in protection of neonatal rhesus macaques against oral SIVmac251 challenge. *J Immunol* **177**, 4028-36.

40. Gomez-Roman, V. R., Patterson, L. J., Venzon, D., Liewehr, D., Aldrich, K., Florese, R. & Robert-Guroff, M. (2005). Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. *J Immunol* **174**, 2185-9.

41. Huber, V. C., Lynch, J. M., Bucher, D. J., Le, J. & Metzger, D. W. (2001). Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J Immunol* **166**, 7381-8.

42. Baldridge, J. R. & Buchmeier, M. J. (1992). Mechanisms of antibody-mediated protection against lymphocytic choriomeningitis virus infection: mother-to-baby transfer of humoral protection. *J Virol* **66**, 4252-7.

43. Holl, V., Peressin, M., Decoville, T., Schmidt, S., Zolla-Pazner, S., Aubertin, A. M. & Moog, C. (2006). Nonneutralizing antibodies are able to inhibit human

immunodeficiency virus type 1 replication in macrophages and immature dendritic cells. *J Virol* **80**, 6177-81.

44. Holl, V., Peressin, M., Schmidt, S., Decoville, T., Zolla-Pazner, S., Aubertin, A. M. & Moog, C. (2006). Efficient inhibition of HIV-1 replication in human immature monocyte-derived dendritic cells by purified anti-HIV-1 IgG without induction of maturation. *Blood* **107**, 4466-74.

45. Bringans, S. D., O'Keefe, B. R., Bray, M., Whitehouse, C. A. & Boyd, M. R. (2004). Development of a fluorescent microplate assay for determining cyanovirin-N levels in plasma. *Anal Bioanal Chem* **380**, 269-74.

46. Stemmer, W. P., Crameri, A., Ha, K. D., Brennan, T. M. & Heyneker, H. L. (1995). Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164**, 49-53.

47. Becktel, W. J. & Schellman, J. A. (1987). Protein stability curves. *Biopolymers* **26**, 1859-77.

48. Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K. M., Bilska, M., Kothe, D. L., Salazar-Gonzalez, J. F., Wei, X. P., Decker, J. M., Hahn, B. H. & Montefiori, D. C. (2005). Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol* **79**, 10108-10125.

49. Gupta, R., Jung E., & Brunak, S. (2004). Prediction of N-glycosylation sites in human proteins. *In preparation*. http://www.cbs.dtu.dk/services/NetNGlyc/.

50. Imperiali, B. & O'Connor, S. E. (1999). Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr Opin Chem Biol* **3**, 643-9.

51. Barrientos, L. G., Louis, J. M., Botos, I., Mori, T., Han, Z., O'Keefe, B. R., Boyd, M. R., Wlodawer, A. & Gronenborn, A. M. (2002). The domain-swapped dimer of cyanovirin-N is in a metastable folded state: reconciliation of X-ray and NMR structures. *Structure* **10**, 673-86.

52. Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R. & Wilson, I. A. (2001). Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design. *Science* **293**, 1155-9.

