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Analysis of Transmembrane Domains and Lipid Modified Peptides with Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry

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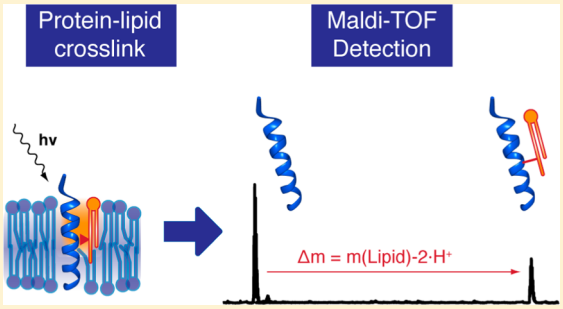
Supporting Information

ABSTRACT: Protein−lipid interactions within the membrane are difficult to detect with mass spectrometry because of the hydrophobicity of tryptic cleavage peptides on the one hand and the noncovalent nature of the protein−lipid interaction on the other hand. Here we describe a proof-of-principle method capable of resolving hydrophobic and acylated (e.g., myristoylated) peptides by optimizing the steps in a mass spectrometric workflow. We then use this optimized workflow to detect a protein−lipid interaction *in vitro* within the hydrophobic phase of the membrane that is preserved via a covalent cross-link using a photoactivatable lipid. This approach can also be used to map the site of a protein−lipid interaction as we identify the peptide in contact with the fatty acid part of ceramide in the START domain of the CERT protein.



Membrane proteins are important drug targets,[1] and their function can be modulated by their lipid environment.[2,3] These interactions have been investigated using structural methods,[4] NMR,[5] Förster resonance energy transfer, and mass spectrometry of intact assemblies.[6] However, detecting the interaction with mass spectrometry on the peptide level is a challenge, due to the strong hydrophobicity of the interacting molecules.[7] For the analysis of intrinsically hydrophobic or lipid modified peptides, several steps in the workflow must be optimized: proteins and their fragments have to be solubilized during the digest.[8,9] The resulting peptides need to be recovered from the digest[10] and from desalting columns[11] and later cocrystallized with the matrix.[9,12]

Here we present an optimized workflow for the detection of hydrophobic peptides. As a proof-of-principle, we analyzed the moderate hydrophobic transmembrane domain (TMD) peptide of the p24 protein[2] (p24), the strongly hydrophobic TMD peptide of the insulin receptor[13] (IR), and the myristoylated peptide of human ADP-ribosylation factor 1 (Arf1).[14] We use this method to detect the protein lipid adduct of a TMD with a photoactivatable lipid. Finally, we map a peptide that interacts with the START domain of the ceramide transport protein (CERT[15]).

MATERIALS AND METHODS

Proteins. The DNA sequence corresponding to amino acids 161−193 of p24 (TMED2_HUMAN, Uniprot Q15363) was cloned into the vector pMal-C5x (New England, Biolabs) via NotI and *Bam*HI sites resulting in MBP-tev-p24(TMD). The protein was expressed in *Escherichia coli* BL21 cells and purified on an amylose resin (NEB).[2] CERT-START (C43BP_HUMAN, Uniprot Q9Y5P4) DNA was kindly donated by Joost Holthuis (Osnabrück, Germany) and expressed analogous to STARD7 as described elsewhere.[16] Full-length human insulin receptor (INSR_HUMAN, Uniprot P06213, Isoform Short, HIR-A) was purified as outlined in the Supporting Information.

Reconstitution of MPB-tev-p24(TMD) in Proteoliposomes. Proteoliposomes (200 nM maltose-binding protein (MBP)-tev-p24(TMD) in 1 mM of lipids) were generated according to Contreras et al.[2] Briefly, the desired lipids (DOPC/PE/pSM, 85:10:2) were dried down, placed in a desiccator for 30 min, and then resuspended in HEPES/KOH buffer (20 mM, pH 7.4, 150 mM NaCl). MBP-tev-p24(TMD) was added in HEPES/KOH OG buffer (containing *n*-octyl-*β*-D-glucoside (OG), final concentration 33 mM) and the detergent was removed by several incubations with Bio-Beads SM-2 (Bio-Rad) at 4 °C. The samples were UV-irradiated for 2 min (Sylvania R 100 W) at a distance of about 5 cm at 25 °C.

