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Gramicidin A-based peptide vector for intracellular protein delivery

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ABSTRACT

The development of the peptide-based vectors for the intracellular delivery of biologically active macromolecules has opened new prospects of their application in research and therapy. Earlier the amphipathic cell-penetrating peptide (CPP) Pep-1 was reported to mediate cellular uptake of proteins without covalent binding to them. In this work we studied the ability of a series of membrane-active amphipathic peptides, based on the gramicidin A sequence, to transport a model protein across the eukaryotic cell membrane. Among them the positively charged Cys-containing peptide P10C demonstrated the most effective β-galactosidase intracellular delivery. Besides, this peptide was shown to form noncovalent associates with β-galactosidase as judged from electrophoresis and enzymatic activity assays. In addition, a series of new gramicidin analogues were prepared and the effect of N-terminus modification of gramicidin on the protein transduction efficiency was studied.

1. Introduction

Impermeability of the cell membrane for hydrophilic molecules is an important factor restricting the use of peptide- and protein-based drugs. To resolve this problem, a number of approaches, e.g. microinjection, electroporation, application of liposomal and viral vectors, have been developed. A series of studies have demonstrated that short fragments of several proteins named "protein transduction domains" (PTDs) are responsible for the internalization of the proteins [1–6], and since then the idea of creating peptide-based delivery systems has attracted considerable interest. In particular, the Tat_{48-60} peptide (the fragment of the HIV-1 Tat protein) and Penetratin, derived from the 60-amino acid residue homeodomain of the *Drosophila* Antennapedia transcription factor, should be mentioned [7]. Along with protein-derived PTDs, a number of chimeric and synthetic peptides, called "cell-penetrating peptides" (CPPs) have been developed [7–11]. It was shown that these peptides can translocate a variety of covalently linked cargo components, such as proteins [12,13], peptides [14], oligonucleotides [15] and even nanoparticles [16] and liposomes [17], across membranes, which led to extensive use of CPPs as delivery systems.

The requirement of covalent binding of a vector peptide to the cargo restricts considerably the use of peptide-based delivery systems. Recently a peptide (named Pep-1) which forms noncovalent complexes with proteins and transfers them across membranes [18], has been described. This peptide is composed of two main fragments: the relatively hydrophobic sequence containing 5 Trp residues (KEWWETWWTEW) and the Lys-rich motif (KKKRKV) derived from the nuclear localization sequence of simian virus 40 large T antigen; two fragments are linked by a short spacer (SQP). C- and N-terminus of the peptide are capped with cysteamine and acetyl groups, respectively.

Another cell-penetrating peptide, YTA2, developed by Lindgren et al. has also been shown to deliver proteins into cells without chemical coupling between the peptide and a cargo protein [19]. The above mentioned CPPs are built according to similar rules and have amphipathic structures.

Antimicrobial peptides represent another class of amphipathic peptides. The differentiation of these two classes of peptides is rather formal and the overlap between the structure and the mode of action of CPPs and antimicrobial peptides has been noted [20,21]. For example, antimicrobial peptides in several cases were shown to enter eukaryotic cells [22]. Moreover, the cell-penetrating peptide Pep-1 is readily transformed to an antibiotic by Glu→Lys substitution in the hydrophobic domain [23]. On the other hand, such CPPs as pVec and TP10 display distinct antimicrobial activity [24].

In preceding communications, we described synthesis and ion channel activity of a series of amphipathic peptides, based on the gramicidin A sequence [25,26]. In the present work we investigated the ability of such peptides to deliver proteins into eukaryotic cells using β-galactosidase as a cargo. The hydrophobic membrane-active

Abbreviations: CPP, cell-penetrating peptide; PTD, protein transduction domain; NLS, nuclear localization sequence; SV-40, simian virus 40; Tat, CPP, derived from HIV-1; β-Gal, β-galactosidase; oNPG, *o*-nitrophenil-β-D-galactoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline solution; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; DIC, diisopropylcarbodiimide; Fmoc, fluorenylmethoxycarbonyl; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis

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sequence of gramicidin A was considered to be an anchor providing affinity to the cell membrane. Besides, we expected the gramicidin motif with its 4 Trp residues to provide the formation of peptide–protein complexes via hydrophobic interactions, similarly to the Trp-rich domain of Pep-1 responsible for binding to the protein cargo [27]. The basic nuclear localization sequence derived from the SV-40 virus (KKKRKV) that was attached through a short spacer (GSG) to the C-terminus of gramicidin A, improved its solubility in aqueous solutions and was expected to induce cellular uptake. Furthermore, new analogues containing NLS at the N-terminus and those with the free N-terminus were prepared and studied.

