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Highlights

The heptad repeats of gp41 form hetero-oligomers in solution and in membranes

The CHR and NHR helices align parallel to the membrane surface

The two domains cause membrane disordering

CHR and NHR play an active role in membrane fusion

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Structure, interactions and membrane topology of HIV gp41 ectodomain sequences

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ABSTRACT

The gp41 type I membrane protein is part of the trimeric *Env* complex forming the spikes at the HIV surface. By interacting with cellular receptors, the *Env* protein complex initiates the infectious cycle of HIV. After the first contact has been established *Env* disassembles by shedding gp120 while the remaining gp41 undergoes a number of conformational changes which drive fusion of the cellular and the viral membranes. Here we investigated the membrane interactions and oligomerization of the two gp41 heptad repeat domains NHR and CHR. While these are thought to form a six-helix bundle in the post-fusion state little is known about their structure and role during prior fusion events. When investigated in aqueous buffer by CD and fluorescence quenching techniques the formation of NHR/CHR hetero-oligomers is detected. An equilibrium of monomers and hetero-oligomers is also observed in membrane environments. Furthermore, the partitioning to POPC or POPC/POPG 3/1 vesicles of the two domains alone or in combination has been studied. The membrane interactions were further characterized by ^{15}N solid-state NMR spectroscopy of uniaxially oriented samples which shows that the polypeptide helices are oriented parallel to the bilayer surface. The ^{31}P solid-state NMR spectra of the same samples are indicative of considerable disordering of the membrane packing. The data support models where NHR and CHR insert in the viral and cellular membranes, respectively, where they exhibit an active role in the membrane fusion events.

ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
CHR	C-terminal heptad repeat
DPC	dodecyl phosphocholine
EDT	ethane dithiol
EM	electron microscopy
<i>Env</i>	viral envelope protein complex
FP	fusion peptide
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
LWHH	line width at half height
MALDI TOF	matrix assisted laser desorption ionization time of flight
MPER	membrane proximal external region
NBD	nitrobenzoxadiazole (4-chloro-7-nitrobenzofurazan)
NMR	nuclear magnetic resonance
NHR	N-terminal heptad repeat
PC	phosphatidylcholine
PG	phosphatidylglycerol
PS	phosphatidylserine
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'-rac-glycerol) sodium salt
POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoserine
TFA	trifluoroacetic acid
TM	transmembrane

INTRODUCTION

Almost 40 years after the discovery of AIDS, no cure or efficient vaccination for HIV infection has been found. Therefore, keeping the virus at stake requires life-long treatment with expensive medicines. Upon HIV infection the viral and the target cell membranes fuse, a process that is mediated by the viral envelope glycoprotein *Env* [1, 2]. *Env* is a trimeric heterodimer made up of three copies of gp120 and gp41, which result from cleavage of gp160. The complex is anchored in the viral membrane by gp41. In the pre-fusion state three gp120 proteins are exposed at the viral surface [3-5] which during the viral life cycle interact with the human cell surface receptor CD4 [6] and one of the chemokine receptors CCR5 or CXCR4 [7, 8]. This docking to the human membrane causes a number of conformational rearrangements of *Env*, shedding of gp120, exposure of gp41 concomitant with major structural changes of the latter to result in an extended pre-fusion state [2, 9].

The following fusion events are then driven by gp41 membrane interactions [10], a protein which has received particular attention also in this work (Figure 1). Whereas in the extended pre-fusion state the gp41 amino-terminal fusion peptide reaches out for the host cell membrane, the transmembrane domain of this protein, being 172 amino acid residues apart, remains anchored in the viral envelope [9, 11].

Figure 1: Schematic illustration of the gp41 sequence: FP: fusion peptide; FPPR: fusion peptide proximal region, NHR and CHR: N-terminal and C-terminal heptad repeat, respectively, MPER: membrane proximal external region; TM: transmembrane anchor; CT: cytoplasmic terminus. The numbering follows the HXB2 subtype of HIV-1 (uniprot P04578).

Table 1: Peptides derived from gp41 (uniprot P04578) and investigated in this paper. ¹⁵N labelled sites are shown in red, ²H₃-alanines in green, tryptophanes in bold. *NBD*-represents 4-Chloro-7-nitrobenzofurazan, the residues in brackets show the additional residues of N36 and C34 investigated e.g. by [12-14].

