Communication

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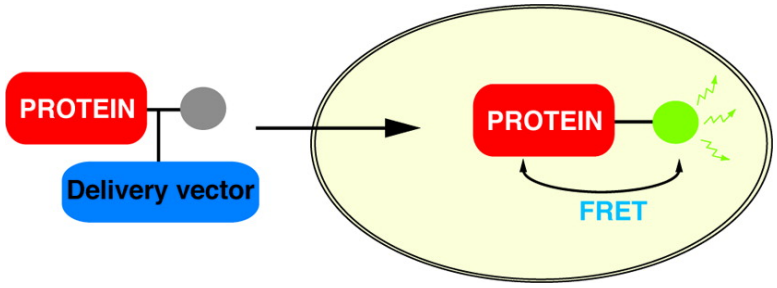

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Real-Time Fluorescence Detection of Protein Transduction into Live Cells

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The ability to deliver proteins across biological barriers not only dramatically expands the therapeutic potential of protein-based drugs but also provides formidable experimental opportunities in basic research. Over the past decade, a collection of delivery vectors (DV) that mediate the translocation of proteins across the plasma membrane of cells has been identified.[1] However, current methods for studying protein translocation into cells are not sufficiently quantitative to evaluate the efficiency of protein transduction.[2] This results in part from two challenges associated with measuring the translocation of a protein into live cells: (1) a protein that appears to have reached the inside of a cell might simply be trapped inside endocytic vesicles from which it cannot escape, and (2) a protein might be degraded during translocation and degradation products might be detected instead of the intact protein. To address these issues, we have developed a chemical DV−protein cargo system in which a combination of three fluorescence signals can be used to distinguish three protein populations after internalization of the cargo inside living cells.

We used a red fluorescent protein labeled with a green fluorophore as a model protein cargo from which a fluorescence resonance energy transfer (FRET) signal can be obtained (Figure 1).[3] A DV of interest is conjugated to a fluorescence quencher and linked to the protein cargo through a disulfide bond. The DV−quencher conjugate both quenches the green fluorophore and promotes transduction of the protein. On the basis of evidence suggesting that disulfide bonds are stable in early endosomes, we reasoned that green fluorescence would not be activated in these organelles.[4] In contrast, protein delivery into the cytosol would result in specific disulfide bond cleavage by cytosolic glutathione (GSH), fluorophore unquenching, and activation of both green fluorescence and FRET.[5] The endocytic and cytosolic fractions of the protein cargo should therefore be easily distinguishable. Furthermore, although denaturation or proteolysis of the protein cargo might yield an activated green fluorophore, protein degradation would result in loss of FRET. Therefore, the FRET signal validates the cytosolic delivery of the intact protein rather than degraded fragments.[6]

The protein transduction probe was synthesized with the red fluorescent protein mCherry.[7] Expressed protein ligation (EPL) was used to introduce the peptide Cys-Lys(Fl) (**1**) (Fl is 6-carboxyfluorescein) to the C-terminus of a recombinant mCherry construct (**2**).[8] The dually fluorescent conjugate mCherry-Cys-Lys(FL) (**3**) produced upon ligation contains a single cysteine that was used for site-specific labeling. We used dabcyl (Dab) as a quenching molecule for Fl and the HIV1-TAT peptide as a model DV.[12] The quencher−DV conjugate was synthesized by solid-phase peptide synthesis and activated for disulfide bond formation by addition of (2-pyridyldithio)propionic acid (PDP) (peptide **4**, PDP-Lys(Dab)-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly, TAT sequence underlined).[9,10] Protein **3** was labeled with **4** to yield the final product mCherry-Cys(SS-Lys(Dab)-TAT)-Lys(FL) (**5**) (Figure 1B).

