Structural Evolution of Trimeric Gamma-Type Retroviral Envelope Proteins

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Abstract

The envelope glycoproteins (Env) of retroviruses are highly diverse due to their relatively rapid evolution and the ancient origin of retroviruses. Sequence similarity among the Env of retroviruses is observed in the transmembrane subunit (TM) of Env mediating membrane fusion, whereas this similarity is more limited or absent in the surface subunit (SU) mediating receptor binding. Recent structural modeling of the SU of the major orthoretroviral groups identified a structurally conserved β-sheet domain in SU and the SU-equivalent of the filoviruses, GP1, that is differentially expanded to generate the structural diversity observed in this protein family. Here, the evolution of the trimeric gamma-type Env of gammaretroviruses and alpharetroviruses is addressed by structural modeling using AlphaFold2. The trimeric murine leukemia virus (MLV) and avian leukemia virus Env models show a close structural relationship between trimeric Env proteins of gammaretroviruses and alpharetroviruses and the filoviral spike protein, with an extended hydrophobic structure anchoring SU into TM in the retroviral gamma-type Env occluding the intersubunit disulfide bond. In addition, the structural model of trimeric MLV Env indicates that the receptor binding domains (RBD) of gammaretroviral Env are positioned on the side of the Env trimer pointing towards the viral envelope membrane. The trimeric MLV Env structural model, including RBD position, is consistent with previous trimeric Env electron-density maps. The structural models provide insights into the structure, function and evolution of trimeric, pre-fusion gamma-type Env of gammaretroviruses and alpharetroviruses.

INTRODUCTION

The envelope glycoprotein (Env) of retroviruses mediates infection by inducing fusion between the viral and host-cell membranes. The Env of retroviruses also plays critical roles in the physiology of reproduction, including humans, where envelope protein genes of integrated endogenous retroviruses encode syncytins that mediate cell-to-cell fusion in placental tissues (Henzy and Johnson, 2013; Mi et al., 2000). Thus, elucidation of retroviral Env structures is relevant for the understanding of both virus-host interactions and basic physiologic mechanisms relevant to human health.

The Env of orthoretroviruses is cleaved by host cell proteases to form two independent subunits, the surface (SU) and transmembrane (TM) subunits, which remain covalently or non-covalently associated (Hogan and Johnson, 2023). The SU subunit binds host-cell receptors whereas TM mediates membrane fusion. The native Env on virions is composed of trimers of SU-TM heterodimers. The native trimeric Env is metastable, with TM assuming significantly different conformations in the pre-fusion and post-fusion states (Hogan and Johnson, 2023). The SU (gp120) and TM (gp41) subunits of the human immunodeficiency type 1 virus (HIV-1) pre-fusion Env trimer are closely associated, with the TM subunit assuming a relatively complex metastable helical fold (Pancera et al., 2014). In contrast, in the post-fusion state the SU and TM subunits are dissociated, with TM assuming a stable a six-helix coiled-coil conformation (Chan et al., 1997). The transition between the two states is triggered by receptor binding. Following receptor binding, the fusion peptide (FP) in the amino-terminus *of TM inserts itself into the target cell membrane and TM refolds itself into the six-helix bundle state, bringing the two membranes together to induce fusion.

Retroviral Env is classified into two major groups according to the manner by which SU and TM interact in the Env trimer (Henzy and Coffin, 2013; Henzy and Johnson, 2013; Hogan and Johnson, 2023). The major Env group is the gamma-type, of which the Env of gammaretroviruses is the prototype but also including the Env of alpharetroviruses, where SU and TM are covalently linked by an intersubunit disulfide bond (Hogan and Johnson, 2023; Leamnson and Halpern, 1976; Pike et al., 2011; Pinter et al., 1997). The third cysteine residue in a conserved CX_nCC motif of TM forms this intersubunit disulfide bond whereas the first and second Cys residues form a disulfide loop within TM. The cysteine in SU forming the intersubunit disulfide bond has a more variable context depending on retroviral group. In the gammaretroviruses, the second Cys residue of a conserved CXXC motif forms the intersubunit disulfide bond whereas in the alpharetroviruses an amino-terminal Cys residue has that function (Pike et al., 2011; Pinter et al., 1997; Sjöberg et al., 2006). Importantly, the GP2 protein of filoviral envelope spike proteins (SP), including Ebola virus (EBOV), share sequence similarity with the TM of retroviruses. In fact, the GP2 subunit of filoviral SP also has a CX_nCC motif that forms a disulfide bond with an amino-terminal cysteine in the SU-equivalent GP1 as in gamma-type Env (Bénit et al., 2001; Gallaher, 1996; Jeffers et al., 2002). The second major retroviral Env class is the beta-type Env of betaretroviruses and lentiviruses (Henzy and Coffin, 2013; Henzy and Johnson, 2013). The beta-type Env lacks a covalent intersubunit disulfide bond and has a CX_nC motif in TM instead.

Beyond the common features of retroviral Env described

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above, the Env of retroviruses is highly diverse. The most intensively studied retroviral Env outside the primate lentiviruses is that of the gammaretroviruses. A recent review describes the structure and function of gammaretroviral Env in detail (Hogan and Johnson, 2023). The most important difference between the Env of gammaretroviruses relative to other retroviral groups is the segregation of SU into two independently-folding domains, the amino-terminal receptor-binding domain and a more conserved carboxy-terminal C-domain thought to interact with TM. These two domains are linked by a relatively long proline-rich region (PRR) that modulates Env fusion (Barnett et al., 2003, 2001; Battini et al., 1992; Fass et al., 1997; Pinter et al., 1997; Valsesia-Wittmann et al., 1997). In contrast, the two domains of HIV-1 gp120 are closely associated structurally, with both domains participating in receptor and co-receptor binding (Kwong et al., 1998).

The high sequence diversity of retroviral Env, especially in its SU region, has precluded more detailed understanding of its evolution. Comparison of Env protein structures, which should evolve more slowly than its underlying protein sequences, should extend our understanding of the evolution of retroviral Env. Unfortunately, Env structural information is highly skewed, with many high-resolution of monomeric and trimeric HIV-1 Env structures but more limited information for the structure for the Env of other retroviral groups. The latter encompass crystal structures for the receptor-binding domain of type-C gammaretroviruses, including the murine (MLV) and feline (FLV) leukemia viruses (Barnett et al., 2003; Fass et al., 1997), lower resolution Env electron density maps of trimeric MLV Env (Förster et al., 2005; Löving et al., 2012; Riedel et al., 2017; Wu et al., 2008), an endogenous syncytin-2 SU structure in complex with its receptor (Martinez-Molledo et al., 2022) and post-fusion TM helical bundle structures of several different retroviruses. However, these structures have not led to broader insights into the evolution retroviral Env. Several reasons make determination of retroviral Env structures experimentally challenging. These include the metastable state of pre-fusion Env and the complex multidomain SU organization probably associated with conformational flexibility. These challenges will continue to hinder detailed understanding of the function of Env proteins in different retroviral groups as well as understanding of the evolution of this complex and highly diverse retroviral protein.

A significant breakthrough in structural biology is the emergence of protein structure prediction tools based on machine learning and artificial intelligence methods. In particular, AlphaFold2 (AF2) has been very successful in generating structural models of proteins with high confidence, including for proteins without structural precedent in the Protein Data Bank (PBD) (Jumper et al., 2021; Tunyasuvunakool et al., 2021). The broad availability of AF2 has provided an opportunity to extend the understanding of retroviral Env structure and function as well as its evolution.

AF2 is a neural network-based method for protein structure prediction (Jumper et al., 2021). The AF2 neural network was trained on protein structures and their sequences present in the PDB until about 2020. While AF2 can make use of templates for modeling, these are not necessary for structural modeling. This *ab initio* modeling can be successfully performed even when similar structures are not present among those used for AF2 training. Importantly, AF2 reports quality scores for its final and intermediate models, allowing interpretation of model reliability and selection of models among those in the output.

AF2 has been applied to the modeling of SU structures lentiviruses and betaretroviruses (Hötzel, 2022, 2021). The SU structural models yielded both expected and unexpected results. Among the expected results was the confirmation of a partial structural similarity between the SU of the beta-type betaretroviruses and lentiviruses limited to a TM-proximal region of SU, named the proximal domain (PD), formed by a complex β-sandwich structure, in agreement with previously determined fragmentary sequence similarities between the SU of these two retroviral groups (Hötzel, 2021, 2008; Hötzel and Cheevers, 2001). The structural models further indicated that the apparent structural diversity of beta-type lentiviral and betaretroviral SU, including its domain configuration, is the result of differential expansion of loops from of the conserved PD in the betaretroviruses and lentiviruses (Hötzel, 2021).

Subsequent application of AF2 to the modeling of the SU of other retroviral groups yielded additional unexpected results. Modeling of the SU of gammaretroviruses revealed that most of the C-domain is comprised of a PD with the same overall β-sandwich structure as the PD of the betaretroviruses and lentiviruses without significant internal expansions (Hötzel, 2022). The RBD and PRR of gammaretroviral SU is an amino-terminal extension of the conserved PD. In addition, the Env of some gammaretroviral and alpharetroviral endogenous elements are comprised of a lone PD without an amino-terminal RBD, including some without any significant expansions and others with relatively long internal PD expansions. Structural modeling of retroviral SU has provided significant insights into the evolution of this protein family, identifying conserved structural domains that are not obvious from sequence alignments and mechanisms of structural diversification based on expansion of the conserved PD.

