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Interactions of histatin 5 and histatin 5-derived peptides with liposome membranes: surface effects, translocation and permeabilization

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A number of cationic antimicrobial peptides, among which are histatin 5 and the derived peptides dhvar4 and dhvar5, enter their target cells and interact with internal organelles. There still are questions about the mechanisms by which antimicrobial peptides translocate across the membrane. We used a liposome model to study membrane binding, translocation and membrane-perturbing capacities of histatin 5, dhvar4 and dhvar5. Despite the differences in amphipathic characters of these peptides, they bound equally well to liposomes, whereas their membrane activities differed remarkably: dhvar4 translocated at the fastest rate, followed by dhvar5, whereas the histatin 5 translocation rate was much lower. The same pattern was seen for the extent of calcein release: highest with dhvar4, less with dhvar5 and almost none with histatin 5. The translocation and disruptive actions of dhvar5 did not seem to be coupled, because translocation occurred on a much longer timescale than calcein release, which ended within a few minutes. We conclude that peptide translocation can occur through peptide–phospholipid interactions, and that this is a possible mechanism by which antimicrobial peptides enter cells. However, the translocation rate was much lower in this model membrane system than that seen in yeast cells. Thus it is likely that, at least for some peptides, additional features promoting the translocation across biological membranes are involved as well.

Key words: antimicrobial peptide, histatin, liposome, LL-37, membrane translocation.

INTRODUCTION

In the last few decades, an expansive number of antimicrobial peptides have been isolated and characterized from virtually all classes of organisms, where they play an important role in the innate defence against microbial and viral infections. Characterization of these peptides has revealed a wide diversity in amino acid sequences, yet with shared characteristic features: they are usually polycationic and amphipathic, containing both a hydrophilic and a hydrophobic side [1–3]. This promotes their binding to and insertion into the cytoplasmic membrane of the target cell, leading to perturbation of the membrane. Although membrane lysis almost certainly is a key event in the bactericidal action of many antimicrobial peptides, including temporins [4], cathelicidins [5–7] and mellitin [8,9], evidence has been produced that, for a number of peptides, this is not the major mechanism of action. For example, human salivary histatins and tenecin 3 have been shown to penetrate fungal pathogens to induce lethal action intracellularly [10,11]. Also for other peptides, including histatin derivatives [12] and lactoferricin B [13], uptake by their target cells and killing apparently are obligatory linked processes. Internalization of these antimicrobial peptides by target cells share some features with uptake of basic peptides in mammalian cells, e.g. the HIV-tat peptides, polyarginine and polylysine [14–18]. Apparently, uptake does not require a distinct amino acid sequence; nor is the chirality of the peptide backbone essential [16,19]. On the other hand, an active metabolic condition for the yeast or mammalian cell in many cases is a prerequisite for internalization, since conditions and agents that diminish metabolism, e.g. oxygen depletion or inhibition of respiration by sodium azide, abrogated the translocation into the cell [10,15,20].

Although there is ample evidence that peptides translocate over biological membranes, the internalization pathway is still a matter of debate. The susceptibility to metabolic inhibitors has been taken as evidence that active, endocytotic internalization pathways are responsible for the uptake. On the other hand, it has been argued that, since a specific primary structure is not essential, internalization does not involve active endocytosis or specific protein transporters. Instead, a direct transport through the lipid bilayer of the membrane, e.g. involving self-promoted uptake via preformed peptide pores, has been suggested as a possible mechanism of translocation [21,22]. Direct experimental evidence in support of either mechanism, however, is scarce. In the present study, we have addressed the question whether antimicrobial peptides with different amphipathic features are able to diffuse passively through an artificial phospholipid bilayer. As model compounds for antimicrobial peptides, we used histatin 5, a natural antimicrobial peptide from human saliva, and two synthetic variants derived from it, with enhanced and decreased amphipathic features respectively [12,23]. Each of these peptides has activity against yeast cells, and is internalized in these cells during the killing process [12]. Translocation of peptides was examined by monitoring peptide degradation by trypsin encapsulated in PC/PS (L-α-phosphatidylcholine/3-*sn*-phosphatidyl-L-serine) liposomes. To improve our understanding, the interaction with the liposome membrane was characterized with regard to the

Abbreviations used: BAPNA, Nα-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; CZE, capillary zone electrophoresis; Fmoc, fluoren-9-ylmethoxycarbonyl; PC, L-α-phosphatidylcholine; PS, 3-*sn*-phosphatidyl-L-serine; RP, reverse phase; SBTI, soya-bean trypsin inhibitor; TFA, trifluoroacetic acid.

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amount of peptides bound to liposomes, the effect on the external electrokinetic (zeta) potential of the phospholipid membrane, and the disturbance of the membrane integrity.