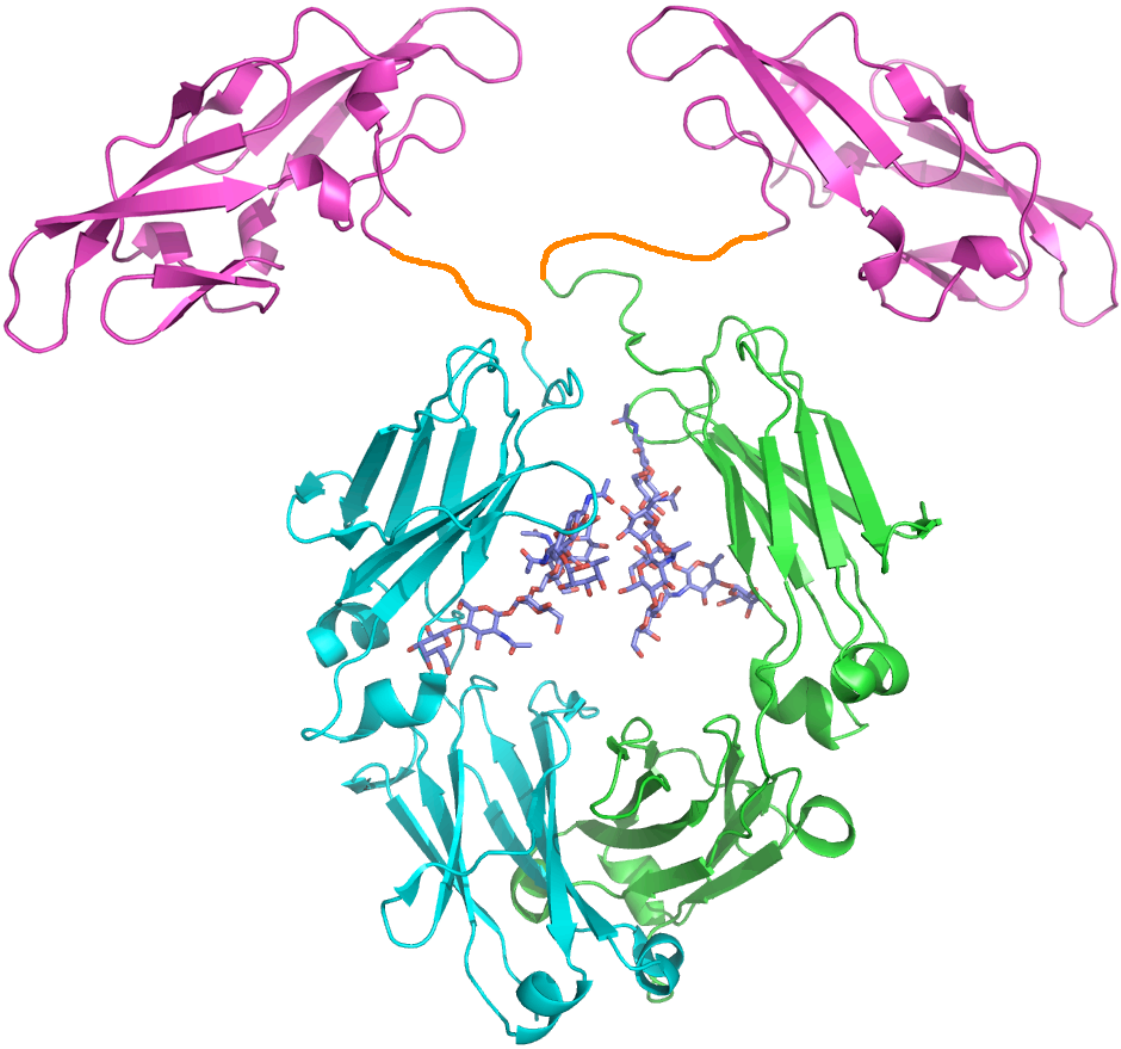

Figure 4-1. Model of the CVN-Fc lectibody. The CVN monomers are shown in magenta attached to the Fc (cyan and green) through flexible polypeptide linkers shown in orange. The Fc glycosylation is shown in stick representation with blue carbons. This model was created by combining a monomeric NMR structure of CVN[32] and the Fc from the IgG1b12 crystal structure (1HZH)[52] in Adobe Photoshop and is not a solved structure of this variant.

