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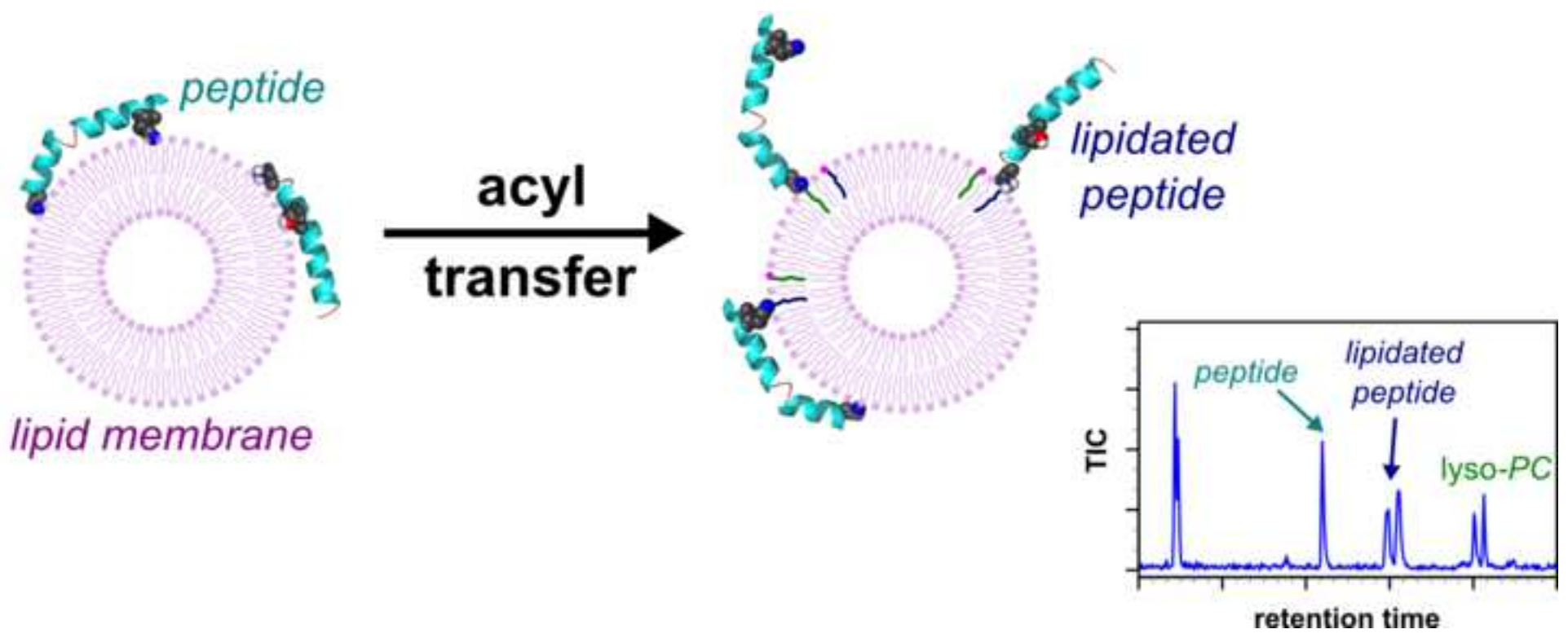
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Abstract: The generality of acyl transfer from phospholipids to membrane-active peptides has been probed using liquid chromatography-mass spectrometry (LC-MS) analysis of peptide-lipid mixtures. The peptides examined include melittin, magainin II, PGLa, LAK1, LAK3 and penetratin. Peptides were added to liposomes with membrane lipid compositions ranging from pure phosphatidylcholine (PC) to mixtures of PC with phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylglycerol (PG). Experiments were typically conducted at pH 7.4 at modest salt concentrations (90 mM NaCl). In favorable cases, lipidated peptides were further characterised by tandem MS methods to determine the sites of acylation. Melittin and magainin II were the most reactive peptides, with significant acyl transfer detected under all conditions and membrane compositions. Both peptides were lipidated at the N-terminus by transfer from PC, PE, PS or PG, as well as at internal sites: lysine for melittin; serine and lysine for magainin II. Acyl transfer could be detected within 3 hours of melittin addition to negatively charged membranes. The other peptides were less reactive, but for each peptide, acylation was found to occur in at least one of the conditions examined. The data demonstrate that acyl transfer is a generic process for peptides bound to membranes composed of diacylglycerophospholipids. Phospholipid membranes cannot therefore be considered as chemically inert towards peptides, and by extension proteins.



HIGHLIGHTS

- The scope of acyl transfer from lipids to peptides has been probed
- Melittin undergoes lipidation over a broad range of conditions
- Magainin II is readily lipidated, but is less reactive than melittin
- Other peptides (PGLa, penetratin, LAK1, LAK3) are lipidated but are less reactive
- Acyl transfer from lipids to peptides, without enzyme catalysis, is a general process

Acyl Transfer From Membrane Lipids To Peptides Is A Generic Process

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ABSTRACT

The generality of acyl transfer from phospholipids to membrane-active peptides has been probed using liquid chromatography-mass spectrometry (LC-MS) analysis of peptide-lipid mixtures. The peptides examined include melittin, magainin II, PGLa, LAK1, LAK3 and penetratin. Peptides were added to liposomes with membrane lipid compositions ranging from pure phosphatidylcholine (PC) to mixtures of PC with phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylglycerol (PG). Experiments were typically conducted at pH 7.4 at modest salt concentrations (90 mM NaCl). In favorable cases, lipidated peptides were further characterised by tandem MS methods to determine the sites of acylation. Melittin and magainin II were the most reactive peptides, with significant acyl transfer detected under all conditions and membrane compositions. Both peptides were lipidated at the N-terminus by transfer from PC, PE, PS or PG, as well as at internal sites: lysine for melittin; serine and lysine for magainin II. Acyl transfer could be detected within 3 hours of melittin addition to negatively charged membranes. The other peptides were less reactive, but for each peptide, acylation was found to occur in at least one of the conditions examined. The data demonstrate that acyl transfer is a generic process for peptides bound to membranes composed of diacylglycerophospholipids. Phospholipid membranes cannot therefore be considered as chemically inert towards peptides, and by extension proteins.

KEYWORDS

phospholipid, membrane, acylation, lipidation, melittin.

ABBREVIATIONS

EIC, extracted ion chromatogram; FTICR, Fourier transform ion-cyclotron resonance; FWHM, full width at half maximum; LC, liquid chromatography; MS, mass spectrometry; P:L, peptide to lipid ratio; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; rt, retention time; SLPE, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; SLPG, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoglycerol; SLPS, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; TFA, 2,2,2-trifluoroacetic acid; TIC, total ion chromatogram.

1. INTRODUCTION

When the fluid mosaic model was proposed for the biological membrane by Singer and Nicolson,¹ the potential for specific (electrostatic) interactions between proteins and lipids was considered, but otherwise these two types of molecule were treated as being chemically inert towards each other. Although it has since become clear that cell membranes are extremely diverse, dynamic and complex structures,² this notion of chemical inertness has remained unchallenged. Diacylphospholipids, however, are potential acyl group donors through reaction with the ester functionality. Recently, acyl group transfer from phospholipids to the peptide melittin was described.^{3,4} This acyl transfer, termed intrinsic lipidation, did not require enzyme catalysis and therefore reflects an innate reactivity of the peptide towards lipids. This reactivity is of interest because it does not require reagents other than the lipid membrane and the peptide itself and is consequently a process to which all membrane-active peptides and proteins are potentially subject.

