Drug Delivery

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Detecting Cytosolic Peptide Delivery with the GFP Complementation Assay in the Low Micromolar Range

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Abstract: *Transfection of cells with a plasmid encoding for the first ten strands of the GFP protein (GFP1-10) provides the means to detect cytosolic peptide import at low micromolar concentrations. Cytosolic import of the eleventh strand of the GFP protein either by electroporation or by cell-penetrating peptide-mediated import leads to formation of the full-length GFP protein and fluorescence. An increase in sensitivity is achieved through structural modifications of the peptide and the expression of GFP1-10 as a fusion protein with mCherry.*

There is great interest in exploiting peptides and peptide-derived molecules as drugs to address the intracellular target space. Cell-penetrating peptides (CPPs) and related molecules are receiving considerable attention as delivery vectors.[1] However, efficient cytoplasmic delivery is still a major hurdle as uptake occurs mostly through endocytosis, and no generic concept has been developed to achieve either direct penetration of the plasma membrane or endosomal release.[2] A key prerequisite in the further development of delivery vectors is the availability of a robust technique to assess cytosolic delivery of the intact molecule. For molecules small enough to pass the nuclear pore complex, which is of a molecular weight of below around 40 kDa, this is also equivalent to nuclear delivery. For the delivery of oligonucleotides, it has been shown that import efficiency as measured by cell-associated fluorescence of various labeled CPP-oligonucleotide complexes does not correlate well with biological activity.[3] Therefore, cytosolic delivery of intact oligonucleotides is determined by functional assays, such as the splice-correction assay.[4] For delivery strategies geared towards enzymes and large molecular weight proteins, import efficiency can be measured by detecting enzymatic activity.[5] Luciferin has been employed as a model compound to detect the delivery of small molecules, and dexamethasone-induced gene expression as a general means to detect endosomal release. Both methods, though, do not provide information about the integrity of the carrier to which the drug is attached.[6]

By contrast, the quantitation of CPP-mediated peptide delivery has mainly relied on the detection of peptide-coupled fluorescence. However, as shown by our own fluorescence correlation spectroscopy (FCS) and Förster energy resonance transfer-based analyses, most cytosolic fluorescence originates from fluorophores coupled to degraded peptide fragments.[7] Therefore, the implementation of functional assays that quantitate the cytosolic delivery of intact peptides is urgently needed. Mass spectrometry-based analyses address some of these problems.[8] But also with this approach, it remains a challenge to distinguish cytosolic delivery from sequestration into endosomes. Furthermore, it is difficult to foresee how this approach could be implemented in preclinical in vivo studies.

Ideally, a functional assay should enable a robust read-out based on signal intensity and should directly probe for the molecular interaction of a functional peptide, as this corresponds to a potential application of peptides as therapeutics. Examples of functional assays include induction or enhancement of apoptosis. However, these are difficult to relate to a certain concentration of active peptide interacting with a specific target protein.[9]

Herein, we demonstrate that complementation of a GFP fragment consisting of the first 10 of the 11 strands of the GFP β-barrel (GFP1-10) with a second fragment, the 16 amino acid long 11th strand can be used to detect cytosolic peptide delivery. The spontaneous association of both fragments is required for the generation of GFP fluorescence.[10] GFP1-10 complementation is detectable at submicromolar concentrations of GFP11 when GFP1-10 is present at low micromolar concentrations.[10,11] This strand complementation assay is one of the so-called fragment complementation assays, and to our knowledge the only one in which one part comprises a fragment of the size of a peptide.[12] We demonstrate that the sensitivity and robustness of the assay can be further increased by generating a fusion protein of GFP1-10 with the red fluorescent mCherry protein, and through structural

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modifications of the GFP11 peptide. Moreover, we identify incubation time as a critical parameter. Overall, we are able to detect complementation at low micromolar peptide concentrations.