Trimeric Env can potentially provide additional insights into the evolution of retroviral Env. After the PD was identified as a universal retroviral SU structure it became evident that the gamma-type SP of filoviruses also has a PD structure in GP1 in the same general location as the PD of retroviruses, closely associated to GP2 in SP trimers (Hötzel, 2022). Remarkably, the PD of filoviruses is oriented significantly different in SP trimers compared to the PD of HIV-1 in Env trimers, with a shift of about 120° around the vertical axis. This results in different GP1 PD regions interacting with GP2 compared to the contacts the PD of HIV-1 gp120 makes with gp41. In addition, the organization of GP2 and gp41 in pre-fusion trimers also differ significantly structurally (Lee et al., 2008; Pancera et al., 2014). Analysis of glycosylation patterns of gammaretroviral SU suggests that the orientation of the PD in the trimeric Env of gammaretroviruses resembles that of filoviruses rather than the lentiviruses (Hötzel, 2022).

A more recent variant of AF2, AF2 multimer, allows modeling of multimeric protein complexes (Evans et al., 2021), providing an opportunity structurally compare the trimeric Env of different retroviral groups. Here, AF2 multimer was used to model Env trimers of the prototype gammaretrovirus, MLV, and the variant gamma-type Env of avian leukemia alpharetrovirus (ALV). These trimeric Env models were compared with the existing structures of trimeric HIV-1 Env and EBOV SP to understand the evolution of trimeric retroviral Env. The trimeric Env models confirm the close structural relationship between gamma-type Env and filoviral SP, with the progressive development of novel intersubunit interactions in the gammaretroviruses, providing insights into the evolution of trimeric gammaretroviral Env.

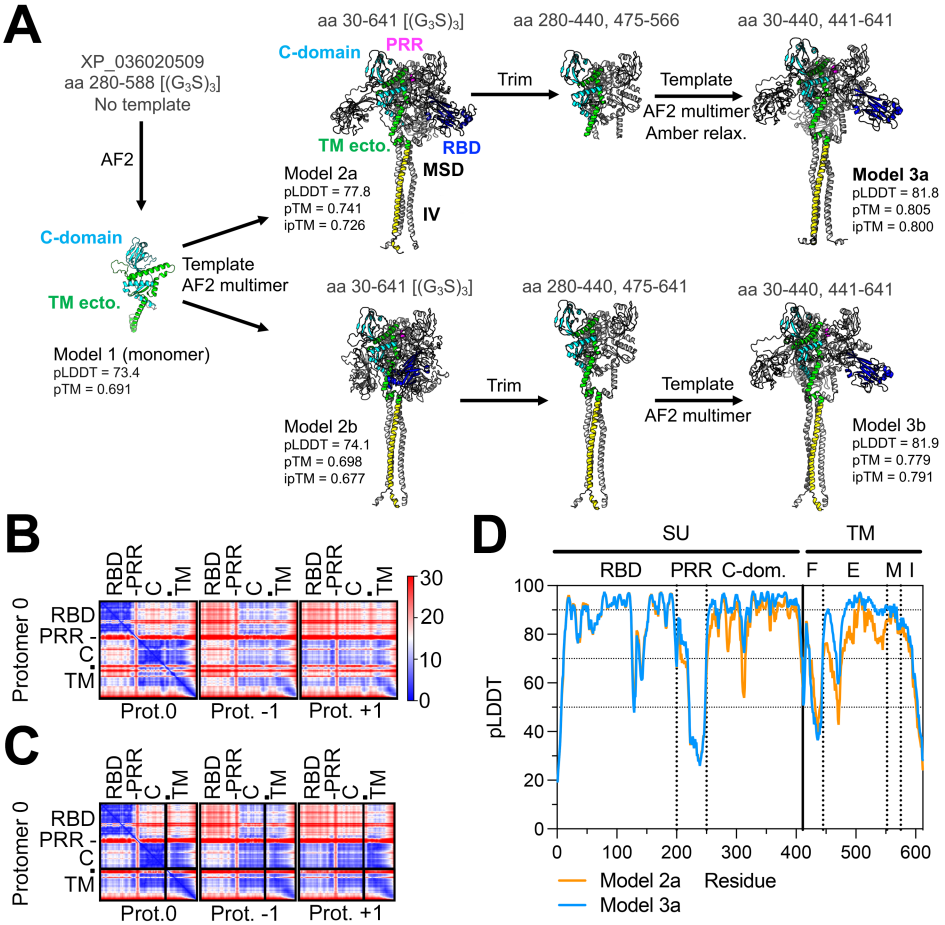

Figure 1 – Trimeric Env modeling strategy and quality scores. (A) Modeling of trimeric MLV Env, showing intermediate models and templates used for each step. The global pTM and ipTM and average local pLDDT quality scores are shown for each model. The amino acid sequence boundaries for each model and template are indicated above the model vignettes. The vignettes visually summarize the structural boundaries of the models and templates. The approximate location of the SU and TM regions are indicated in the model 2a vignette. The $[(G_3S)_3]$ notation indicates the linker added between residues 411 and 412 in models 1, 2a and 2b. Amber relaxation was only performed for model 3b. The "TM ecto", "MSD" and "IV" labels indicate the ectodomain, membrane spanning domain and intraviral regions of TM. (B) PAE scores trimeric Env model 2a. The leftmost panel indicates PAE scores within each protomer ("Protomer 0"), with the approximate location of the main Env regions shown on the left and top of the matrix. The dot indicates the location of the fusion peptide in the matrix and C indicates the C-domain of SU. The middle and right panels show the PAE of "protomer 0" relative to the protomers in the counterclockwise (-1) and clockwise (+1) directions as observed from the top of the trimer. The PAE scale, in Å, is shown to the right of the panels. The other effectively redundant matrices for each of the two other protomers in the trimer are not shown for simplicity. (C) PAE matrix for model 3a, as shown in (B). (D) Positional pLDDT scores of models 2a and 3a. The boundaries or the major SU and TM regions are shown by vertical dotted lines. F, E, M, I indicate the fusion peptide, ectodomain, membrane-spanning domain and intraviral regions of TM. The threshold pLDDT scores of 50, 70 and 90 are shown by dotted lines.

RESULTS

Modeling of trimeric MLV Env with AF2 multimer. The AlphaFold Structure Database (AFDB) (Varadi et al., 2021) includes structural models for a large number of endogenous, uncleaved Env elements from multiple vertebrate species of varying quality. However, multimeric protein complexes, including retroviral Env, are not yet part of the AFDB. Therefore, structural modeling of trimeric Env was performed here using AF2 multimer.

Selection and vetting of models were guided by the quality and reliability scores yielded by AF2 and by visual examination of models to exclude those with artifacts incompatible with previously described biochemical data. AF2 and AF2 multimer has four metrics for structural model reliability (Evans et al., 2021; Tunyasuvunakool et al., 2021). One is a metric of local structural confidence, the predicted local distance difference test (pLDDT), which ranges from 0 to 100. In practice, pLDDT scores of 90 or above indicate high side-chain conformation confidence, whereas scores of 70 and above indicate good main-chain reliability. Scores between 50 and 70 reflect lower confidence and, below 50, poor confidence or effectively unfolded states. The second metric is the predicted template modelling (pTM) score, a more global metric of model reliability, with pTM scores above 0.5 (in a scale from 0 to 1) indicating same fold. A related metric is the interprotomer pTM, or ipTM, which provides a similar global metric for reliability between different protomers in the model. The fourth metric is the positional alignment error (PAE). This is calculated on a per-residue manner, yielding a matrix where the predicted error, in Å, in the position of residue *x* on models aligned on residue *y*. This metric is useful to identify domains and subdomains with

variable relative position, or interdomain flexibility.

Several modeling strategies were explored in preliminary trimeric MLV Env modeling runs, including control modeling runs to interpret the role of different input parameters and potential biases embedded in the AF2 neural networks. These preliminary modeling runs were performed with the Env sequences of different MLV strains with different linker sequence lengths between the SU and TM regions, including different subsets of the membrane-spanning domain (MSD) and intraviral (IV) TM regions and different templating strategies. Structural models with low quality scores, TM in conformations resembling post-fusion states, unfolded or with different types of domain swaps in which the polypeptide chain in the TM regions cross the trimer axis into an adjacent Env protomer (defined here as a single SU/TM heterodimer) that are incompatible with MLV Env intersubunit disulfide biochemical data (Löving et al., 2012) were not further pursued. A stepwise modeling strategy successfully yielding a mature MLV Env trimer structural model is described below (Figure 1A).