Enzyme-catalyzed modification of proteins with groups found in lipids, such as fatty acids, has been well characterised.^{5,6} These lipidation reactions use fatty acyl thioesters as precursors and occur either co-translationally (N-myristoylation) or post-translationally (S-palmitoylation). Autoacylation processes without a requirement for enzymes have also been described.⁷⁻⁹ Other modes of protein acylation, distinct from the major enzyme-mediated methods, have been identified for lipidation at internal sites in proteins. Lipidation *via* ester formation at serine or threonine residues occurs for some proteins, including myelin¹⁰ and ion channels.¹¹ Acylation of internal lysine side

chains has been documented for phospholipases,^{12,13} cytokines,^{14,15} tumour necrosis factor,¹⁵ membrane immunoglobulin heavy chain (μ_m),¹⁶ cytochrome C oxidase,¹⁷ adenylate cyclase toxin from *B. pertussis*,¹⁸ lung surfactant protein C¹⁹ and ocular lens aquaporin, the latter protein being oleoylated site-specifically.²⁰ Hemolysin from *E. coli* (HlyA) is acylated on the side chain of two specific lysine residues.^{21–23} Although an enzyme catalyzing this acylation has been identified, HlyA isolated in its native form from *E. coli* is modified on each lysine with variety of acyl groups of 14-, 15- or 17-carbon atoms.²⁴ Similar diversity has been found for leukotoxin A (LtxA) when isolated from *Aggregatibacter actinomycetemcomitans* or expressed in *E. coli*. LtxA is modified at two internal lysines with a mixture of fatty acyl groups (palmitoleoyl, palmitoyl, myristoyl and stearoyl) within 4 h of the induction of expression.²⁵ By contrast, the A-type inclusion protein from vaccinia virus was found to be randomly myristoylated at six different arginine and lysine residues in a sequence-independent manner 20 h after the administration of labeled myristate to the infection medium.²⁶ For most of the examples described above (with the exception of surfactant protein C), lipids have not been considered as reactive acyl transfer agents. However, the observation of the intrinsic lipidation of melittin raises the prospect that, in some cases, lipids may be precursors for protein lipidation. Melittin lipidation was found to be a moderately slow process, occurring on a timescale of hours to days. This timescale is significant for a number of processes involving membrane-active peptides and proteins *in vivo*, such as amyloidogenesis²⁷ and the turnover of plasma membrane and mitochondrial proteins,²⁸ as well as for experimental methods that employ long timescales for data acquisition, such as neutron reflectometry²⁹ and NMR spectroscopy.^{30,31} The upper limit for the rate

of intrinsic lipidation has yet to be determined, but should peptides be discovered that undergo lipidation considerably faster than melittin, intrinsic lipidation will be brought into the same timescale as many routine laboratory methods for studying peptide-lipid binding, as well as becoming a method for generating peptides with novel membrane activity. It is therefore of considerable interest to determine the scope of intrinsic lipidation.

2. RESULTS

Our primary objectives were twofold: first to characterise **the ability of melittin to undergo** acylation under a broad range of conditions of salt, temperature, peptide to lipid ratio (P:L) and membrane composition; second, to determine whether other peptides are subject to similar lipidation reactions. The peptides used for this work are given in Table 1 and include, alongside melittin, antimicrobial peptides (magainin II, PGLa), a cell penetrating peptide (penetratin) and designer peptides used for NMR studies of peripheral (LAK3) and integral (LAK1) peptide association with membranes.³²

2.1 MELITTIN

Experiments to examine melittin acylation used membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and were conducted at pH 7.4 in aqueous solutions at a selection of ionic strengths (from water to 150 mM NaCl), P:L and temperature (20 °C and 37 °C). Bicarbonate (10 mM) was employed as a volatile buffer in most cases, the exceptions being control experiments and experiments conducted in water. Membranes of mixed lipid composition were also examined: POPC/1-stearoyl-2-

linoleoyl-*sn*-glycero-3-phosphoethanolamine (SLPE), POPC/1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoserine (SLPS) and POPC/1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoglycerol (SLPG). All were used at a molar ratio of 4:1, with POPC as the major component. These combinations of lipids were designed such that acyl transfer from each position of both lipids could be distinguished. Analysis was conducted directly on peptide-lipid mixtures, without any extraction or enrichment steps. This minimised the loss of material during sample analysis, at the expense of restricting the range of P:L accessible before either the concentrations of peptides dropped below the detection limit, or the amount of salt and lipid in the sample became detrimental to product separation and compound detection.

Intrinsic lipidation of melittin occurred in all the conditions of temperature, pH, ionic strength and membrane composition that were examined (Figure 1), with the major sites of acylation being the side chain of Lys-23 (K23; Peak iv, Figure 1B) and the N-terminal amino group (Peak v, Figure 1B) in increasing order of product abundance. Acylation at minor sites could also be detected (Figure 1B, Peaks i-iii, corresponding respectively to acylation at Ser-18, Lys-21 and Lys-7). Product assignment has been described elsewhere.⁴ At the peptide concentrations used, detection of products by UV absorbance was not feasible, preventing precise determination of the degree of conversion of melittin to lipidated products. Using ion chromatograms, quantification of each species was not facile, in part because the concentration dependence of the ionisation efficiency for all species was uncharacterised, and in part because the elution of some products was overlapping, which would be expected to bias peak areas in favor

of the species with higher ionisation efficiency. Nevertheless, some semi-quantitative comparisons could be made. With membranes composed of POPC, high salt concentrations and raised temperatures (37 °C) both favored the reaction, yielding significantly increased levels of conversion to lipidated products than samples prepared at lower temperatures and ionic strengths. The presence of bicarbonate had a negligible effect on the rate of reaction. It is notable that significant levels of intrinsic lipidation occurred at a P:L of 1:100 (see Figure S1, supporting information), lower than the critical value for toroidal pore formation (1.62 in POPC at 30 °C).³³ The hydrochloride salt of melittin yielded higher acylation activity than the salt with trifluoroacetic acid (TFA), with a higher propensity for acylation at K23.

High levels of lipidation resulted from the addition of melittin to membranes composed of binary lipid mixtures, including those with a negative electrostatic surface potential (Figure 1 (d)-(e)), complementary to the net positive charge of the peptide at pH 7.4, or different intrinsic curvature (Figure 1 (f)). In each case where melittin was added to a binary lipid mixture, lipidation was detectable after 3 h (although earlier times were not examined). Lipidation was not selective for lipid class, as the levels of transfer to the N-terminal amino group of melittin from SLPS, SLPE or SLPG were in accordance with the composition of the membranes, being ~25% of the transfer from PC. Products resulting from transfer of stearyl and linoleoyl groups to the side chain of Lys-23 could not be deciphered due to chromatographic peak overlap. For the majority of the experiments with melittin, no significant selectivity was observed for transfer from the *sn*-1 position versus the *sn*-2 position of glycerol, as evidenced by both the extracted ion

chromatograms for modification at each position, and the relative intensities of the *lyso*-PC peaks.

2.2 MAGAININ II

Lipidation reactions of magainin II were examined in a parallel fashion to those of melittin (Figure 2). As with melittin, magainin II lipidation was found under all conditions examined. The rates of magainin II lipidation, as determined by the levels of lipidated peptides after comparable periods of time, were slower than those observed for melittin. Lipidation was promoted by high salt conditions and was notably enhanced in samples prepared using the hydrochloride salt of magainin II.

Lipidated products were further characterised by LC combined with FTICR-MS (Figure 3 A, B) and tandem MS (MS/MS and MS³, Figure 3C). The former method provided data, through the observation of molecular ion charge state series $[M + nH]^{n+}$ (where $n = 3-5$), which confirmed that transfer of single acyl groups had occurred. The full scan mass resolution (100,000 FWHM at m/z 400), mass accuracy and precision (< 3 ppm) was sufficiently high that assignments could be made with confidence. The tandem MS methods enabled sites of acylation to be identified from the presence of sequence ladders, particularly for b- and y-type ions. As with melittin, the main lipidated products arose by reaction of the N-terminal amino group, with no discernable preference for transfer from the *sn*-1 or *sn*-2 position of glycerol. The second most abundant product was formed by acyl transfer to Ser-8 (S8). The tandem MS data support this assignment through the sequence ladders that are generated, and through the observation, in MS³ spectra, of an abundant product ion formed by C–O bond cleavage in the ester. This

product ion has the same m/z for both S8-palmitoyl and S8-oleoyl magainin II precursor ions. Two further products could be identified, particularly in the extracted ion chromatogram for palmitoyl-magainin II. These both yielded sequence ladders that matched acylation at a site in the N-terminal eight residues of the peptide. These were tentatively assigned as the products of acyl transfer to Lys-4 and His-7 (Peaks ii_P and iv_P respectively, Figure 2B). There is a precedent for acylation on the side chain of histidine, and subsequent transfer to neighbouring residues such as serine, from side reactions that occur at unprotected histidine residues during peptide synthesis.³⁴

Lipidation of magainin II in binary mixtures containing a lipid with net negative electrostatic charge (PS or PG, Figure 2 chromatograms a and b) was notably more efficient than single component PC membranes. Lipidation in binary mixtures containing POPC and SLPE displayed selectivity for palmitoyl transfer from the *sn*-1 position of POPC to the N-terminal amino group and (tentatively) His-7, but otherwise the level of reactivity in the presence of PE was lower than for binary mixtures with PG or PS.

2.3 OTHER PEPTIDES

The remaining peptides in Table 1 may be grouped together as being less reactive than melittin or magainin II. Each of these peptides yielded lipidated products under some, but not all, of the conditions examined (Figure 4A). The antimicrobial peptide PGLa was acylated at two positions when added at moderate salt concentrations (≥ 90 mM) to membranes composed of POPC or POPC/SLPS, but not other lipid compositions (POPC/SLPG, POPC/SLPE) or POPC in water. Tandem MS data were consistent with

the N-terminal amino group as the major site of modification (Peak ii, Figure 4B), although reaction at Ser-4 or Lys-5 could not be ruled out. Penetratin yielded acylated products (Figure 4C) with POPC in high salt conditions, although detection of these products was challenging due to the low levels of lipidated peptide present and the presence of multiply-sodiated ions in their mass spectra. LAK3, which associates with membranes in a peripheral orientation,³² only yielded detectable lipidation in binary POPC/SLPS mixtures (Figure 4D). By contrast, LAK1, which adopts a transmembrane orientation, was lipidated in high salt conditions (Figure 4E). LAK1 underwent acyl transfer to three locations on the peptide, of which the major site was identified by tandem MS as the lysine at position 2.