Ceramide Labeling of CERT-START. CERT-START was loaded with liposomes similar to Hanada et al.[15] DOPC

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liposomes with 5% photoactivatable ceramide were created. To this end, a total of 1 μmol of lipids was dried down with nitrogen and placed in a desiccator for 30 min. Buffer A (10 mM Hepes pH = 7.4, 150 mM NaCl) (200 μL) was added, vortexed, and set for 1 h at 55 °C at 750 rpm. Liposomes were subjected to 10 rounds of sonication and freeze/thaw cycles (liquid nitrogen/55 °C water bath) until the solution became almost transparent. START-CERT (30 μg) protein was diluted to 20 μL with buffer A. Of the liposome solution, 20 μL was added and incubated for 30 min at 37 °C. Samples were UV-irradiated (Sylvania R 100 W) at a distance of about 5 cm for 2.5 min on ice.

Tryptic Digest. Of the respective protein, 10 μg was mixed with trypsin (Promega) in a ratio of 1:30 in 40 mM ammonium bicarbonate, adjusted to 10% acetonitrile and incubated at 37 °C overnight in an Eppendorf tube. When protein had to be precipitated,[17] it was first solubilized in 25 μL of 6 M urea, shaken at 40 °C for 10 min, and then adjusted to a final concentration of 1 M urea for digestion.

Partitioning of Hydrophobic Peptides. The extraction protocol was adapted from Folch et al.[18] To the tryptic digest, 250 μL of MeOH and 500 μL of chloroform were added and vigorously mixed. Ammonium bicarbonate (150 mM) was added to a total of 150 μL of aqueous input. The samples were shaken at 1400 rpm for 30 min at room temperature (RT), and the phases were separated by benchtop centrifugation (10 500 rpm, 5 min). The organic phase was dried down in a SpeedVac and taken up in acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA).

Desalting. When indicated, samples were concentrated and desalted using C_4 ZipTips (Millipore). The column beds were treated with the following concentrations of ACN/0.1%TFA in H_2O/0.1% TFA: wetting 3× in 20 μL 100%, equilibration 2× in 20 μL 40%, samples binding in 20 μL 40%, washing in 3× 20 μL 50%, and elution in 3× 1 μL 100% on the target. The acetonitrile concentration dependency of the ZipTip elution was tested by wetting, equilibrating, and loading the ZipTips as above. After washing the ZipTips with 40% ACN, the peptides were eluted with 2 μL of the indicated concentration of ACN on the target.

Target Preparation. α-Cyano-4-hydroxycinnamic acid (Bruker) was dissolved in a mixture of ACN/0.1% TFA (2:1) to saturation (modified from Redeby et al.[12]) and was adjusted to 6 mM OG with a 1% aqueous solution. 4-Chloro-α-cyanocinnamic acid (Cl-CCA) was kindly donated by Thorsten Jaskolla (University of Muenster)[19] and used at a concentration of 20 mM in 70% ACN/0.1% TFA. After 1 μL of matrix solution was applied to a steel target plate, 1 μL of sample solution was added to the matrix droplet and dried by a gentle vacuum.

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Analysis was performed on a Bruker Reflex III MALDI-TOF instrument in reflector mode. Identification of protein bands was achieved by peptide mass fingerprinting using Mascot (Matrix Science) and the MSDB mass spectrometry protein database. LIFT spectra were recorded on a ULTRAFLEX TOF/TOF (Bruker). After the spectra were obtained, the following peptide standards (Sigma-Aldrich) were added to the spot for internal calibration: Angiotensin_II (1046.5418), Angiotensin I (1296.6848), Bombesin (1619.8223), ACTH_clip (18−39, 2465.1983), Somatostatin (28, 3147.4710).

