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RESEARCH ARTICLE

A QUANTITATIVE METHOD TO DISTINGUISH CYTOSOLIC FROM ENDOSOME-TRAPPED CELL-PENETRATING PEPTIDES

Françoise Illien[a], Zoltán Bánóczi[b], Sandrine Sagan*[a]

[a] Françoise Illien, Dr. Sandrine Sagan
Department of Chemistry, Laboratory of Biomolecules
Sorbonne University, Faculty of Sciences and Engineering
4 place Jussieu, 75005 Paris cedex, France
E-mail: sandrine.sagan@sorbonne-universite.fr

[b] Dr. Zoltán Bánóczi
ELTE Eötvös Loránd University, Budapest, Hungary
Institute of Chemistry, Faculty of Science, Pázmány Péter sétány. 1/A, Budapest H-1117, Hungary
HUN-REN-ELTE Research Group of Peptide Chemistry, 1117 Budapest, Hungary, Hungary

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Abstract: Cell-penetrating peptides are known to penetrate cells through endocytosis and translocation. The two pathways are hardly distinguished in current cell assays. We developed a reliable, simple and robust method to distinguish and quantify independently the two routes. The assay requires (DABCYL) 4-(dimethylaminoazo)benzene-4-carboxylic acid- and (CF) carboxyfluorescein-labeled peptides. When the labeled peptide is intact, the fluorescence signal is weak thanks to the dark quenching property of DABCYL. A 10-fold higher fluorescence signal is measured when the labeled peptide is degraded. By referring to a standard fluorescent curve according to the concentration of the hydrolyzed peptide, we have access to the internalized peptide quantity. Therefore, cell lysis after internalization permits to determine the total quantity of intracellular peptide. The molecular state of the internalized peptide (intact or degraded), depends on its location in cells (cytosol vs endo-lysosomes), and can be blocked by boiling cells. This boiling step results indeed in denaturation and inhibition of the cellular enzymes. The advantage of this method is the possibility to quantify translocation at 37°C and to compare it to the 4°C condition, where all endocytosis processes are inhibited. We found that ranking of the translocation efficacy is DABCYL-R_6-(εCF)K >> DABCYL-R_4-(εCF)K ≥ CF-R_9.

Introduction

Cell-penetrating peptides have been described for the first time in the 1990's [1]. For about 30-years, research world-wide has been focusing on the mechanisms behind the unique property of these peptides to internalize into cells and convey cargo molecules with them, either covalently conjugated or through non-covalent complexes. The more common current vision is that to enter cells, these peptides can also use direct translocation across the cell membrane in addition to endocytosis pathways [2]. Albeit many progresses have been done in that regard, translocation is still hardly understood [3], since this cell process is complex to be visualized and quantitatively evaluated and distinguished from endosomal escape. Translocation is however not only endosomal release, since this phenomenon can be observed at 4°C. Moreover, translocation is a route that can be used at low micromolecular concentrations [2a]. Distinguishing cytosolic localization is however very important since various biological and therapeutic targets are localized there. There have been many reports that describe biophysical strategies to evidence translocation [2e]. In addition, recent developments in cell engineering rely on methods that permit to study specifically cytosolic location. Teo and coworkers [4] describe the NanoLuc assay applied to R_9 and Tat, demonstrating that these CPPs can translocate across cell membrane, rather than being endowed with endosomal escape efficiency. On their side, Lucchino and co-workers [5] have developed a SNAP-tag assay and report that Tat peptide translocation can occur at low μM concentrations, as previously reported by MS analysis [2a]. Albeit these two examples, there is still some space for a simple and robust method to specifically address the translocation potency of CPPs. Herein, we have developed a new protocol based on FRET dark quenching and release through cell enzyme hydrolysis of DABCYL- and CF-labeled peptides [6]. DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K have been reported as efficient CPPs thanks to the presence of the DABCYL moiety, a well-known azobenzene push–pull system [7]. Its conjugated π-system in the phenyl ring and the absence of polar substituents make this molecule quite hydrophobic. In addition, the absorption band of the chromophore DABCYL is in the range of 350-550 nm that overlaps with the emission band of the fluorophore carboxyfluorescein (maximal λ_{em} at about 520-525 nm in the peptides). We took advantage of this property to develop a method to quantify at 37°C independently translocation and endocytosis.

Results and Discussion

PEPTIDE SYNTHESIS AND CYTOTOXICITY

The peptides DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K were synthesized by solid-phase Fmoc strategy as previously reported [7] and CF-R_9 was purchased from a peptide supplier (Eurogentec). Their sequence is shown Figure 1. The peptides were first tested for their cytotoxicity after 1 hr incubation with

CHO-K1 cells. As shown in Figure S1, the three peptides are not cytotoxic up to 20 μM.

