Binding of the C-terminal domain of the HIV-1 capsid protein to lipid membranes: a biophysical characterization

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The capsid protein, CA, of HIV-1 forms a capsid that surrounds the viral genome. However, recent studies have shown that an important proportion of the CA molecule does not form part of this capsid, and its location and function are still unknown. In the present work we show, by using fluorescence, differential scanning calorimetry and Fourier-transform infrared spectroscopy, that the C-terminal region of CA, CA-C, is able to bind lipid vesicles *in vitro* in a peripheral fashion. CA-C had a greater affinity for negatively charged lipids (phosphatidic acid and phosphatidylserine) than for zwitterionic lipids [PC/Cho/SM (equimolar mixture of phosphatidylcholine, cholesterol and sphingomyelin) and phosphatidylcholine]. The interaction of CA-C with

lipid membranes was supported by theoretical studies, which predicted that different regions, occurring close in the three-dimensional CA-C structure, were responsible for the binding. These results show the flexibility of CA-C to undergo conformational rearrangements in the presence of different binding partners. We hypothesize that the CA molecules that do not form part of the mature capsid might be involved in lipid-binding interactions in the inner leaflet of the virion envelope.

Key words: capsid protein, differential scanning calorimetry (DSC), Fourier-transform infrared (FTIR) spectroscopy, HIV-1, lipid binding.

INTRODUCTION

Several thousand copies of retroviral precursor protein Gag self-associate on the plasma membrane of the infected cell and bud to form an immature virion surrounded by a lipid envelope. Subsequent to budding, Gag is proteolytically cleaved by the viral protease into the mature proteins: the MA (matrix), CA (capsid), NC (nucleocapsid) and p6, as well as into the spacer peptides p2 and p1 [1–3]. After maturation, CA proteins assemble into the conical core particle [4]. Interestingly, recent studies have shown that not all the molecules of CA are involved in capsid formation, and their location and function are still unknown [5,6]. On the other hand, the lipid composition of the particle envelope in several retroviruses is different to that of the plasma membrane of the host cells [7–9]. This suggests that viruses might bud from specific plasma membrane microdomains where some lipids are more abundant than others [8–11]. Furthermore, it is not well established whether after proteolysis of the Gag protein, the MA protein, which mediates membrane binding and insertion of envelope glycoproteins into the virions [1,3], is the unique protein bound to membranes or there are other lipid-binding proteins. Thus a complete understanding of the lipid-binding processes requires a quantitative study of the several Gag proteolytically produced proteins and their domains in the presence of lipids.

The CA protein of HIV-1 is formed by two independently folded domains separated by a flexible linker [12–15]. The N-terminal domain (residues 1–146 of the intact protein), CA-N, is composed of five coiled-coil α -helices, with two additional short α -helices following an extended proline-rich loop [12–14]. The C-terminal domain (residues 147–231), CA-C, occurs as a dimer both in solution and in the crystallized form [15,16]. Each CA-C monomer is composed of a short 3_{10} -helix followed by an

extended strand and four α -helices connected by short loops. The dimerization interface is largely formed by the mutual docking of α -helix 2 from each monomer (residues Ser¹⁷⁸–Val¹⁹¹). The CA-C domain also has a region of 20 amino acids at the beginning of the polypeptide chain called the MHR (major homology region), which is highly conserved among retroviruses.

In the present study, we focus on several questions relating to the possible interaction between CA-C and lipids. (i) Is CA-C specifically targeted to lipids? (ii) If so, is there any preference for the charge of the lipid? And, finally, (iii) does the lipid charge alter the secondary structure of CA-C? Our results showed that CA-C was able to bind to lipids, and the lipid affinity decreased in the order: DMPA (dimyristoyl phosphatidic acid), DMPS (dimyristoyl phosphatidylserine) and DMPC (dimyristoyl phosphatidylcholine). The secondary structure of CA-C changed upon binding to the lipids, as shown by FTIR (Fourier-transform infrared) measurements, but lipid binding did not modify the dimerization state of the protein, as concluded from thermal FTIR experiments. Theoretical predictions suggested that the lipid–CA-C interface contained Tyr¹⁶⁴, Tyr¹⁶⁹ and Trp¹⁸⁴, in agreement with the well-known tendency of such aromatic residues to be involved in the membrane–water interface of membrane proteins. These results highlight the flexibility of CA-C to undergo conformational rearrangements in the presence of different binding partners.

EXPERIMENTAL

Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.), except Cho (cholesterol) which was purchased from Sigma. Dialysis tubing was from Spectrapore, with a

Abbreviations used: CA, capsid (protein); CA-C, C-terminal domain of CA; CA-N, N-terminal domain of CA; Cho, cholesterol; DMPA, dimyristoyl phosphatidic acid; DMPC, dimyristoyl phosphatidylcholine; DMPS, dimyristoyl phosphatidylserine; DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; ΔH , enthalpy of the calorimetric transition; MA, matrix (protein); MHR, major homology region; MLV, multilamellar vesicle; PA, phosphatidic acid; PC, phosphatidylcholine; PC/Cho/SM, equimolar mixture of PC, Cho and SM; PS, phosphatidylserine; SM, sphingomyelin; SUV, small unilamellar vesicle; T_c , temperature at the peak maximum of the calorimetric transition.