NHR ¹⁵N-L11, ²H₃-A13: SGIVQQQNNL LRAIEAQQHL LQLTV**W**GIKQ LQAR (IL)
 NBD-NHR: NBD-SGIVQQQNNL LRAIEAQQHL LQLTV**W**GIKQ LQAR
 CHR ¹⁵N-L18: **W**MEWDREINN YTSLIHS**L**IE ESQNQQEK (NEQELL)
 NBD-G-CHR: NBD-G**W**MEWDREINN YTSLIHS**L**IE ESQNQQEK

Whereas the fusion events that follow occur via transient structural rearrangements and remain largely speculative the post-fusion conformation of the gp41 ectodomain consists of a six-helix bundle bringing the fusion peptide and the transmembrane domain, although far removed in the primary sequence, into close physical proximity [15, 16]. High resolution structures of the six-helix bundle show an antiparallel packing of the C-terminal heptad repeat (CHR) helices into the hydrophobic grooves formed on the surface of a central trimeric helical bundle of N-terminal heptad repeats (NHR) [12, 16, 17]. In contrast an X-ray structure at 4.8 Å and EM structures of the soluble *Env* trimer representing the native spike indicate an almost perpendicular alignment of CHR relative to NHR when gp41 is associated with gp120 [5, 18, 19].

Cryo EM images of the native spike show a trimeric NHR core and upon activation of *Env* during receptor binding subtle conformation changes of gp120 that release the fusion peptide from its hydrophobic burial site [4, 5, 20, 21]. Upon complexation with CD4 and 17b antibody (or B12) additional interactions of the gp41 NHR helices with gp120 are observed concomitant with conformational rearrangements of the helical bundle [18].

However, it remains obscure how the high-energy barrier of fusion is overcome by the pre-hairpin intermediate of gp41 which has to change from the extended prefusion arrangement to the compact post-fusion bundle of helical hairpins made of six helices in antiparallel arrangement [12, 16, 17]. These conformational changes bring the membranes in close contact [2, 11, 22]. The exposed pre-hairpin conformation is of particular interest because it is accessible to the humoral immune response during a relatively long lag phase of HIV infection [23, 24] and has become the target of antiviral fusion inhibitor drugs [25-27].

Fusion intermediates of gp41 are commonly depicted by extended trimers of linear CHR and NHR helices [10, 28, 29], however, there is increasing evidence that not only the TM domains and the N-terminal fusion peptide [30] but also NHR directly interacts with membranes and actively participates in the fusion process [14, 31]. NHR derived peptides have been shown to interact with phospholipid vesicles [14] and the membrane interactions of FP is much increased when covalently linked to NHR [31]. Furthermore, NHR-mimicking peptides that were designed to compete with NHR insertion into the six helix-bundle are much more efficient in inhibiting this

process when carrying an alkyl chain as a membrane anchor [32]. Notably, the NHR- and CHR-derived polypeptides have been shown to interact with membranes and their oligomerization state is affected by the presence of phospholipid vesicles or zwitterionic detergents [13, 14, 33]. Thus, it seems that these amphipathic domains of gp41 can help to lower the energetic barrier of membrane fusion as do other amphipathic peptides [30, 34].

When constructs representing NHR and CHR were investigated by solution NMR spectroscopy in the presence of DPC micelles [28, 33] the data provide a somewhat divergent view/interpretation for the intermediate state. Roche and coworkers find a monomeric flexibly linked two-helix structure where NHR and CHR lay on the surface of DPC micelles [33]. Lakomek et al. investigated gp41 (27-194) a longer construct comprising also the full immunodominant loop between CHR and NHR as well as the MPER and TM regions [28], i.e. this protein is shortened only by deletion of the FP and the FP proximal region when compared to a prior study [35]. This investigation revealed high internal dynamics of the N-terminal parts of the protein and a more static C-terminal region suggesting a predominantly extended conformation such as suggested for the prefusion intermediate [28]. Furthermore, strong lipid interactions of the loop region and several residues of NHR as well as a lipid-dependent monomer – trimer equilibrium have been measured [28].

Thus, whereas the data by Roche et al. suggested that CHR and NHR reside in different membranes when the prefusion intermediate forms, the findings of Lakomek are suggestive of an extended conformation where only FP and TM are membrane inserted. This latter conformation is in equilibrium with a structure where the protein folds back onto itself [28, 33]. The models diverge further in that Roche et al. propose gp41 trimerisation due to interactions within the TMD [33] while it is driven by NHR in the Lakomek model [28].

Notably, in the presence of 100 mM DPC the chemical shifts recorded for the NHR-CHR construct are very close to those of individual NHR or CHR polypeptides indicating that the two domains interact with membranes as independent units [33]. Therefore, in this paper we have investigated the membrane interactions of the NHR and CHR domains further using CD-, fluorescence- and oriented solid-state NMR spectroscopies. Whereas CD spectroscopy has been used to test the secondary structure preferences of the polypeptides, solid-state NMR spectroscopy of ^{15}N and/or ^2H labelled polypeptides provides valuable information about the structure, dynamics

and topologies of membrane-associated polypeptides [36]. The fluorescence quenching technique has been developed to test for oligomerisation and/or other supramolecular arrangements within lipid bilayer environments [37]. Furthermore, spectral changes during lipid titration experiments have been used to get a quantitative view on membrane association [13, 14, 38]. The combined information thus provides valuable information about the structural arrangements and interactions of CHR and NHR in membranes and about their role in membrane fusion and HIV infectivity.

