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Mini review

Gateways and tools for drug delivery: Endocytic pathways and the cellular dynamics of cell penetrating peptides

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Abstract

A major goal in drug delivery is to be able to design a macromolecular entity that utilises an endocytic pathway to deliver a bioactive payload into a malfunctioning cell. However, the effectiveness of this approach may be constrained by insufficient information regarding the fate of the delivery vector within the confines of the endo-lysosomal network. Successful drug delivery through this mechanism is therefore dependent on an equal high level of understanding of the specific endocytic pathways that are inherent in the target cell and the traffic and fate of the macromolecule within endocytic organelles. Cell penetrating peptides (CPPs) are promising candidate vectors for delivering macromolecules, however, there is little consensus regarding their exact mechanism of uptake. This review highlights the numerous endocytic pathways and sorting mechanisms that may deliver CPPs to a number of cellular destinations. Our use of non-adherent leukaemia cell lines to study the cellular dynamics of CPPs HIV-TAT and octaarginine is also discussed.

Keywords: Cell penetrating peptides; Drug delivery; Endocytosis; Endosomes; Lysosomes

Contents

1. The wonders and complexities of endocytic pathways

The unravelling of the human genome, together with proteomic and genomic databases of specific diseases identifies a wealth of targets for macromolecular therapeutics such as genes and proteins. In a number of cases, the design strategy is to allow the vector molecule and its payload to be internalised by the cells endocytic pathways where translocation to the cytosol is then a prerequisite for immediate targeting or secondary localisation to in the nucleus. This strategy is appealing as it utilises highly efficient processes, fundamental to cell physiology. This is demonstrated when natural ligands recognising plasma membrane receptors are incubated with cells, and Fig. 1 shows the abundance of transferrin positive structures that become apparent in a single HeLa cell when incubated with this ligand for only 5 min. Endocytic pathways are however complex and the current relative inefficiency of intracellular delivery and targeting, most notably using non-viral vectors, may be due to our lack of understanding of the dynamics of molecules downstream of the plasma membrane and within the endo-lysosomal system (Jones, 2001; Jones et al., 2003; Watson et al., 2005).

The fate of a molecule that interacts within the exofacial leaflet of the plasma membrane may in part be immediately predetermined via its localisation or sequestration into a specific domain such as a lipid raft (London, 2005; Parton and Simons, 2007). It is likely that domains are also present on the membranes of downstream organelles and that internalised

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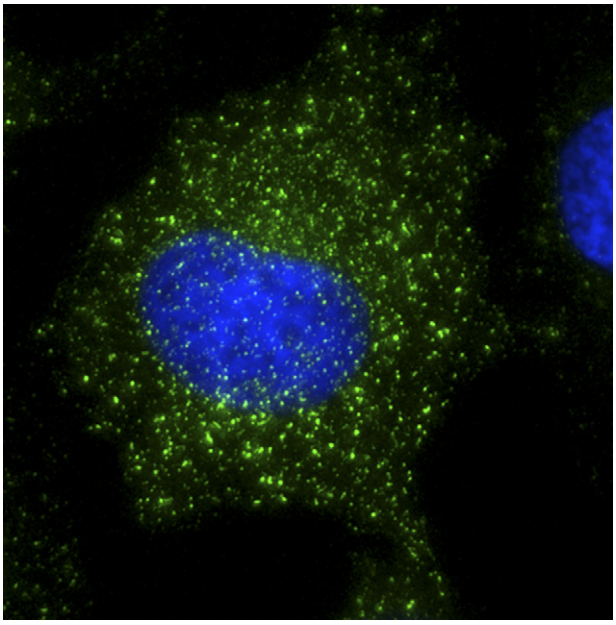

Fig. 1. Alexa488-transferrin labelled vesicles in a HeLa cell incubated with the probe for 5 min at 37 °C, nucleus is labelled with Hoechst 33352.