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molecular-mass cut-off of 3500 Da. Standard suppliers were used for all other chemicals. Water was de-ionized and purified using a Millipore system.

Protein expression and purification

The wild-type CA-C protein (residues 147–231 of CA) was expressed in *Escherichia coli* BL21(DE3) and purified as described previously [17]. Protein stocks were run on SDS/PAGE gels and found to be >97% pure. Purity was also confirmed by MS analysis. Samples were dialysed thoroughly to remove any metal ions associated with the protein. Protein concentrations were calculated from the absorbance measured at 280 nm, by using the molar absorption coefficients of amino acids [18].

Fluorescence experiments

All the experiments were carried out at 25 °C, using 2 µM protein, in 10 mM Hepes/100 mM KCl (pH 7). Fluorescence spectra were obtained by using an excitation wavelength of 280 nm, and a cross-oriented configuration of the polarizers ($Ex_{pol} = 90^\circ$ and $Em_{pol} = 0^\circ$), which provides maximal suppression of the scattering artefacts [19]. Spectra were also recorded by excitation at 295 nm to check for the possible influence of tyrosine residues; no differences were observed in the binding curves when compared with those acquired by excitation at 280 nm, except for a lower signal-to-noise ratio (results not shown). Spectra were collected in an Aminco Bowman SLM 8000 spectrofluorometer (Spectronics Instruments, Urbana, IL, U.S.A.) interfaced to a Haake water bath. SUVs (small unilamellar vesicles) were used in the fluorescence experiments as they introduced smaller scattering contributions [20]; therefore, they were more appropriate to detect CA-C lipid binding and to compare the relative affinities for the different lipids. SUVs were prepared by sonication [21], mixed with the corresponding volumes of protein, buffer solution and water, and equilibrated for 60 min to give the final lipid/protein samples. No differences were observed with control samples equilibrated overnight, indicating that the equilibrium had been reached. The average fluorescence emission intensity, $\langle\lambda\rangle$, was calculated from [22]:

$$\langle\lambda\rangle = \frac{\sum_1^n \frac{1}{\lambda_i} I_i}{\sum_1^n I_i} \quad (1)$$

where I_i is the fluorescence intensity measured at a wavelength λ_i . The Nernst partition coefficient (K_p) [23] is described by:

$$K_p = \frac{n_l/V_l}{n_w/V_w} \quad (2)$$

where n_l and n_w are the number of moles of the protein bound to the lipid and in aqueous solution respectively; and V_l and V_w are the volumes of the lipid and aqueous phase respectively. The K_p can be determined from any spectroscopic parameter that changes with the concentration of protein bound to a membrane [24], according to:

$$Y = \frac{Y_{\max} K_p \gamma [\text{lipid}]}{1 + K_p \gamma [\text{lipid}]} \quad (3)$$

where Y is the fluorescence parameter that changes upon addition of increasing amounts of lipid, $Y_{\max}$ is the maximum value of Y , and γ is the molar volume of the lipid {0.6 M⁻¹ for chicken egg PA (phosphatidic acid), 0.75 M⁻¹ for bovine brain PS (phosphoserine), 0.8 M⁻¹ for chicken egg PC (phosphocholine), and

0.65 M⁻¹ for PC/Cho/SM [an equimolar mixture of PC, Cho and SM (sphingomyelin)] [25]}. In the K_p calculation, only 60% of the lipid, which is the amount assumed to be in the outer hemilayer of the SUVs, was taken into account [26]. The molar fraction of protein remaining in the aqueous solution, X_w , can be calculated by [27]:

$$X_w = \frac{1}{1 + K_p \gamma [\text{lipid}]} \quad (4)$$

Fittings of the data by non-linear least-squares analysis to eqns (3) and (4) were carried out by using the general curve fit option of Kaleidagraph (Abelbeck Software) on a PC.

DSC (Differential scanning calorimetry)

Purified protein was dialysed against 2 litres of buffer containing 5 mM Hepes/10 mM KCl (pH 7). The MLVs (multilamellar vesicles) used in the experiments were prepared by resuspension of the dried lipids in buffer, heating at 10 °C above the respective lipid transitions, and vortexing. Different amounts of protein were incubated with a fixed quantity of lipids for 1 h at room temperature, to give a final lipid concentration of 0.5 mM. Samples were degassed under vacuum for 10–15 min, with gentle stirring, prior to being loaded into the calorimetric cell. DSC experiments were performed in a VP-DSC differential scanning calorimeter (MicroCal) under a constant external pressure of 207 kPa (30 p.s.i.) in order to avoid bubble formation, and samples were heated at a constant scan rate of 60 °C/h. Experimental data were corrected from small mismatches between the two cells by subtracting a buffer baseline prior to data analysis. The excess heat capacity functions were then analysed by using the software package Origin 7.0 (Microcal Software). The error in the determination of the T_c (temperature at the peak maximum of the calorimetric transition) was in all cases $\pm 0.2^\circ\text{C}$, and the error in the determination of the ΔH (enthalpy of the calorimetric transition) was ± 0.5 kJ/mol.

