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**FLIM-FRET and FRAP reveal association
of influenza virus hemagglutinin with membrane rafts**

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short title: raft association of influenza HA

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Synopsis

It has been supposed that the hemagglutinin (HA) of influenza virus must be recruited to membrane rafts to perform its function in membrane fusion and virus budding. Here, we aimed at substantiating this association in living cells by biophysical methods. To this end, we fused the cyan fluorescent protein Cerulean to the cytoplasmic tail of HA. Upon expression in CHO cells HA-Cer was glycosylated and transported to the plasma membrane similarly as authentic HA. We measured Förster's resonance energy transfer by fluorescence lifetime imaging microscopy (FLIM-FRET) and showed strong association of HA-Cer with Myr-Pal-YFP, an established marker for rafts of the inner-leaflet of the plasma membrane. Clustering was significantly reduced when rafts were disintegrated by cholesterol extraction and when the known raft-targeting signals of HA, the palmitoylation sites and amino acids in its transmembrane region, were removed. Fluorescence recovery after photobleaching (FRAP) showed that removal of raft-targeting signals moderately increased the mobility of HA in the plasma membrane indicating that the signals influence access of HA to slowly diffusing rafts. However, Myr-Pal-YFP exhibited a much faster mobility compared to HA-Cer, demonstrating that HA and the raft marker do not diffuse together in a stable raft-complex for long periods of time.

key words: hemagglutinin, raft, palmitoylation, transmembrane region, budding

Introduction

Membrane rafts are very small, dynamic liquid-ordered assemblies enriched in cholesterol and sphingolipids, which can selectively incorporate proteins, most notably GPI-anchored proteins to the outer leaflet, doubly-acylated proteins to the inner leaflet as well as several transmembrane proteins. Rafts recruit certain membrane proteins, which should favour interactions between the raft components. Rafts are also considered as point of entry as well as assembly and budding sites of a wide range of viruses. Published results demonstrating the occurrence of most proteins in rafts were obtained biochemically using cold extraction with Triton. It is, however, highly controversial whether partitioning of proteins into these detergent-resistant membranes (DRMs) reflects their association with rafts inside living cells [1-5].

If molecules are present in rafts, they should form cholesterol-sensitive clusters largely independent of their cell surface density. Fluorescence resonance energy transfer (FRET) is exceptionally well suited to demonstrate clustering of proteins, because it occurs only if two molecules tagged with a fluorophore are in close proximity (2–5 nm), which is in the range of the size of a membrane raft. However, mixed results were obtained when the FRET-technique was applied to probe cells for the existence of rafts, i.e. for the clustering of proteins which had been shown to partition into DRMs. Significant energy transfer between fluorophore-labelled antibodies against GPI-anchored proteins was detected. However, FRET correlated with the surface density of the raft marker suggesting that FRET was due to random interaction of mobile proteins in the membrane, but not to clustering of proteins [6-8]. Using a homo-FRET approach others have shown that GPI-anchored proteins form cholesterol-sensitive clusters independent of their expression levels. Only a small, but significant (20–40%) fraction of GPI-anchored proteins form high density clusters of nanometer size (4–5nm), each consisting of only a few molecules [9]. Zacharias et al. fused the yellow- and cyan fluorescent protein (YFP and CFP) to peptide sequences directed to membranes by dual acylation. FRET independent of the expression level was observed at the inner leaflet of the membrane and was sensitive to cholesterol depletion [10].