An AF2 structural model of the C-domain of SU and TM ectodomain from a polytropic MLV strain joined by a $(G_3S)_3$ linker sequence at the Env cleavage site was obtained without templates. The resulting model was very similar to a polytropic Env model in the AFD (UniProt accession P10404) but with slightly better pLDDT scores in the TM region. This monomeric Env fragment model was then used as a template for full-length trimeric Env modeling including a $(G_3S)_3$ linker between SU and TM in two independent runs at different dates to account for changes in random seeds and the stochastic nature of AF2 structural modeling. The top-scoring trimeric Env models 2a and 2b from each run were selected for further analysis and modeling. The main difference between the monomeric Env fragment template and the trimeric Env models was observed at the top and center of the trimer, where a kinked helix corresponding to the center of the trimer in the monomeric Env template is observed as a longer straight α-helix in the trimeric models. In addition, although the PD and TM regions and the relative position of different elements are similar in different models, RBD position varies (compare overall shape of models 2a and 2b in Figure 1A vignettes for an overall view). These correlations between PD and TM regions within and between protomers and the lack of correlation between the RBDs and other parts or the trimer are reflected in the PAE scoring matrices (Fig. 1B). Local pLDDT scores of the top structural models were relatively high except in the PRR, fusion peptide, a short region at the top and center of the trimer in TM and in the intraviral region of TM (Fig. 1D). Visual inspection of the models revealed that the $(G_3S)_3$ linker between SU and TM is sandwiched between the RBDs and TM regions in slightly different conformations in different models, potentially interfering with RBD positioning. Thus, a final round of modeling of cleaved, mature trimeric Env without linkers was performed.

Modeling of mature trimeric Env was performed by providing the SU and TM sequences separately. Modeling using the monomeric Env Model 1 as template resulted in TM domain-swapped models only. Therefore, sections of the trimeric models 2a and 2b were used as templates. The first model used only the C-domain and TM ectodomain regions of the trimer in model 2a as template, without the signal sequence, $(G_3S)_3$ linker and TM membrane-proximal (MPER) regions as template. The template for an independent modeling run had the same regions of model 2b but also included the MSD and IV sections of TM. The top-scoring models 3a and 3b from this second round from each different template converged on highly similar structures within the PD and TM regions, differing in those regions only by minor shifts in the positions of some helices in the TM region ectodomain. However, the positioning of the RBD's relative to the rest of the trimer remained variable even without the linker sequence, although in a generally similar orientation among models, indicating that the variations in RBD position in the models were not due to artifacts introduced by linker regions and represent true uncertainty in the models. The uncertainty of RBD position and the higher correlation between the PD and TM regions of the trimers are reflected in the PAE scoring matrices for the final model 3 (Fig. 1C). The reliability scores of models 3a and 3b were generally higher than for the first round (Fig. 1A-D), with the caveat that these may be overconfident due to high template and model sequence identity (Roney and Ovchinnikov, 2022). However, convergence of top models 3a and 3b obtained from slightly different templates indicates the mature trimeric Env models from as probably being better than the preceding models with the $(G_3S)_3$ linker.

The top model from the third round (model 3a) was further processed by amber relaxation to resolve residue clashes. The major differences between the relaxed and unrelaxed models were in residues Glu-481, Leu-464 and Thr-465 (numbering from the first residue of the Env model, residue 30 in XP_036020509, ignoring linker region residues) in the turn between two TM α-helices close to the distal end of TM adjacent to the trimer axis and the nearby SU His-314. Extensive clashes are observed among these residues from different protomers in the unrelaxed model whereas in the relaxed model many of these clashes are resolved by changes in side-chain rotameric conformation of these residues that break the local trimer symmetry in that region of the model. These TM residues are located in the region of the chain that crosses the trimer axis to form the major domain swap observed in some models. This suggests that the artifactual domain swaps in several trimeric Env models are a consequence of local clashes among axis-proximal residues that arise from symmetrical modeling of the protomers by AF2 multimer. Analysis of relaxed model 3a using the MolProbity server (Davis et al., 2007) indicated good overall model structure quality, with 36 unique residue clashes (77 total in the three protomers) within and between chains and 5 unique Ramachandran main-chain angle outliers (11 total). Importantly, clashes between protomers in the model are located mostly in the periphery of the trimer involving regions that are likely flexible, the PRR and fusion peptide, none in the hydrophobic inter-protomer interface in the trimer axis (see below).

It is possible that AF2 uses previously known viral structures in its training dataset as implicit templates for MLV Env trimer modeling that might bias the resulting models rather than yielding models closer to the true trimeric MLV Env structure. The most obvious implicit templates are the EBOV SP and HIV-1 Env trimer structures. This was addressed by control modeling of the Zaire strain EBOV trimeric SP without templates, as it was done for MLV Env trimer modeling. However, initial modeling of the GP1 base and head subdomains that comprise the PD of GP1 (Hötzel, 2022; Lee et al., 2008), without templates, resulted in unfolded models even though several GP1 structures were presumably included in AF2 training. Similar results were obtained when attempting to model monomeric GP1/GP2, even when using a GP1 crystal structure as a partial template. Models of the EBOV SP trimer in the pre-fusion state were only obtained when enabling automatic selection of templates from the PDB. These results

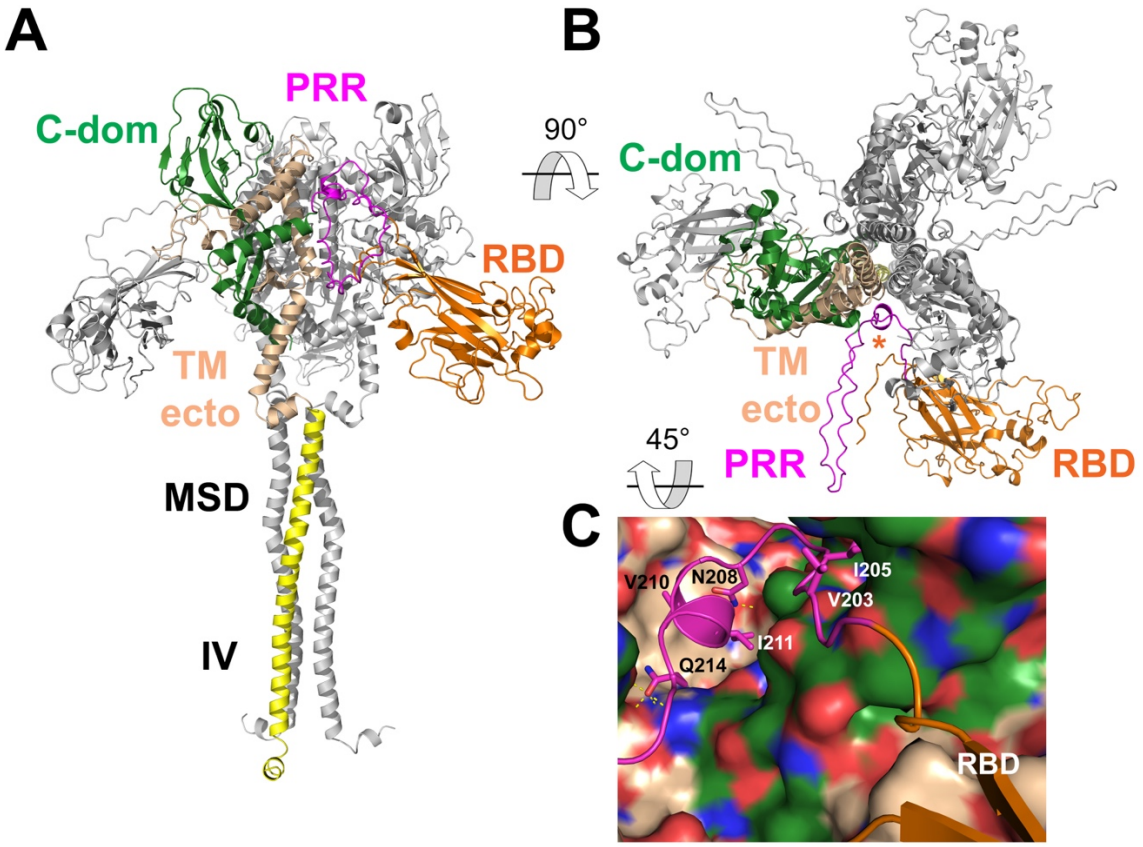

Figure 2 – Overall view of the trimeric MLV Env model and PRR anchoring. (A) Lateral view of trimeric MLV Env model 3a. The regions of a single protomer are shown in color for clarity, with color-coded labels. MSD and IV indicate the membrane-spanning domain and intraviral regions of TM, in yellow. (B) Top view of the trimeric MLV Env model, with the same color scheme as in (A). The orange asterisk indicates the helical PRR anchor along the central TM helices. (C) detailed view of the anchoring residues of the PRR (magenta), with the TM (salmon) and C-domain (green) regions shown in surface rendering. Red and blue areas indicate negative and positive charges. The RBD preceding the PRR is shown in orange. Dotted lines indicate potential hydrogen bonds. The rotation angles and directions from panels (A) to (B) and (B) to (C) (approximate) are indicated.

indicate that it is unlikely that structural information embedded in AF2 was "recalled" to model the MLV Env trimer and the intermediate models used as templates. Thus, the trimeric MLV Env *ab initio* model obtained here arises from the sequence information within the input, the sequence diversity of retroviral Env sequences in the multiple sequence alignments, used by AF2 to determine residue covariations and co-evolution, and general protein folding information embedded in AF2.

Structural alignments of the MLV Env trimer model to PDB structures and models in the AFDB were used to assess its uniqueness. A single protomer of the trimeric polytropic MLV Env model excluding the RBDs and PRR was used in structural alignment searches to identify homologs in the PDB and the AFDB using the Foldseek server (Kempen et al., 2023). Although several monomeric endogenous gammaretroviral Env models in the AFDB were identified as close homologs, no structural homologs were identified in the PDB. The DALI Protein Structure Comparison server (Holm, 2020) identified, among 635 spurious hits unrelated to viral proteins, 3 viral envelope protein structures. In decreasing hit score order these were an EBOV SP trimer (PDB 6VKM), a post-fusion structure for a baculovirus gp64 envelope protein (PDB 3DUZ) and a trimeric human immunodeficiency virus type 1 (HIV-1) Env structure (PDB 6PWU). The latter aligned with the MLV Env trimer model mostly in the PD regions previously shown to be conserved among retroviruses with very limited alignment in the TM regions. Therefore, the trimeric MLV Env structure is unprecedented in its details and distinct from previous structures.