Octanol-Partioning. Proteins were digested *in silico* and their hydrophobicity was calculated using the Wimley-White scale.[20] In the case of hArf1, the water-to-octanol partitioning free energy (ΔG_{wo}) of the myristoyl anchor was estimated as an alanine (0.5 kcal/mol) plus 11 × ΔG_{wo} for a methylene group (which was estimated as −0.716 kcal/mol from Patton et al.[21]). Finally, the ΔG_{wo} for the charged N-terminus (5.5 kcal/mol) was subtracted.[21]

RESULTS AND DISCUSSION

To study hydrophobic peptides and their interaction with lipids in a mass spectrometric approach, we started with a TMD containing stretch of the p24 protein fused to maltose-binding protein (MBP-tev-p24(TMD), Figure 1A).[2] A standard peptide mass fingerprint after SDS-PAGE[22] resulted in 77% sequence

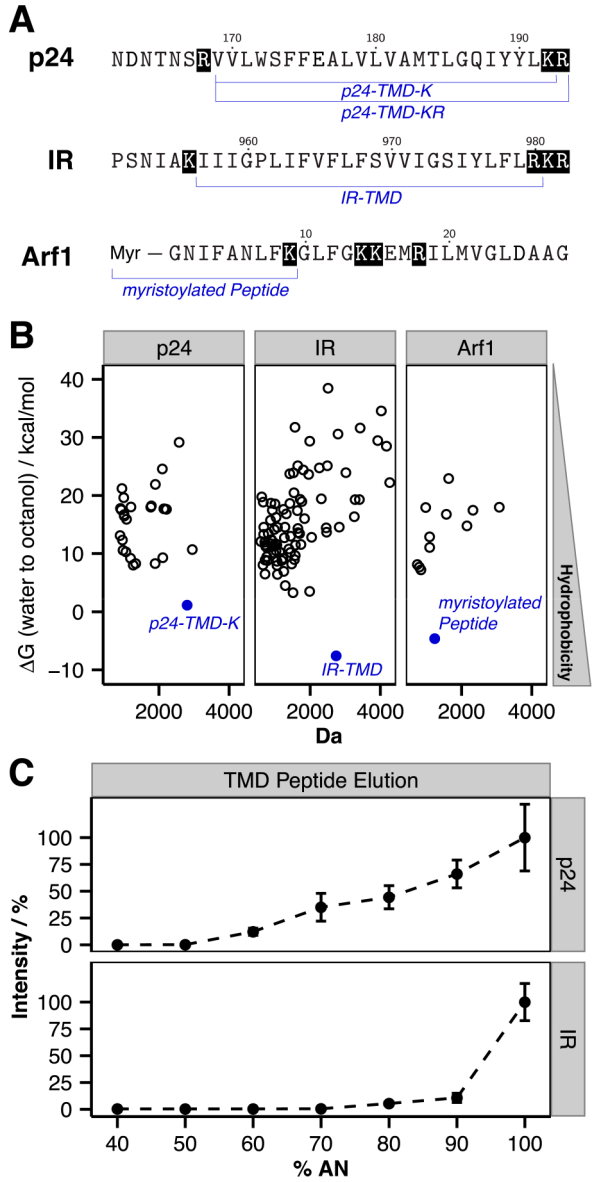

Figure 1. Hydrophobic tryptic peptides: (A) hydrophobic peptides used in this study: MBP-tev-p24(TMD) (p24), the human insulin receptor (IR), and human Arf1. Sites of tryptic cleavage are indicated in black. (B) Hydrophobicity plot of the *in silico* tryptic digests. The peptide's m/z (assuming $z = 1$) plotted against their free energies of water-to-octanol partitioning (ΔG_{wo}). Peptides from part A are indicated in blue. (C) Elution profile of hydrophobic peptides from ZipTips depending on the ACN concentration as MALDI-TOF MS intensities of the spectra obtained for peptides with the lowest [ΔG_{wo} (p24-TMD-KR) = 2.96 kcal/mol] and highest [ΔG_{wo} (IR-TMD) = −7.58 kcal/mol] hydrophobicity in the study.

