**Assembly and Characterization of gp160-Nanodiscs: A New Platform for
Biochemical Characterization of HIV Envelope Spikes**

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Abstract

The human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS) and is thus responsible for significant morbidity and mortality worldwide. Despite considerable effort, preparation of an effective vaccine for AIDS has been elusive and it has become clear that a fundamental understanding of the relevant antigenic targets on HIV is essential. The Env trimer spike is the only viral antigen present on the surface of the viral particle and it is the target of all broadly neutralizing antibodies isolated to date. Thus, a soluble, homogeneous, and well-defined preparation of Env trimers is an important first step towards biochemical and structural characterization of the antigenic spike. Phospholipid bilayer nanodiscs represent a relatively new technology that can serve as a platform for the assembly of membrane proteins into a native membrane-like environment. Here we describe the preparation and characterization of unprocessed full-length, natively glycosylated gp160 Env proteins incorporated into nanodiscs (gp160-ND). The particles are soluble and well defined in the absence of detergent, and possess a morphology anticipated of Env incorporated into a lipid ND. Importantly, the gp160-NDs retain CD4 and Env antibody binding characteristics expected of a functional trimer spike and their incorporation into a lipid membrane allows interrogation of epitopes associated with the membrane-proximal ectodomain region of gp41. These studies provide the groundwork for the use of gp160-ND in more detailed biochemical and structural studies that may set the stage for their use in vaccine development.

Keywords

HIV; AIDS; Env, gp160; glycoprotein; lipid nanodiscs; membrane protein

Graphical Abstract

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1. Introduction

By the end of 2013 an estimated 35.3 million individuals in the world were living with the human immunodeficiency virus (HIV), the infectious agent causing acquired immune deficiency syndrome (AIDS; <http://www.unaids.org>). Approximately 1.6 million people died that year from AIDS-related complications, which makes HIV infection a major global health concern. While antiviral therapeutics have been effective in controlling the progression of the disease, durable control of the global epidemic will likely depend on the development of a safe and effective vaccine. Although results of the RV144 trial have indicated the feasibility of vaccine protection against HIV acquisition (Rerks-Ngarm et al., 2009), it is generally accepted that a better understanding of the relevant biochemical and structural features of the HIV particle is needed to develop a more effective vaccine.

The infectious HIV particle is composed of two RNA genomes protected by a capsid shell, a matrix protein, and a lipid bilayer that displays the viral envelope glycoprotein (Env, Figure 1A) (Freed and Martin). Env is expressed as a heavily glycosylated 160 kDa precursor (gp160) that initially self-assembles as a membrane-bound homotrimer in the lipid bilayer of the *trans* Golgi network. Host proteases cleave the protein into a 41 kDa transmembrane subunit (gp41) that remains non-covalently associated with a 120 kDa surface subunit (gp120) (Moulard and Decroly, 2000). This yields the mature trimeric spike that is

ultimately incorporated into the viral envelope during budding from the cell. Env spikes decorate the viral surface and mediate events required for cell entry, including CD4/co-receptor binding and membrane fusion steps (Melikyan, 2014; Wilen et al., 2012). While proteolytic maturation of gp160 is not strictly required for CD4 binding, the immature gp160 trimer spike does not support fusion (Gu et al., 1995; Moulard and Decroly, 2000). Thus, infectious viral particles isolated from COS-1 and PBMC cells contain predominantly fully processed gp41:gp120 Env spikes (Dubay et al., 1995; Herrera et al., 2005; Iwatani et al., 2001; McCune et al., 1988). In contrast, the immature gp160 trimer spike comprises a sizeable fraction of Env found within the plasma membrane of HEK 293T cells infected with HIV, as well as pseudovirions derived from these cells (Blay et al., 2007; Moore et al., 2006). These data indicate that full-length, unprocessed gp160 can be efficiently transported to the cell membrane, and that it can be incorporated into viral particles during budding in some, but not all cell types.

Env is the sole viral protein displayed on the surface of the HIV particle and it is the target for all known broadly neutralizing antibodies (bNAbs) characterized to date (Burton et al., 2012; Corti and Lanzavecchia, 2013; Hoxie, 2010; Kwong and Mascola, 2012; Schief et al., 2009; van Gils and Sanders, 2013). Thus, this protein represents an important target for vaccine development. Initial studies utilized recombinant, monomeric gp120 as a target antigen but these approaches have been unsuccessful in generating bNAbs to HIV (Hoxie, 2010). Subsequent efforts employed soluble truncated molecules of gp160 in an attempt to maintain quaternary features of the “native” Env trimer spike. Specifically, gp140 fusion

constructs composed of gp120 and the ectodomain of gp41 (Figure 1A) have been used as vaccine candidates. While somewhat more effective they nevertheless similarly fail in generating bNAbs (Hoxie, 2010; Phogat and Wyatt, 2007), most likely due to the failure of gp140 to assemble into a native trimeric structure (Guttman and Lee, 2013). Given the presence of immature gp160 trimers in some HIV particles (*vide supra*), it has been suggested that unprocessed Env may serve as an antigen that is recognized by some bNAbs (Leaman et al., 2010; Moore et al., 2006; Poignard et al., 2003). Within this context, we have demonstrated that full-length gp160 used in a prime-boost immunization protocol induces low levels of neutralizing antibodies and protection in a SIV model (Hu et al., 1992; Polacino et al., 1999a; Polacino et al., 1999b). More recently, a novel design of unprocessed Env, called SOSIP, has been shown to possess the structural and antigenic properties of a functional trimer (Julien et al., 2013a; Julien et al., 2013b); However, despite considerable effort to define an effective antigen, all “rationally designed” immunogens have so far failed to elicit bNAbs to diverse primary isolates of HIV. It is now generally accepted that (i) there is a fundamental gap in our understanding of how the membrane-anchored Env protein is presented in the HIV particle and that (ii) a better understanding of the biochemical and structural features of the spike may better guide our attempts towards the development of an effective vaccine (Hu and Stamatatos, 2007; Karlsson-Hedestam et al., 2008; Walker and Burton, 2008).