3. DISCUSSION

This work was conducted to examine whether acyl transfer from membrane lipids to peptides is a general process, or a process that is restricted to one peptide, melittin, over a limited range of conditions. **Intrinsic lipidation was found to be a robust process.** Melittin was lipidated in all the conditions that were examined, and acyl transfer to all peptides was enhanced at temperatures and ionic strengths that most closely resemble physiological conditions. Any enhancement of lipidation by the presence of TFA in the sample (a common ion pair reagent for LC purification of cationic peptides) was ruled out. In fact, lipidation reactions using the hydrochloride salts of melittin and magainin II yielded increased levels of acylated products. **The reasons for these differences in reactivity are uncertain, but it is reasonable to suggest that they arise from the relative abilities of the TFA and chloride salts to partition into the membrane interface.**

Membrane properties exerted an important influence on reactivity. In particular, complementarity between the net charge of the peptide and the electrostatic surface potential of the membrane promoted lipidation in most cases. In general, membranes containing PG were more reactive towards cationic peptides than those containing PS, which indicates that factors other than charge-charge complementarity have some effect. Melittin had a notably higher relative reactivity in membranes containing PE than magainin II, which points to roles for other properties in controlling reactivity, such as intrinsic curvature and the disposition of the membrane-embedded peptide.

Three of the peptides in this study, melittin, magainin II and PGLa, were lipidated at an N-terminal glycine residue. It is unclear whether the lower steric demand offered by glycine favours reaction, but upon examination of helical wheel representations it is apparent that for the most reactive peptide melittin, the N-terminal residue lies at the edge of the hydrophilic surface of the peptide (Figure 5A), whereas for less reactive magainin II, the N-terminal amino group resides further from the hydrophobic-hydrophilic interface (Figure 5B). In the case of PGLa (Figure 5C), with lower reactivity than magainin II, the N-terminal glycine is in the middle of the hydrophilic surface of the peptide. For LAK1 (Figure 5D), the most reactive lysine residue is exposed on an otherwise hydrophobic face of the peptide. The prevalence of lipidation of N-terminal glycine residues is of potential significance in cell biology, as protein cleavage by caspases produces a number of peptides with N-terminal glycine residues, some of which are subject to post-translational lipidation prior during apoptosis.³⁵ It is further noteworthy that the second major sites of acylation for melittin and magainin II are either exposed on the hydrophobic surface of the peptide in the case of melittin K23, or

proximal to the N-terminal glycine in the case of magainin II S8. This suggests that proximity to the hydrophobic surface favours intrinsic lipidation for amphiphilic peptides.

When all potential acylation sites are considered, the potential for chemical complexity is significant. If acylation only at the N-terminus and the side chains of lysine and serine is considered, melittin, for example, has five potential lipidation sites. Therefore, a mixture that starts with just two pure components, peptide and lipid, will evolve into a mixture with up to 10 additional singly-acylated peptides and four *lyso*-lipids (as acyl group migration occurs in *lyso*-lipids). In a mixture of melittin with binary lipid mixtures, such as those used in this work, there are 20 potential lipidated melittin products, 11 of which have been identified.

Of the remaining peptides, the observation of acyl transfer to LAK1 demonstrates that intrinsic lipidation can occur with transmembrane helices, expanding its relevance to a wide range of membrane proteins. Although it may be possible to account for acylation at Lys-2 of LAK1 in terms of the membrane disposition of a transmembrane helix, with Lys-2 being interfacial,³² selectivity for acylation at the N-terminal end of the peptide remains unaccounted for (as Lys-21 and Lys-22 are also interfacial). Both LAK3 and penetratin yielded acylated products, but tandem MS data of sufficient quality to identify acylation sites could not be obtained.

4. CONCLUSIONS

The data presented here demonstrate that intrinsic lipidation is a robust process to which all membrane-active peptides are potentially subject. Peptide reactivity is controlled by a combination of factors such as peptide structure and membrane composition that are not yet fully understood. Nevertheless, there is evidently the potential that membrane proteins and other membrane-active agents undergo similar reactions with lipids.

5. MATERIALS AND METHODS

Materials. Synthetic melittin (Alexis brand) was obtained from Enzo Life Sciences (Exeter, UK). Lipids were obtained as dry powders from Avanti Polar Lipids *via* Instruchemie B.V., The Netherlands. Penetratin was obtained from Jena Bioscience, Jena, Germany *via* Bioquote Ltd., York UK. The syntheses of all other peptides (LAK1, LAK3, magainin II and PGLa) have been described elsewhere.^{32,36} Hydrochloride salts of peptides were prepared by lyophilising each peptide twice from a frozen solution prepared at a peptide concentration of 10 μ M in 20 mM HCl. Liposomes were prepared by drying a solution of the lipid (typically 165 μ l of a 2 mg/ml solution in CHCl_3) *in vacuo* to form a thin film around the side of a round bottomed flask. This film was then hydrated with 1 ml of buffer and after thorough mixing was subjected to five freeze thaw cycles. The vesicles were then extruded 10 times through laser-etched polycarbonate membranes (Whatman, 100 nm pore size) at 50 °C using a thermobarrel extruder (Northern Lipids, Burnaby, Canada) under a positive pressure of N_2 .

Lipidation. Standard acylation conditions employed aqueous buffers at pH 7.4 containing sodium bicarbonate (10 mM) and sodium chloride (90 mM). Mixtures (50–100 μ L) of peptides (30 μ M) with liposomes (0.3 mM) were prepared by adding a solution of the peptide to the liposome dispersion in buffer. Following mixing, mixtures were incubated at 37 °C. The pH values of the solutions (pH 7.4) were unchanged after mixing of peptide and liposomes. For analysis by LC-MS, 20 μ L of the reaction mixture was removed and diluted into 80 μ L aqueous medium (20 mM ammonium bicarbonate at pH 7.4).

Sample analysis. An Xbridge 3.5 μ m C18 column (Waters UK, Manchester, UK) of dimensions 100 x 2.1 mm was used for all LC work. Two eluants were used: solvent A: H₂O; solvent B: MeCN. Both eluants contained 0.1% v/v formic acid. Standard LC-MS analysis was performed using a QToF Premier (Waters UK, Manchester, UK), with 5 μ L aliquots injected onto the column and a 10 minute reverse phase gradient of 5% B to 95% B at a flow rate of 300 μ L min⁻¹. MS data were recorded and processed using MassLynx version 4.1 (Waters UK, Manchester, UK). LC-MSⁿ was performed with a Surveyor HPLC coupled to an LTQFT MS (ThermoFinnigan Corp, Bremen, Germany). LC was performed using a 12 minute gradient of 5% B to 90% B at a flow rate of 200 μ L min⁻¹. All full scan MS data were measured in the Fourier transform-ion cyclotron resonance mass analyser surrounded by a 7 Tesla superconducting magnet. Collision-induced dissociation (CID) experiments were performed entirely within the linear ion trap with a fixed isolation window of 4 m/z , using helium as a collision gas and an optimised normalised collision energy level of 25%. All MSⁿ experiments were

performed with alternating MS and MSⁿ scans throughout the chromatographic run. MS Data were recorded using Xcalibur version 2.0.7 (ThermoFisher Corp, Bremen, Germany) and processed using the embedded program Qual Browser. Further processing was done using the XCMS package³⁷ in the R Statistical Programming Environment.³⁸

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8. FIGURE LEGENDS

Figure 1. LC-MS analysis of melittin/liposome mixtures. Reaction conditions unless otherwise stated: [melittin], 26 μM ; [POPC], 0.26 mM (P:L 1:10), [NaCl], 90 mM; NaHCO_3 , 10 mM; pH 7.4, 37 $^\circ\text{C}$. Key: rt, retention time. (a) no NaHCO_3 , 72 h; (b) 20 $^\circ\text{C}$, 150 mM NaCl, 72 h; (c) water (no NaHCO_3 or NaCl), 48 h; (d) POPC/SLPS (4:1), 72 h; (e) POPC/SLPG (4:1), 48 h; (f) POPC/SLPE (4:1), 72 h. (A) Total ion chromatograms (TICs; area normalised). The dashed box indicates the region in (B). (B) Extracted ion chromatograms (EICs; area normalised) for palmitoyl-melittin (blue) and oleoyl-melittin (red). For (d)-(f), combined EICs for oleoyl/stearoyl/linoleoyl-melittin are shown in red. The peak indicated by an asterisk is from a polymeric impurity. Chromatographic peak identities are annotated using a roman numeral to indicate the main site of peptide modification responsible for the peak, with a subscript to identify the acyl group.