FTIR experiments

Samples of CA-C and the different lipids were dried in a Speed Vac concentrator (Savant, Farmingdale, NY, U.S.A.) and dissolved in the deuterated working buffer. The CA-C samples were resuspended in ²H₂O and incubated at 4 °C overnight to maximize the H–²H exchange of the protein. These samples were dried again and resuspended in a final volume of 25 µl of deuterated buffer. The total absence of amide II band in Figure 4 (see below) indicates that the isotopic exchange was complete. In the FTIR experiments in the presence of lipids, the deuterated CA-C sample was used to resuspend the previously dried lipids. The resulting samples were placed amid a pair of CaF₂ windows separated by a 50 µm thick spacer in a Harrick demountable cell (Ossining, NY, U.S.A.). The final concentrations were: CA-C at 1 mM (where the protein was a dimer [15–17]), and DMPC, DMPs and DMPA at 35 mM. Spectra were acquired on a Bruker FTIR-66S instrument equipped with a deuterated triglycine sulphate detector and fitted with a Thermo Haake water bath. The cell container was continuously filled with dry air. Usually, 500 scans/sample were taken, averaged, apodized with a Happ–Genzel function, and Fourier transformed to give a final resolution of 2 cm⁻¹. The contributions of the buffer spectra were subtracted, and the resulting spectra were used for analysis as described previously [28]. Experiments either in the absence or in the presence of CA-C were repeated three times with fresh new samples to test the reproducibility of the measurements. In all cases, the differences among the three experiments were lower than 5%. The error

Figure 1 Changes in the CA-C fluorescence in the presence of different lipids

(A) CA-C emission spectra in aqueous solution (thick continuous line) and in the presence of PS (thin continuous line), PA (broken line), PC (dashed dotted line) or PC/Cho/SM in a 1:1:1 ratio (dotted line). Lipid concentrations were 1 mM. (B) Changes in the $\langle \lambda \rangle$ (left-hand axis: ●, ○) and fluorescence intensity (F.I.) at 340 nm (right-hand axis: ▲, △) at increasing concentrations of the different lipids: PS (○), PA (●), PC (▲) and PC/Cho/SM (△). The lines are fit to eqn (3). The reasons for the poor fittings of PA and PS data are unknown, but they could be related to processes of co-operative partitioning, which is usual for the lipid binding of oligomeric species [20]. All the experiments were carried out at 25 °C in 10 mM Hepes/100 mM KCl (pH 7). CA-C concentration was 2 μ M. Fluorescence intensity is in arbitrary units.

in estimation of the percentage of secondary structure depends mainly on the removal of spectral noise, and it was estimated to be 2% [29]. To be conservative, we have used an estimated error of 4% in our band decomposition.

In the thermal unfolding experiments, the scanning rate was 50 °C/h, the spectra were acquired every 2.5 °C and 50 scans/sample were recorded. The errors in the determination of the thermal midpoints of the second transition of CA-C to the two-state equation [30] were ± 2 °C; this value is high due to the short native and unfolded baselines (see Figure 5, below). Every thermal scan was repeated three times with fresh new samples; the differences in the fitting errors of the calculated thermal midpoints were always lower than 0.5 °C.

RESULTS

Fluorescence experiments

Fluorescence is highly sensitive to changes in the environment of the protein aromatic residues, and thus it has been widely used to monitor the interaction of proteins with lipid membranes [19]. The emission spectrum of CA-C (at 2 μ M, where the monomer is the main species [15–17]) in solution showed a maximum at 342 nm (Figure 1A), which indicates that the sole tryptophan residue, Trp¹⁸⁴, was partially solvent exposed [15,31]. We observed that in the presence of the different lipid vesicles, the emission spectrum of CA-C underwent spectral changes. A decrease in the fluorescence intensity and a spectral red-shift (maximum at 345 nm) were observed for either the zwitterionic lipid PC and for a lipid mixture that mimics the biologically relevant rafts, i.e. ordered lipid domains highly enriched in sphingolipids and cholesterol (PC/Cho/SM, 1:1:1 molar stoichiometry). However, after incubation with the negatively charged lipids, PS and PA, a different behaviour was observed. In PS liposomes, the emission spectrum of CA-C showed a marked increase in the fluorescence intensity and a blue-shift (maximum at 330 nm); whereas in the presence of PA liposomes, a large blue-shift (maximum at 321 nm) and a decrease in the fluorescence intensity were observed.