The hemagglutinin (HA) of influenza virus was the first transmembrane protein described as a component of DRMs [11]. Hydrophobic amino acids in the outer leaflet of the transmembrane region (TMR) and palmitoylation at cytoplasmic and transmembrane cysteines are required for partitioning of HA into DRMs [12, 13]. HA acquires detergent resistance at a late stage during its transport to the cell surface, probably in the trans-Golgi-network [14]. It was postulated that cholesterol-sphingolipid enriched domains form vesicles in the trans-Golgi-network (TGN), which serve as carriers for lipids and entrapped proteins to the apical membrane in epithelial cells [15]. Not only does HA require rafts for its transport to the plasma membrane, but influenza virus also buds from raft-domains and HA needs to be concentrated in membrane rafts to perform its fusion activity efficiently [16-18]. However, attempts to demonstrate the association of HA with membrane rafts with methods other than Triton extraction have produced ambiguous results: Using fluorescence recovery after photobleaching (FRAP) it was demonstrated that wild-type HA diffused more slowly compared to a non-raft HA mutant, but its diffusion rate was elevated to non-raft HA values after disruption of rafts by depletion of cholesterol [19]. In contrast, authors performing large-scale confocal FRAP assays have shown that the diffusion coefficients of proteins localized in DRMs (including HA) do not differ from those of proteins present in the bulk phase of the membrane [20]. More consistent data for clustering of HA were obtained by quantitative immunoelectron microscopy and by fluorescence-photoactivation-localization microscopy (FPALM). It was shown that HA is not randomly distributed in the plasma membrane, but forms irregular clusters ranging in size from a few nanometers up to many micrometers. However, only clusters at the nanometer length scale, i.e. with the size of rafts, could be disintegrated by extraction of cholesterol [21-23].

Using FLIM-FRET we have shown recently that a construct consisting of the cytoplasmic tail and the transmembrane region of HA fused to variants of GFP clusters with a GPI-linked peptide, a marker for outer-leaflet rafts [24]. Here we have analyzed whether *authentic* HA equipped at its cytoplasmic tail with a fluorophore associates with a doubly-acylated peptide, a marker for inner-leaflet rafts [10].

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Materials and methods

Cloning of fluorescent proteins

The plasmids pEYFP (Clontech) and pECerulean [25] were used for cloning. The A206K monomeric mutation (10) was made in YFP using overlap extension PCR. The HA gene from influenza virus strain A/FPV/Rostock/34 (H7N1) was inserted into the plasmid such that the resulting construct contains the amino acid sequence LRPEAPRARDPPVAT between the end of HA and the start of YFP. For FLIM-FRET experiments we used HA which contains glycine instead of arginine at position 339 at the cleavage site of HA₁ [26]. The non-acylated HA contains serine instead of cysteine at positions 551, 559 and 562 at the C-terminus of HA₂ [27]. The amino acids VIL at position 527 to 530 were converted to alanines to delete the other signal for association of HA with DRMs [12, 17]. Myr-Pal-YFP contains the amino acid sequence MGCIKSKRKDNLNDDEPPVAT derived from the N-terminus of the Lyn kinase before the start of YFP.

Transfection, metabolic labeling and treatment of cells

CHO-K1 cells were transfected using Lipofectamine 2000 (Invitrogen) 24 hours prior to performing the experiments. Metabolic labelling, immunoprecipitation with 2 µl anti-fowl plaque virus (FPV) antiserum, SDS-PAGE and fluorography were carried out as described previously [27]. Endoglycosidase-H and PNGase-F digestions of immunoprecipitated samples were performed as described by the manufacturer (New England Biolabs). For cholesterol depletion, cells were incubated for 30 min at 37 °C with 5 mM or with 10 mM methyl-β-cyclodextrin (Sigma) as indicated in the figure legends. To estimate the amount of extracted cholesterol, cells were labelled with [1α, 2α(n)-³H]-cholesterol (1mCi/ml, GE-Healthcare) for about 4 h. Subsequent to extraction, the supernatant was incubated with scintillation cocktail (Lumasafe Plus, Lumac Lsc.) and cells were lysed with NaOH (0,1M, 20 min) and also incubated with scintillation cocktail. Radioactivity (in counts per minute, CPM) was measured with a luminescence counter (1600 RT, Packard) and the percentage of cholesterol extracted was calculated as: $CPM_s / (CPM_s + CPM_c)$ where CPM_s are the CPM in the supernatant and CPM_c are the CPM in the cell lysate.

Confocal fluorescence microscopy

Confocal microscopy with live cells was performed using an Olympus FluoView 1000 microscope equipped with a UPLSAPO 60x/1.35 NA oil objective (Olympus) at 25 °C. Images were obtained by sequential excitation at 440 nm (Cerulean) using a laser diode and at 515 nm (YFP) using an argon laser. Emission was recorded between 460 and 490 nm for Cerulean and between 535 and 575 nm for YFP.