MATERIALS AND METHODS

Organic solvents of 99% purity were from Sigma-Aldrich (division of Merck KGaA, Darmstadt, Germany). The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Larodan Solna, Sweden and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) sodium salt (POPG) from Avanti Polar Lipids (Birmingham, AL, USA).

Peptide synthesis

Peptides were assembled on solid supports using Fmoc chemistry and the standard cycles of a Millipore 9050 automatic peptide synthesizer (Millipore, Billerica, MA, USA, today division of Merck KGaA, Darmstadt, Germany). Fmoc-protected amino acids were from NovaBiochem (division of Merck KGaA, Darmstadt, Germany) and used at four-fold excess. The Tentagel-R-RAM resin was from Rapp Polymere (Tübingen, Germany). Cleavage of the peptide was performed in 95% (94%) TFA, 2.5% (2.5%) water, (2.5% EDT) and (1%) 2.5% triethylsilane for 4 hours (in parentheses the conditions for CHR). After solubilization and ether precipitation the TFA counter ions were exchanged by re-solubilization in 4% acetic acid and drying under high vacuum. For purification by semipreparative HPLC an acetonitrile/water gradient on a C18 reverse-phase column (Luna 5u c18(2) 100 Å; 150 x 30 mm, Phenomenex, Torrance, CA, USA) was used. Finally, the identity of the products was confirmed by MALDI TOF mass spectrometry.

NBD-Labeling of the peptides

NBD was attached to the amino terminus when the peptide was still on the resin and the side chains protected. 10 equivalents of 4-chloro-7-nitrobenzofurazan (NBD) (Fluka, Sigma-Aldrich, St. Louis, Missouri, USA) were dissolved in 50% acetonitrile containing 50 mM NaHCO₃ and the resin was incubated for 8h (typically 2 ml / 100 mg resin) [39]. After cleavage from the resin the peptides were purified by reverse phase HPLC. The identity and purity of the products were confirmed by MALDI mass spectrometry.

CD spectroscopy

Stock solutions of 0.5 mM CHR or NHR were prepared by dissolving the peptides in water. The concentration was verified by tryptophan absorption spectroscopy at 280 nm. Samples were prepared by dilution of the stock solution into the indicated buffers.

CD spectra were acquired on a JASCO-810 spectrometer (Jasco Tokyo, Japan) using cuvettes of 1 mm path length at 20 °C between 190 and 250 nm with an optical resolution of 3 nm. The spectra were analyzed for secondary structure composition of the peptides using CDPRO and the CONTIN/LL algorithm [40].

Self-quenching and tryptophan fluorescence measurements:

Fluorescence measurements were performed in 10 mM Tris, pH 7. Fluorescence spectroscopy was performed using a FluoroLog spectrophotometer (HORIBA, Ltd., Kyoto, Japan) with the polarization filters always at the magic angle. The slit size was 5 nm for excitation and emission. For NBD fluorescence the emission was scanned from 470 nm to 650 nm at an excitation wavelength of 465 nm. For tryptophan measurements the emission was scanned from 300 nm to 450 nm with an excitation wavelength of 280 nm.

Self-quenching analysis:

For every series of measurements, the label was diluted with unlabeled analogues of the peptides. The resulting series contained 100 %, 66.7 %, 50 %, 33.3 % and 20 % (per mole) of labeled analogues. The intensities of the fluorescence signal was

determined with a principal component analysis and fitted assuming an equilibrium between monomer and multimers:

$$P(A_{mon}, A_{multi}, n, \lambda) = A_{mon} \lambda + A_{multi} \lambda (1 - \lambda)^{n-1}, \text{ where}$$

$\lambda(1 - \lambda)^{n-1}$ is the probability that exactly one of the n peptides in a multimer is labeled. A_{mon} and A_{mult} correspond to the intensity of the monomer and multimer respectively in absence of self-quenching, n is the number of monomers in the multimer (fitting parameter can be non-integral) and λ is the fraction of labeling.