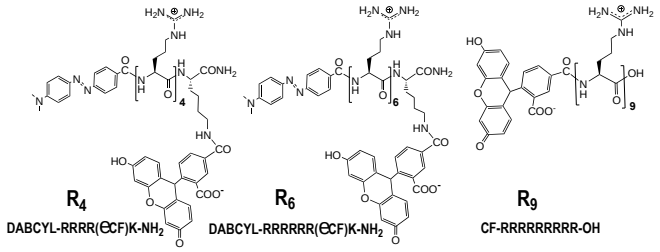


Figure 1. Structure of the fluorescent peptides used in this study. Abbreviations, DABCYL: 4-(dimethylaminoazo)-benzene-4-carboxylic acid; CF: carboxyfluorescein; K: lysine; R: arginine. MW of peptides: 1379.1 g/mol for DABCYL-R_4-(εCF)K, 1691.9 g/mol for DABCYL-R_6-(εCF)K and 1792 g/mol for CF-R_9.

FLUORESCENCE OF DABCYL-R_n-(εCF)K PEPTIDES IN DIFFERENT EXPERIMENTAL CONDITIONS

The protocol to quantify intracellular peptide is briefly the following : i) incubation of 10^6 cells with the peptide in culture medium for 1 hr, either at 37°C or 4°C; ii) after washings and trypsin digestion at 37°C (or pronase at 4°C), to eliminate membrane-bound peptides and detach cells, cells are mixed with the lysis buffer and immediately subjected to sonification for 15 min at RT (intracellular peptide hydrolysis by the released enzymes), in order to get the total intracellular peptide concentration, or to boiling for 15 min (intracellular enzyme inactivation), in order to get the peptide internalized by endocytosis only; iii) centrifugation for 10 min to remove cell debris and collect the supernatant; iv) fluorometry of the supernatant (λ_{ex} = 495 nm; λ_{em} = 525 nm).

We first examined the fluorescence behavior of DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K in the different conditions used (Fig. 2), to establish at which steps of the protocol we could measure fluorescence release resulting from peptide hydrolysis. As seen in Figure 2, the intact DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K peptides are weakly fluorescent, unless they are hydrolyzed by enzymes, either trypsin alone or enzymes released from cellular compartments after cell lysis (Fig. 2b). As controls, sonification and boiling steps that are used in the protocol to lyse cells, have no effect on the peptide integrity (Fig. 2c). When cell lysates are kept intact at 4°C or in the presence of enzyme inhibitors, there is no degradation of DABCYL-R_4-(εCF)K (Fig. 2d). Identical results were obtained when lysis buffer is added to cells and they are immediately boiled (Fig. 2d). On its side DABCYL-R_6-(εCF)K was insensitive to enzymes from boiled cells (Fig. 2d). A significant degradation of this peptide is however observed with cells kept at 4°C or in the presence of protease inhibitors. This increase in the fluorescence signal is likely the result of peptide degradation by still active cell enzymes since in the absence of cells, we did not observe any effect of sonification, boiling or combination of both treatments (Fig. 2c).

In contrast, when cells are lysed by sonification and kept at room temperature, the enzymes released from intracellular organelles, mainly endosomes and lysosomes, quickly digest the two peptides (Fig. 2b). The fluorescence signal read at 525 nm increases about 10-times between the intact and enzymatically degraded peptides. This difference of fluorescence signal between the intact and degraded peptides is sufficiently different to discriminate between the two molecular states. The fluorescence signal is linearly related to the concentration used, both for the intact and the degraded peptides (Scheme S1). Altogether, these observations opened the possibility to use fluorescence to quantify these peptides inside cells, by using a standard curve of the same peptide, done in parallel with the internalization experiments. In particular, we observe that boiling at 100°C or cooling at 4°C, are conditions that preserve peptide molecular integrity, allowing to block specific cellular situations and studying the mechanisms of cell entry of these peptides. We studied next the internalization of the peptides in varying conditions.

