
29 Imaging the Influx of Cell-Penetrating Peptides into the Cytosol of Individual Live Cells

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29.1 INTRODUCTION

One of the most surprising recent findings in biology is that certain naturally occurring and synthetic, short peptide sequences (cell penetrating peptides (CPPs)) have the ability to cross the plasma membrane of living cells even when linked to a wide variety of peptides, proteins, and molecular cargo.^{1,2} The main effort on CPPs in our laboratory is on tumor-specific, activatable CPPs (ACPPs), in which the CPPs are attached via protease-cleavable linkers to sequences that veto cell uptake.³ In normal tissues, the linkers remain intact, whereas in tumors expressing appropriate proteases, the linkers are cleaved, allowing the released CPPs to accumulate on and in cells. For imaging in whole animals or patients, the entry mechanism and final location of the cargo are of minor importance, because in vivo imaging lacks subcellular spatial resolution. However, the same mechanism of tumor targeting should also work for chemotherapeutic or radiation-sensitizing cargoes, for which delivery to cell nuclei would be optimal. There is still considerable debate about the mechanism of cell entry and the quantitative efficacy of the many CPPs now

available.⁴ This is, in part, due to the limitations in methods available to quantitatively measure and discriminate the uptake of CPPs into specific cell compartments such as the cytosol, nucleus, and endosomes. Ideally, such a method would continuously monitor the uptake into individual live cells with high spatial and temporal resolution. We have developed such a method which uses fluorescence resonance energy transfer (FRET) from a genetically encoded cytosolic receptor to a biarsenical fluorophore conjugated to a CPP. Such biarsenicals have a picomolar affinity for short (~10 amino acids) tetracysteine peptides containing the general sequence CCPGCC and have been used to specifically label and visualize recombinant proteins containing this sequence in living cells.^{5–8} By targeting a tetracysteine-tagged fluorescent protein to a specific cell compartment, such as the cytoplasm, by standard molecular biology techniques, changes in FRET upon incubation with CPP–biarsenicals permit real-time quantitative measurement of cytosolic uptake in single cells.

29.2 EXPERIMENTAL

29.2.1 SYNTHESIS OF FLAsH–OLIGOARGININE CONJUGATES

Peptides, H₂N-aha-R₇-CONH₂, and H₂N-GGR₁₀-CONH₂ were synthesized by standard Fmoc solid-phase techniques using a Pioneer peptide synthesizer (Perceptive Biosystems, Framingham, MA). Peptides were cleaved from the support with trifluoroacetic acid (TFA) triisopropylsilane–thioanisole (96:2:2 v/v) overnight and precipitated with cold ether–hexanes. The crude peptides (~2 mM in water, 30 μL) were treated with 5-carboxyFLAsH–EDT₂ succinimidyl ester (2 mM in dry dimethylformamide (DMF); 30 μL), bicine buffer pH 8.5 (50 mM in water; 40 μL) and 1,2-ethanedithiol (10 mM in DMF, 6 μL) at 4°C overnight.⁷ Alternatively, DMF can be used as a solvent with *N*-methylmorpholine as the base. The red precipitated crude product was collected by centrifugation, washed with water and dissolved in 50% acetonitrile–water–0.1% TFA and purified by HPLC (Dionex, Sunnyvale, CA) on C₁₈ columns (Phenomenex, Torrance, CA) with an acetonitrile–H₂O–0.1% TFA gradient followed by lyophilization. Electrospray mass spectroscopy (ES-MS) (50% MeOH 1% HOAc) positive mode gave for FLAsH-aha-R₇-CONH₂, 638.7 (M + 3H⁺), 479.3 (M + 4H⁺), 383.9 (M + 5H⁺) indicating a mass of 1912.38, calculated for C₇₃H₁₁₄As₂N₃₀O₁₄S₄, 1912.64. ES-MS (50% MeOH 1% HOAc) positive mode gave for FLAsH-GGR₁₀-CONH₂, 596.7 (M + 4H⁺), 477.6 (M + 5H⁺) indicating a molecular mass of 2381.84, calculated for C₈₉H₁₄₅As₂N₄₃O₁₈S₄, 2381.91.