The fluorescence emission properties of **3** and **5** were first tested in vitro. When excited at 488 nm, **3** showed FRET from the green donor to the red acceptor (denaturation of the protein in urea led to the loss of the 610 nm emission and an increase of the 525 emission indicating a FRET efficiency of 35%).[11] In contrast, efficient intramolecular quenching of Fl and suppression of FRET could be observed in **5** (Figure 1C). Treatment with GSH (5 mM, approximate cytosolic concentration) resulted in the rapid release of the quencher−DV and was accompanied by 35- and 20-fold increases in emission at 525 and 610 nm, respectively. Interestingly, when excited at 586 nm, the fluorescence emission of mCherry at 610 nm was essentially unaffected by the presence of dabcyl, suggesting specific quenching of Fl and not of mCherry. Thus, the FRET signal was observed only in reducing (i.e., cytoplasmic-like) conditions and only when the protein was properly folded.

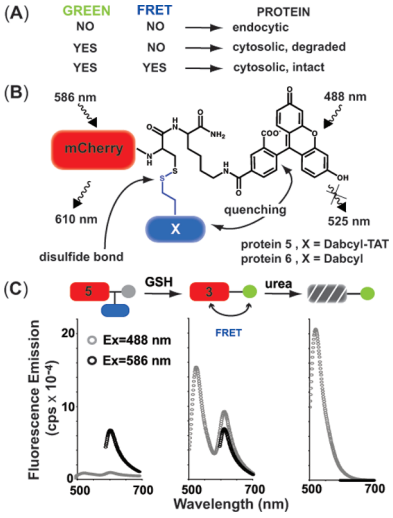

Figure 1. (A) Rationale for the fluorescence detection of multiple protein populations inside living cells. Green and FRET represent the fluorescence signals emitted by the protein. (B) Schematic representation of a DV−protein cargo probe. The properties of the DV of interest X are measured by monitoring the transduction of the mCherry−fluorescein cargo. (C) Fluorescence spectra of **5** (1 μM) before and 1 min after reduction with GSH (5 mM). Upon disulfide bond cleavage, both green (Ex/Em = 488/525 nm) and FRET (Ex/Em = 488/610 nm) signals are activated, while the red signal (Ex/Em = 586/610 nm) remains constant. Upon denaturation of the protein with urea, only the green signal is detected.

We next examined the in vivo fluorescence properties of **5**. We microinjected **5** into the cytosol of live HeLa cells. By monitoring donor and FRET activation, we observed that approximately 60% of the donor was unquenched within 20 s after microinjection and disulfide bond cleavage was completed within 2 min (Supporting Information Figure S6). Interestingly, the fluorescence intensity ratios (donor/FRET = 2.0 ± 0.3 and acceptor/FRET = 1.6 ± 0.2) and cellular distribution of reduced **5** were similar to those of microinjected **3** (Supporting Information Figures S2 and S3). The intracellular fluorescence intensities measured were constant over a period of 3 h following reduction. Thus, the protein was not degraded in the cytosol, and the observed fluorescence ratios were specific to a homogeneous population of reduced and intact **3**.

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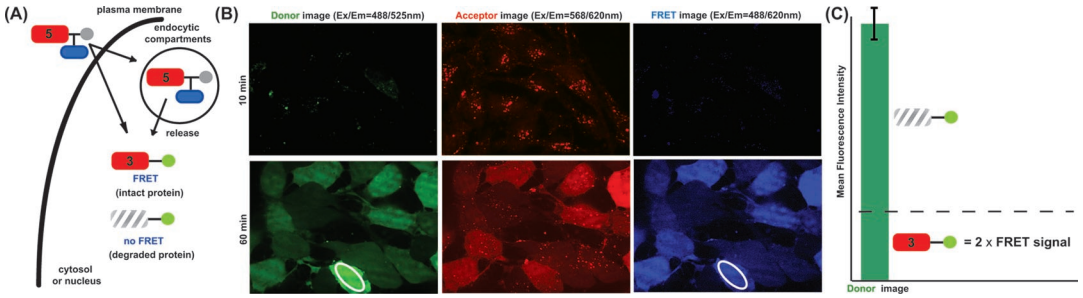

Figure 2. Monitoring of three protein populations by using three fluorescence signals. (A) Scheme illustrating the different populations of protein that can be expected in cells incubated with **5**. The degraded protein represents any denatured or proteolyzed protein fragment in which the green fluorophore does not undergo FRET. (B) Representative fluorescence images from cells incubated with **5** for 10 min and imaged at 10 and 60 min. (C) Example of mean fluorescence intensity measured from cells incubated with **5** (circled cell in B). The contribution of **3** in the donor image is estimated from the FRET image (the FRET signal is unique to **3**, and the donor/FRET intensity ratio was determined as 2 by direct microinjection of **3**). The fraction of degraded protein can be evaluated by subtracting the contribution of **3** from the total donor signal. Because no degradation is observed when the protein is directly microinjected in the cytosol, these results suggest that the transduced protein is degraded before it is released into the cytosol.