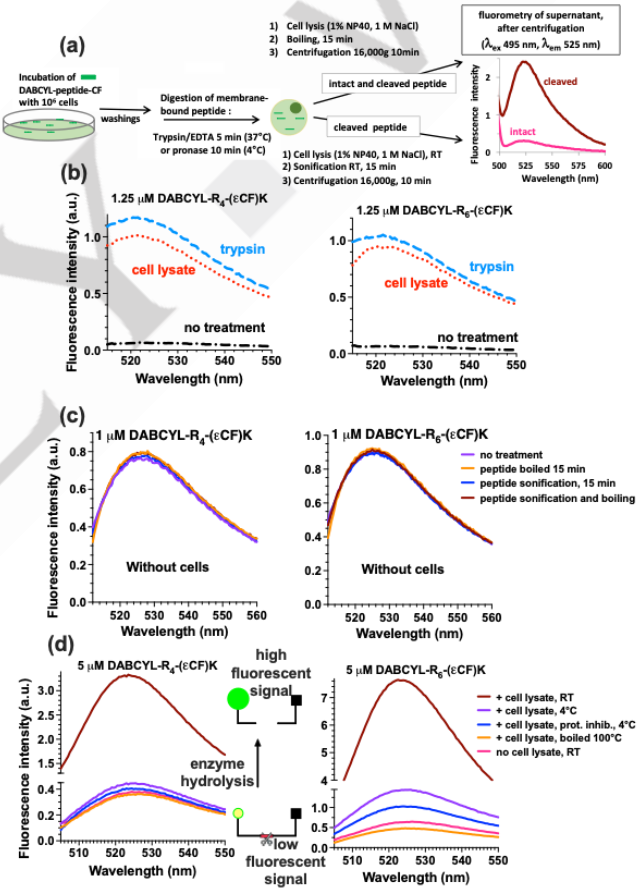


Figure 2. FRET dark quenching and fluorescence properties of the intact and enzymatically cleaved DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K. **(a)** Protocol developed in the study to quantify the internalization efficacy; **(b)** 1.25 μM (250 pmole/200 μL) peptide was incubated for 1 hr : i) in the lysis buffer alone as a negative control (intact peptide, black line); ii) as shown in (a), with 10^6 cells in 200 μL lysis buffer followed by sonification for 15 min at RT (cell lysate, red line); iii) with trypsin plus EDTA (0.008%), in the lysis buffer at 37°C as a positive control (degraded peptide, blue line); samples were then centrifugated at 16,000g, before fluorescence was recorded in the supernatant (λ_{ex} = 495 nm); **(c)** Effect of 15 min sonification and/or 15 min boiling on the fluorescence signal of 1 μM (200 pmole), intact peptide. **(d)** Control experiments to visualize the impact of the different treatments (lysis buffer, boiling,

sonification and combined), on the stability of the peptides: incubation of 5 μM (1000 pmole/200 μL lysis buffer), peptide for 1 hr : in lysis buffer alone at room temperature (intact peptide); with 10^6 intact cells in 200 μL lysis buffer, then sonification on ice (4°C), for 15 min in the presence (cells, prot. inhib.), or absence (cells), of protease inhibitors (Roche Complete Mini); with 10^6 intact cells in 200 μL lysis buffer, then boiled (100°C), for 15 min (cells, boiled), or lysed by sonification for 15 min at RT (cell lysate), before fluorescence intensity recording; all experiments have been replicated 3 times independently. A representative experiment is shown in every panel.

INTERNALIZATION ASSAYS AND DETERMINATION OF THE ENTRY ROUTES OF DABCYL-R_n-(εCF)K PEPTIDES

We analyzed first whether the cell enzymes are sufficiently active to degrade all the peptide internalized, whatever the intracellular amount. We incubated for one hour at 37°C, one million cells with 1, 2.5, 5 and 10μM concentrations of the fluorescent peptides.

After washings and trypsin treatment necessary to detach cells from their plastic support and to remove membrane-bound peptides [8], cells were then lysed in a specific buffer (containing or not trypsin), by sonification at room temperature. Then, centrifugation of cell lysates permits to measure fluorescence signals from clear supernatants (Fig. 3).

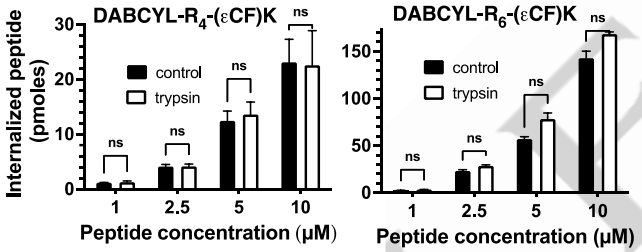
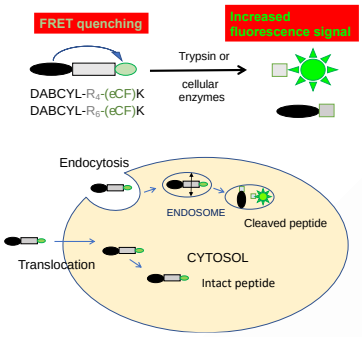


Figure 3. Quantification of the total internalization of DABCYL-R_n-(εCF)K peptides at 37°C. The total internalized peptides are measured by total fluorescence of cleaved peptides, in enzymatically active cell lysates in the absence or the presence of additional trypsin. The experiments have been replicated 3 times independently.

We verified that the peptides internalized at 37°C can be completely cleaved in cells during the lysis. We obtained similar results (multiple t-test, not significant), with or without trypsin in the lysis buffer (Fig. 3). This result indicates that within this concentration range and under these experimental conditions, the internalized peptides studied herein, are completely cleaved during cell lysis. In addition, we confirmed by MALDI-MS analysis for DABCYL-R_4-(εCF)K (Figure S2) that the maximal fluorescence intensity we record for the trypsin-treated peptide corresponds to complete hydrolysis of the peptide.

We established then the protocol described above and summarized in Scheme 1, to analyze the entry routes, translocation versus endocytosis, of the two peptides, CF-R_9 being used as a control peptide. The results are given in Table 1.

The interesting breakthrough provided by this protocol is that it allows easily to quantify translocation at 37°C, without cell engineering or treatment with endocytosis inhibitors.



