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Biophysical Chemistry 114 (2005) 169–179

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Membrane destabilizing properties of cell-penetrating peptides

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Received 8 July 2004; received in revised form 25 November 2004; accepted 26 November 2004
Available online 15 December 2004

Abstract

Although cell-penetrating peptides (CPPs), also denoted protein transduction domains (PTDs), have been widely used for intracellular delivery of large and hydrophilic molecules, the mechanism of uptake is still poorly understood. In a recent live cell study of the uptake of penetratin and tryptophan-containing analogues of Tat(48–60) and oligoarginine, denoted TatP59W, TatLysP59W and R_7W, respectively, it was found that both endocytotic and non-endocytotic uptake pathways are involved [Thorén et al., Biochem. Biophys. Res. Commun. 307 (2003) 100–107]. Non-endocytotic uptake was only observed for the arginine-rich peptides TatP59W and R_7W. In this paper, the interactions of penetratin, R_7W, TatP59W and TatLysP59W with phospholipid vesicles are compared in the search for an understanding of the mechanisms for cellular uptake. While R_7W, TatP59W and TatLysP59W are found to promote vesicle fusion, indicated by mixing of membrane components, penetratin merely induces vesicle aggregation. Studies of the leakage from dye-loaded vesicles indicate that none of the peptides forms membrane pores and that vesicle fusion is not accompanied by leakage of the aqueous contents of the vesicles. These observations are important for a proper interpretation of future experiments on the interactions of these peptides with model membranes. We suggest that the discovered variations in propensity to destabilize phospholipid bilayers between the peptides investigated, in some cases sufficient to induce fusion, may be related to their different cellular uptake properties.

Keywords: Protein transduction domain; Inverted micelle; Cellular delivery; Antisense

1. Introduction

Due to the barrier properties of the plasma membrane, intracellular delivery of gene-targeted drugs has turned out to be one of the major obstacles for the application of such molecules both in vitro and in vivo. However, with the recent findings that the cellular uptake of large and hydrophilic molecules such as oligonucleotides and proteins can be improved by covalent linkage to short peptides capable of traversing the plasma membrane, their usefulness is likely to improve. Such cell-penetrating peptides (CPPs[1]), also denoted protein transduction domains (PTDs), have been widely used over the last decade to accomplish cellular delivery of various otherwise cell-impermeable compounds (for recent reviews, see Refs. [1–4]). The most well-known CPPs are penetratin, corresponding to the third helix of the Antennapedia homeodomain [5,6], Tat(48–60), derived from the HIV-1 Tat protein [7,8] and oligoarginines [9–11]. CPPs have been reported to be taken up by cells via a

Abbreviations: CF, 5- (and 6-) carboxyfluorescein; CHO, Chinese hamster ovary; CPP, cell-penetrating peptide; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocoline; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000]; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; LUVs, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; P/L ratio, peptide to lipid molar ratio; PNA, peptide nucleic acid; PTD, protein transduction domain; Rh-PE, *N*-(lissamine rhodamine B)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; RET, resonance energy transfer; SDS, sodium dodecyl sulfate; SUVs, small unilamellar vesicles; UV, ultraviolet.

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doi:10.1016/j.bpc.2004.11.016

yet unknown, energy- and receptor-independent uptake pathway [1–4]. The lack of signs of internalization via known uptake pathways has led to the suggestion that direct interactions with the lipid matrix of the plasma membrane is responsible for the uptake of CPPs. Based on this assumption, a model for the transmembrane translocation of penetratin has been proposed [6], in which interactions between the charged residues of the peptide and the phospholipid headgroups leads to local invaginations of the plasma membranes. Inverted micelles would then be formed within the bilayer, allowing passage of the peptide and its cargo without exposure to the hydrophobic interior of the membrane. A similar uptake route has also been proposed for the Tat peptide [7]. Since peptide–lipid interactions have been suggested to play a key role for the cellular uptake of CPPs, penetratin has been extensively studied in a variety of lipid model systems, including vesicles [12–20], SDS micelles [15,21,22], phospholipid monolayers [23] and deposited phospholipid bilayers [24]. Although translocation of penetratin across vesicle membranes has been reported [12], the role of the plasma membrane lipids in the uptake mechanism has remained unclear.

Most studies of the cellular uptake of CPPs found in the literature are based on fluorescence microscopy on fixed cells and/or flow cytometry analysis. Over the years, it has frequently been assumed that washing the cells with buffer after incubation causes desorption of peptide associated with the plasma membrane. This is, however, unlikely to occur for peptides with a high affinity for the cell surface. Thus, it was recently demonstrated that the use of standard cell fixation protocols leads to an artificial uptake of peptide associated with the plasma membrane, despite repeated washings [25,26]. The possible contributions from membrane-associated peptide should also be considered in flow cytometry studies, but this has generally not been the case [25,26]. Although it is unlikely that every observation of CPP uptake stems from experimental artifacts, these recent findings call for a reevaluation of much of the published investigations of the uptake mechanisms of CPPs. In the future, such studies should thus be made on live (unfixed) cells or be based on flow cytometry assays that may discriminate between internalized peptide and peptide bound to the plasma membrane. In live CHO and HeLa cells, uptake via endocytosis has now been reported for Tat(48–60) and oligoarginine [26] and the same observation was also made for VP22, another CPP [25].