29.2.2 CYTOSOLIC UPTAKE OF FLAsH–OLIGOARGININE CONJUGATES

HeLa cells were transfected with a Cyan Fluorescent Protein (CFP) tetracysteine construct (CFP-AEAAAREACCPGCCARA) using Fugene (Roche Diagnostics, Alameda, CA).⁷ Imaging experiments were performed 24 h after transfection with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). Fluorescence was monitored in three channels; CFP, excite at 440 nm (20 nm bandwidth) with emission

at 480 nm (30 nm bandwidth); FRET, excite CFP with emission at 635 nm (55 nm bandwidth); FIAsh (Fluorescein arsenical hairpin binder), excite at 495 nm (10 nm bandwidth) with emission at 535 nm (25 nm bandwidth). The 455 nm dichroic mirror used passed sufficient light at 495 nm. Cells were washed with Hanks buffered saline solution (HBSS) containing glucose and treated with 1–2.5 μM FIAsh oligoarginine conjugate at room temperature. The adduct of two 2,3-dimercaptopropanesulfonate (DMPS) with FIAsh-GGR₁₀-CONH₂ was made by incubation with 100 μM DMPS in HBSS for 15 min. After staining with FIAsh oligoarginine conjugate for 30–60 min, the cells were washed with HBSS, then treated with 5 mM 2,3-dimercaptopropanol (BAL) to disrupt binding of FIAsh conjugates to the CFP tetracysteine. The percentage of CFP tetracysteine bound with FIAsh conjugate was determined by the increase of CFP fluorescence emission after adding BAL. Stoichiometric binding of FIAsh to this construct reduces the CFP emission by 65%.⁵ Cellular CFP tetracysteine concentrations were determined by comparison with microcuvets of purified protein of known concentration.⁷

29.3 RESULTS AND DISCUSSION

29.3.1 DETERMINING CYTOPLASMIC UPTAKE RATES OF OLIGOARGININE CONJUGATES

To specifically measure entry of oligoarginine CPPs into the cytoplasm of individual live cells we devised a method (Figure 29.1) utilizing the highly specific interaction of FIAsh, a fluorescein biarsenical for a tetracysteine-containing peptide.^{5,7} This genetically encoded tag was fused to CFP and transiently transfected into living

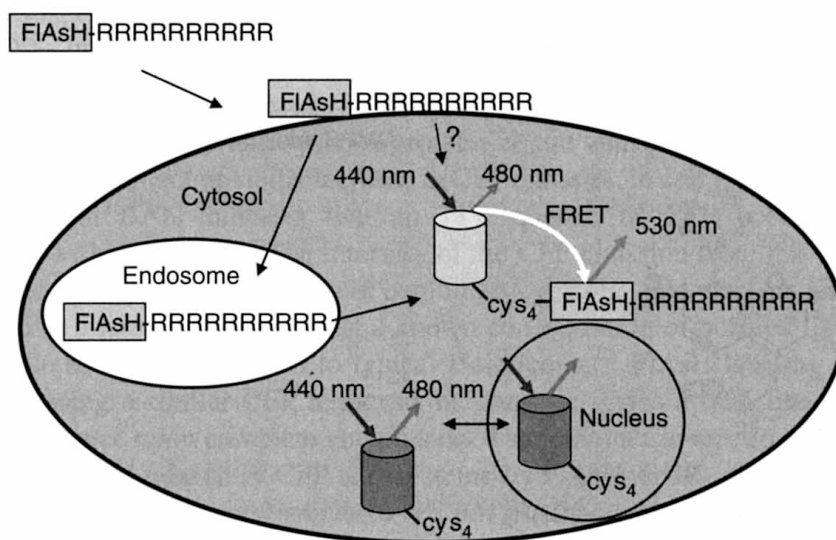


FIGURE 29.1 Scheme depicting the use of a cytoplasmic CFP tetracysteine to detect transduction to the cytosol and nucleus of FIAsh-labeled oligoarginine CPPs. The CFP fluorescence is quenched upon FIAsh binding due to FRET and gives a real-time measurement in single cells of the rate and amount of CPP transduction.