Figure 2. LC-MS analysis of magainin II/liposome mixtures. Reaction conditions unless otherwise stated: [magainin II], 35 μM ; [POPC], 0.35 mM (P:L 1:10); [NaCl], 90 mM; NaHCO_3 , 10 mM; pH 7.4, 37 $^\circ\text{C}$. (a) POPC/SLPS (1:4); (b) POPC/SLPG (1:4); (c) POPC/SLPE (1:4); (d) [NaCl], 150 mM, 144 h. (A) TICs (area normalised) after 72 h. (B) EICs (area normalised, 72 h) for palmitoyl-magainin II (blue) and oleoyl/linoleoyl/stearoyl-magainin II (red). Arrows identify the positions of MS and MS^n spectra in Figure 3. Chromatographic peak identities are annotated in the same manner as Figure 1.

Figure 3. Exemplar MS data for lipidated magainin II. FTICR-MS spectra at retention times corresponding to (A) arrow 1 and (B) arrow 2 in Figure 2B. Selected ions are shown in expanded form as insets with arbitrarily expanded x-axis scales. (C) MS³ spectrum of oleoyl magainin II at a retention time corresponding to arrow 2 in Figure 2B (MS/MS precursor ion b17*, $z=2$, m/z 1042). Peaks in ion series marked with an asterisk match ions modified with an oleoyl group. The i-ion series in (C) corresponds to internal fragments terminating at residue 17 of magainin II (*e.g.* i7 corresponds to the sequence from residues 7 to 17 of magainin II). Other notable ions: m/z 787.1 (i4-NH₃, $z=2$), 795.7 (i4, $z=2$), 823.8 (i3, $z=2$), 871.7 (i2-H₂O, $z=2$), 1572.7 (i4-NH₃, $z=1$). Other assignments are provided in the supporting information (Figures S21–44).

Figure 4. LC-MS analysis of liposome mixtures with PGLa, penetratin, LAK1 and LAK3. Conditions unless otherwise stated, [peptide], 31–34 μ M; [POPC], 0.31–0.34 mM (P:L 1:10), [NaCl], 90 mM; NaHCO₃, 10 mM; pH 7.4, 37 °C. (A) TICs (area normalised, 72 h) for (a) LAK1; (b) LAK3, POPC/SLPS; (c) penetratin, 150 mM NaCl; (d) PGLa, POPC/SLPS. (B)-(E) EICs (area normalised, 72 h) for palmitoyl-peptides (blue) and oleoyl/linoleoyl/stearoyl-peptides (red), corresponding to the TICs in (A). Chromatographic peak identities are annotated in the same manner as Figure 1.

Figure 5. Helical wheel projections of melittin (A), magainin II (B), PGLa (C) and LAK1 (D). Key: hydrophobic residues are in yellow, negatively charged residues in red, positively charged residues in blue and polar uncharged in green; major sites of acylation are represented as magenta diamonds.

Acyl Transfer From Membrane Lipids To Peptides Is A Generic Process

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ABSTRACT

The generality of acyl transfer from phospholipids to membrane-active peptides has been probed using liquid chromatography-mass spectrometry (LC-MS) analysis of peptide-lipid mixtures. The peptides examined include melittin, magainin II, PGLa, LAK1, LAK3 and penetratin. Peptides were added to liposomes with membrane lipid compositions ranging from pure phosphatidylcholine (PC) to mixtures of PC with phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylglycerol (PG). Experiments were typically conducted at pH 7.4 at modest salt concentrations (90 mM NaCl). In favorable cases, lipidated peptides were further characterised by tandem MS methods to determine the sites of acylation. Melittin and magainin II were the most reactive peptides, with significant acyl transfer detected under all conditions and membrane compositions. Both peptides were lipidated at the N-terminus by transfer from PC, PE, PS or PG, as well as at internal sites: lysine for melittin; serine and lysine for magainin II. Acyl transfer could be detected within 3 hours of melittin addition to negatively charged membranes. The other peptides were less reactive, but for each peptide, acylation was found to occur in at least one of the conditions examined. The data demonstrate that acyl transfer is a generic process for peptides bound to membranes composed of diacylglycerophospholipids. Phospholipid membranes cannot therefore be considered as chemically inert towards peptides, and by extension proteins.

KEYWORDS

phospholipid, membrane, acylation, lipidation, melittin.

ABBREVIATIONS

EIC, extracted ion chromatogram; FTICR, Fourier transform ion-cyclotron resonance; FWHM, full width at half maximum; LC, liquid chromatography; MS, mass spectrometry; P:L, peptide to lipid ratio; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; rt, retention time; SLPE, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; SLPG, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoglycerol; SLPS, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; TFA, 2,2,2-trifluoroacetic acid; TIC, total ion chromatogram.

1. INTRODUCTION

When the fluid mosaic model was proposed for the biological membrane by Singer and Nicolson,¹ the potential for specific (electrostatic) interactions between proteins and lipids was considered, but otherwise these two types of molecule were treated as being chemically inert towards each other. Although it has since become clear that cell membranes are extremely diverse, dynamic and complex structures,² this notion of chemical inertness has remained unchallenged. Diacylphospholipids, however, are potential acyl group donors through reaction with the ester functionality. Recently, acyl group transfer from phospholipids to the peptide melittin was described.^{3,4} This acyl transfer, termed intrinsic lipidation, did not require enzyme catalysis and therefore reflects an innate reactivity of the peptide towards lipids. This reactivity is of interest because it does not require reagents other than the lipid membrane and the peptide itself and is consequently a process to which all membrane-active peptides and proteins are potentially subject.

Enzyme-catalyzed modification of proteins with groups found in lipids, such as fatty acids, has been well characterised.^{5,6} These lipidation reactions use fatty acyl thioesters as precursors and occur either co-translationally (N-myristoylation) or post-translationally (S-palmitoylation). Autoacylation processes without a requirement for enzymes have also been described.⁷⁻⁹ Other modes of protein acylation, distinct from the major enzyme-mediated methods, have been identified for lipidation at internal sites in proteins. Lipidation *via* ester formation at serine or threonine residues occurs for some proteins, including myelin¹⁰ and ion channels.¹¹ Acylation of internal lysine side

chains has been documented for phospholipases,^{12,13} cytokines,^{14,15} tumour necrosis factor,¹⁵ membrane immunoglobulin heavy chain (μ_m),¹⁶ cytochrome C oxidase,¹⁷ adenylate cyclase toxin from *B. pertussis*,¹⁸ lung surfactant protein C¹⁹ and ocular lens aquaporin, the latter protein being oleoylated site-specifically.²⁰ Hemolysin from *E. coli* (HlyA) is acylated on the side chain of two specific lysine residues.²¹⁻²³ Although an enzyme catalyzing this acylation has been identified, HlyA isolated in its native form from *E. coli* is modified on each lysine with variety of acyl groups of 14-, 15- or 17-carbon atoms.²⁴ Similar diversity has been found for leukotoxin A (LtxA) when isolated from *Aggregatibacter actinomycetemcomitans* or expressed in *E. coli*. LtxA is modified at two internal lysines with a mixture of fatty acyl groups (palmitoleoyl, palmitoyl, myristoyl and stearoyl) within 4 h of the induction of expression.²⁵ By contrast, the A-type inclusion protein from vaccinia virus was found to be randomly myristoylated at six different arginine and lysine residues in a sequence-independent manner 20 h after the administration of labeled myristate to the infection medium.²⁶ For most of the examples described above (with the exception of surfactant protein C), lipids have not been considered as reactive acyl transfer agents. However, the observation of the intrinsic lipidation of melittin raises the prospect that, in some cases, lipids may be precursors for protein lipidation. Melittin lipidation was found to be a moderately slow process, occurring on a timescale of hours to days. This timescale is significant for a number of processes involving membrane-active peptides and proteins *in vivo*, such as amyloidogenesis²⁷ and the turnover of plasma membrane and mitochondrial proteins,²⁸ as well as for experimental methods that employ long timescales for data acquisition, such as neutron reflectometry²⁹ and NMR spectroscopy.^{30,31} The upper limit for the rate

of intrinsic lipidation has yet to be determined, but should peptides be discovered that undergo lipidation considerably faster than melittin, intrinsic lipidation will be brought into the same timescale as many routine laboratory methods for studying peptide-lipid binding, as well as becoming a method for generating peptides with novel membrane activity. It is therefore of considerable interest to determine the scope of intrinsic lipidation.