Overall SU structure in trimeric Env. The SU region in the MLV Env trimer is very similar to previously described structures and models. The RBD assumes the same L-shaped Ig-like fold a previously described ecotropic MLV RBD crystal structure (PDB 1AOL) (Fass et al., 1997). The polytropic Env RBD model and ecotropic MLV strain RBD structure aligned with a root mean-squared deviation (rmsd) of 2.11 Å and TM-score of 0.70. As mentioned above, the RBD was located not at the top of the trimer in the final model or the best intermediate models, as might be expected, but rather on the sides of the trimer pointing towards the viral envelope membrane with varying angles in different models. Each RBD was associated with the PD and TM regions of the Env protomer in the counter-clockwise direction when observed from the top of the trimer, consistent with the cross-protomer PAE scores between the RBD and PD and TM regions (Fig. 1B and 1C, protomer -1). The orientation of the RBD along its main axis also varied among models. In most models the RBD variable regions A and B (VRA and VRB) point in the counter-clockwise direction when the trimer is observed from the top, but in some models the RBD is rotated about 180° along its main axis, resulting in the VRA and VRB regions pointing in a clockwise direction. In some lower-scoring models

the RBDs are positioned on top of the trimer pointing away from the envelope membrane. The uncertainty about the RBD position is not unexpected given that the RBD is linked to the rest of the SU region by the relatively long PRR. Residues 203, 205, 208, 210, 211 and 214 in a more conserved region of the PRR are anchored by hydrophobic and hydrogen bond interactions in pockets in a larger crevice formed by TM and the PD near the center of the trimer axis (Fig. 2B and C). The rest of the PRR from that anchor region to the C-domain assumes an unfolded conformation with low pLDDT scores in all models (Fig. 1D and 2A).

Unsurprisingly, the PD region is also essentially identical to the previously reported monomeric PD AF2 model (Hötzel, 2022), without any significant differences due to the trimeric context. As described in detail below, the C-domain section between the PRR and PD regions forms an α-helix (αPD1) that is closely associated with TM and the terminal PD regions rather than the core PD regions. In addition, the C-terminus of SU also forms an α-helix (αPD5) closely associated with TM (Fig. 2A and 3C).

Pre-fusion MLV TM assumes a conformation similar to pre-fusion filoviral GP2 in trimers. The TM ectodomain regions form the center of the trimer model along its vertical axis with a mostly helical structure. The MLV Env trimer has four α-helices in the TM ectodomain, designated here αTM1 to αTM4 (Fig. 3C and G). The αTM2 helices from different protomers are positioned around the vertical trimer axis in a parallel configuration and form extensive, mostly hydrophobic, interprotomer contacts along the axis. The central αTM2 helices align with an rmsd of 0.407 Å to the corresponding regions of a xenotropic MLV TM structure in the post-fusion state (PDB 4JGS) (Aydin et al., 2013) even though these structures were not used as templates, indicating that inter-protomer interactions in this region remain essentially unchanged in the fusion process. Helix αTM2 includes all 17-aa residues of the immunosuppressive domain preceding the CX_6CC motif (Bénit et al., 2001; Cianciolo et al., 1985; Henzy and Johnson, 2013; Hogan and Johnson, 2023), which remains fully occluded in the pre-fusion state. Its location in the central axis of the TM trimer in both the pre- and post-fusion states partially explains the high conservation of this region. Helix αTM2 is preceded by helix αTM1, which projects from the periphery towards the center of the trimer in an approximately 60° angle relative to the vertical axis. Helix αTM1 is in turn preceded by the fusion peptide. Helices αTM1 and αTM2 in pre-fusion TM form the first

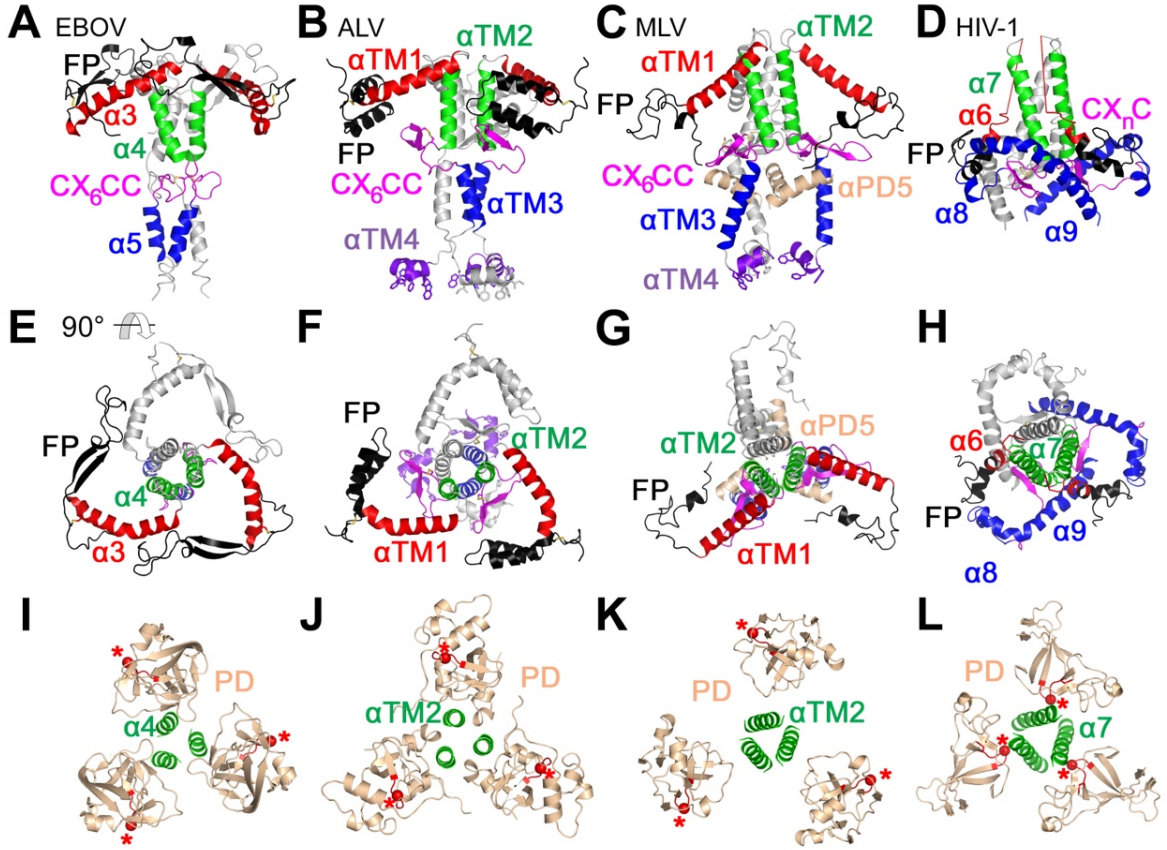

Figure 3 – Structural similarity between pre-fusion gammaretroviral TM and filoviral GP2. The structures and structural models of the pre-fusion, trimeric EBOV GP2 and retroviral TM are shown in panels A-H, with the lateral (panels A-D) and top (panels E-H) views shown. The viral membrane is located towards the bottom of the figures in the lateral views. The virus for the structures and models in each column (e.g., panels A, E and I) are indicated in panels A-D. The homologous GP2 and TM helices and regions are shown in different colors with color-coded labels. Only two of the protomers are colored for clarity, with the third protomer shown in gray. Panels I to L show the orientation of SU and GP1 PD in the trimeric structures and models from a top view as in panels E-H. Only the αTM2 homologues are shown for the TM and GP2 regions. The red asterisks show the location of the PD turn between the conserved β-strands 4 and 5. Note the outward-facing orientation of the β4/β5 turn for the EBOV SP and ALV and MLV Env models and the inward-facing orientation of the same turn in the HIV-1 trimeric Env structure. Note also the large hydrophobic side-chains of αTM4 (shown as sticks) in the MLV and ALV models that project towards the envelope membrane in panels B and C. EBOV SP structures, PDB 5JQ3. HIV-1 Env structures, PDB 4TVP.

contiguous helix of the post-fusion TM coiled-coil. Helix αTM2 is followed by a loop including the CX_6CC motif and helix αTM3. The αTM3 helices from different protomers are displaced away from the vertical trimer axis, resting on the αPD5 helices formed by the last residues of SU before the cleavage site. The last SU residue before the cleavage site is positioned close to the trimer axis, implying that Env cleavage must occur prior to final assembly of that region of the Env trimer.

The overall configuration of the αTM1, αTM2 and αTM3 helices is similar to that of corresponding α-helices α3, α4 and α5 in the GP2 of the EBOV SP trimer, but different from the α-helices of HIV-1 gp41 except for the central α-helix α7 that corresponds to MLV Env αTM2 and EBOV SP α4 (Fig. 3A, E, D and H). As previously expected from the analysis of gammaretroviral SU glycosylation patterns, the orientation of the PD in the MLV Env trimer model is essentially the same as the PD of EBOV GP2 in the trimeric structures and different from the orientation of the HIV-1 PD in Env trimers (Fig. 3I, K and L), confirming the closer structural similarity between trimeric MLV Env and EBOV SP trimers that includes both TM organization and SU orientation.

