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siRNA and pharmacological inhibition of endocytic pathways to characterize the differential role of macropinocytosis and the actin cytoskeleton on cellular uptake of dextran and cationic cell penetrating peptides octaarginine (R8) and HIV-Tat

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ABSTRACT

Cell penetrating peptides (CPPs) have been extensively studied as vectors for cellular delivery of therapeutic macromolecules. It is widely accepted that they can enter cells directly across the plasma membrane but also gain access through endocytic pathways that are yet to be fully defined. Here we developed siRNA methods in epithelial cell lines, HeLa and A431, to inhibit endocytic pathways regulated by clathrin heavy chain, flotillin-1, caveolin-1, dynamin-2 and Pak-1. In each case, functional uptake assays were developed to characterize the requirement for these proteins, and the pathways they regulate, in the internalisation of defined endocytic probes and also the CPPs octaarginine and HIV-Tat. Peptide uptake was only inhibited in A431 cells depleted of the macropinocytosis regulator Pak-1, but experimental variables including choice of cell line, pharmacological inhibitor, macropinocytic probe and serum starvation significantly influence our ability to assess and assign this pathway as an important route for CPP uptake. Actin disruption with Cytochalasin D inhibited peptide entry in both cell lines but the effects of this agent on dextran uptake was cell line dependent, reducing uptake in HeLa cells and increasing uptake in A431 cells. This was further supported in experiments inducing actin stabilisation by Jasplakinolide, emphasising that the actin cytoskeleton can both promote and hinder endocytosis. Overall the data identify important aspects regarding the comparative mechanisms of CPP uptake and macropinocytosis, and accentuate the significant methodological challenges of studying this pathway as an endocytic portal and an entry route for drug delivery vectors.

1. Introduction

Cell penetrating peptides (CPPs) are short peptide sequences, typically less than 30 amino acids that have a universal ability to translocate across biological membranes. They are of considerable interest owing to their capacity to co-internalise an expanding repertoire of cargo from small molecule drugs and fluorophores to peptides, proteins, nucleotides and nanoparticles. Thus they are of considerable interest as potential vectors for delivering therapeutic entities to the inside of cells and intracellular organelles [1–4]. The mechanism of membrane penetration of a few variants such as HIV-Tat, oligoarginines and penetratin has been extensively studied using both cells in culture and synthetic membranes. Depending on factors such as temperature and extracellular concentration they have a capacity, at least attached to small probes, to gain cell entry via endocytosis and by direct translocation across the plasma membrane [5–9].

It is now appreciated that a single cell has the capacity to internalize material through a number of different endocytic pathways including those regulated by clathrin, caveolin and flotillin [10]. Macropinocytosis represents another pinocytic pathway, though differentiating this from phagocytosis is problematic [8,11,12]. Macropinocytosis gained prominence when it was shown to be induced in cells treated with growth factors such as epidermal growth factor (EGF), that initially induce extensive plasma membrane ruffling [13]. Downstream of ruffling is a rapid increase in cell uptake of fluid phase endocytic probes such as horseradish peroxidase and dextran, that were shown to be internalized into large macropinosomes [14,15]. It has also been proposed that some cell types are able to do this constitutively, in the absence of growth factor activation [8]. Macropinocytosis has emerged as a prominent uptake route for cationic CPPs attached to small molecular weight fluorophores or larger cargo [16–20]. Assigning the involvement of this pathway was mostly based on (1) an ability to inhibit this pathway via a largely undefined mechanism with amiloride and amiloride analogues, (2) CPP-

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mediated stimulation of dextran endocytosis and (3) the effects of a number of CPPs on the dynamics of the actin cytoskeleton that is required for macropinocytosis to proceed. A role for actin has however been shown for a number of different endocytic pathways [21] and chemical inhibitors like amiloride are prone to having multiple effects on cells [22,23]. The effects of amiloride and amiloride analogues on CPP uptake are inconsistent in the literature as these agents have been shown to have no effects or to even stimulate peptide internalisation [5,24–26]. Indeed the requirement for any endocytic pathway for CPP internalisation has been questioned [27]. Overall the data highlight the problems, with existing analytical tools, of identifying the importance of macropinocytosis for CPP entry, and indeed endocytosis; the same could be said for many other drug delivery vectors. The use of siRNA depletion of endocytic proteins that regulate endocytic pathways is an alternative to the use of pharmacological inhibitors and we have recently made use of this technology to study the endocytic mechanisms of polyplex uptake in Retinal Pigment Epithelial cells [28].

Here we use further developed siRNA models to deplete pathways regulated by Clathrin Heavy Chain (CHC), Flotillin-1 (Flot-1), Dynamin-2 (DNM2), Caveolin-1 (Cav-1) and p21-activated kinase 1 (Pak-1) and we further characterize each pathway in terms of their effects on the time-dependent uptake of various endocytic probes. Using time course experiments we investigated the roles for these proteins and the pathways they regulate in mediating the uptake of fluorescent conjugates of Tat and R8. Despite having defined endocytic defects, cells depleted of CHC, Flot-1, Cav-1 and DNM2 had control levels of CPP uptake. Cells incubated with the actin-disrupting agent Cytochalasin D, and depleted of the macropinocytosis regulator Pak-1 had reduced uptake of CPPs confirming a role for this pathway in peptide uptake. However, the effects of actin disruption on the uptake of dextran was highly dependent on the choice of cell line as it was found to reduce dextran uptake in HeLa cells and stimulate uptake in A431 cells. Thus, the well established effects of actin disruption on CPP uptake may not lie solely at the level of inhibition of endocytosis. It may be a more general manifestation of the collapse of the cell cytoskeleton, and thus cellular morphology and mechanics, to inhibit entry either directly across the plasma membrane or into additional unidentified endocytic routes.