To test whether elongation of the GFP11 peptide with a CPP interfered with complementation, HEK293T cells were transfected with GFP1-10 while GFP11 peptides were introduced directly into the cytoplasm by electroporation. Next to GFP11, we tested GFP11 elongated with R9 and an R9-GFP11 analogue elongated with the C-terminal PEG-moiety Ado (8-amino-3,6-dioxaoctanoic acid) and in which the methionine was replaced by a sterically very similar norleucine (Nle) (Supporting Information, Figure S5 A, B). The C-terminal Ado elongation is expected to increase proteolytic stability and solubility of the peptide, while replacement of the methionine residue should circumvent problems associated with the oxidation of methionine.

Following electroporation, cells could recover for 20 min and 2 h. The 2 h recovery was selected in accordance with the previously reported kinetics for GFP complementation.[13] The 20 min recovery time was selected because inside cells proteolysis can lead to a rapid depletion of complementation-active molecules.

For the 2 h recovery, there was a dose-dependent increase in GFP fluorescence for all three peptides (Figure 1 A), which was the result of a uniform shift of the whole cell population (Supporting Information, Figure S1 A). Our previous work had shown that using electroporation, the concentration of peptide inside the cell is always lower than the concentration outside.[14] Therefore, the concentrations in the electroporation solution are an upper limit for the concentration expected in the cytosol.

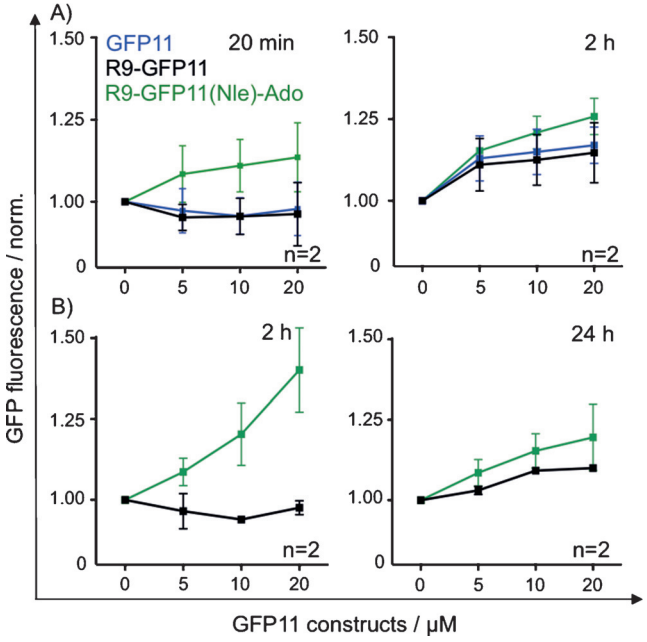

Figure 1. Dose-dependence of GFP complementation. GFP11 peptides were introduced into HEK293T cells transfected with standard GFP1-10 either by A) electroporation or by B) penetration. Fluorescence of GFP was detected by flow cytometry from morphologically intact cells gated based on forward versus sideward scatter at the indicated time points. The GFP fluorescence of the GFP11 variants was normalized to the background signal acquired from mock-treated cells. Curves indicate the average of the normalized medians of two independent experiments. Error bars = standard error of the mean (SEM).

There was no difference in complementation efficiency for GFP11 and the R9-elongated peptide (Figure 1 A), demonstrating that elongation with the CPP was compatible with complementation. The (Nle)-Ado modified peptide was slightly more efficient than the non-modified peptide.

In contrast, for the short 20 min recovery time, only for the modified peptide, an increase in fluorescence could be observed while there was no activity for the unmodified peptides (Figure 1 A; Supporting Information, Figure S2 A). This difference in activity was not a result of a difference in import efficiency as carboxyfluorescein-labeled analogues of all the peptides showed only slight differences in the dose-dependent electroporation efficiencies (Supporting Information, Figure S3).