In a recently published live cell study, the cellular uptake of carboxyfluorescein (CF)-labeled analogues of penetratin, Tat(48–60) and oligoarginine in V79 and PC-12 cells was examined [27]. In the Tat(48–60) and oligoarginine analogues, denoted TatP59W and R_7W, respectively, a tryptophan residue was inserted in order to be able to take advantage of the useful spectroscopic properties of tryptophan for a comparison of the same peptides in lipid model systems, without the carboxyfluorescein tag [28]. The uptake of penetratin was found to be inhibited by incubation at low temperature or by depletion of intracellular ATP, indicating that penetratin was taken up via endocytosis. R_7W, on the other hand, was efficiently taken up by cells even under such conditions. This suggests a different, energy-independent uptake mechanism for R_7W. For TatP59W, the uptake characteristics were somewhere in between those of penetratin and R_7W. Uptake was inhibited by depletion of intracellular ATP, but not by incubation at low temperature, suggesting that competing uptake pathways may be involved. Strikingly different results were obtained for a Tat analogue with the arginine residues substituted for lysines, TatLysP59W. This peptide was not taken up by live cells and even exhibited a very low affinity for the cell surface. The same result was obtained for a penetratin analogue, where the arginines were substituted for lysines, indicating that arginines play a key role in the cellular uptake of these peptides. Since arginine to lysine substitution does not alter the peptide charge, specific interactions between the amino acids and plasma membrane components may be responsible for the observed differences between arginine-rich and lysine-rich peptides. It has been suggested that bidentate hydrogen bonds formed between the guanidinium group of arginine and membrane moieties containing phosphate and sulfate groups might be the basis for the unique membrane interactions of arginine-rich peptides [9], although it is unclear by which mechanism such interactions may affect the cellular uptake.

In this paper, the interactions of penetratin, R_7W, TatP59W and TatLysP59W (Table 1) with phospholipid vesicles at elevated peptide to lipid molar ratios are examined. While R_7W, TatP59W and TatLysP59W are found to promote vesicle fusion, indicated by mixing of membrane components, penetratin merely induces vesicle aggregation. Studies of the leakage from dye-loaded vesicles indicate that none of the peptides forms membrane pores, and that vesicle fusion is not accompanied by leakage of the aqueous contents of the vesicles. Since vesicle aggregation and fusion can lead to a large increase in turbidity, which complicates spectroscopic measurements, the observations reported here are important for a proper interpretation of future experiments on the interactions of these peptides with model membranes. The discovered variations in propensity to destabilize phospholipid bilayers between the peptides investigated, in some cases sufficient to induce fusion, may be related to the different cellular uptake properties of the peptides.

Table 1
Names and amino acid sequences of peptides studied in this work

Name	Sequence
Penetratin	Ac-RQIKIWFQNRRMKWKK-NH_2
TatP59W	Ac-GRKKRRQRRRPWQ-NH_2
TatLysP59W	Ac-GKKKKKQKKKPWQ-NH_2
R_7W	Ac-RRRRRRRW-NH_2

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocoline (DOPC) was purchased from Larodan. 1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were obtained from Sigma. Polar lipid extract of soybean lecithin, consisting of a mixture of phosphatidylcholine (45.7%), phosphatidylethanolamine (22.1%), phosphatidylinositol (18.4%), phosphatidic acid (6.9%) and others (6.9%), was obtained from Avanti Polar Lipids. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (DSPE-PEG) was from BioTrend. 5- (and 6-) carboxyfluorescein, 5- (and 6-) carboxyfluorescein succinimidyl ester, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and *N*-(lissamine rhodamine B)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE) were purchased from Molecular Probes. Standard Fmoc-protected amino acids were obtained from Nova Biochem (Arg, Lys, Met, Trp, Phe), Alexis (Gln, Asn) and Perseptive Biosystems (Ile). For turbidity and lipid mixing experiments, the buffer was 10 mM HEPES, 5 mM NaOH, 1 mM EDTA and 0.1 M NaCl (pH 7.4). For the induced leakage experiments, the buffer used was 10 mM HEPES, 5 mM NaOH, 1 mM EDTA and 0.107 M NaCl (pH 7.4). Deionized water from a Milli-Q system (Millipore) was used.

2.2. Peptide synthesis

Peptides were synthesized on a Pioneer Peptide synthesizer (Perseptive Biosystems). Fmoc solid-phase synthesis was carried out on an Fmoc-PAL-PEG-PS support, resulting in an amidated carboxyl terminus after cleavage from the resin. After the synthesis was complete, peptides used for spectroscopic studies were capped with an acetyl group at the amino terminus. Penetratin was cleaved from the resin with trifluoroacetic acid/1,2-ethanedithiol/water/triisopropylsilane (94:2.5:2.5:1) for 2 h. For the Tat analogues and R_7W, a solution of trifluoroacetic acid/water/triisopropylsilane (95:2.5:2.5) and a cleavage time of 4 h was used. After precipitation by addition of cold ether, the peptides were collected by centrifugation, washed twice with ether, dried, dissolved in water and lyophilized. Preparative reversed-phase HPLC (Kromasil C8 column, Eka Chemicals) was used to further purify the peptides (isocratic elution: water/trifluoroacetic acid/isopropanol or water/trifluoroacetic acid/acetonitril). Peptide concentrations in aqueous solutions were determined by UV absorbance at 280 nm, using a molar extinction coefficient of 5690 M^{-1} cm^{-1} [29].

2.3. Preparation of large unilamellar vesicles

Chloroform solutions of lipid were mixed to obtain the desired ratio of zwitterionic and negatively charged lipids and the solvent was removed with a rotary evaporator. The dry phospholipid film was placed in high vacuum for 2 h to remove trace amounts of chloroform. Vesicles were prepared by dispersion of the lipid film in buffer by vigorous vortexing. The dispersion was subjected to five freeze–thaw cycles [30] before extrusion 21 times through two 100-nm polycarbonate filters on a LiposoFast-Pneumatic extruder (Avestin, Canada) to obtain large unilamellar vesicles (LUVs). A homogeneous size distribution around 100 nm was confirmed by dynamic light scattering analysis. Dynamic light scattering measurements were performed using an ALV-6010/EPP Multiple Tau Digital Correlator and an ALV/CGS-8F goniometer at a wavelength of 632.8 nm (HeNe-Laser). The scattering angle was maintained at 90° and the temperature kept at 25 °C. The lipid concentration was determined by the Stewart assay [31]. Vesicles with encapsulated CF were prepared as described above, except that the lipid film was dispersed in a solution of 10 mM HEPES, 50 mM CF, 10 mM NaCl, 148 mM NaOH and 1 mM EDTA (pH 7.4).

