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Cellular uptake and biophysical properties of galactose and/or tryptophan containing cell-penetrating peptides

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ABSTRACT

Glycosylated cell penetrating peptides (CPPs) have been conjugated to a peptide cargo and the efficiency of cargo delivery into wild type Chinese hamster ovary (CHO) and proteoglycan deficient CHO cells has been quantified by MALDI-TOF mass spectrometry and compared to tryptophan- or alanine containing CPPs. In parallel, the behavior of these CPPs in contact with model membranes has been characterized by different biophysical techniques: Differential Scanning and Isothermal Titration Calorimetries, Imaging Ellipsometry and Attenuated Total Reflectance IR spectroscopy. With these CPPs we have demonstrated that tryptophan residues play a key role in the insertion of a CPP and its conjugate into the membrane: galactosyl residues hampered the internalization when introduced in the middle of the amphipathic secondary structure of a CPP but not when added to the *N*-terminus, as long as the tryptophan residues were still present in the sequence. The insertion of these CPPs into membrane models was enthalpy driven and was related to the number of tryptophans in the sequence of these secondary amphipathic CPPs. Additionally, we have observed a certain propensity of the investigated CPP analogs to aggregate in contact with the lipid surface.

1. Introduction

Cell penetrating peptides (CPPs) with their capacity to shuttle cargoes inside cells are regarded as a sort of "magic bullets" for intracellular delivery of bioactive molecules, even though their intracellular localization is dependent on their mechanism(s) of entrance: direct translocation of the plasma membrane or endocytotic pathways [1–6]. In search of some specificity in addressing CPPs, we have studied the effect of CPP myristoylation on the delivery of a peptide cargo. Myristoylation of commonly used CPPs and synthetic dendrimers showed an increase in conveying a cargo inside CHO cells [7,8]. Recently, we have explored the influence of glycosylation of a CPP sequence on cargo delivery, taking as a starting point our lead CPP: Ac-RRWWRRWRR-NH_2 [9]. Glycosylation of bioactive compounds has been used mainly for increasing the hydrophilicity, the enzymatic stability, and/or the delivery into the brain [10–12]. In the context of CPPs, we have hypothesized that glycosylation could regulate the binding to the plasma membrane of CPPs and conjugates and consequently their internalization pathway and/or specificity.

In our previous study, the pro-apoptotic KLAK peptide $(KLAKLAK)_2$, was conjugated via a labile disulfide linker to analogs of the CPP Ac-C-RRWWRRWRR-NH_2, these analogs possessing galactosyl unit(s) instead of the indole side-chains of the tryptophans [13]. Cell viability studies showed an inverse relationship with the number of galactosyl moieties, the conjugate, Ac-C-RRA(βtGal)A(βtGal)RRA(βtGal)RR-NH_2, with three galactoses and no tryptophan, did not induce CHO cell death. Furthermore, fluorescence studies performed in parallel showed that the KLAK peptide was not delivered inside the cells with this galactosyl-substituted peptide. This correlation between cell death and cellular uptake of the cargo was also observed for the other glycosylated vectors studied [13]. Rothbard et al. have shown that analogs of

Abbreviations: ATR Spectroscopy, Attenuated Total Reflection Spectroscopy; CPP, cell-penetrating peptide; CHO, Chinese hamster ovary; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycine; DSC, differential scanning calorimetry; DSPG, distearylphosphatidylglycine; GAG, glycosaminoglycan; DMEM, Dulbecco's modified Eagle's medium; ITC, isothermal titration calorimetry; KLAK peptide, $(KLAKLAK)_2$; LUVs, large unilamellar vesicles; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MLVs, multilamellar vesicles; MS, mass spectrometry; NMP, *N*-methylpyrrolidone; PKCi, peptide inhibitor of protein kinase C; RP-HPLC, reverse-phase high-pressure chromatography; RT, room temperature; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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$(Arg)_{10}$ were as efficient as $(Arg)_{10}$ to enter Jurkat cells when simultaneously modified in positions 2, 5 and 8 by proteinogenic or modified amino acids (Trp and Cys were not tested), except the two analogs with acidic residues: Arg_7/Asp_3 and Arg_7/Glu_3, which "entered cells relatively poorly" [14]. The aim of the present study was to aid in the identification of the structural, physico-chemical and thermodynamic parameters relevant for cellular uptake. For this, we have quantified the efficiency of cargo delivery of glycosylated CPPs into cells and analyzed by different techniques their interactions with model membranes. The glycosylated CPPs studied previously ((**3**) and (**4**), (Table 1)) were conjugated to a peptide inhibitor of protein kinase C (PKCi) [7,15–17]. We have also synthesized a new glycosylated CPP (**5**) with three galactose units on the *N*-terminus of the Arg/Trp sequence, and a control Arg/Ala peptide (**2**), suspecting that tryptophan residues were playing an essential role in the internalization process.