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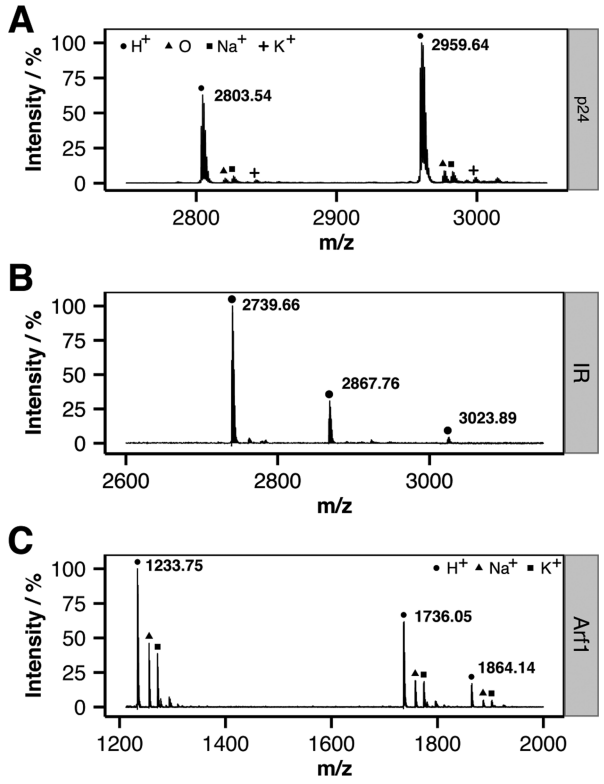

Figure 2. MALDI-TOF MS spectra of hydrophobic peptides and lipid peptide adducts. Proteins indicated were digested with trypsin, Folch partitioned, desalted, and mixed with Cl-CCA: (A) TMD of p24, (B) TMD of the insulin receptor (IR) in three peptides, (C) myristoylated peptide of human Arf1 (Arf1) as three peaks. Peptide details are show in Table S-1 in the Supporting Information.

coverage. However, the TMD peptide was not recovered. Free energies of water-to-octanol partitioning ΔG_{wo}[20] of the *in silico* generated tryptic peptides of MBP-tev-p24(TMD) indicated the TMD-peptide to be very hydrophobic (Figure 1B, left panel), most likely the cause for the lack of its enrichment and detection. We therefore adjusted our workflow to the peptide of interest.

Digest. Digestion conditions were changed from in-gel to solution. If the proteins to be digested were provided in detergent, they were precipitated with organic solvents beforehand.[17] In order to keep the hydrophobic proteins or peptides soluble, organic solvents or urea were applied.[9] This solubilization is most likely important, because with continuous digestion peripheral hydrophilic peptides are removed, increasingly exposing the hydrophobic sites that become more and more insoluble. Because of this effect, the protein often appears in multiple forms with missed cleavages (Figure 2). If the effect is too pronounced, then the peptide might be represented by a high number of derivatives and drop below the detection limit.

Peptide Recovery. Hydrophobic peptides were recovered by direct Folch partitioning[18] of the tryptic digest to also recover precipitated peptides, similar to earlier attempts.[10] MBP-tev-p24(TMD) peptides partitioning into the organic and aqueous phases were profiled by MALDI-TOF MS: Nearly all peptides were found in the aqueous phase, while peptides with a ΔG_{wo} < 9.5 kcal/mol were occasionally detected in the organic phase. The TMD peptide was only recovered in the organic phase and represented the most abundant peak there (Figure 2A and Figure S-1, Table S-1 in the Supporting Information). Its sequence was confirmed using LIFT TOF/TOF (Figure S-2A, Table S-3 in the Supporting Information). Besides their solubilization, the organic partitioning also isolates the lower ionizing hydrophobic peptides from their hydrophilic counterparts, which might help to prevent ion suppression.