Experimental conditions	Cell incubation with peptide, 37°C	Cell incubation with peptide, 4°C
Cell lysate RT	Total internalization measured (E + T)	Translocation measured (cleaved peptide) (T)
Cell lysate boiled	Endocytosis measured (E)	Translocation (intact peptide) (T)

E (endocytosis); T (translocation)

Scheme 1. Scheme of the expected internalization routes according to the experimental conditions. After cells are incubated at 37°C (endocytosis and translocation), with peptides, cell lysis at RT permits quantification of the total internalized peptide (endocytosis and translocation), when cells boiling blocks the intracellular fate of peptides and gives access to peptide that used endocytosis only. After cells are incubated at 4°C (translocation only), cell lysis at RT permits quantification of translocation only. One can thus compare translocation quantification measured at 37°C or 4°C.

As seen in Table 1, DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K behave differently in terms of the major route they take to reach the cytoplasm of cells. At low concentrations (1 and 2.5 μM), DABCYL-R_4-(εCF)K internalizes equally by translocation and endocytosis, while at higher concentrations (5 and 10 μM), the contribution of endocytosis is predominant. Interestingly, the contribution of translocation determined at 37°C or 4°C (Table 1, columns c and d, respectively), were similar, showing that lowering temperature, thus affecting the fluidity of the membrane, does not impact negatively the translocation process for this peptide. Concomitantly the endocytosis contribution is also similar when determined at 37°C or deduced from complementary experiments (Table 1, columns b, a-d or a-e).

On its side, DABCYL-R_6-(εCF)K is more efficiently internalized into cells than DABCYL-R_4-(εCF)K (Table 1, column a). The peptide uses concomitantly half endocytosis and half translocation routes during its internalization into cells (Table 1, columns b and c). The quantification of translocation gave different amounts when comparing the amount of DABCYL-R_6-(εCF)K obtained at 37°C (column c), with the one measured at 4°C with the use of the calibration curve with the digested peptide (column d). We could assume that lowering the temperature impact the translocation process in that case. However, when using the calibration curve with the intact DABCYL-R_6-(εCF)K (column e), we measured similar amounts of peptide as the 37°C condition (column c). We must hypothesize in that case that cell enzyme activity in the cell lysate (sonification 15 min at RT), is affected by the 4°C storage of cells for one hour, even for the trypsin added exogenously. This was not the case for DABCYL-R_4-(εCF)K, but the amounts of peptide to hydrolyze were much

less. Finally, CF-R_9 internalization mostly relies on endocytosis for all concentrations. Altogether, DABCYL-R_6-(εCF)K internalizes in similar amounts by translocation and endocytosis, while CF-R_9 preferred way is endocytosis and DABCYL-R_4-(εCF)K, a mixture of both depending on the extracellular concentration (Fig. 4a).

Table 1. Quantity (pmoles) of DABCYL-R_4-(εCF)K, DABCYL-R_6-(εCF)K and CF-R_9 internalized into cells, according to the experimental conditions described in the table of Scheme 1. Different amounts (pmoles) of peptides were incubated for 1 hr with 10^6 cells, either at 37°C (endocytosis and translocation, E+T) or 4°C (translocation, T). After washings, cells were submitted to lysis in conditions allowing complete peptide hydrolysis (columns a and d) or not (columns b and e). Column e corresponds to the quantification of the translocation process, calculated with the calibration curve of the intact peptide (see Scheme S1). Comparison, between columns permits to quantify only endocytosis (E) or translocation (T) contribution. Depending on the described conditions, experiments have been replicated from 4 to 29 times, independently. The quantities of peptide incubated with cells at 200, 500, 1000 and 2000 pmoles correspond respectively to 1, 2.5, 5 and 10 μM concentrations. Measurements of intracellular pmoles of peptides correspond to μM intracellular concentrations.

Peptide pmoles [g]	(a) 37°C Lysate +trypsin RT (E+T)	(b) 37°C Boiled lysate (E)	(c) 37°C a-b[h] (T)	(d) 4°C Lysate +trypsin RT (T)	(e) 4°C Lysate 4°C (T)	a-d[i] a-e (E)
R4 (200)	0.7 ± 1.0	0.5 ± 0.06	0.2*	0.4 ± 0.06	-	0.3**
R4 (500)	2.0 ± 0.2	1.0 ± 0.06	1***	1.0 ± 0.1	-	1.0***
R4 (1000)	8.0 ± 0.9	5.0 ± 0.8	3*	2.0 ± 0.1	-	6.0***
R4 (2000)	30 ± 3.3	23 ± 7.6	7	4.0 ± 0.3	-	26***
R6 (200)	2.5 ± 0.2	2.0 ± 0.2	0.5*	0.6 ± 0.04	2 ± 0.01	0.5
R6 (500)	24 ± 1.3	15 ± 1.8	9***	2.0 ± 0.3	9 ± 0.3	15
R6 (1000)	59 ± 2.4	31 ± 1.9	28***	6.0 ± 0.8	34 ± 4.5	25
R6 (2000)	149 ± 5.9	69 ± 3.5	80***	18 ± 2.2	83 ± 8.3	66
R9[j] (200)	2.9 ± 1.3	3.5 ± 1.2	-	0.6 ± 0.1	-	2.3
R9 (500)	20 ± 3.5	19 ± 1.9	-	1.6 ± 0.2	-	18.4
R9 (1000)	34 ± 4.3	36 ± 1.7	-	4.0 ± 0.5	-	30
R9 (2000)	52 ± 6.2	55 ± 2.5	-	10 ± 1.9	-	42

[g] In brackets, picomoles amounts of peptide incubated with cells; 200, 500, 1000 and 2000 correspond respectively to 1, 2.5, 5 and 10 μM concentrations.