2.4. Measurements of optical density

Peptide-induced vesicle aggregation, with or without subsequent fusion, was detected by monitoring the time course of the optical density at 436 nm on a Cary 4B UV–Vis spectrometer. All measurements were performed at room temperature in a 1×1 cm quartz cell with a sample volume of 3 mL. The lipid concentration was 100 μM in all experiments and the measured optical density was corrected for dilution upon peptide addition.

2.5. Fluorescence

Fluorescence was measured on a Spex Fluorolog τ-3 spectrofluorometer (JY Horiba) using a 1×1 cm quartz cell thermostated at 25.0 °C and a sample volume of 3 mL. The bandpass of the excitation slit was optimized in order to obtain an optimal signal to noise ratio without photodegradation.

2.6. Lipid mixing

Fusion of vesicles detected as mixing of membrane lipids was monitored using the probe dilution assay of Struck et al. [32]. The assay is based on resonance energy transfer (RET), i.e. the radiationless transfer of excitation energy between a donor and an acceptor molecule. RET results in quenching of the donor fluorescence and, when a fluorescent acceptor is used, an increase of its fluorescence. Since the extent of RET is strongly dependent on the distance between the donor and the acceptor, changes in the surface densities of lipid-anchored probes can be monitored as changes in fluorescence intensity.

Vesicles containing 0.6 mol% each of NBD-PE (donor) and Rh-PE (acceptor) were mixed with unlabeled vesicles at

a 1:4 ratio at a total lipid concentration of 100 μM. The time course of the fluorescence intensity at 530 nm was monitored, using an excitation wavelength of 460 nm. Peptide was added from aqueous stock solution to yield a defined peptide to lipid molar ratio. Fusion of vesicle membranes resulted in dilution of the probes and was detected as an increase in NBD fluorescence at 530 nm, due to diminishing RET. In the concentration range used, the NBD-PE fluorescence intensity increases linearly with the dilution of the probes [32]. The extent of lipid mixing was therefore calculated according to

$$\%\,\text{NBD fluorescence} = 100 \times \left(\frac{F_t - F_0}{F_{100} - F_0}\right) \quad (1)$$

where F_t is the fluorescence intensity at time t after peptide addition, corrected for dilution, F_0 is the fluorescence intensity before peptide addition and F_{100} is the fluorescence intensity corresponding to complete dilution of the probes. F_{100} was recorded in a separate experiment in the absence of peptide, using vesicles with a lipid composition corresponding to that used in the lipid mixing experiments but containing only 0.12 mol% each of NBD-PE and Rh-PE. The total lipid concentration was the same in both types of experiments. Comparison of emission spectra recorded between 470 and 700 nm prior to addition of peptide and 30 min after addition showed that when an increase in NBD fluorescence intensity was observed, it was always accompanied by a decrease in Rh fluorescence intensity, detected at the emission maximum at 590 nm.

2.7. Induced leakage assay

Vesicles loaded with CF were separated from non-entrapped dye on a Sephadex G-50 column (Amersham Pharmacia Biotech), using an isoosmolar buffer (10 mM HEPES, 5 mM NaOH, 1 mM EDTA and 0.107 M NaCl, pH 7.4). The vesicles were diluted to a lipid concentration of 25 μM with isoosmolar buffer in a 1×1 cm cuvette before addition of peptide. Peptide-induced leakage of vesicle-entrapped CF was monitored using excitation and emission wavelengths of 490 and 520 nm, respectively. Starting with a self-quenching concentration of CF (50 mM) inside the vesicles, any leakage of dye can be detected as an increase in fluorescence intensity, due to dilution below self-quenching concentrations. The spontaneous leakage of CF in the absence of peptide was found to be negligible. The extent of leakage was taken to be equal to the corresponding increase in fluorescence intensity according to

$$\%\,\text{Leakage} = 100 \times \left(\frac{I(t) - I_0}{I_T - I_0}\right) \quad (2)$$

where $I(t)$ is the fluorescence intensity at time t, I_0 is the fluorescence intensity before peptide addition and I_T is the fluorescence intensity after lysing the vesicles by addition of Triton X-100 to a final concentration of 0.1 vol.%.

3. Results

3.1. Aggregation/fusion monitored by optical density

Aggregation of large unilamellar vesicles can readily be monitored as an increase in the turbidity of the sample [33]. The effect of adding penetratin, R_7W, TatP59W or TatLysP59W to a vesicle population was investigated by continuously monitoring the optical density at 436 nm. Fig. 1 shows the time course of the optical density in samples containing DOPC/DOPG (60/40) vesicles before and after addition of R_7W (A), TatP59W (B), TatLysP59W (C) and penetratin (D) at the peptide to lipid (P/L) molar ratios indicated in the figure. As evident in Fig. 1, the extent of increase in optical density was highly dependent on the P/L ratio. For R_7W (Fig. 1A), no increase was observed at P/L ratios below 1:150. At higher P/L ratios, a rapid increase in optical density was obtained upon mixing of peptide and vesicles. After the initial, steep slope of the optical density curve, up to ~5 min after mixing, the rate of the increase in signal was slower. After each measurement, the sample was investigated by visual inspection. The samples with the highest peptide to lipid molar ratios, corresponding to the upper two curves in Fig. 1A, were opaque and, after prolonged incubation, the particles were large enough to be observed with the naked eye. For TatP59W and TatLysP59W (Fig. 1B and C), results similar to those of R_7W were obtained. However, the Tat peptides were generally found to induce a larger increase in optical density at similar P/L ratios. Strikingly different results were obtained for penetratin (Fig. 1D). At a P/L ratio of 1:50, the optical density of the sample was not affected by the addition of peptide. When P/L ratios above 1:50 were used, the optical density first increased, indicating rapid vesicle aggregation. The optical density then went through a maximum and eventually decreased. After long times, the optical density of the samples reached the level observed prior to peptide addition, indicating a more or less complete dissociation of the vesicle aggregates. The origin of the discontinuity observed after approximately 100 min at P/L ratios 1:45 and 1:40 is unclear, but notably, it appears only at P/L ratios close to the critical P/L ratio needed to induce aggregation. Dynamic light scattering experiments before peptide addition showed that the vesicles exhibit a homogeneous size distribution around 100 nm in the absence of peptide (data not shown). Upon peptide addition, the particle size was found to increase and the size distribution became heterogeneous. As the optical density decreased, the DLS measurements showed a gradual return to a homogeneous size distribution around 100 nm, indicating restitution of non-aggregated vesicles (data not shown). Very similar results were obtained in an earlier study using DOPG vesicles [13]. By visual inspection of the samples, it was confirmed that the samples were transparent and that no flocculation had occurred. The order of addition (adding peptide to a solution containing vesicles or adding

