Journal of Controlled Release 200 (2015) 13–22

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

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

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ARTICLE INFO

Article history:
Received 14 September 2014
Received in revised form 13 December 2014
Accepted 16 December 2014
Available online 17 December 2014

Keywords:
Anthrax toxin
Pseudomonas exotoxin A
Protein engineering
Cytoplasmic delivery
Designed ankyrin repeat protein
Biotin ligase

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.

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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http://dx.doi.org/10.1016/j.jconrel.2014.12.019
0168-3659/

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_3C (a fusion protein consisting of anti-EpCAM DARPin Ec1, *Pseudomonas* exotoxin A of the residues indicated and DARPin cargo NI_3C) 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_3C 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_3C, 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_2C variants, a model structure was obtained through the removal of the central 33 -amino -acid internal repeat from 2QY1, by least-

squares aligning internal repeats 1 and 2 of one copy of the structure to repeats 2 and 3 of a second copy, and then combining the N-cap and 1st internal repeat of the first copy with the 3rd internal repeat and C-cap of the second copy. The model was relaxed with all atom constraints and ΔΔG calculations for the introduced mutations were performed as described above.

3. Results

3.1. Development of an assay to measure cytosolic protein delivery

A major aim in the development of intracellular protein therapy is the construction of generic, modular protein transport systems geared towards cytosolic delivery. However, as a first step, an unbiased assay that provides quantitative information on cytosolic protein delivery is urgently needed. The method of our choice relies on the prokaryotic enzyme biotin ligase (BirA) which modifies a peptide tag that can be fused to any protein cargo. With an extreme specificity, BirA adds biotin to the lysine of a 15-amino-acid peptide sequence (GLNDIFEAQKIEWHE) referred to as the avi tag [17,18].

We reasoned that, since this enzyme can be expressed exclusively in the cytosol, we could distinguish between the two steps in the uptake process: endocytic uptake and translocation into the cytosol. Biotinylation of the cargo would point towards cytosolic delivery, whereas total cellular uptake — i.e. cargo located either in the cytosol or in the endosomes — would be indicated by an independent antibody-detectable peptide tag (Fig. 1a, b). Both detection strategies would be independent of the protein cargo and thus be applicable for any proteinaceous cargo.

To establish the assay, we first tested whether transiently expressed prokaryotic BirA was capable of specifically biotinylating the unselected DARPin E3_5 carrying a C-terminal avi tag when co-expressed from the same vector in the cytosol of Flp-In 293 cells. The high specificity of the enzyme and the lack of eukaryotic biotin ligases able to biotinylate the avi tag were confirmed in a titration experiment (Fig. 1c): quantification of band intensities revealed a linear relationship between sample