[h] Statistical significance ($p<0.05$), has been calculated by unpaired t-tests with: $^{ns}p>0.05$, *$0.01<p<0.05$, **$0.0001<P<0.01$, ***$p<0.0001$.

[i] (a-d) was used for R4 and (a-e) for R6.

[j] for R9 columns b and c give total (T+E) internalization. Endocytosis is obtained by subtracting column d from column a.

We had to take great care when we wished to compare the internalization efficacy of the three peptides, as it has been reported that within a CF-labeled peptide sequence, the pKa value of fluorescein decreases with the increasing number of arginyl residues at proximity of the fluorophore [9]. With this principle, fluorescence intensity ranking should be $R_9 > R_6 > R_4$, which is indeed the case in our measurements. This difference in fluorescence intensity can be established from the fluorescence standard curve of each peptide : Y= 0.0029X for R_4, Y=0.0037X for R_6 and Y=0.0067X for R_9, which are run in parallel of the internalization assays (Scheme S1). This is important to run standard curve in parallel of every internalization experiment, since cell preparation and the efficacy of intracellular enzymes may vary from one time to the other. We found as mean values that R_9 is respectively 2.3-fold and 1.8-fold more fluorescent than R_6 and R_4. On its side R_6 is 1.3-fold more fluorescent than R_4.

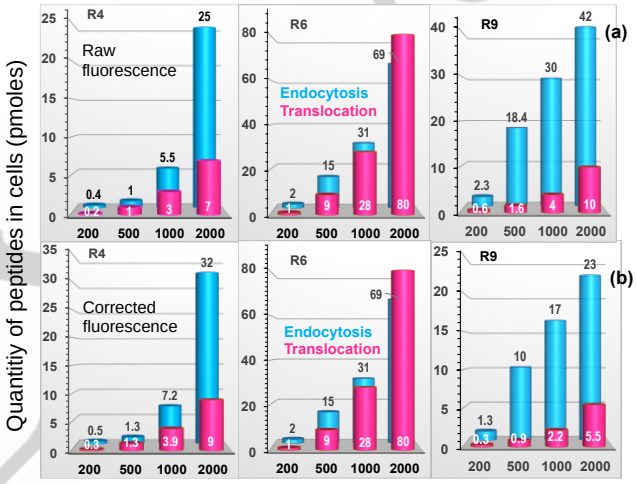


Figure 4. Quantity of peptide internalized by endocytosis (light blue) or translocation (pink). **(a)** endocytosis and translocation are determined from raw data (fluorescence arbitrary units); **(b)** endocytosis and translocation are determined from corrected fluorescence values since the three peptides have different intrinsic fluorescence (see text): R_9 is respectively 2.3-fold and 1.8-fold more fluorescent than R_6 and R_4; R_6 is 1.3-fold more fluorescent than R_4. Fluorescence has been corrected for R_4 and R_9.

Taking into account the difference of fluorescence intensity between peptides led us to revise the intensity values measured in internalization assays (Figure 4b). The revised values indicate that the efficacy ranking of total internalization is still : DABCYL-R_6-(εCF)K > CF-R_9 > DABCYL-R_4-(εCF)K. But for translocation only, the ranking became DABCYL-R_6-(εCF)K > DABCYL-R_4-(εCF)K and CF-R_9. These two latter peptides translocate in similar amounts or even better for DABCYL-R_4-(εCF)K used at 5 and 10 μM extracellular concentrations (Figure 4b).

COMPARISON OF FLUOROMETRY AND FLOW CYTOMETRY QUANTITATIVE DATA IN THE ANALYSIS OF THE PEPTIDES INTERNALIZATION ROUTES

We have previously reported that flow cytometry must be used with cautious to compare cell-penetrating peptide internalization efficacy and pathways [8]. In the present flow cytometry analysis, the fluorescence of intact DABCYL-R_n-(εCF)K peptides can be hardly detected because of FRET quenching. As shown, the fluorescence intensity of the hydrolyzed peptides is about 10-times higher than the intact one (Fig. 2d). In addition, when the peptides are cleaved in cells, the fluorescence signal can be weakened in endo-lysosomal vesicles (acidic pH values), or at high fluorescein concentrations (self-quenching). Therefore, interpretation of flow cytometry data can be complex to appreciate the real presence and intracellular concentration of labeled peptides. As shown in Figure 5, flow cytometry of intact cells incubated at 37°C with the three peptides gives however an internalization efficacy pattern similar to that of fluorometry: DABCYL-R_6-(εCF)K > CF-R_9 > DABCYL-R_4-(εCF)K. To ensure that all membrane-associated peptides are completely removed by enzymatic digestion (trypsin at 37°C or pronase at 4°C), thus that we measure only intracellular peptides, we used trypan blue to quench membrane fluorescein [10]. In Figure S3, we visualize that the same amount of internalized peptide is detected in the absence or the presence of trypan blue, showing that trypsin treatment (or pronase at 4°C), is sufficient to remove the membrane-bound peptides.