2. RESULTS

Our primary objectives were twofold: first to characterise the ability of melittin to undergo acylation under a broad range of conditions of salt, temperature, peptide to lipid ratio (P:L) and membrane composition; second, to determine whether other peptides are subject to similar lipidation reactions. The peptides used for this work are given in Table 1 and include, alongside melittin, antimicrobial peptides (magainin II, PGLa), a cell penetrating peptide (penetratin) and designer peptides used for NMR studies of peripheral (LAK3) and integral (LAK1) peptide association with membranes.³²

2.1 MELITTIN

Experiments to examine melittin acylation used membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and were conducted at pH 7.4 in aqueous solutions at a selection of ionic strengths (from water to 150 mM NaCl), P:L and temperature (20 °C and 37 °C). Bicarbonate (10 mM) was employed as a volatile buffer in most cases, the exceptions being control experiments and experiments conducted in water. Membranes of mixed lipid composition were also examined: POPC/1-stearoyl-2-

linoleoyl-*sn*-glycero-3-phosphoethanolamine (SLPE), POPC/1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoserine (SLPS) and POPC/1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoglycerol (SLPG). All were used at a molar ratio of 4:1, with POPC as the major component. These combinations of lipids were designed such that acyl transfer from each position of both lipids could be distinguished. Analysis was conducted directly on peptide-lipid mixtures, without any extraction or enrichment steps. This minimised the loss of material during sample analysis, at the expense of restricting the range of P:L accessible before either the concentrations of peptides dropped below the detection limit, or the amount of salt and lipid in the sample became detrimental to product separation and compound detection.

Intrinsic lipidation of melittin occurred in all the conditions of temperature, pH, ionic strength and membrane composition that were examined (Figure 1), with the major sites of acylation being the side chain of Lys-23 (K23; Peak iv, Figure 1B) and the N-terminal amino group (Peak v, Figure 1B) in increasing order of product abundance. Acylation at minor sites could also be detected (Figure 1B, Peaks i-iii, corresponding respectively to acylation at Ser-18, Lys-21 and Lys-7). Product assignment has been described elsewhere.⁴ At the peptide concentrations used, detection of products by UV absorbance was not feasible, preventing precise determination of the degree of conversion of melittin to lipidated products. Using ion chromatograms, quantification of each species was not facile, in part because the concentration dependence of the ionisation efficiency for all species was uncharacterised, and in part because the elution of some products was overlapping, which would be expected to bias peak areas in favor

of the species with higher ionisation efficiency. Nevertheless, some semi-quantitative comparisons could be made. With membranes composed of POPC, high salt concentrations and raised temperatures (37 °C) both favored the reaction, yielding significantly increased levels of conversion to lipidated products than samples prepared at lower temperatures and ionic strengths. The presence of bicarbonate had a negligible effect on the rate of reaction. It is notable that significant levels of intrinsic lipidation occurred at a P:L of 1:100 (see Figure S1, supporting information), lower than the critical value for toroidal pore formation (1:62 in POPC at 30 °C).³³ The hydrochloride salt of melittin yielded higher acylation activity than the salt with trifluoroacetic acid (TFA), with a higher propensity for acylation at K23.

High levels of lipidation resulted from the addition of melittin to membranes composed of binary lipid mixtures, including those with a negative electrostatic surface potential (Figure 1 (d)-(e)), complementary to the net positive charge of the peptide at pH 7.4, or different intrinsic curvature (Figure 1 (f)). In each case where melittin was added to a binary lipid mixture, lipidation was detectable after 3 h (although earlier times were not examined). Lipidation was not selective for lipid class, as the levels of transfer to the N-terminal amino group of melittin from SLPS, SLPE or SLPG were in accordance with the composition of the membranes, being ~25% of the transfer from PC. Products resulting from transfer of stearoyl and linoleoyl groups to the side chain of Lys-23 could not be deciphered due to chromatographic peak overlap. For the majority of the experiments with melittin, no significant selectivity was observed for transfer from the *sn*-1 position versus the *sn*-2 position of glycerol, as evidenced by both the extracted ion

chromatograms for modification at each position, and the relative intensities of the *lyso*-PC peaks.

2.2 MAGAININ II

Lipidation reactions of magainin II were examined in a parallel fashion to those of melittin (Figure 2). As with melittin, magainin II lipidation was found under all conditions examined. The rates of magainin II lipidation, as determined by the levels of lipidated peptides after comparable periods of time, were slower than those observed for melittin. Lipidation was promoted by high salt conditions and was notably enhanced in samples prepared using the hydrochloride salt of magainin II.

Lipidated products were further characterised by LC combined with FTICR-MS (Figure 3 A, B) and tandem MS (MS/MS and MS³, Figure 3C). The former method provided data, through the observation of molecular ion charge state series $[M + nH]^{n+}$ (where $n = 3-5$), which confirmed that transfer of single acyl groups had occurred. The full scan mass resolution (100,000 FWHM at m/z 400), mass accuracy and precision (< 3 ppm) was sufficiently high that assignments could be made with confidence. The tandem MS methods enabled sites of acylation to be identified from the presence of sequence ladders, particularly for b- and y-type ions. As with melittin, the main lipidated products arose by reaction of the N-terminal amino group, with no discernable preference for transfer from the *sn*-1 or *sn*-2 position of glycerol. The second most abundant product was formed by acyl transfer to Ser-8 (S8). The tandem MS data support this assignment through the sequence ladders that are generated, and through the observation, in MS³ spectra, of an abundant product ion formed by C–O bond cleavage in the ester. This

product ion has the same m/z for both S8-palmitoyl and S8-oleoyl magainin II precursor ions. Two further products could be identified, particularly in the extracted ion chromatogram for palmitoyl-magainin II. These both yielded sequence ladders that matched acylation at a site in the N-terminal eight residues of the peptide. These were tentatively assigned as the products of acyl transfer to Lys-4 and His-7 (Peaks ii_P and iv_P respectively, Figure 2B). There is a precedent for acylation on the side chain of histidine, and subsequent transfer to neighbouring residues such as serine, from side reactions that occur at unprotected histidine residues during peptide synthesis.³⁴

Lipidation of magainin II in binary mixtures containing a lipid with net negative electrostatic charge (PS or PG, Figure 2 chromatograms a and b) was notably more efficient than single component PC membranes. Lipidation in binary mixtures containing POPC and SLPE displayed selectivity for palmitoyl transfer from the *sn*-1 position of POPC to the N-terminal amino group and (tentatively) His-7, but otherwise the level of reactivity in the presence of PE was lower than for binary mixtures with PG or PS.

2.3 OTHER PEPTIDES

The remaining peptides in Table 1 may be grouped together as being less reactive than melittin or magainin II. Each of these peptides yielded lipidated products under some, but not all, of the conditions examined (Figure 4A). The antimicrobial peptide PGLa was acylated at two positions when added at moderate salt concentrations (≥ 90 mM) to membranes composed of POPC or POPC/SLPS, but not other lipid compositions (POPC/SLPG, POPC/SLPE) or POPC in water. Tandem MS data were consistent with

the N-terminal amino group as the major site of modification (Peak ii, Figure 4B), although reaction at Ser-4 or Lys-5 could not be ruled out. Penetratin yielded acylated products (Figure 4C) with POPC in high salt conditions, although detection of these products was challenging due to the low levels of lipidated peptide present and the presence of multiply-sodiated ions in their mass spectra. LAK3, which associates with membranes in a peripheral orientation,³² only yielded detectable lipidation in binary POPC/SLPS mixtures (Figure 4D). By contrast, LAK1, which adopts a transmembrane orientation, was lipidated in high salt conditions (Figure 4E). LAK1 underwent acyl transfer to three locations on the peptide, of which the major site was identified by tandem MS as the lysine at position 2.

3. DISCUSSION

This work was conducted to examine whether acyl transfer from membrane lipids to peptides is a general process, or a process that is restricted to one peptide, melittin, over a limited range of conditions. Intrinsic lipidation was found to be a robust process. Melittin was lipidated in all the conditions that were examined, and acyl transfer to all peptides was enhanced at temperatures and ionic strengths that most closely resemble physiological conditions. Any enhancement of lipidation by the presence of TFA in the sample (a common ion pair reagent for LC purification of cationic peptides) was ruled out. In fact, lipidation reactions using the hydrochloride salts of melittin and magainin II yielded increased levels of acylated products. The reasons for these differences in reactivity are uncertain, but it is reasonable to suggest that they arise from the relative abilities of the TFA and chloride salts to partition into the membrane interface.

Membrane properties exerted an important influence on reactivity. In particular, complementarity between the net charge of the peptide and the electrostatic surface potential of the membrane promoted lipidation in most cases. In general, membranes containing PG were more reactive towards cationic peptides than those containing PS, which indicates that factors other than charge-charge complementarity have some effect. Melittin had a notably higher relative reactivity in membranes containing PE than magainin II, which points to roles for other properties in controlling reactivity, such as intrinsic curvature and the disposition of the membrane-embedded peptide.

