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Harnessing post-translational modifications for next generation HIV immunogens

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Abstract

The extensive post-translational modifications of the envelope spikes of the human immunodeficiency virus (HIV) present considerable challenges and opportunities for HIV vaccine design. These oligomeric glycoproteins typically have over thirty disulphide bonds, around a hundred N-linked glycosylation sites and are functionally dependent on protease cleavage within the secretory system. The resulting mature structure adopts a compact fold with the vast majority of its surface obscured by a protective shield of glycans which can be targeted by broadly neutralizing antibodies. Despite the notorious heterogeneity of glycosylation, rare B-cell lineages can evolve to utilize and cope with viral glycan diversity and these structures therefore present promising targets for vaccine design. The latest generation of recombinant envelope spike mimetics contain reengineered post-translational modifications to present stable antigens to guide the development of broadly neutralizing antibodies by vaccination.

Keywords

virus; glycosylation; vaccine; immunogen; glycans

Introduction

The immense variation of the genome of HIV presents a considerable obstacle to the development of an effective, sterilizing vaccine. The failure of classical vaccination strategies has driven advances in our understanding of the molecular basis of viral infectivity and the host immune response. Lines of enquiry have included investigating correlates of protection arising from the RV144 trial such as the role of non-neutralizing antibodies [1–3]. Here, however, we focus on vaccination strategies aiming to develop immunogens capable of eliciting a protective broadly neutralizing antibody (bnAb) response.

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Innovations towards the development of bnAb-eliciting immunogens have included the production of native-like trimers that mimic the envelope glycoprotein (Env) spikes expressed on the surface of the virion [4–7]. These soluble glycoproteins have revolutionized our understanding of the viral glycoprotein structure and have stimulated the design of new immunogens. Encouragingly, these mimetics bind to a growing number of bnAbs isolated from infected patients and they are being investigated as a platform for the next-generation of immunogens. Armed with the detailed structure of the envelope spike mimetics, detailed knowledge of the intricate network of post-translational modifications has been revealed [8–15]. Exploiting and targeting the post-translational modifications of the viral spike has enabled the design of improved immunogens that are able to elicit protective neutralizing antibodies against a narrow but growing range of viral isolates [16–18].

Engineering the envelope spike

Env is the sole viral protein expressed on the surface of the HIV virion and antibodies capable of binding the functional form of the viral spike can effectively neutralize viral infectivity [19–25]. As a class I viral fusion glycoprotein, Env consists of a trimer of heterodimers comprising gp120, which consists of five constant domains interspersed with hypervariable loops (Figure 1A), responsible for receptor recognition, and the transmembrane fusion glycoprotein, gp41. Env enables viral particle recognition of CD4+ T-cells, binding to the chemokine co-receptor, and ultimately fusion of the viral membrane with that of the target cell. To generate infectious viral particles, the viral spike must be extensively post-translationally modified by the host cell. These modifications include the formation of disulphide bonds (Figure 1B), extensive N-linked glycosylation (Figure 1C and 1D) and two proteolytic cleavage events, one after the signal peptide and the other between gp120 and gp41 (Figure 1E)[26].

Successful attempts to recombinantly mimic the Env-based epitopes of broadly neutralizing antibodies have involved solubilizing and stabilizing glycoprotein trimers utilising post-translational modifications and amino acid substitutions. Crucially, native-like Env trimers have been generated by the addition of a disulphide bond between gp120 and gp41 subunits and an isoleucine to proline mutation in gp41 which stabilise the prefusion conformation of Env. These immunogens are termed “SOSIPs” [5,27,28]. These stable soluble mimetics are not in themselves sufficient to generate a broad and protective immune response. Strategies to improve the breadth and potency of the antibody response has included the design of immunogens to stimulate the precursor lineage of potent bnAbs, for example by the targeted elimination of key glycans and the hyperstabilization of trimers through disulphide bond engineering [27–31].

Disulphides in infection and hyperstabilization

The quaternary structure of natively folded viral spikes requires the correct formation of disulphide bonds in the mammalian secretory pathway. Slow folding events allow time for the extensive network of disulphide bonds to form and to “shuffle” using protein disulphide isomerase (PDI) to ensure the optimal disulphide bond configuration is achieved [32–34]. The correct pairing of disulphides is an essential requirement for infectious Env, as by knocking

out PDIs *in vitro* the resultant protein is able to bind to CD4 but not undergo the conformational changes required for membrane fusion[35]. The incorrect formation of disulphides is also promoted when viral spike mimetics remain uncleaved by furin, this not only impacts the resultant quaternary structure of the viral spike it also perturbs the glycan shield and therefore bnAb binding[36].