EXPERIMENTAL

Peptide synthesis and purification

Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY), dhvar4 (KRLFKKLLFSLRKY), dhvar5 (LLLFLLKKRKKRKY) and the control peptides cysS1–13 (SSSKEENRIIPGG: the 13 N-terminal amino acids of cystatin S) and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES: the human cathelicidin antimicrobial peptide, which is strongly membrane active [24,25]) were synthesized by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry on a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, MA, U.S.A.) according to the manufacturer's procedures. *N*-α-Fmoc-protected amino acids and preloaded PEG–PS [poly(ethylene glycol)–polystyrene] supports were obtained from Applied Biosystems (Foster City, CA, U.S.A.). Peptides were purified by preparative RP (reverse phase)-HPLC on a Jasco HPLC System (Jasco Corporation, Tokyo, Japan) using a VYDAC C_{18}-column (218TP; 1.0 cm $\times$ 25 cm, 10 μm particles, Hesperia, CA, U.S.A.), as described previously [26]. Briefly, peptides were dissolved in 0.1 % (v/v) TFA (trifluoroacetic acid) and eluted with a linear gradient [from 15–60 % (v/v) acetonitrile containing 0.1 % (v/v) TFA] in 20 min at a flow rate of 4 ml/min. The absorbance of the column effluent was monitored at 214 nm, and peak fractions were pooled, freeze-dried and reanalysed by RP-HPLC using an analytic column, and also by CZE (capillary zone electrophoresis; see below). The purity of the peptides was at least 95 %, and the authenticity of the peptides was confirmed by ion-trap MS (mass spectrometry) on a LCQ Deca XP (Thermo Finnigan, San Jose, CA, U.S.A.).

CZE

Purity and degradation of peptides was analysed by CZE on a BioFocus 2000 Capillary Electrophoresis System (Bio-Rad, Hercules, CA, U.S.A.) equipped with an uncoated fused silica capillary of internal diameter 50 μm, and a length of 24 cm. Samples were loaded by pressure injection at 20 $\mathrm{lbf \cdot in^2 \cdot s}$ (where 1 $\mathrm{lbf \cdot in^2} = 6.9$ kPa), and peptide separation was performed according to the manufacturer's instructions at 10 kV (anode at the detector side) and 20 °C using 0.1 M phosphate buffer, pH 2.5, as the electrolyte. On-line UV detection was performed at 200 nm. The running time of the analyses was 7 min, and data were analysed using BioFocus Integrator software. Peptide degradation was quantified by comparing the relevant peak heights with those of the internal standard imidazole.

Liposome preparation

Unilamellar liposomes were prepared from a 9:1 (w/w) mixture of egg-yolk PC and PS from bovine brain (Sigma–Aldrich, St Louis, MO, U.S.A.). This liposome composition containing negatively charged phospholipids (i.e. PS) was chosen as a model for microbial membranes. The lipid mixture in chloroform was vacuum-dried and hydrated in 1 ml of 10 mM Hepes buffer, pH 7.2, to a final concentration of 2.5 mg/ml. After five cycles of freeze-thawing, liposomes were extruded 21 times through two stacked 400 nm filters, as described previously [27].

Calcein-loaded liposomes and trypsin-loaded liposomes were prepared by hydration of the lipid mixture in 10 mM Hepes buffer, pH 7.2, containing either 0.5 mM calcein or 100 μg/ml trypsin (EC 3.4.21.4, sequencing grade; Roche Diagnostics GmbH, Mannheim, Germany) and processed further, as described above. Free calcein and trypsin were removed by centrifugation on a Sephadex G-50 column, essentially as described previously [28]. Liposomes were stored at 4 °C and used within 48 h.

Zeta potential measurements

Effects of peptides on the zeta potential of liposomes were examined as follows: dhvar4, dhvar5 or histatin 5, in concentrations ranging from 2.5 to 20 μM, were incubated for 5 min with liposomes suspended in 10 mM Hepes, pH 7.2, at a lipid concentration of 37.5 μg/ml. The zeta potentials derived from the electrophoretic mobilities of the liposome preparations were measured on a DELSA 440 (Coulter Electronics, Inc., Hialeah, FL, U.S.A.), which employs a four-angle detection of the Doppler shift of frequency of scattered laser light. Data were analysed with the accompanying analysis software (the DELSA 440SX control program version 2.0.1) according to the manufacturer's instruction manual, essentially as described previously [29].

Peptide binding to liposomes

Liposomes were suspended at 250 μg/ml lipid in Hepes buffer. Binding (including uptake) was measured as the depletion of peptides in supernatants of liposomes incubated for 15 min with 5–20 μM dhvar4, dhvar5, histatin 5 or cysS1–13. Samples were centrifuged at 200 000 ***g*** at 4 °C for 30 min, and the concentrations of the remaining peptides in supernatants were measured by CZE.

Peptide-induced liposome permeabilization

Calcein-loaded liposomes were used to measure peptide-induced permeation of liposomes. To a suspension of calcein-loaded liposomes (250 μg/ml lipid) in Hepes buffer, 40 μM dhvar4, dhvar5 or histatin 5 was added. The changes in fluorescence intensities were determined as a percentage of the maximal fluorescence intensity obtained after total lysis of the liposomes by 0.5 % (v/v) Triton X-100. Calcein fluorescence was monitored continuously over time at excitation and emission wavelengths of 494 nm and 520 nm respectively, on an SLM-Aminco SPF-500 fluorescence spectrometer. Because of an increase in turbidity of the liposome suspension upon peptide addition, another method was also used, in which the release of calcein was determined in liposome supernatants. In this assay, the calcein-loaded liposomes were centrifuged at 200 000 ***g*** for 30 min after a 15 min peptide incubation, and the calcein fluorescence in the supernatant was determined.