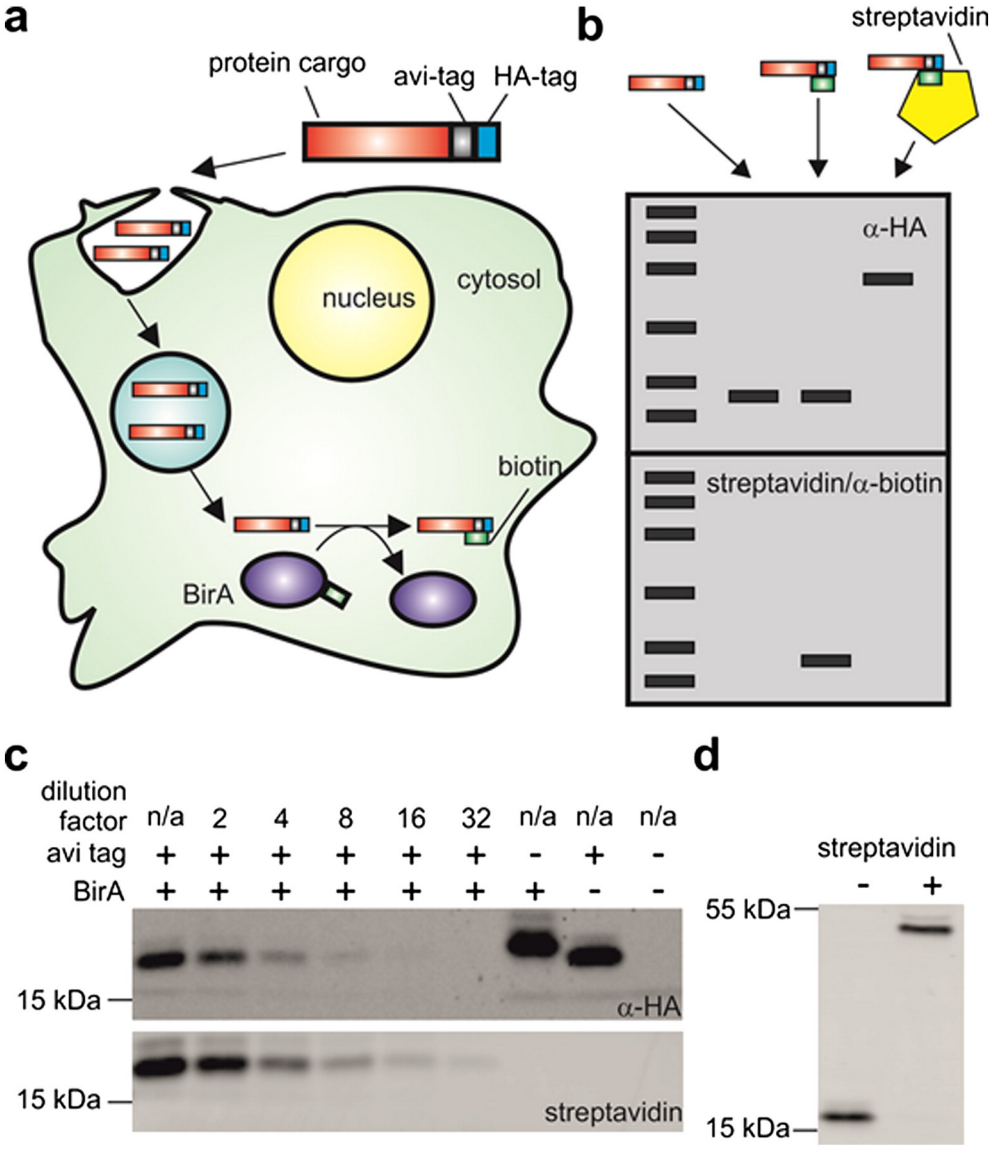

Fig. 1. Establishment of a BirA assay for quantifying cytosolic delivery. (a) Schematic representation of the uptake of avi-tagged cargo in BirA-overexpressing cells. (b) Schematic depiction of possible sample analyses via western blotting. (c) Western blot from Flp-In 293 cells that were transiently transfected with the unselected control DARPin E3_5 containing an N-terminal HA tag and a C-terminal avi tag. The control without the avi tag contains a TEV-cleavable myc tag instead. An α-HA-tag antibody and streptavidin IRDye 680LT were used for detection. (d) Western blot of a lysate from Flp-In 293 cells stably expressing EpCAM and BirA that were transiently transfected with E3_5 containing a C-terminal avi tag. Lysates were incubated with streptavidin or left untreated before being separated via SDS-PAGE. The signal was detected with α-DARPin serum. n/a; not applicable. BirA, *E. coli* biotin ligase; HA, peptide from human influenza hemagglutinin.

loaded and signal obtained (Supplementary results, Supplementary Fig. S1a–c), as well as the absence of background activity, supporting the use of band intensities for signal quantification in subsequent experiments.

Having confirmed enzyme specificity, we generated stable cell lines that could be used for cargo uptake studies. Together with BirA, we chose to overexpress the tumor-cell marker EpCAM as a model receptor [19]. The fact that a cytosolic protein can be completely biotinylated by BirA was confirmed by transiently transfecting the BirA/EpCAM expressing cells with an avi-tagged DARPin (Fig. 1d and Supplementary Fig. S1d) and detection of a western blot shift according to the method described by Petris et al. [20]. For quantification purposes, however, the direct detection of biotinylated cargo with a fluorescently labeled streptavidin was preferable because of the higher sensitivity obtained with the labeled streptavidin than with the antibody against the HA tag (Fig. 1c). Moreover, when incubating a partially biotinylated construct having adjacent avi and HA tags (as present in all the constructs described below) with streptavidin before loading, we noticed that the HA tag was less well detected (~40% less signal) in streptavidin-bound samples as compared to unbound (i.e. unbiotinylated) samples (Supplementary Fig. S1e, f). The most likely reason is steric hindrance, preventing the efficient binding of the anti-HA antibody to the HA tag when in proximity to a streptavidin-bound avi tag.

