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Efficient cell-specific uptake of binding proteins into the cytoplasm through engineered modular transport systems

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ABSTRACT

Through advances in protein scaffold engineering and selection technologies, highly specific binding proteins, which fold under reducing conditions, can be generated against virtually all targets. Despite tremendous therapeutic opportunities, intracellular applications are hindered by difficulties associated with achieving cytosolic delivery, compounded by even correctly measuring it. Here, we addressed cytosolic delivery systematically through the development of a biotin ligase-based assay that objectively quantifies cytosolic delivery in a generic fashion. We developed modular transport systems that consist of a designed ankyrin repeat protein (DARPin) for receptor targeting and a different DARPin for intracellular recognition and a bacterial toxin-derived component for cytosolic translocation. We show that both anthrax pores and the translocation domain of *Pseudomonas* exotoxin A (ETA) efficiently deliver DARPins into the cytosol. We found that the cargo must not exceed a threshold thermodynamic stability for anthrax pores, which can be addressed by engineering, while the ETA pathway does not appear to have this restriction.

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1. Introduction

Advances in the understanding of the biology of disease and better diagnostic tools have made the rationale for developing drugs that inhibit intracellular protein–protein interactions in aberrantly activated signaling pathways stronger than ever, particularly for cancer [1,2]. One way to achieve this would be through the direct delivery of intact inhibitory molecules. Ideally, such an approach would be combined with targeting only particular cells, thereby adding another layer of selectivity. Inhibitory binding proteins would solve the problem of specific intracellular binding, and progress in engineering scaffolds that work under reducing conditions and in selection technologies now allow for the development of protein binders against virtually all intracellular targets [3–5]. Compared to small molecules, a major advantage of proteins as drugs is that they could provide a much greater specificity to members of large protein families (e.g. protein kinases), by binding to characteristic surface features, or target otherwise undruggable molecules, which may not have a cavity for small molecules.

Since proteins are in general not cell-permeable by themselves, intracellular applications require that the delivery is accomplished by dedicated delivery technologies. To date, however, there are no available systems that combine the features of high-level delivery of proteinaceous cargo to the cytosol, easy restriction to cell types of choice and compatibility with the delivery of highly specific binding proteins, indicating a need for novel efficient, more generic delivery systems.

Uptake of external proteins is a two-step process. In the first step, which occurs after receptor binding, the cargo is internalized via endocytosis, either actively via induction of receptor-mediated endocytosis or passively via membrane recycling. In the second step, the protein would need to translocate across a lipid bilayer to reach the cytosol.

In contrast to endocytosis, translocation from an endosome or another intracellular compartment into the cytosol is typically very inefficient and the mechanisms are poorly understood. Although a number of approaches to improve translocation have been attempted over the years, varying from endosomal fusion or disruption through lipids, polymers or peptides to the exploitation of pathogenic virus- or bacteria-derived mechanisms, there is currently no consensus on what is the best approach for the delivery of a proteinaceous cargo [6].

One reason for the slow progress has been the inability to objectively measure the cytoplasmic delivery of cargo. For proteins, there are

Abbreviations: Ac2, EpCAM-binding DARPin; ATCC, American Type Culture Collection; Avi tag, peptide tag that is biotinylated by BirA; BirA, *E. coli* biotin ligase; CPP, cell-penetrating peptide; DARPin, designed ankyrin repeat protein; Ec1, EpCAM-binding DARPin; EpCAM, epithelial cell adhesion molecule; ETA, *Pseudomonas* exotoxin A; E3_5, non-binding control DARPin; GFP, green fluorescent protein; HA tag, peptide from human influenza hemagglutinin; HEK293/T17 cells, human embryonic kidney 293/T17 cells; Flp-in 293 cells, cell line for recombinase-based genome integration; IMAC, immobilized metal ion affinity chromatography; KDEL, Lys-Asp-Glu-Leu tail; LF, anthrax lethal factor; MBP, maltose-binding protein; MG-132, proteasome inhibitor; NI_1C, NI_2C, NI_3C, DARPin with 1, 2 or 3 internal consensus repeats between capping repeats; Off7, control DARPin binding to MBP; PA, protective antigen (pore-forming unit) of anthrax toxin; PA_m, PA with inactivating mutations; PA_{wt}, PA with wild-type sequence; TEV protease, Tobacco Etch Virus protease.

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several methods to determine cytosolic delivery, but most rely on the delivery of a particular entire protein that needs to be functional [7,8], and thus such assays are not generalizable. Other approaches rely on fluorescence or subcellular fractionation, but these are prone to artifacts induced by preparing the cells for measurements and thus difficult to reliably quantify, or they need to be optimized for each individual cell type [9,10]. To our knowledge, there are presently no established assays that accurately measure cytosolic delivery of proteins in a manner independent of folding and/or of a particular protein function.

In this study, our first aim was thus to develop an assay that would work in conjunction with any protein cargo, which we could then apply for an unbiased comparison of the efficiency of various engineered modular protein transport systems and cargoes and for their further development. For the assay, we chose to employ the *Escherichia coli*-derived biotin ligase (BirA), which highly specifically biotinylates a short peptide sequence that is not a substrate of intrinsic eukaryotic biotin ligases.

To achieve cytosolic delivery of cargo proteins, we opted for a protein-based modular transport system composed of three components: a receptor-targeting binding protein, a module for translocating cargo into the cytosol and a model intracellular binding protein. Using *Pseudomonas* exotoxin A (ETA) and anthrax toxin-derived mechanisms for translocation, and designed ankyrin repeat proteins (DARPins) as model binding proteins and for targeting to the epithelial cell adhesion molecule (EpCAM), we found that the subset of small DARPins that had a low enough thermodynamic stability was translocated very efficiently by both toxins, whereas highly stable DARPins could only be delivered efficiently by the translocation domain of ETA (252–412). The most likely reason is that, whereas ETA (252–412) exploits the host translocation machinery for actively unfolding the cargo, anthrax toxin relies on the unfolding force generated by its own translocation pore, which has a limited capacity to unfold cargo. We could confirm the role of too high a thermodynamic stability as a limiting factor for translocation by gradually destabilizing the DARPin framework, which restored efficient translocation via anthrax toxin.