Determination of trypsin activity

Trypsin-loaded liposomes were suspended at a lipid concentration of 250 μg/ml. The internal trypsin activity of trypsin-loaded liposomes and the concentration of SBTI (soya-bean trypsin inhibitor) required to block this activity was determined using FITC-labelled gelatin (DQ-gelatin; Molecular Probes, Eugene, OR, U.S.A.) as substrate. This substrate consists of highly quenched FITC-labelled gelatin that, upon proteolytic digestion, releases fluorescent fragments. Trypsin-loaded liposomes were lysed with Triton X-100 and incubated with FITC-gelatin in Hepes buffer supplemented with various concentrations of SBTI. Fluorescence

Table 1 Sequences and characteristics of the peptides investigated

Data shown in the column labelled 'Charge' represent the total net charge based on the number of charged amino acids at pH 7. $\langle\mu\rangle$ represents the mean hydrophobic moment of each respective peptide in an α-helical conformation. LC_{50}, peptide concentration (μM) at which 50 % of *C. albicans* cells are killed [12].

Peptide	Sequence	Mass (M_r)	Charge	Mean hydrophobic moment ($\langle\mu\rangle$)	LC_{50} (μM)
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	3037.1	5+	0.09	8.3
dhvar4*	KRLFKKLLFSLRKY	1840.7	6+	0.44	0.1
dhvar5	LLLFLLKKRKKRKY	1847.8	7+	0.06	2.0

* This study includes dhvar4 (a more amphipathic variant derived from the active domain of histatin 5) and dhvar5 (a peptide in which the hydrophobic residues are concentrated in the N-terminal part and the hydrophilic residues in the C-terminal part of the peptide).

was monitored in a fluorescence microplate reader (FLUOstar Galaxy; BMG Laboratories GmbH, Offenburg, Germany) at excitation and emission wavelengths of 485 nm and 520 nm respectively. The capacity of 20 μg/ml SBTI to inhibit all trypsin in the samples was confirmed by incubation of 40 μM peptides with 1 μg/ml trypsin or lysed trypsin-loaded liposomes in the presence of 20 μg/ml SBTI. After different time intervals, aliquots were taken and analysed by CZE, revealing that the peptides were not degraded, and confirming that the SBTI concentration was high enough to effectively inhibit all trypsin.

Liposome-bound peptide degradation

To study whether liposome-bound peptides were susceptible to degradation, 10 μM peptides were incubated in Hepes buffer with liposomes at 250 μg/ml lipid, with addition of 10 μg/ml imidazole. After 15 min of incubation, binding of the peptides to the liposomes was determined as described above. Thereafter, 1 μg/ml trypsin was added to the liposome/peptide mixture and degradation was followed in time by CZE. As a reference, peptides were incubated with trypsin in buffer without liposomes.

Degradation of peptides by trypsin-loaded liposomes

Trypsin-loaded liposomes were suspended in Hepes buffer supplemented with 20 μg/ml SBTI and 10 μg/ml imidazole, as an internal standard for CZE, at a lipid concentration of 250 μg/ml. The concentration of SBTI was sufficient to effectively inhibit all trypsin activity after liposomes were lysed with 0.1 % (v/v) Triton X-100. The suspension was subsequently incubated with 40 μM each of histatin 5, dhvar4, dhvar5 or cysS1–13. After several time intervals, 50 μl aliquots were taken, boiled for 5 min to inhibit all trypsin activity, lysed in 0.1 % Triton X-100 and analysed by CZE. Degradation of peptide implied digestion of the internalized peptide by the trypsin within the liposomes.

Furthermore, trypsin-loaded liposomes were incubated with peptides in the absence of SBTI to study peptide-induced leakage of trypsin, and peptide degradation due to this leakage. After these peptide incubations, the liposomes were centrifuged, and the trypsin activity was measured in the supernatants in a colorimetric assay.

Peptide-induced trypsin leakage from liposomes

Trypsin-loaded liposomes were incubated for 15 min with 40 μM peptides and centrifuged at 200000 ***g*** at 4 °C for 30 min. Aliquots (90 μl) of the supernatants were mixed with 10 μl of 10 mM BAPNA (*N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; Sigma–Aldrich) in 200 mM Tris/HCl, pH 9, containing 10 mM $CaCl_2$. After 30 min of incubation at 37 °C, colour development was measured at 405 nm.

RESULTS

A number of cationic antimicrobial peptides, including histatin 5 and designed variants derived from its active domain, are able to translocate across the cell membrane of their target organisms. However, it is unknown whether these peptides diffuse passively over the membrane, or are actively taken up via transport proteins. In the present study, the membrane-translocating properties of histatin 5 and derived peptides (Table 1) were examined and compared with their membrane-binding and membrane-disrupting activities using unilamellar liposomes as a model system for phospholipid membranes. All experiments were repeated at least three times, yielding identical results.