The partition coefficients (K_p values), which quantify the interaction of CA-C with the different lipids, were determined. To maximize the signal-to-noise ratio, the fluorescence parameter that experienced greater variations upon lipid binding (i.e. $\langle \lambda \rangle$) for

Table 1 Partition coefficients (K_p) of lipids for CA-C

n.d., not determined.

Lipid	K_p	
	100 mM KCl	1 M KCl
PA	$15\,352 \pm 1660^*$	$2830 \pm 160^\dagger$
PS	$6629 \pm 845^*$	$10\,300 \pm 1800^\dagger$
PC	$1776 \pm 92^\dagger$	n.d.
PC/Cho/SM	$3442 \pm 185^\dagger$	n.d.

* Calculated from the changes in $\langle \lambda \rangle$. Similar values of the K_p were obtained by following the changes in the fluorescence intensity at 340 nm.

† Calculated from the changes in the fluorescence intensity at 340 nm. Similar K_p values were obtained by following the changes in the $\langle \lambda \rangle$ and also by measuring at 360 nm, where a hypothetical scattering contribution would be greatly reduced.

PA and PS, and fluorescence emission intensity at 340 nm for PC and PC/Cho/SM) was used to determine the K_p values (Figure 1B). The K_p values obtained indicated that CA-C had a higher affinity for negatively charged lipids (PA and PS) rather than for non-charged lipids (PC/Cho/SM and PC) (Table 1). The K_p values for PA and PS were also determined at high ionic strength (1 M KCl) to elucidate the importance of the electrostatic contributions to the binding. Our results suggest that: (i) in PA, the binding was fundamentally governed by electrostatic interactions, as indicated by the decrease in K_p at 1 M KCl; and, (ii) the binding of CA-C to PS was not reduced at high ionic strength; the K_p appeared to be higher at 1 M KCl, although the large fitting error precluded a reliable conclusion on the variation of the partition coefficient. These findings suggest that, although PA and PS are both anionic lipids, the electrostatic contribution to the binding of CA-C is different.

DSC experiments

The effect of CA-C on the phase transition of the lipids was studied by using DSC, which gives information about the type of interaction and the degree of penetration of a protein into a lipid bilayer [32]. For this purpose, the dimyristoylated forms of PC, PA and PS (DMPC, DMPA and DMPS respectively), which exhibit co-operative phase transitions at mild temperatures, were

Figure 2 DSC of the lipids in the presence of CA-C

Thermograms of samples containing DMPC, DMPA and DMPS at increasing (from top to bottom) concentrations of CA-C, expressed as the lipid-to-protein molar ratio. (A) DMPC in buffer, and in the presence of 250:1 and 50:1 CA-C. (B) DMPA in buffer, and in the presence of 250:1 and 10:1 CA-C. (C) DMPS in buffer, and in the presence of 250:1, 50:1 and 10:1 CA-C. Lipid concentration was 0.5 mM in all cases. The buffer used was 10 mM Hepes/100 mM KCl (pH 7). 1 cal = 4.18 J.

used. DSC experiments were not carried out on the lipid mixture mimicking the rafts, since, as it is a complex lipid mixture, no simple co-operative phase transition could be observed [33]. The DSC scans of the pure lipids showed a sharp peak corresponding to their main phase transition, accompanied, in the case of DMPC, by a minor peak at lower temperatures, the so-called pretransition (Figure 2). The values for ΔH , estimated from the area under the peaks (26.7, 23.4 and 29.6 kJ/mol for DMPC, DMPA and DMPS respectively), and T_c (24, 52.1 and 37.3 °C for DMPC, DMPA and DMPS respectively) were similar to those previously reported for the pure lipids [34,35].

The presence of CA-C induced changes in the phase transitions of the lipids (Figure 2). In DMPC, a decrease in the co-operativity and in the ΔH of the main lipid phase transition were detected (23.8 versus 26.7 kJ/mol), whereas the T_c value was not altered. Conversely, large changes were observed in the negatively charged lipids. For DMPA, CA-C induced a broadening of the peak, a moderate increase in the ΔH (24.6 versus 23.4 kJ/mol) and a small raise of the T_c value (52.7 versus 52.1 °C). A more complex behaviour was detected in DMPS. At a lipid-to-protein ratio of 250:1, a 'shoulder', that broadened the main transition and that appeared at higher temperatures, was observed; at lower ratios (that is, at higher CA-C concentrations), a decrease in the co-operativity and an increase in the T_c of the main peak (38 versus 37.3 °C), concomitantly with an increase in the values of the cooperativity and the T_c of the shoulder, were observed. These findings suggest the presence of at least two different DMPS populations in the presence of CA-C.

FTIR experiments

The interaction of CA-C with liposomes of different composition was further characterized by the study of the absorption of the lipids and the protein in the infrared region. The analysis of the protein-lipid interaction by FTIR (either the lipid carbonyl band or the CA-C amide I' band) was carried out at 25 °C. This value is close to the phase-transition temperature of pure DMPC (see above); however, the same results described here were obtained at temperatures lower than 25 °C (results not shown).