Fitting of apparent partition equilibrium:

Partition isotherms were fitted with the equation: $I([lip]) = I_0 + \Delta I \frac{1}{1 + 1/k_{lip}}$, which

follows directly from the relations:

$$k = \frac{[pep_{bound}]}{[lip][pep_{free}]}, \text{ where } k \text{ is the equilibrium constant, } [pep_{free}] + [pep_{bound}] = [pep]$$

$$\text{and } I = I_0 \frac{[pep_{free}]}{[pep]} + (I_0 + \Delta I) \frac{[pep_{bound}]}{[pep]}.$$

The equations are formally identical to a binding in the sense of the mass action law. The interpretation of $c_{lip}^{pep} = \frac{[pep_{bound}]}{[lip]}$ as the concentration of the peptide within the membrane allows to read the equations as a partition equilibrium.

Preparation of small unilamellar vesicles:

POPC (Larodan Solna, Sweden) and POPG (Avanti Polar Lipids, Alabaster, Alabama, USA) were co-dissolved in chloroform/methanol 2/1 v/v in a small test tube and the solvent was evaporated under a stream of nitrogen. The remaining traces of organic solvent were removed by exposure to high vacuum overnight. The appropriate amount of 10 mM Tris, pH 7 was added in order to obtain a 10 mM lipid suspension. The samples were first vortexed and then homogenized by four freeze-thaw cycles. Small unilamellar vesicles were produced by extrusion 21 times through a poly-carbonate film of 100 nm using a syringe extruder (Avanti Polar Lipids, Alabaster, Alabama, USA).

Preparation of supported lipid bilayers

A homogenous solution of peptides and lipids was prepared in chloroform/methanol 2/1. The solvent was slowly evaporated under a stream of nitrogen until a viscous solution was obtained (~ 200 μ L). This was applied to 22 ultrathin glass plates (8 x 12 mm, thickness 00, Marienfeld, Lauda-Königshofen, Germany), dried first in air, and finally in high vacuum overnight. The glass plates were equilibrated at 93% relative humidity at ambient temperature, stacked on top of each other and sealed in plastic wrapping (illustrated protocol in [41]).

Solid-state NMR spectroscopy

Membrane samples uniaxially oriented on glass plates were inserted into flat-coil NMR probes [42] with the sample normal being aligned parallel to the magnetic field direction of the NMR spectrometer.

Proton-decoupled ^{31}P solid-state NMR spectra were recorded at a Larmor frequency of 121.570 MHz using a Bruker Avance 300 solid-state NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) and a Hahn-echo pulse sequence [43]. The acquisition parameters were: 90° pulse of 5 μ s, echo and recycle delays of 10 μ s and 3 s, respectively, spectral width 46 kHz. Before Fourier transformation an exponential line broadening of 30 Hz was applied. 85% phosphoric acid was used as an external reference (0 ppm).

Proton-decoupled ^{15}N solid-state NMR spectra were recorded at 76.01 MHz on a Bruker Avance 750 solid-state NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) using cross polarization [44]. The following parameters were used: spectral width 100 kHz, acquisition time 3.9 ms, CP contact time 1 ms, recycle delay 3s, B_1 field 40 kHz, number of scans typically 40k. An exponential multiplication with line broadening of 300 Hz was applied before Fourier transformation. The spectra were calibrated relative to external ammonium chloride (40 ppm; [45]).

^2H solid-state NMR spectra were recorded on a Bruker Avance 750 solid-state NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) at 115.16 MHz using a quadrupolar echo pulse sequence [46]. The spectral window, acquisition time and recycle delay were 100 kHz, 10 ms and 0.5 s, respectively. 130k scans were accumulated. An exponential multiplication with line broadening of 300 Hz was applied before Fourier transformation. D_2O was used as an external reference.

RESULTS

The CHR and NHR domains of gp41 exhibit important functions during the HIV viral life cycle. They are thought to undergo considerable conformational transitions during the fusion of the viral and cellular membranes and are targets of viral inhibitors. Therefore, the NHR and CHR polypeptides representing functionally important domains of gp41 were prepared by solid-phase peptide synthesis and HPLC purification (Table 1). In order to investigate their membrane interactions ^{15}N and ^2H isotopic labels were included into the sequences for solid-state NMR structural investigations. Furthermore, fluorophores were attached to their amino terminus which opens their investigation to a wide variety of spectroscopic investigations [47].

In a first step CHR and NHR were investigated in solution (Figure 2). Figure 2A shows circular dichroism (CD) spectra of 50 μM CHR (green) 50 μM NHR (red) or of the mixture (black). The CD spectrum obtained from NHR shows characteristics of disordered/polyproline II conformations with a minimum ~ 202 nm whereas CHR exhibits a minimum around 215 nm. The mixture shows a broad minimum between 210 nm and 220 nm typical for helical conformations albeit at a lower intensity than expected for a typical α -helix covering the full sequence. Indeed, CD spectral line shape analysis [40] shows that in solution CHR exhibits a high degree of disordered and β -sheet/turn contributions (Fig. 2A). The calculated sum of the CHR and NHR spectra (dashed black line) does not correspond to the measured spectrum of the mixture indicating interactions between the peptides that result in structural changes.