3.2. Design of a generic modular transport system for delivery of proteins to the cytoplasm

We aimed for a generic design, consisting of three exchangeable components: a receptor-targeting moiety, a protein-based mechanism for membrane translocation to the cytosol and an intracellularly acting binding protein. For both the receptor-targeting module and as model therapeutic cargo proteins, we applied distinct DARPins, which are binding proteins based on the ankyrin scaffold [4,13]. The favorable biophysical properties of DARPins allow for a wide range of engineering procedures such as the construction of well-folding fusion proteins [4], required here — formats that are not well tolerated by antibodies as they tend to aggregate then [21]. Moreover, due to an absence of cysteines and a general lack of aggregation tendencies, DARPins fold equally well in the cytoplasm [15,22].

For achieving translocation through the lipid bilayer into the cytosol, we opted to use nature's mechanisms as a guide, since protein toxins are known to be delivered to the cytoplasm, where they act as enzymes and thus need to be present in a folded state. We have used bacterial toxins from two distinct families which use very different translocation mechanisms: anthrax toxin and *Pseudomonas aeruginosa* exotoxin A (ETA). Both translocation systems can in principle deliver heterologous cargo and be retargeted, but cargo requirements are poorly understood [23–26]. In all cases investigated here, the catalytic domains that are responsible for the cellular toxicity of these toxins (i.e., for *Pseudomonas* Exotoxin A: ADP-ribosylation of elongation factor-2; for anthrax lethal factor: cleavage of mitogen-activated protein kinase kinase; for anthrax edema factor: cAMP-level increases) have been either eliminated or inactivated.

ETA is thought to deliver its catalytic domain to the cytosol via retrograde transport to the ER, where it hijacks the host (retro)translocation machinery for misfolded proteins [27]. During retrotransport, the receptor-targeting DARPin and the first 27 amino acids of the translocation domain are cleaved off by a furin-like protease in the endosome, which cleaves between R279 and G280, implying that only part of the translocation domain and the C-terminal cargo reach the cytosol [27]. In this study, we define the translocation domain of ETA as amino acids 252–412, which corresponds to the structural domains II, Ib and the beginning of structural domain III.

Anthrax toxin instead forms its own hepta- or octameric pH-dependent translocation channel: an 83-kDa precursor binds to one of its receptors, capillary morphogenesis gene 2 or the anthrax toxin receptor 1, is cleaved by furin and the C-terminal 63-kDa fragment is released and oligomerizes to form a channel, which is then endocytosed [28].

Both translocation mechanisms require unfolding of the cargo protein at least to some extent. We therefore chose to build transport systems containing consensus model DARPins as a cargo with varying degrees of thermodynamic stability [29]. Consensus DARPins consisting of either one ($T_m = 60$ °C), two ($T_m = 90$ °C) or three ($T_m > 100$ °C) internal repeats were used.

For ETA, the EpCAM-targeting DARPin Ec1 was fused at the N-terminus [12]. The positioning of the receptor-targeting moiety at the N-terminus is crucial because this part (where the natural receptor-binding domain is also situated) is cleaved off by furin during intracellular processing and does not get translocated into the cytosol. Cargo DARPins were fused C-terminally to either a truncated ETA (aa 252–608 of the mature native exotoxin) containing the inactivating mutation E553D [30], or to the translocation domain of ETA only (aa 252–412 of the mature protein), in both cases followed by avi and HA tags (Fig. 2a, b). All constructs ended with a C-terminal KDEL sequence for retrograde transport to the endoplasmic reticulum. Controls without C-terminal DARPin cargo were constructed and tested as well (Fig. 3 and Supplementary Table ST1). Since fusions at the C-terminus, unlike those at the N-terminus, are meant to be transported into the cytosol, proteins added C-terminally are referred to as 'cargo proteins'. It is thus at this position that DARPins with an intracellular activity should be positioned. ETA fusion proteins could be expressed at very high levels in the cytosol of *E. coli* (50–100 mg/L medium in shake flasks) in soluble form, and are known to form disulfides there within the ETA domain [31].