Three of the peptides in this study, melittin, magainin II and PGLa, were lipidated at an N-terminal glycine residue. It is unclear whether the lower steric demand offered by glycine favours reaction, but upon examination of helical wheel representations it is apparent that for the most reactive peptide melittin, the N-terminal residue lies at the edge of the hydrophilic surface of the peptide (Figure 5A), whereas for less reactive magainin II, the N-terminal amino group resides further from the hydrophobic-hydrophilic interface (Figure 5B). In the case of PGLa (Figure 5C), with lower reactivity than magainin II, the N-terminal glycine is in the middle of the hydrophilic surface of the peptide. For LAK1 (Figure 5D), the most reactive lysine residue is exposed on an otherwise hydrophobic face of the peptide. The prevalence of lipidation of N-terminal glycine residues is of potential significance in cell biology, as protein cleavage by caspases produces a number of peptides with N-terminal glycine residues, some of which are subject to post-translational lipidation prior during apoptosis.³⁵ It is further noteworthy that the second major sites of acylation for melittin and magainin II are either exposed on the hydrophobic surface of the peptide in the case of melittin K23, or

proximal to the N-terminal glycine in the case of magainin II S8. This suggests that proximity to the hydrophobic surface favours intrinsic lipidation for amphiphilic peptides.

When all potential acylation sites are considered, the potential for chemical complexity is significant. If acylation only at the N-terminus and the side chains of lysine and serine is considered, melittin, for example, has five potential lipidation sites. Therefore, a mixture that starts with just two pure components, peptide and lipid, will evolve into a mixture with up to 10 additional singly-acylated peptides and four *lyso*-lipids (as acyl group migration occurs in *lyso*-lipids). In a mixture of melittin with binary lipid mixtures, such as those used in this work, there are 20 potential lipidated melittin products, 11 of which have been identified.

Of the remaining peptides, the observation of acyl transfer to LAK1 demonstrates that intrinsic lipidation can occur with transmembrane helices, expanding its relevance to a wide range of membrane proteins. Although it may be possible to account for acylation at Lys-2 of LAK1 in terms of the membrane disposition of a transmembrane helix, with Lys-2 being interfacial,³² selectivity for acylation at the N-terminal end of the peptide remains unaccounted for (as Lys-21 and Lys-22 are also interfacial). Both LAK3 and penetratin yielded acylated products, but tandem MS data of sufficient quality to identify acylation sites could not be obtained.

4. CONCLUSIONS

The data presented here demonstrate that intrinsic lipidation is a robust process to which all membrane-active peptides are potentially subject. Peptide reactivity is controlled by a combination of factors such as peptide structure and membrane composition that are not yet fully understood. Nevertheless, there is evidently the potential that membrane proteins and other membrane-active agents undergo similar reactions with lipids.

5. MATERIALS AND METHODS

Materials. Synthetic melittin (Alexis brand) was obtained from Enzo Life Sciences (Exeter, UK). Lipids were obtained as dry powders from Avanti Polar Lipids *via* Instruchemie B.V., The Netherlands. Penetratin was obtained from Jena Bioscience, Jena, Germany *via* Bioquote Ltd., York UK. The syntheses of all other peptides (LAK1, LAK3, magainin II and PGLa) have been described elsewhere.^{32,36} Hydrochloride salts of peptides were prepared by lyophilising each peptide twice from a frozen solution prepared at a peptide concentration of 10 μ M in 20 mM HCl. Liposomes were prepared by drying a solution of the lipid (typically 165 μ l of a 2 mg/ml solution in CHCl_3) *in vacuo* to form a thin film around the side of a round bottomed flask. This film was then hydrated with 1 ml of buffer and after thorough mixing was subjected to five freeze thaw cycles. The vesicles were then extruded 10 times through laser-etched polycarbonate membranes (Whatman, 100 nm pore size) at 50 °C using a thermobarrel extruder (Northern Lipids, Burnaby, Canada) under a positive pressure of N_2 .

Lipidation. Standard acylation conditions employed aqueous buffers at pH 7.4 containing sodium bicarbonate (10 mM) and sodium chloride (90 mM). Mixtures (50–100 μ L) of peptides (30 μ M) with liposomes (0.3 mM) were prepared by adding a solution of the peptide to the liposome dispersion in buffer. Following mixing, mixtures were incubated at 37 °C. The pH values of the solutions (pH 7.4) were unchanged after mixing of peptide and liposomes. For analysis by LC-MS, 20 μ L of the reaction mixture was removed and diluted into 80 μ L aqueous medium (20 mM ammonium bicarbonate at pH 7.4).

Sample analysis. An Xbridge 3.5 μ m C18 column (Waters UK, Manchester, UK) of dimensions 100 x 2.1 mm was used for all LC work. Two eluants were used: solvent A: H₂O; solvent B: MeCN. Both eluants contained 0.1% v/v formic acid. Standard LC-MS analysis was performed using a QToF Premier (Waters UK, Manchester, UK), with 5 μ L aliquots injected onto the column and a 10 minute reverse phase gradient of 5% B to 95% B at a flow rate of 300 μ L min⁻¹. MS data were recorded and processed using MassLynx version 4.1 (Waters UK, Manchester, UK). LC-MSⁿ was performed with a Surveyor HPLC coupled to an LTQFT MS (ThermoFinnigan Corp, Bremen, Germany). LC was performed using a 12 minute gradient of 5% B to 90% B at a flow rate of 200 μ L min⁻¹. All full scan MS data were measured in the Fourier transform-ion cyclotron resonance mass analyser surrounded by a 7 Tesla superconducting magnet. Collision-induced dissociation (CID) experiments were performed entirely within the linear ion trap with a fixed isolation window of 4 m/z , using helium as a collision gas and an optimised normalised collision energy level of 25%. All MSⁿ experiments were

performed with alternating MS and MSⁿ scans throughout the chromatographic run. MS Data were recorded using Xcalibur version 2.0.7 (ThermoFisher Corp, Bremen, Germany) and processed using the embedded program Qual Browser. Further processing was done using the XCMS package³⁷ in the R Statistical Programming Environment.³⁸

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8. FIGURE LEGENDS

Figure 1. LC-MS analysis of melittin/liposome mixtures. Reaction conditions unless otherwise stated: [melittin], 26 μM ; [POPC], 0.26 mM (P:L 1:10), [NaCl], 90 mM; NaHCO_3 , 10 mM; pH 7.4, 37 $^\circ\text{C}$. Key: rt, retention time. (a) no NaHCO_3 , 72 h; (b) 20 $^\circ\text{C}$, 150 mM NaCl, 72 h; (c) water (no NaHCO_3 or NaCl), 48 h; (d) POPC/SLPS (4:1), 72 h; (e) POPC/SLPG (4:1), 48 h; (f) POPC/SLPE (4:1), 72 h. (A) Total ion chromatograms (TICs; area normalised). The dashed box indicates the region in (B). (B) Extracted ion chromatograms (EICs; area normalised) for palmitoyl-melittin (blue) and oleoyl-melittin (red). For (d)-(f), combined EICs for oleoyl/stearoyl/linoleoyl-melittin are shown in red. The peak indicated by an asterisk is from a polymeric impurity. Chromatographic peak identities are annotated using a roman numeral to indicate the main site of peptide modification responsible for the peak, with a subscript to identify the acyl group.

Figure 2. LC-MS analysis of magainin II/liposome mixtures. Reaction conditions unless otherwise stated: [magainin II], 35 μM ; [POPC], 0.35 mM (P:L 1:10); [NaCl], 90 mM; NaHCO_3 , 10 mM; pH 7.4, 37 $^\circ\text{C}$. (a) POPC/SLPS (1:4); (b) POPC/SLPG (1:4); (c) POPC/SLPE (1:4); (d) [NaCl], 150 mM, 144 h. (A) TICs (area normalised) after 72 h. (B) EICs (area normalised, 72 h) for palmitoyl-magainin II (blue) and oleoyl/linoleoyl/stearoyl-magainin II (red). Arrows identify the positions of MS and MS^n spectra in Figure 3. Chromatographic peak identities are annotated in the same manner as Figure 1.

Figure 3. Exemplar MS data for lipidated magainin II. FTICR-MS spectra at retention times corresponding to (A) arrow 1 and (B) arrow 2 in Figure 2B. Selected ions are shown in expanded form as insets with arbitrarily expanded x-axis scales. (C) MS³ spectrum of oleoyl magainin II at a retention time corresponding to arrow 2 in Figure 2B (MS/MS precursor ion b17*, $z=2$, m/z 1042). Peaks in ion series marked with an asterisk match ions modified with an oleoyl group. The i-ion series in (C) corresponds to internal fragments terminating at residue 17 of magainin II (*e.g.* i7 corresponds to the sequence from residues 7 to 17 of magainin II). Other notable ions: m/z 787.1 (i4-NH₃, $z=2$), 795.7 (i4, $z=2$), 823.8 (i3, $z=2$), 871.7 (i2-H₂O, $z=2$), 1572.7 (i4-NH₃, $z=1$). Other assignments are provided in the supporting information (Figures S21–44).