CD spectral line shape analysis [40] also reveals 25-30% helical structure for both NHR and CHR (Fig. 2A). A small degree of helicity was also observed for somewhat extended polypeptide sequences [13, 32]. Thus, the propensity for helix formation is already present in aqueous solution and is enhanced when the domains associate in the context of larger polypeptide constructs [33, 48], in a crystal [12] and/or when interacting with micelles [28, 33, 35, 48].

Additional investigations in solution were performed with peptides carrying the NBD fluorophore at the N-terminus. This chromophore quenches when another one is close within 1 nm [37]. Therefore, it is possible to deduce spatial relationships by mixing labeled and unlabeled peptides. In order to avoid quenching interactions between tryptophan and NBD a glycine spacer was added to the CHR peptide (Table 1). In a first series of experiments the individual NBD-G-CHR peptides were mixed at

varying proportion with unlabeled CHR (Figure 2B, black line). The fluorescence signal shows a linear dependence with the amount of NBD label indicating the absence of multimers [37]. A related observation was made with NBD-NHR (data not shown).

However, a deviation from linearity occurs for both NBD-G-CHR and NBD-NHR when the complementary peptide in its unlabeled form is added (Figure 2B for NBD-G-CHR). This indicates that the labels are in close proximity and their fluorescence progressively quenched when the degree of labeling increases. The data can be fitted by a model where monomers and trimers coexist [12, 16, 17]. The fraction of NHR in the trimer state in the presence of increasing amounts of CHR is shown in Figure 2C, while the fraction of trimeric CHR in the presence of increasing amounts of NHR is represented in Figure 2D. In both experiments about half the peptides occur in the multimeric state once an equimolar amount of the binding partner has been added. The data shows clearly that in aqueous solution the peptides form hetero-oligomers, possibly hetero-hexamers [12, 16, 17]. The somewhat smoother curve of CHR (Figure 2D) could be due to the presence of additional dimers of CHR.

Figure 2: **A.** Normalized circular dichroism spectra of 50 μM CHR (green), 50 μM NHR (red) and 25 μM NHR + 25 μM CHR (black) the dashed black line is calculated from the individual spectra (half NHR + half CHR). **B.** Fluorescence intensity of 10 μM NBD-G-CHR as a function of dilution with unlabeled CHR and in the presence of increasing amounts of NHR. The solid lines are fitted with a model of a mixture of monomer and trimer. **C.** shows the fraction of 10 μM NHR in the trimeric state for increasing amounts of CHR. **D.** shows the fraction of 10 μM of CHR in the trimeric state for increasing amounts of NHR. All experiments were performed in 10 mM Tris/HCl, pH 7.

Because the NBD fluorescence is sensitive to the environment it can be used to quantitatively measure the adsorption of the peptide to the lipid membrane [13]. Figure 3 shows titration curves of 20% labeled NBD-NHR/80% NHR (A,B) and 20% NBD-G-CHR/80% CHR (C,D) with neutral POPC vesicles (B,D) or with anionic vesicles containing 25% negative surface charge (A,C). On the one hand NBD-NHR binds well to negatively charged vesicles (Figure 3A) with a partitioning constant of $(2.8 \pm 0.7) 10^4 \text{ M}^{-1}$ whereas binding to neutral vesicles cannot be detected within the concentration range of the experiment (Figure 3B). On the other hand, NBD-G-CHR binds only weakly to both charged (Figure 3C) and neutral vesicles (Figure 3D). The slope of the binding isotherm seems to be somewhat steeper for neutral POPC vesicles, but since the saturation is not reached the data cannot be analyzed in a quantitative fashion.

Interestingly, the presence of CHR not only quenches the signal of NBD-NHR in solution but also in the POPC/POPG membrane (compare the black and red traces of Figure 3A). For NBD-G-CHR the quenching effect of NHR is most pronounced in the presence of neutral vesicles. (Figure 3D).

When charged vesicles are added (Figure 3C) NHR initially increases the membrane affinity of NBD-G-CHR before the fluorescence follows the curve in the absence of NHR. Indeed, the initial shape resembles the binding isotherm in Figure 3A suggesting a high affinity of heterooligomers for charged vesicles followed by the weak binding of isolated CHR also shown in Figure 3C.

For neutral vesicles the binding of NBD-G-CHR in mixture with NHR is weaker than association of free NBD-G-CHR (Figure 3D).