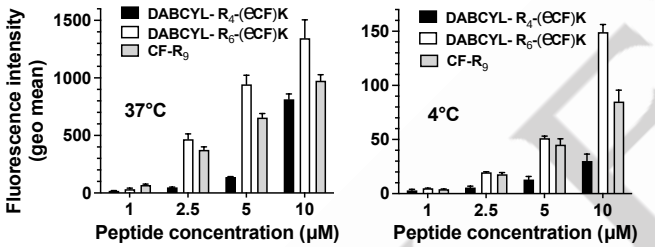


Figure 5. Flow cytometry detection of the internalization of DABCYL-R_n-(εCF)K and CF-R_9 peptides at 37°C and 4°C. The experiments have been replicated from 6 to 9 times independently.

We further compared for each peptide concentration, the relative internalization efficacy between the three peptides, measured by fluorometry or flow cytometry (Table S1). At 37°C, it can be noticed that the relative internalization efficacy between peptides is similar with the two fluorescence techniques, for concentrations 2.5 and 5 μM. For 1 μM and 10 μM concentrations, the differences between peptides in their internalization efficacy is however underestimated by flow cytometry measurements. The reason for such observation is probably the small quantities to be detected at 1 μM and concentration quenching phenomenon likely occurring at 10 μM. More interestingly at 4°C, the relative internalization efficacies between peptides evaluated by the two types of fluorescence measurements, are totally convergent, when we used values of DABCYL-R_6-(εCF)K translocation from column (e) of Table 1. This latter assessment implies that a strong fluorescence quenching process may occur for this peptide inside cells.

Conclusion

Altogether, we show herein with this FRET-based method that, depending on their size, oligoarginines internalize into cells according to different contributions of endocytosis and translocation routes. It also evidences a point of focus in the design and interpretation of data when comparing CF-labeled CPPs with each other: the pKa of CF strongly depends on its environment and may vary from one peptide sequence to the other, endowing peptides with different fluorescence intensity. This is a major point to pay attention to since, as we show herein, it can bias the relative internalization efficacy between peptides. Another major point of vigilance is to determine for every internalization experiment, a calibration curve in parallel, which corresponds to the fluorescence intensity according to concentrations of the same peptide sequence hydrolyzed by enzymes from the released enzymes in cell lysates. One must obtain a linear regression relationship, at least for quantities found in cells. Proceeding in this manner prevents biased results coming from possible uncomplete digestion within one particular experiment. In the present study we found a slight deviation between standard curves obtained from trypsin hydrolysis or cells (10^6) lysis, starting above 100 picomoles peptide.

Whatever the concentration, DABCYL-R_6-(εCF)K is the more efficient peptide and uses equally translocation and endocytosis to enter cells. On its side, DABCYL-R_4-(εCF)K, is internalized by translocation similarly or even better than CF-R_9. Compared to the DABCYL-labeled peptides, CF-R_9 uses endocytosis the more, whatever its extracellular concentration, thus confirming that the hydrophobic moiety DABCYL advantageously replaces arginyl residues to favor translocation [7]. Finally, the quantity of internalized peptide shows that translocation is far from being simple diffusion since the peptides can be concentrated inside cells: at 2.5 μM extracellular concentrations, after fluorescence correction we measured intracellularly, 1.3 μM DABCYL-R_4-(εCF)K, 0.9 μM CF-R_9 and 9 μM DABCYL-R_6-(εCF)K.

Altogether, we designed a new quantification protocol that relies on FRET quenching and release upon exposure of peptides to enzyme degradation. This method is easy since it does not require any cell engineering and can be adapted very quickly to various, if not all, cell types. In addition, the DABCYL- and CF-modified peptides can be easily obtained by solid-phase peptide synthesis. We developed herein this method with oligoarginine peptides that are easily cleaved by trypsin. As shown in Figure S2, cleavage is complete for this oligoarginine sequences. It can be used for different peptide sequences since endosomes contain more than 60 different enzymes with a wide spectrum of substrate activity. Amongst major hydrolases found in lysosomes, there are indeed papain-like or pepsin-related proteases [11]. The two types of enzymes can digest broadly, with preferential cleavage sites at Lys, Arg, Leu and Gly for papain and Tyr, Phe, and Trp for pepsin. In addition, pronase instead of trypsin can be used to check for peptide hydrolysis and run standard curves in parallel.

We examplified in Table S3 that the method can be applied to another sequence, DABCYL-R_2WR_2-(εCF)K peptide [12]. This peptide can also be fully degraded by enzymes as shown in

Figure S4. In that case, we also observed that the quantity of direct translocation from the extracellular medium to the cytosol of cells measured at 37°C, is similar to the quantity of translocated peptide measured at 4°C, except for the 10μM extracellular concentration. At 10 μM, the quantity of translocated peptide is indeed 8-times higher than at 4°C. We could assume that at 10 μM concentration and within 1 hr incubation, DABCYL-R_2WR_2-(εCF)K can escape from endosomes to reach the cytosol or that the temperature affects its translocation at this concentration. More work has to be done to decipher which hypothesis is the correct one.