The short amphipathic α-helix αTM4 forms an MPER that lies nearly parallel to the presumed envelope membrane, projecting large hydrophobic side-chains into the membrane as judged from the position of the MSD in the model just prior to the MSD (Fig. 2A and 3B). Thus, the MPER extends the hydrophobic surface of the MSD. A similar helix-turn-helix structure has been described for the EBOV SP MPER/MSD region (Lee et al., 2017). An amphipathic α-helix in the influenza virus M2 protein partially embedded in the intracellular side of the membrane has been shown to enrich the M2 protein to the neck of budding virions by sensing membrane curvature and to promote the membrane lipid phase transitions necessary for virion release (Martyna et al., 2016; Schmidt et al., 2013). The topologically opposite MLV MPER may thus promote enrichment of Env on budding virions and facilitate the last steps of membrane fusion during infection. The N-linked glycosylation site of TM is located in the MPER αTM4 close to the trimer axis, with the side-chain of the asparagine pointing in the distal direction, away from the membrane. The MSD and IV regions of TM form contiguous α-helices that are closely associated among the protomers along the trimer axis (Fig. 2A).

A unique structure in the MLV Env trimer anchoring SU to TM. Despite the overall similarity between the trimeric MLV TM model and EBOV GP2 structures, there are significant structural differences between the structural elements that anchor MLV SU to TM and EBOV GP1 to GP2 in the trimeric pre-fusion state. EBOV GP1 anchors in a hydrophobic structure formed by GP2, in a manner analogous to the anchoring of gp120 anchor to gp41 (Pancera et al., 2014). The SU anchoring structure in the MLV Env trimer model is similar to the one observed in the EBOV SP trimer but with additional structural elements unique to MLV Env that extend the anchoring structure in a manner similar, although not homologous, to HIV-1 Env. The main anchoring elements for the EBOV GP1 PD β-strands 2 and 11 homologues are GP2 α-helices α3 and α4 and parts of the fusion peptide (Fig. 4A and E). Similar interactions are observed

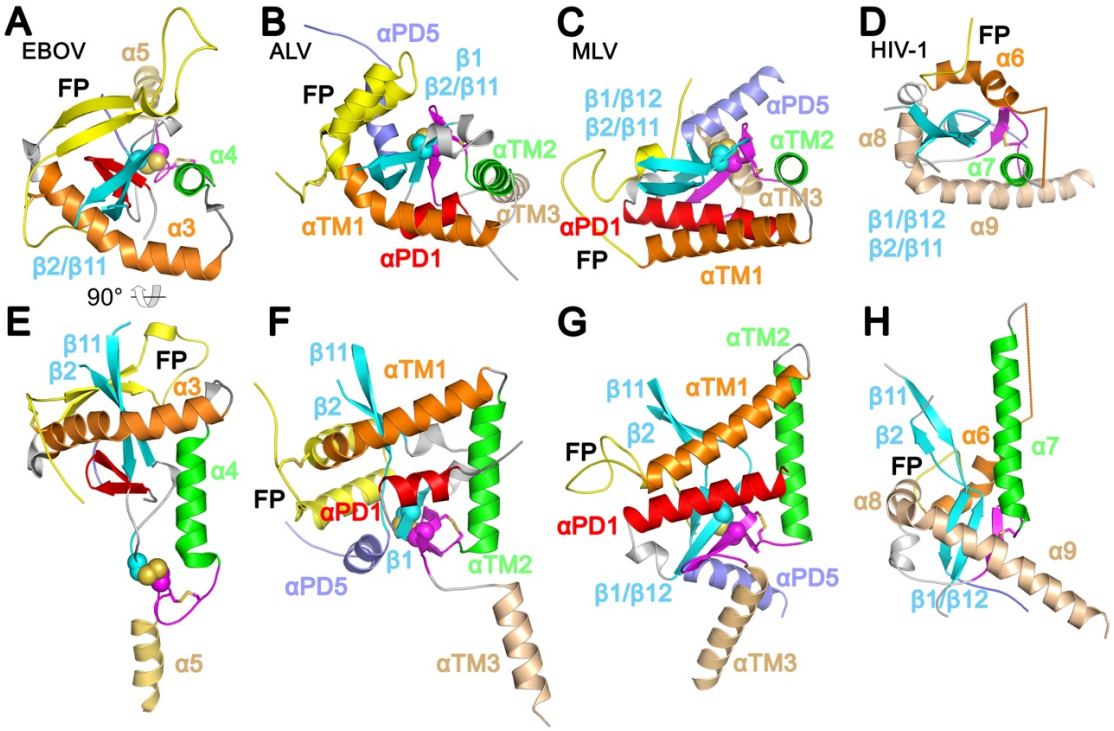

Figure 4 – Structure of the hydrophobic SU and GP1 anchoring structures in retroviral Env and filoviral SP. Panels A-D show a top view of the anchoring structures for the different viruses as indicated in the panels. Only one protomer of the SP and Env structures and models is shown for clarity. The α4, αTM2 and α7 homologues in green are located in the center of the trimeric structures and models. Panels E-H shown the corresponding lateral view of the structures, rotated relative to the top panels as indicated in panel A, with the viral envelope towards the bottom of the figure. The homologous TM and GP2 structures are shown in the same colors in different panels and indicated in color-coded labels. The terminal PD β-strands of GP1 and SU are shown in cyan. The Cys residues mediating the intersubunit disulfide bonds in EBOV SP and ALV and MLV Env are shown as spheres. The straight line connecting α6 and α7 in the HIV-1 structure indicates an unresolved region of the structure. PDB structures as in Figure 3.

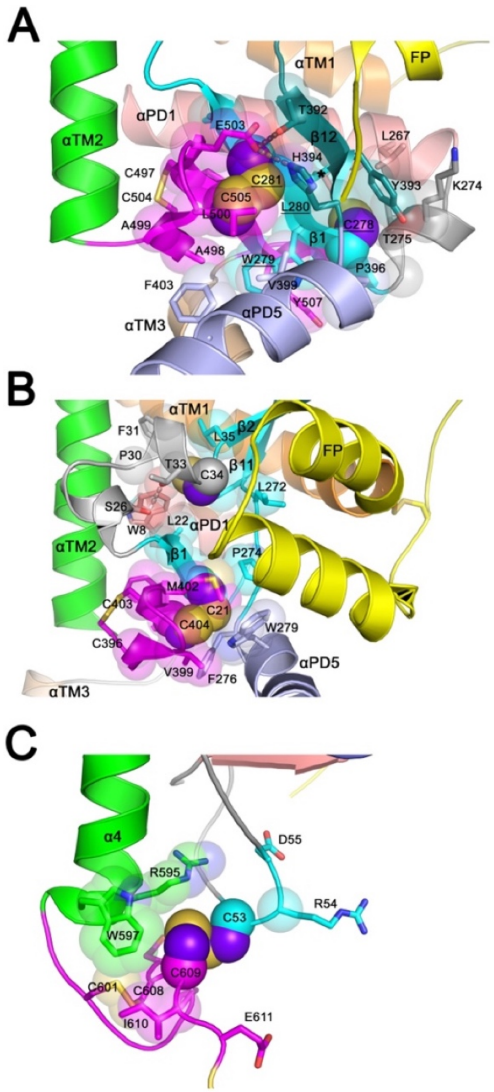

Figure 5 – Structure of the intersubunit disulfide bond in trimeric gammaretroviral Env and EBOV SP. (A) MLV Env intersubunit disulfide. The disulfide bond between SU Cys-281 and TM Cys-505 is shown as spheres. Atoms surrounding the intersubunit disulfide are shown as transparent spheres superposed on the corresponding residues shown as sticks and numbered. The different structural elements of TM and SU are shown in different colors and indicated with labels. The side-chain of unpaired Cys-278 in the CWLC motif is also shown as spheres with surrounding atoms shown as transparent spheres. The model is shown from the side of one protomer with the viral envelope towards the bottom. (B) ALV Env intersubunit disulfide structure. The ALV Env model is shown in the same orientation as in panel A, with similar rendering and labeling scheme. Conserved unpaired Cys-34 is also shown as spheres. (C) EBOV SP intersubunit disulfide bond (PDB 5JQ3).

in the MLV Env trimer model, with PD β-strands 2 and 11 contacting the GP2 α3 homologue, αTM1, and the signal peptide (Fig. 4C and G). However, unlike EBOV GP1 and similar to HIV-1 gp120, the MLV PD region of SU has the β-strands 1 and 12 extensions emanating from the core region of the PD in a virion-proximal direction (Fig. 4C and G). These two β-strands, as the homologous β-strands in the trimeric HIV-1 Env structure (Fig 4D and H), are almost entirely embedded in a hydrophobic shell in the MLV Env trimer model. Several elements form this hydrophobic shell, including the fusion peptide, αTM2 and αTM3 in TM and the SU C-domain helix αPD1, which is positioned in on the virion-proximal side of αTM1, and αPD5. An amino-terminal GP1 β-hairpin occupies a similar space in the EBOV SP trimers as αPD1 in trimeric MLV Env. The HIV-1 Env trimer does not have a structure homologous to MLV αPD1. The region occupied by αPD1 in MLV Env is instead occupied by gp41 helix α9 in HIV-1 Env (Fig. 4D and H). Thus, MLV Env has an SU anchoring structure that extends interactions homologous to those of EBOV SP in the proximal direction to include PD β-strands 1 and 12, similar to as in HIV-1 Env. However, the structures forming the virion-proximal section of the hydrophobic anchoring structure. around PD β-strands 1 and 12 differ between the MLV Env trimer model and HIV-1 Env trimer structures, including a major MLV SU α-helix in that structure that is not observed in HIV-1 gp120.

