

is currently unknown. In this work, we use density gradient centrifugation and liposome coflotation analysis to demonstrate that the anchoring energy of the Dengue E protein in anionic membranes composed of POPC+POPG is much greater than in membranes composed of POPC+POPE.

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HIV Fusion Peptide Perturbs Membrane Structure in a Cholesterol Dependent Fashion

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The fusion between viral envelopes with host cell membranes is required for viral entry and infection, which is mediated by special glycoproteins anchored on the viral membrane. Fusion peptide (FP) is the domain that initiates membrane fusion. However, the mechanism of membrane fusion is still unclear. We previously found that the influenza hemagglutinin FP increases the order of DMPC lipid bilayer. We hypothesize that inducing lipid ordering might be a critical step in fusion caused by a variety of fusion proteins. HIV gp41 FP plays a similar role as influenza HA FP. However, gp41 FP is polymorphic and changes from alpha helix to beta aggregation as cholesterol concentration in lipid increases. We used PC spin labels on the lipid head group and different positions on the acyl chain to detect the perturbation by gp41 FP to POPC/POPG lipid bilayers with different cholesterol concentration (0% to 30%) by CW-ESR. Our data show that 1) gp41 FP affects the lipid order in the same pattern as HA FP does, i.e., a cooperative effect vs. lipid/peptide ratio, thus supporting our hypothesis; 2) gp41 FP induces membrane ordering in all tested lipid compositions, consistent with promoting membrane fusion in these compositions; 3) in the high cholesterol containing lipid bilayers, whereas gp41 FP is in the beta aggregation conformation, its effect on the lipid ordering reaches deeper into the bilayer, consistent with deeper membrane insertion for gp41 FP in this conformation. We are extending the ESR studies to look for coexisting membrane microdomains induced by different conformations of FPs and the FP partitioning between them and for precise separation of the effects of ordering and molecular motion.

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CD4 Binding Induces an Asymmetric Transition of HIV-1 Env from its Native Conformation into the Prehairpin Intermediate State

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HIV-1 membrane fusion is mediated by sequential binding of the viral homotrimeric Env (gp120/gp41) to cellular CD4 and chemokine receptor (CXCR4 or CCR5). These binding events coordinate conformational transitions of the gp41 trimer from its native conformation into a prehairpin intermediate state (PHI) and, subsequently, into its fusogenic trimer-of-hairpins structure (TOH). In the PHI, the gp41 trimer assumes an extended conformation that bridges the viral and cellular membranes, and exposes the N-HR and C-HR domains which are targeted by HIV-1 fusion inhibitors T20 and 5-Helix, respectively. Our previous work suggests that the PHI-to-TOH transition occurs in a concerted (symmetric) fashion. Here, we investigate the transition from the native state to the PHI using a functional complementation strategy that employed heterotrimeric Envs containing only one or two functional CD4- and chemokine receptor-binding sites. Additionally, by incorporating T20- and 5-Helix- resistance mutations into individual Env protomers, we were able to interrogate exposure of discrete gp41 N-HR and C-HR domains. Our data indicate that a single CD4 binding to an Env trimer is sufficient to promote fusion. Moreover, the first CD4 binding event appeared to expose only one of three possible binding sites for each fusion inhibitor, suggesting an asymmetric transition into the PHI. This asymmetry was significantly more pronounced in the gp41 N-HR region compared to the gp41 C-HR region. These results can be used to explain the multiphasic titration of mutant Env homotrimers that naturally form asymmetric N-HR domain structures. Taken together, these data suggest that the native state-to-PHI transition of Env occurs in a multistep (asymmetric) fashion.

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Evolution of HIV-1 Resistance to a Cholesterol-Linked D-Peptide Fusion Inhibitor

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During HIV-1 entry, the N-HR and C-HR regions of the gp41 viral glycoprotein associate to form a trimer-of-hairpins critical for membrane fusion. The N-HR region contains a highly conserved hydrophobic pocket that is the target for a multivalent, D-peptide fusion inhibitor, PIE12-trimer. Recently, a modified

form of this inhibitor with a conjugated cholesterol (chol-PIE12-trimer) was shown to possess up to 160-fold increased potency, presumably due to membrane targeting. Here, we determined the resistance profile of chol-PIE12-trimer starting from either inhibitor-naïve or PIE12-trimer-resistant HIV-1 (NL4-3 strain). Viral propagation in increasing inhibitor concentrations produced HIV-1 populations with 50- to 100-fold reduced sensitivity chol-PIE12-trimer. These viruses were also resistant to the parental, unconjugated inhibitor PIE12-trimer. Using HIV-1 fusion inhibitors di-C37 and 5-Helix, we were able to interrogate the effect of escape mutations on the exposure of the gp41 N-HR and C-HR regions, respectively. Resistance to chol-PIE12-trimer resulted in a slight decrease in the temporal window of N-HR exposure, regardless of the starting viral population. Exposure of the C-HR region was unchanged for resistant viruses generated from the inhibitor-naïve viral pool. By contrast, the temporal window of C-HR exposure was significantly increased (>20-fold) for resistant viruses that were generated from the PIE12-trimer-resistant viral pool. Efforts to identify the mechanisms of chol-PIE12-trimer resistance and the decoupling of N-HR and C-HR exposure are underway.

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Fusogenic Activity of the HIV-1 Gp41 MPER-TMD Region: Mechanism and Targeting by Immunogens and Inhibitors

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Fusion of the viral envelope with the cell membrane marks the beginning of the HIV-1 replicative cycle. This event is targeted by inhibitors currently in clinical use and by preventive vaccines under development. Antibodies 4E10 and 10E8 bind to the gp41 membrane proximal external region (MPER)-transmembrane domain (TMD) junction and block fusion. These antibodies display the broadest viral neutralization known to date, which underscores the conservation and functionality of the MPER-TMD region. In recent work, we have described that peptides representing this region have potent membrane-destabilizing effects. Here, based on the outcome of vesicle assays, atomic force microscopy studies and molecular dynamics simulations, we propose a mechanism for the involvement of the MPER-TMD region in HIV-1 fusion. In addition, we provide evidence that underpins the potential use of its activity as a new target for inhibitor and immunogen development.

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Studies on the Membrane-Active Behavior of Outer Membrane Vesicles from a Gram Negative Bacterium

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Gram-negative bacteria are known to produce small ~50-200 nm vesicles by "blebbing" of their outer membranes. These outer membrane vesicles (OMV) have been implicated in activities such as transmission of virulence factors, horizontal gene transfer and development of biofilms. In this investigation, the interactions of OMV from *Lysobacter enzymogenes* (strain C3) with other membranes have been monitored using fluorescent assays for association and/or fusion. Defined composition large unilamellar vesicles labeled with the fluorescence resonance energy transfer (FRET) pair 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE) were incubated with isolated OMV to observe the interaction via the FRET ratio of donor/acceptor. OMV substantially increased this ratio over the course of about an hour ($t_{1/2}$ ~10-20 min.) at 30°C, when added directly to vesicles comprising disordered or fluid lipids, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). This apparent fusion was much more limited or absent with liquid ordered membranes of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/cholesterol (DPPC/chol). In the case of POPG/POPE 1/3 vesicles, apparent fusion was also confirmed by testing vesicles with exclusively inner monolayer fluorescent probes to diminish any fluorescence changes from external interactions of outer monolayer fluorophores. When the OMV were labeled with the fluorophore 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, and incubated with a natural host, yeast cells (strain S30), association and/or fusion was observed by co-sedimentation of the OMV label with yeast cells. These studies may help to elucidate the mechanism of natural host-pathogen interactions and define the lipid specificity for