Ideally, an Env preparation suitable to biochemical, biophysical, and structural interrogation would be a membrane-bound Env trimer spike that is soluble,

monodisperse, and stable in solution for prolonged storage. A variety of approaches have been utilized to characterize “native” Env trimers in a native-like lipid environment, including reconstitution of uncleaved gp160 into virosomes (Cornet et al., 1992) and the use of pseudovirions or infectious virus (Crooks et al., 2007; Liu et al., 2008; Poon et al., 2005). A recent technology involves the use of phospholipid bilayer nanodiscs (ND) as a platform for the assembly of membrane proteins into a native lipid environment (Nath et al., 2007; Ritchie et al., 2009). These particles consist of discoidal portions of phospholipid bilayer circumscribed by a protein “belt” composed of two copies of a membrane scaffold protein (MSP, Figure 1B). These are amphipathic helical proteins originally derived from human apolipoprotein A-I and NDs closely resemble the nascent, pre- β high-density lipoprotein particle. NDs self-assemble upon removal of detergent from a mixture of detergent-solubilized lipid and MSP; if a detergent-solubilized membrane protein is present in the reconstitution mixture, it may partition into the disc upon removal of the detergent. Once integrated, the proteins are situated in a lipid bilayer that mimics the membrane of a cell or the envelope of a viral particle. When purified, they contain a defined ratio of one functional target protein unit (monomer or multimer) per disc, as opposed to highly variable detergent solubilized, liposomal, and pseudovirus preparations. This lends an obvious advantage for use with techniques in which sample solubility and homogeneity is a strict requirement. Thus, NDs can provide a soluble, stable, and monodisperse preparation of a membrane protein embedded in a native-like lipid bilayer, which provides a valuable tool for the study of

membrane bound proteins in solution (Nath et al., 2007; Ritchie et al., 2009). To date, a number of membrane proteins have been incorporated into lipid NDs including monomeric and trimeric bacteriorhodopsin, G-protein coupled receptors, Tar bacterial chemoreceptor, and cytochrome P450 3A4 (Bayburt and Sligar, 2010).

Here we describe the preparation and characterization of unprocessed full-length, natively glycosylated gp160 Env proteins incorporated into lipid nanodiscs (gp160-ND). The particles are soluble, homogeneous, and stable as compared to the detergent-solubilized protein preparations. The isolated gp-160 NDs retain bNAb-binding activity comparable to the detergent-solubilized form of gp160, while allowing for segregation of oligomeric forms and a more defined preparation. The results of these studies provide the groundwork for the use of gp160-ND in more detailed biochemical studies and potentially provide well-defined particles for use in vaccine development.

2. Materials & Methods

2.1. Materials. Methyl α -D-mannopyranoside, β -octyl glucopyranoside, and 1% sodium deoxycholate were purchased from Sigma-Aldrich, USA. Agarose-bound lens culinaris agglutinin (lentil lectin affinity media) was purchased from Vector Labs, USA. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids. Bio-Beads SM-2 was purchased from BioRad. NuPAGE and NativePAGE Novex Bis-Tris gradient gels were purchased from Invitrogen. TMB chromogen solution, goat anti-mouse HRP (#62-6520), goat

anti-human HRP (#62-8420), and recombinant protein G-HRP (#10-1223) were obtained from Life Technologies. All other materials were of the highest quality available.

Human HIV-immunoglobulin (HIV-IG) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog #3957, HIV-IG from NABI and NHLBI. Recombinant four domain soluble human CD4 (sCD4) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Soluble Human CD4 from Progenics, Catalog #4615, from Progenics. Human/human x mouse anti-CD4 antibody OKT4 (cell supernatant) was obtained from the American Type Culture Collection (#HB-10895). Sheep Anti HIV-1 gp120 polyclonal antibody was purchased from Aalto Bioreagents, Ireland (#D7324). Human VRC01 (Catalog #12033) and VRC03 (Catalog #12032) anti-HIV gp120 monoclonal antibodies were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1gp120 Monoclonal (VRC01 and VRC03), from Dr. John Mascola. Recombinant human monoclonal antibody b12 (Catalog #2640) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas. Recombinant human monoclonal antibody 4E10 (#10091) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 gp41 Monoclonal (4E10) from Dr. Hermann Katinger.

2.2. Protein Purification. All protein purifications utilized an Amersham Biosciences AKTApurifier core 10 System and all chromatography media was

purchased from GE Healthcare, USA. Absorbance spectra were recorded on a NanoDrop 2000c UV-Vis spectrophotometer.

HexaHistidine-tagged MSP-1D1 protein was expressed and purified according to published protocols (Ritchie et al., 2009). We use the generic term H6-MSP to denote this construct in the remainder of this work. Detergent solubilized, full-length, recombinant gp160 (1084i) was purified from BSC40 cells using vaccinia as the expression vector (Hu et al., 1986). Unless otherwise specified, all steps were carried out on ice or at 4°C using ice-cold buffers. Cell lysis was performed in Buffer A (20 mM Tris buffer, pH 8, 500 mM NaCl containing 1% Triton X-100) and the clarified lysate was applied to a lentil lectin column (5 mL) equilibrated and washed with Buffer B (20 mM Tris buffer, pH 8, containing 500 mM NaCl, 1% β -octyl glucopyranoside and 1% sodium deoxycholate). Bound protein was then eluted with Buffer B containing 500 mM methyl α -D-mannopyranoside and the gp160-containing fractions were pooled. The crude gp160 was applied to a HiTrap Q HP column (2 mL) equilibrated with Buffer B, except that the NaCl was reduced to 50 mM in the load and wash steps. Bound protein was then eluted with Buffer B and the gp160-containing fractions were pooled to afford a stock solution of crude gp160 solubilized in 1% β -octyl glucopyranoside and 1% sodium deoxycholate (Fraction I, 4-5 mg total protein). Unless otherwise indicated, we use generic term “detergent” to specifically mean 1% β -octyl glucopyranoside plus 1% sodium deoxycholate.

Detergent solubilized gp160 was further purified by size exclusion chromatography (SEC) for use as an Env standard in the ELISAs. Fraction I (1 mg)

was applied to a Superdex 200 10/300 GL column equilibrated and developed with Buffer B. The majority of the material eluted in the void volume of the column (8.7 mL, MW >1,300,000 Da) and the gp160-containing fractions were pooled and stored at 4°C until further use (Fraction II). The concentration of gp160 was determined spectrally using the calculated absorbance extinction coefficient ($178,760 \text{ M}^{-1} \text{ cm}^{-1}$), which typically afforded 900 µg purified protein (90% yield).

2.3. Assembly of gp160 Nanodiscs (gp160-ND). The ND assembly protocol was based upon previously published methods (Ritchie et al., 2009) and is outlined in Figure 1C. Briefly, appropriate volumes of POPC (20 mM solubilized in 200 mM sodium cholate), gp160 (~ 10 µM Fraction I solubilized in detergent) and purified H6-MSP were mixed to give final concentrations of 2 mM POPC, 4.5 µM gp160, 32 µM H6-MSP, and 20 mM sodium cholate (~ 3 mL reaction volume, 2 mg crude gp160). This represents a 125: 0.28: 2 molar ratio of lipid : gp160 : H6-MSP, which in principle should afford a 10-fold excess of empty ND relative to gp160-ND, assuming complete incorporation of gp160 as trimer spikes. To prepare “empty” ND, gp160 was omitted from the reaction mixture and replaced with an equal volume of Buffer B. The assembly mixtures were incubated for one hour at 4°C with gentle agitation. Bio-Beads, a nonpolar polystyrene material that adsorbs detergent, was used as an alternative to dialysis to remove the bulk of the detergent. Bio-Beads were added to a final concentration of 0.8 gm/mL and the reaction mixtures were incubated at 4°C for an additional four hours with gentle agitation. The supernatant was aspirated from the Bio-Beads and residual detergent was removed by overnight dialysis against at least 100 volumes of

Buffer C (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 50 mM imidazole) with a minimum of one buffer change to afford a crude ND preparation.