FLIM-FRET

FLIM images were acquired using the time-resolved LSM-upgrade Kit (PicoQuant) which uses the time-correlated single photon counting (TCSPC) technique. Cerulean was excited at 440 nm using a pulsed laser diode. The emitted fluorescence was detected by a single photon avalanche photodiode and a 470 ± 15 nm filter. Electrical signals were processed by the TimeHarp 200 PC card. FLIM pictures of cells were accumulated for 90 seconds using the SymPhoTime software. A TCSPC-histogram containing the accumulated decay signals only from the plasma membrane was created and fitted to calculate the average lifetime. For the fitting the instrument response function was taken into account. Since Cerulean has been reported to show either mono- or biexponential fluorescence decay [25, 28], both fits were tested. We achieved the best fitting by assuming a biexponential decay, which yields a short and a long lifetime. The actual lifetime was calculated as the average of the two lifetimes. To calculate the lifetime of the donor fluorophore in the absence of acceptor, ten cells expressing only the donor were analyzed on each day an experiment was performed.

The FRET efficiency (E) was calculated using the equation:

$$E = 1 - (\tau_{DA} / \tau_{D_{av}})$$

where τ_{DA} is the lifetime of a single cell co-expressing donor and acceptor and $\tau_{D_{av}}$ is the average lifetime of the ten cells expressing only the donor.

FLIM measurements and the acquisition of the picture to determine the fluorescence intensity of the acceptor (Myr-Pal-YFP) and of the donor (HA-Cer) were performed on the same confocal picture. To determine the amount of acceptor and donor at the plasma membrane, the mean intensity of their fluorescence was calculated with ImageJ. A cell, where HA-Cer was clearly visible at the plasma membrane (see Fig. 1B) was selected and the software recognized the plasma membrane as a continuous structure with (almost) the same fluorescence intensity. To ensure that the amount of fluorophore is directly related to its fluorescence intensity, we included the instrument settings, i.e., laser intensity and gain settings in the analysis as follows:

$$Ac = \{ [(M - bg) / \text{laser intensity}] / \text{gain} \}$$

Ac is the acceptor concentration, M represents the mean of the measured fluorescence intensity at the plasma membrane and bg the background.

Intensities of HA-Cer and of its mutants were in the same range, i.e. the variation in their fluorescence intensity was larger between individual cells expressing the same construct than between different HA-constructs.

The FRET efficiency E of every cell co-expressing donor and acceptor was plotted against the fluorescence intensity of the acceptor F in this cell. The data points were fitted according to the equation:

$$E = E\%_{\max} * F / F + K_D$$

assuming that E is a hyperbolic function of the amount of acceptor. $E\%_{\max}$ is the maximal FRET efficiency calculated from the fitting. The equation gives a dissociation constant K_D as a parameter to assess the associative properties of donor and acceptor [10]. K_D values should be compared with the acceptor intensities within the same experiment rather than to K_D 's from other experiments whose absolute intensities scales are not directly comparable as described above.

Results for each acceptor/donor pair were compiled from one to nine different transfections, either sister cultures, i. e. cells grown in different dishes but transfected and measured in parallel or (mostly) cells transfected on different days, therefore representing completely independent experiments. The actual data for each graph are listed in the figure legends. In each transfected cell culture dish 10 to 20 different cells were measured. Each cell was included in the evaluation, no "outstanders" were eliminated.

Fluorescence recovery after photobleaching (FRAP)

We first established the laser intensity at 515 nm to photobleach about 60–70% of the fluorescence of YFP. FRAP was then performed at the plasma membrane above the nucleus of cells expressing HA-YFP. After 10 scans at low laser intensity, a circle of $15.11 \mu\text{m}^2$ was bleached for 100 ms. Subsequently, scanning of the bleached circle was continued at 515 nm at low laser intensity for three minutes. Analysis was performed in Origin 7.5 (OriginLab Corp.). Recovery curves were fitted with an approximation of the theoretical recovery curve:

$$F(t) = (F_0 + F_r t) / (t + \tau)$$

where t is the time after bleach, F(t) is the fluorescence as a function of t, F_0 is the fluorescence immediately after bleach, F_r is the amount of fluorescence recovery, and τ is the time of half-maximal recovery [29, 30]. The fits accurately match the experimental curves.