Hela cells to express the recombinant protein in the cytoplasm (and nucleus). The cells were incubated with FIAsh chemically conjugated by its benzoic acid moiety (which does not interfere with binding to tetracysteine peptides) to oligoarginine CPPs (Figure 29.2). If peptide translocation occurs to the cytoplasm, binding of the FIAsh conjugate to the CFP tetracysteine construct will result and be measured by a quench of the CFP fluorescence emission due to FRET to FIAsh. Cytoplasmic CFP concentrations of cells can be estimated by comparison with known standards to give values for the rates of cytoplasmic uptake of FIAsh–oligoarginine conjugates.

F2

Figure 29.3 shows typical cell images and time-courses of such an experiment with the FIAsh–GGR₁₀ conjugate. After an initial stable baseline, addition of the peptide results slowly in a slow but steady decrease in CFP emission over approximately 30 min at room temperature. After washing of the cells, the binding of CFP tetracysteine to FIAsh conjugates is rapidly reversed by the addition of a high concentration of the competing dithiol, BAL. The resulting rapid increase of CFP emission is a direct measure of the extent of FRET between CFP and FIAsh.

F3

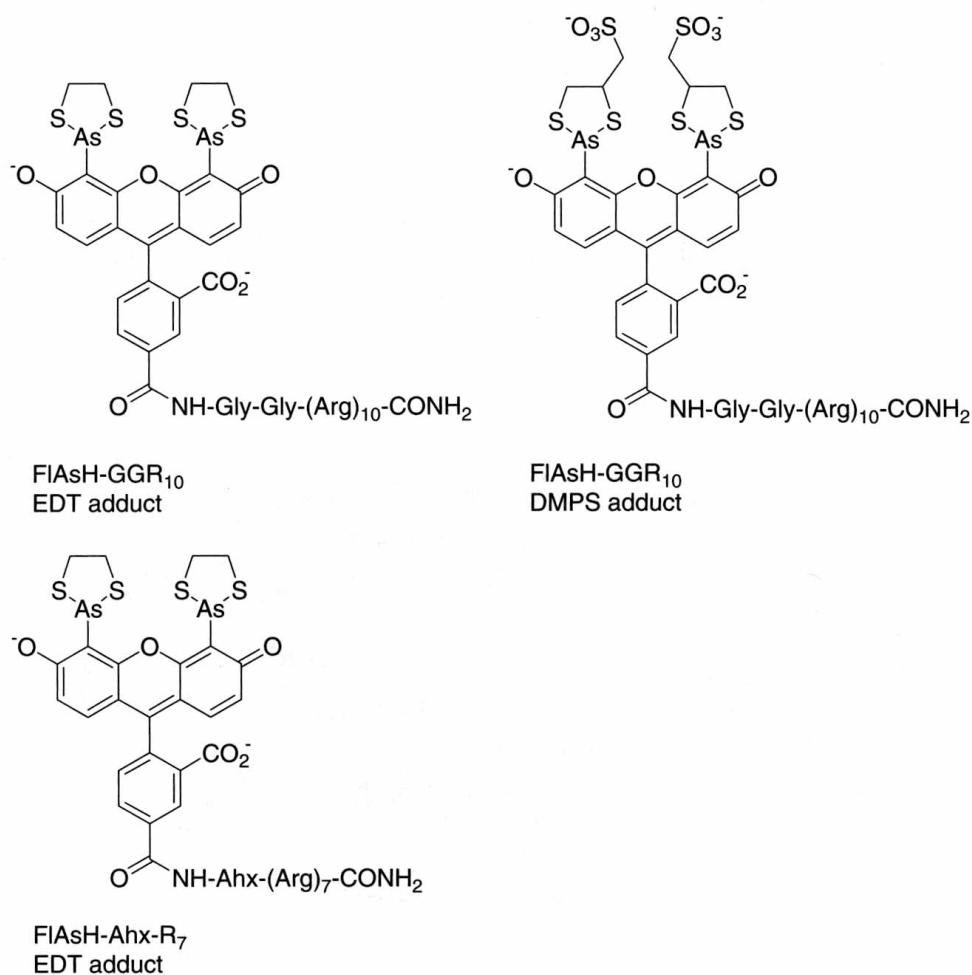


FIGURE 29.2 FIAsh conjugates of R₁₀ and R₇ CPPs. Each arsenic (III) is complexed to a uncharged (EDT) or negatively charged (DMPS) dithiol.

