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X-pep, a novel cell-penetrating peptide motif derived from the hepatitis B virus

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ABSTRACT

Cell-penetrating peptides (CPPs) are able to penetrate the plasma membrane and gain access to the interior of any replicating or non-replicating cell, and are being considered as drug delivery agents. Here we describe the serendipitous discovery of a novel CPP motif (MAARLCCQ), designated X-pep, located at the extreme N-terminus of the X-protein of the hepatitis B virus. X-pep, and a C-terminally truncated form of the peptide (MAARL), readily penetrated HepG2 cells. Further truncation by removal of the terminal leucine residue impaired the cell-penetrating activity of peptide, indicating that MAARL is the active core of the peptide. X-pep is located adjacent to another CPP, namely Xentry, and like Xentry is unable to penetrate unactivated resting lymphocytes suggesting selective cell uptake. A D-isomeric form of the MAARL peptide was not cell-permeable, indicating that the cell-penetrating function of the peptide involves stereoselective interaction with a chiral receptor. The discovery of X-pep, which bears no resemblance to known CPPs, allows studies to be undertaken to determine additional characteristics of this novel CPP.

1. Introduction

Over the past three decades more than 100 short peptides, commonly referred to as cell-penetrating peptides (CPPs), have been discovered to be capable of translocating the plasma membrane of cells [1–6]. CPPs are generally 10–30 amino acid (aa) residues in length, and are either arginine-rich, amphipathic and lysine-rich, or hydrophobic [7]. Many have been shown to have the ability to deliver cargo molecules into intracellular compartments, without causing significant damage to the plasma membrane, thus making them attractive non-viral vectors for delivering biologically active molecules into cells [1–6].

We recently described the serendipitous discovery of an entirely new class of CPP represented by the short peptide Xentry (LCLRPVG) derived from the N-terminal aa 16–20 of the X-protein [8]. The X-protein is a short 154 aa residue protein of 17 kDa encoded by the hepatitis B virus (HBV) [9], which is necessary for viral replication, regulates cell apoptosis, and contributes to the development of HBV-induced hepatocellular carcinoma [10–13]. Xentry differs from previously described CPPs in that it is very short, being only 7 aa residues in length with the active cell-penetrating core comprising just the 4 aa LCLR [14]. Further, it is not arginine-rich, amphipathic and lysine-rich, or uniformly hydrophobic [8]. The ability of Xentry to permeate adherent cells is dependent on the ubiquitously expressed heparan sulphate proteoglycan syndecan-4 [8]. Xentry is unique amongst CPPs as it is unable to passively enter syndecan-deficient, non-adherent cells, such as resting blood cells [8]. This feature offers a therapeutic advantage as Xentry is not sequestered and diluted by blood cells when injected intravenously. Xentry was able to deliver proteins, antibodies and siRNA in a biologically active form to the intracellular compartment of cells, and to tissues in mice [8].

At the time of discovery of Xentry, we became aware of a second CPP sequence almost immediately adjacent to Xentry, located at the extreme N-terminus of the X-protein. Here we describe a preliminary report on some of the features of this novel CPP, which has been designated X-pep.

2. Materials and methods

2.1. Cells and peptides

The human HepG2 (liver cancer) and mouse TK-1 (thymic lymphoma) cell lines were obtained from the American Type Culture Collection (ATCC). The HepG2 cell line was propagated in

Abbreviations: aa, amino acid; CPP, cell-penetrating peptide; HBV, hepatitis B virus.

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full MEM medium at 37 °C and 5% CO2, while TK-1 cells were propagated in full RPMI 1640 medium at 37 °C and 5% CO2. All peptides were synthesized by Peptide 2.0 Inc., Chantilly, VA. L-isomeric forms of peptides are written in uppercase, and D-isomeric forms in lowercase, according to convention.

2.2. Assay to test the cell-penetrating ability of peptides

The HepG2 cell line was seeded into 8-well chamber slides at 1×10^5 cells per well in MEM medium (Gibco, Life Technologies New Zealand Ltd) containing 10% FCS and penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (0.29 mg/ml). The cells were cultured overnight at 37 °C in a 5% CO_2 atmosphere, and washed thrice with serum-free MEM medium. FITC-labelled L-isomeric peptides were diluted in 500 μl of MEM medium without FCS, whereas FITC-labelled D-isomeric peptides were diluted in the same media containing FCS as D-isomeric peptides are resistant to serum proteases. Peptides were added to cells at a final concentration of 10 μM, or as indicated. The cells were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere, washed with PBS, fixed with 4% formaldehyde for 30 min, and washed thrice with PBS. A drop of Prolong Gold anti-fade reagent with DAPI (Invitrogen, Life Technologies New Zealand Ltd) was added, and the cells mounted and examined with a Nikon E600 fluorescence microscope or a Leica TCS-SP2 confocal microscope.