Structure of the intersubunit covalent bond in the MLV Env trimer model. A major defining feature of gamma-type Env is the covalent disulfide bond between SU and TM and, in the gammaretroviruses specifically, the SU CXXC motif (CWLC in MLV) mediating this interaction. The MLV Env CWLC motif is located in PD β-strand 1, which is almost completely buried with the virion-proximal anchoring hydrophobic structure (Fig. 4G and 5A). The second Cys residue of this motif forms a disulfide bond with the third Cys residue of the CX_6CC motif of TM within the same Env protomer in the trimeric model (Fig. 5A), consistent with previous biochemical data (Löving et al., 2012; Sjöberg et al., 2006). The first and second Cys residues of the CX_6CC motif in TM form the expected disulfide loop near the trimer axis in the Env model (Fig. 5A). The two last Cys residues in the CX_6CC motif are part of a β-strand that forms an extended β-sheet with PD β-strands 1 and 12 and a fourth β-strand within the TM cysteine loop (Fig. 5A). As expected, the first Cys residue of the CWLC motif, which is also entirely buried within the hydrophobic anchor, does not form a disulfide bond in the pre-fusion trimeric Env model (Fig. 5A). Thus, the isomerization of Env disulfides that occurs upon receptor binding required for cell infection (Hogan and Johnson, 2023; Wallin et al., 2005) must be preceded by significant conformational changes in SU and TM to expose both Cys residues in the CWLC motif. In contrast, the intersubunit disulfide bond between GP1 and GP2 in the EBOV SP is accessible on the surface of the trimer (Fig. 5C), thus defining another key structural difference between retroviral and filoviral gamma-type envelope proteins.

Fitting of models into trimeric MLV Env electron density maps. The MLV Env model was assessed by fitting to previously described electron density maps of mature MLV Env. Several electron density maps have been described (Förster et al., 2005; Löving et al., 2012; Riedel et al., 2017; Wu et al., 2008). The best published map has a resolution of 15 Å and shows the Env trimer as a tripod-shaped structure with a region of low electron density in the center (Riedel et al., 2017). Four contiguous regions of high electron density in the periphery and top of the trimer were previously named the foot, heel, knee and head regions, starting at the base of the trimer (Fig. 6D). Previous attempts to fit the RBD structure to the Env electron density maps have put the RBD at the top of the trimer in a location expected to be available for receptor binding.

The MLV Env trimer model without the RBD and most of the PRR, which have low-confidence positions in the model, was fitted to a 15 Å resolution electron density MLV Env trimer

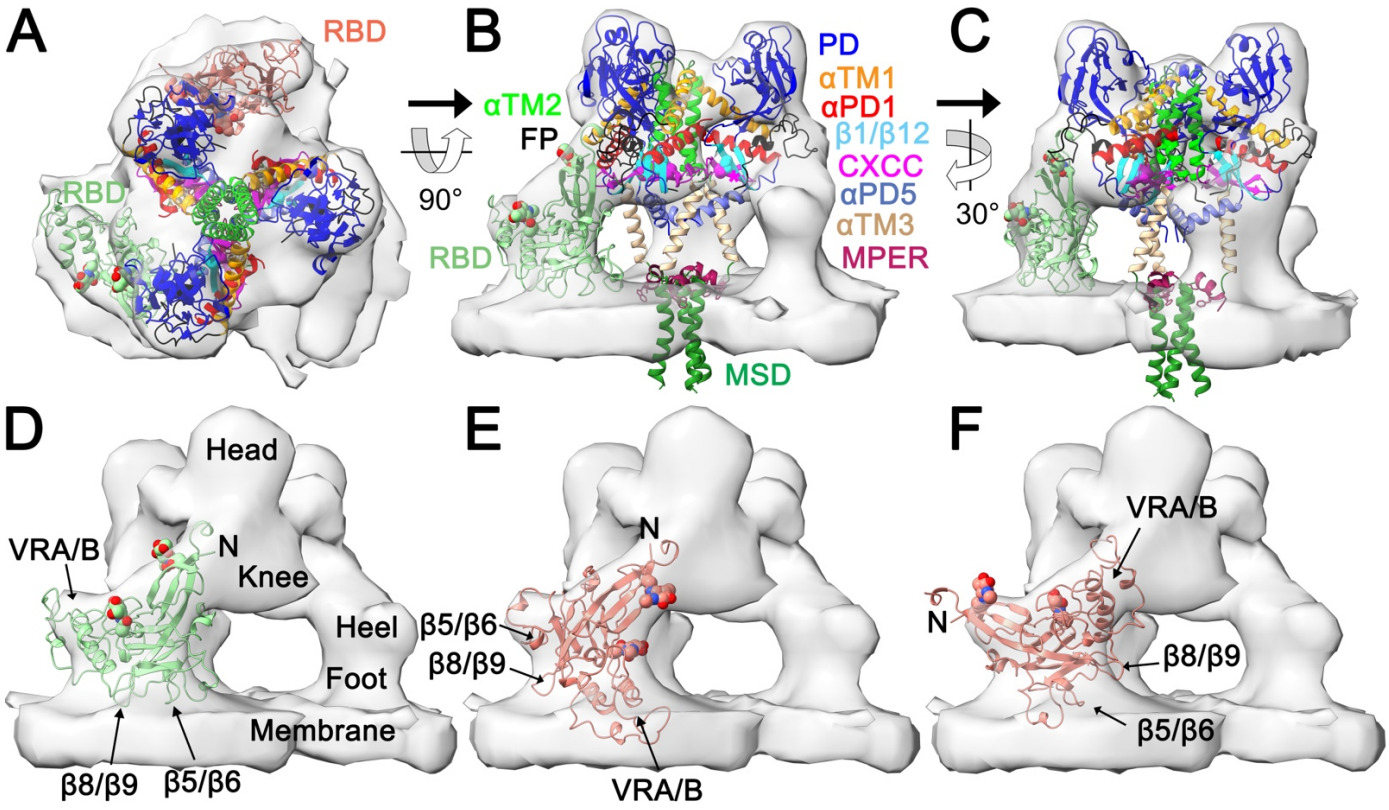

Figure 6 – Fitting of the trimeric Env MLV model and RBD structure in trimeric MLV Env electron-density maps. Panels A to C show top (A) and side (B and C) views of the MLV Env trimer model without the RBD and PRR regions fitted to the composite trimeric MLV Env electron-density map (EMDB EMD-3373). Panel A also shows the structure of the MLV SU (PDB 1AOL) fitted in two different poses. The SU and TM sub-regions are shown in different colors indicated with color-coded labels. Panel B omits the second RBD pose shown in salmon in panel A for clarity. The structures and models are rotated as indicated between panels. The electron-density maps in panels D-F are rotated 60° to the right on the vertical axis relative to panel C to show alternative fittings of the RBD in the maps. The position of the VRA/B regions and loops between RBD β-strands 5/6 and 8/9 and the amino-terminus of SU (N) are indicated to show the relative RBD orientation. The location of the previously described head, knee, heel and foot regions of the electron-density maps are shown in panel D. The magenta asterisks in the middle section, right of center of panel B indicate the location of the intersubunit disulfide bonds shown as spheres.

map (EMDB accession EMD_3373) (Riedel et al., 2017) using ChimeraX (Goddard et al., 2017). The automatically optimized fit positioned the αTM4 helices and membrane-spanning regions of the model just above and within the membrane, respectively, and the SU PD region from strands β2 to β11 in the head regions of the maps at the top of the trimer (Fig. 6A-C). The central depression at the top of the trimer is formed by αTM1 and the distal end of αTM2 (Fig. 6A-C). The knee region includes the TM structure anchoring PD β-strands 1 and 12, with the CWLC motif and the inter-subunit disulfide bond in its core. The TM regions closer to the trimer axis are largely in the central regions of low electron density in the maps. However, a weak but clear electron density was described in the central region of some the MLV Env maps (Riedel et al., 2017). These lower-density map regions fit well visually with the central α-helices of TM and αPD5, including an intra-membrane density along the trimer axis that would correspond to the location of the membrane-spanning helices in the trimer model. The lack of strong density corresponding to the central TM regions at the resolutions attained for the MLV Env electron density maps was previously discussed (Riedel et al., 2017).