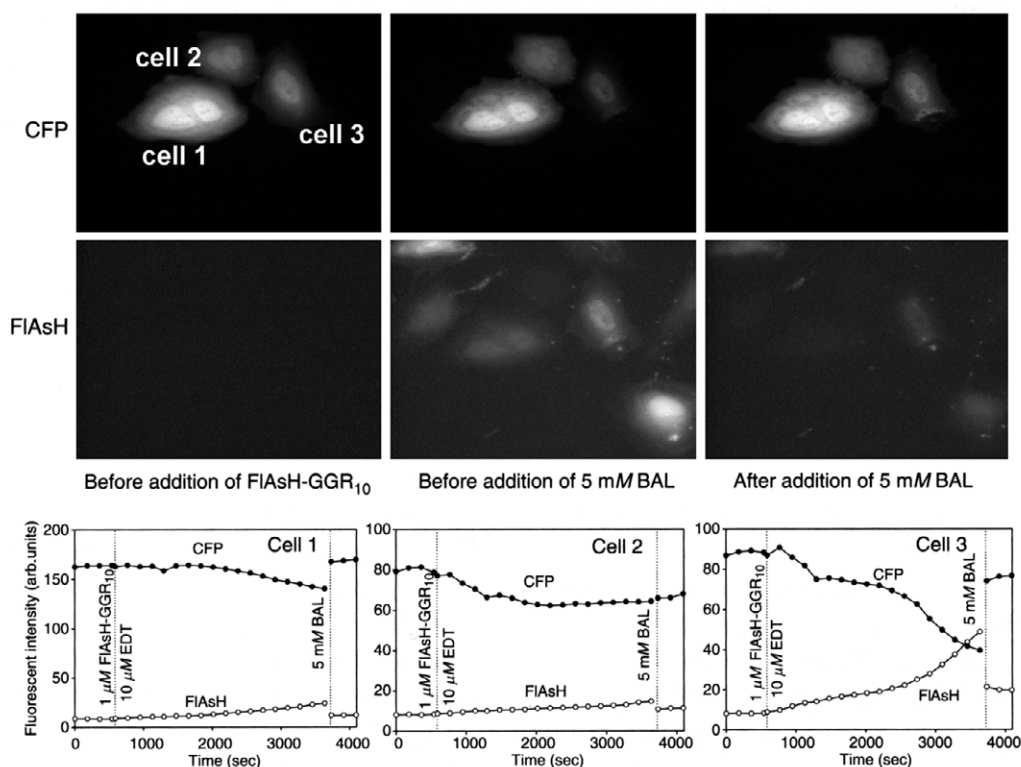


FIGURE 29.3 A typical experiment to monitor cell uptake and cytoplasmic binding of FIAsh-GGR₁₀ to CFP tetracysteine (CFP-AEAAAREACCPGCCARA) transiently expressed in HeLa cells. Upper panels: CFP fluorescence (upper images) and corresponding FIAsh fluorescence (lower images) from a field of cells before and after incubation with 1 μ M FIAsh-GGR₁₀ and 10 μ M EDT, and after acute reversal of binding by addition of the dithiol, BAL. Lower panels. Time courses of the average CFP and FIAsh fluorescence from regions encompassing each of the single transfected cells shown in the upper panels.

Knowing the maximal value of FRET upon stoichiometric labeling with FIAsh gives the fraction of CFP tetracysteine proteins bound with FIAsh conjugate during the incubation. For example, the increase in CFP emission in cell 1 in Figure 29.3 on treatment with BAL indicates that an 18% quench of CFP occurred during incubation with the peptide. The intensity of the CFP emission from this entire cell corresponds to approximately 7 μ M combined cytosolic and nuclear CFP when compared to microcuvets containing a known concentration of purified CFP with a comparable thickness ($\sim 5 \mu$ m) to typical HeLa cells.^{7,9} FIAsh labeling of living cells expressing a similar CFP tetracysteine construct gives a 65% quench of the CFP fluorescence upon complete and presumed stoichiometric reaction.⁵ Assuming a similar maximal quench of CFP tetracysteine by FIAsh-GGR₁₀, the 18% quench in cell 1 after incubation corresponds to $\sim 2 \mu$ M FIAsh conjugate bound to cytosolic and nuclear CFP tetracysteine after approximately 50 min of continual incubation at room temperature.

