CELL REGENERATION

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Characterization of a novel cell penetrating peptide derived from human Oct4

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Abstract

Background: Oct4 is a transcription factor that plays a major role for the preservation of the pluripotent state in embryonic stem cells as well as for efficient reprogramming of somatic cells to induced pluripotent stem cells (iPSC) or other progenitors. Protein-based reprogramming methods mainly rely on the addition of a fused cell penetrating peptide. This study describes that Oct4 inherently carries a protein transduction domain, which can translocate into human and mouse cells.

Results: A 16 amino acid peptide representing the third helix of the human Oct4 homeodomain, referred to as Oct4 protein transduction domain (Oct4-PTD), can internalize in mammalian cells upon conjugation to a fluorescence moiety thereby acting as a cell penetrating peptide (CPP). The cellular distribution of Oct4-PTD shows diffuse cytosolic and nuclear staining, whereas penetratin is strictly localized to a punctuate pattern in the cytoplasm. By using a Cre/loxP-based reporter system, we show that this peptide also drives translocation of a functionally active Oct4-PTD-Cre-fusion protein. We further provide evidence for translocation of full length Oct4 into human and mouse cell lines without the addition of any kind of cationic fusion tag. Finally, physico-chemical properties of the novel CPP are characterized, showing that in contrast to penetratin a helical structure of Oct4-PTD is only observed if the FITC label is present on the N-terminus of the peptide.

Conclusions: Oct4 is a key transcription factor in stem cell research and cellular reprogramming. Since it has been shown that recombinant Oct4 fused to a cationic fusion tag can drive generation of iPSCs, our finding might contribute to further development of protein-based methods to generate iPSCs.
Moreover, our data support the idea that transcription factors might be part of an alternative paracrine signalling pathway, where the proteins are transferred to neighbouring cells thereby actively changing the behaviour of the recipient cell.

Keywords: Cell penetrating peptides, Oct4, Penetratin, Homeodomain transcription factors, Cellular internalization, Reprogramming

Introduction

Cell penetrating peptides (CPPs) - also known as protein transduction domains (PTDs) - are an intensively studied, yet diverse class of peptides in regard to amino acid composition, size, charge and structure. Their joint feature is the mediation of the internalization of a peptide or a protein and, in most cases, a conjugated cargo. Classifications are either based on the physico-chemical nature of the sequence (primary-, secondary-, or non-amphiphatic) [1] or their origin (protein-derived, chimeric or synthetic peptides) [2].

The first CPPs, Tat and penetratin, were described already in the late 1980s and early 1990s, and are still among the most studied CPPs today [3]. Tat is derived from amino acids 48-60 of HIV-1 trans-activator of the transcription (Tat) protein [4,5], while penetratin derives from the *Drosophila melanogaster* homeodomain protein Antennapedia (Antp). Antp is a transcription factor and its 60 aa homeodomain (pAntp) can be unconventionally secreted without the need of a signal peptide [6]. Consequently, it can be

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uptaken by neighbouring cells in a receptor-independent way [7]. Penetratin, a 16 amino acid peptide, corresponds to the third helix of the Antp homeodomain and has been shown to be sufficient for uptake of the whole protein [8]. In previous studies it was shown that internalization of penetratin relies mainly on endocytosis [9], but direct translocation has also been proposed [10-12]. Still, the mechanism of uptake remains debatable [3].

Detailed characterization of the mechanisms guiding peptide internalization is desired, and i.e. the interaction of penetratin and its derivatives with cellular membrans has been intesively studied using lipid model systems [13]. These studies have clarified that upon binding of penetratin to anionic lipid membranes, the peptide changes its secondary structure and adopts either an α-helical or β-sheet shaped structure, depending on the peptide/lipid ratio [14,15].