molecules associate with these as they are delivered towards their final destination. A class of proteins that have been extensively studied for analysis of distinct endocytic pathways, membrane trafficking organelles and endocytic domains are Rabs proteins, belonging to the Ras superfamily of small GTPases (Pfeffer, 2005; Spang, 2004; Stenmark and Olkkonen, 2001; Zerial and Mcbride, 2001). These are chaperoned in the cytosol and loaded via their lipid tails on to organelles where they then become activated by switching from GDP to GTP bound forms. Via interaction with a host of effector molecules they then mediate classical trafficking functions such as budding, fusion and directed transport along the cell cytoskeleton. Approximately 14 of the 60 Rab variants contained in the human genome are now known to be located on endocytic organelles and these regulate endocytic pathways and numerous other cellular processes (Simpson and Jones, 2005; Stein et al., 2003). Studies from the Zerial Laboratory have proposed that a molecule such as transferrin, entering a cell via clathrin-coated vesicles, moves through specific Rab-enriched domains as it traffics from, and sorted through early and recycling vesicles (Sonnichsen et al., 2000; Zerial and Mcbride, 2001). Other molecules such as epidermal growth factor and its receptor can also be internalised via clathrin-coated vesicles but these are more likely to be delivered to late endosomes and lysosomes. This in part is due to the internal sorting of the receptor that is known to exist early in an endocytic pathway, into intravesicular organelles or multivesicular bodies (Gruenberg, 2001). Ubiquitination plays a major part in this sorting process that is controlled from yeast to man by multisubunit complexes termed as endosomal sorting complexes required for transport (ESCRT) (Bishop, 2003; Piper and Katzmann, 2007). Similarly, traffic from endosomes to the *trans*-Golgi network, utilised by toxins such as ricin, is reliant on a different complex called retromer (Bonifacino and Rojas, 2006).

This reliance on sorting machinery and multisubunit complexes reflects on the expenditure the cell places to ensure that proteins, possibly internalised via a common mechanism, are sorted to different destinations. Researchers interested in drug delivery will undoubtedly benefit from this increased understanding of the regulation of endocytosis and may aim to influence the traffic and sorting of their candidate delivery vectors to give enhanced biological responses.

Uptake via clathrin-coated vesicles has been the most studied pathway but molecules can also be internalised by one or more clathrin-independent systems, macropinocytosis and via caveolae (Johannes and Lamaze, 2002; Kirkham and Parton, 2005; Mayor and Pagano, 2007). Relatively little is known about these other pathways that are undefined with respect to an absolute requirement for the recruitment of coat proteins with functions analogous to clathrin and adaptins (Robinson, 2004). Macropinocytosis was recently reviewed by this author especially with regards to its importance in the uptake of cell penetrating peptides (Jones, 2007)-discussed below.

The earliest separated station from the plasma membrane is called the sorting endosome or early endosome and temporally it can be loosely defined as an organelle containing material that has been internalised by endocytosis for between 2 and 5 min. Classically, early endosomes have pH in the range 6.0–6.5 but caveosomes, have been shown to have neutral pH (Pelkmans et al., 2004). These are pre-existing intermediate cytosolic stations that are rich in a protein called caveolin. Thus, all molecules internalised into cellular endocytic pathways may not be exposed to low pH, and for drug delivery this may be important if processing such as cleavage of a pH responsive linker is required for release of the therapeutic into the cytosol.

2. Cell penetrating peptides and their cellular entry mechanisms

Experiments that have illuminated endocytic pathways for cell biologists have also been utilised to study uptake and intracellular traffic of a host of vector molecules that are designed to enhance intracellular delivery of therapeutic molecules. These are often synthetic molecules such as polymers and liposome formulations and may therefore not follow conventional mechanisms of cell association, uptake and trafficking along endocytic pathways. The use of labelled endocytic probes such as transferrin, dextrans and toxins, defining uptake via clathrin, fluid phase/macropinocytosis and caveolae, respectively, especially in association with fluorescent microscopy, has revealed considerable information regarding their cellular association, uptake and fate inside the cell. Pharmacological inhibition of endocytosis may also support these data in unravelling the pathways traversed, though a lack of specificity hampers their use as specific inhibitors of defined pathways. It is however often the case that for a molecule of interest, a simple endocytic scheme rarely emerges, and this may be due to the fact that a single entity could use a number of different uptake routes. A recent review distinguishing the many forms of clathrin-independent endocytosis suggests a single cell may have available to it several different mechanisms

for internalisation, and for each there is a requirement for unique and universal proteins and lipids. (Mayor and Pagano, 2007)