The interesting point of the present study is definitely the possibility to compare translocation at 37°C and 4°C and establish whether lowering temperature, which affects membrane fluidity and dynamics, impacts also the translocation process of a given peptide sequence. For the present oligoarginine peptides, we did not observe any impact of the temperature drop on their translocation efficacy. Two possible explanations to rationalize this observation: either the temperature impact is too marginal to be significantly detected or it does not exist for these peptides. It would be very interesting to study other various peptide sequences and see whether this is also the case since, if it is a general observation for all peptides, our method could also be useful to quantify the endosomal escape of a given peptide, either at incubation time longer than 1 hr or different concentrations as for DABCYL-R_2WR_2-(εCF)K. As described in Table S2, the present protocol could indeed permit to determine the relative contribution of endocytosis, translocation and endosomal escape in the measured quantity of peptide inside cells.

Finally, this dark FRET quenching method offers the possibility to measure translocation at 37°C, by simply adding a DABCYL and CF in the peptide sequence, does not require cell engineering and gives the possibility to study endosomal escape that likely occurs according to the concentration of peptide and/or kinetics of the internalization.

Materials and Methods

PEPTIDE SYNTHESIS

The peptides were synthesized manually by solid-phase on Rink-amide MBHA resin (0.250 g, 0.69 mmol/g) using Fmoc/tBu strategy [7]. After the last arginine, the Fmoc protecting group was removed and the free *N*-terminal α-amino group reacted with DABCYL–OH using DIC-HOBt reagents (2 eq. from each). Peptides were cleaved from the resin with 5 mL TFA-containing 0.375 g phenol, 0.25 mL distilled water, 0.25 mL thioanisole and 0.125 mL 1,2–ethanedithiol as scavengers. Crude products were precipitated by dry diethylether, dissolved in 10% acetic acid and freeze-dried. Peptides were then purified by RP–HPLC on a semipreparative Phenomenex Jupiter C18 column (250×10 mm I.D.) with 10 μm silica (300 Å pore size). Flow rate was 4 mL/min. Linear gradient elution was applied. The coupling of carboxyfluorescein (CF) was done in DMF using DIC-HOBt coupling reagents in 1.1 eq. to the peptide. The conjugate was isolated from the reaction mixture by RP–HPLC. The purified compounds were characterized by analytical RP–HPLC and ESI–MS as DABCYL-R_4-(εCF)K and DABCYL-R_6-(εCF)K [7].

CELL CULTURE

Wild type hamster ovary CHO-K1 cells (CCL-61, ATCC, LGC Standards Sarl, France), were cultured in DMEM-F12 growth medium supplemented with 10% fetal calf serum (FCS), penicillin (100,000 IU/L), streptomycin (100,000 IU/L), and amphotericin B (1mg/L) in a humidified atmosphere containing 5 % CO_2 at 37°C.

CYTOTOXICITY ASSAYS

Cytotoxicity was determined with the Cell Counting Kit 8 (CCK-8) colorimetric assay from Dojindo Molecular Technologies. 96-well plates were inoculated with $2x10^4$ CHO cells/100 μL/well. After 24 hrs incubation (37°C, 5% CO_2), peptides were added to obtain 1, 2.5, 5, 10 and 20 μM in DMEM-F12 and the plate further incubated for 1 hr at 37°C. After washing, cells were further incubated with 100 μL 10% CCK-8 in DMEM for 3 hrs before reading the at 450 nm (Polarstar Optima). Controls were cells in DMEM (negative control, 100% viability) and cells in 0.1% of triton X-100 (positive control, 0% viability).

FRET QUENCHING AND RELEASE

After incubation of 10^6 cells with the peptide in culture medium for 1 hr, either at 37°C or 4°C, cells were washed and trypsin (0.05%, or pronase at 4°C), used to eliminate membrane-bound peptides and detach cells. Cells were mixed with 200 μl lysis buffer (50 mM Tris pH 7.4, 1 M NaCl, 1% NP40), and immediately subjected to sonification for 15 min at RT (intracellular peptide hydrolysis by the released enzymes), in order to get the total intracellular peptide concentration, or to boiling for 15 min (intracellular enzyme inactivation), in order to get the peptide internalized by endocytosis only. Samples were centrifugated at 16,000 g for 10 min to remove cell debris and collect the supernatant and fluorometry of the supernatant was recorded at λ_{ex} = 495 nm and λ_{em} = 525 nm. In parallel for standard curves, digestion by trypsin was used to measure the total fluorescence of the DABCYL-R_n-(εCF)K peptides. We used different amounts of DABCYL-R_4-(εCF)K or DABCYL-R_6-(εCF)K in 137μL lysis buffer (50 mM Tris pH 7.4, 0.15 M NaCl, 1% NP40) in the presence or absence of trypsin (0.008%). The samples were analyzed without cells or in the presence of 10^6 cells (1 hr at 37°C). For the sample with trypsin, NaCl was then added to the sample after digestion to obtain 1M final concentration. The samples were then sonicated 15 min and centrifuged at 16,000 g for 10 min. Fluorescence was measured in the supernatant using a MOS 200M fluorimeter (BioLogic SA, France). The fluorescence signal of peptides only

was obtained by subtraction of the fluorescence intensity of cell lysates (cell autofluorescence) from the fluorescence intensity of the sample.