3. Results

3.1. The extreme N-terminus of the X-protein contains a cell-penetrating peptide motif

During an investigation of the functions of short peptides spanning the entire length of the X-protein, it was revealed that the FITC-labelled N-terminal peptide spanning aa residues 1–20 (MAARLCCQLDPARDVLCLRP) was spontaneously taken up within minutes by HepG2 cells in a similar fashion to the overlapping Xentry (LCLRPVG)-containing FITC-labelled peptide spanning aa residues 16–35 (LCLRPVGAESRGRPVSGPFG) (Fig. 1A). Fluorescence and confocal imaging localised the peptide to both the cytoplasm and nucleus (Fig. 1A). It was expected that peptide aa 1–20 would be cell-penetrating as it contains the active motif of Xentry (LCLR) at its C-terminus, however there remained the possibility that a second cell-permeable motif was contained within the N-terminal region of this peptide. The peptide was C-terminally truncated to remove Xentry to test this notion, giving the peptide aa 1–15 (MAARLCCQLDPARDV). Conventional and confocal microscopy revealed that the truncated peptide aa-1–15 at 10 μM penetrated HepG2 cells (Fig. 1B). The truncated peptide was divided into two short peptides, containing aa 1–8 (MAARLCCQ) and aa 9–15 (LDPARDV). Peptide MAARLCCQ was readily taken up by HepG2 cells at a final concentration of 10 μM, whereas peptide LDPARDV was not cell-permeable (Fig. 2). Peptide MAARLCCQ was divided to