After electroporation, we assessed complementation for detecting the CPP-mediated import of peptides. Again, the modified peptide (R9-GFP11(Nle)-Ado) was more active than the unmodified one (R9-GFP11), and this difference was more pronounced for the 2 h than for the 24 h penetration. Importantly, a reproducible increase in fluorescence at 5 μM could be observed, with a stronger intensity after the 2 h compared to the 24 h incubation (Figure 1; Supporting Information, Figures S1 B and S2 B). As for introduction by electroporation, the enhanced signal increase by the modified peptide could not be explained by differences in uptake efficiency or subcellular distribution. Carboxyfluorescein-labeled analogues of R9-GFP11 and R9-GFP11(Nle)-Ado showed equal levels of CPP-mediated uptake over the tested concentration range of 2.5 to 20 μM (Supporting Information, Figure S4). For both peptides, in addition to some punctate staining, fluorescence was distributed homogeneously throughout the cytoplasm and nucleus over the whole concentration range, indicating that the peptides were available for complementation.

Even though we found a high number of fluorescent cells, the implementation of the assay as such still provided insufficient information on the efficiency of delivery. In particular, in transient expression procedures, expression levels between cells are variable and many cells may not express the transfected protein at all. Low fluorescence can therefore be a consequence of poor peptide delivery or of poor expression of GFP1-10. Therefore, to obtain reliable information on GFP1-10 expression, we created a fusion protein with mCherry at the N-terminus of GFP1-10 (Supporting Information, Figure S5). We reasoned that in this configuration, the mCherry would also stabilize the GFP1-10.

Consistent with a stabilizing role of mCherry, electroporation of peptides into cells expressing the fusion protein yielded stronger increases in fluorescence than for GFP1-10, and this was particularly the case for the 20 min recovery time with a maximum increase of 1.75 versus 1.15 seen for the (Nle)-Ado modified peptide (Figures 1 A and 2 A). Gating on mCherry-positive cells (Supporting Information, Figure S6) increased the average fluorescence, resulting in a detection of complementation at 5 μM for all peptides (Figure 2).