Peptide translocation

To investigate the membrane translocation of peptides, an enzyme digestion assay was developed in which peptides were incubated with trypsin-loaded liposomes. It was verified that all peptides used were susceptible to trypsin, and that peptide binding to liposomes did only slightly decrease the degradation by trypsin. This is shown for dhvar4 and dhvar5 (Figure 1). Precautions were taken to prevent degradation by any free trypsin by performing the incubations in the presence of the trypsin inhibitor SBTI (at a concentration of 20 μg/ml, which was sufficient to completely inhibit any external trypsin activity). Thus peptide degradation by trypsin was taken to imply that translocation over the liposome membrane had occurred. A second experiment was performed in the absence of SBTI to study the capacity of peptides to cause trypsin leakage.

Histatin 5, dhvar4 and dhvar5 were incubated with trypsin-loaded liposomes in the presence or absence of 20 μg/ml SBTI. At several time points aliquots were taken, boiled to inactivate the trypsin and then analysed by CZE (Figure 2A). The extent of degradation was quantified in the electropherogram by measuring the peptide peak height of the sample and comparing it with the initial peptide peak height (Figure 2B). Both in the presence and absence of SBTI, the rate of degradation was highest for dhvar4, less for dhvar5, whereas histatin 5 was hardly degraded. Within 5 min, the dhvar4 peak had decreased by 40 %. Concomitantly, in the electropherogram a number of novel peaks appeared, demonstrating the formation of dhvar4 degradation products. After 2 h incubation, dhvar4 was virtually completely degraded, and the number and height of the peaks corresponding to degradation products had increased further. At a 10-fold-lower dhvar4/lipid ratio, degradation also occurred within 5 min (results not shown).

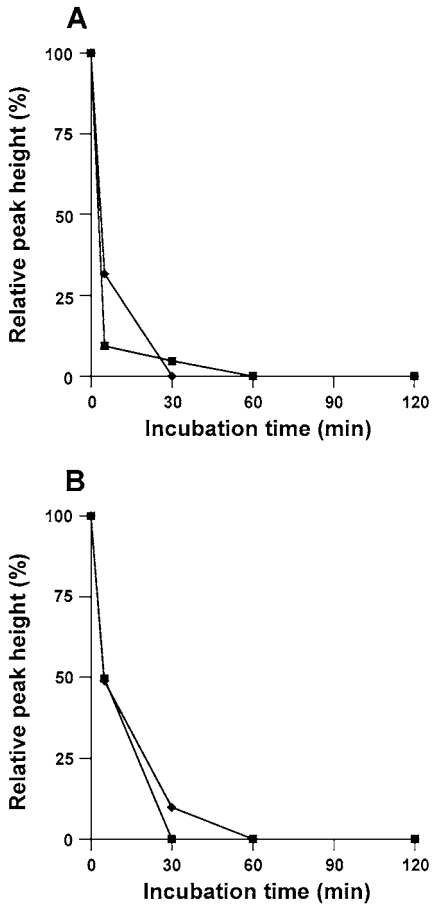

Figure 1 Liposome-bound peptide degradation

Unloaded liposomes were incubated with 10 μM dhvar4 (**A**) or dhvar5 (**B**) and incubated for 15 min. Trypsin (1 μg/ml) was added, and degradation was measured by CZE after 5, 30, 60 and 120 min (◆) and compared with degradation of peptides without liposomes (■).

Dhvar5 was degraded more slowly than dhvar4, exhibiting little degradation after 5 min of incubation, and only 40 % after 2 h. Histatin 5 was only slightly degraded within 2 h. After 24 h, approx. 50 % of the histatin 5 peak had disappeared (results not shown).

As control peptides, LL-37, known to have strong membrane effects, and the inactive cysS1–13 were included. cysS1–13 was only slightly degraded in both types of experiments, indicating that the external amount of trypsin was negligible. Strikingly, LL-37 was not degraded in the presence of SBTI, but was directly and completely degraded in the absence of SBTI, indicating disruption of the membrane integrity. This was supported further by the high trypsin activity found outside the liposomes after incubation with LL-37 (Figure 3). Thus, in contrast with the other peptides, the damage caused by LL-37 made the liposomes permeable for molecules as large as trypsin or SBTI.

Peptide binding to liposomes

To examine whether the low translocation observed with histatin 5 and dhvar5 was caused by differences in lipid avidity of the various peptides, association of peptides with (unloaded) liposomes was studied by measuring the depletion of peptides from liposome supernatants after incubation (Figure 4). All peptides displayed a comparable dose–response behaviour in the concentration range tested. Binding reached saturation at a peptide concentration of approx. 10 μM. Despite the similarity in charge and size, the peptides had very different effects on the zeta potential of the peptide–liposome complexes formed (Figure 5). An increase in zeta potential of the liposomes from -50 mV to -10 mV was induced by histatin 5, and a zeta potential of 0 mV was obtained by incubation with dhvar4. Upon incubation with dhvar5, the zeta potential of the liposomes became positive at concentrations above 5 μM, and reached a maximal value of $+20$ mV at concentrations above 20 μM. Since dhvar5 possesses just one more positive charge than dhvar4, this suggests that these peptides insert differently into the liposome surface.