2.4. Purification of gp160 Nanodiscs. The gp160-ND purification scheme is outlined in Figure 1C. Briefly, the assembled NDs (above) were applied to a Ni Sepharose 6 FF (IMAC) column (0.5 mL media per 1.5 mL reaction mixture) equilibrated and washed with Buffer C. Bound material, which is associated with H6-MSP, was then eluted with Buffer C containing 500 mM imidazole. The gp160-containing fractions were pooled to afford a partially purified gp160-ND mixture containing ~ 300 µg gp160.

The mixture was next applied to a Superose 6 10/300 GL gel filtration column equilibrated and developed with Buffer D (20 mM Tris buffer, pH 8, 500 mM NaCl). The major gp160-containing fractions were pooled to afford a preparation containing 160 µg purified gp160 incorporated into nanodiscs. The gp160-NDs thus purified were stored at 4°C (1 - 2 weeks) or at -20°C (up to 8 weeks) until used.

2.5. Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting. Nanodisc samples for BN (native) PAGE were mixed with an appropriate volume of NativePAGE™ Sample Buffer (4x) and then fractionated by electrophoresis at 4°C on a 3-12% NativePAGE Novex Bis-Tris gradient gel. Samples for SDS (denaturing) PAGE analysis were first dissociated by incubation with 0.15% sodium cholate for 5 minutes at room temperature, followed by protein precipitation in 6% trichloroacetic acid. The protein pellet was resuspended in

200 mM Tris buffer, pH 8, containing 4% SDS and 150 mM NaOH, and then fractionated by electrophoresis on a 4-12% NuPAGE™ Bis-Tris gradient gel. In both cases, protein was visualized by staining of the gel with Coomassie R-250.

For Western blot analysis, the proteins were transferred from the gel to PVDF membranes according to gel manufacturer instructions. Tris-buffered saline containing 0.05% Tween-20 (TBST) and 4% nonfat dry milk was used for blocking of the membrane and for the dilution of all sample and antibody preparations. The membranes were blotted with HIV-IG (1:2,000 dilution of a 50 mg/mL stock solution) and protein G-HRP was used for gp160 detection (1:10,000 dilution of the manufacturer's stock solution). Immuno reactive bands were developed using the Pierce ECL Plus Western Blotting Substrate (Thermo Pierce) according to manufacturer instructions.

2.6. Calculation of the gp160:MSP Ratio in gp160 Nanodiscs. The protein content of the nanodisc preparations was estimated by video densitometry. Briefly, the Coomassie-stained gels were imaged using an EpiChemi³ darkroom system (UVP Bioimaging Systems) equipped with a Hamamatsu camera. The intensities of the gp160 and H6-MSP protein bands were quantified using the Image Quant software package (Molecular Dynamics) as previously described (Maluf et al., 2005). To account for possible staining differences between the two proteins, a standardization protocol was employed as follows. Mixtures of detergent-solubilized gp160 (Fraction II) and H6-MSP were prepared to afford molar ratios of 1:2, 2:2, and 3:2, corresponding to one, two and three gp160 subunits per nanodisc. The samples were fractionated by SDS-PAGE and the

ratio of the Coomassie stained proteins quantified by video densitometry to afford a standard curve, which was used to calculate the gp160:H6-MSP ratio in each nanodisc preparation.

2.7. Quantitation of Nanodisc Phospholipid Content. ND samples were dialyzed against 10 mM Tris pH 8, 20 mM NaCl overnight at 4°C and the solvent was then evaporated by heating to 200°C for ~30 minutes. The total phospholipid content of the resulting material was quantified using a published phosphomolybdate assay (Chen et al., 1956).

2.8. Electron Microscopy. The samples were applied to 300 mesh carbon coated copper grids treated by negative glow discharge and then negatively stained with Nano-W (Nanoprobes). Transmission electron microscopy was performed on a FEI Tecnai G2 F20 S-Twin TEM.

2.9. CD4 Binding Assay (ELISA). Unless otherwise indicated, all samples were diluted with phosphate-buffered saline containing 0.005% Tween-20 (PBST) supplemented with 2% nonfat dry milk. Sheep anti HIV-1 gp120 polyclonal antibody was rehydrated according to manufacturer instructions (1 mg/ml), diluted 1:100 in PBS and immobilized in Costar high binding EIA/RIA flat bottom 96-well plates (Corning); the wells were then blocked with 5% nonfat dry milk in PBST. Detergent solubilized gp160 (Fraction II), purified gp160-NDs, and empty nanodiscs were diluted to 3 µg/ml, 3 µg/ml gp160, and 0.3 µg/ml MSP, respectively, the latter corresponding to the anticipated MSP content of the gp160-ND. The diluted proteins were added to the wells and the samples were

incubated for two hours at room temperature. Human sCD4 was then added to each well at the indicated concentration and incubated for one hour at room temperature, followed by human/human x mouse anti-CD4 antibody OKT4 (a 1:100 dilution of OKT4 cell supernatant), which was incubated for 2 hours at room temperature. The wells were washed with PBST, goat-anti-mouse HRP conjugate (1:1,000 dilution of a 1.5 mg/mL stock solution) was added to each well, and the samples were incubated for an additional hour. Finally, each well was washed with PBST in preparation for ELISA.

2.10. CD4 Binding Competition Assay (ELISA). The sCD4 binding competition ELISA was performed as above except that the competition antibody (VRC01 or VRC03) at the indicated concentration was added to immobilized gp160 concurrent with the addition of a saturating concentration of sCD4 (5 µg/ml).

2.11. bNAb Binding Assay (ELISA). Detergent solubilized gp160 (Fraction II), purified gp160-NDs, and empty NDs were diluted as described above except that PBS was used as the diluent. The samples were directly immobilized in the 96-well plate and the wells were blocked and washed as above. Each bNAb was added at the concentration indicated in each individual experiment and incubated for one hour at room temperature. The wells were washed, goat anti-human HRP conjugate (1:10,000 of a 1 mg/mL stock solution) was added, and the samples incubated for one hour. Finally, each well was washed with PBST in preparation for ELISA.

2.12. Enzyme-Linked Immunosorbent Assay (ELISA). Unless otherwise

indicated all ELISAs were carried out at room temperature in PBST. TMB substrate was added to each well according to the manufactures instructions and the mixtures were incubated for 15 minutes. The reactions were then quenched with 2 M H₂SO₄ and the absorbance at 450 nm was measured on an Infinite plate reader (Tecan). All binding data were analyzed using non-linear least squares fitting according to a simple hyperbolic curve function to obtain a $K_{D,app}$. The CD4 competition binding data were similarly analyzed according to a simple hyperbolic curve function to obtain an IC₅₀ for each antibody.