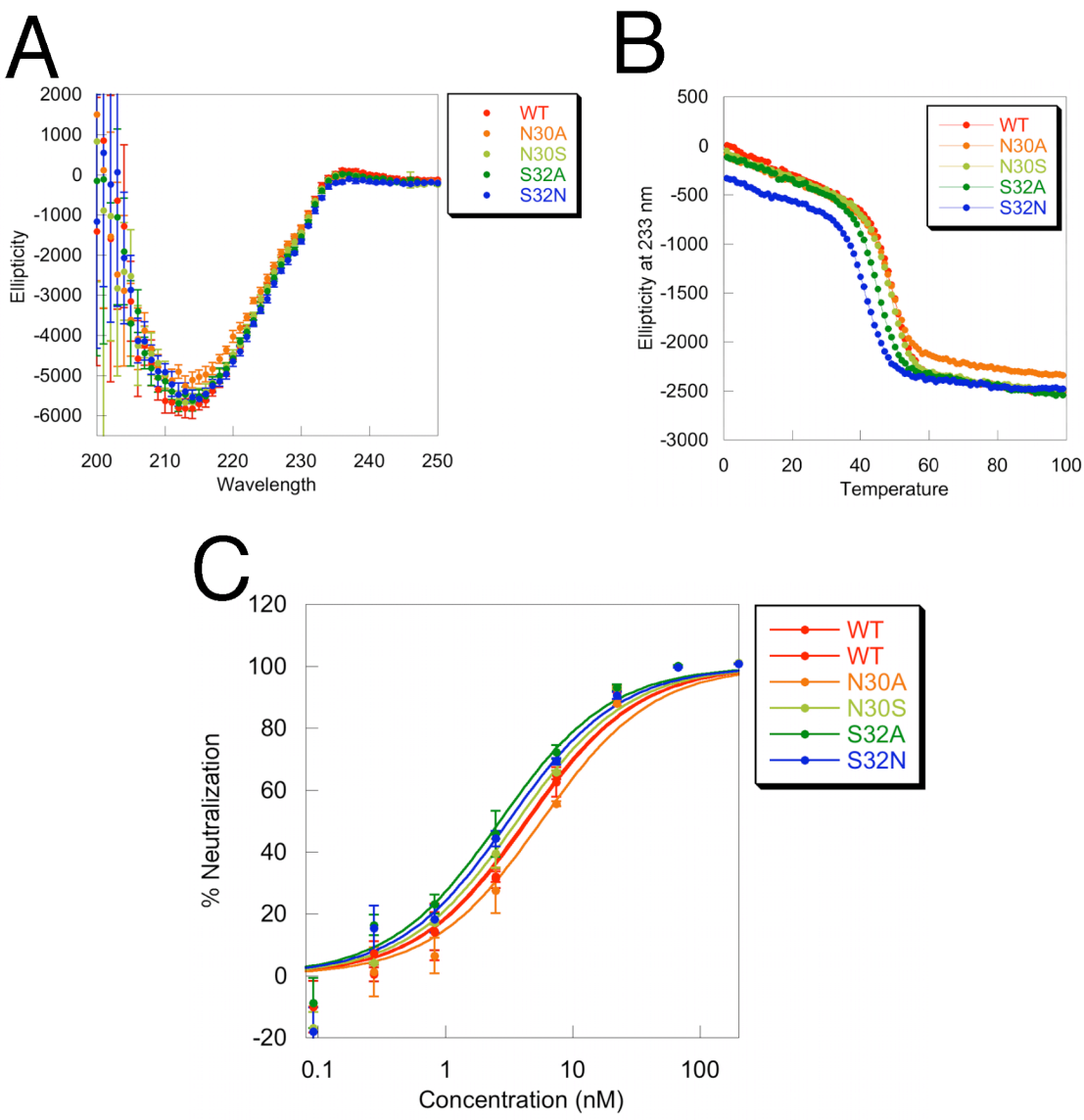

Figure 4-2. Assessment of glycosylation site deletion variants. (A) CD wavelength scans of the four variants compared to WT CVN. (B) Thermal denaturation of WT and the variants monitored by CD at 233 nm. (C) HIV neutralization curves of glycosylation site variants and WT.