For anthrax toxin, the EpCAM-targeting DARPin Ac2 was fused C-terminally of the full-length pore-forming protein of anthrax toxin, termed anthrax "protective antigen" or PA (carrying the mutations N682A/D683A, numbering of the mature protein) [24], (Fig. 2c). DARPin fusions had to be C-terminal in this case, since the N-terminal 20 kDa is cleaved off during processing by furin, which occurs at the plasma membrane before internalization. Different EpCAM-binding DARPins were chosen, because, even though Ec1 is the binder of choice with the highest affinity (68 pM) [12], it is thought to require its N-terminus for EpCAM binding (N. Stefan, personal communication), meaning it is less suitable when positioned as a C-terminal fusion. We considered Ac2, with an affinity of 1.2 nM, a suitable alternative [12]. The consensus DARPins serving as cargo were fused to the C-terminus of lethal factor (LF) 1–254, which is the channel-binding part of the natural cargo of anthrax toxin, and does not carry toxic activity (Fig. 2d). LF and PA fusions were fused behind MBP and expressed in the cytosol of *E. coli* also at 50–100 mg/L medium in shake flasks. MBP was cleaved off from LF before use. The general structure of the transport systems is depicted in Fig. 2e and a complete list of used constructs is included as Supplementary Table ST1.

3.3. Performance of transport system in BirA/EpCAM Flp-In 293 cells

Uptake efficiency of the aforementioned constructs was compared after a 4-h incubation at 37 °C in the Flp-In 293 cell line stably expressing EpCAM and BirA. All avi-tagged cargo proteins, i.e. the LF-variants and the ETA-based variants, were incubated at 200 nM, whereas the pore-forming component of anthrax toxin, protective antigen (PA), was used at 20 nM. The 10-fold lower concentration of the pore-forming protein PA (compared to LF) was to ensure that anthrax (pre)pores were saturated as much as possible with LF before being internalized, while keeping the LF concentration similar to the 200 nM used for ETA constructs.

As our initial focus was on the translocation efficiency of these toxins, first experiments were performed in the presence of the proteasome inhibitor MG-132, which would minimize the influence of differences in intracellular stability after transport, i.e. through proteolysis. For each of the three tested consensus DARPin cargoes, cytosolic delivery