The use of experiments utilising fluorescent endocytic probes and inhibitors of endocytosis has attempted to unravel the mechanism(s) by which cell penetrating peptides (CPPs) are able to enter cells with such a high degree of efficiency. CPPs are of considerable interest to those interested in drug delivery as a number have shown an ability in vitro and in vivo to deliver small-molecule drugs through polypeptides, proteins, RNA and DNA in to cells, allowing them to mediate a biological response (Fischer et al., 2005; Futaki, 2006; Henriques et al., 2006; Martin and Rice, 2007; Wagstaff and Jans, 2006). A series of reviews discussing their mechanisms of cell entry and delivery capacity have recently been published in a single issue of Biochemical Society Transactions (Howl et al., 2007).

The most extensively studied of these peptides are characterised by their richness in positive arginines and lysines, residues that play major roles in enhancing their internalisation and capacity to promote the intracellular delivery of associated macromolecules. Extensively studied variants include the penetratin peptide from the *Drosophila* Antennapedia homeoprotein, the HIV-TAT peptide, and arginine peptides typically R4-R16. Numerous studies have now reported on the interaction of CPPs with cells, their internalisation and downstream fate. However the increase in recent publications is not paralleled by increased consensus on the mechanism by which they initially cross the plasma membrane. There is sufficient data to suggest that internalisation can occur by translocation through the plasma membrane and by one of a number of endocytic pathways.

Most of these analyses have utilised fluorescence microscopy and flow cytometry as analytical tools, and though they are invaluable techniques they also can give misleading information. This was most dramatically shown when a number of the effects of the peptides to localise to the nucleus were shown to be due to fixation artefacts (Lundberg and Johansson, 2002; Richard et al., 2003). Less use is also now being made of fluorescein isothiocyanite (FITC) as a fluorescence label, as any such molecule, sensitive to pH in the plasma membrane to lysosome range of 7.4–4.5, can also give misleading data. The effect of the probe on the cellular dynamics of the peptide cannot be overlooked (Szeto et al., 2005) and there is also the possibility of extensive intracellular peptide degradation; this can often be reduced by using D-amino acids (Burlina et al., 2005; Elmquist and Langel, 2003; Fischer et al., 2004; Gammon et al., 2003). Imaging a number of CPP in cells using fluorescent microscopy-based methods has also been difficult on account of their strong interaction with the plasma membrane and their binding to tissue culture material.

We recently analysed the intracellular dynamics of two CPPs, HIV-TAT and octaarginine in leukaemia cell models (Al-Taei et al., 2006; Fretz et al., 2007). These non-adherent cells are removed from the original incubation material on to multi-well slides prior to microscopical analysis and therefore the problem of background staining is eliminated. Their small size, and large nucleus relative to cell area however, makes high-resolution fluorescence microscopy a challenge. We were surprised that there was very little evidence of interaction of these CPPs with the plasma membrane suggesting that the commonly observed plasma membrane binding is not a universal feature. Rather, in two cell models for acute and chronic myeloid leukaemia, the peptides were located to distinct endocytic structures with different distribution profiles (Fig. 2), and we identified these as lysosomes. Intriguingly, whereas the peptide was localised to vesicles at physiological temperatures, they were diffusely distributed throughout the cytoplasm of KG1a cells when peptide incubations were performed on ice (Al-Taei et al., 2006; Fretz et al., 2007). This difference in peptide distribution between ice and 37 °C was previously noted (Thoren et al., 2003) and this highlights the notion that uptake may proceed, in a single cell, via very different mechanisms. There