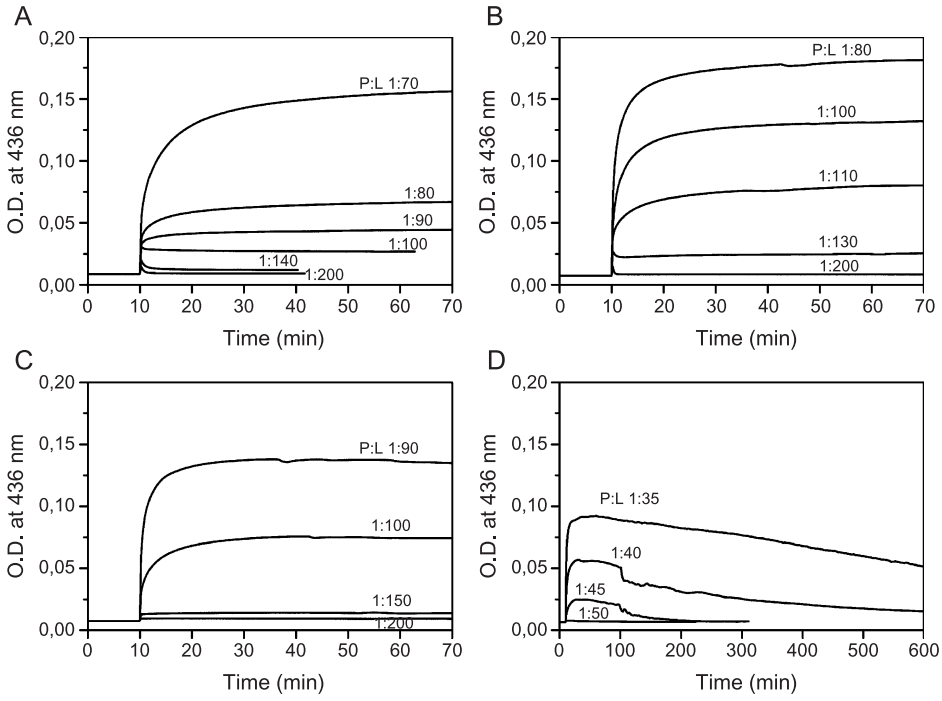

Fig. 1. Time course of the optical density at 436 nm in samples containing DOPC/DOPG (60/40) vesicles at a lipid concentration of 100 μM. Peptide was added after 10 min to yield peptide to lipid molar ratios as indicated in the figures. (A) R_7W, (B) TatP59W, (C) TatLysP59W and (D) penetratin. For penetratin, the optical density eventually returned to the level before peptide addition, indicating dissociation of the vesicle aggregates. The kinetics of dissociation were highly dependent on the peptide to lipid molar ratio. For clarity, the full time course of the decrease in optical density for the upper curves is therefore not shown in the figure. When those samples were left overnight, the optical density had reached the level before peptide addition.

vesicles to a peptide solution) had no effect on the results (data not shown).

The same experiments were also performed using vesicles formed from a soybean lecithin extract, consisting of a heterogeneous mixture of lipids (see Section 2). Results very similar to those presented in Fig. 1 were obtained (data not shown). For zwitterionic vesicles (100% DOPC), on the other hand, none of the peptides induced vesicle aggregation even at a P/L ratio of 1:25 (data not shown).

3.2. Lipid mixing

Measurements of the optical density increase upon mixing vesicles with peptide cannot discriminate between aggregation and fusion of vesicles. Therefore, fluorescence spectroscopy experiments were performed in order to determine whether the peptides were capable of fusing the vesicle membranes. The most widely used assay for detection of mixing of membrane components is based on RET, using the lipid-anchored probes NBD-PE and Rh-PE as donor and acceptor, respectively [32]. Two versions of the assay exist, based on either probe mixing or probe dilution. In the former method, the two probes are incorporated into separate vesicle populations and fusion of the vesicles is detected as the emergence of RET. In the probe dilution assay, on the other hand, both probes are incorporated into one set of vesicles. Subsequent fusion with unlabeled vesicles, often added in excess, then results in dilution of donors and acceptors and, consequently, diminishing RET. It has been shown, that the probe mixing assay is sensitive to aggregation, since close contact of vesicle bilayers during aggregation leads to energy transfer between contacting bilayers even in the absence of fusion [34]. The probe dilution assay, on the other hand, has been shown not to be affected by mere aggregation of vesicles. No significant transfer of probe from labeled vesicles to unlabeled vesicles takes place, unless the vesicle membranes fuse [34]. For discrimination between vesicle aggregation and fusion, this assay is thus more suitable.