With the structural characterisation by X-ray crystallography and mass spectrometry using the BG505 SOSIP.664 construct it has been possible to map the locations of the network of disulphide bonds[36,37]. As well as understanding the locations of the disulphide bonds in 3-dimensional space it is also possible to utilise the biophysical analysis to design next-generation hyperstabilized constructs that employ additional disulphide bonds to minimise the intrinsic flexibility and instability of Env. Torrents de la Peña *et al.* describe additional mutations to the original BG505 SOSIP.664 of which the most prominent is the addition of an interprotomer gp120 disulphide bond[27]. This effectively stabilizes the trimeric Env oligomeric state and also prevents the possibility of gp120 shedding. These modifications do not impact upon previously characterised bnAb binding and do not influence other post translational modifications such as N-linked glycosylation and therefore demonstrate the ability to configure Env post-translational modifications for superior immunogen design[27].

Glycan engineering of germline targeting immunogens

In addition to their role in protein folding, the high density of N-linked glycans is thought to be driven by immune selection whereby glycans mask underlying conserved protein epitopes. This is evidenced by their accumulation during longitudinal infection and depletion during collapse of an effective adaptive immune response in late infection[38–42]. The high density of glycans means that the evolution of the glycan shield can be understood as holes being formed and being filled rather than there being a continuum of options for glycan locations[43]. This is less so in the variable loops where there is very low conservation of glycan positions (Figure 1C and 1D)[44]. One interesting consequence of the trimeric structure of the viral spike is the role of conserved glycans at the protomer interface. Presumably the interfacial glycans act to obscure important surface features and may help stabilize the trimeric structure[45]. The highly conserved N-glycan sites across the surface of Env also form epitopes for a range of bnAbs and with the characterisation of one such bnAb that is able to recognise the highly conserved N262 and N448 glycans the entire surface of Env forms bnAb epitopes[46,47].

The conservation of potential N-linked glycan sites across the envelope spike far exceeds other regions of the envelope spike and present a robust platform for immunogen design[47,48]. In addition, the high density of glycosylation and the trimeric architecture places steric restrictions on glycan processing which drives the formation of a population of under-processed oligomannose-type glycans[9,49–53]. However, the extensive heterogeneity inherent to N-linked glycan processing means that these steric constraints alone are not entirely sufficient for native-like glycosylation[49].

The producer cell of the Env spike, either viral or recombinant, can lead to changes in glycosylation that may impact on bnAb binding. In one extreme, when the glycans of

macrophage-derived HIV particles are compared to those from peripheral blood mononuclear cells (PBMCs) there was a significant shift in the composition of the population of complex-type glycans with the macrophage-derived glycans exhibiting large polylactosamine structures[54]. Importantly, the viruses exhibited different sensitivities to antibody neutralization and this may be attributed to glycosylation.

Glycan heterogeneity is an important parameter when assessing recombinant Env spike mimetics. Such heterogeneity has been shown to contribute to partial neutralization by glycan-dependent bnAbs [55–57]. Encouragingly, trimeric SOSIPs exhibit native-like levels of oligomannose-type glycans. However, there will be potentially important variations in glycan structure between recombinant immunogens and their viral counterparts. For example, between PBMC and HEK293T derived gp120 there are subtle differences such as the sialic acid linkages of complex type glycans, with PBMCs presenting a larger population of α2,6 linkages. Such differences between viral and recombinant glycosylation may result in diminished presentation of potential glycan epitopes to the immune system when recombinant constructs are used as immunogens[58]. Despite this caveat, promising immunogens are being developed that exploit viral glycosylation.

Despite the broad neutralization and high affinities of bnAbs to the viral spike, few patients develop bnAb lineages before the genetic diversity of the viral spike overwhelm the immune system. The original goal of mimicking the natively glycosylated viral spike has produced strong autologous responses but have not managed to elicit broadly neutralizing antibodies in macaques[17]. The focus of immunogen design has therefore shifted and now focuses on manipulating the post-translational modifications of Env to guide the immune system to mature the rare B cell lineages of broadly neutralizing antibodies. This approach has been greatly stimulated by the observation that patients that have eventually developed a potent bnAb response often have virus missing key glycan sites[59]. Many investigators are now examining immunogens displaying glycan holes[28–31,60,61].