Desalting. When hydrophobic peptides are purified on ZipTips to remove residual salts or urea, the conditions must be adjusted to match the hydrophobic character of these peptides ensuring efficient loading, washing, and elution. Therefore peptides are solubilized and loaded with a substantial fraction of organic solvents and eluted with an even higher solvent concentration.[11] To illustrate the retention of TMD peptides on ZipTips, we monitored their elution with increasing ACN concentrations for the least and most hydrophobic peptide used in this study (Figure 1C). The ACN concentrations needed for TMD release are usually beyond the 50−75% ACN used in standard protocols.

Matrixes. Hydrophobic peptides need to be solubilized for the process of crystallization on the target by organic solvents[12] or detergents. The p24-TMD peptide was only detected with α-cyano-4-hydroxycinnamic acid matrix when >5 mM *n*-octyl-β-D-glucoside (OG) was used in the saturated matrix (Figure S-1 in the Supporting Information).[9,23] However, the detection of hydrophobic peptides also depends on the right choice of matrix.[24] We observed good signal intensities with the Cl-CCA matrix in 70% ACN[19] while the sample was provided in 100% ACN/0.1% TFA (e.g., Figures 2−4).

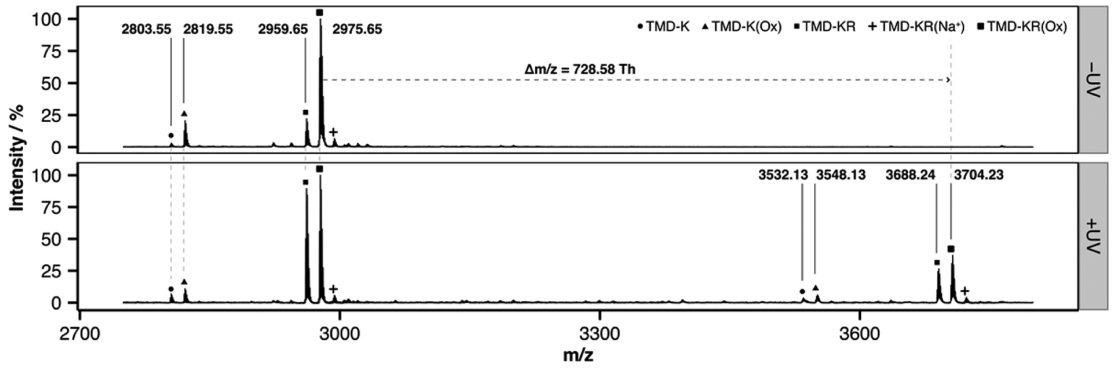

Figure 3. Detection of a TMD−lipid interaction. Spectra of the MBP-tev-p24(TMD) transmembrane peptide recovered from proteoliposomes with photoactivatable sphingomyelin. The protein was precipitated with organic solvents, digested with trypsin, Folch partitioning, and desalted. When no UV was applied, TMD-K, TMD-KR, and the oxidized peptides were detected. The UV-treated sample exhibited an additional set of peaks that were shifted to a higher mass ($\Delta m/z$ = 728.58 Th) for all four peaks.