The diffusion coefficient (D) was calculated using the equation:

$$D = (\gamma/4) \frac{\omega^2}{\tau}$$

where γ is related to the bleaching depth and is approximately 1.3 under our experimental conditions [31], ω^2 is the radius, and τ is the calculated time for half-maximal recovery. The mobile fraction (M_f) was calculated using the equation:

$$M_f = (F_r - F_0)/(1 - F_0)$$

Results and Discussion

Construction and testing of fluorescent HA and raft marker

As an established constituent of membrane rafts of the inner leaflet of the plasma membrane, we created Myr-Pal-YFP. This construct consists of a myristoylated and palmitoylated peptide from the Lyn kinase fused to the N-terminus of the yellow-fluorescent protein (YFP), which can serve as an acceptor in FRET experiments [10]. As a donor fluorophore we fused Cerulean (Cer), a variant of CFP with improved quantum yield and a higher excitation coefficient [25], to the cytoplasmic tail of HA (H7 subtype). We used HA with a mutation at its cleavage site since proteolytical cleavage of the HA-subtype H7 by a cellular enzyme causes instability of the protein [32, 33]. Two mutants were generated by removing either of the known signals for incorporation of HA into DRMs. The three palmitoylated cysteines were exchanged by serines (HA-Cer-C3S) and three bulky, hydrophobic side chains (VIL) present at the N-terminal end of the external leaflet of the transmembrane region were converted to alanines (HA-Cer-VILA3). A similar mutation in the transmembrane region of a H3-subtype HA renders the molecule completely soluble in cold detergent [17]. In another mutant (HA-Cer-C3S+VIL3A) both raft-targeting signals were deleted simultaneously (see Fig. 1A for a schematic view of all proteins used in this study). All our constructs contain the A206K mutation in the fluorescent proteins. This prevents their dimerization and thus avoids the occurrence of a false-positive dimerization-induced FRET signal [10]. Upon expression in CHO cells all constructs received Endo-H resistant carbohydrates (Fig. 1C) and were transported to the cell surface as visualized by confocal microscopy (Fig. 1B). Thus, attachment of additional sequences to the cytoplasmic tail of HA does not interfere with its processing and its transport to the plasma membrane.

FLIM-FRET is suitable to demonstrate clustering of proteins in the membrane

We used FLIM to study possible energy transfer between HA-Cer (donor) and the raft marker Myr-Pal-YFP (acceptor). FLIM-FRET has several advantages compared to conventional FRET methods, such as sensitised emission or acceptor photobleaching. FRET measurements done by FLIM are more robust and quantitative and no corrections are needed for donor fluorophore emission bleed through in the acceptor emission channel [34]. If FRET occurs, the lifetime of the excited state of the donor will be shortened in the presence of the acceptor in close proximity. From these measurements the FRET efficiency, defined as the fraction of donor excitation events that result in energy transfer to the acceptor, can be calculated. However, analyzing FRET in a two-dimensional system, such as the plasma membrane, requires a more systematic evaluation of the data [35]. Since HA-Cer as well as Myr-Pal-YFP are mobile in the plane of the membrane, energy transfer can simply occur by random collision of both molecules. In this case the FRET efficiency is a linear function of the concentration of the acceptor protein at the membrane, i.e. it is absent at very low expression levels, but FRET-efficiencies increase linearly with increasing protein concentration. In contrast, if FRET is due to clustering of HA with the raft marker, the FRET efficiency is largely independent of the concentration of the acceptor protein. To evaluate our FLIM data we used a model applied by Zacharias et al. to demonstrate clustering of the double-acylated raft marker [10]. In this model, which describes the binding of a ligand to its receptor, the FRET efficiency is a hyperbolic function of the concentration of the acceptor protein, i.e. its fluorescence intensity. The respective equation yields a dissociation constant K_D as a parameter to assess the associative properties of donor and acceptor. If K_D is very small compared to the intensity of the acceptor, clustering of acceptor and donor occurs, but if K_D is in the same range as or larger than the acceptor intensity, FRET is due to random collision of both molecules. However, K_D values cannot be compared between different protein pairs, even if they are attached to the same acceptor and donor fluorophore. The FRET efficiency depends on the distance between the donor and the acceptor and on the relative orientation of

the donor emission dipole moment and the acceptor absorption dipole moment, parameters which can not be measured [10].