3. Results

3.1. Purification and Characterization of Detergent-Solubilized gp160. Isolation of gp160 as described in Materials and Methods affords a highly enriched preparation of detergent-solubilized gp160 (Fraction I, 4-5 mg total protein). It is noteworthy, however, that a large and variable amount of the protein appears as high-order complexes that do not enter a SDS (denaturing/reducing) polyacrylamide gel, even upon prolonged boiling of the preparation in SDS loading buffer (Figure 2A). Where indicated, this material was further purified by size exclusion chromatography using a Superdex S200 column. As shown in Figure 2B, the majority of detergent solubilized protein elutes near the void volume of the column, which indicates the presence of higher-order gp160 oligomers¹. This fraction was collected as Fraction II with a yield of 90% total protein. Analysis of Fraction II by native (non-denaturing) gel electrophoresis

¹ Given that the exclusion volume of the column is > 1,300 kDa, the oligomers must be octamers of gp160 or larger.

(Figure 2C) demonstrates that roughly 30% of the protein migrates as an *apparent* ~ 760 kDa band, while the majority is further associated into higher-order complexes².

3.2. Preparation and Purification of Detergent Free gp160-Nanodiscs. The nanodisc assembly reaction was performed using detergent solubilized gp160 (Fraction I, above) as described in Materials and Methods and outlined in Figure 1C. The *detergent-free* products were first fractionated by Immobilized Metal ion Affinity Chromatography (IMAC) to separate excess lipid and gp160 protein that is *not* associated with the H6- MSP protein. Of note, detergent-free gp160 found in the IMAC flow-through fraction is highly aggregated and elutes in the void volume of a Superose 6 size exclusion column (Figure 3A, *dashed line*). This material is resistant to dissociation by detergents, denaturants, and prolonged boiling in SDS-PAGE load buffer (data not shown). In contrast, material that is retained by and subsequently eluted from the IMAC column is fully soluble in the absence of detergent. This material was next fractionated on a Superose 6 column and the chromatogram is displayed in Figure 3A (*solid line*). The majority of the material elutes as a large, broad peak between 9 -14 mL followed by two additional species eluting at 16 mL and 18 mL. SDS-PAGE analysis confirms that the later fractions contain free H6-MSP (Figure 3C) and their elution positions confirm that they are H6-MSP dimers and monomers, respectively. Importantly, little to no

² We note that the migration of a protein in a *non-denaturing* gel reflects not only the size, but also the overall *charge* of the complex. The molecular weight standards thus provide an estimation of *apparent* molecular weight.

material is observed in the SEC chromatogram when the scaffold protein is excluded from the nanodisc assembly reaction mixture (not shown). This demonstrates that the preceding IMAC step effectively removes all gp160 that is *not* associated with H6-MSP (i.e., not incorporated into a nanodisc). In the absence of gp160, the reaction mixture affords empty nanodiscs that elute from the column at their expected position (not shown) (Ritchie et al., 2009).

Selected fractions eluting from the SEC column were analyzed by Western blot, which reveals that an *apparent* ~740 kDa complex represents the major gp160 immunoreactive species (>90%; Figure 3B)³. Based upon the chromatogram and the Western blot data, SEC fractions 11 - 13 mL were combined to afford the major nanodisc species, which we refer to as gp160-ND and that contains ~ 160 µg gp160, (~ 8% yield). The pooled fractions were concentrated and analyzed by SDS-PAGE for their protein content. This confirms that gp160-ND contains both gp160 (~160 kDa) and MSP (~25 kDa) proteins, as expected for a gp160 nanodisc (Figure 3C). As noted above, detergent-free gp160 that is *not* incorporated into nanodiscs contains highly aggregated, insoluble protein. Importantly, neither this aggregated material nor the larger oligomers observed in detergent-solubilized Fraction II (Figure 2C) are observed in the gp160-ND preparation. This indicates that gp160 incorporation into a ND effectively excludes higher-order gp160 oligomeric species (compare Figures 2A and 3C). Moreover, unlike detergent-

³ The calculated MW for a trimeric gp160-ND is ~ 630 kDa; three gp160's (480 kDa), two MSP's (50 kDa), and ca. 130 copies of POPC molecules (~ 99 kDa).

solubilized gp160, the purified gp160 nanodiscs remain soluble even upon prolonged storage (~ 8 weeks at -20°C; data not shown).

3.3. Biochemical and Structural Characterization of gp160-ND. A functional nanodisc contains two copies of the MSP protein and this constraint allows quantitation of the number of gp160 subunits per nanodisc by densitometric analysis of Coomassie-stained SDS-PAGE gels. Analysis of the data presented in Figure 3C indicates that gp160-ND is enriched with nanodiscs that contain apparent trimers of gp160 (Table 2).

The structural features gp160-ND were examined by transmission electron microscopy, which demonstrates that the particles are distinctly different in morphology from empty nanodiscs (Figure 4A). Rather, the particles more closely resemble those found in the detergent solubilized gp160 preparation. Quite importantly, however, the particles appear less aggregated and much more homogeneous. Indeed, the particles resemble published micrographs of trimeric Env spikes and of trimeric gp140 examined by EM ([Center et al., 2002](#); [Iyer et al., 2007](#); [Ringe et al., 2013](#)) and possess a morphology that is expected for a gp160 trimer incorporated into a nanodisc (see Figure 4B) ([Dörr et al., 2014](#); [Pandit et al., 2011](#)). We interpret the ensemble of data presented above to indicate that detergent free gp160-ND are composed of full-length, natively glycosylated, trimeric Env protein incorporated into a soluble, well-defined ~ 10 nm lipid nanodisc.

3.4. Biological Activity of the gp160-Nanodiscs: CD4 Binding Assay. The biological activity of purified gp160-ND was first evaluated by their capacity to recognize the cognate CD4 receptor. ELISAs were conducted with the four domain soluble CD4 receptor (sCD4) as described in Materials and Methods and the results are presented in Figure 5A. Quantitation of the binding data indicates that the affinity of CD4 for *detergent-free* gp160-ND is essentially identical to that obtained for heterogeneous detergent-solubilized gp160 preparation (Table 3). Importantly, empty nanodiscs do not react with sCD4 in this assay, demonstrating that the ELISA is specific for gp160 incorporated into the disc (Figure 5A, Table 3).

We next employed a competition ELISA to evaluate the *specificity* of sCD4 binding to the gp160-NDs. These studies utilized VRC01, an antibody that recognizes gp120 epitopes required for CD4 binding (Georgiev et al., 2013; Wu et al., 2010). Immobilized gp160-ND was incubated with saturating a concentration of sCD4 (5 µg/ml) and the indicated concentration of VRC01. Inhibition of sCD4 binding to gp160-ND was quantified by ELISA and the data presented in Figure 5B demonstrates that VRC01 strongly inhibits sCD4 binding to both detergent-solubilized gp160 and to the detergent-free gp160-ND preparation in a concentration dependent manner, and with a similar IC₅₀ (Table 3). We also evaluated VRC03 in the competition ELISA as a negative control; this antibody has a limited breadth of neutralization of clade C pseudoviruses compared to VCR01 and it does not interfere with sCD4 binding to gp160 from the HIV 1084i isolated used in these studies (Wu et al., 2010). As anticipated, the VRC03

antibody neither affects sCD4 binding to detergent-solubilized gp160 nor to the detergent-free gp160-ND (data not shown). This is consistent with the observation that while VRC01 efficiently neutralizes 1084i pseudovirus, the VRC03 antibody has little effect (Townsend and Hu, unpublished). In sum, the data indicate gp160 incorporated into nanodiscs retains the ability to recognize CD4 and a specific antibody that recognizes CD4 binding epitopes on gp120.