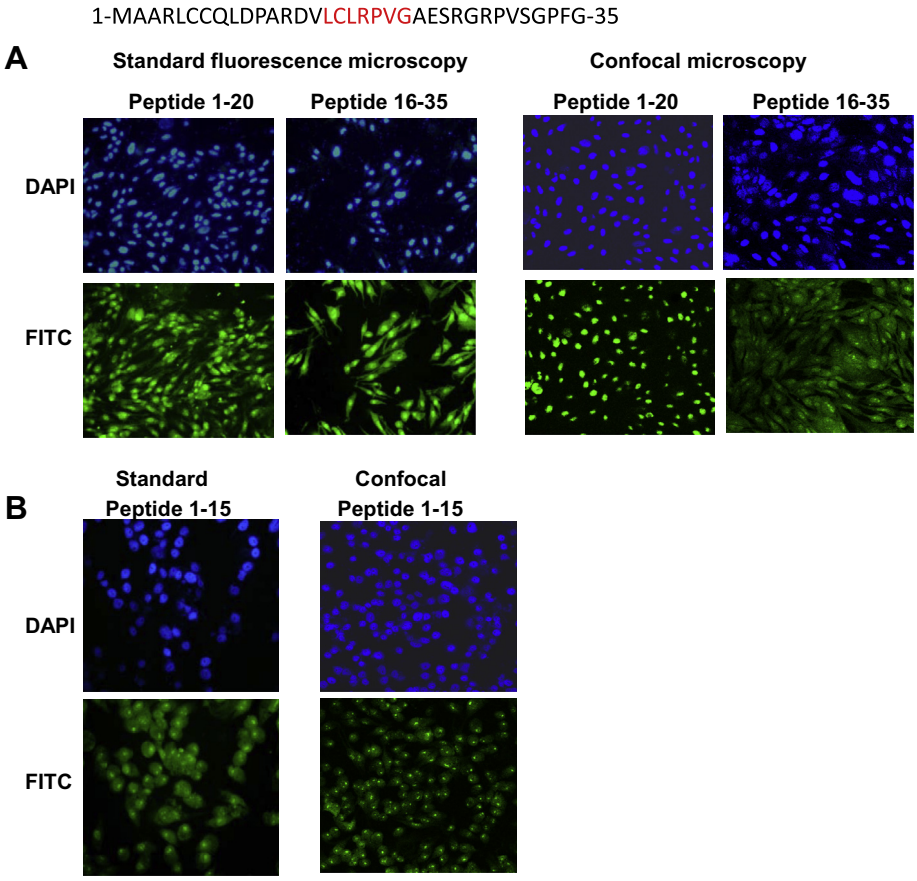

Fig. 1. The N-terminal X-protein peptide aa 1–15 is cell-permeable. The sequence of the first 35 N-terminal residues of the X-protein is shown with the sequence of Xentry (LCLRPVG; residues 16–22) highlighted in red. FITC-labelled peptides encompassing (A) aa 1–20 (MAARLCCQLDPARDVLCLRP) and 16–35 (LCLRPVGAESRGRPVSGPFG), and (B) aa 1–15 (MAARLCCQLDPARDV) from the N-terminal region of the X-protein were incubated with HepG2 cells for 3 h at a final concentration of 10 μM. Cell nuclei were stained blue with DAPI. Peptide uptake by the cells was recorded by standard fluorescence or confocal microscopy, as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

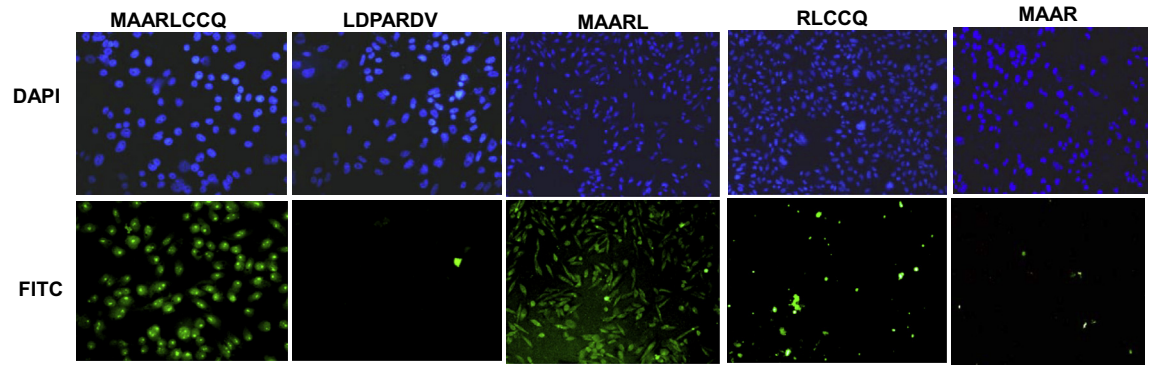

Fig. 2. Cell-penetrating ability of truncated X-protein peptides. FITC-labelled truncated peptides were incubated with HepG2 cells for 3 h at a final concentration of 10 μM. Cell nuclei were stained blue with DAPI. Peptide uptake by the cells was recorded by fluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

give peptides containing aa 1–5 (MAARL) and aa 4–8 (RLCCQ). Peptide MAARL was readily taken up by HepG2 cells at a final concentration of 10 μM, whereas peptide RLCCQ was not permeable (Fig. 2). Removal of the C-terminal leucine to give the short peptide aa 1–4 (MAAR) impaired the cell-penetrating activity of the peptide (Fig. 2). Thus, the octapeptide MAARLCCQ represents a second cell-penetrating motif found within the N-terminal region of the X-protein, with MAARL being the active core. Here, the MAARLCCQ peptide has been designated X-pep to distinguish it from Xentry.

3.2. X-pep is unable to penetrate non-adherent lymphocytes

Endocytic cell uptake of Xentry and several other CPPs is dependent on the heparan sulphate proteoglycan syndecan-4 [8,15]. X-pep (MAARLCCQ) was examined for its ability to penetrate the non-adherent mouse thymic lymphoma cell line TK-1, which is deficient in syndecans [8]. The CPP polyarginine (R9) was used as a control for comparison as it readily penetrates syndecan-deficient lymphocytes due to the fact that it can passively penetrate cells independently of syndecans [16]. X-pep was not taken up by TK-1 cells, whereas as expected the R9 peptide readily penetrated the lymphocyte cell line (Fig. 3). Thus, X-pep displays cell-selectivity.

