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Research paper

Identification and characterization of novel protein-derived arginine-rich cell-penetrating peptides

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ABSTRACT

Cell-penetrating peptides (CPPs) have proven their potential as an efficient delivery system due to their intrinsic ability to traverse biological membranes and transport various cargoes into the cells. In the present study, we have identified novel natural protein-derived CPPs using an integrated (*in silico* and experimental) approach. First, using bioinformatics approach, arginine-rich peptide segments were extracted from SwissProt proteins and their cell-penetrating properties were predicted. Finally, eight peptides were selected and their internalization was validated using experimental techniques. Laser scanning confocal microscopy and flow cytometry confirmed that seven out of eight peptides were internalized into live cells with varying efficiencies without significant cytotoxicity. Three peptides have shown higher internalization efficacy than TAT peptide, the most widely used CPP. Among these three peptides, one peptide (P8), derived from voltage-dependent L-type calcium channel subunit alpha-1D, was able to accumulate inside in a variety of cell types very efficiently through a rapid dose-dependent process. Further, experiments involving inhibition with various endocytic inhibitors along with co-localization studies indicate that the uptake mechanism of P8 is macropinocytosis, a fluid-phase endocytosis process. In addition, competitive inhibition with heparin revealed the involvement of cell-surface proteoglycans in P8 uptake. In summary, the present study provides evidence that an integrated *in silico* and experimental approach is an effective strategy for the identification of novel CPPs and CPPs identified in the present study have promising perspectives for future drug delivery applications.

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1. Introduction

Most of the therapeutic lead molecules, despite their high therapeutic properties, fail to enter in the clinical trials due to their poor delivery and low bioavailability. Thus considerable efforts have been made to overcome these limitations. In this direction, small cationic peptides known as cell-penetrating peptides (CPPs) have drawn significant attention [1] and provided solutions to the above limitations of poor delivery and low bioavailability. Owing to their intrinsic cell internalization properties, CPPs have improved the intracellular delivery of various therapeutic molecules, including oligonucleotides [2–4], small molecules [5,6], proteins [7], peptides [8], siRNAs [9], nanoparticles [10], etc., most of which otherwise cannot cross the plasma membrane barrier by their own.

The journey of CPP was started almost two decades ago [11] with the discovery of TAT(48–60) [12] and penetratin peptides [13]. Since then hundreds of new CPPs have been discovered [14] and characterized for various therapeutic applications. Recently, a systematic cataloging of these CPPs has been carried out [14], and their analysis has revealed that CPPs constitute a family of diverse peptides; a few CPPs are derived from viral proteins such as VP22 (derived from herpesvirus tegument protein) [15], some are derived from snake venom protein such as CyLOP-1 (derived from crotamine) [16], a few are part of cell adhesion glycoprotein such as pVEC (derived from murine vascular endothelial-cadherin protein), and others are synthetic or designed such as oligoarginine. Though CPPs are very heterogeneous, they share some common features such as CPPs are often cationic, and/or amphipathic in nature [17]. Most of the CPPs are derived from natural proteins

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Table 1
List of peptides examined.[a]

Designation	Sequence
P1	FITC-ahx-KKKKKKNKKLQQRGD
P2	FITC-ahx-RGDGPRRRPRKRRGR
P3	FITC-ahx-RRRQKRIVVRRRLIR
P4	FITC-ahx-RRVWRRYRRQRWCRR
P5	FITC-ahx-RRARRPRRLRPAPGR
P6	FITC-ahx-LLRARWRRRRSRRFR
P7	FITC-ahx-RGPRRQPRRHRRPRR
P8	FITC-ahx-RRWRRWNRFNRRRCR
TAT	FITC-ahx-GRKKRRQRRRPPQ

[a] All peptides were FITC labeled at the N-terminus. An aminohexanoic acid linker was placed between the FITC and the peptide. The C-terminus of all peptides is free.

and contain high arginine content, which play crucial roles in mediating their internalization into the cells [18,19]. Despite this heterogeneity in their sequences and structures, CPPs have been suggested to be internalized mainly by endocytosis [20,21]. However, a few CPPs have been reported to be internalized via a non-endocytic process [22,23]. Moreover, internalization mechanism has been shown to be dependent on various factors, including cell types, peptide sequence, peptide concentration, type of conjugated cargo, temperature, incubation time, etc. [24].

Although many CPPs have been identified so far, most of them have shown relatively low uptake efficiencies. Therefore, in the present study, we have used an integrated *in silico* and experimental approache to identify novel and efficient CPPs. To achieve this, first, using bioinformatics approach; we have extracted arginine-rich segments (length 15 amino acid) in all the proteins available in SwissProt database. Subsequently, various filters were applied to define CPPs. To further achieve higher success rate, cell-penetrating properties of peptides were predicted using CellPPD [25], an *in silico* algorithm for the CPP prediction, recently developed by our group. Finally, eight predicted peptides were selected (Table 1) and their cell-penetrating properties were validated experimentally on human epithelial cervical carcinoma cells (HeLa) cells using fluorescence-activated cell sorting (FACS) and confocal-laser scanning microscopy (CLSM). Results demonstrate that seven out of eight peptides internalized into live cells with varying efficiencies without significant toxicities. Three peptides internalized more efficiently than the TAT peptide, a widely used intracellular delivery vehicle. Among these, one peptide derived from human voltage-dependent L-type calcium channel subunit alpha-1D (P8, RRWRRWNRFNRRRCR) was found to be approximately 10 times more efficient than TAT and it internalized into the live cells very efficiently via an endocytic process, making P8 a promising candidate for further molecular and cellular investigations. This investigation also provided a validation of the prediction algorithm to identify new CPP sequences.