Peptide-induced liposome permeabilization

The effect of peptides on the integrity of liposomes was studied using calcein-loaded liposomes. The release of calcein was measured as the 'dequenching' of the calcein fluorescence and monitored continuously. Total (100 %) calcein release was determined as the fluorescence signal after addition of 0.1 % Triton X-100 to the liposome suspension (Figure 6A). Directly after peptide addition, a decrease in fluorescence was observed, immediately followed by an increase in fluorescence, which was maximally 30 % for dhvar4 and happened within 2 min. Histatin 5 and dhvar5 only marginally increased calcein fluorescence. We noted that the initial decreases in fluorescence were due to an increase in turbidity of the samples. As the turbidity persisted throughout the measurements, all fluorescence levels were underestimated. Therefore an alternative method was used, in which, after 15 min of incubation, the calcein fluorescence was determined in the supernatants, obtained by centrifugation of the liposomes (Figure 6B). We found the same pattern in membrane-disrupting properties as in the continuous measurements; however, the increases in fluorescence were substantially higher, being 100 % for 20 μM dhvar4, 70 % for 20 μM dhvar5 and 10 % for 20 μM histatin 5 respectively. The control peptide cysS1–13 induced no release at all.

DISCUSSION

Antimicrobial peptides are able to interact with, and insert into, negatively charged cytoplasmic membranes of their target cells due to the presence of spatially segregated hydrophobic and cationic domains. The microbial specificity of cationic antimicrobial peptides has been ascribed to the fact that microbial membranes contain more negatively charged phospholipids than host membranes. The peptide–membrane interaction is thought to interfere with the barrier function of the membrane, resulting in killing of the target cells. Indeed, there is a considerable body of data indicating that such peptides cause leakage in model membrane systems, as well as in bacteria and yeast cells. Hence several membrane-permeabilization mechanisms have been proposed [1,30–32]. Although these mechanisms differ with regard to the precise molecular events involved, in each of these the formation of peptide–membrane complexes is assumed. Strikingly, studies addressing the localization of antimicrobial peptides in their target cells indicate that, in most cases, these are internalized, implying transmembrane migration, rather than remaining associated with the cytoplasmic membrane of their target cells [30]. This has been demonstrated not only for widely different natural, as well as artificial, antimicrobial peptides [10–13,33,34], but also for homopolymers [16]. In fact, only for a few cationic peptides has stable binding to the plasma membrane of their target cell been demonstrated unequivocally [6,35].