3.3. Cell-penetrating function of X-pep displays chiral selectivity

The D-isomeric protease-resistant form of Xentry is readily cell-permeable [8] (Fig. 4). In contrast, a D-isomeric form of the peptide maarl was unable to penetrate HepG2 cells, even at concentrations as high as 100 μM (Fig. 4). Further, a D-isomeric form of the parental peptide spanning aa 1–22 of the X-protein (maarlrlccq-pardvlclrpvg) also did not penetrate HepG2 cells, despite containing Xentry at its C-terminus (Fig. 4). The latter results demonstrate that fusion of the D-isomeric peptide maarl to Xentry inhibits the cell-penetrating ability of Xentry. In contrast, an equimolar concentration of the isolated D-isomeric peptide maarl did not competitively inhibit cell uptake of Xentry by HepG2 cells (Fig. 4), indicating that the maarl peptide must be fused to Xentry within the same peptide to effectively exert its inhibitory effect.

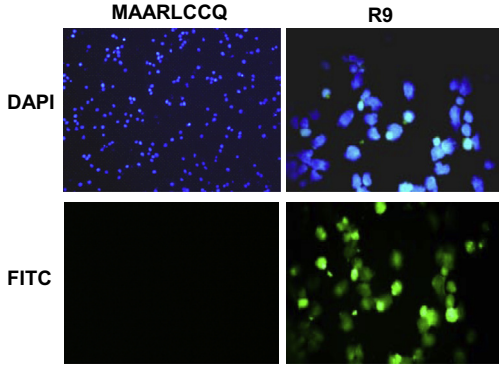

Fig. 3. X-pep is unable to penetrate the syndecan-deficient T lymphocytes. FITC-labelled X-pep (magnification 20×) and the CPP R9 (magnification 40×) were incubated with TK-1 cells in suspension for 3 h at a final concentration of 10 μM, and peptide uptake recorded by fluorescence microscopy.

4. Discussion

Here we have demonstrated that a second CPP sequence (aa 1–8; MAARLCCQ) resides within the N-terminal region of the X-protein. The peptide has been designated X-pep to distinguish it from Xentry. X-pep shares with Xentry [8] an inability to permeate non-adherent unactivated lymphocytes, which indicates that it does not passively enter cells, and displays receptor-dependent uptake. The pentamer MAARL represents the most active core of X-pep as cell-permeability was impaired upon removal of the C-terminal leucine residue from MAARL. We cannot exclude the possibility of a shorter active core as N-terminal truncation of this peptide was not carried out. X-pep is structurally similar to Xentry in that it contains a single arginine residue flanked by hydrophobic residues, but is otherwise very different in sequence. Importantly, the active core lacks a cysteine residue which was shown to be critical for the cell-penetrating ability of Xentry [14]; albeit X-pep contains two C-terminal cysteine residues. There are just two short β-strands within the first 40 aa residues of the X-protein, where the second strand overlaps the active core of Xentry, and the first strand overlaps the last 5 aa residues of X-pep [14]; which is interesting as β-strands participate in protein-protein interactions.

The D-isomeric form of the maarl core peptide failed to retain the cell-penetrating ability of the L-enantiomer. Chiral selectivity is indicative of receptor-mediated uptake [17]. Enantiomers are non-superimposable mirror images of one another, and do not always display identical properties. Both L- and D-isomers of the CPP octaarginine are cell-permeable, however there are large differences in the rates of cell penetration and binding to vesicle walls between various stereoisomers containing different proportions of L- and D- amino acids [17]. A D-isomeric form of the parental peptide maarlccqldpardvlclrpvg was also inactive, despite the fact that it contains Xentry at its C-terminus. Xentry is fully functional as a D-isomer [8], indicating that the D-isomeric motif maarl blocks Xentry-mediated cell uptake. The maarl peptide in solution was