2. Materials and methods

Rink amide resin and Trityl chloride resin for peptide synthesis was purchased from PepChem (Tubingen, Germany), Fmoc-protected amino acids were from Novabiochem (Bad Soden, Germany), standard chemicals for peptide chemistry were obtained from Fluka (Deisenhofen, Germany) and Chimmed (Moscow, Russia). Solvents were pure analytical grade. β-Galactosidase, *o*-nitrophenil-β-D-galactoside and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) were obtained from Sigma Aldrich (Steinheim, Germany), 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was from Bethesda Research Laboratories (Gaithersburg, MD). glutaraldehyde, 2-mercaptoethanol and Triton X-100 were purchased from Fluka (Deisenhofen, Germany).

Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, fetal bovine serum (FBS), trypsin–EDTA solution, streptomycin and penicillin, were purchased from Gibco Invitrogen Corp. (Carlsbad, CA).

2.1. Peptide synthesis

The synthetic peptides used in this study are listed in Table 1. Synthesis of the peptides P10C, N1C, P5C, P4C has been described in our previous reports [25,26]. Synthesis of new peptides was carried out by the solid phase method on Rink amide resin [4(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin] following the standard Fmoc chemistry procedure by using HOBt/DIC/*N*,*N*-dimethylformamide coupling reactions. Pep-1 was synthesized by means of standard solid phase Fmoc chemistry on trityl chloride resin, preloaded with Fmoc-cysteamine. N-terminal formylation (P4C) and N-terminal acetylation (Pep-1, P10C, P5C, N1C) of peptides were carried out using 4-nitrophenyl formate and acetic anhydride respectively, in the presence of N-ethyldiisopropylamine. The peptide resins were treated with trifluoroacetic acid–ethandithiol–water (94:3:3) for 2.5h. Peptides were purified by preparative HPLC on a C4 column with ethanol gradient in 0.1% trifluoroacetic acid in water and characterized by analytical HPLC and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis. The overall yields starting from the aminoacid–resin conjugates were 9–25%.

2.2. Cells and cell cultures

Adherent HeLa, L-929 and NIH-3T3 cells were routinely cultured as exponentionally growing subconfluent monolayers in 25-cm^2 cultural flasks in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (growth medium) in a humidified 5% CO_2 (37 °C) incubator. Cells were split in 1/4 dilution every 3–4 days, after they reached confluency. Jurkat lymphoid and Sp2/0 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in a humidified 5% CO_2 (37 °C) incubator.

Table 1
Amino acid sequences and molecular masses of the peptides examined in this study

Peptide	Sequence	Molecular mass (Da)	
		Calculated	Measured from mass-spectra
P10C	Ac-V G A l A v V v W l W l W l W βAGSGPKKKRKVC-amide	3091.8	3092.0
P4C	Form-V G A l A v V v W l W l W l W βAGSGPKKKRKVC-amide	3077.8	3076.8
P5C	Ac-V G A l A v V v W l W l W l W βAGSGPKKKRKVG-amide	3046.7	3046.5
N1C	Ac-V G A l A v V v W l W l W l W βAGSGEEEESQS	2843.9	2843.4
P11C	V G A l A v V v W l W l W l W βAGSGPKKKRKVC-amide	3049.8	3050.5
P1N	CPKKKRKVGSGV G A l A v V v W l W l W l W βA	3048.8	3050.6
P3N	CVKRKKKPGSGV G A l A v V v W l W l W l W βA	3048.8	3049.8

D-amino acids are printed in the lower-case letters.
Symbol βA denotes β-alanine residue.