Mixing of membrane lipids was investigated by mixing vesicles containing NBD-PE and Rh-PE with unlabeled vesicles at a 1:4 ratio. In a series of experiments, peptide was added at various P/L ratios and the time course of the fluorescence intensity of NBD-PE (donor) was monitored. Fig. 2 shows the extent of lipid mixing, calculated according to Eq. (1), for DOPC/DOPG/NBD-PE/Rh-PE (60/38.8./0.6/0.6) and DOPC/DOPG (60/40) vesicles in the presence of R_7W at various P/L ratios. Peptides were added after 5 min. In the absence of peptide, no fusion occurs. At P/L ratios too low to induce vesicle aggregation (cf. Fig. 1A), no fusion is detected (Fig. 2, lower trace). At higher P/L ratios, on the other hand, the NBD fluorescence intensity increases with time, indicating that fusion of the vesicle membranes is induced by the peptide. Both the kinetics and the extent of lipid mixing are dependent on the P/L ratio. The corresponding experiments were also performed for

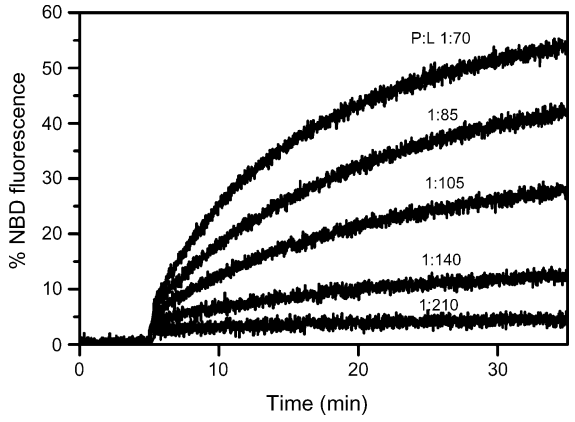

Fig. 2. Extent of lipid mixing detected by the RET assay upon addition of R_7W to vesicles composed of DOPC/DOPG/NBD-PE/Rh-PE (60/38.8/0.6/0.6) and DOPC/DOPG (60/40). The ratio of labeled and unlabeled vesicles was 1:4 and the total lipid concentration was 100 μM. Peptide was added after 5 min to yield the peptide to lipid molar ratio indicated above each trace in the figure. The extent of mixing was calculated according to Eq. (1).

TatP59W, TatLysP59W and penetratin. The two Tat peptides were also found to able to induce membrane fusion. In order to be able to compare the peptides investigated, the calculated extent of lipid mixing 30 min after peptide addition was plotted against the P/L ratio (Fig. 3A). Evidently, the efficiency of peptide-induced fusion decreases in the order R_7W>TatP59W>TatLysP59W, although the Tat peptides aggregate vesicles with a higher efficiency than does R_7W (cf. Fig. 1). Interestingly, no lipid mixing was observed for penetratin even at high P/L ratios. In fact, a small decrease (~5%) in NBD fluorescence intensity was obtained immediately after mixing of penetratin with the vesicles (data not shown).

In order to investigate the influence of the lipid composition on the peptide-induced membrane fusion, the lipid mixing assay was also applied on vesicles formed from the soybean lipid extract described in the previous section (Fig. 3B). R_7W, TatP59W and TatLysP59W were found to induce fusion to a similar extent as in the case of DOPC/DOPG vesicles. The relative fusion efficiency of the peptides was the same as for the DOPC/DOPG vesicles. As was the case for DOPC/DOPG vesicles, penetratin did not induce membrane fusion, only vesicle aggregation/disaggregation (see previous section).

It has been shown that inclusion of poly(ethylene glycol)-lipid conjugates in the lipid bilayers can inhibit fusion of vesicles induced by, e.g., Ca^{2+} [35] and phospholipase C [36]. In the present study, the effect of including phosphatidylethanolamine conjugated to PEG_{2000} (DSPE-PEG) was investigated. When using vesicles composed of DOPC/DOPG/DSPE-PEG (60/35/5) and DOPC/DOPG/DSPE-PEG/NBD-PE/Rh-PE (60/33.8/5/0.6/0.6),

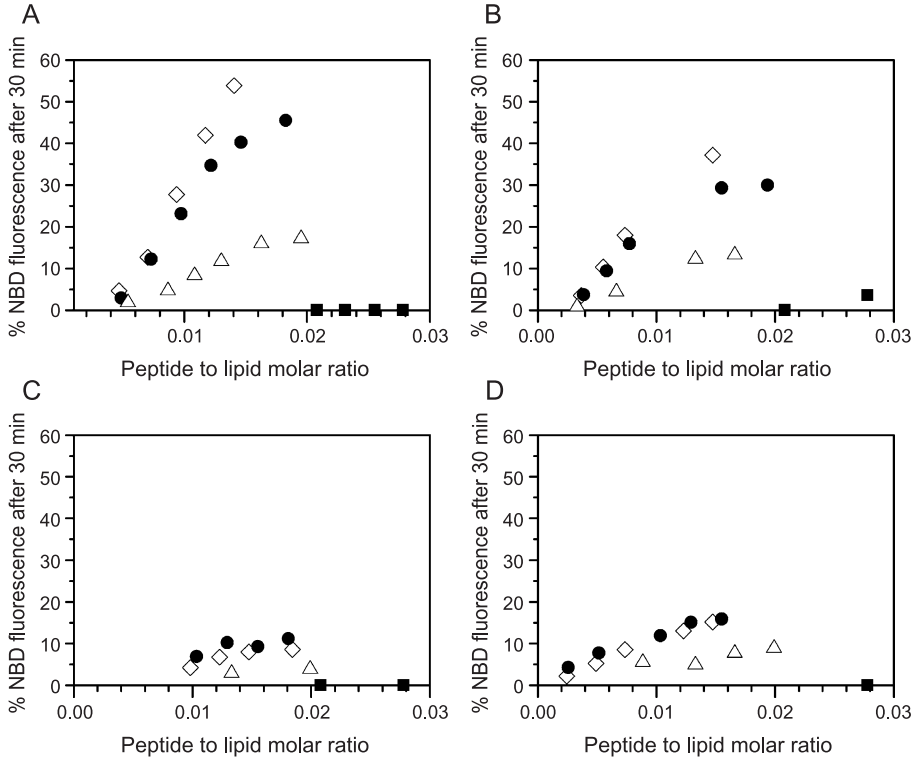

Fig. 3. Extent of lipid mixing, calculated according to Eq. (1) for vesicles with varying lipid composition. Experiments were performed as described in Fig. 2, and the NBD fluorescence intensity 30 min after peptide addition was used as a measure of membrane fusion in order to compare the peptides. R_7W (◇), TatP59W (●),TatLysP59W (△) or penetratin (■) was added to vesicles composed of (A) DOPC/DOPG/NBD-PE/Rh-PE (60/38.8/0.6/0.6) and DOPC/DOPG (60/40); (B) soybean lipid extract (described in Section 2) with and without 0.6 mol% each of NBD-PE and Rh-PE (C) DOPC/DOPG/DSPE-PEG/NBD-PE/Rh-PE (60/33.8/5/0.6/0.6) and DOPC/DOPG/DSPE-PEG (60/35/5) and (D) DOPC/DPPG/NBD-PE/Rh-PE (60/38.8/0.6/0.6) and DOPC/DPPG (60/40).