3.5. Antigenic Properties of the gp160-Nanodiscs: Broadly Neutralizing Antibody (bNAb) Binding Assay. VRC01 recognizes epitopes presented in monomeric gp120 and does not rely on quaternary, conformation-dependent epitopes that would be present in a gp160 trimer spike (Georgiev et al., 2013). We therefore employed bNAbs that recognize conformation-dependent epitopes to confirm the structural integrity and biological activity of the gp160-ND preparations. We first examined b12, a bNAb that recognizes an epitope overlapping the CD4 binding site on gp120 (Burton et al., 1994; Pantophlet and Burton, 2006). The data presented in Figure 5C demonstrate that b12 binds to detergent solubilized gp160 and to detergent-free gp160-ND with essentially equal affinity (Table 3). This is consistent with the sCD4 binding and VCR01 competition data presented above. We next examined 4E10, a bNAb that recognizes membrane proximal epitopes on Env and that is effective against over 90 isolates of HIV (Binley et al., 2004; Stiegler et al., 2001). The data presented in Figure 5D demonstrate that 4E10 binds to both detergent solubilized gp160 and to detergent-free gp160-ND. Quantitation of the data indicates that 4E10 binds gp160-ND with a modest increase in affinity. Importantly, neither b12 nor 4E10

bind to empty nanodiscs in this concentration range (Figure 5, Table 3).

4. Discussion

Current efforts in HIV vaccine development have focused on soluble forms of Env (gp120 and gp140 constructs) because they represent soluble and comparatively homogeneous preparations, as necessary for biochemical and structural interrogation. In contrast, relatively little effort have been directed to membrane-bound or full-length forms of Env as potential vaccine immunogens. In part, this is because gp160 must be solubilized with detergent and the preparations nevertheless present a heterogeneous population of high-order complexes that complicate biochemical and immunological analysis. Moreover, strong interaction of detergent to specific sites on proteins can be a significant issue, complicating biochemical and biophysical analysis of protein-protein and protein-substrate interactions in detergent solubilized systems (Hanagan et al., 1998; Li-Blatter et al., 2009).

As a first step towards the isolation of a detergent free, soluble, stable, and homogeneous gp160 preparation, we have developed a protocol for the incorporation of HIV gp160 into lipid nanodiscs. The data suggest that full-length, natively glycosylated gp160 trimers can be selectively incorporated into the nanodiscs during assembly, effectively excluding higher-order gp160 species present in the detergent-solubilized preparations and that aggregate upon detergent removal. The detergent-free gp160-ND show a dramatic improvement in Env solubility and homogeneity when compared to detergent-solubilized protein.

Moreover the gp160-ND can be stored for longer times without evidence of aggregation in solution. These features provide preparations that are far superior for biochemical, biophysical, and structural interrogation, a future goal of this work. Equally important, the NDs provide a preparation that imbeds native Env in a lipid environment whose size and composition can be modified at will. This allows for (i) interrogation of the role of the envelope in membrane-proximal ectodomain region (MPER) epitopes and (ii) the incorporation of additional molecules, such as co-stimulating molecules or molecular adjuvants, for ND use as an immunogen.

The biological activity of the gp160-ND was also examined, which did not show any appreciable difference in sCD4 or bNAb binding between the detergent solubilized and detergent-free gp160-ND protein preparations. That the antigenic properties are similar is an important demonstration of functionality, indicating that gp160 incorporation into NDs did not in any way perturb major structural features critical for antibody or receptor binding interactions. We note that the polydispersity and variability of oligomeric species in the detergent solubilized gp160 preparations complicates interpretation of biochemical results, from EM to ELISA, as it is unclear which of the oligomeric species are actually binding bNAbs and with what affinity. The relative homogeneity of the gp160-ND preparations provides a significant improvement in this regard. Moreover, the capture of trimeric Env in a defined lipid environment has potential use as an immunogen to elicit antibody responses directed to epitopes that are only presented in the native trimer form of Env. Importantly, trimeric Env in the lipid

bound form (i.e., a ND) may facilitate the analyses of critical gp41 epitopes in the MPER that are not possible with soluble forms of Env such as gp140.

Finally, we note that the yield of purified gp160-ND in this study is modest, which likely reflects the minor population of trimeric Env present in the detergent-solubilized preparation that is captured during nanodisc assembly (see Figure 2C). We believe that a dramatic improvement in yield, homogeneity, and biological activity is likely by (i) optimizing conditions that afford a greater fraction of trimer in the crude, detergent solubilized gp160 starting material, (ii) utilizing alternate MSP proteins to afford particles with smaller and/or larger membrane footprints (9 - 17 nm; (Ritchie et al., 2009)), (iii) optimization of the nanodisc assembly reaction conditions such as the ratio of lipid:gp160:MSP, and (iv) optimization of the gp160-ND purification protocol. Moreover, improvements in the biological activity of the gp160-ND are also possible with the use of gp160 from alternative HIV clades and by modification of the type of lipid used to assemble the discs. With respect to the latter, the lipid composition of the gp160-ND preparation can be varied to simulate different types of biological membrane including the cell and viral envelope membranes. Thus, this study sets the stage for detailed biochemical and immunological interrogation of the protein, glycan, and lipid features that mediate Env recognition in a defined *in vitro* system.

Tables

Table 1. Purification Yield of gp160-ND

Assembly/Purification Step	gp160 Yield	
	<i>Mass</i>	<i>Percent</i>
gp160-ND assembly mixture (starting material)	2 mg*	100%
Ni Sepharose 6 FF (IMAC) column	300 µg	15%
Superose 6 10/300 GL (SEC) column	160 µg	8%

***Fraction I (starting material) was essentially pure detergent solubilized gp160 (see Figure 2). Product yields at each step was determined spectroscopically. See text for details.**

Table 2. Composition of the gp160-ND Fraction.

Fraction	gp160 (mole)	Lipid	MSP (mole)
Empty-ND	-	++	2
gp160-ND	2.7 ± 0.8	++	2

The data represents the average of four replicates with standard deviations indicated. We note that construction of the standard curve is complicated by the observation that a sizable and variable fraction of detergent-solubilized gp160 is highly self-associated and does not migrate far into the gel (see Figure 2A). This feature introduced a significant error into the quantitation of gp160 on the gel.

Table 3. $K_{D,app}$ for sCD4 and bNAbs, measured by ELISA

Sample	sCD4 $K_{D,app}$ ($\mu\text{g/mL}$)	VRC01^a IC_{50} ($\mu\text{g/mL}$)	b12 $K_{D,app}$ ($\mu\text{g/mL}$)	4E10 $K_{D,app}$ ($\mu\text{g/mL}$)
Empty ND	ND^b	-	ND^b	ND^b
Detergent-solubilized gp160	0.10 ± 0.04	7 ± 2^a	0.042 ± 0.002	8.1 ± 1
gp160-ND	0.07 ± 0.03	8 ± 5^a	0.034 ± 0.006	4.7 ± 1.7

The data presented in Figure 5 was quantified as described in Materials and Methods.

