*Proceedings of the **4th International Peptide Symposium** in conjunction with the **7th Australian Peptide Conference** and the **2nd Asia-Pacific International Peptide Symposium, 2007***

Jackie Wilce (Editor) on behalf of the Australian Peptide Association

Identification and biological applications of rhegnylogically-organized cell penetrating peptides

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Introduction

Many different cell penetrating peptides (CPPs) have been utilized as vectors to affect the highly efficient intracellular delivery of bioactive moieties. A majority of such studies employ sychnologically-organized tandem combinations of a cargo (*message)* and a CPP (*address)*. To date, bioactive cargoes have included peptides (Fig. 1), proteins and a range of oligonucleotides attached either by direct chemical conjugation or as a component of a larger macromolecular complex. Moreover, a majority of CPPs, including the commonly used sequences Tat and penetratin, are designed to be both biologically and toxicologically inert.

More recently, a QSAR-based algorithm has been developed to predict cryptic polycationic CPP motifs within the primary sequences of proteins [1]. As described here, this novel technology has enabled the study of rhegnylogic CPPs in which multiple pharmacophores for cellular penetration and desirable biological activities are discontinuously organized within the primary sequence of single peptide. This organization differs from the more commonly utilized sychnologic strategy which joins functionally discrete and continous address and messages together in a tandem construct.

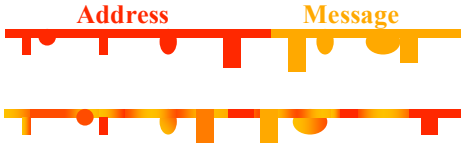

Fig. 1. Comparative organization of sychnologic (Top) and rhegnylogic (Bottom) CPP constructs

Results and Discussion

A cell penetrant modulator of heterotrimeric G proteins

Type 2 G protein-coupled receptors include a family of proteins that bind peptide ligands which are structurally related to calcitonin. The 20 amino acid sequence, *H*-RKLTTIFPLNWKYRKALSLG-*NH2*, predicted as a highly probable CPP, is located within the first intracellular loop of the human type a calcitonin receptor hCTRa. This sequence, hCTRa$^{174\text{-}193}$, includes a splice variant 16 amino acid insert that modulates the pharmacology of hCTRs [2].When added exogenously to cells expressing hCTRa, the peptide hCTRa$^{174\text{-}193}$ (1 mM) independently stimulated cAMP formation and also augmented calcitonin-induced cAMP synthesis. This mechanism involves the direct activation of heterotrimeric G proteins by hCTRa$^{174\text{-}193}$. Confocal microscopy, employing hCTRa$^{174\text{-}193}$ labeled with fluorescein at the amino terminal, confirmed that hCTRa$^{174\text{-}193}$ efficiently translocated the plasma membrane of EVC304 human bladder cancer cells (Fig. 2). Additional investigations to determine the G protein selectivity of hCTRa$^{174\text{-}193}$ are currently in progress.

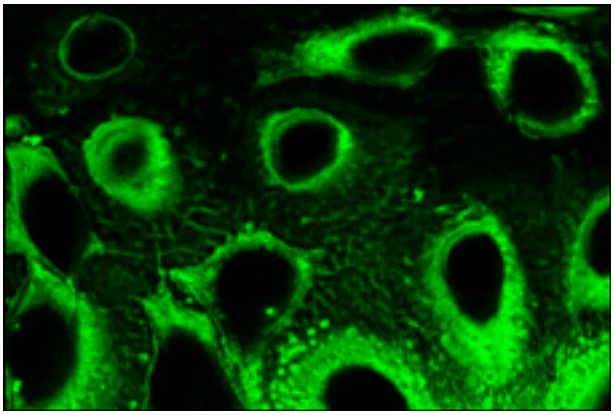

Fig. 2. Intracellular distribution of flouresceinyl-hCTRa$^{174\text{-}193}$

Human Cytochrome c as a CPP prediction template

The rhegnylogic strategy was also applied to cytochrome c (Cyt c), a signaling protein that is integral to intrinsic apoptotic events. As illustrated by the examples below, highly probable CPPs predicted within the primary sequence of Cyt c were restricted in distribution to major helical domains at the amino- and carboxyl- termini of the protein (Fig. 3).

Sequence	Position
EKGKKIFIMK	Cyt c$^{4\text{-}13}$
KKKEERADLIAYLKKA	Cyt c$^{86\text{-}101}$
GTKMIFVGIKKKEERADLIAYLKKA	Cyt c$^{77\text{-}101}$
KMIFVGIKKKEERA	Cyt c$^{79\text{-}92}$

Fig. 3. Predicted CPPs within human cytochrome c

The properties of two CPPs from the C-terminal helix of human Cyt c, Cyt c$^{77\text{-}101}$ and the shorter homologue Cyt c$^{86\text{-}101}$, indicate that these CPPs can effectively target different intracellular domains. Thus, confocal imaging revealed that rhodamine-conjugated Cyt c$^{77\text{-}101}$ assumed a cytoplasmic distribution and was co-localized with both endoplasmic reticulum and mitochondria. Moreover, Cyt c$^{77\text{-}101}$ did not co-localize with the intracellular

endosomal compartment, a feature that may restrict the utility of other CPPs including Tat. Cyt c^{77-101} was also excluded from the nucleus of living cells (Fig. 4). In contrast, rhodamine-conjugated Cyt c^{86-101} specifically located within the nucleus of U373MG astrocytoma cells and comparative little fluorescent peptide was observed in other cellular compartments (Fig. 4).