vesicle fusion was strongly inhibited for all the peptides (Fig. 3C). Only a small, instantaneous increase in NBD fluorescence was obtained at elevated P/L ratios. When the optical density was investigated, using unlabeled vesicles and the protocol described in the previous section, only a minute increase could be observed at elevated P/L ratios (data not shown). This shows that not only membrane fusion but also vesicle aggregation was inhibited by the presence of 5 mol% DSPE-PEG. When 2.5 mol% DSPE-PEG was used, R_7W, TatP59W and TatLysP59W were still able to induce vesicle aggregation and fusion, albeit to a much smaller extent than without the PEG-lipid (data not shown). For penetratin, on the other hand, vesicle aggregation was more or less completely inhibited already at 2.5 mol% DSPE-PEG (data not shown).

In another series of experiments, the negatively charged lipid DOPG was substituted for dipalmitoylphosphatidylglycerol (DPPG), which has the same polar headgroup. The only difference between the two lipids is that while the hydrocarbon chains of DOPG have 18 carbon atoms and a double bond between carbon atoms 9 and 10, those of DPPG have 16 carbon atoms and are saturated. Fig. 3D shows the extent of lipid mixing for DOPC/DPPG/NBD-PE/Rh-PE (60/38.8./0.6/0.6) and DOPC/DPPG (60/40) vesicles. Interestingly, the extent of lipid mixing was strongly reduced for R_7W, TatP59W as well as TatLysP59W, compared to the vesicles containing the same fraction of DOPG (Fig. 3A). Penetratin did not induce lipid mixing in the vesicles containing DPPG either.

3.3. Leakage of vesicle contents

Penetratin-induced dye leakage has been examined previously in vesicles of various lipid compositions [12,14,18], and only a minute leakage has been observed, even at high P/L ratios. It is thus obvious that penetratin does not form membrane pores and that penetratin-induced vesicle aggregation only results in a very small extent of release of vesicle contents. Here, a comparative study of the leakage from carboxyfluorescein-loaded DOPC/DOPG (60/40) vesicles in the presence of penetratin, R_7W, TatP59W and TatLysP59W was performed, as described in Section 2. When peptide was added to the vesicles, a small, instantaneous increase in fluorescence intensity was obtained but no significant further increase was observed with time (data not shown). The carboxyfluorescein fluorescence intensity 15 min after peptide addition was used as a measure of the peptide-induced dye leakage. In line with previous findings [12,14,18], the extent of penetratin-induced leakage, calculated according to Eq. (2), was found be only 1.6% at the highest P/L ratio examined (1:18) (data not shown). For R_7W, the leakage was no more than 2.8% for the highest P/L ratio examined (1:17), while the extent of leakage in the corresponding experiments for TatP59W and TatLysP59W was 1.9% and 0.8%, respectively (data not shown). Since the classical pore-forming peptide melittin has been found to induce a CF leakage of >80% in the corresponding experiments (Thorén et al., unpublished results), it is clear that none of the peptides in this study forms membrane pores. Furthermore, even under conditions where R_7W, TatP59W and TatLysP59W promote lipid mixing, the leakage of vesicle contents is minute.

4. Discussion

The promising prospect of using cell-penetrating peptides for the delivery of gene-targeted drugs necessitates an understanding of the underlying uptake mechanism. Important clues regarding the internalization process can be obtained in studies of the interaction of CPPs with lipid model systems. This paper describes the membrane interactions of four different peptides: penetratin, R_7W, TatP59W and TatLysP59W. The experiments presented here show that the peptides under investigation interact differently with phospholipid vesicles. An interesting comparison can be made with the recently presented live cell study, where these four peptides exhibited vastly different uptake characteristics [27]. R_7W, which exhibited the most efficient cellular uptake in live cells and was taken up by an apparently energy-independent pathway [27], has the strongest ability to induce vesicle fusion. For TatP59W, which was taken up mainly via endocytosis but also exhibited an efficient uptake at 4 °C [27], vesicle fusion is also efficient. Penetratin, on the other hand, which was shown to enter cells via endocytosis [27], lacks fusogenic properties. Based on these three observations, there thus appears to be a correlation between the cellular uptake efficiency of these peptides and their ability to destabilize phospholipid membranes sufficiently to induce membrane fusion. TatLysP59W breaks the pattern, however. The peptide exhibited no cellular uptake and a very low affinity for the surfaces of the cells examined [27]. In the study presented here, on the other hand, it is clear that membrane fusion is induced, although to a smaller extent than for TatP59W. If the membrane destabilization effect observed in this study is relevant for the cellular uptake of these peptides, it is possible that the difference in cellular uptake between TatP59W and TatLysP59W lies mainly in their different affinity for the cell surface. One could speculate that TatP59W has a much stronger affinity than TatLysP59W for some component present in cell membranes but not in the membrane model system employed here. Indeed, studies have shown that the lipid affinity of these peptides is of the same magnitude [28]. As to the interesting observation of subsequent dissociation of vesicle aggregates formed upon addition of penetratin, it can be noted that the aggregation/disaggregation process resembles that observed in pure DOPG vesicles [13], except that it takes place on a longer time scale and at lower peptide to lipid molar ratios. The latter observation can be related to the smaller fraction of negatively charged lipids used in the present study, lowering the bound peptide concentration

required for charge neutralization. The origin of the disaggregation phenomenon is not known, but it was speculated that the process might involve transmembrane translocation of peptide molecules in aggregated vesicles [13]. Notably, the aggregation/dissociation phenomenon was not only observed in DOPC/DOPG vesicles, but also in vesicles formed from a soybean lipid extract, showing that it is not only present in vesicles formed from synthetic lipids. It remains to be established whether this peculiar property of penetratin has any biological relevance.