One such platform is the glycan depleted trimer, termed BG505 SOSIP.v4.1-GT1 which has been designed to trigger B cells corresponding to germline PGT121 and germline VRC01. This trimer lacks 15 glycans across the viral spike and initiates antibody responses in knock-in mice expressing the predicted germlines for these bnAbs[62]. In addition, a comparative glycan analysis revealed that the remaining oligomannose-type glycans were largely unaffected by the glycan deletions with changes in their mannose trimming localised to regions proximal to a depleted glycan[63]. The overall integrity of the mannose patch is a promising observation and broadens the possibilities of using extensive glycan holes as a design feature of new immunogens.

It is also possible to harness the microheterogeneity of conserved glycan sites to present glycan epitopes that result in the proliferation of the rare B-cell lineages of bnAbs. In trimers derived from *env* sequences from a prolonged infection such as the clade A BG505 sequence used for BG505 SOSIP.664 the apex sites N160 and N156 display oligomannose glycans[50–52]. Andrabi *et al.* demonstrated that bnAb precursors require sialic acid to bind to the apex viral spike and that Env sequences derived from early infection appear to have sialic acid glycans at N160 and N156[64]. Although the bnAb precursors require sialic acid

the resultant mature bnAbs are able to bind to trimers containing apex sites that are well defined as oligomannose. These observations teach us that glycan heterogeneity can evolve during the course of infection and that this feature can potentially be exploited in vaccine design.

Optimising and bypassing furin cleavage

Another critical post-translational modification of Env is furin cleavage of the gp160 pro-protein. Furin cleavage is thought to occur in the trans Golgi network and is an essential step in the formation of functional correctly folded trimers. Furin cleaves between the gp120 and gp41 subunits of the gp160 polypeptide, recognising the amino acid motif Arg-X-(Arg/Lys)-Arg[65]. Negative stain electron microscopy of uncleaved trimers, which have been explored as viral spike mimetics, revealed that they do not naturally adopt the classical trimeric architecture[58,66]. The perturbation of the structural integrity resulting from a lack of furin cleavage also has implications for the post-translational modifications of candidate immunogens. Analysis of N-linked glycosylation on a global and a site-specific level on a number of uncleaved structures described an elevation in glycan processing, most likely resulting from aberrant trimerization[9,67]. As broadly neutralizing antibody epitopes frequently contain N-linked glycans the aberrant glycosylation resulting from a lack of furin cleavage will have knock-on effects on the antigenicity of those trimers. Furthermore, the destabilization of the quaternary structure ablates the binding of quaternary-specific bnAbs such as PGT151 and also reduces the affinity of other quaternary specific bnAbs such as PGT145[68,69].

The requirement for furin cleavage for correct assembly is an important feature in the expression of BG505 SOSIP.664 as the low levels of endogenous furin result in large populations of uncleaved trimers. Binley *et al.* circumvented this problem by co-transfecting plasmid containing the *furin* gene concomitantly with BG505 SOSIP.664. In addition, it is important to optimise the protease cleavage step as much as possible. The majority of HIV strains present a furin cleavage site consisting of a REKR motif. A panel of mutations introduced to this region demonstrated that this is not the optimal motif for furin mediated Env glycoprotein cleavage. By replacing the REKR motif with 6 arginine residues the proteolytic separation of gp120 and gp41 is greatly improved[70]. By modifying the amino acid sequence in this way, it is possible to manipulate post-translational protease cleavage to allow for a larger amount of native-like material. Impressively, efficient furin cleavage has also now been achieved in production of clinical grade BG505 SOSIP.664 using a stable CHO cell line containing the target SOSIP, possessing an optimised furin cleavage site, and also the gene encoding furin[71].