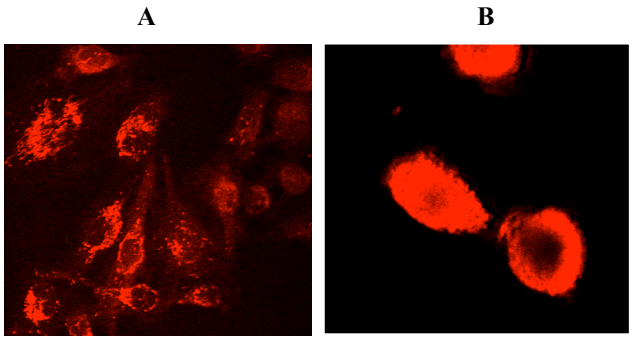

Fig. 4. Intracellular distribution of Cyt c^{77-101}(A) and Cyt c^{86-101}(B) in living U373MG cells

Additional studies indicated that both Cyt c^{77-101} and Cyt c^{86-101} reduced the viability of U373MG astrocytoma by the specific induction of apoptosis. These findings indicate that these peptides can mimic the apoptogenic activity of the native protein

As human Cyt c contains at least two domains that harbor cryptomic cell penetrant motifs, we also investigated whether exogenously applied Cyt c could penetrate cells. Horse spleen Cyt c was labeled with FITC [3] and purified by dialysis. As shown below (Fig. 5), mono-labeled Cyt c protein readily translocated the plasma membrane of U373 MG cells to assume both a cytoplasmic and nuclear distribution. In contrast to the observed distribution of Cyt c-derived peptides (see above), a major component of translocated Cyt c was distributed within the endosomal compartment of living cells.

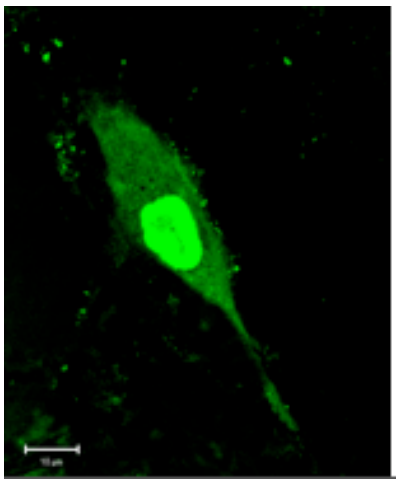

Fig. 5. Intracellular distribution of Cyt c protein in living U373 MG cells

These observations indicate that the native Cyt c protein enters cells by a predominantly endosomal route of import, whilst the cell penetrant fragments Cyt c^{77-101} and Cyt c^{86-101} utilize an alternative route. However, it is obvious that a significant fraction of translocated Cyt c protein is able to escape the endosomal compartment to re-locate within the nucleus. This intracellular trafficking reflects the known movement of Cyt c when it is released from mitochondria during apotosis [4].

Endothelial cell modulation by $eNOS^{492-507}$

The calcium-calmodulin (CaM) complex modulates the activity of a variety of protein targets including the endothelial isozyme of nitric oxide synthase (eNOS). More recently, x-ray crystallographic structures have indicated that a 20 amino acid peptide from eNOS forms a stable complex with CaM [5]. Using the QSAR-based prediction algorithm [1], we determined that a 4 amino acid-deleted homologue of this peptide, $eNOS^{492-507}$, would have efficient cell penetrating properties. Moreover, this peptide (*H*-RKKTFKEVANAVKISA-NH_2) has unique properties as a novel modulator of endothelial cell function. Thus, $eNOS^{492-507}$ potently (10-100 nM) inhibits the proliferation, migration and tube forming capacity of bovine aortic endothelial cells stimulated with fibroblast growth factor-2 (FGF-2). Furthermore, the same peptide inhibits FGF-2 stimulated angiogenesis *in vivo* and could, therefore, have therapeutic potential.

We conclude that the rhegnylogically-organized CPPs are valuable probes of biological phenomena.

Acknowledgments

We are extremely grateful to Ülo Langel (Stockholm) who provided access to the CPP prediction algorithm. This work also benefited from collaborations with Shant Kumar (Manchester) and Matjaž Zorko (Ljubljana). These investigations were financially supported in part by the Samantha Dickson Brain Tumour Trust.

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