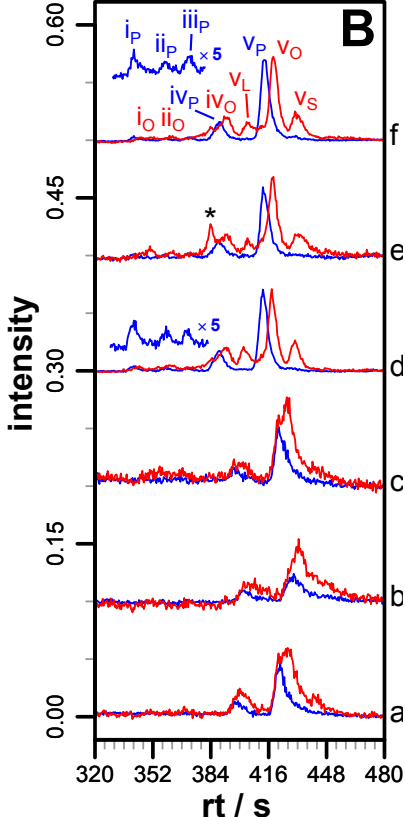
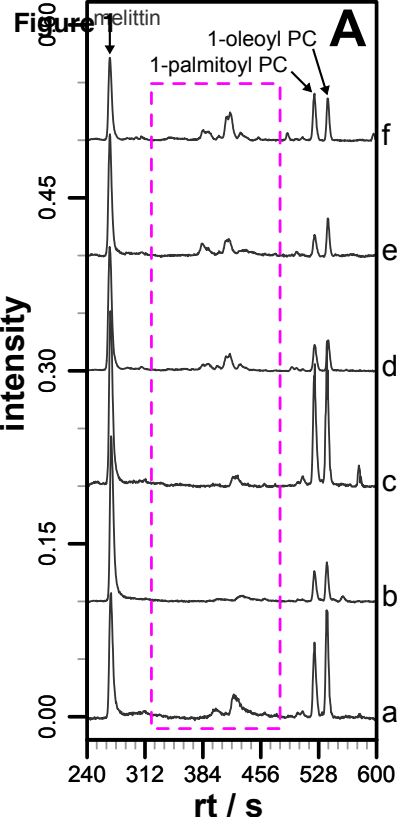
Figure 4. LC-MS analysis of liposome mixtures with PGLa, penetratin, LAK1 and LAK3. Conditions unless otherwise stated, [peptide], 31–34 μ M; [POPC], 0.31–0.34 mM (P:L 1:10), [NaCl], 90 mM; NaHCO₃, 10 mM; pH 7.4, 37 °C. (A) TICs (area normalised, 72 h) for (a) LAK1; (b) LAK3, POPC/SLPS; (c) penetratin, 150 mM NaCl; (d) PGLa, POPC/SLPS. (B)-(E) EICs (area normalised, 72 h) for palmitoyl-peptides (blue) and oleoyl/linoleoyl/stearoyl-peptides (red), corresponding to the TICs in (A). Chromatographic peak identities are annotated in the same manner as Figure 1.

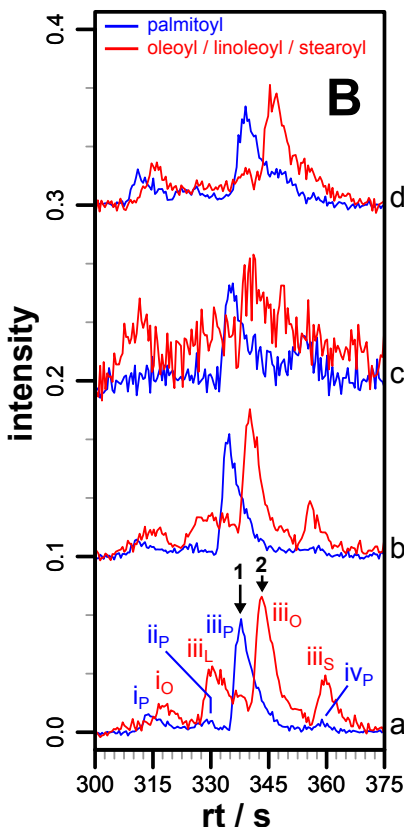
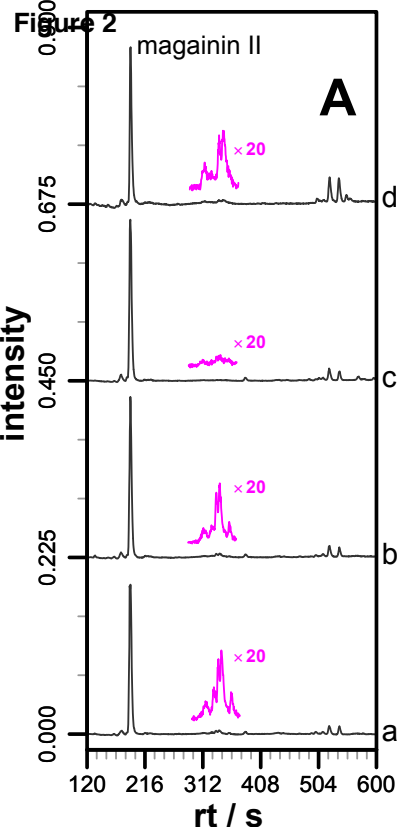
Figure 5. Helical wheel projections of melittin (A), magainin II (B), PGLa (C) and LAK1 (D). Key: hydrophobic residues are in yellow, negatively charged residues in red, positively charged residues in blue and polar uncharged in green; major sites of acylation are represented as magenta diamonds.

Table 1. Peptides used in this work.

Peptide	Sequence ^a
Melittin	H-GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂
Magainin II	H-GIGKFLHSAKKFGKAFVGEIMNS-OH
PGLa	H-GMASKAGAI A GKIAKVALKAL-NH ₂
LAK1	H-KKLALALAL <u>A</u> LKALALALALKKA-NH ₂
LAK3	H-KKLALALAK <u>A</u> LAKALKLALALAKK-NH ₂
Penetratin	H-RQIKIWFQNRRMKWKK-OH

^a Labeled residues are underlined (for ¹⁵N) or emboldened (for 3 × ³H in the side chain).