2.3. Cellular uptake of P10C/β–Gal complexes

Peptide/β-Gal complexes were formed by titration of 0.25 μM protein with the peptide in PBS with subsequent incubation for 30 min at ambient temperature. Cells were washed with DMEM and treated with the preformed protein/peptide complexes solutions (the molar ratio from 7 to 600 and from 1 to 1000 for Pep-1 and P10C, respectively) diluted with DMEM. The amount of internalized β-Gal was determined by measuring its enzymatic activity using two substrates. Before the X-Gal staining procedure, cells were washed thoroughly with phosphate buffer and fixed with 0.25% glutaraldehyde solution. In the case of oNPG staining, in order to remove the protein that could be associated with the outer membrane, after the washing step cells were treated with trypsin–EDTA solution for 10 min as described [28], lysed with 0.2% Triton-40 μM mercaptoethanol solution, and oNPG solution (4 mg/ml) was added. The internalized protein was detected by the OD measurement at 405 nm.

2.4. Cytotoxicity assay

Cell viability was determined by the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, HeLa cells grown in 96-well plates to 75% confluency were incubated with 0.76–48 μM P10C or P4C in 30 μl DMEM for 2 h, then peptide solutions were aspirated and 10% serum was added. After 24 h 30 μl of MTT (5 mg/mL) was added to each well. The product (MTT formazan) was solubilized in dimethyl sulfoxide (DMSO) after 2 h, and cytotoxicity was evaluated by colorimetric assay at 540 nm and a reference wavelength of 620 nm as described [29] using Titertek Multiscan (Labsytems, Finland).

2.5. Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE was used for the protein/peptide complex formation analysis. It was performed by incubating 5.2 μM β-Gal with increasing amounts of P10C for 30 min at ambient temperature in a final volume of 10 μl, made up with 0.125 M Tris–HCl buffer (pH 7.5) containing 1% (v/v) of glycerol. Samples were applied onto the gel composed of 5% stacking gel and 10% resolving gel, and run under native conditions, in 25 mM Tris, 19.2 mM glycine, pH 8.3 at 20 mA for 2 h at 4 °C. Gels were fixed in 50% ethanol solution containing 20% of acetic acid and stained in 0.12% Coomasie brilliant blue R, 45% methanol, 10% acetic acid for 20 min and destained in 45% methanol, 10% acetic acid. The gels were scanned and the amount of free protein was estimated by gel image analysis based on measuring of total protein bands intensities. The analysis was performed using home-made software.

2.6. Enzymatic assay of β-galactosidase

Enzymatic activity of β-Gal was estimated by colorimetric assay, detecting at 405 nm the product of the hydrolysis of *o*-nitrophenil-β-D-galactoside (oNPG). The peptide solution was added at peptide/protein ratios from 1 to 30,000 to the enzyme dissolved in PBS (pH 7.4) and incubated for 40 min at 37 °C. The substrate solution was added at nonlimiting concentrations in PBS (pH 7.4) and the assay was followed by the optical density measurement.

3. Results

3.1. Intracellular delivery of β-Galactosidase induced by gramicidin A derivatives

To examine the ability of the amphipathic gramicidin analogues described in our previous reports (P4C, P5C, P10C, N1C) and the new gramicidin derivatives (P11C, P3N) to translocate proteins across the cell membrane, the reporter protein β-galactosidase was used because of its convenient detection via enzymatic activity. The β-Gal uptake induced by gramicidin analogues was studied in human carcinoma cell line (HeLa) and the cell-transducing activity of all the derivatives was compared. Complexes of the peptides with the protein were formed as described above. Two hours proved to be the optimal time of incubation with HeLa cells (data not shown) as measured by two different staining procedures. X-Gal substrate is degraded by β-Gal giving rise to a blue-colored water-insoluble product which is observed in the cells directly (Fig. 1A). To estimate the efficiency of cellular uptake of β-Gal, we used oNPG staining (Fig. 1B). In order to exclude the contribution of the protein associated with the external membrane, cells were treated with 0.25% trypsin solution before oNPG staining [28,30].

It was found that the analogue N1C modified with a negatively charged sequence showed no cell-transducing activity (Fig. 1A), whereas analogues containing the basic motif (P5C, P10C, P11C, P1N, P3N) transported the protein across the membrane (Fig. 1B, C, D).