Fusion of biological membranes has been the subject of a large number of studies over the last decades (for recent reviews, see Refs. [37–39]). Because of their defined structure and the vast possibilities of varying lipid composition as well as experimental conditions, phospholipid vesicles have been frequently employed as model systems. The most rigorous definition of vesicle fusion is mixing of the membrane components of contacting vesicles and the concomitant coalescence of their internal compartments [40]. In the present study, however, the mixing of membrane lipids was taken as the only criterion for fusion, distinguishing fusion from vesicle aggregation. Whether mixing of aqueous contents takes place was not investigated.

Since lipid bilayers are generally stable dynamic structures that do not fuse spontaneously, a destabilization is required for fusion to occur, enabling rearrangement of lipids from that of two juxtaposed bilayers to a final state as a single bilayer. Generally, this transition has been shown to be promoted by the presence of lipids with a negative spontaneous curvature, while lipids with a positive spontaneous curvature are able to inhibit fusion (see, for example, Refs. [37,41] and references therein). This implies that the fusion process involves intermediate stages, where membrane lipids transiently adopt highly curved nonbilayer structures [37,41]. Two main models for the bilayer fusion process have been postulated, both involving structural alterations in a small part of the bilayer, in the region of contact between fusing vesicles. In the first model, it is suggested that the merging of bilayers occurs via the formation of inverted micellar intermediates (see Ref. [40] and references therein). The second proposed mechanism for the bilayer transition involves the formation of semitoroidal structures, so-called "stalk" intermediates [42–44]. The stalk intermediate is depicted as an hourglass shape resulting from the merging of the proximal leaflets (*cis* monolayers) of the interacting membranes, while the distal leaflets (*trans* monolayers) remain relatively undisturbed. The stalk subsequently evolves into a fusion pore, connecting the aqueous compartments. It should be noted, however, that there is yet no direct experimental evidence for any of the proposed intermediate structures. This has been attributed to their transient nature [37].

In this context, it is of interest to consider the forces acting between two approaching bilayers. The interaction energy of vesicles containing negatively charged lipids is determined mainly by three types of forces: electrostatic repulsion, van der Waals attraction and hydration forces. The first two forces are long-range forces, while hydration forces become dominating at shorter surface separations, below ~2–3 nm. Hydration forces arise due to the work involved in removing water molecules tightly bound to the phospholipid headgroups (see Refs. [40,45] and references therein). Cations can induce aggregation by screening the charges on the vesicle surface or by causing charge neutralization upon binding to the vesicle surfaces, thereby decreasing the electrostatic repulsion [40]. There are also examples where polycations have been shown to induce vesicle aggregation by dehydrating the phospholipid headgroups [45–47]. For fusion to occur, the membranes must be brought in close contact. As mentioned above, the repulsive hydration forces dominate at short intermembrane distances, and removal of interfacial water should thus be necessary for molecular contact to be established [40,45]. Since hydration forces are proportional to membrane area, less work is necessary if close contact is established at a localized point [37]. Perturbation of the bilayer structure at the site of contact can subsequently lead to merging of the membranes. It is thus not necessarily true that a defined intermediate structure is required for fusion to occur [40].

The ability of peptides such as R_7W and TatP59W to promote fusion of LUVs, while penetratin only induces vesicle aggregation, is remarkable. Polycations without hydrophobic character, such as polylysine and polyhistidine, have previously been reported to induce fusion of small unilamellar vesicles (SUVs) [48,49]. However, these observations more likely only reflect an intrinsic instability of SUV membranes rather than fusogenic properties of the polycations, since LUVs with a similar lipid composition were found to merely aggregate in the presence of polylysine [40,50]. SUVs have a built-in strain due to the strong curvature and are therefore more prone to fuse than LUVs (see Ref. [50] and references therein), the polycation merely serving as a promoter of vesicle aggregation. LUVs are thus obviously a better membrane model system, making the observation that R_7W promotes fusion of LUVs even more interesting. Since all the peptides studied are capped in both the N-terminus and the C-terminus, penetratin and R_7W carry seven positive charges, while the Tat peptides carry eight positive charges at neutral pH. For all these highly charged peptides, binding to the negatively charged bilayers results in charge neutralization, which facilitates vesicle aggregation. There is, however, no clear correlation between peptide charge and the P/L ratio, at which aggregation occurs (cf. Fig. 1A–D). The finding that the Tat peptides induce aggregation of DOPC/DOPG (60/40) vesicles at lower P/L ratios than R_7W is consistent with their higher charge. However, R_7W induced strong aggregation at a P/L ratio of ~1:100, while penetratin-induced aggregation occurs at a P/L ratio of ~1:45, suggesting that additional factors such as the distance between the charges within the peptides and dehydration effects play an important role. The effect of peptide binding on the