Figure 3: NBD fluorescence at 540 nm of 20% labeled NHR (A,B) and of 20% labeled CHR (C,D) upon titration with POPC (B,D) or with POPC/POPG 3/1 mole/mole vesicles (A,C) in the absence (black) or in the presence (red) of the other unlabeled peptide.

In the crystal structure of the CHR-NHR six helix-bundle the tryptophanes are located in the interface between both peptides [12]. This can make tryptophan fluorescence a test if hetero-dimers also exist within lipid membranes. Figure 4A shows tryptophan fluorescence spectra of NHR in the presence of increasing amounts of POPC/POPG 3/1 mole/mole. Upon lipid addition a clear blue shift from 348 nm to 333 nm and an increase in signal intensity upon addition of vesicles becomes visible. In contrast, the tryptophan fluorescence of CHR is only slightly quenched during the titration (Figure 4B). Repeating the titration experiment with the mixture of both peptides results in a first quenching of the fluorescence signal before it slowly increases at higher lipid concentrations (Figure 4C where the green and blue curves represent intermediate lipid concentrations). The maximum at 340 nm does not shift during the titration. When the spectrum of the mixtures (Fig. 4C solid lines) are compared to spectra calculated from measurements of isolated NHR and CHR (Figure

4C dashed lines) the real spectra do not show the noticeable shift observed in the simulation and a more pronounced increase in fluorescence signal than expected.

Figure 4: Tryptophan fluorescence emission spectra of 10 μM NHR (A), 10 μM CHR (B) and 5 μM CHR + 5 μM NHR (C) in the presence of increasing amounts of POPC/POPG 3/1 mole/mole. The dashed lines in C corresponds to the calculated spectrum (i.e. the sum of A and B divided by 2).

In a next step the CHR and NHR domains carrying isotopic labels at specific sites were reconstituted into supported POPC membranes, inserted into the solid-state NMR spectrometer with the sample normal parallel to the magnetic field direction and ^{15}N and ^2H solid-state NMR spectra were recorded. The resulting ^{15}N chemical shift of the peptide amides correlates with the helical tilt angle and/or the alignment of the ^{15}N - ^1H vector [49]. Whereas ^{15}N - ^1H vectors that are oriented parallel to the membrane normal (i.e. transmembrane helices) exhibit ^{15}N chemical shifts around 200 ppm those that are aligned parallel to the bilayer surface resonate at < 100 ppm. Finally, unstructured domains either aggregate and show broad powder pattern line shapes (50 to 230 ppm) or they realign fast and exhibit isotropic peak positions (ca 120 ppm) [50].

In a first series of experiments the [^{15}N -Leu18]-CHR and [^{15}N -Leu11, $^2\text{H}_3$ -Ala13]-NHR domains were reconstituted into oriented phospholipid bilayers, inserted into the NMR coil with the sample normal parallel to the magnetic field and investigated by ^{15}N solid-state NMR spectroscopy (Figure 5). The ^{15}N chemical shifts of NHR and CHR in POPC membrane are 81 ppm (LWHH ± 17 ppm) and 81 ± 50 ppm, respectively (Fig. 5A,B). These ^{15}N chemical shift values are indicative that the helices of both peptides [12, 13, 16, 51] are oriented parallel to the membrane surface in agreement with their amphipathic character [49]. The CHR spectra are broader and show additional powder pattern contributions (spectral components reaching into the 200 ppm region) suggesting less stable membrane association of this negatively charged peptide (net -3). When both peptides are added at equimolar ratio a sharper resonance at 77 ± 32 ppm is observed (Fig. 5C).

In POPC/POPG 3/1 mole/mole membranes the peak maxima are 80 ± 44 ppm and 90 ± 50 ppm for NHR and CHR, respectively (Fig. 5D,E). In the presence of both peptides the peaks add up to a line shape around 85 ± 45 ppm (Fig. 5F). Also in the presence of POPG the in-planar alignment dominates although contributions from other peptide orientations become more abundant. The ^2H solid-state NMR spectrum of [^{15}N -Leu11, $^2\text{H}_3$ -Ala13]-NHR shows a rather broad spectral distributions with discontinuities corresponding to quadrupolar splittings of 58 kHz, 41 kHz and 30 kHz (Fig. 6A).

Figure 5: Proton-decoupled ^{15}N solid-state NMR spectra of oriented samples of 1 mole% [^{15}N -Leu11]-NHR (A, D) 1 mole% [^{15}N -Leu18]-CHR (B,E) and 0.5 mole% [^{15}N -Leu11]-NHR + 0.5 mole% [^{15}N -Leu18]-CHR (C,F) in uniaxially oriented POPC (A-C) or POPC/POPG 3/1(mole/mole) membranes (D-F). The membrane normal is parallel to the magnetic field direction. The sum of the spectra from individual peptides is shown in red in panels C and F.