Changes in the lipid signals upon CA-C binding

In Figure 3, the carbonyl bands of DMPA, DMPC and DMPS in the absence (continuous line) and in the presence (broken line) of CA-C are shown. In DMPA, the presence of CA-C led to a

Figure 3 FTIR spectra of the lipid carbonyl region

Spectra of (A) DMPA, (B) DPMC and (C) DMPS were recorded in the absence (continuous line) and in the presence (dotted line) of CA-C. Lipid concentration was 35 mM. CA-C concentration was 1 mM. Normalized spectra at the largest height are shown. The buffer was 10 mM Hepes/100 mM KCl (pH 7). Experiments were carried out at 25 °C.

loss of absorbance at 1725 cm⁻¹ when compared with the peak maximum, centred at 1740 cm⁻¹. These two frequencies correspond to the absorption maxima of carbonyl moieties of the lipid that are either hydrogen bonded or non-hydrogen bonded with the solvent respectively. These results indicate that the presence of CA-C reduced the degree of hydrogen bonding of the DMPA with the solvent. In DMPS and DMPC, CA-C binding also caused a reduction in the level of hydrogen bonding of the carbonyl moieties, although the magnitude of the changes was smaller.

The influence of CA-C on the phase transition of the different lipids was also studied by monitoring the temperature-induced changes in the 2850 and 2920 cm⁻¹ bands, which correspond to the symmetric and asymmetric stretching of the methylene groups respectively. The T_c values determined were similar to those obtained by DSC (see above).

Figure 4 FTIR amide I' and amide II bands

(A) Amide I' and amide II bands of CA-C (A), of CA-C in the presence of DMPC (B), and of CA-C in the presence of DMPA (C). The amide II band, which typically appears as a wide band in the 1570–1510 cm^{-1} range [56], is no longer present, indicating that the isotopic exchange of the protein under any conditions has been completed. The band with a maximum at 1581 cm^{-1} and a small shoulder at approx. 1570 cm^{-1} can be assigned to the side chains of aspartate, glutamate and arginine residues, and the band appearing at 1515 cm^{-1} to the protonated form of the phenolic group of the tyrosine residue [57]. The reconstruction of the amide I' band from the observed spectral components is shown, but it typically overlaps and cannot be distinguished from the recorded amide I' band. The component bands are assigned to: turns/loops/ 3_{10} -helix (dashed lines), either that at 1675 cm^{-1} (appearing only in DMPC) and those at 1662 cm^{-1} ; α -helix (dotted lines), those appearing at 1648 and 1638 cm^{-1} ; extended chains (dotted-dashed lines) appearing at 1625 cm^{-1} . The band centred at 1613 cm^{-1} , which has not been indicated in Table 2, has been assigned to amino acid side chains [51] and it is also shown as a thin line. All spectra have been normalized to the unity, to allow comparison among the different peaks and deconvoluted bands. Lipid concentration was 35 mM. CA-C concentration was 1 mM. The buffer used was 10 mM HEPES/100 mM KCl (pH 7). Experiments were carried out at 25 °C.

Changes in the structure of CA-C upon lipid binding

The CA-C secondary structure in aqueous solution was estimated by analysing the absorption in the region from 1600 to 1700 cm^{-1} , the so-called amide I' band (Figure 4 and Table 2). The calculated total percentage of α -helix (that is, the sum of the 1638 and the 1648 cm^{-1} bands) for CA-C in aqueous solution (51.4 %), agrees well with that obtained from the crystal structure (54.6 %) [13,14]. The amide I' band of CA-C in the presence of DMPC and DMPA was studied to detect possible changes in its structure. Our results show that the presence of both lipids induced similar changes; an increase in the total percentage of α -helix, with 62 % in DMPC and 79.3 % in DMPA. The percentage of secondary structure could not be determined in the presence of DMPS, as the serine moiety strongly absorbs at 1622 cm^{-1} [36]. To rule out that these changes among FTIR spectra acquired under different conditions were due to partial H^2H exchange, we kept all the CA-C or CA-C plus lipid samples overnight in $^2\text{H}_2\text{O}$ (see the

Table 2 Secondary structure analysis of CA-C as determined by FTIR

Errors in the wavenumbers are estimated to be $\pm 2 \text{ cm}^{-1}$. Errors in the estimation of the percentage of secondary structure are 4%. The analysis was carried out at 25 °C. There was one more band present, centred at 1613 cm^{-1} , which has not been shown in the Table (see Figure 4). This band is assigned to the amino acids side chains [51] and it accounted for 1–9 % of the total area of the amide I' bands. The percentages of total secondary structure shown in the Table do not take into account the contribution of this band.

Wavenumber (cm^{-1})	Percentage of total secondary structure			Structural assignment	Reference
	CA-C	CA-C + DMPC	CA-C + DMPA		
1675	–	5.9	–	Turns	[52]
1662	32	20.2	18.7	Loops/turns/ 3_{10} -helix	[53,54]
1648	18.2	40	46.5	α -Helix	[51,52]
1638*	33.2	22	32.8	α -Helix	[55]
1625	16.5	11.7	1.8	Extended chains	[54]

* Absorptions between 1640 and 1630 cm^{-1} can be associated with distorted helical structure [55].