The only region in the electron density maps left for the RBDs based on these fitting results are in the heel and foot regions close to the viral membrane, away from the target cell. That is, the RBDs in the electron density maps appear to be in the same general position as the RBDs in the best quality AF2 Env trimer models. Indeed, that region of the electron density maps is L- shaped and should accommodate a similarly-shaped RBD. The MLV RBD crystal structure (PDB 1AOL) was manually fitted into that region while avoiding major obvious clashes with the PD and TM Env model regions fitted to the same maps (Fig. 6). The shape of the RBD and the heel and foot regions allow several possible fittings. In the first fitting the RBD was oriented with the VRA and VRB regions in the heel region pointing in the clockwise direction as seen from the top of the trimer (Fig. 6A-D, light green RBD moiety), in the opposite orientation as the RBD in MLV Env models 3a and 3b. In that orientation, the RBD touches the membrane and both RBD glycosylation sites point towards the outside of the trimer. In the second fitting with the RBD turned 180° along its main axis relative to the first fitting, significant portions of the RBD embed in the membrane and one the RBD glycans points towards the

center of the trimer (Fig. 6A and E, salmon RBD moiety). In a third alternative fitting, the RBD termini are located in the heel region, with the VRA/B regions pointing towards the knee region (Fig. 6F). In the last fitting, the RBD in the third fitting is turned around 180° along its main axis, resulting the VRA/B region being deeply embedded in the membrane (not shown) and, therefore, unplausible. Other possible arrangements, such as with the RBD termini in the foot region pointing towards the membrane (not shown), are also biologically and structurally unplausible. Although the first fitting with the VRA/B region located in the heel region is the most likely of the three poses, the shape of the RBD allows alternative fittings in the electron density map corresponding to the heel and foot regions that are not occupied by other trimeric Env model regions.

Trimeric alpharetroviral Env models have composite structural features. The major differences between the Env of alpharetroviruses and gammaretroviruses is the absence of an independent RBD and CXXC motif in the SU of alpharetroviruses. Instead, an amino-terminal Cys residue in the SU of alpharetroviruses forms a covalent disulfide bond with TM, similar to EBOV GP1 (Jeffers et al., 2002; Pike et al., 2011). Another similarity between alpharetroviral Env and filoviral SP not shared by the gammaretroviral Env is the presence of a disulfide loop in the fusion peptide in TM and GP2 (Delos and White, 2000; Lee et al., 2008). Modeling of trimeric alpharetroviral Env was performed to compare its structure to the two other major gamma-type trimeric proteins. Modeling was performed using the Env sequence of an avian leukemia virus (ALV) strain as described for the MLV Env trimer, retaining a linker between SU and TM during modeling.

Reliability scores for the trimeric ALV Env trimer model were generally lower than for the MLV Env model (Fig. 7A, C and D). In fact, only a small subset of ALV sequences tested yielded SU or trimeric Env models of reasonable quality. Despite its lower reliability scores, the *ab initio* trimeric ALV Env structural model provides sufficient information when interpreted in the context of the trimeric EBOV SP structure and MLV Env model.

The trimeric ALV Env model is similar to the trimeric MLV Env model without the RBD (Fig. 7A). In the ALV Env trimer model, two discontinuous loops corresponding to host-range regions 1 and 2 (hr1 and hr2) (Holmen and Federspiel, 2000; Taplitz and Coffin, 1997) emanate from the PD distally to form a nearly contiguous surface on the distal end of Env in a position that would be available for receptor binding (Fig. 7A and B). The relative arrangement of α-helical regions in the ALV Env trimer model is more similar to the trimeric EBOV SP than to the MLV Env trimer model, especially in the αTM3 helices which, similar to the corresponding α-helices of the EBOV SP trimer, are located immediately around the vertical trimer axis and interact with each other through hydrophobic interactions (Fig. 3B and F, compare with Fig. 3A and E). The ALV Env trimer model also has short hydropathic membrane-proximal α-helix αTM4 homologues (Fig. 3B). These are positioned parallel to the envelope membrane and extend the hydrophobic surface of the membrane-spanning region as in the MLV Env trimer model, with large hydrophobic residues projecting towards the membrane, suggesting that this structure is a more general

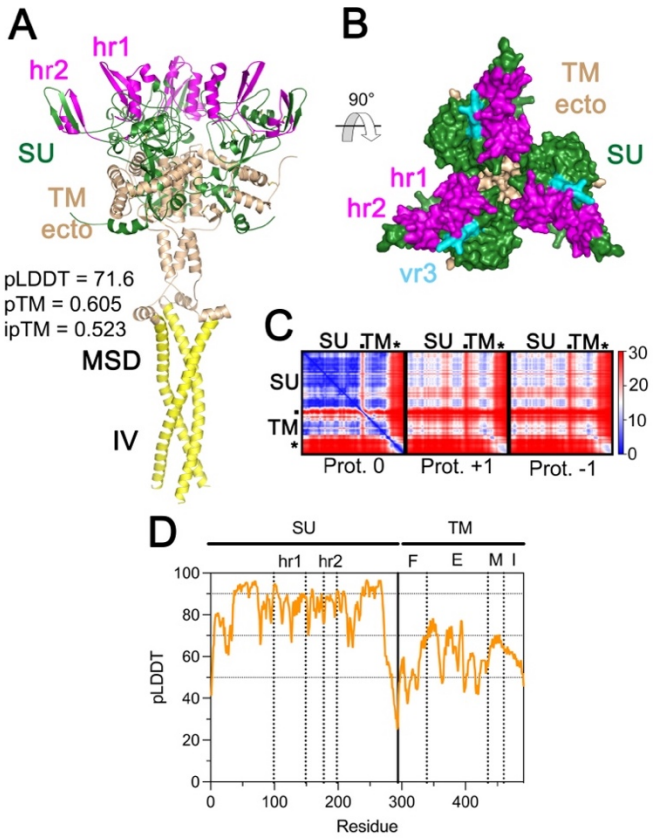

Figure 7 – Trimeric ALV Env model. (A) Side view of the trimeric ALV Env model. SU is shown in green, with the host receptor interacting regions 1 and 2 (hr1 and hr2) of SU highlighted in magenta. The extracellular region of TM (TM ecto) is shown in wheat and the MSD and IV regions in yellow. AF2 quality scores for trimeric Env model are indicated in the panel. (B) Top view of the ALV Env trimer model shown in surface rendering. The extended variable region vr3 is shown in cyan. The $(SG_3)_3$ linker between SU and TM is omitted in panels A and B. (C) PAE scores of the trimeric ALV Env model. The leftmost panel indicates PAE scores within each protomer ("Protomer 0"), with the approximate location of the main Env regions shown on the left and top of the matrix. The dot indicates the location of the fusion peptide in the matrix and the asterisk the MSD. The middle and right panels show the PAE of "protomer 0" relative to the protomers in the counterclockwise (-1) and clockwise (+1) directions as observed from the top of the trimer. The PAE scale, in Å, is shown to the right of the matrices. The other effectively redundant matrices for each of the two other protomers in the trimer are not shown for simplicity. (D) pLDDT scores of the ALV Env model. The boundaries or the major SU and TM regions are shown by vertical dotted lines. F, E, M, I indicate the fusion peptide, ectodomain, membrane-spanning domain and intraviral regions of TM. The threshold pLDDT scores of 50, 70 and 90 are shown by dotted lines.

feature of gamma-type envelope proteins. The PD orientation in the ALV Env trimer model is the same as for the MLV Env trimer model and EBOV SP trimer structures (Fig. 3J). The fusion peptide forms a disulfide loop similar to the fusion peptide of EBOV GP2 on the surface of the trimer (Fig. 4E and F). The low pLDDT scores of that region may reflect flexibility of the signal peptide in trimeric Env.

The ALV SU model has a short amino-terminal α-helix homologous to the MLV αPD1 helix that is positioned in a similar location and orientation in the trimer as MLV αPD1 (Fig. 4F and G). The PD-proximal hydrophobic anchor around PD β-strands 2 and 11 is similar to the other two trimer structures and models (Fig. 4B and F). The virion-proximal region of the hydrophobic anchor is similar in the ALV and MLV Env trimer models, with the intersubunit disulfide bond located in a similar position in the ALV and MLV trimer models (Fig. 4B, C, F and G). In fact, the SU cysteine mediating the intersubunit disulfide bond (Pike et al., 2011) is located in a β-strand homologous to MLV PD β-strand 1, where the CWLC motif is located in MLV Env. Thus, the intersubunit disulfides are structurally homologous in MLV and ALV Env. However, the ALV Env model does not have a well-defined terminal PD β-strand 12 and the corresponding region in ALV Env adopts an extended conformation instead (Fig. 4F). The intersubunit disulfide is occluded in the virion-proximal hydrophobic anchor by an α-helix in the carboxy-terminal region of SU (Fig. 5B). Thus, the ALV Env trimer model has a structure that is intermediate between the MLV Env trimer model EBOV SP trimer structures, with an SU and intersubunit disulfide anchoring similar to the former and fusion peptide and αTM3 configuration more similar to the latter. A highly conserved unpaired ALV SU Cys residue (Cys-34 in the model) that is critical for infectivity and is exposed only upon receptor binding (Smith and Cunningham, 2007) is occluded in a mostly hydrophobic shell within the virion-proximal anchor, about 10 Å from the intersubunit disulfide bond in the virion-distal direction (Fig. 5B).

DISCUSSION

Here, AF2 multimer was used to understand the structure and evolution of trimeric retroviral Env. The quality of the trimeric Env models described here vary, high for the MLV and lower for the ALV Env trimer models, as assessed by the AF2 quality scores. The models nonetheless can be used to understand certain functional aspects and the evolution of trimeric retroviral Env when considered together and in combination with experimental EBOV SP and HIV-1 Env trimer structures. The results show structural features that, in hindsight, are not particularly surprising and fit what might be expected by assuming certain key structural features to be highly conserved among gamma-type Env variants, including EBOV SP trimers, generally supporting the validity of the trimeric Env models. Furthermore, the structural models are compatible with all available structural and biochemical biological available for these proteins. The most surprising finding, the position and orientation of the RBD in MLV Env trimers, does not contradict any previous data, but simply expectations of its position in the trimer. Thus, the models also reveal structural and functional features and evolutionary connections of retroviral Env that were unexpected.