Herein, we report the quantification by MALDI-TOF mass spectrometry [18,19] of the PKCi cargo internalized into wild type CHO and proteoglycan deficient CHO cells [4]. Indeed many studies showed that CPPs bind to glycosaminoglycans (GAGs) with significant affinity [20–22] and that GAGs are involved in CPPs internalization pathways [4,23]. In parallel, we have characterized by different biophysical techniques (DSC, ITC, Imaging Ellipsometry and ATR) the behavior of the glycosylated CPPs in contact with LUVs and lipid monolayers. Our results show a clear correlation between the efficiency of internalization, the presence of tryptophan residues on the CPP sequence and an enthalpy gain resulting from the energetically favorable transfer of Trp residues from the aqueous solution to the interior of the lipid bilayer.

2. Material and methods

2.1. Peptide syntheses

Rink amide (*p*-methylbenzhydrylamine)-resin (MBHA-resin, 100–200 mesh), (4-(2′-4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA polystyrene resin, 0.64 mmol/g), *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-Cys(*S*-*St*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Ala-OH were purchased from Novabiochem. Fmoc-Pra-OH was purchased from IRIS Biotech. Peptides were characterized by MALDI-TOF MS (DE-Pro, ABI) in positive ion reflector mode using the matrix CHCA. The m/z of the protonated molecules is given as experimental and calculated in Table 2. Proteomix 4 (Laserbio Labs) [500–3500 Da] was used for calibration.

Table 1
Sequences of the cell-penetrating peptides and the corresponding PKCi conjugates.

N°	Sequence
(**1**)	Ac-C(*S*-Acm)*RRWWRRWRR-NH$_2$*
(**2**)	Ac-C(*S*-Acm)*RRAARRARR-NH$_2$*
(**3**)	Ac-C(*S*-Acm)*RRWA(βtGal)RRWRR-NH$_2$*
(**4**)	Ac-C(*S*-Acm)*RRA(βtGal)A(βtGal)RRA(βtGal)RR-NH$_2$*
(**5**)	Ac-*A(βtGal)A(βtGal)A(βtGal)*C(*S*-Acm) *RRWWRRWRR-NH$_2$*
(**1′**)	Biot(O_2)-GGGGC(S-S(*NAc*)*CRRWWRRWRR-NH$_2$*)-RFARKGALRQKNV-NH_2
(**2′**)	Biot(O_2)-GGGGC(S-S(*NAc*)*CRRAARRARR-NH$_2$*)-RFARKGALRQKNV-NH_2
(**3′**)	Biot(O_2)-GGGGC(S-S(*NAc*)*CRRWA(βtGal)RRWRR-NH$_2$*)-RFARKGALRQKNV-NH_2
(**4′**)	Biot(O_2)-GGGGC(S-S(*NAc*)*CRR(βtGalA)(βtGalA)RR(βtGalA)RR-NH$_2$*)-RFARKGALRQKNV-NH_2
(**5′**)	Biot(O_2)GGGGC(S-S(*NAc-A(βtGal)A(βtGal)A(βtGal))CRRWWRRWRRNH$_2$*)-RFARKGALRQKNV-NH_2

Acm: $-CH_2-C(O)-NH_2$; Biot(O_2): biotine sulfone; A(βtGal): β-(1H,3-galactosyl-[1-3] triazol)alanine and PKCi: RFARKGALRQKNV. CPP sequences are represented in italic in the table for a better visualization within the sequences of the conjugates with PKCi.