To analyze whether this method is suitable to evaluate FLIM-FRET data we first analyzed a donor-acceptor pair which is known to form stable clusters. We co-expressed HA-Cer with HA fused in identical manner to YFP. As known, HA trimerizes in the ER, which is a prerequisite for its intracellular transport along the exocytic pathway [36]. If mixed trimers containing HA-Cer and HA-YFP will be present at the plasma membrane, both fluorophores should be in close proximity, since the cytoplasmic tail of HA consists of only eleven amino acids. Hence a significant FRET signal would be observed, which should be independent of the expression level of HA. CHO cells transfected with HA-Cer alone and cells co-transfected with HA-Cer and HA-YFP were selected and the lifetime of the donor fluorophore and the intensity of the acceptor fluorophore were analyzed at the plasma membrane. From these data the FRET efficiency was calculated and plotted against the intensity of the acceptor (Fig. 2A). The FRET-efficiencies were clearly independent of the intensity of the acceptor fluorescence and hence the expression level of HA-YFP. Fitting the data points to the described equation revealed a very low K_D of 1×10^{-16} indicating the situation of strong clustering and thus the presence of mixed trimers.

We then asked whether FLIM-FRET can also be used to detect clusters such as those formed by the double-acylated raft marker with itself. Cells coexpressing Myr-Pal-Cer and Myr-Pal-YFP showed significant FLIM-FRET independent of their expression levels with a K_D of 120, which is clearly lower than the fluorescence intensity of the acceptor (Fig. 2B). Incubation of cells with cyclodextrin to remove cholesterol increased the K_D by an order of magnitude suggesting that clustering is reduced by disintegration of membrane rafts (Fig. 2C). Thus, we could confirm by FLIM-FRET clustering of Myr-Pal-YFP at the plasma membrane as has been described by others using acceptor photobleaching FRET [10]. This clearly demonstrates that FLIM-FRET is suitable for studying raft clustering.

FLIM-FRET reveals clustering of HA with the raft marker dependent on DRM-targeting signals and intact rafts

As a negative control, we measured FLIM-FRET between HA-Cer and YFP, which is localized all over the cell. In individual CHO cells transfected with HA-Cer alone and cells co-transfected with HA-Cer and YFP, the lifetime of the donor fluorophore and the intensity of the acceptor fluorophore were measured at the plasma membrane. From these data the FRET efficiency was calculated and plotted against the fluorescence intensity of the acceptor. Although a mean FRET efficiency of 12% was determined, the almost linear shape of the curve and the high K_D of about 4500 indicate no clustering, as expected (Fig. 3A + B).

We then measured FLIM-FRET of HA-Cer-wt with Myr-Pal-YFP to assess the clustering of HA with membrane rafts. We observed a high FRET efficiency (52%, Fig. 3A) clearly independent of the intensity of the acceptor fluorescence and hence the expression level of Myr-Pal-YFP (Fig. 3C). Fitting the data points to the described equation revealed a very low K_D of 1.6×10^{-16} .