Figure 5 FTIR thermal denaturations following the half height of the amide I' band

Thermal transitions of 1 mM CA-C alone ($\square$), in the presence of 35 mM DMPA ($\blacksquare$) and 35 mM DMPC ($\circ$). The buffer used was 10 mM HEPES/100 mM KCl (pH 7). Thermal midpoints were obtained by fitting of the data to a two-state equation [30].

Experimental section). This time allowed for exchange agrees with the low conformational stability of CA-C [17], and with our own experience in H^2H NMR experiments, where all the NH protons of CA-C exchanged in 5 h at 5 °C (results not shown).

Thermal denaturation experiments

Thermal denaturation experiments were carried out to test for the presence of the dimeric species of CA-C under the experimental conditions used. It has been shown that FTIR and NMR are the only techniques able to monitor CA-C monomerization occurring at $\sim 30^\circ\text{C}$ [30]. The thermal FTIR experiments in the presence of DMPA and DMPC showed the same low-temperature transition as CA-C in solution (Figure 5), suggesting that CA-C was dimeric in the presence of lipids, and that the spectral changes detected by FTIR were not due to dimer dissociation. The second sigmoidal transition obtained by FTIR reports the unfolding of the CA-C monomer, occurring at 61 °C in the absence of lipids. This temperature was decreased in the presence of DMPA (57 °C), whereas it remained unchanged in DMPC (60 °C) (Figure 5).

DISCUSSION

Binding of CA-C to lipids

As the HIV-1 particle buds from the infected cell, it recruits lipid molecules from the plasma membrane to form its envelope. However, the lipid composition of the viral envelope is different from that of the host plasma membrane [10]. The more significant differences are: (i) an increase in the presence of Cho and SM; and (ii) a change in the relative presence of the different phospholipids – there is an increase in the content of negatively charged lipids (the PA and PS content of the viral envelope is 3.5 and 1.5 times respectively that of the host cell plasma membrane) and a reduction in PC (in the viral envelope, it is 0.6 times the value of the plasma membrane). In the present work, we have shown, using fluorescence spectroscopy, DSC and FTIR, that CA-C was able to bind to lipid model membranes. The K_p values determined (Table 1) were similar to those reported for other lipid–protein models [23,27], and they indicate that CA-C had a higher affinity for liposomes of the negatively charged type, PA and PS, than for those of the zwitterionic type, PC/Cho/SM and PC. Interestingly, the variations observed in the relative content of phospholipids in the viral envelope were coincident with the relative phospholipid affinities of CA-C (PA > PS > PC).

The changes in the fluorescence emission spectra of CA-C caused by the PC and PC/Cho/SM liposomes were very similar: a reduction in the fluorescence intensity and a small red shift. These results suggest that Trp¹⁸⁴ of CA-C was subject to small changes in its environment caused by either PC and PC/Cho/SM binding when compared with aqueous solution. However, when CA-C was bound to both PA and PS liposomes, significant blue shifts in the spectra maxima were observed, indicating that Trp¹⁸⁴ was transferred to a more hydrophobic environment. The significant differences in the fluorescence spectra of CA-C when bound to negative and zwitterionic lipids suggest that there were differences in: (i) the structure of CA-C and/or (ii) the type of interaction of CA-C with both lipids.

The analysis of the FTIR amide I' band of CA-C showed that the binding of CA-C to the DMPA and DMPC liposomes caused an increase in the α -helix content of CA-C [in conditions where, according to eqn (4), only the 2.4% (for PC) and the 0.2% (for PA) of the protein partitioned into the aqueous solution]. These findings indicate that the secondary structure of CA-C changed in the presence of DMPA. Moreover, they also indicate that, although the tryptophan environment of the PC-bound CA-C was not greatly modified (see above), the secondary structure of CA-C was altered (Table 2).

The interaction of CA-C with DMPC membranes, as shown by FTIR and DSC, caused only minor changes in the carbonyl region hydration state and in the acyl chain packing. This probably means that there was not a large degree of penetration of CA-C in the DMPC vesicles. In DMPA, there were increases in the ΔH and the T_c values of the calorimetric transition; these changes are characteristic of proteins that have a simple electrostatic binding to the lipid bilayer surface, without penetration into the hydrocarbon region [32]. The changes in the infrared carbonyl band of the DMPA indicate that the interaction caused dehydration of the lipid surface, suggesting that CA-C binding to the DMPA vesicles hampered the access of the solvent to the water–lipid interphase. Moreover, the variation in the K_p at high ionic strength further supported the theory that the interaction between DMPA and CA-C was predominantly electrostatic. In DMPS, there were also changes in the thermotropic parameters when compared with those of the lipid alone. The lipid phase transition showed a reduction in the intensity of the main peak, and the appearance of a second broader peak at higher temperatures (Figure 2). The

presence of two peaks suggested that CA-C must interact preferentially with a fraction of the total DMPS population. Alternatively, the small variations in the infrared DMPS carbonyl band in the presence of CA-C show that the binding caused only a minor reduction in the degree of hydration of the DMPS polar heads. Thus the differences in the degree of the lipid hydration and the insertion of the protein into the lipid could explain the differences in the fluorescence spectra and the DSC experiments in the presence of DMPA and DMPS.