2.1.1. Ac-C-RRAARRARR-NH_2

Ac-C(*S*-*St*Bu)-RRAARRARR-NH_2 was synthesized manually by solid-phase methodology on a Rink amide resin (0.64 mmol/g) by Fmoc strategy in a fritted syringe. Fmoc protection was removed with a solution of piperidine in NMP (20%, 3×5min). Stepwise coupling reactions were performed with Fmoc-protected amino acids, HBTU and DIPEA (3/2.85/3 eq) at RT. After removal of the last Fmoc protecting group, the peptide was *N*-acetylated with a solution of acetic anhydride in NMP (20%, 45 min). The resin was washed with NMP, CH_2Cl_2, CH_3OH and dried under *vacuum*. The resin was then treated with TFA/H_2O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), the cleaved peptide precipitated in diethyl ether, lyophilized and purified by RP-HPLC on a preparative C8 column using a 30 min linear methanol (0.1% TFA) gradient in an aqueous solution (0.1% TFA) to yield the product as a white powder after lyophilisation (20% yield). The purity of the peptide was determined by analytical RP-HPLC on a C8 column using a linear methanol (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 15.7 min (0–100% methanol over 30 min). The peptide was characterized by MALDI-TOF MS: (m/z) $[MH]^+$ calcd 1400.80, found 1400.90.

DTT (20 eq) was added to Ac-C(*S*-*St*Bu)-RRAARRARR-NH_2, dissolved in a degassed solution of Tris–HCl 50 mM (~2 mM), and the reaction was stirred at RT for 2 h. The reaction was monitored by RP-HPLC. Crude thiol peptide was then purified by RP-HPLC on a preparative C8 column using a linear methanol (0.1% TFA) gradient in an aqueous solution (0.1% TFA) to yield the product as a white powder after lyophilisation (9% yield). Peptide purity was analyzed by analytical RP-HPLC on a C8 column using a linear methanol (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 9.8 min (0–100% methanol over 30 min). The peptide was characterized by MALDI-TOF MS: (m/z) $[MH]^+$ calcd 1312.76, found 1312.56.

2.1.2. Ac-A(βtGal)A(βtGal)A(βtGal)-C-RRWWRRWRR-NH_2

Ac-Pra-Pra-Pra-C(*S*-*St*Bu)-RRWWRRWRR-NH_2 was synthesized manually by solid-phase methodology on a Rink amide resin (0.64 mmol/g) by Fmoc strategy in a fritted syringe. Fmoc protection was removed with a solution of piperidine in NMP (20%, 3×5 min). Stepwise coupling reactions were performed with Fmoc-protected amino acids, HBTU and DIPEA (3/2.85/3 eq) at RT. After removal of the last Fmoc protecting group, the peptide was *N*-acetylated with a solution of acetic anhydride in NMP (20%, 45 min). The resin was washed with NMP, CH_2Cl_2, MeOH and dried under vacuum. For analysis, approximately 5 mg of the resin was treated with TFA/H_2O/TIS: 95/2.5/2.5 (15 mL/g resin, 2 h, RT), the peptide precipitated in diethyl ether, lyophilized and analyzed by analytical RP-HPLC on a C8 column using a linear methanol (0.1% TFA) gradient in an aqueous solution (0.1% TFA). Retention time of the product was 9.8 min (0–100% methanol over 30 min). The pure peptide was characterized by MALDI-TOF MS (m/z) [MH] + calcd 2031.04, found 2031.1.

In a fritted syringe CuI (15 eq), ascorbic acid (15 eq), the azido-sugar: 1-azido-2,3,4,6-tetra-*O*-acetyl-β-(D)-galactopyranoside (6 eq) [13] and a degassed solution of DMF(anhydrous)/2,6-lutidine (7/3) containing DIPEA (21 eq) were added to the dried Pra containing-peptidyl-resin, Ac-Pra-Pra-Pra-C(*S*-*St*Bu)-RRWWRRWRR-resin (0.023 M). The solution was sonicated for 5 min and shaken for 15 h at RT. The resin was then washed with DMF, DMF/Pyridine (6/5) containing ascorbic acid (0.02 g/mL), CH_2Cl_2 and MeOH. The resin was dried under vacuum and then cleaved using TFA/H_2O/TIS: 95/2.5/2.5. Analytical RP-HPLC showed complete conversion of the peptide into the glycopeptide. The major peak in the HPLC spectra was identified as the expected product. RP-HPLC retention time of the crude product was ~16.5 min (30–100% methanol over 30 min). MALDI-TOF MS: (m/z) $[MH]^+$ calcd 3150.37, found 3150.56. This peptide, Ac-A(βtGal(*OAc*))A(βtGal(*OAc*)A(βtGal(*OAc*))-C(*S*-*St*Bu)-RRWWRRWRR-NH_2 was used directly for the next step.