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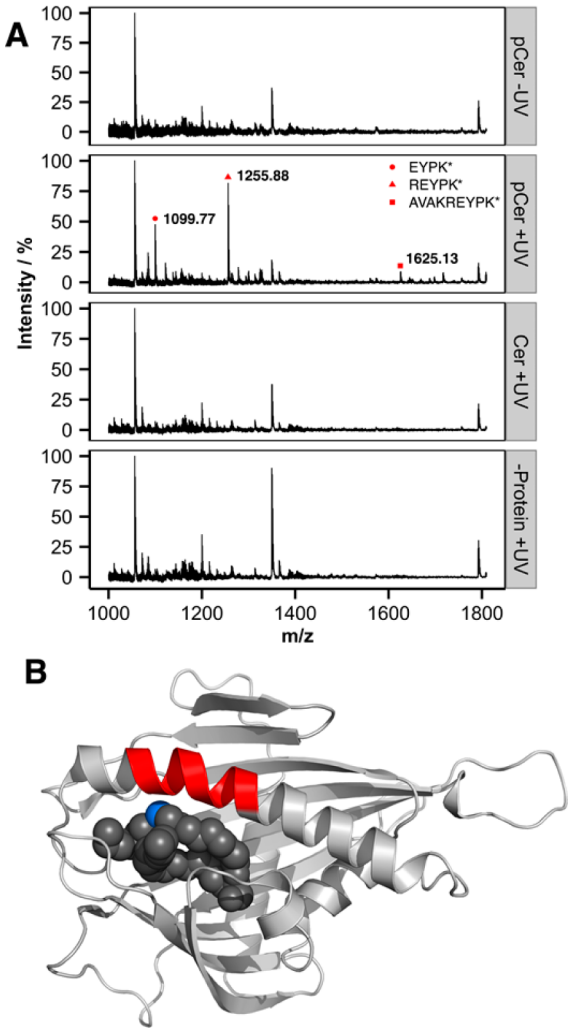

Figure 4. Mapping of a lipid transfer protein−lipid interaction. (A) Spectra of the peptide−lipid cross-links (indicated with ∗) of the CERT-START domain after incubation with PC liposomes containing 5% photoactivatable Cer and UV-irradition. The protein was precipitated with organic solvents, digested with trypsin, Folch partitioning, and desalted. The labeled peptides were not found without UV-irradiation (pCer -UV) or when not-photoactivatable ceramide (Cer + UV) was used. The background was also detected when the CERT-START domain was omitted (− Protein + UV). (B) Crystal structure of the CERT-START domain with Cer (d18:1/18:0) bound.[28] The position of the diazirine on the photoactivatable ceramide (blue) and the corresponding overlapping peptides (red) that were detected as protein−lipid adducts in part A are indicated.

In summary, we set up a method that is optimized for detecting hydrophobic peptides by adjusting every step in the MALDI-TOF MS workflow to meet the specific characteristics of the hydrophobic peptides. With this approach we were still able to detect 8 fmol of TMD-KR peptide at a signal-to-noise ratio >10, when the MBP-tev-p24 digest extract was serially diluted.

In order to challenge our method, we applied it to other hydrophobic peptides. The tryptic digest of the human insulin receptor (IR),[13] a single-spanning membrane protein of 1370 amino acids, results in a 24 amino acid TMD containing peptide with a ΔG_{wo} = −7.58 kcal/mol (Figure 1A,B), which is significantly more hydrophobic than the p24 TMD (Figure 1B, middle panel). After digest, extraction and ZipTip cleanup the major peptides in the organic phase were three TMD containing peptides (Figure 2B and Table S-1 in the Supporting Information), the most intense of which was confirmed by fragmentation (Figure S-2B and Table S-4 in the Supporting Information).

Furthermore, we investigated a membrane anchor peptide that comprises the myristoylated N-terminal amphipathic α-helix[25] of the human ADP-ribosylation factor 1 (Arf1).[14] Three intense peaks were found in the organic phase (Figure 2C), the major of which was fragmented (Figure S-2C and Table S-5 in the Supporting Information). The nonmyristoylated N-terminal tryptic peptide is calculated to be mildly hydrophobic (ΔG_{wo} = +8.26 kcal/mol), but its hydrophobicity increases to a calculated ΔG_{wo} = −4.62 kcal/mol due to the addition of the myristoyl-anchor and the concomitant loss of the charged N-terminus (Figure 1B, left panel). Therefore, the method can also be applied to fatty-acylated peptides.