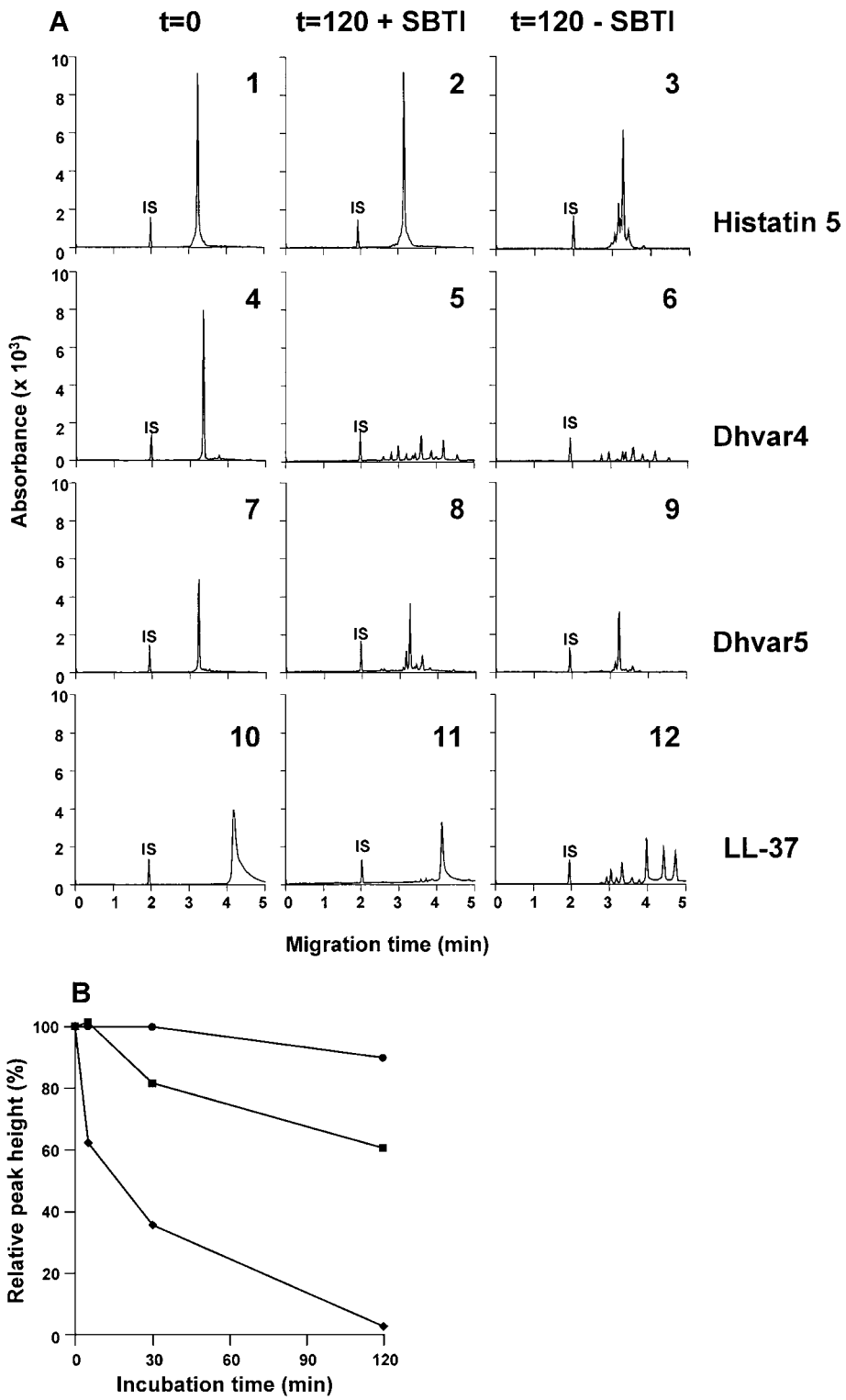

Figure 2 Detection of peptide translocation into liposomes using the enzymic digestion assay

(**A**) Trypsin-loaded liposomes (250 μg/ml) were incubated with 40 μM peptides. At the zero time point (left column) and after 120 min, aliquots were taken, boiled for 5 min, and lysed with 0.1 % Triton X-100. Incubations were carried out either in the presence (middle column) or absence (right column) of SBTI. Samples were analysed by CZE using imidazole as internal standard (IS). Histatin 5 (panel 1) showed virtually no translocation into the liposomes within 2 h (panel 2). Dhvar4 (panel 4) translocated completely within 2 h (panel 5), whereas dhvar5 (panel 7) translocation was intermediate. A substantial part of the dhvar5 peak remained after 2 h incubation (panel 8). LL-37 (panel 10) apparently showed no translocation (panel 11), although it has well-known membrane activity. In the incubations without SBTI, LL-37 was completely degraded (panel 12), indicating that this peptide rapidly caused severe membrane damage, enabling inactivation of trypsin by SBTI in an early stage of the experiment. In this experiment, the other peptides showed CZE profiles similar to those in the experiment with SBTI, indicating that these peptides do not cause such severe membrane damage as LL-37, and demonstrating that the low degradation of histatin 5 truly represents its low ability to translocate over the lipid membrane. (**B**) Kinetics of the peptide translocation into liposomes in the presence of 20 μg/ml SBTI. Histatin 5 (●) showed almost no translocation into liposomes, dhvar5 (■) showed intermediate translocation, whereas dhvar4 (◆) showed almost complete degradation within 2 h.

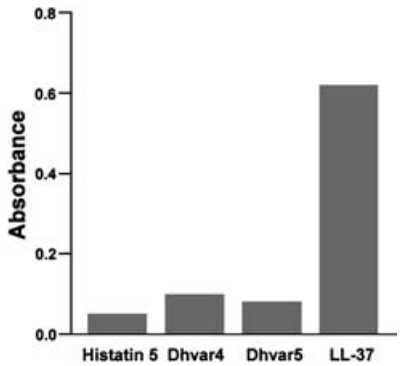

Figure 3 Peptide-induced trypsin leakage from liposomes

Trypsin-loaded liposomes were incubated for 15 min with 40 μM peptides. Liposomes were centrifuged, and trypsin activity was determined in the supernatants using the chromogenic substrate BAPNA (1 mM). After a 30 min incubation at 37 °C, colour development was measured at 405 nm. LL-37 was the only peptide to cause massive efflux of trypsin from the liposomes.

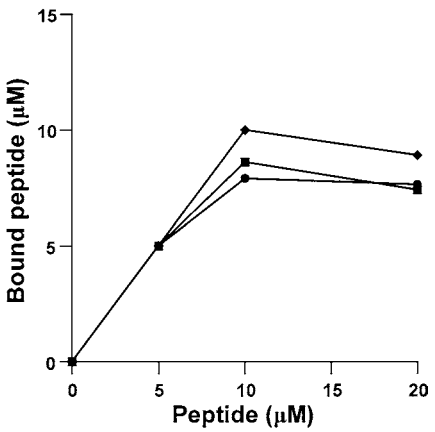

Figure 4 Peptide binding to liposomes

Liposomes were incubated for 15 min with 5, 10 or 20 μM histatin 5 (●), dhvar4 (◆) or dhvar5 (■) and centrifuged for 30 min at 200000 ***g***. Peptide binding to liposomes was determined by CZE by comparing the peak heights of the supernatants with those of the initial peptide concentration in running buffer. All three peptides showed a maximal binding of $\pm$10 μM, achieved within 15 min.