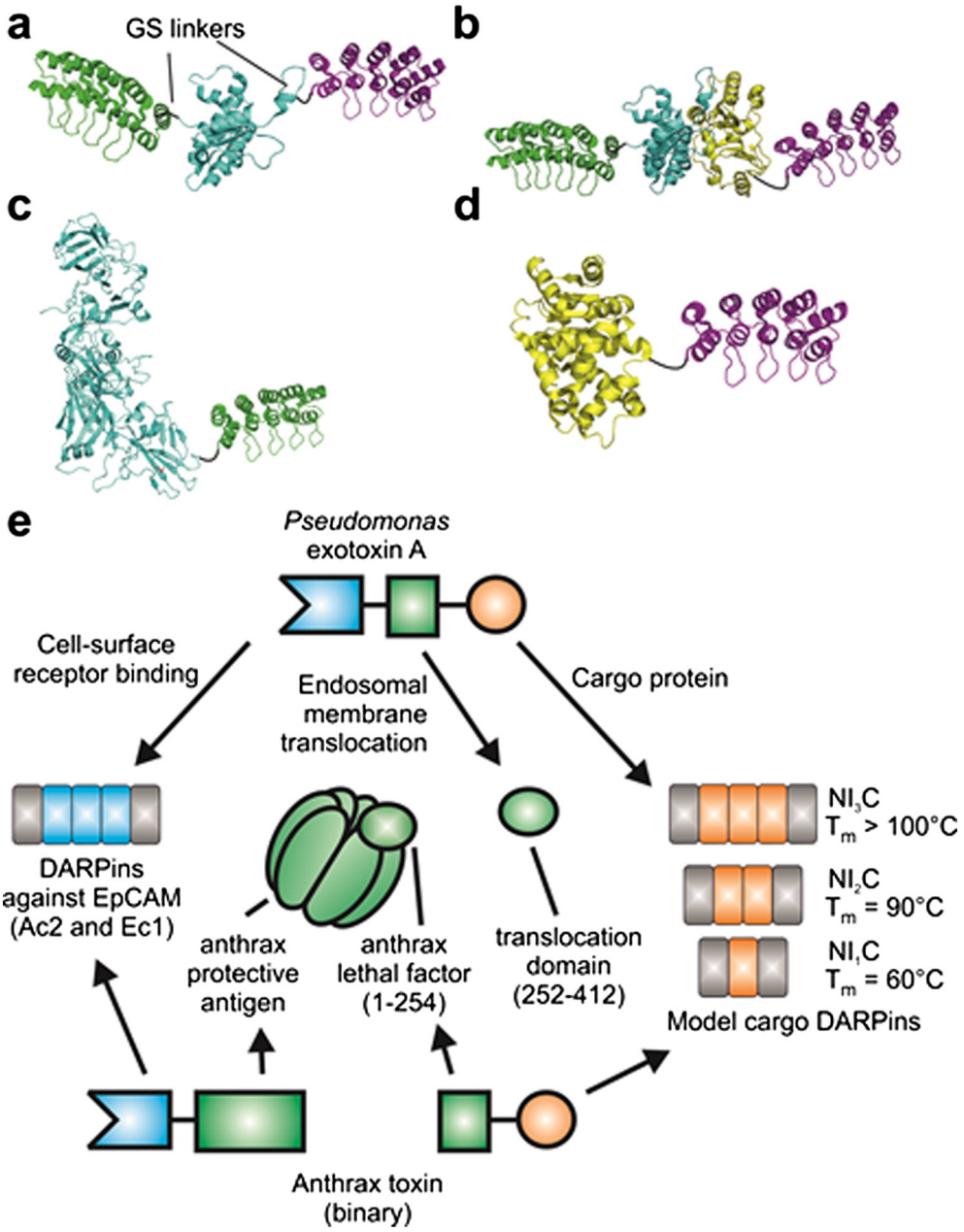

Fig. 2. Schematic structural models of the transport systems designed in this study. The targeting DARPin (recognizing EpCAM) is in green and the cargo DARPin in purple, the translocation domain in cyan and the additional natural cargo domain in yellow: (a) a DARPin-ETA(252–412)-DARPin fusion, (b) a DARPin-ETA(252–608)-DARPin fusion, (c) anthrax protective antigen-DARPin fusion and (d) a lethal factor 1–254-DARPin fusion. Structures used were PDB ID: 2XEE for the DARPin [32], PDB ID: 1IKQ for *Pseudomonas* exotoxin A [33], PDB ID: 1ACC for protective antigen [34] and PDB ID: 1J7N for lethal factor [35]. (e) Schematic drawing of the design of the modular engineered transport systems and the main components used in this study. DARPin, designed ankyrin repeat protein; ETA, *Pseudomonas* exotoxin A; EpCAM; epithelial cell adhesion molecule; T_m, melting temperature.

mediated by the translocation domain of ETA was most efficient, without a clear dependency on the thermodynamic stability of the cargo (Fig. 3a, c). Rather unexpectedly, the inclusion of the natural inactivated catalytic domain of ETA severely restricted cytosolic delivery, even without C-terminal DARPin cargo (Fig. 3a, c). It should be noted that almost exclusively the cleaved portion of the fusion protein appeared to reach the cytosol, in agreement with its generally assumed mechanism of action, and corroborating the notion that biotinylation exclusively occurs in the cytosol (Fig. 3a, b) — otherwise biotinylation should be observable on other protein bands as well. The cytosolically expressed HA-E3_5-avi, which served as a positive control in every experiment, again showed complete biotinylation. In all experiments, we also added avi-tagged protein directly to the lysate to confirm the absence of biotinylation activity after lysis. In contrast to cytosolic delivery, the total cellular internalization of the ETA fusions (i.e., corresponding mostly to localization in endosomes) was higher in the presence of the inactive catalytic domain (Fig. 3b, d).