There are substantial cell-to-cell differences in such time-courses even within a single microscopic field that probably reflect cell variability in the kinetics of CPP uptake. For example, cell 1 in Figure 29.3 shows a steady decline in CFP

fluorescence after a lag of 20 min. Cell 3 shows a more biphasic response with an immediate slow drop in fluorescence followed by faster uptake. In both of these cases, the fluorescence decrease shows no sign of leveling off even after almost 1 h of incubation with the CPP. However, decreases in CFP emission can also be due to slow changes in cell shape or focus during this prolonged treatment with peptide. For example, cell 2 in Figure 29.3 shows an initial decrease in CFP emission that stabilizes after 30 min but no dequench is revealed on treatment with BAL.

The changes in FIASH fluorescence during such experiments are generally less informative and harder to quantify than those occurring from CFP. Extracellular FIASH-GGR₁₀, such as FIASH-EDT₂, is nonfluorescent so is not visible upon addition to the cells in solution or upon the subsequent rapid binding to the plasma membrane typical with CPPs. Similarly, uptake into acidic endosomes is unlikely to generate significant fluorescence as FIASH reacts only with reduced thiols in a neutral environment. FIASH fluorescence will primarily result (after endosomal release) from formation of complexes with CFP tetracysteine and from nonspecific binding to endogenous cytoplasmic thiols. The latter can be seen in the untransfected cells in the panel of Figure 29.3 that are only visible in the FIASH image.

To control for any endosomal proteolysis of FIASH-GGR₁₀ and release of a membrane-permeable FIASH fragment that could bind to the cytoplasmic CFP tetracysteine, and masquerade as cytosolic translocation, we pre-incubated FIASH-GGR₁₀ with excess DMPS. Dithiol exchange rapidly occurs to produce the DMPS bis-adduct containing two additional negatively charged sulfonate groups (Figure 29.2). As expected, FIASH-DMPS₂, prepared similarly from FIASH-EDT₂ and DMPS, is not membrane permeable and when incubated with cells expressing CFP tetracysteine, no binding is detectable (data not shown). However, when incubated with cells, the DMPS adduct of FIASH-GGR₁₀ shows a rate of binding to

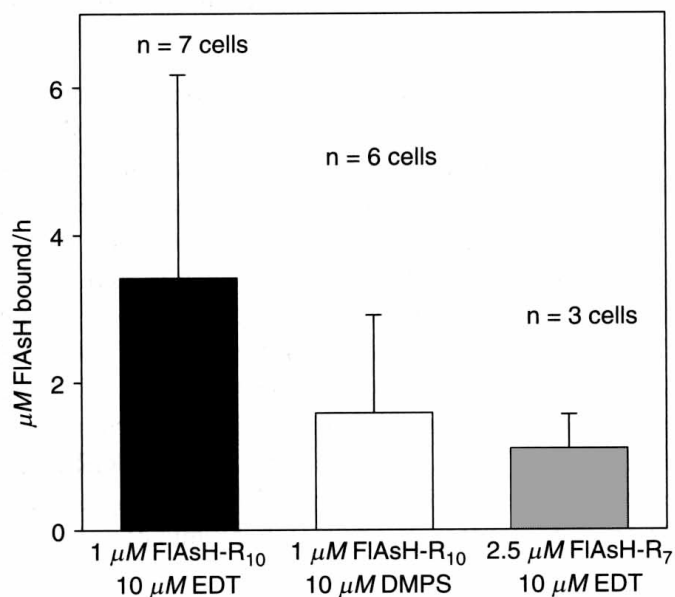


FIGURE 29.4 Rates of cytoplasmic and nuclear uptake of different FIASH-oligoarginine conjugates into HeLa cells at room temperature.