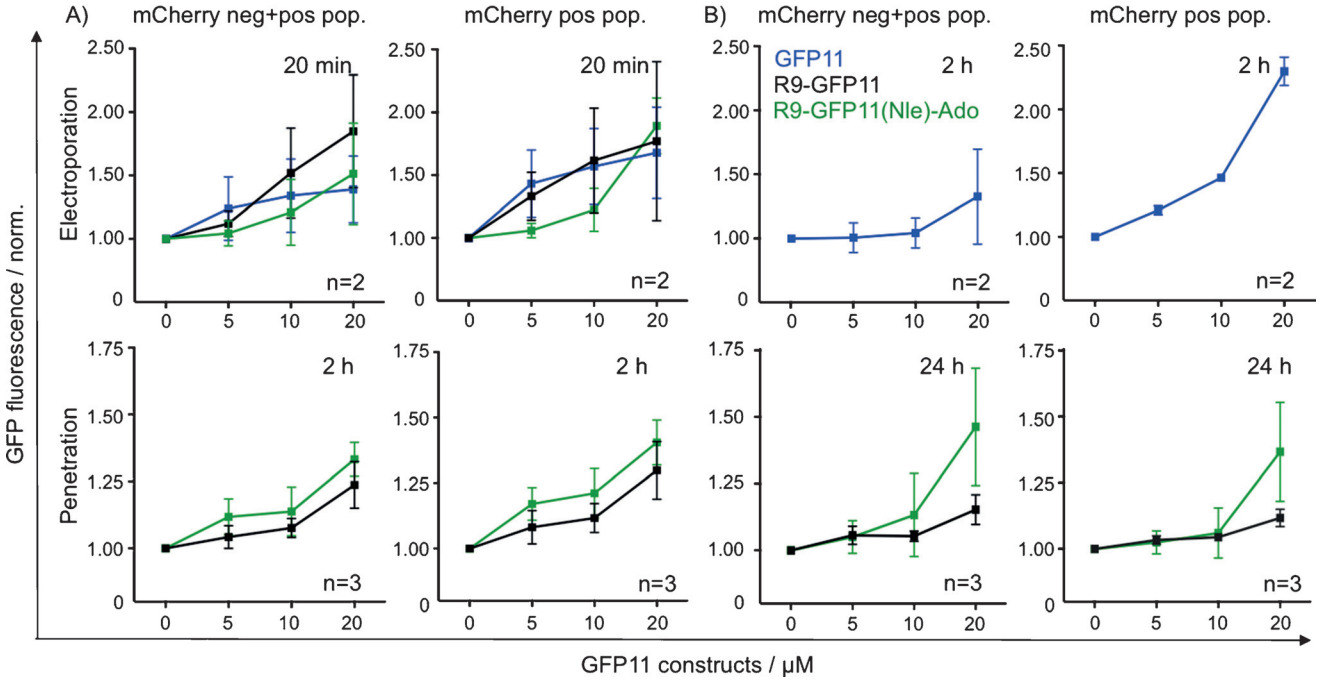

Figure 2. Dose-dependence of GFP complementation for the mCherry-GFP1-10 fusion protein after short (A) or long term (B) incubation. GFP11 peptides were introduced into HEK293T cells transfected with mCherry-GFP1-10. Fluorescence for both mCherry and GFP was detected by flow cytometry from morphologically intact cells gated based on forward versus sideward scatter after A) 20 min or B) 2 h (electroporation, top) or A) 2 h or B) 24 h (penetration, bottom). Incubation for 24 h was performed in FCS-containing medium. The GFP fluorescence of the GFP11 variants either for all cells (left) or for mCherry-positive cells was normalized to the background signal acquired from mock-treated cells. Curves indicate the average of the normalized medians of at least two independent experiments. Error bars = standard error of the mean (SEM).

At this point we were also interested in defining in more detail the dose-response function of GFP complementation. For this purpose, pairs of samples were electroporated either with the fluorescein-labeled peptide and cell-associated fluorescence determined by flow cytometry, or with the non-fluorescent counterpart to determine GFP formation. For the fluorescein-labeled peptide, there was a nearly linear increase in cellular fluorescence with peptide concentration. In contrast, for GFP complementation, from 10 to 20 μM of peptide the increase in fluorescence was weaker than expected, indicating saturation effects of the complementation reaction (Supporting Information, Figure S7).

For CPP-mediated import, again, the increase in fluorescence was stronger for the (Nle)-Ado analogue than for the unmodified peptide (Figure 2; Supporting Information, Figure S6).

A further analysis of the subpopulations of mCherry-positive and -negative cells also revealed that the population of cells with little mCherry expression showed a dose-dependent increase in GFP signal (Supporting Information, Figures S6 and S8). The molecular basis for this observation is not clear. Since differences in reporter protein expression may reflect a stronger propensity of cells to take up material, we validated that uptake of R9-GFP11 was independent of mCherry-GFP1-10 expression (Supporting Information, Figure S9). Furthermore, treatment of mCherry-GFP1-10 expressing cells with R9 alone, or of mock-transfected cells with R9-GFP11, did not show any signal (Supporting Information, Figure S10), validating that the observed increase in GFP fluorescence was a consequence of complementation (Figure 2).

For both GFP1-10 constructs, complementation was more efficient after 2 h incubation than after 24 h incubation (Figures 1B and 2; Supporting Information, Figures S6B and S8B). In addition to the degradation of peptide and protein turnover, another factor contributing to this difference might be the presence of serum in the incubation medium, which reduces the bioavailability of peptide.[15] The 2 h incubations were conducted in the absence of serum, while the 24 h incubation was performed in the presence of serum to maintain cell viability.

After having identified a 2 h incubation period as the optimal condition for detecting complementation, we were interested to learn whether complementation could also be detected at lower concentrations of peptide. In fact, GFP fluorescence could already be detected at 0.6 μM, which was slightly more pronounced for the (Nle)-Ado modified peptide than for the unmodified R9-GFP11. However, between 0.6 and 2.5 μM there was no clear dose-dependent increase of fluorescence. Incubation of cells with the R9-GFP11 peptides at very high concentrations (40 μM) resulted in a further increase of GFP fluorescence, demonstrating that the assay can also be used at even higher concentrations, even though at these concentrations CPP-mediated toxicity may become a concern (Supporting Information, Figure S11).