With respect to the anthrax toxin-derived translocation system, protective antigen (the pore-forming protein) was able to translocate its own natural substrate LF, and LF when fused to the smallest and least stable DARPin NI_1C with comparable efficiency, suggesting that the fusion of NI_1C does not affect the translocation process much. In contrast, fusions of LF with NI_2C or NI_3C were hardly detected in the cytosol, indicating that these more stable DARPins block translocation (Fig. 3a, c). Notably, LF-NI_2C and LF-NI_3C also showed a lower HA signal in comparison to LF and LF-NI_1C, implying that less intact protein was inside the cell at the time of lysis (Fig. 3b, d), presumably due to the rapid degradation of translocation-incompetent complexes in the lysosomes or just cleavage of the HA tag. The rapid degradation of LF in lysosomes

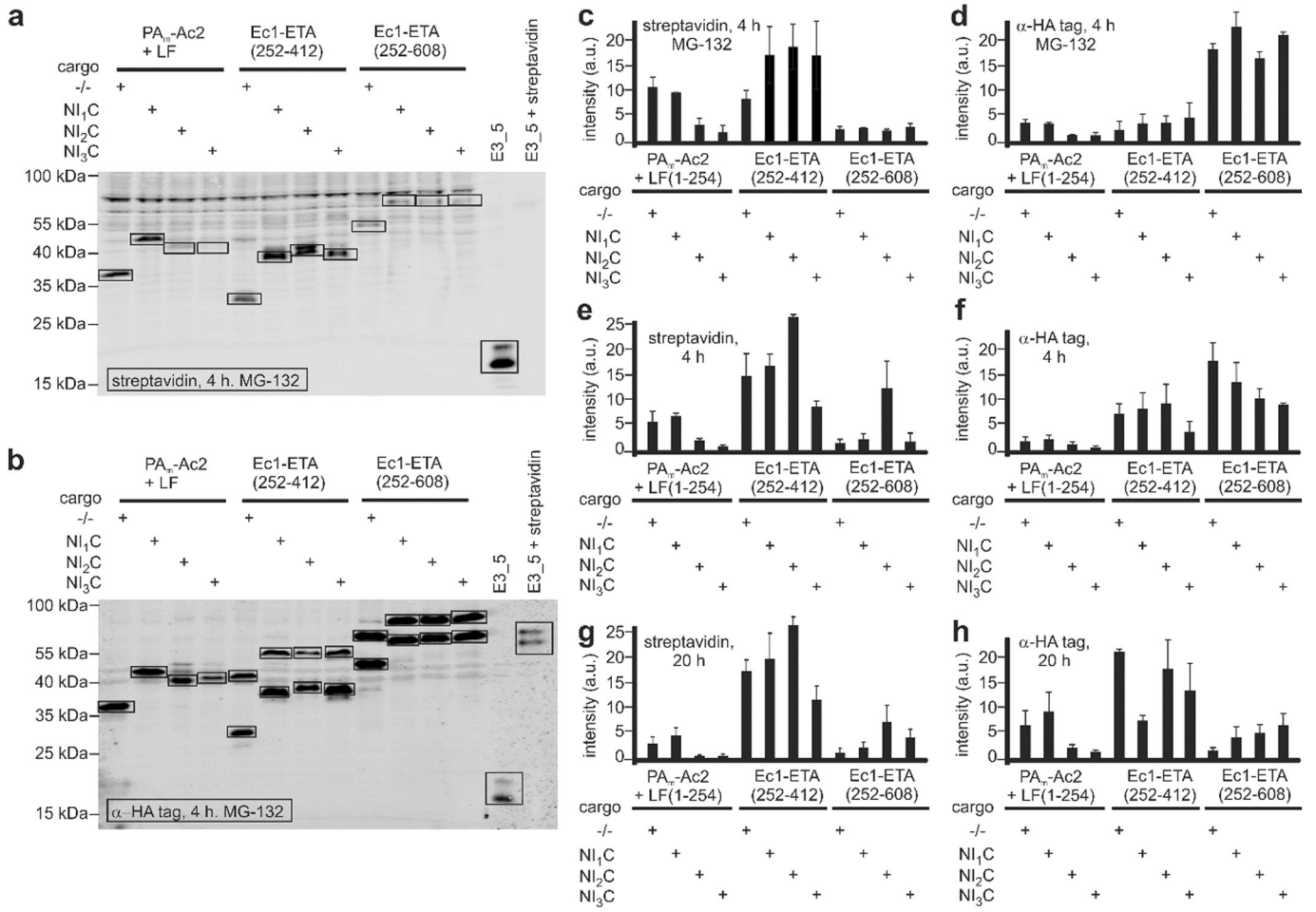

Fig. 3. Cytosolic delivery of DARPins via modular transport systems. (a) A representative streptavidin IRDye 680LT western blot of a 4-h-uptake experiment of avi-tagged toxins with or without cargo at 200 nM and MBP–PA$_m$–Ac2 at 20 nM in the presence of the proteasome inhibitor MG-132 in Flp-In 293 cells stably expressing BirA and EpCAM. Boxes indicate the bands of interest (i.e. furin-cleaved and uncleaved fusion toxins) that show the expected cargo molecular weight. These bands (only furin-cleaved for ETA constructs) were used for quantification purposes. The band at ~70 kDa is a non-specific signal and probably derives from Hsp70, which is also recognized by streptavidin and ubiquitously expressed. Detection of this band correlates well with the actin signal (Supplementary Fig. S2a). (b) A representative α-HA tag western blot with the same samples as in (a). (c, d) Quantification of western blot bands from (a), with (c) showing the streptavidin IRDye 680LT signal and (d) the α-HA tag signal. Normalized average and range are shown. N = 2. (e, f) Quantification of streptavidin IRDye 680LT (e) and α-HA tag signal (f) from a 4-h incubation in the absence of proteasome inhibition. Average and the standard error of the mean (SEM) are shown. N = 3. (g, h) Quantification of streptavidin IRDye 680LT (g) and α-HA tag signal (h) 20-h uptake experiments. Average and SEM are shown. N = 3. Please note that the highly stable NI$_2$C and NI$_3$C DARPins do not completely unfold during SDS-PAGE sample preparation, leading to additional faster running bands that may erroneously suggest a lower molecular weight. BirA, *E. coli* biotin ligase; ETA, *Pseudomonas* exotoxin A: HA tag, peptide from human influenza hemagglutinin; LF, anthrax lethal factor; PA, protective antigen.