interfacial hydration is, however, difficult to predict. While it was recently shown that the peptides bind to vesicles with a comparable affinity and that the tryptophan residues of these peptides are located at approximately the same depth in the lipid bilayer [28], it is possible that the membrane penetration depth of the rest of the amino acid residues of the peptides is quite different. One could speculate that penetratin, which has a higher content of apolar amino acid residues, adopts an orientation below the level of the phosphate groups of the membrane lipids, while R_7W, TatP59W and TatLysP59W preferentially assume a position with their charged residues mainly in the aqueous region containing the lipid headgroups. A lack of significant penetration into lipid bilayers has been suggested for other oligopeptides lacking hydrophobic residues, such as oligolysine [51,52]. Such a difference in the positioning of the peptides might result in a different impact on the hydration of the lipid headgroups. A deeper penetration for penetratin could result in less dehydration of the membrane lipids, which would be consistent with the observed lack of peptide-induced vesicle fusion. The shallow membrane penetration of R_7W, TatP59W and TatLysP59W suggested above implies that the C-terminus, which contains the tryptophan residue and the amidated C-terminus, must "dip down" into the membrane interior in order to position the tryptophan at a depth equal to that of the tryptophan residues in penetratin. Such a peptide orientation could create defects in the lipid packing, which would further promote vesicle fusion, in addition to the dehydration effects discussed. Insertion of peptides at an oblique angle has been shown to promote negative curvature in lipid bilayers and is a common feature of many membrane fusion peptides (see Ref. [53] and references therein). It is thus possible that peptides with the corresponding sequences, but lacking the tryptophan residue, would not be as fusogenic as the ones studied here. Notably, while R_7W, TatP59W and TatLysP59W do not form an ordered structure upon membrane binding [28], penetratin exhibits an interesting conformational behavior in the presence of vesicles containing anionic lipids. Penetratin has been shown to undergo an α-helix to β-sheet transition in DOPG vesicles at elevated P/L ratios, simultaneous with vesicle aggregation [13]. Since then, it has been shown by using vesicles containing 2.5 mol% DSPE-PEG, sufficient to prevent penetratin-induced vesicle aggregation, that no β-sheet formation is obtained at high P/L ratios in the absence of aggregation [54]. It is thus likely that it is the vesicle aggregation that drives the α-helix to β-sheet transition, rather than vice versa. It is possible that the larger conformational flexibility of R_7W, TatP59W and TatLysP59W plays a role in the fusion process.

The investigation of the influence of lipid composition on the peptide-induced vesicle fusion yielded some interesting results. The presence of 5 mol% of PEG_{2000}-conjugated lipids was found to efficiently inhibit peptide-induced fusion of vesicles. Inclusion of PEG-lipid conjugates has previously been reported to prevent calcium-mediated fusion [35] and phospholipase C-induced fusion [36]. The ability of PEG-lipids to inhibit fusion has been attributed to steric repulsion of contacting bilayers [35] as well as stabilization of the lamellar phase, preventing the formation of highly curved intermediate structures involved in the fusion mechanism [36]. Importantly, since not only fusion, but also vesicle aggregation is inhibited for the peptides examined here, vesicles containing PEG-lipids can be used for spectroscopic studies of peptide–lipid interactions in the absence of extensive vesicle aggregation, as in the recent binding study mentioned above [28].

Interestingly, when the negatively charged lipid DOPG was substituted for DPPG, vesicle fusion was strongly inhibited for R_7W, TatP59W as well as TatLysP59W. Because the two lipids have the same polar headgroup, the observed inhibition of fusion must be attributed to their acyl chains. The difference between the two lipids can be discussed in terms of molecular geometry. Since the acyl chains of DOPG are unsaturated, the cross-sectional area at the headgroup region is smaller than at the hydrocarbon region and the molecule has a slightly cone-shaped structure. DPPG, on the other hand, has two saturated acyl chains and thus a similar cross-sectional area at the headgroup and hydrocarbon regions, giving the molecule a cylinder shape [55]. The inhibitory effect of using DPPG instead of DOPG suggests that induction of negative curvature in the lipid bilayers, which is favored by the use of slightly cone-shaped lipids like DOPG, may be involved in the membrane fusion mechanism for R_7W, TatP59W and TatLysP59W. It is likely that lipids with a larger propensity to form structures with negative curvature, such as phosphatidylethanolamine, would further promote fusion.

No detailed examination of the kinetics of aggregation and fusion was performed in this study. The rate of lipid mixing (Fig. 2) is apparently considerably slower than the corresponding increase in optical density (Fig. 1A), suggesting that vesicle aggregation is rapid, while the fusion process is slower. It has been suggested that the bulky headgroups of NBD-PE and Rh-PE may hinder diffusion of the labeled lipids across the contact points between fusing vesicles [56]. This would slow the probe dilution down, compared to the overall lipid mixing process. It is thus possible that membrane fusion is much more rapid than indicated by the RET assay. Since the geometry of the fusion pore is unknown, the probability of hindered probe diffusion is difficult to estimate.

How can the present study help us understand the mechanism of uptake of CPPs? For R_7W, an efficient cellular uptake has been observed both at low temperature and with depletion of intracellular ATP [27]. One could speculate that the peptide is able to enter cells via a local destabilization of the plasma membrane, as indicated by the fusion assay and the indirect evidence of induction of inverted membrane structures. This may take place at points of transient invaginations in the plasma membrane, which are known to appear even at low temperature [57]. TatP59W

is also taken up by cells at low temperature, while the uptake of penetratin was inhibited under such conditions [27]. The latter observation may be related to its lack of bilayer-destabilizing properties, indicated by the lipid mixing assay. Importantly, membrane destabilization in this context is not synonymous with pore formation, which would be toxic to cells. The leakage experiments show that none of the peptides examined here form membrane pores. Furthermore, a very low toxicity has been reported for both oligoarginines and the Tat CPP, and it has been well established that neither of these peptides cause leakage of cell contents [7,11,58].

Although endocytosis apparently plays a significant role in the uptake of at least some of the CPPs [25–27], the fact that CPPs have been successfully employed to deliver oligonucleotides, PNAs and proteins to the cytoplasm and nucleus of cells [4,59–61] implies that at least a fraction of the conjugates taken up by endocytosis is able to escape from the endosomes. The differences observed in the lipid mixing assay could reflect the relative abilities of the peptides investigated to escape from endosomal compartments. The results presented here may thus help to explain, e.g., the higher carrier efficiency reported for the Tat peptide compared to penetratin [4].

Another interesting aspect of the present study is the possibility of using CPPs to merge drug-containing vesicles with cell membranes. Although possible artifacts emanating from the use of cell fixation cannot be ruled out, liposomes with Tat(48–60) attached to the liposome surface via PEG-conjugated lipids have been demonstrated to be efficiently internalized by cells [62]. It is possible that CPPs capable of fusing membranes have a huge potential in facilitating the intracellular release of vesicle-encapsulated drugs.

Acknowledgements

This work was supported by the Strategic Nucleic Acid Research Program and the Swedish Research Council.

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