To demonstrate that cytosolic delivery is the decisive factor for detection of complementation, we manipulated the composition of the plasma membrane by treatment of HeLa cells with bacterial sphingomyelinase. Bacterial sphingomyelinase converts sphingomyelin into ceramide, which in turn leads to the rapid cytosolic delivery of arginine-rich CPPs and

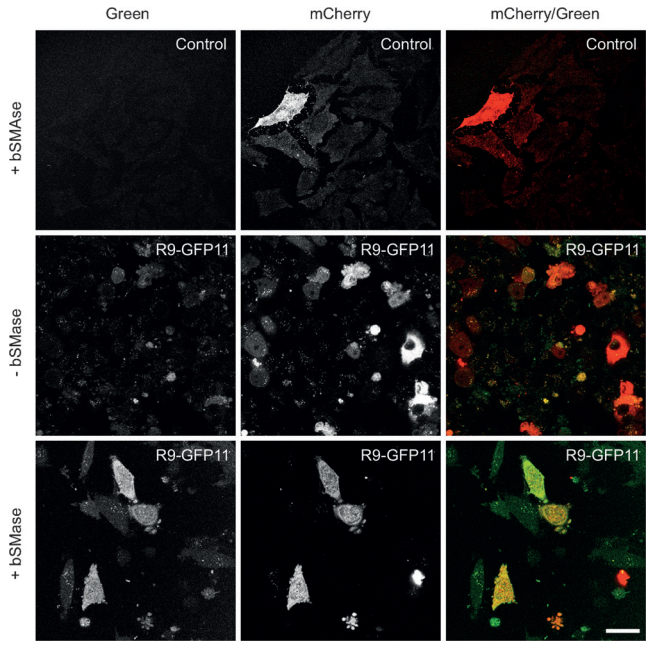

Figure 3. Change in membrane structure through induction of ceramide formation induces an increase in GFP complementation. HeLa cells were transfected with mCherry-GFP1-10 and incubated with 5 μM R9-GFP11 in the presence or absence of bSMase (1200 mU mL^{-1}) for 20 min at 37 °C followed by confocal microscopy. As a background control, transfected cells were incubated only in the presence of bSMAse (1.200 mU mL^{-1}). Green fluorescence indicates the presence of complemented GFP. Scale bar = 30 μm.

conjugates.[16] We selected HeLa cells because they showed a stronger endosomal confinement of fluorescence than HEK293T cells. For the unmodified peptide, complementation efficiency was clearly increased for the sphingomyelinase-treated cells (Figure 3).

To conclude, we presented a complementation assay to detect CPP-mediated delivery of intact peptides into the cytoplasm, based on the formation of a fluorescent GFP by integration of a peptide corresponding to the eleventh β-strand. Improvements in sensitivity were achieved by modifying the original GFP11 sequence and by expressing a two-domain construct in which GFP1-10 is linked to the C-terminus of mCherry. As a first application, we demonstrate that sphingomyelinase treatment of cells increases the permeability of the plasma membrane for direct cytoplasmic delivery of the peptide. We foresee future applications in testing strategies to enhance endosomal release, or characterization of carriers with respect to their capacity to mediate direct crossing of the plasma membrane. With the generation of stable cell lines, the assay should be readily compatible with high-throughput formats. Even though the assay does not report on the integrity of the carrier, research in delivery typically aims at the integrity of the cargo, and this is captured by the GFP complementation. The extent of complementation obtained by electroporation was not qualitatively different from the one obtained by penetration and also the sensitivity was comparable. A concentration of 5 μM in the electroporation cuvette yields a low micromolar peptide concentration inside the cytosol.[14] This result demonstrates that CPP-mediated import for HEK cells yielded similar cytoplasmic concentrations. The sensitivity of this assay is rather controlled by the affinity of the GFP1-10 fragment for the peptide than by lack of cytosolic delivery.

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