^a sCD4 included at saturating concentrations (5 $\mu\text{g/mL}$).

^b ND, not detectable. Essentially no signal above background was observed in the ELISA (See Figure 5).

Figure Legends

Figure 1. **Panel A.** The HIV-1 Envelope (Env) Glycoprotein. Env is expressed as a 160 kDa precursor (**top figure**) which is cleaved in the *trans*-Golgi network by host proteases such as furin. The resulting surface (gp120) and transmembrane (gp41) subunits remain noncovalently associated (**bottom figure**). In both, gp120 is shown in blue, the membrane proximal ectodomain region (MPER), the transmembrane domain, and the cytoplasmic domain of gp41 are shown in dark orange, gray and light orange, respectively. Soluble gp140 fusion constructs composed of gp120 and the ectodomain of gp41 span the region indicated at bottom. A cartoon for an infectious HIV particle is shown at **right**, with mature gp120/gp41 trimer spikes (blue/red) imbedded in the viral envelope (gray). The matrix protein (yellow) and capsid shell (dark green) surround the two RNA genomes (dark blue). Details are discussed in the Text. **Panel B.** Cartoon of a lipid nanodisc composed of a lipid bilayer (grey circles) circumscribed by a helical amphipathic membrane scaffold protein (MSP, dark blue bands). The MSP1D1 protein used in this study affords nanodiscs ~10 nm in diameter. Membrane proteins (green) can be incorporated into the discs, solubilizing them in a native-like bilayer. **Panel C.** Assembly of gp160 Nanodiscs. Detergent-solubilized lipid, detergent solubilized gp160, and MSP are mixed and nanodisc self-assembly is initiated by removal of detergent using Bio-Beads. Free lipid and unincorporated gp160 are eliminated by IMAC and the resulting mixture of products is fractionated by size exclusion chromatography (SEC). Details are provided in the Text.

Figure 2. Purification of Detergent Solubilized gp160. Panel A. Detergent solubilized gp160 (Fraction I) purified as described in Materials and Methods was analyzed by SDS (reducing, denaturing) PAGE. The gel was stained with Coomassie blue. The position of the loading well and the 160 kDa molecular weight marker are indicated at the right of the gel. Note the presence of gp160 retained in the well indicating highly aggregated material in the detergent-solubilized preparation. **Panel B.** Detergent-solubilized gp160 (Fraction I) was fractionated on a Superdex S200 gel filtration column and eluting material was monitored by absorbance. The majority of gp160 elutes near the void volume of the column (8 – 10 mLs). These fractions were pooled as detergent solubilized gp160 Fraction II. Material eluting at 13.5 ml, 15 ml, and 20.5 mL in the chromatogram does not contain protein detectable by SDS-PAGE (not shown). **Panel C.** Native PAGE analysis of detergent solubilized gp160 Fraction II. The gel was stained with Coomassie blue.

Figure 3. Assembly and Purification of gp160 Nanodiscs (gp160-ND). Panel A. Detergent-free gp160-ND were assembled and partially purified by IMAC as described in Materials and Methods and pictorially in Figure 1C. The crude nanodiscs were fractionated on a Superose 6 gel filtration column and eluting material was monitored by absorbance (*solid line*). Detergent-free gp160 that is *not* retained by the IMAC column (i.e., flow through that is not associated with a H6-MSP) elutes in the void volume of the SEC column (*dashed line*) indicating that the material is highly aggregated. **Panel B.** The indicated SEC fractions were

fractionated by native (nondenaturing) PAGE and analyzed by Western blot using HIV-IG. The fraction numbers and the migration position of molecular weight standards are indicated at the top and at left of the gel, respectively. The major gp160-immunoreactive species is indicated with an arrow at the right of the gel.

Panel C. *SDS-PAGE Analysis of the Purified gp160-ND Preparation.* Pooled fractions were fractionated by SDS-PAGE and were visualized with Coomassie blue. *gp160-ND lane*, pooled fraction 11-13 mL; *MSP lane*, pooled fractions 15-18 mL. The identities of gp160 and H6-MSP1D1 contained in this pooled fraction were confirmed by Western blot analysis (data not shown).

Figure 4. Electron Micrographs of Pooled SEC Fractions stained with Nano-W.

Panel A. *Left*, detergent-solubilized gp160 containing ~5 nm Nanogold spheres (nanoprobes) for a size reference; *Middle*, purified gp160-ND; *Right*, empty nanodiscs. **Panel B.** *Upper*, CryoEM structure of soluble BG505 SOSIP.R6 gp140 trimers showing side and top views, as indicated; *Lower*, Collage of EM images of purified gp160-NDs showing side and top views, as indicated. Size bars indicate 50 nm.

Figure 5. Soluble Human CD4 (sCD4) and bNAbs Bind to gp160-NDs. ELISAs were performed as described in Materials and Methods using detergent solubilized gp160 (○), detergent-free gp160-NDs (●), and detergent-free empty nanodiscs (◇). The data were fit to a simple Langmuir binding model, or in the case of the VRC01 competition assay a simple competition binding model as described in Materials and Methods to afford $K_{D,app}$ and IC_{50} values, respectively (see Table 3). The best-fit curves are displayed as dashed lines for detergent-solubilized gp160

and solid lines for gp160-ND and empty nanodisc samples. *Panel A*. ELISA showing that sCD4 binds to detergent-solubilized gp160 and detergent-free gp160-ND, but not to empty nanodiscs. *Panel B*. ELISA showing that VRC01 inhibits sCD4 binding. VRC01 antibody was added to the binding mixture at the indicated concentration and sCD4 binding quantified. *Panel C*. ELISA showing that b12 binds to detergent-solubilized gp160 and detergent-free gp160-ND, but not to empty nanodiscs. *Panel D*. ELISA showing that 4E10 binds to detergent-solubilized gp160 and detergent-free gp160-ND, but not to empty nanodiscs. In all cases, each data point represents the average of at least duplicate reaction wells with error bars indicated.

Figures

Figure 1

Figure 2

Figure 3.

Figure 4.

Figure 5.