Ever since the discovery of penetratin, a number of other peptides as well as proteins from the large family of homeodomain transcriptional regulators have been assessed for their capacity to cross cellular membranes. Prominent examples of internalized whole proteins or the homeodomain helix in combination with cargo molecules are Hoxa5, Hoxc8, PDX-1 or Engrailed-2 [16-19]. However, not all homeodomain peptides or proteins are efficiently taken up by cells [20]. In contrast, uptake of Pax-4, a paired-box transcription factor also containing a homeodomain, has been shown to depend upon the paired domain rather than the homeodomain [21].

One homeodomain protein that previously has not been tested for containing a functional PTD is human Oct4. Oct4 is a prominent member of the POU-family of transcription factors containing two distinct DNA-binding domains, the POU-specific domain and the homeodomain [22]. It is necessary for maintaining the pluripotent state of embryonic stem cells [23], but has probably raised most interest as a key factor for cellular reprogramming of somatic cells into induced pluripotent stem cells (iPS) [24].

While several techniques to non-integratively transfect cells with reprogramming factors like mRNA or CPP-tagged proteins [25-29] have been reported, a simple and safe approach adding no genetically altered authentic recombinant protein has not been tested so far.

Therefore, we explored if the third helix of the human Oct4 homeodomain (Oct4-PTD) might in principle be able to translocate into living cells and mediate cargo uptake. We compared this activity as well as its secondary structure, membrane interaction and cytotoxicity to the well characterized CPP penetratin.

We report that Oct4-PTD-mediated uptake is very efficient, and that already 1 hour after exposure, FITC-labelled Oct4-PTD localizes to the nucleus and diffusely to the cytoplasm. Structural analysis of the peptides using circular dichroism (CD) showed that upon binding to large unilamellar vesicles (LUVs), a helical structure of Oct4-PTD is only observed if the FITC label was present on the N-terminus of the peptide. In contrast, penetratin shows a helical structure independently of the fluorescence moiety.

Moreover, we show that recombinantly expressed and purified Oct4-PTD-Cre fusion protein successfully translocates into reporter cells when added to the medium and recombines loxP-modified marker genes. Finally, a weak but significant uptake of recombinantly expressed native human Oct4 was observed. We therefore suggest that uptake of Oct4 might be a further contribution towards establishing safe methods to generate iPSCs.

Material and methods

Materials

Peptides (>95% purity) were purchased from Biomatik (Cambridge, Ontario, Canada). For labelled peptides the N-terminal fluorescein isothiocyanate (FITC) label was conjugated by an Acp (amino caproic acid) linker. The peptides were delivered as lyophilized acetate salts, dissolved in MQ-water and stored at -20°C as aliquots. Cell culture media were from PAA Laboratories and Biochrom AG (Berlin, Germany), heparin sodium was purchased from Serva (Heidelberg, Germany) and buffer salts were from Sigma. FM 4-64 dye and 7-aminoactinomycin D (7-AAD) were from Invitrogen. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-Palimitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were from Larodan Fine Chemicals (Malmö, Sweden).

Cell culture

Chinese Hamster Ovary cells (CHO-K1) were cultured in Ham's F12 medium supplemented with 10% fetal calf serum and 2 mM L-Glutamine. RPTEC/TERT1 cells [30] were cultivated in ProxUp medium (Evercyte GmbH, Austria). CV1-5B cells and BJ foreskin fibrobrasts were cultured in DMEM supplemented with 10% FCS, 1× NEAA, 1× Sod.-Pyruvat (all Life technologies, Carlsbad, CA). Cell lines were maintained under antibiotic free conditions at 37°C and 5% CO2.

Flow cytometry

For quantitative analysis of uptake 10,000 CHO-K1 cells were seeded per well of a 96-well plate 48 h prior to the experiment and cultured to approximately 80% confluence. The cells were washed with serum-free (SF) Ham's F12 and subsequently incubated with indicated concentrations of FITC-labelled peptides diluted in SF-medium for 60 min at 37°C, 5% CO_2.

Following incubation cells were washed twice with 20 mM HEPES/150 mM NaCl, pH 7.4, supplemented with 100 μg/ml Heparin in the first wash step to remove membrane bound peptide from the cell surface [31].