The key model described here is the one for trimeric MLV Env, which has extensive experimental data available to assess its validity. Although the trimeric MLV Env electron density maps were not used to inform modeling of the MLV Env trimer, the resulting model fits remarkably well to the best electron density maps. The model fitting results show that the head region of trimeric Env includes the C-domain rather than the RBD, as previously proposed (Riedel et al., 2017; Wu et al., 2008). The location of the PD in the head region rather than in more membrane-proximal regions of Env as previously proposed is consistent with the location of the C-domain homolog of EBOV GP1 (the PD domain) in the trimeric SP structure. This would be expected, given the importance of this region in interacting with TM, which should be evolutionarily conserved across widely divergent gamma-type Env structures.

The trimeric Env models provide a detailed picture of the evolution of Env trimers in the retroviruses and filoviruses. First, as might be expected, the gamma-type Env share a common structural plan, including disposition of α-helical regions within pre-fusion TM/GP2, location of the intersubunit covalent disulfide bond in the trimer, configuration of the upper, PD-proximal hydrophobic SU anchor and PD orientation within the trimer. This conserved structural plan arises *de novo* in modeling of trimeric MLV and ALV Env, without templates from known gamma-type Env structures, supporting these as shared features of gamma-type Env. However, there are clear distinctions between the different gamma-type Env models within the retroviruses and between these and the EBOV SP trimer. The retroviral gamma-type Env models have an additional SU helical region, αPD1, that replaces a small β-hairpin loop observed in a similar position in the EBOV SP. This α-helix, along with other slight rearrangements in TM, extend the hydrophobic anchor towards the virus surface, forming the hydrophobic shell encasing the intersubunit covalent bond in the retroviruses. The ALV Env trimer seems to represent a structural intermediate between EBOV SP and MLV Env. The trimeric ALV Env has essentially the same general structure as the EBOV SP trimer, including the fusion peptide with in a loop largely within a disulfide bond, adding only a relatively short αPD1 helix in SU and minor changes in the SU carboxy-terminus that bury the intersubunit disulfide bond. The MLV Env trimer extends these modifications to more fully bury the intersubunit disulfide bond in a complete virion-proximal extension of the hydrophobic anchor and adds a cysteine in SU to form the CWLC motif within that structure. The distal anchoring structure is absent in HIV-1 Env, which instead has only a virion-proximal anchor in a similar position as the virion-proximal section of the anchor in MLV Env, although not completely homologous structurally, especially in the gp41/TM region. Of note, the apparent sequence of structural changes described here do not imply direct descent of the SP/Env between these major viral groups.

The main objective in this study was understanding retroviral Env structural evolution. However, the models provide some surprising functional features of retroviral Env. Here, only the main functional consequences suggested by the models are briefly discussed.

The most important unexpected functional consequence that emerges from the models described here is the apparent functional RBD occlusion in trimeric MLV Env. The results of model fitting into trimeric MLV Env electron density maps indicate that the unusual tripod shape of the MLV Env trimer is given by the RBD, which can only fit in the outer regions of the tripod-shaped trimer, with the receptor-binding regions pointing away from the target cell. A lower-resolution electron density

map of MLV Env bound to cell receptors in the context of target cell membranes show a chalice-like Env structure without the outer tripod legs, but rather with an extended density beyond the head region presumed to correspond to the RBD bound to the cell receptor (Riedel et al., 2017). This suggests a functional model in which the resting RBDs in trimeric Env sit retracted on the periphery of the trimer, shifting to an extended configuration for receptor binding, where they would be available to interact with the C-domain in the head region of the trimer to trigger fusion (Lavillette et al., 2001). In addition, gammaretroviral Env can also be functionally complemented by soluble RBD provided in *trans*, even when trimeric Env retains its own RBD moieties (Barnett et al., 2003, 2001; Barnett and Cunningham, 2001; Lavillette et al., 2001). The retracted RBD conformation would allow the soluble RBS moieties to directly interact with non-RBD Env regions, most likely the exposed loop D region of PD exposed on the head region of the trimer (Hötzel, 2022; Lavillette et al., 2001), to induce conformational changes in Env that trigger membrane fusion. The signals and structural mechanisms would trigger the RBD of Env to move from the side to the apical end of Env to interact with cell receptors are not clear from the models or inspection of the lower-quality Env trimer models with RBDs extending distally at the top of the trimer but may involve the PRR (Lavillette et al., 1998). RBD retraction may participate in immune evasion by sequestration of receptor-binding sites away from neutralizing antibodies until the virus is close to the target cell, where antibodies may not be able to access the critical epitopes. Of note, the cleavage site between SU and TM is buried by the RBD in the retracted state. This suggests that not only positioning of αPD5 close to the trimer axis but also the retracted RBD state may depend on Env cleavage by cellular proteases.

The results presented here show the value of novel deep-learning-based methods for structure prediction to advance the understanding of retroviral Env evolution and function, beyond what is possible by sequence analysis. Modeling of trimeric Env structures remains significantly challenging however, dependent in most cases on the identification of optimal starting sequences and sequence fragments and modeling strategies empirically, analogous to experimental approaches for structure determination. The methods described here should inform strategies for modeling of other trimeric retroviral Env and syncytin structures. The structural models should also provide useful information to guide future efforts to experimental determination of monomeric and trimeric Env structures, a major challenge in understanding the structure, function and evolution of these proteins.

MATERIALS AND METHODS

Trimeric MLV Env structural modeling. Structural modeling of trimeric polytropic MLV Env (GenBank XP_036020509) was performed with AF2 and AF2 multimer using the ColabFold server (Mirdita et al., 2022), accessible at https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb with A100 GPU enabled. Selection of the MLV for Env modeling was based on preliminary monomeric and trimeric Env structural modeling using a diverse set of sequences of MLV Env strains, choosing the sequence that yielded the highest AF2 quality scores and lowest rate of modeling artifacts such as domain swaps. The segments used for modeling monomeric and trimeric Env the corresponding templates and linker sequences between SU and TM, when used, are shown in Figure 1A. Monomeric Env modeling with standard AF2 used three recycles for each of the 5 modeling runs, without amber relaxation. The highest-scoring model (by pLDDT score) was selected as template for the next round trimeric Env structural modeling. Model regions omitted from templates were removed from PDB files using PyMol version 2.5.1 (Schrödinger LLC) prior to modeling of the next round of modeling. Trimeric Env modeling was performed using AF2 multimer v3. Two different trimeric Env modeling runs were performed in different dates to avoid re-use of the same random seed in different runs that result in identical models. The user-specified parameters used for modeling were the following: no amber relaxation; msa_mode: mmseqs2_uniref_env; model_type: alphafold2_multimer_v3; num_models: 5: num_recycles: null; recycle_early_stop_tolerance: null (default, 0.5 Å); rank_by: multimer; pair_mode: unpaired_paired; stop_at_score: 100.0; random_seed: 0; num_seeds: 1; use_dropout: false; use_cluster_profile: true. The second run to obtain models 3a and 3b used the same parameters except that it used 2 seeds for modeling (for a total of 10 final models). Ranked models were visually inspected to exclude models with TM resembling post-fusion states, if any, and chain-swapping artifacts between αTM1 and αTM2 or between αTM2 and the CX_6CC motif loop. Modeling of trimeric EBOV SP was performed in a similar manner, to the trimeric Env modeling up to models 2a and 2b, using templates as described in the results section. Models were visualized with PyMol version 2.5.1. The coordinate file of trimeric MLV Env model 3a is available upon request.

Modeling of trimeric ALV Env. Modeling of trimeric ALV Env was performed as described for MLV Env models 2a and 2b, followed by amber relaxation of the top-scoring model. An ALV subgroup J Env sequence (GenBank QGW52078, residues 69 to 559) was used for trimeric Env modeling based on preliminary SU modeling with a diverse set of ALV Env sequences, selecting the sequence yielding the highest AF2 quality scores. An $(SG_3)_3$ linker sequence was inserted between the SU and TM sequences for monomeric and trimeric Env modeling. Model residue numbering starts in residue 69 of QGW52078 set as 1, excluding the linker sequence for numbering of TM regions. The coordinate file of the trimeric ALV Env model is available upon request.

Assessment of structural model quality. The final, amber-relaxed, trimeric Env models were assessed for structural clashes and overall structural quality attributes using the Molprobity server (http://molprobity.biochem.duke.edu) (Davis et al., 2007).

Electron-density model fitting. Fitting of trimeric MLV model 3a excluding the RBD and PRR regions to the electron-density map of EMDB accession EMD_3373 was done using ChimeraX 1.6.1 (Goddard et al., 2017). The trimeric Env model section was initially fitted manually followed by automated fitting based

on local alignment between the model and the electron-density map. Fitting of MLV RBD (PDB 1AOL) to the electron-density map in different alternative poses was performed manually.

Structural alignment database searches. PDB files of single Env protomers from the trimeric Env models were used to search for similar structures in the PDB and AFDB using the DALI (http://ekhidna2.biocenter.helsinki.fi/dali/) (Holm, 2020) and FoldSeek servers (https://search.foldseek.com/search) (Kempen et al., 2023).

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