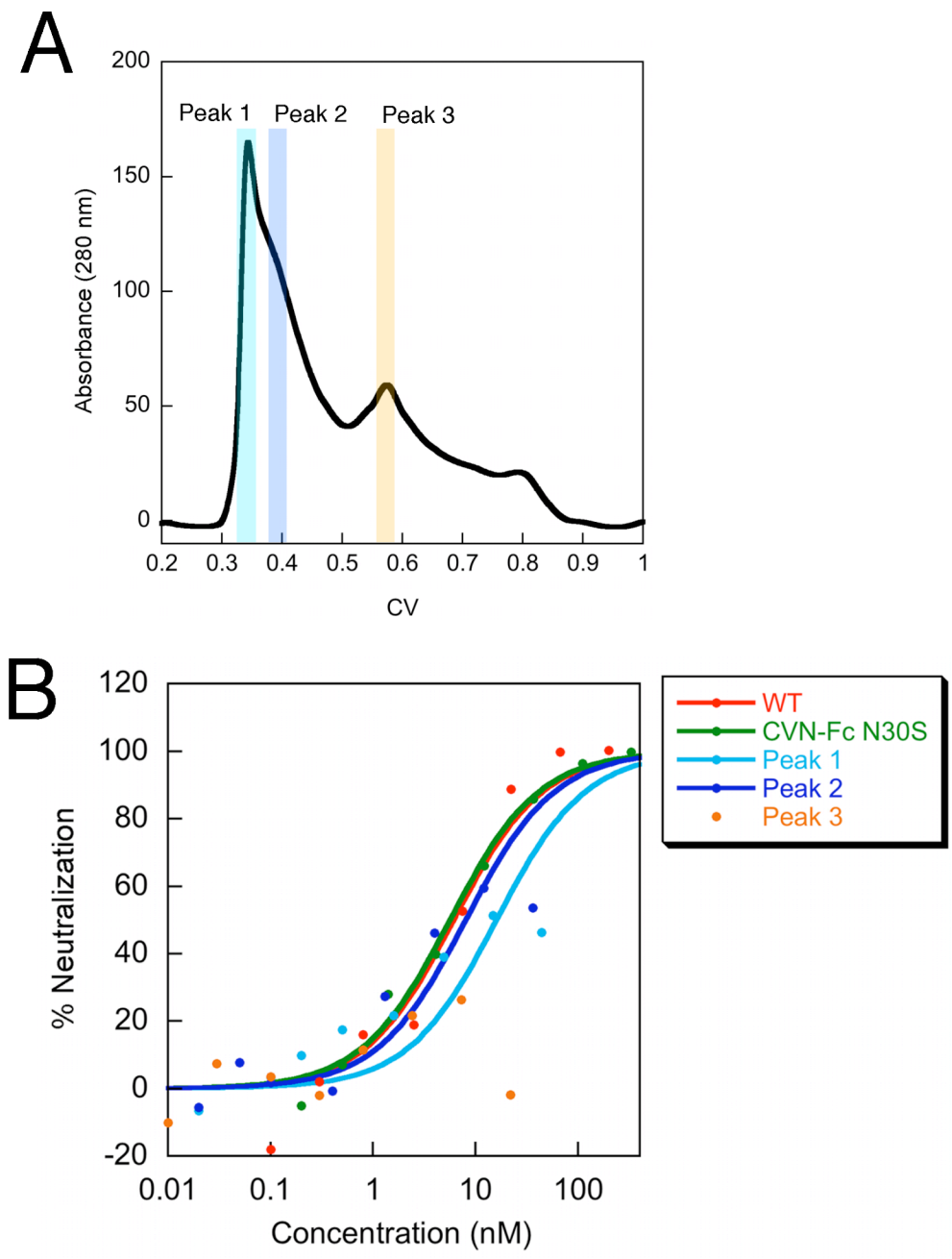

Figure 4-3. CVN-Fc N30S purification and activity. (A) A gel filtration trace of CVN-Fc N30S shows that the majority of the protein forms high order oligomers. (B) CVN-Fc N30S has WT-like HIV neutralization activity, but the active protein is the high molecular weight species and not from Peak 3 which corresponds to dimeric lectibody. No curve fit is shown for Peak 3 due to the low neutralization activity.

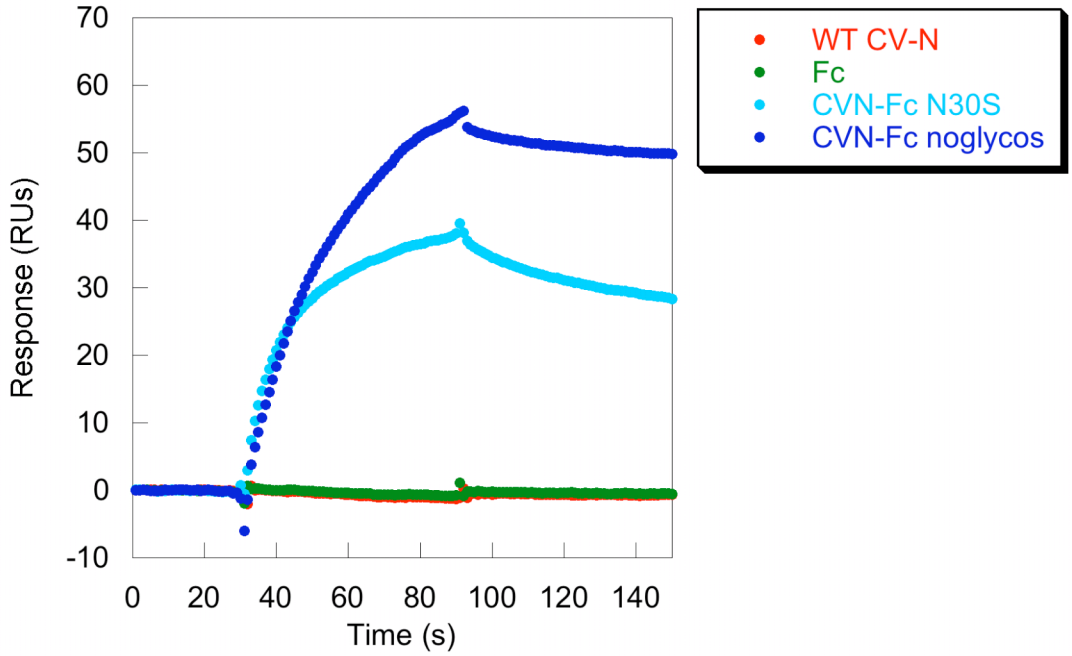

Figure 4-4. Surface plasmon resonance assays of lectibodies and Fc. WT CVN was immobilized on the surface and various proteins were analyzed for binding. WT CVN and human glycosylated Fc did not bind to the surface. However, both lectibody constructs (CVN-Fc N30S and CVN-Fc noglycos) showed significant interaction with the WT CVN surface that could not be regenerated.