FLUOROMETRY ASSAYS

We used the quantification method described previously [8]. Briefly, one million CHO-K1 cells were incubated for 1 hr at 37°C (or 4°C), with the peptide DABCYL-R_4-(εCF)K or DABCYL-R_6-(εCF)K or CF-R_9 at 1, 2.5, 5 and 10 µM in 500 µL DMEM-F12. After washings with HBSS, 500 µL 0.05% trypsin/EDTA (37°C), or 0.05% pronase in 100 mM Tris pH 7.4 (4°C) was added for 5 min to degrade the remaining extracellular and membrane-bound peptide and to detach cells. After addition of 100 µL enzyme inhibitors (complete mini at 4°C (Roche) or trypsin inhibitor (soybean inhibitor 5 mg/mL) at 37°C mixed with 100 µL bovine serum albumin (1 mg/mL), cells were transferred into a microtube, centrifuged, washed with 1 mL 50 mM Tris-buffer pH 7.4, 0.1% BSA, and lysed in 200 µL 50 mM Tris pH 7.4, 1 M NaCl, 1% NP40. The samples were then sonicated for 15 min at room temperature (to homogenize samples), or boiled for 15 min (to inhibit proteases). Samples were then centrifugated for 10 min at 16.000 g. Fluorescence intensity in the supernatants was monitored with a MOS 200M fluorimeter (Biologic SAS) and the maximal intensity was detected around λ = 525 nm. The maximal intensity was retained for the standard curve and for quantification of samples. The amounts of internalized peptides were calculated by comparing the fluorescence intensity of the sample with the standard curve. For the standard curve, we prepared a range of peptide amounts (from 2 to 500 pmoles) in the lysis buffer (50 mM Tris pH 7.4, 1 M NaCl, 1% Nonidet P40) in the absence or the presence of one million suspended cells. The samples were sonicated 15 min and centrifuged at 16.000 g for 10 min. Fluorescence intensity in the supernatants was monitored with a MOS 200M fluorimeter (Biologic SAS, France) and the maximal intensity was detected at λ_{em} = 525 nm (λ_{ex} = 495 nm). The fluorescence signal of peptides only was obtained by subtraction of the fluorescence intensity of cell lysates (autofluorescence) from the fluorescence intensity of the sample**.**

FLOW CYTOMETRY ASSAYS

One million cells were incubated one hour at 37°C (or 4°C) with the peptides at 1 µM, 2.5 µM, 5 µM or 10 µM in 500 µL DMEM-F12. After washing cells with HBSS, 500 µL trypsin/EDTA 0.05% (37°C experiments) or pronase 0.05% (4°C experiments) in 100 mM Tris-buffer was added for 5 min to degrade the remaining extracellular and membrane-bound peptide and to detach cells. After addition of 100µL of 5 mg/mL soybean trypsin inhibitor (37°C), or Roche Complete Mini (4°C), and mixed with 100 µL bovine serum albumin (1 mg/mL). Cells were transferred into a microtube, centrifuged, washed with 1 mL of 50 mM Tris-buffer pH 7.4, 0.1% BSA. The cell pellet was suspended in 400 µL of PBS. The fluorescence of individual cells was analyzed with a FACSCalibur flow cytometer. 20,000 cells were measured for each experimental condition. The mean fluorescence of a sample was obtained by subtracting the autofluorescence of cells from the measured mean fluorescence of cells in the presence of the fluorescent peptide.

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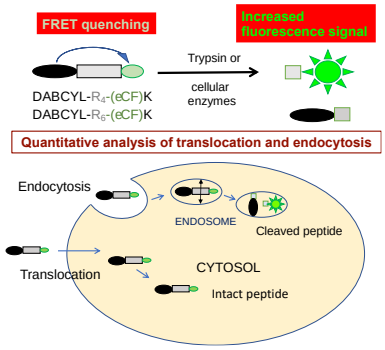

This study reports a Förster resonance energy transfer (FRET) fluorescence protocol to determine the quantity of a cell-penetrating peptide internalized at 37°C either by direct translocation across the cell membrane or endocytosis. The protocol can be used with any cell type and only requires (DABCYL) 4-(dimethylaminoazo)benzene-4-carboxylic acid- and (CF) carboxyfluorescein-labeled peptides. Its application to tetra- and hexaarginines highlights their difference of internalization.

Institute and/or researcher X usernames: Laboratoire des Biomolécules (@LBM_lab) ; Société Chimique de France Division de Chémobiologie (@SCF_ChemBio)

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