To investigate the interaction of a TMD with a lipid, we cross-linked the MBP-tev-p24(TMD) fusion protein in proteoliposomes with a sphingomyelin that contains a photolabile diazirine group. Upon UV irradiation at 350 nm, diazirines yield a highly reactive carbene that covalently links to molecules in closest proximity (<3 Å).[26] The resulting covalent protein−lipid cross-link product was separated from the remaining lipids by protein precipitation with organic solvents,[17] was then digested by trypsin, and was extracted by Folch partitioning. MALDI-TOF MS analysis of the not UV irradiated control proteoliposomes yielded peaks representing the p24 TMD (Figure 3, upper panel, Table S-1 in the Supporting Information). During preparation of the proteoliposomes, the methionine in the TMD was oxidized, which resulted in a total of 4 peaks. Even the presence of 1 mM tris(2-carboxyethyl)phosphine (TCEP) in the proteoliposome preparation could not prevent this oxidation. The UV irradiated sample also contained the TMD peaks (Figure 3, lower panel) and in addition 4 peaks that were shifted to higher m/z by a mean of 728.60 ± 0.02. This shift is consistent with the insertion of a SM(d18:1/18:0) carbene into a residue of the transmembrane peptide (theoretical value 728.583, Figure 3).

Thus, our method detects the cross-link product of a photoactivatable lipid with a transmembrane domain of a protein and thus allows analyzing the interaction of proteins with individual molecular lipid species by mass spectrometry. TMD lipid cross-links have been also investigated with photoactivatable peptides incorporated into liposomes and electrospray ionization of complete proteoliposomes.[27] However, we find our method is specifically improved by separation of the MBP tagged protein from the liposomal lipids before digest and extraction, which allows low protein to lipid ratios and removes the lipid excess from the spectrum.

In order to map the site of a lipid protein cross-link, we investigated the C-terminal START domain of the ceramide transporter CERT,[15] which catalyzes the transport of ceramide between the ER and the Golgi. We loaded the CERT START[28] protein by incubation with PC liposomes that contain photoactivatable ceramide and irradiated the complex with UV light, thereby cross-linking the lipid to the protein.[16] The protein was again precipitated and digested with trypsin, and hydrophobic peptides were extracted. In the irradiated sample, a series of peptides was found that were not present in the control. Their masses are consistent with a Cer(d18:1/18:0) cross-link to the peptide AVAKREYPK and its smaller tryptic fragments (Figure 4A, Table S-1 in the Supporting Information). The identified peptide is positioned right next to Cer(d18:1/18:0) in the CERT START crystal structure[28] (Figure 4B). A control sample loaded

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with natural, nonphotoactivatable ceramide showed a spectrum comparable to the −UV control. The background seems independent of the CERT protein, as it is also detected when no CERT was applied to the assay (Figure 4B).

CONCLUSION

We have developed a method to detect hydrophobic peptides such as TMDs and acylated peptides. These peptides are usually difficult to measure due to their size and hydrophobicity. This method is also applicable to study protein−lipid interactions by mass spectrometry when photoactivatable lipids are used. Here we show that the process works *in vitro*; however, cells are able to form all kinds of photoactivatable sphingolipids from a diazirine sphingosine.[29] Although, processes like the isolation, separation, and MS/MS identification of the cellular adducts still need to be optimized, the logical next step is to apply the method to cellular assays to map the protein−lipid interactions of transmembrane proteins[2] and lipid transport proteins[30,31] that have yet not been assigned.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.J.G., B.B., and F.W. designed the research; M.J.G. conceived the ideas, performed the experimental research, and analyzed the data; Ü.C. and M.G. purified the insulin receptor; T.S. synthesized the diazirine lipids; and M.J.G., B.B., and F.W. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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