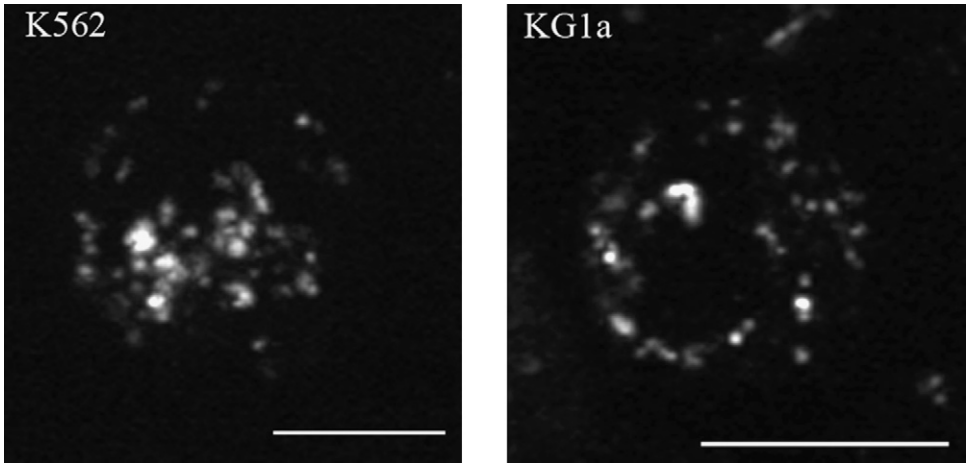

Fig. 2. Confocal maximum projection images showing the distribution of Alaexa488-TAT in single K562 and KG1a cells; models for chronic and acute myeloid leukaemia, respectively. The cells were incubated with the peptides (1 μM) for 1 h at 37 °C and the image shows distinct distribution profiles with the K562 harbouring the peptide in lysosomes that are enriched in a juxtanuclear region (Al-Taei et al., 2006). Both cell lines show minimal labelling of the plasma membrane and the stringent trypsinisation and washing that is required to reduce surface binding in other cell types has very little effect in these cells as the vast majority of labelling is intracellular (Al-Taei et al., 2006; Fretz et al., 2007). Scale bars 10 μm.

is also an evidence that CPP modified liposomes can breach the plasma membrane at low (4 °C) temperatures (Iwasa et al., 2006).

The use of agents deemed to inhibit specific endocytic pathways have provided much information on the uptake of CPPs but they have not pinpointed an agreed uptake mechanism or pathway (Duchardt et al., 2007; Fischer et al., 2001; Kaplan et al., 2005; Nakase et al., 2004; Richard et al., 2003; Zaro et al., 2006). Greater use of cell lines with genes of endocytic proteins silenced or mutated with resulting defects in defined pathways will undoubtedly provide more information (Benmerah, 2004; Huang et al., 2004). It is, however, likely that at the concentrations utilised for most studies, the peptides gain entry via multiple pathways and associated cargo is also likely to have a major influence. Indeed recent studies from our group and from the Roland Brock group have shown that increasing the peptide concentration (HIV-TAT and R8/9) above a certain threshold results in extensive cytosolic labelling that is unlikely to be attributable to any form of endocytosis (Duchardt et al., 2007; Fretz et al., 2007). A challenge is to determine whether any pathway could be selected for more efficient translocation of the peptide and associated cargo, to the cytosol and nucleus.

3. Conclusions

Its is likely that major advances in the understanding of the molecular machinery that control endocytic pathways will lead to parallel increased understanding of the mechanisms by which cells interact with and internalise the ever increasing armoury of macromolecules that are designed to transport macromolecular therapeutics to target cells. Though the uptake and fate of CPP as single entities or fluorescent conjugates has been extensively studied, the same degree of scrutiny is rarely given to unravelling endocytic pathways utilised by CPP associated with bioactive cargo. What are the relationships between CPP-based delivery systems and multivesicular bodies, do they in any way affect the integrity of endocytic organelles or the direction of trafficking pathways? How can we modify endocytic pathways and CPPs to enhance cytosolic delivery? These questions are not easy to address but are fascinating challenges for the future.

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