When the ^{31}P solid-state NMR spectra of the same uniaxially oriented samples shown in Figure 5 are recorded the 30 ppm contributions dominate indicating a large proportion of phospholipids oriented with the long axis parallel to the sample normal (Fig. 6B,C). However significant contributions extending up to -15 ppm are also observed indicative of different lipid alignments and/or conformational heterogeneity at the level of the phospholipid head group [52]. This heterogeneity is particularly pronounced for NHR and CHR in POPC but alleviated in the presence of POPG or when both peptides are mixed (Fig. 6B,C)

Figure 6: **A.** ^2H solid-state NMR spectrum of $[\text{}^{15}\text{N}\text{-Leu11}, \text{}^2\text{H}_3\text{-Ala13}\text{]-NHR}$ reconstituted at 1 mole% into uniaxially aligned POPC lipid bilayers. The membrane normal was parallel to the magnetic field of the NMR spectrometer and the experiments were performed at room temperature. **B. and C.** Representative ^{31}P spectra of uniaxially oriented samples of POPC in the presence of 1 mole % CHR (B) or 0.5 mole % NHR + 0.5 mole% CHR (C). The membrane normal is parallel to the magnetic field direction.

DISCUSSION

The *Env* protein complex of HIV is of utmost biomedical importance because it mediates the contact of the virus with the target cell and assures the fusion of the viral and cellular membranes. Therefore, gp41 or gp120, the building blocks of *Env*, are prime targets to interfere with the infections cycle of the virus [10, 53, 54].

Furthermore, a number of broadly neutralizing antibodies have been identified that target conserved regions of the protein complex [55-57]. Despite intensive research and impressive progress in the investigation of the *Env* protein a great number of questions remain [10, 18, 53]. This is due not only to the complex nature of *Env*, its limited stability and the fact that gp41 is a type-I membrane protein but also due to its highly dynamic nature which results in multiple conformational intermediates during membrane fusion. In particular the role, structures and membrane interactions of gp41 during viral fusion remain a subject of intense research.

In this context a number of NMR investigations of gp41 domains have been conducted in membrane environments [58, 59] including the structures of the ectodomain when interacting with membranes [28, 33]. Based on x-ray crystallographic data which are thought to represent the post-fusion conformation the NHR-CHR sequence has been suggested to form a six-helix bundle. This bundle is made up from helices of the heptad repeats which fold back onto each other in an antiparallel fashion. A trimer of those then assembles into a six-helix bundle [12, 16, 17]. In contrast, very different structural arrangements have been revealed in more recent structures of *Env* complexes representing the native spike as well as structural changes due to interactions with receptors and/or antibodies [5, 18, 19]. Therefore, the questions remain what structural transitions occur during the infectious cycle and how the NHR and CHR domains contribute to membrane fusion. Notably, NMR structures of extended parts of gp41 in DPC micelles reveal helical conformations for NHR and CHR [28, 33, 35].

Here, fluorescence quenching experiments were designed to investigate the aggregation state of NHR and CHR as well as the mixture in different environments (Fig. 2). In aqueous solution at pH 7 neither NHR nor CHR alone show indications for pronounced oligomerization whereas when mixed together about 50% of the peptide is in an oligomeric state (Fig. 2C,D). The *Env* protein is indeed a trimeric structure and fragments encompassing parts of the ectodomain have been shown to

associate as trimers [5, 18]. The transmembrane domain has also been postulated to drive trimerization [60] a finding that may need further evaluation [58].

Upon addition of liposomes to the preformed hetero-oligomers several observations suggest that in the studies presented in this paper interactions between NHR and CHR may persist. Thus, the mutual quenching of NBD fluorescence by the other peptide persists even when the peptides associate with membranes (Fig. 3). Furthermore, the tryptophan fluorescence of the mixture (Fig. 4C) is not merely the addition of the spectra from the individual peptides (Fig. 4A,B). Finally, the strong disordering effect on the lipid bilayers of the individual peptides is attenuated when NHR and CHR are added in combination (Fig. 6B,C), whereas the ^{15}N spectra of the same samples appear additive within experimental error (Fig. 5).

This seems in contradiction to observations made by Yechiel Shai and co-workers, where a dissociation of complexes made of N36 and C34 (sequences shown in Table 1) has been observed in the presence of PC/PS 1/1 vesicles [13, 14]. However, it should be kept in mind that also in our experiments oligomerization is incomplete and governed by an equilibrium (Fig. 2C,D) [28]. Thus, the degree of oligomerization can be tuned by the peptide concentration (which is an order of magnitude higher here than in the earlier experiments), the detailed peptide sequence, the salt concentration (which is lower in the experiments presented here), pH and the membrane lipid composition, in particular the amount of negative charges has been found important [13, 14].