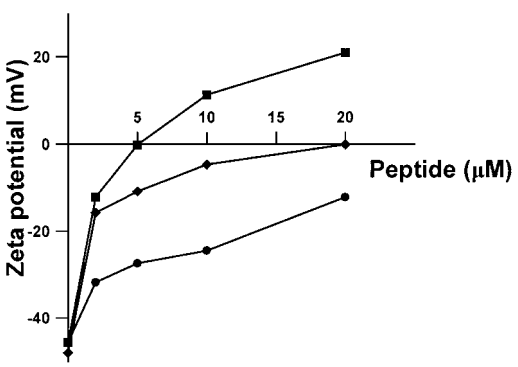

Figure 5 Zeta potential of liposomes incubated with peptides

Zeta potential of liposomes was measured as a function of the peptide concentration (37.5 μg/ml liposomes; 5 min of incubation). All peptides caused a rise in zeta potential. dhvar4 (20 μM; ◆) completely neutralized the negative charge of the liposomes, whereas dhvar5 (■) even led to a positive zeta potential of 20 mV. Histatin 5 (●) showed the least neutralizing activity.

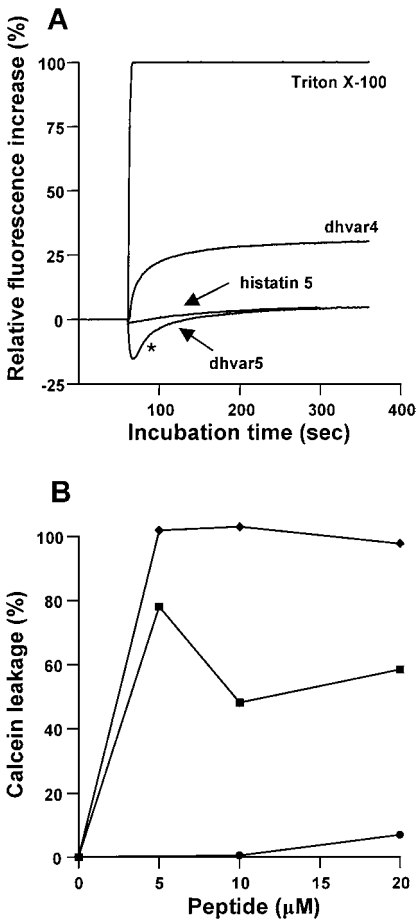

Figure 6 Peptide-induced calcein release from liposomes

(**A**) Calcein-loaded liposomes were incubated with 20 μM peptides. The increase in fluorescence, caused by dequenching of the calcein upon release from the liposomes, was monitored continuously over time. Triton X-100 (0.1 %) was added to liposomes to determine the maximal increase in fluorescence. Upon addition of the peptides, the turbidity of the suspension increased, which interfered with the fluorescence signal. Note the decrease in fluorescence upon dhvar5 addition (shown by the asterisk) caused by this phenomenon. (**B**) To obviate this interference, the peptide/liposome incubation mixtures were centrifuged, and the calcein fluorescence in the supernatants was measured. Histatin 5 (●) induced little, if any calcein release, dhvar5 (■) induced 70 % release, and dhvar4 (◆) caused 100 % release. For both dhvar4 and dhvar5, maximal calcein release was obtained at 5 μM.

In the present study, we used a liposome model to address the question of whether spontaneous diffusion over a phospholipid double layer is feasible for histatin 5, dhvar4 and dhvar5. These peptides differ in fungicidal potency (Table 1), but all translocate over the plasma membrane of their target cells [36]. There is some debate on the translocation mechanism involved, i.e. whether an active endocytotic pathway is involved or whether translocation occurs via passive diffusion; the internalization of these peptides is sensitive to metabolic inhibitors, although an all-D variant of a histatin fragment was equally well internalized [19,37,38], excluding the involvement of a stereospecific receptor.

We used liposomes, composed of PC and PS, as a simplified model for the microbial membrane. Peptide translocation was studied over phospholipid bilayers by monitoring their degradation by trypsin entrapped in liposomes. To prevent peptide degradation by external trypsin, incubations were carried out in the presence of a trypsin inhibitor (SBTI). It should be pointed out that large membrane defects, allowing influx of inhibitor or efflux of trypsin, result in instantaneous neutralization of trypsin activity,

which erroneously may be interpreted as lack of membrane translocation. For instance, the cathelicidin LL-37, which induces sufficiently large membrane defects to allow efflux of trypsin (Figure 3), was not degraded in the presence of the externally added SBTI (Figure 2A, panel 11). Since translocation in this system involves various steps, namely association, translocation and trypsin degradation, it is necessary to identify the rate-limiting step. We found that the binding of peptides to the liposomes was completed within 15 min (results not shown), excluding the possibility that this step was rate-limiting. Neither was trypsin degradation of (liposome-bound) peptides rate-limiting: liposome-bound dhvar4 and dhvar5 were readily degraded [half-life ($t_{1/2}$) $\leqslant$ 5 min; Figure 1], even at a trypsin concentration 100 times lower than in the trypsin-loaded liposomes. In contrast, degradation of peptides by trypsin-loaded liposomes occurred much more slowly ($t_{1/2}$ of 20 min and $>$ 2 h for dhvar4 and dhvar5 respectively). We therefore conclude that translocation of peptide from the outer to the inner leaflet represents the rate-limiting step.