The Gag protein is preferentially targeted to lipid rafts. The localization of Gag in lipid rafts is preferentially mediated by the N-terminal region of Gag, although the association is greatly facilitated by the Gag–Gag interaction domain in the NC (nucleocapsid) protein [11]. In our studies, the lipid mixture (PC/Cho/SM) resembling the rafts did show a smaller affinity than PS and PA, but larger than PC (Table 1). We do not know yet, however, whether this increase is due to the high-ordered state of the rafts, or to the specific interaction of CA-C with the SM or Cho of this lipid mixture.

Theoretical predictions

To predict the regions of CA-C that mediate its binding to lipid membranes, the whole-residue interfacial scale described by Wimley and White [37] was used. In the calculations, we considered that aspartate and glutamate residues had a negative charge (and thus both would cause repulsion for biological membranes), and we used sequences of 7–9 residues; under these conditions, significant favourable lipid-binding propensities were detected for three regions of CA-C. Since the Wimley and White scale used was obtained in POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) membranes [37], it could be thought that our results could be skewed for the binding to negatively charged lipids. It is interesting to note that this scale has been widely used for prediction of the interfacial binding of a protein to a membrane. Furthermore, this scale correlates very well with the octanol hydrophobicity scale [38], and with the 'biological hydrophobicity' scale [39] obtained recently in dog pancreas rough microsomes, which are known to have a varied lipid composition. Thus the general conclusions described below can be safely assumed to occur in a lipidic biological system.

The first region, Y¹⁶⁴VDRFYKTL¹⁷², is located in α -helix 1 and belongs to the MHR (Figure 6). The theoretical prediction at this region is supported by previous experimental studies on the ability of Gag protein to bind acidic phospholipid vesicles: mutation of Arg¹⁶⁷ or the deletion of the entire MHR reduced the membrane affinity when compared with the wild-type Gag [40]. The second region, N¹⁸³WMTETLLV¹⁹¹, is part of α -helix 2, and the third region, L²⁰²KALGPGA²⁰⁹, comprises the second half of α -helix 3 and some interhelical residues. A fourth region, A²¹⁷CQGVGG²²³, also showed a tendency to interact with the membrane interface, but it produced non-significant scores. According to this prediction, the tryptophan and tyrosine residues of CA-C (Tyr¹⁶⁴, Tyr¹⁶⁹ and Trp¹⁸⁴) would be part of the membrane-interacting zones, in agreement with the well-known propensity of both types of residues to be located in the water–lipid interface of membrane proteins [41].

A similarity search of the HIV-1 CA-C sequence was performed to study the theoretical membrane affinities of similar proteins, and only the sequences of the capsid proteins of different retroviruses produced significant alignments. Accordingly, the propensities of the C-terminal domains of these proteins to interact with the membrane interface were estimated (Figure 6). The highly related CA-C sequences of HIV-2 and SIV (simian immunodeficiency virus) showed very similar regions to those

Figure 6 Sequences of HIV-1 CA-C and similar regions in the capsid proteins of other retroviruses

Zones predicted to have a significant tendency to interact with the membrane interface are in italics and underlined. The region of HIV-1 CA-C predicted with non-significant scores ($A^{217}CQGVGG^{223}$) is depicted in italics and with broken underline. The different helices (h1–h4) of CA-C are indicated at the top of the sequences, and the highly conserved MHR region is boxed. The 3_{10} helix involves residues $T^{148}SILD^{152}$ in HIV-1. The retroviruses and the accession numbers of the capsid proteins are: HIV-1 (strain 00TCD.2064), CAD36299; HIV-2 (strain AG1612), AAQ99634; SIV (simian immunodeficiency virus), AAL18230; BIV (bovine immunodeficiency virus), P19559; FIV (feline immunodeficiency virus), AAN52130; CAEV (caprine arthritis-encephalitis virus), AAQ21499; VV (Visna–Maedi ovine progressive pneumonia virus), AAM51649; EIAV (equine infectious anaemia virus), 2EIA.B; SRL (small-ruminant lentivirus), AAR22615; HTLV (human T-cell lymphotropic virus 1), 1QRJ.B; Herv-K (human endogenous retrovirus K), Q7LDI9. All belong to the genera lentivirus, except HTLV, which is a deltaretrovirus, and Herv-K, which is a provirus. The similarity search was performed with BLAST [58] and the sequence alignment with ClustalW [59].