In previous NMR experiments paramagnetic relaxation enhancement and studies of internal dynamics of gp41 starting with NHR and ending with the TM domain show that the membrane anchor and some of the NHR interact with the membranes, whereas the CHR remains solvent exposed [28]. Whereas in the presence of > 135 mM DPC the TM region is the least dynamic element the fusion peptide is the most mobile structured region in this investigation, a finding being incompatible with a six-helix bundle arrangement [28, 35]. The authors of this study therefore assign the structure to the conformation of a pre-fusion intermediate in which only the TM domain and the FP but not NHR or CHR interact with opposing membranes [28] (Figure 7A).

This contrasts another study from which a more active role in membrane fusion of the heptad repeat sequences was postulated [33]. In this work a construct involving NHR and CHR connected by a shortened loop was studied in 100 mM DPC

revealing clear indications of strong interactions of both NHR and CHR with detergents and with liposomes mimicking the composition of the T-cell membrane. In the presence of detergent or phospholipid vesicles the helices dissociate from each other thus a monomer made of two independent helical domains is formed [13, 14, 33]. In this manner the amphipathic helices of NHR and CHR insert into the cellular and viral membranes (Figure 7B) which are destabilized thereby lowering the energetic barrier for fusion [13, 14, 31] an effect that is particularly enhanced by the kinked helical structure of NHR due to Q562 [33].

Figure 7: Models of gp41 pre-fusion intermediate states that have been suggested by **A.** Lakomek et al. [28] and **B.** Roche et al. [33]. The CHR domain is shown in yellow, the NHR domain in green. The net negative and positive charges of the domains are also indicated and as well as the potential electrostatic and direct attractive interactions of these membrane-associated domains are sketched by the double-arrow in B. Notably, the fusion peptide has been shown to exhibit a high degree of plasticity and to adopt α -helical or antiparallel β -sheet conformations in a lipid dependent manner [61].

Notably the data obtained here support such a more active role in the membrane fusion events. First of all, peptides representing the NHR and CHR domains were found to strongly associate with a variety of membranes (Fig. 3A) where partitioning constants of similar orders of magnitude have been observed for related polypeptides to PC/PS bilayers or vesicles containing cholesterol [13, 14]. Notably, the negative surface charge much promotes the association of the slightly cationic NHR (net +1) to

PC/PG membranes (Fig. 3A) but not of the anionic CHR sequence (net -3), which associates only weakly with both PC and PC/PG membranes (Fig. 3C,D). Stronger membrane interactions of NHR when compared to CHR have also been observed when PC/PS 1/1 mole/mole membranes were studied [13].

The mutual influence of the CHR and NHR domains when their membrane interactions are studied (Fig. 3) may thus derive from the changes of the bilayer surface charge density upon membrane partitioning [62] concomitant with alterations of the membrane physico-chemical properties while direct interactions between the peptides in membranes cannot be excluded (Figure 7B).

Furthermore, the ^{15}N chemical shifts obtained from oriented solid-state NMR spectra are all < 100 ppm, clearly indicating that the NHR and CHR domains both interact with and align parallel to the membrane surface (Fig. 5). The same samples exhibit pronounced disordering effects of the phospholipid head groups when investigated by ^{31}P NMR spectroscopy, even at the relatively small peptide-to-lipid ratios of 1/100 (Fig. 6B,C). This observation is also in line with prior conclusion where chemical modifications of related sequences were investigated [63].

Notably, using oriented and MAS solid state NMR spectroscopy a strong disordering effect at the level of the phospholipid headgroup has also been observed for the fusion peptide [64, 65]. The fusion peptide shifts in a lipid-dependent manner between α -helical and β -sheet conformations where the latter population exhibits a wide distribution of N-H alignments relative to the membrane surface [61].

During infection the gp41 protein undergoes through several intermediates [66]. It is therefore possible that during the viral life cycle the gp41 NHR and CHR domains encounter many different environments where NHR forms a trimeric core without tight CHR interactions [5, 18], where the hexarepeat sequences dissociate completely [13, 14, 33], or where a six-helix bundle is formed of both sequences [12, 16, 17]. In most of the current models the N-terminal region of gp41 interacts with the cellular membrane whereas the C-terminal domains are anchored in the viral membrane. Thereby NHR makes the surface charge of the cellular membrane more positive and CHR the one of the cellular membrane negative (Figure 7B).

Furthermore, both peptides have been shown to have a strong disordering effect on the lipid packing of the membrane, an effect that can be modulated by interactions between the two peptides. Such changes of the physico-chemical properties of the

membranes as well as direct interactions between these domains potentially promote the membrane fusion process.

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