2. Materials and methods

2.1. Reagents

Alexa488 transferrin (Alexa488-Tf), Alexa488 10 kDa Dextran (Alexa488-Dex10), tetramethylrhodamine-70 kDa neutral Dextran (TMR-Dex70), Oligofectamine, Opti-MEM I serum reduced media and BODIPY® (4,4-difluoro-4-bora-3a,4 adiaza-s-indacene)-LacCer (lactosylceramide) (BODIPY-LacCer) were purchased from Invitrogen (Paisley, U.K). FITC-40 kDa Dextran (FITC-Dex40), epidermal growth factor (EGF), bovine serum albumin (BSA), fatty acid-free BSA, and rhodamine-phalloidin were from Sigma-Aldrich (Dorset, UK). R8 (RRRRRRRRGC) and HIV-Tat peptide (Tat) GRKKRRQRRRPPQGC were obtained from American Peptide Company and labelled at the C-terminus with Alexa488 C_5 maleimide (Invitrogen), purified by RP-HPLC and characterised by mass spectrometry as previously described [24]. Complete mini protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). FITC-Anti-CD59 antibodies (clone A35) were kindly provided by Dr Rossen Donev, Cardiff University. Single siRNA sequences of 21–23 residues (Table 1) were purchased from Eurofins MWG Operon (Ebesburg, Germany). siRNA cocktail targeting Dynamin II was from Dharmacon (See legend to Supplementary Fig. 5 for cocktail sequences).

2.2. Pharmacological inhibitors

Dynasore, Chlorpromazine (CPZ), Cytochalasin D (CytD), Blebbistatin (Blebb) and 5-ethylisopropyl amiloride (EIPA) were obtained from Sigma-Aldrich (Dorset, UK), Jasplakinolide was purchased from Invitrogen (Paisley, U.K).

Table 1
siRNA sequences used in this study.

siRNA target	Sequence	Reference
CHC	UAAUCCAAUUCGAAGACCAAUdTdT	Based on [29]
Flot-1	AGAUGCACGGAUUGGAGAAdTdT	Custom designed
cav-1	AGACGAGCUGAGCGAGAAGdTdT	Custom designed
DNM2	GGACAUGAUCCUGCAGUUdTdT	[30]
Pak-1	AUAACGGCCUAGACAUUCAdTdT	Custom designed
Lamin-A/C	CUGGACUUCCAGAAGAACAdTdT	[31]
GFP	GGCUACGUCCAGGAGCGCAdTdT	Custom designed

2.3. Cell culture

HeLa (cervical) and A431 (epidermal) cancer cell lines were maintained as a subconfluent monolayer in D-MEM supplemented with 10% (v/v) heat inactivated foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C. All tissue culture reagents were from Invitrogen (Paisley, U.K.).

2.4. Antibodies

Antibodies recognising clathrin heavy chain, dynamin-2 (DNM2) and Flot-1 were from BD Transduction Laboratories (Oxford, UK). Anti-cav-1 was from Cell Signalling Technology (Hertfordshire, UK), anti-Pak-1 was from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) and anti-∝-tubulin was from Sigma (Dorset, UK). Anti-EGFR and secondary HRP conjugated goat-anti-mouse and goat-anti-rabbit antibodies were from Thermo Scientific- Pierce (Loughborough, UK) and Alexa488 conjugated chicken-anti-rabbit antibodies were from Invitrogen (Paisley, U.K.).

2.5. Transfection of cells with siRNAs

Cells were seeded onto 6- or 12-well plates, and cultured for 24 h to be 50%–60% confluent on the day of transfection. The siRNA procedure was performed according to a manufacturer's protocol using the following volumes and concentrations for each well of a 12-well plate: 0.5 μl of a 50 μM stock siRNA was diluted in 89.5 μl of Opti-MEM I and 2.0 μl of Oligofectamine was gently mixed with 8.0 μl of Opti-MEM I. The diluted siRNA and diluted Oligofectamine were then combined and mixed gently and incubated at room temperature for 30 min. For each well, the medium was removed and replaced with 400 μl of Opti-MEM I medium (without antibiotic and serum). siRNA-oligofectamine complex was then added dropwise to the wells, prior to incubating at 37 °C and 5% CO_2 for 4 h. Finally, 250 μl of Opti-MEM growth medium containing 30% (v/v) serum was added directly to the transfection mixture and the cells were incubated for 48 or 72 h as indicated.

2.6. Internalisation of Alexa-488-Tf, FITC/Alexa488/TMR-dextran, FITC-anti-CD59 antibody, and BODIPY-LacCer

On the day of the experiment, siRNA transfected or non-transfected cells were washed 2× with phosphate buffered saline (PBS, pH 7.50) at room temperature and then incubated with serum-free medium (SFM) containing 0.2% (w/v) BSA (SFM/BSA) for 30 min. The cells were then washed 2× with PBS at room temperature and incubated for various periods of time with SFM/BSA containing either 100 nM Alexa488-Tf, 2 μg/ml anti-CD59 antibody, 5.0 mg/ml FITC-Dex40, 0.2 mg/ml Alexa488-Dex10 or 0.75 mg/ml neutral TMR-Dex70. The plates were then placed on ice to inhibit further uptake and washed 2× with ice-cold PBS followed by a 1 min incubation in ice-cold acid wash (0.2 M acetic acid,