Literature Cited

- Bayburt, T.H., Sligar, S.G., 2010. Membrane protein assembly into Nanodiscs. *FEBS Letters* 584, 1721-1727.
- Binley, J.M., Wrin, T., Korber, B., Zwick, M.B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C.J., Burton, D.R., 2004. Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies. *Journal of Virology* 78, 13232-13252.
- Blay, W.M., Kasprzyk, T., Misher, L., Richardson, B.A., Haigwood, N.L., 2007. Mutations in Envelope gp120 Can Impact Proteolytic Processing of the gp160 Precursor and Thereby Affect Neutralization Sensitivity of Human Immunodeficiency Virus Type 1 Pseudoviruses. *Journal of Virology* 81, 13037-13049.
- Burton, D.R., Ahmed, R., Barouch, D.H., Butera, S.T., Crotty, S., Godzik, A., Kaufmann, D.E., McElrath, M.J., Nussenzweig, M.C., Pulendran, B., Scanlan, C.N., Schief, W.R., Silvestri, G., Streeck, H., Walker, B.D., Walker, L.M., Ward, A.B., Wilson, I.A., Wyatt, R., 2012. A Blueprint for HIV Vaccine Discovery. *Cell Host and Microbe* 12, 396-407.
- Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., Nara, P.L., al., e., 1994. Efficient Neutralization of Primary Isolates of HIV-1 by a Recombinant Human Monoclonal Antibody. *Science* 266, 1024-1027.
- Center, R.J., Leapman, R.D., Lebowitz, J., Arthur, L.O., Earl, P.L., Moss, B., 2002. Oligomeric Structure of the Human Immunodeficiency Virus Type 1 Envelope Protein on the Virion Surface. *Journal of Virology* 76, 7863-7867.
- Chen, P.S., Toribara, T.Y., Warner, H., 1956. Microdetermination of Phosphorus. *Analytical Chemistry* 28, 1756-1759.

- Cornet, B., Decroly, E., Thines-Sempoux, D., Ruyschaert, J.M., Vandenbraden, M., 1992. Properties of HIV membrane reconstituted from its recombinant gp160 envelope glycoprotein. *AIDS Res. Hum. Retroviruses* 8, 1823-1831.
- Corti, D., Lanzavecchia, A., 2013. Broadly Neutralizing Antiviral Antibodies. *Annual Review of Immunology* 31, 705-742.
- Crooks, E.T., Moore, P.L., Franti, M., Cayan, C.S., Zhu, P., Jiang, P., de Vries, R.P., Wiley, C., Zharkikh, I., Schülke, N., Roux, K.H., Montefiori, D.C., Burton, D.R., Binley, J.M., 2007. A Comparative Immunogenicity Study of HIV-1 Virus-Like Particles Bearing Various Forms of Envelope Proteins, Particles Bearing no Envelope and soluble monomeric gp120. *Virology* 366, 245-262.
- Dörr, J.M., Koorengevel, M.C., Schäfer, M., Prokofyev, A.V., Scheidelaar, S., van der Cruisen, E.A.W., Dafforn, T.R., Baldus, M., Killian, J.A., 2014. Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K⁺ channel: The power of native nanodiscs. *Proceedings of the National Academy of Sciences* 111, 18607-18612.
- Dubay, J.W., Dubay, S.R., Shin, H.J., Hunter, E., 1995. Analysis of the cleavage site of the human immunodeficiency virus type 1 glycoprotein: requirement of precursor cleavage for glycoprotein incorporation. *Journal of Virology* 69, 4675-4682.
- Freed, E.O., Martin, M.A., 2005. HIVs and Their Replication, in: Knipe, D.M., Howley, P.M. (Eds.), *Fields Virology*, Fifth ed. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, PA, pp. 2107-2185.
- Georgiev, I.S., Joyce, M.G., Zhou, T., Kwong, P.D., 2013. Elicitation of HIV-1-neutralizing antibodies against the CD4-binding site. *Current opinion in HIV and AIDS* 8, 382-392.
- Gu, M., Rappaport, J., Leppla, S.H., 1995. Furin is Important but Not Essential for the Proteolytic Maturation of gp160 of HIV-1. *FEBS Letters* 365, 95-97.

- Guttman, M., Lee, K.K., 2013. A Functional Interaction between gp41 and gp120 Is Observed for Monomeric but Not Oligomeric, Uncleaved HIV-1 Env gp140. *Journal of Virology* 87, 11462-11475.
- Hanagan, A., Meyer, J.D., Johnson, L., Manning, M.C., Catalano, C.E., 1998. The phage lambda terminase enzyme: 2. Refolding of the gpNu1 subunit from the detergent-denatured and guanidinium hydrochloride-denatured state yields different oligomerization states and altered protein stabilities. *Int J Biol Macromol* 23, 37-48.
- Herrera, C., Klasse, P.J., Michael, E., Kake, S., Barnes, K., Kibler, C.W., Campbell-Gardener, L., Si, Z., Sodroski, J., Moore, J.P., Beddows, S., 2005. The impact of envelope glycoprotein cleavage on the antigenicity, infectivity, and neutralization sensitivity of Env-pseudotyped human immunodeficiency virus type 1 particles. *Virology* 338, 154-172.
- Hoxie, J.A., 2010. Toward an Antibody-Based HIV-1 Vaccine. *Annual Review of Medicine* 61, 135-152.
- Hu, S.-L., Abrams, K., Barber, G.N., Moran, P., Zarling, J.M., Langlois, A.J., Kuller, L., Morton, W.R., Benveniste, R.E., 1992. Protection of macaques against SIV infection by recombinant subunit vaccines of SIV envelope glycoprotein gp160. *Science* 255, 456-459.
- Hu, S.-L., Kosowski, S.G., Dalrymple, J.M., 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. *Nature* 320, 537-540.
- Hu, S.-L., Stamatatos, L., 2007. Prospects of HIV Env Modification as an Approach to HIV Vaccine Design. *Current HIV Research* 5, 507-513.
- Iwatani, Y., Kawano, K., Ueno, T., Tanaka, M., Ishimoto, A., Ito, M., Sakai, H., 2001. Analysis of Dominant-Negative Effects of Mutant Env Proteins of Human Immunodeficiency Virus Type 1. *Virology* 286, 45-53.
- Iyer, S.P.N., Franti, M., Krauchuk, A.A., Fisch, D.N., Ouattara, A.A., Roux, K.H., Krawiec, L., Dey, A.K., Beddows, S., Maddon, P.J., Moore, J.P., Olson, W.C.,

2007. Purified, Proteolytically Mature HIV Type 1 SOSIP gp140 Envelope Trimers. *AIDS Research and Human Retroviruses* 23, 817-828.