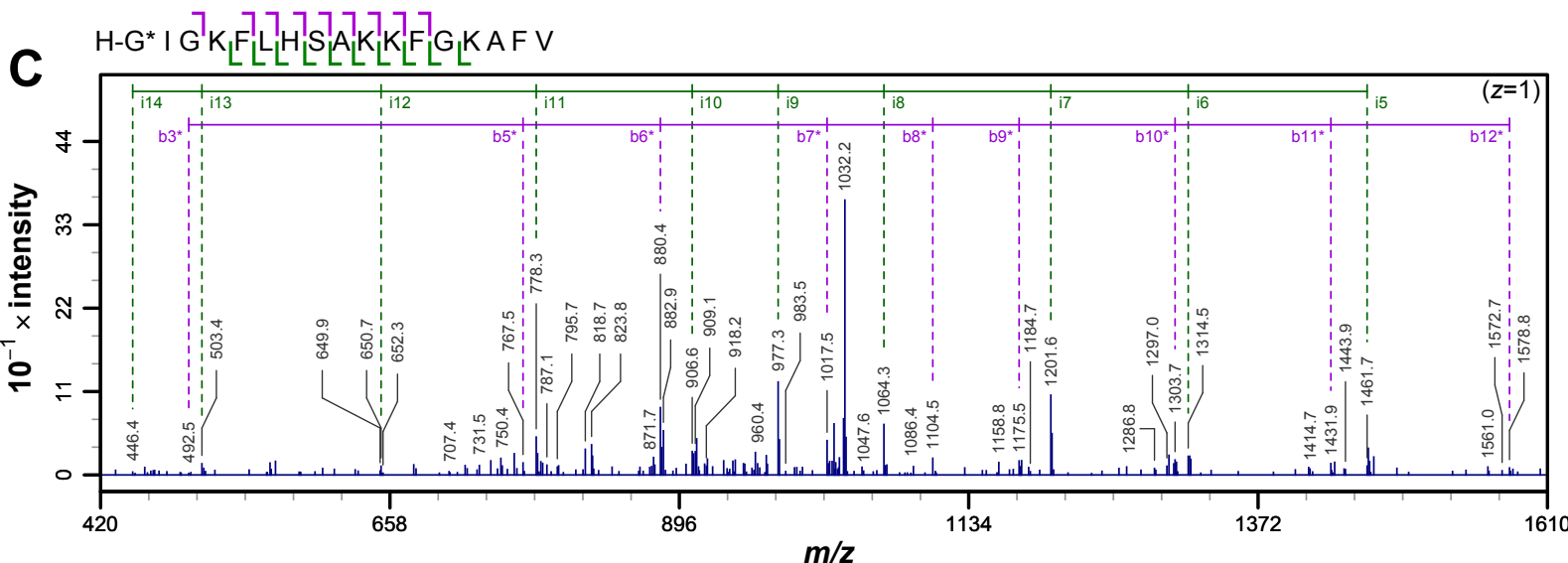
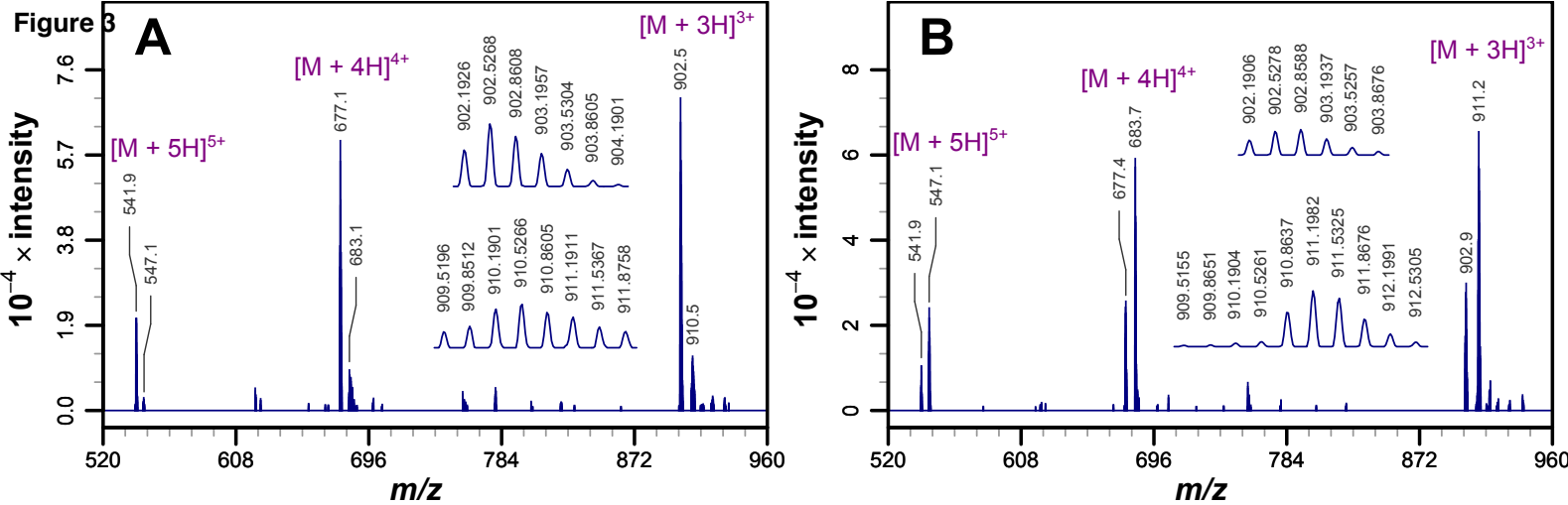


Figure 4

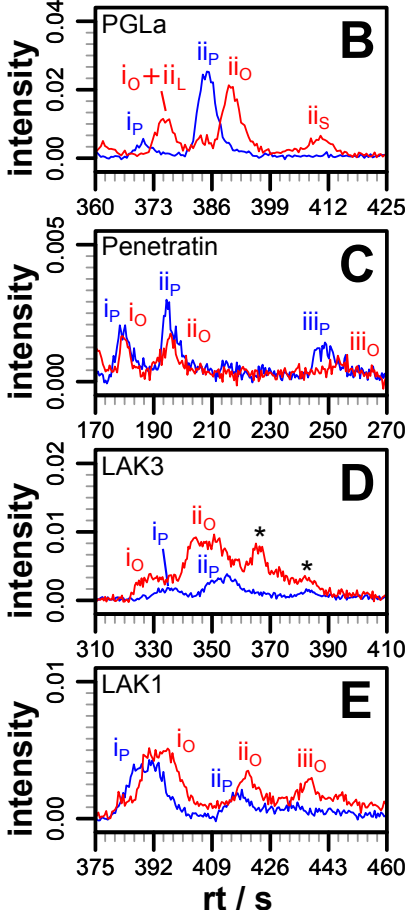
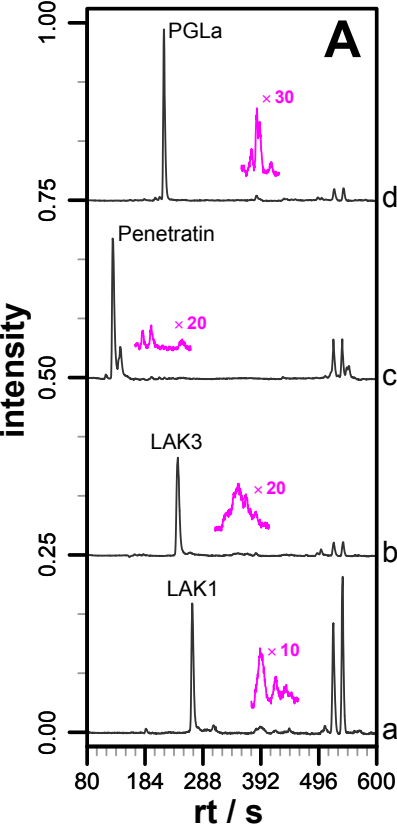
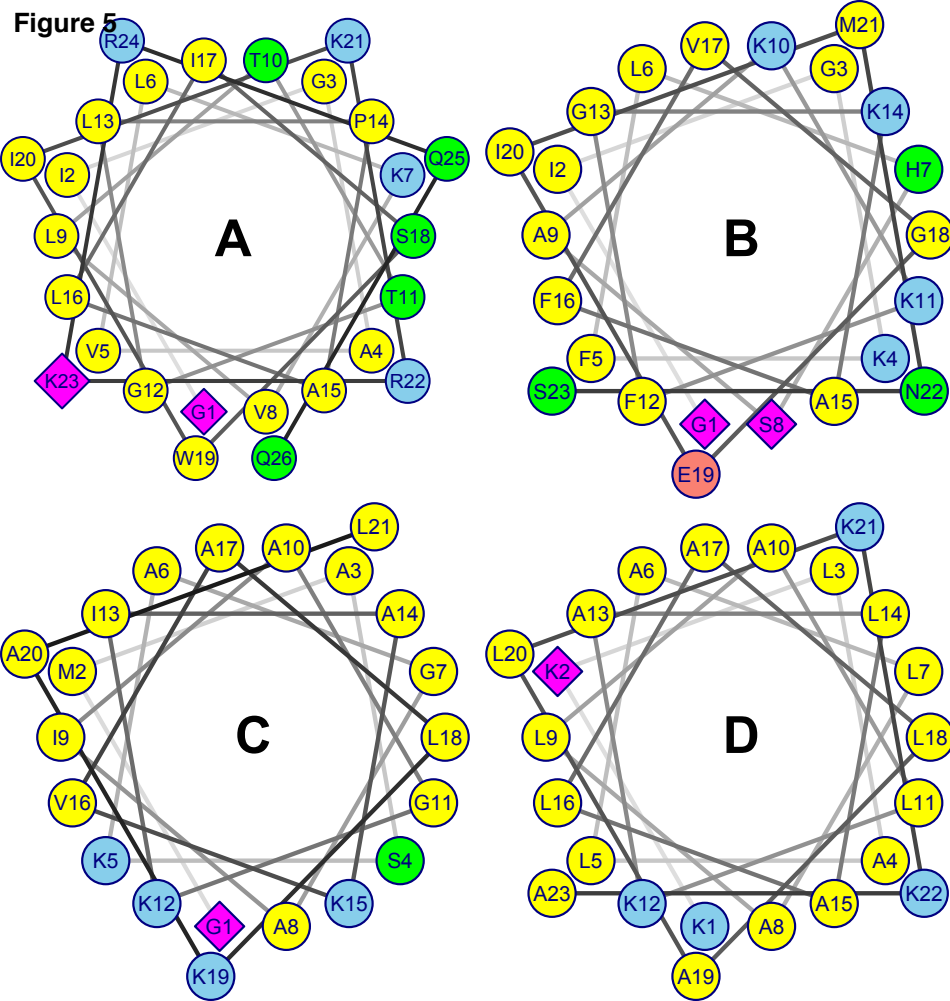


Figure 5



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