intracellular CFP tetracysteine that was only slightly lower than with the corresponding EDT adduct (Figure 29.4). This decrease may just reflect the effect of two negative additional charges on the cell uptake of FIAsh-GGR₁₀ rather than an indication of endosomal hydrolysis. In support of this, cell uptake of FIAsh-aha-R₇ that has a similar net positive charge (+5 compared to +6 for FIAsh-GGR₁₀) was only detectable at higher labeling concentrations of 2.5 μ M (Figure 29.4). The lower efficacy of heptaarginine is in agreement with previous studies with fluorescein conjugates.¹⁰

The major finding of this study is that the overall rates of CPP uptake under these conditions are quite modest, in the range of a few micromolar over a time-course of an hour at room temperature. Therefore, final cytoplasmic concentrations only match, or slightly exceed, the initial extracellular concentration of the conjugates. The extent of CPP uptake is also quite variable between even adjacent and apparently identical cells in the same microscopic field.

29.3.2 COMPARISON WITH OTHER METHODS

Two general approaches used to quantify cytosolic and/or nuclear uptake of CPPs are the detection of fluorescent conjugates by nondestructive optical methods or subcellular fractionation of these two compartments upon cell lysis. One of the original, and still most popular, is the measurement of the uptake of fluorescently labeled CPPs by fluorescence activated cell sorter (FACS). This analysis measures the total amount of CPP bound per cell; trypsin treatment following labeling of cells can remove any extracellular peptide bound to the surface (but only if the CPP contains L-amino acids), but signals from endosomal and cytosolic peptide cannot be differentiated. Many early fluorescence microscopy studies were flawed by fixation effects on subcellular distribution on CPPs, but the recent use of confocal microscopy of living cells has proven more reliable, for example, in revealing the endosomal staining predominately found following incubation with CPPs such as Tat and oligoarginine.^{11,12} However, quantitatively distinguishing cytosolic/nuclear localization from that of endosomal is difficult because these organelles are below the resolution of light microscopy and the endosomal CPPs are likely to be partly self-quenched by molecular crowding or by low pH. A similar FRET-based method uses CPPs containing a quenching nitrotyrosine group attached to fluorophore by a disulfide linker.¹³ Fluorescence was generated only when the disulfide bond is cleaved by entry of the CPP into a reducing environment, assumed to be the cytoplasm. Using this method, millimolar intracellular concentrations of CPPs were measured, in contrast to our results. However, disulfide bonds can be reduced within endosomes, not just in the cytoplasm, so the difference in apparent uptake may reflect CPPs remaining within endosomes.¹⁴ Furthermore, the low fluorescence and UV excitation of the aminobenzoyl fluorophore limits this method to cell suspensions.

A recently reported quantitative single-cell assay for cytosolic uptake detects phosphorylation of Tat-conjugated peptide substrates of cytoplasmic kinases such as CaMKII and PKB.¹⁵ A laser pulse disrupts individual cells and the intracellular peptides are separated and quantified by capillary electrophoresis. The rates of CPP

uptake, consistent with our results, showed micromolar cytoplasmic levels of the peptides achieved by a 10 min loading followed by 1 h of incubation to complete endosomal to cytoplasmic trafficking. Zaro and Shen¹⁶ describe a method for determining the extent of endosomal and cytoplasmic CPP using subcellular fractionation of radiolabeled oligoarginine peptides followed by size-exclusion chromatography. A fluorescent dextran applied with the CPP was used as a marker of endocytosis and to correct for the amount of endosomes lysed during processing, but the final cytoplasmic concentration was not calculated. This method is a destructive assay applicable only to large populations of cells and critically dependent on the quality of subcellular fractionation.

One limitation of our method is that cytosolic trapping of CPP would reduce or suppress any efflux from the cytosol, so only unidirectional entry is measurable. Also, any nonspecific binding of the biarsenical to sites other than the CFP tetracysteine would cause an underestimation of the amount of cargo delivered.⁶

Future work with this method will investigate the dependence of cytosolic uptake upon CPP concentration and endosomal function to probe the molecularity of uptake and its biophysical mechanism. The effect of different cargoes on CPP uptake, and a comparison of oligoarginines with other CPPs such as Tat or penetratin, would also be of interest. Judicious modifications of the cargo may also permit us to monitor endosomal versus cytosolic CPPs simultaneously.

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