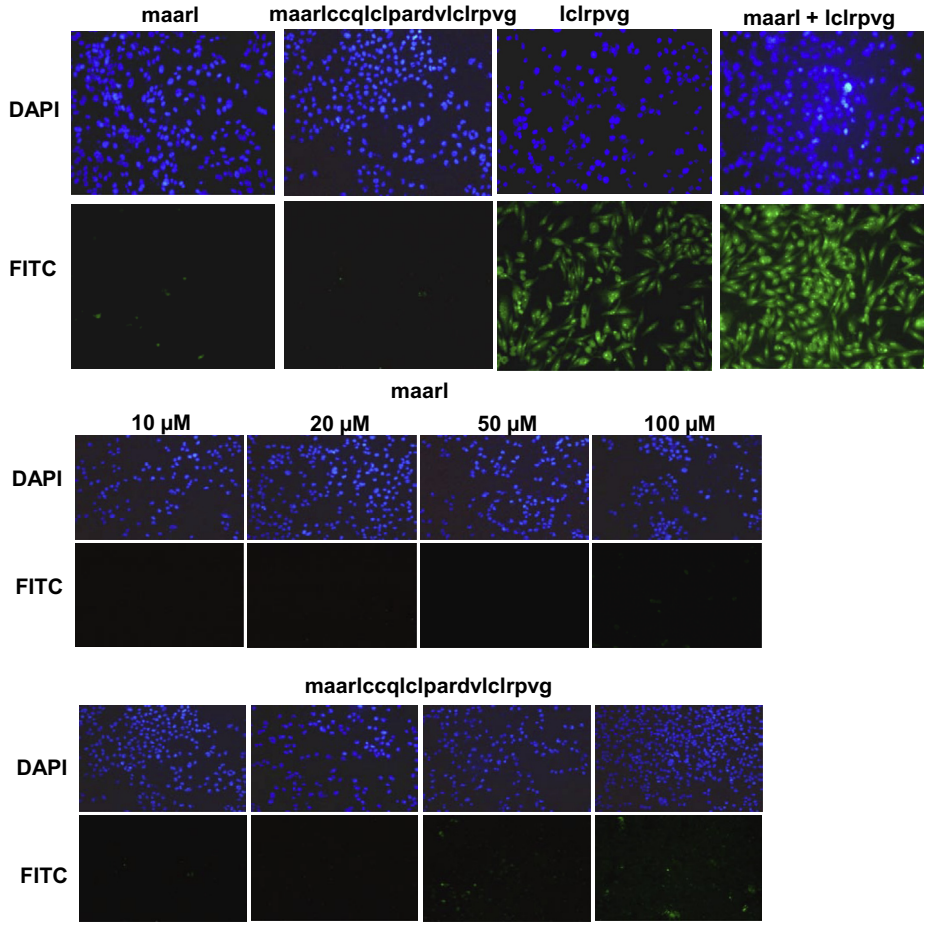

Fig. 4. A D-isomeric form of the maarl peptide is not cell-permeable, and antagonizes cell uptake of Xentry. D-isomeric forms of the maarl peptide, the parental peptide (maarlccqlclpardvlclrpvg), Xentry, and Xentry in combination with the maarl peptide were incubated with HepG2 cells for 3 h at concentrations of 10 μM (upper panel), or at increasing concentrations (lower 2 panels). Cell nuclei were stained blue with DAPI. Peptide uptake by the cells was recorded by fluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unable to inhibit cell uptake of an equimolar concentration of the D-isomer of Xentry, revealing that the maarl peptide has to be fused with Xentry in the same peptide in order to exert its inhibitory affect.

We have recently demonstrated that placement of two Xentry motifs adjacent to one another in the same peptide renders the resulting peptide unable to penetrate cells [14]. This feature provided the opportunity to create protease-activatable forms of Xentry by placement of a protease-cleavable linker between the adjacent peptides. Protease cleavage of the linker released the Xentry peptides allowing them to be taken up by cells [14]. X-pep could potentially be used as a blocking moiety to inhibit cell uptake of Xentry in a similar fashion.

It is curious that the X-protein should bear two cell-penetrating peptide motifs within close proximity. The cell-penetrating activity of the Tat protein is thought to be important in HIV infection, as the Tat protein is able to leave infected cells to enter surrounding cells and induce viral gene transcription, immunosuppression, and cell death [18,19]. The native X-protein is not cell-permeable [20], indicating that the X-pep and Xentry sequences must be masked in some way. It remains to be seen whether any of the many natural splice variants of the X-protein are cell-penetrating, and also contribute to viral gene transcription and tumorigenesis.

In summary, this study has identified X-pep as a second cell-penetrating motif within the N-terminal region of the X-protein. Chiral selective cell uptake of X-pep indicates that X-pep enters cells via a cell-surface receptor. X-pep does not bear a resemblance to any previously described CPP, and hence may possess other novel functions that remain to be uncovered.

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