- Julien, J.-P., Cupo, A., Sok, D., Stanfield, R.L., Lyumkis, D., Deller, M.C., Klasse, P.-J., Burton, D.R., Sanders, R.W., Moore, J.P., Ward, A.B., Wilson, I.A., 2013a. Crystal structure of a soluble cleaved HIV-1 envelope trimer. *Science* (New York, N.Y.) 342, 10.1126/science.1245625.
- Julien, J.-P., Lee, J.H., Cupo, A., Murin, C.D., Derking, R., Hoffenberg, S., Caulfield, M.J., King, C.R., Marozsan, A.J., Klasse, P.J., Sanders, R.W., Moore, J.P., Wilson, I.A., Ward, A.B., 2013b. Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9. *Proceedings of the National Academy of Sciences* 110, 4351-4356.
- Karlsson-Hedestam, G.B., Fouchier, R.A.M., Phogat, S., Burton, D.R., Sodroski, J., Wyatt, R.T., 2008. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. *Nat Rev Micro* 6, 143-155.
- Kwong, Peter D., Mascola, John R., 2012. Human Antibodies that Neutralize HIV-1: Identification, Structures, and B Cell Ontogenies. *Immunity* 37, 412-425.
- Leaman, D.P., Kinhead, H., Zwick, M.B., 2010. In-Solution Virus Capture Assay Helps Deconstruct Heterogeneous Antibody Recognition of Human Immunodeficiency Virus Type 1. *Journal of Virology* 84, 3382-3395.
- Li-Blatter, X., Nervi, P., Seelig, A., 2009. Detergents as intrinsic P-glycoprotein substrates and inhibitors. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1788, 2335-2344.
- Liu, J., Bartesaghi, A., Borgnia, M.J., Sapiro, G., Subramaniam, S., 2008. Molecular architecture of native HIV-1 gp 120 trimers. *Nature* 455, 109-113.
- Maluf, N.K., Yang, Q., Catalano, C.E., 2005. Self-association properties of the bacteriophage lambda terminase holoenzyme: implications for the DNA packaging motor. *J Mol Biol* 347, 523-542.

- McCune, J.M., Rabin, L.B., Feinberg, M.B., Lieberman, M., Kosek, J.C., Reyes, G.R., Weissman, I.L., 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* 53, 55-67.
- Melikyan, G.B., 2014. HIV entry: a game of hide-and-fuse? *Current opinion in virology* 4, 1-7.
- Moore, P.L., Crooks, E.T., Porter, L., Zhu, P., Cayanan, C.S., Grise, H., Corcoran, P., Zwick, M.B., Franti, M., Morris, L., Roux, K.H., Burton, D.R., Binley, J.M., 2006. Nature of Nonfunctional Envelope Proteins on the Surface of Human Immunodeficiency Virus Type 1. *Journal of Virology* 80, 2515-2528.
- Moulard, M., Decroly, E., 2000. Maturation of HIV envelope glycoprotein precursors by cellular endoproteases. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes* 1469, 121-132.
- Nath, A., Atkins, W.M., Sligar, S.G., 2007. Applications of Phospholipid Bilayer Nanodiscs in the Study of Membranes and Membrane Proteins†. *Biochemistry* 46, 2059-2069.
- Pandit, A., Shirzad-Wasei, N., Wlodarczyk, L.M., van Roon, H., Boekema, Egbert J., Dekker, Jan P., de Grip, Willem J., 2011. Assembly of the Major Light-Harvesting Complex II in Lipid Nanodiscs. *Biophysical Journal* 101, 2507-2515.
- Pantophlet, R., Burton, D.R., 2006. GP120: Target for Neutralizing HIV-1 Antibodies. *Annual Review of Immunology* 24, 739-769.
- Phogat, S., Wyatt, R., 2007. Rational Modifications of HIV-1 Envelope Glycoproteins for Immunogen Design. *Current Pharmaceutical Design* 13, 213-227.
- Poignard, P., Moulard, M., Golez, E., Vivona, V., Franti, M., Venturini, S., Wang, M., Parren, P.W.H.I., Burton, D.R., 2003. Heterogeneity of Envelope Molecules Expressed on Primary Human Immunodeficiency Virus Type 1 Particles as Probed by the Binding of Neutralizing and Nonneutralizing Antibodies. *Journal of Virology* 77, 353-365.

- Polacino, P., Stallard, V., Klaniecki, J.E., Montefiori, D.C., Langlois, A.J., Richardson, B.A., Overbaugh, J., Morton, W.R., Benveniste, R.E., Hu, S.-L., 1999a. Limited Breadth of the Protective Immunity Elicited by Simian Immunodeficiency Virus SIV_{mac} gp160 Vaccines in a Combination Immunization Regimen. *Journal of Virology* 73, 618-630.**
- Polacino, P., Stallard, V., Montefiori, D.C., Brown, C.R., Richardson, B.A., Morton, W.R., Benveniste, R.E., Hu, S.-L., 1999b. Protection of Macaques against Intrarectal Infection by a Combination Immunization Regimen with Recombinant Simian Immunodeficiency Virus SIV_{mac} gp160 Vaccines. *Journal of Virology* 73, 3134-3146.**
- Poon, B., Hsu, J.F., Gudeman, V., Chen, I.S.Y., Grovit-Ferbas, K., 2005. Formaldehyde-Treated, Heat-Inactivated Virions with Increased Human Immunodeficiency Virus Type 1 Env Can Be Used To Induce High-Titer Neutralizing Antibody Responses. *Journal of Virology* 79, 10210-10217.**
- Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Prem Sri, N., Namwat, C., de Souza, M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J.G., Francis, D.P., Stablein, D., Birx, D.L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M.L., Michael, N.L., Kunasol, P., Kim, J.H., 2009. Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand. *New England Journal of Medicine* 361, 2209-2220.**
- Ringe, R.P., Sanders, R.W., Yasmeen, A., Kim, H.J., Lee, J.H., Cupo, A., Korzun, J., Derking, R., van Montfort, T., Julien, J.-P., Wilson, I.A., Klasse, P.J., Ward, A.B., Moore, J.P., 2013. Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. *Proceedings of the National Academy of Sciences of the United States of America* 110, 18256-18261.**

- Ritchie, T.K., Grinkova, Y.V., Bayburt, T.H., Denisov, I.G., Zolnerciks, J.K., Atkins, W.M., Sligar, S.G., 2009. Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs. *Methods in enzymology* 464, 211-231.
- Schief, W.R., Ban, Y.-E.A., Stamatatos, L., 2009. Challenges for Structure-Based HIV Vaccine Design. *Current Opinion in HIV and AIDS* 4, 431-440.
- Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F., Katinger, H., 2001. A Potent Cross-Clade Neutralizing Human Monoclonal Antibody against a Novel Epitope on gp41 of Human Immunodeficiency Virus Type 1. *AIDS Research and Human Retroviruses* 17, 1757-1765.
- van Gils, M.J., Sanders, R.W., 2013. Broadly neutralizing antibodies against HIV-1: Templates for a vaccine. *Virology* 435, 46-56.
- Walker, B.D., Burton, D.R., 2008. Towards an AIDS Vaccine. *Science* 320, 760-764.
- Wilens, C.B., Tilton, J.C., Doms, R.W., 2012. HIV: Cell Binding and Entry. *Cold Spring Harbor Perspectives in Medicine* 2, a006866.
- Wu, X., Yang, Z.-Y., Li, Y., Hogerkorp, C.-M., Schief, W.R., Seaman, M.S., Zhou, T., Schmidt, S.D., Wu, L., Xu, L., Longo, N.S., McKee, K., O'Dell, S., Louder, M.K., Wycuff, D.L., Feng, Y., Nason, M., Doria-Rose, N., Connors, M., Kwong, P.D., Roederer, M., Wyatt, R.T., Nabel, G.J., Mascola, J.R., 2010. Rational Design of Envelope Identifies Broadly Neutralizing Human Monoclonal Antibodies to HIV-1. *Science (New York, N.Y.)* 329, 856-861.