Several experiments were performed to analyze whether clustering is indeed due to partitioning of HA-Cer into membrane rafts. Extraction of cholesterol with methyl- β -cyclodextrin led to a minor, but statistically significant lowering of the FRET efficiency (46%, Fig. 3A) and to considerable reduction of clustering since the K_D calculated for this association was increased significantly (Fig. 3D). Likewise, expression of non-acylated HA-Cer-C3S (Fig. 3E), of the HA mutant with a deletion of the DRM-targeting signal in its transmembrane region (Fig. 3F) and of the HA mutant devoid of both DRM-targeting signals (Fig. 3G) caused substantial disintegration of clustered HA arrangement with the raft marker. It should be noted that even HA with both DRM-targeting signals deleted showed high FRET-efficiencies with the raft marker (Fig. 3A) and that a few cells in individual graphs showed

FRET even at very low acceptor intensities. Measurements of the fluorescence intensities of donors and acceptors revealed that Myr-Pal-YFP was approximately 10-fold overexpressed compared to HA-Cer. The overexpression of the acceptor renders a FRET pair prone to high transfer rates, since only a few interactions of HA-Cer with the raft marker yield a high FRET signal. On the other hand, since the acceptor concentrations are not limiting, plotting of the FRET-efficiencies against the acceptor concentration is a suitable method to evaluate the data.

FRAP demonstrates transient interactions of HA with rafts

Next, we attempted to verify our conclusions with a second method, fluorescence recovery after photobleaching (FRAP). Proteins present in rafts should have a lower diffusion rate compared to non-raft proteins, since under conditions where viscosity is limiting, lateral diffusion varies inversely with the logarithm of the radius of the transmembrane portion of the diffusing species, for example the accumulated radii of all transmembrane proteins diffusing together in a raft [37]. An exception are apical membranes of polar cells, where the raft domain is the percolating phase in the membrane [38]. For the FRAP-experiments HA fused to YFP was used, since its fluorescence can be bleached more easily. HA-YFP-wt exhibited a diffusion coefficient of $0.14 \mu\text{m}^2/\text{sec}$ and a mobile fraction of more than 80% (Fig. 4A), which is in agreement with published studies on other HA-subtypes not fused with a fluorescent protein (27, 28). Successive removal of the DRM-targeting signals moderately increased the diffusion coefficient of HA from $0.18 \mu\text{m}^2/\text{sec}$ (HA-YFP-C3S) to $0.20 \mu\text{m}^2/\text{sec}$ (HA-YFP-VIL3A) up to $0.30 \mu\text{m}^2/\text{sec}$ if both signals are deleted (Fig. 4A). Likewise, the mobile fraction decreases to slightly over 70% suggesting that HA outside rafts might be prone to aggregation.

Finally, we analyzed the temporal stability of the association of HA with membrane rafts. If HA and the raft marker are both present in a stable raft complex, they should diffuse together for minutes [39] and thus both molecules should exhibit a similar mobility in FRAP-experiments. However, Myr-Pal-YFP exhibited a diffusion coefficient of $0.7 \mu\text{m}^2/\text{sec}$, which is consistent with previous studies on proteins anchored by fatty acids to the inner leaflet of membranes [20], but much faster compared to HA-YFP (Fig. 4B).

Thus, HA and the raft-maker did not diffuse together for minutes, the time period in which their mobilities are observed in FRAP experiments. Indeed, it has been shown that the type of membrane anchorage, i. e. by a transmembrane region versus fatty acids, has a much greater effect on the mobility of a protein compared to its distribution in rafts or non-raft domains of the membrane [20]. Our results are consistent with a model of dynamic partitioning of proteins into and out of raft domains, which permits raft proteins to transiently populate raft domains as well as to undergo diffusion outside of rafts [9, 20]. Since wild-type HA associates with rafts more strongly than HA lacking DRM-targeting signals (as evidenced in this study by FLIM-FRET), it probably remains inside rafts for longer periods of time, which would retard its overall mobility in the membrane. It remains to be shown how such a rather transient interaction of HA with rafts leads to the formation of the viral budzone, a nanodomain of the plasma membrane where assembly and finally budding of virus particles occurs [40].

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Abbreviations

Cer: Cerulean, DRM: detergent-resistant membrane, FLIM: Fluorescence lifetime imaging microscopy, FRET: fluorescence (or Förster's) resonance energy transfer, FRAP: Fluorescence recovery after photobleaching, HA: hemagglutinin, M β CD: methyl-beta-cyclodextrin, Myr-Pal-YFP: myristoylated and palmitoylated peptide fused to YFP, SEM: standard error of the mean, XFP: any variant of the green fluorescent protein, YFP: yellow fluorescent protein.