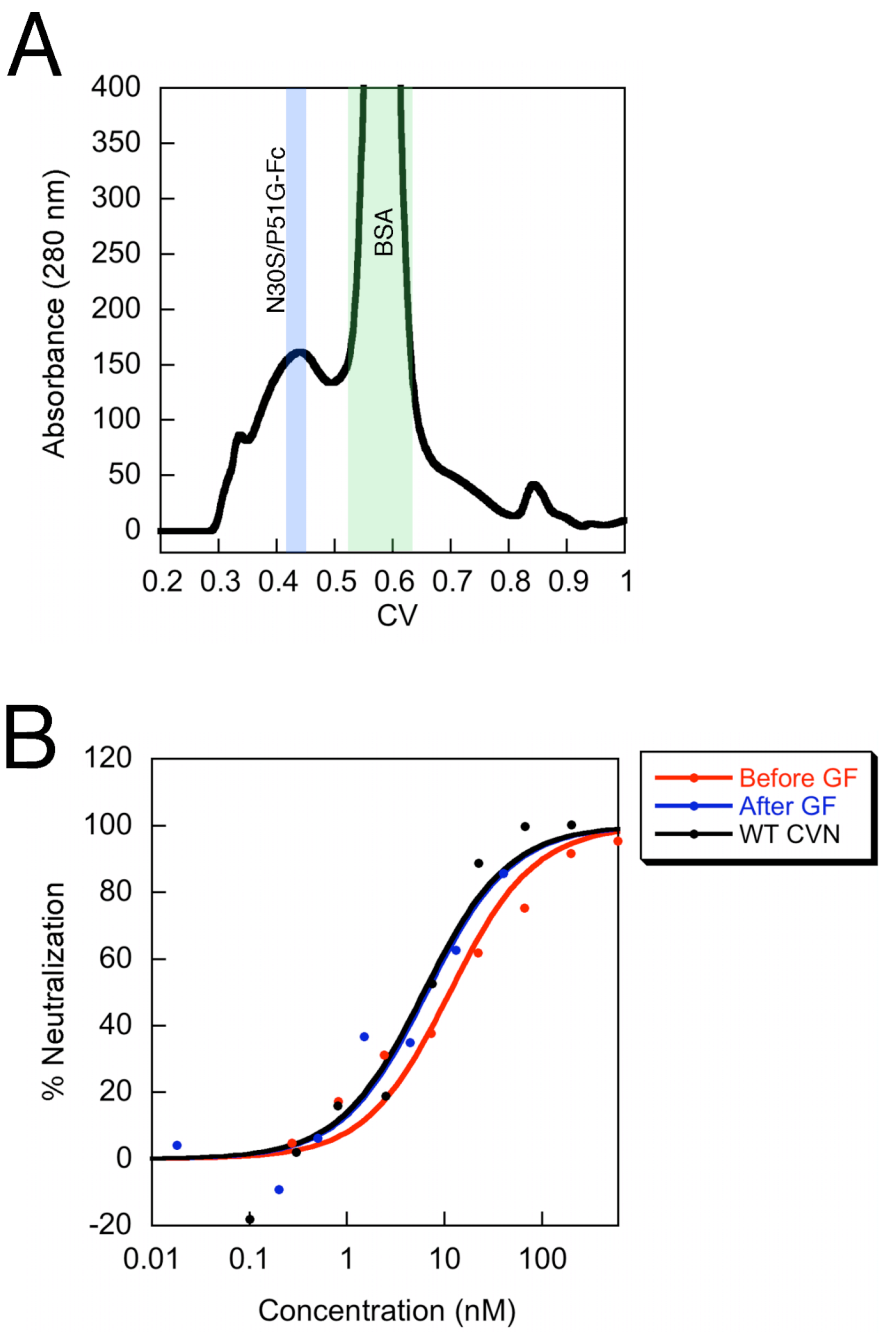

Figure 4-5. N30S/P51G-Fc purification and activity. (A) Gel filtration of N30S/P51G-Fc shows a broad peak corresponding to the lectibody around 0.44 CV. This sample was heavily contaminated by BSA from the expression (0.58 CV). (B) N30S/P51G-Fc is active both before (red data) and after (blue data) gel filtration. Due to lack of space on the assay plate, WT CVN was not run on this plate. A reference curve from a previous assay for WT is shown in black. The small void volume peak and the BSA peak both showed no anti-HIV activity (data not shown).