Figure 7 Proposed model of CA-C-lipid interactions

Crystal structure of the CA-C monomer [15,16]. Tyrosine and tryptophan residues are shown in stick form. The regions with predicted membrane interface affinity are depicted in dark grey, including the region $A^{217}CQGVGG^{223}$ which is indicated by an asterisk (note that the last residues $V^{221}GG^{223}$ are not present in the resolved structure). The Figure was created from the PDB file 1A43 with PyMOL (<http://www.pymol.org>).

calculated for HIV-1 CA-C, although the fourth region had in these two cases a significant affinity for the membrane interface. The number of predicted membrane-interacting regions decreased in the evolutionarily more distant viruses.

The regions with high propensity to interact with the membrane are indicated in dark grey in Figure 7, whereas the fourth one, with a lower propensity, is indicated with an asterisk. Interestingly, all these regions appear to be located in a close area in the tertiary structure of CA-C. However, as they do not form a continuous surface that could easily mediate membrane anchoring, there would be necessary structural rearrangements, which are probably indicated by the FTIR spectra (Table 2). We do not know, however, the exact nature of the arrangements necessary to bring these regions as close as possible, but from Figure 7, it seems that the movements of the N-terminus of helix 2 and the C-terminal region of helix 3 towards helix 1 could bring those

regions closer to facilitate binding to membranes. Recently, the X-ray crystallography structure of a complex formed by a helical peptide and CA-C has been solved [42]. To allocate the peptide, the dimeric structure of CA-C is altered by, among others, a movement of the N-terminus of helix 2. This shift brings together the N-terminus of α -helix 1 and the central region of α -helix 2, as expected when interaction with membranes occur. Interestingly, although this movement changes the orientation of α -helix 2 (mainly responsible for the dimerization interface), it does not disrupt the dimeric state of CA-C, as the interaction with membranes did not alter them as well (Figure 5). Furthermore, the highly hydrophobic peptide (sequence ITFEDLLDYGGF) has contacts with the CA-C residues Val¹⁶⁵, Phe¹⁶⁸, Tyr¹⁶⁹, Leu¹⁷², Lys¹⁸², Asn¹⁸³, Thr¹⁸⁶, Thr²¹⁰, Leu²¹¹ and Met²¹⁵, which are close or involved in the above predicted regions in our studies. Thus it is tempting to suggest that the contacts of the peptide with the protein could resemble the interactions anchoring CA-C to the membrane.

Possible biological implications

It could be thought that the lipid affinity described in the present work is an artifact of the different techniques used. However, there are several pieces of evidence which suggest that the lipid-binding affinity of CA-C is an intrinsic property of the domain. Firstly, we have exploited several techniques which use different protein concentrations (from micromolar in fluorescence spectroscopy to millimolar in FTIR), and lipid binding was observed with all the techniques. Secondly, we used different liposome preparations (SUVs and MLVs) with different protein/lipid ratios which produced similar results in all cases. And finally, we have used theoretical predictions, which indicate several lipid-binding regions in CA-C.

Several HIV-1 proteins interact with the membranes of the host cell in key stages of the infective cycle. Probably, the best characterized of these stages is the binding of the Gag polyprotein to the inner leaflet of the plasma membrane through MA [4,43], although the role of other Gag proteins in binding to membranes is still a matter of debate [44–48]. A hypothetical role of CA in the binding to membranes also remains controversial. An early report [44] shows that the entire CA interacts with lipids only at high ionic strength. However, the later studies show that myristoyl-moiety-mediated membrane binding of Gag occurs in proteins lacking the CA-C terminal domain [11,45]. The

hypothesis that CA might interact with membranes has been strengthened by recent discoveries. Several groups have shown that, contrary to what was previously thought, not all the CA molecules resulting from the Gag proteolysis form the capsid of the virion [5,6]. In fact, it is estimated that between one half and two-thirds of the total population of CA do not form part of the mature capsid. The results presented in the present study might favour the possibility that after cleavage-induced maturation of Gag, a population of the CA protein could bind to the inner leaflet of the viral envelope via its C-terminal domain. The MA proteins attached to the envelope in the mature capsid would not hamper the interaction between CA and the membrane, because, even if MA forms a continuous homogeneous network of trimers, there would be gaps wide enough to accommodate the CA molecules [49].

On the other hand, it has been shown that CA-C is able to allocate an α -helix peptide without disrupting the dimeric species and forming a five helical bundle, instead of the native four helical bundle [42]. Furthermore, the MHR and the immediately adjoining regions of CA-C have been suggested to participate in domain swapping between two close Gag proteins during assembly of the immature particle [50]. These data, together with those from the present study, indicate that CA-C is a versatile domain that can adapt to different environments in the HIV particles. This flexibility of CA-C to adopt slightly different conformations depending on the environment might be a consequence of the severe gene economy in retroviruses, such as HIV. This requirement means that the same region of the polypeptide chain must perform different functions depending on: (i) the different environment in which CA-C is immersed; or (ii) whether the protein belongs to a macromolecular assembly.

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