2. Material and methods

2.1. Materials

Human cervical cancer cell line HeLa, human prostate carcinoma cell line PC3, human normal prostate epithelium cell line RWPE-1 and Chinese hamster ovary cell line CHO-K1 were all obtained from American Type Culture Collection (ATCC; Manassas, VA). Dulbecco's Modified Eagle's Media (DMEM), RPMI-1640, Ham's F12 nutrient mix, Keratinocyte-SFM, fetal bovine serum (FBS), trypsin, penicillin, streptomycin, Opti-MEM, phosphate buffer solution (PBS, pH 7.4), Tetramethylrhodamine-dextran 70 kDa (marker for macropinocytosis), Tetramethylrhodamine-transferrin (marker for clathrin-coated pits and vesicles), LysoTracker RED (marker for lysosomes), DAPI (marker for nucleus) and antifade reagent were purchased from Molecular Probes (Eugene, OR). Methyl-β-cyclodextrin (MβCD), chlorpromazine (CPZ), cytochalasin D (CytD), amiloride, sodium azide and 2-deoxyglucose (DOG), heparin and fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich (USA). MTS cell proliferation assay kit was purchased from Promega (Madison, USA). All the peptide synthetic reagents were procured from Sigma–Aldrich (USA).

2.2. Methods

2.2.1. Peptide synthesis

All CPPs were synthesized by solid phase peptide synthesis strategy using Fmoc (N-(9-fluronyl)-methoxycarbonyl) chemistry in 0.01 mmole scale on a Protein Technologies Inc, USA, PS-3 peptide synthesizer. Fmoc-amino acid was loaded on 2-Chlorotrityl chloride resin manually and then chain elongation was done on the synthesizer and mass was confirmed by MALDI-TOF. In brief, Fmoc-amino acid, 2 equiv., was anchored on 2-Chlorotrityl chloride resin (substitution 1.01 mmole/g, 100–200 mesh, 1%DVB) manually using diisopropylethylamine (5 equiv.) in dichloromethane. Free 2-Chlorotrityl chloride linkers were capped by the treatment of the resin with a solution of dichloromethane (DCM)/methanol/diisopropylethylamine (17:2:1; v/v/v) twice and subsequently with a solution of dimethylformamide (DMF)/diisopropylethylamine/acetic anhydride (8:1:1; v/v/v) twice. After this, the resin was washed three times each with DMF and DCM respectively and finally dried in vacuum for 4 h. The chain elongation of the peptide was done by using four equiv. of the protected Fmoc-amino acid with HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as a coupling reagent and HOBT (n-hydroxybenzotriazole) or COMU (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate) for suppressing racemization with 1 h reaction time. Following side chain protection was employed for Fmoc-protected amino acids, Asparagine, Glutamine, Histidine, Serine: Trityl (trt); Glutamic acid, Aspartic acid: Tert butyl ester (OtBu); Arginine: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl (Pbf); Lysine, Tryptophan: tert-butoxycarbonyl (Boc); Threonine, Tyrosine: tert-butyl (tBu). For C-terminal activation, 0.4 M NMM in DMF was used and for N-terminal Fmoc-group deprotection, 20% piperidine in DMF was used. COMU was used specifically when two or more hydrophobic amino acids occur in succession in the sequence to have better yield. Reaction time was increased from 1.0 h to 1.5 h during coupling of hydrophobic amino acids. The completion of the reaction was monitored by performing Kaiser Test.

2.2.2. Peptide labeling with FITC and purification

N-terminal of the peptide(s) was attached to a spacer amino acid Fmoc-ε-amino hexanoic acid to avoid fluorescent thiohydantoin (FTH) formation from the last α-amino acid of the peptide sequence. Final Fmoc-group was removed with 20% piperidine in DMF for 15 min twice and was thoroughly washed with DMF six times. Then the resin was swelled with DCM and drained. Prepared 1:1 equivalent of FITC dye in pyridine/DMF/DCM (12:7:5) and 500 μl of the solution was added to the resin and mixed it for overnight. Completion of the labeling was checked by performing Kaiser Test. After the completion of the reaction, the resin was washed with DMF twice, isopropanol twice, DCM twice and dried on a vacuum. Thereafter, the peptide was cleaved from the resin by treating the resin with a cleavage cocktail Trifluoroacetic acid/1,2-Ethanedithiol/water/Thioanisole/phenol/Triisopropylsilane (89:2.5:2.5:2.5:2.5:1, v/v/v/v/v/v) for 6 h. Then it was filtered, dried under air pressure and subsequently on a vacuum to yield

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