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Figure Legends

Figure 1: Characterization of HA-Cer constructs expressed in CHO cells.

A: Scheme and abbreviations of the constructs used in this study.

B: Confocal fluorescence microscopy demonstrates transport of constructs to the plasma membrane. Scale bars: 10 μm .

C: Glycosylation: CHO cells expressing the indicated construct were labeled for four hours with [^{35}S]-methionine/cysteine. Samples were then subjected to immunoprecipitation with anti-GFP antibodies, digestion with Endo-H (H), PNGase-F (F) or were left undigested (-) prior to SDS-PAGE (10% gel) and fluorography. C: untransfected control cells. Numbers on the left represent the mobility of molecular weight markers. HA-Cer*: deglycosylated HA.

Figure 2: FLIM-FRET to demonstrate clustering within HA trimers and between raft markers.

For each CHO cell the calculated FRET efficiency (FRET-E) in percent is plotted against the fluorescence intensity of the acceptor. Continuous line: Data were fitted to the saturable one-site binding model as described in "materials and methods". The dashed line is the 95% confidence interval. K_D : dissociation constant, describing the associative properties. A low K_D relative to the acceptor intensity indicates high clustering.

A: HA-Cer-wt and HA-YFP-wt were coexpressed. n: cells analyzed: 66, K_D : $1 \times 10^{-16} \pm 7$.

B: Coexpression of Myr-Pal-Cer with Myr-Pal-YFP. n: 58, K_D : 120 ± 57 .

C: Coexpression of Myr-Pal-Cer with Myr-Pal-YFP. Prior to FLIM-FRET cholesterol was extracted with cyclodextrin (M β CD, 5 mM, 30 min, 37°C). This treatment removed about 50% of cholesterol from cellular membranes (see "materials and methods"). n:12, K_D : 1180 ± 1188 .

Figure 3: FLIM-FRET reveals clustering of HA-Cer with raft markers dependent on DRM-targeting signals.

A: Mean of the FRET-efficiencies for each experiment described in B to G. Error bars indicate the standard error of the mean (SEM). Statistics: Results are statistically significantly different (three asterisks: $p < 0.001$, two asterisks: $p < 0.01$) from HA-Cer-wt as calculated with a one-tailed unpaired Student's *t*-test.

B: Coexpression of HA-Cer-wt and YFP. K_D : 4442 ± 856 , number of analyzed cells (n): 41, number of cell culture dishes (d): 4, number of different experiments, i.e. independent transfections (t): 1.

C–D: Coexpression of HA-Cer-wt and Myr-Pal-YFP.

C: untreated cells. K_D : 1.6×10^{-16} , n: 39, d: 9, t: 8.

D: Cholesterol was extracted with 10 mM cyclodextrin (M β CD, 30 min, 37°C) prior to FLIM-FRET. K_D : 784 ± 139 , n: 48, d: 3, t: 3.

E: Coexpression of HA-Cer-C3S and Myr-Pal-YFP, K_D : 872 ± 287 , n: 53, d: 8, t: 4.

F: Coexpression of HA-Cer-VIL3A and Myr-Pal-YFP, K_D : 422 ± 180 , n: 35, d: 4, t: 3.

G: Coexpression of HA-Cer-C3S+VIL3A and Myr-Pal-YFP, K_D : 739 ± 460 , n: 38, d: 4, t: 3.

Figure 4: FRAP indicates transient interaction of HA with rafts.

FRAP was performed at the plasma membrane of CHO cells expressing HA-YFP (wt or mutants) or Myr-Pal-YFP as indicated. Left part: Calculation of the diffusion coefficient ($\mu\text{m}^2/\text{sec}$) and right part: Calculation of the mobile fraction. Bars represent the mean \pm SEM of at least 30 individual cells.

Statistics: Results are statistically significantly different (three asterisks: $p < 0.001$, two asterisks: $p < 0.01$, one asterisk: $p < 0.05$) from HA-Cer-wt as calculated with a two-tailed unpaired Student's *t*-test.