As well using recombinant glycoproteins as immunogens, a further strategy currently under investigation to boost the neutralizing antibody titre is to use DNA-based approaches. For DNA vaccines, the *env* gene is administered and the trimers are expressed by the host. In order to utilise this approach, it is favourable to bypass the furin cleavage stage as it removes the requirement of co-transfection with furin. Although previously defined uncleaved trimers have produced large populations of misfolded trimers, several constructs now exist that are able to form native-like trimers without the need for furin cleavage. By replacing the furin

cleavage site with a flexible linker it is possible to generate native-like trimers. With additional stabilizing mutations native flexibly linked (NFL) trimer display native-like bnAb binding and analogous glycosylation to BG505 SOSIP.664[6,30,72]. Using *in silico* methods Kong *et al.* redesigned the HR1 loop to generate uncleaved trimers that were stabilized in the pre-fusion conformation (UFO) and present trimers with greater stability that the equivalent SOSIP construct[73]. The ability to redesign fundamental post-translational modifications with little impact on the overall glycosylation and topology of the envelope spike further highlights the robustness of the glycan shield as a target for bnAb elicitation by immunization.

Perspective

As immunogen strategies continue to move towards activating precursor B cells, innovations are increasingly exploiting or bypassing post-translational modifications of the envelope glycoprotein. These advances may well stimulate developments of efficacious vaccines against a much wider range of pathogens where classical vaccine design strategies have proven ineffective.

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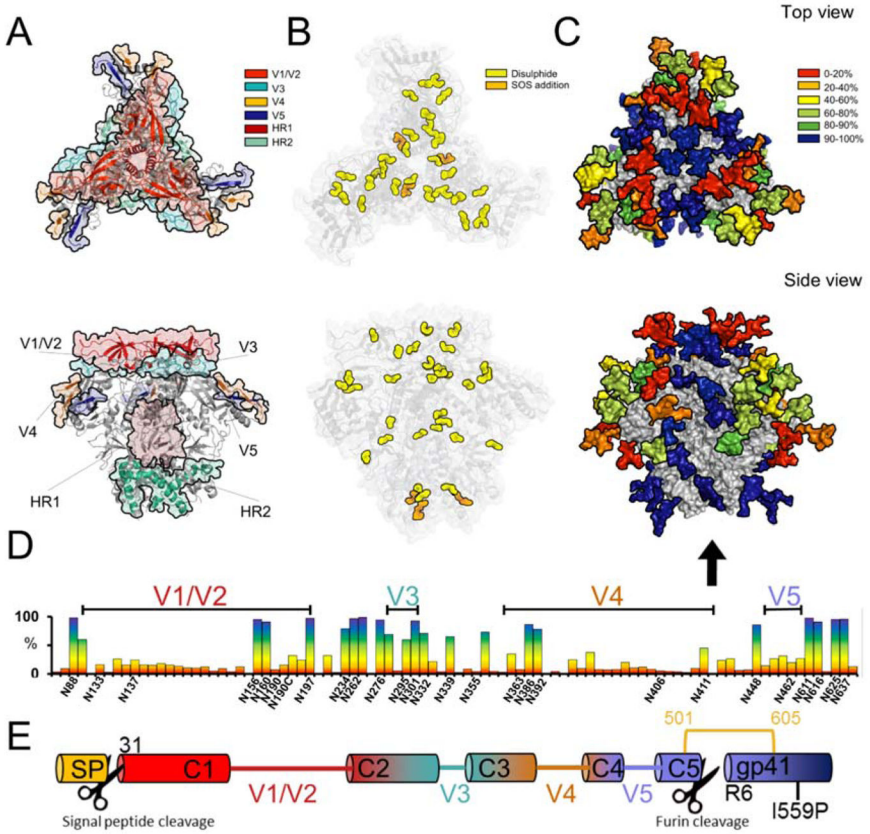

Figure 1. Representation of the post-translational modifications of BG505 clade A envelope glycoprotein

Models were generated using the cryo-EM structure of BG505 SOSIP.664 PDB ID:5ACO [12]. A) 3D representation of the variable loops on gp120 and the heptad repeats of gp41. B) Canonical disulphide bonds found in BG505[36] with the additional stabilizing disulphide bond found in BG505 SOSIP.664 shown in orange. C) Conservation map of the glycans of BG505 SOSIP.664. The glycans were coloured according to their conservation across 4000 Env strains Huang et al.[44]. BG505 crystal structure with N-linked glycans modelled by Behrens et al.[52] D) The frequency of potential N-linked glycan sites across the Env sequence with the PNGs found in BG505 SOSIP.664 labelled on the X axis. E) Schematic showing the locations of proteolytic cleavage for the signal peptide and the furin cleavage site.