We established that passive diffusion over liposome membranes, without the involvement of transporters, was possible, but found large differences in translocation rates. The amphipathic peptide dhvar4 translocated fastest over the phospholipid double layer, followed by dhvar5, and then histatin 5, which was hardly translocated. The same ranking order was also found for the amount of peptide-induced calcein release, which reflects the ability of peptides to induce small defects in the phospholipid double layer (Figure 6). The calcein release occurred much faster than the translocation, e.g. whereas dhvar4-induced calcein release was essentially completed within 2 min, after 5 min only 40 % of dhvar4 had translocated over the membrane. Furthermore, dhvar5 caused maximally 60 % calcein release, in a process that essentially was completed within 2 min. At this time-point little, if any, degradation of dhvar5 by trypsin-loaded liposomes had occurred (Figure 2B). This suggests that the actions of dhvar5 differ from the pore-forming model, in which pore forming and translocation are coupled processes [31]. This may be due to the structure of dhvar5, which exhibits 'head-to-tail' amphipathicity, with all positive residues concentrated in the C-terminal domain of the peptide and the hydrophobic residues in the N-terminal domain, as opposed to the laterally amphipathic peptides, such as dhvar4.

Histatin 5, dhvar4 and dhvar5 exhibited similar binding to liposomes, both with regard to the number of molecules bound and the rate of binding, which was completed within 15 min (Figure 4). However, analysis of the electrostatic properties of the liposome surface by zeta potential measurement, after incubation with the peptides, revealed interesting differences (Figure 5). Liposomes saturated with dhvar4 exhibited a nearly neutral zeta potential, whereas that of dhvar5-saturated liposomes was +20 mV. This can be explained on basis of the different structural features of these peptides: the segregation in hydrophilic and hydrophobic domains promotes a perpendicular orientation of dhvar5 molecules in the phospholipid double layer, with its hydrophobic domain inserted into the core of the double layer, and the positively charged residues exposed at the surface, possibly protruding into solution. On the other hand, in dhvar4 the cationic residues are evenly distributed over the peptide, but the peptide can form an α-helix with lateral amphipathicity. It is assumed that, upon initial interaction with the membrane, dhvar4 and similar peptides adopt an amphipathic α-helical structure that is oriented parallel to the membrane, with the cationic side of the helix electrostatically bound to the negatively charged head groups of phospholipids [39].

Artificial membranes and liposomes are generally considered as a paradigm for biological membranes, in order to study the mechanism of action of antimicrobial peptides. There is no doubt that, with this approach, valuable insight has been gained into the molecular forces that control the interaction between amphipathic peptides and phospholipid bilayers. We show that the used peptides are able to translocate over a phospholipid bilayer and may enter cells without intervention of an (active) membrane-transport system. However, in line with previous reports, the present study shows that any kind of membrane activity of a peptide measured in the liposome model system does not necessarily occur to the same extent in biological systems [40,41]. This is illustrated best for histatin 5, which exhibits low, if any, membrane activity in the model system, whereas this peptide exhibits a number of membrane-related activities against bacteria and fungi. For instance, antimicrobial activity of histatin 5 is coupled with translocation over the membrane of the target cells, as well as with disruption of the membrane. The latter is highlighted by efflux of ions and nucleotides, dissipation of transmembrane gradients and influx of propidium iodide [10,12,42–44]. Strikingly, histatin 5 has not been located at the cytoplasmic membrane of its target cells [10,12,36], contrary to what would be expected from the liposome studies. Essentially similar observations have been made for dhvar4 and dhvar5, which exhibit membrane activities in biological systems that are faster and more pronounced than in liposomes. This points to the involvement of additional features promoting the lipid translocation of peptides across biological membranes. For instance, it is conceivable that a negative-inside potential over the membrane of a living cell will act as a driving force, accelerating translocation of cationic peptides. Indeed, application of an artificial membrane potential in liposomes leads to enhanced transmigration [45–47]. In addition, biological membranes contain proteins or specific lipids that may function as primary receptors for this type of peptides, thereby facilitating their entry into the cell. It has been claimed that entry of histatin 5 into *Candida albicans* is mediated by a receptor protein in the cell wall, identified as the heat-shock protein Ssa1p [48,49]. On the basis of the present data we conclude that classical amphipathic peptides like dhvar4 can enter their target cells by passive diffusion, driven by the peptide gradient, over the phospholipid bilayer, with concomitant formation of temporary pores. In contrast, cell entry of peptides with a less pronounced amphipathic character, such as histatin 5, requires additional involvement of intrinsic membrane features.

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