2. Materials and methods

2.1. Cell lines

Experiments were performed either in human embryonic kidney (HEK) 293T/17 cells (obtained from the American Type Culture Collection (ATCC)), in MCF7 (breast cancer) cells (ATCC) or in Flp-In 293 host cells (Invitrogen), stably overexpressing both EpCAM and BirA. The stable cell line was generated according to the standard Flp-In stable cell line protocol (Invitrogen). Previously described pcDNA5/FRT-derived mammalian expression vectors (Invitrogen) were used, in which the expression cassette was duplicated [11].

2.2. Antibodies

Anti-HA-tag IgG (peptide from human influenza hemagglutinin) and anti-actin IgG2a were obtained from Sigma-Aldrich (Cat. No. H6908 and A3853); anti-avi-tag IgG (peptide sequence that is biotinylated by BirA) from Genscript (Cat. No. A00674-200); anti-biotin IgG Alexa Fluor 680 from Jackson Immunoresearch (Cat. No. 200-622-211); streptavidin IRDye 680LT from LI-COR Biosciences (Cat. No. 926-68031); anti-DARPin rabbit serum was homemade (B. Dreier, unpublished data).

2.3. Transient transfections

Transient transfections were performed in HEK293T/17, stably transfected Flp-In 293 cells or MCF7 cells in 24-well plates. The transfection reagents TransIT 293 (293 cells; Myrus) and TransIT X2 (MCF7 cells; Myrus) were used according to the manufacturer's protocol. pcDNA5/FRT vectors used for transient overexpression contained *birA* (obtained from Avidity) together with full-length *EpCAM* [12], *birA* together with full-length (*HA*)*-tagged Anthrax toxin receptor 1* (Genscript), *birA* together with *HA-E3_5-avi* [13], *birA* together with *HA-E3_5-TEV-myc* (TEV denotes the cleavage site (ENLYFQS) for Tobacco Etch Virus protease; myc denotes the peptide EQKLISEEDL) and, *GFP1-10* [14] together with *HA-E3_5-avi*. A pcDNA3.1 vector containing *E3_5-eGFP* was used for assessing the transfection efficiency in MCF7 cells [15].

2.4. Cellular uptake experiments

Cells were seeded in 24-well plates for uptake experiments. As a positive control for cytosolic biotinylation, HA-E3_5-avi was expressed in the cytosol. To inhibit the proteasome in experiments aimed at quantifying cytosolic delivery, the inhibitor MG-132 was included at a concentration of 50 μM. Experiments were performed with 20 nM, 200 nM or 2 μM of the avi-tagged protein to be translocated and 20 nM of MBP-PA$_{N682A/D683A}$-Ac2 fusion protein (consisting of maltose-binding protein (MBP), anthrax protective antigen (PA) with the two indicated mutations and EpCAM-binding DARPin Ac2), unless mentioned otherwise. Analysis of cellular uptake and cytosolic delivery was achieved via western blotting. For further details, please see Supplementary information materials and methods.

2.5. Cloning, expression and purification of recombinant fusion toxins and Tobacco Etch Virus protease

Anthrax lethal factor (LF) and PA fusions were cloned behind MBP for solubility enhancement. Cloning of recombinant fusion proteins was achieved via standard procedures. Fusions containing the translocation domain of ETA (252–412) or the translocation domains and the inactivated catalytic domain (252–608; E553D) were expressed in soluble form in the cytoplasm of *E. coli* Origami B(DE3) (Novagen). Protective antigen and lethal factor fusion proteins were expressed in soluble form in the cytoplasm of *E. coli* BL21(DE3). For details, please see Supplementary information materials and methods. Purification was achieved via immobilized metal ion affinity chromatography (IMAC) for all constructs. Fusions between MBP and LF-DARPin constructs were cleaved with TEV protease and further purified via reverse IMAC and size-exclusion chromatography. Fusion proteins containing protective antigen were purified directly via size-exclusion chromatography after IMAC. TEV protease was purified via IMAC. For details on cloning, expression and purification, please see Supplementary information materials and methods.

2.6. In vitro biotinylation

Partial in vitro biotinylation of Ec1-ETA(252–608)-NI$_3$C (a fusion protein consisting of anti-EpCAM DARPin Ec1, *Pseudomonas* exotoxin A of the residues indicated and DARPin cargo NI$_3$C) was achieved in a volume of 100 μl with a protein concentration of 30 μM in a buffer containing 50 mM bicine, 50 mM NaCl, 10 mM ATP, 10 mM MgAc, 50 μM biotin and 2.5 μg biotin ligase (kind gift of N. Stefan). The mixture was incubated for 60 min at 30 °C and then buffer-exchanged twice against PBS using Zeba Spin Columns (Pierce Biotechnology, Inc.).

2.7. ΔΔG calculations

The crystal structure 2QYJ of the consensus DARPin NI$_3$C was used as a starting point for Rosetta 3.5 ΔΔG prediction [16]. After relaxation with all atom constraints, for each individual mutation and combination of mutations in NI$_3$C, 50 individual repacking runs with limited backbone flexibility were performed using the high-resolution protocol. For estimating the ΔΔG values for the mutations introduced in consensus NI$_2$C variants, a model structure was obtained through the removal of the central 33 -amino -acid internal repeat from 2QY1, by least-

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