We next tested whether **5** is reduced in endocytic compartments during the first hour of incubation. This is important because the internalization of TAT−protein conjugates has been reported to involve endocytosis.[12] We first labeled the cysteine of **3** with a thiol−dabcyl conjugate to generate **6**, a protein with similar fluorescence properties as **5** but lacking the TAT peptide (Figure 1B). When incubated with live cells, **6** was internalized but remained trapped inside endocytic vesicles (Supporting Information Figure S7). Moreover, internalized **6** showed only acceptor fluorescence, indicating that no green and FRET signals could be detected 1 h after incubation.[13] Thus, there is no FRET signal from the protein trapped in endocytic vesicles under these conditions.[14]

We next examined the transduction of **5** using live-cell confocal microscopy to achieve high spatial and temporal resolution. After incubation of HeLa cells with **5** for 10 min and subsequent washing, endocytic vesicles emitting only acceptor fluorescence could be detected (Figure 2B, 10 min). At later time points, however (Figure 2B, 60 min), the cells showed intense donor and FRET signals and a homogeneous distribution of internalized material (characteristics similar to that of cells microinjected with **3**). The acceptor image differed, however, by displaying the additional punctate distribution seen in cells incubated with **6**. Analysis of the fluorescence intensities in this image showed that the punctate distribution represented as much as 90% of the acceptor signal. Overall, these results suggest that a large fraction of the protein remained trapped in endocytic compartments, while a smaller fraction was released in the cytosol.

To determine if the delivered protein was intact or degraded during the translocation process, we examined the donor fluorescence and FRET signal of over 100 cells incubated with **5** for 1 h. Because FRET is conditional to the red fluorescent protein being folded and linked to the green fluorophore, detection of this signal validates the delivery of **3** (denoted intact protein).[15] In contrast, green fluorescence can be expected not only from the intact protein but also from any degradation products. The donor image therefore results from the contribution of the donor that undergoes FRET with the acceptor (intact protein) and of the donor that does not (degradation products). Because it is unique to the intact protein, the FRET signal can, however, be used to determine the contribution of the intact protein in the donor image by using the donor/FRET ratio established from microinjected **3**. The contribution of the degraded protein population can then be evaluated by subtracting the signal of **3** in the donor image from the total green signal. By applying this procedure (Figure 2C), up to 73% of the donor signal was attributed to degradation products while only 27% was attributed to the intact cytosolic protein.[16]

In conclusion, we have developed an approach that allows the monitoring of protein transduction with high spatial and temporal resolution. Our experiments reveal that the efficiency of TAT-mediated delivery of protein **3** in the cytosol is low because large fractions of the protein were either trapped in endocytic compartments or degraded before being released into the cytosol. To our knowledge, this represents the first time that the extent of protein degradation during transduction has been evaluated. Because our probe design is modular, it should be applicable to the rapid characterization of delivery vectors with different properties and complementary to reported transduction assays.[17]

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Supporting Information Available: Full experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Control experiments with **3** demonstrated that the donor signal could be detected in the acidic lumen of endocytic compartments after 1 h.
(14) These results suggest that no reduction could be detected at this time point but do not indicate that endocytic compartments are non-reducing environments. See: Yang, J.; Chen, H.; Vlahov, I. R.; Cheng, J. X.; Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13872−13877.
(15) The FRET signal cannot account for partial proteolysis of the protein at a site N-terminal of the protein chromophore (residues 70−72 in a protein sequence of 248 residues), but it validates that protein fragments consisting of residues 70−248 remain folded and fluorescent.
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