associated with translocation-incompetent pores (where protective antigen was mutated; F427A) has been reported before [36]. To confirm the observations made with fluorescently labeled streptavidin, the detection of biotinylated proteins was also performed via an anti-biotin antibody, which showed similar results (Supplementary Fig. S2b).

When 4-h- and 20-h-incubations were performed in the absence of a proteasome inhibitor, a generally similar outcome was seen (Fig. 3e–h and Supplementary Fig. S2c–f). Nevertheless, a number of subtly distinct observations were made. First, the cytosolic quantities of LF and LF-NI$_1$C had decreased relative to the ETA translocation domain fusions, indicating a lower stability of the former in the cytosol. A low stability in the cytosol has been reported before for full-length LF [36]. The reduced LF and LF-NI$_1$C concentrations in the absence of MG-132 were also confirmed in a direct comparison (Supplementary Fig. S2g). In this experiment, we also directly demonstrated the necessity of the presence of the pore-forming MBP–PA$_m$–Ac2 for cytosolic translocation.

Delivery of LF-NI$_1$C via MBP–PA$_m$–Ac2, targeting EpCAM, was also compared with delivery via MBP-PA$_{wt}$, targeting the anthrax toxin receptor 1, in an experiment where HEK293/T17 cells were transiently transfected with either *birA* and *EpCAM* or *birA* and *Anthrax toxin receptor 1*. The rationale behind this experiment was to see whether the presence of C-terminal DARPin in the hepta- or octameric anthrax pores would interfere with pore functionality. As translocation efficiencies were comparable, it can be concluded that pore functionality is not significantly affected, despite the physical presence of several DARPins at the pore (Supplementary Fig. S2h).

When absolute cytosolic quantities were estimated using a fully biotinylated MBP as a reference on a western blot, quantities found after 4 h were similar to those found after 20 h for all fusion toxins. To estimate the cytosolic concentration of the protein translocated to the cytosol, we assumed an average Flp-In 293 cell diameter of 13 μm and a spherical shape, which led to estimations of be $4.8 \pm 1.6 \times 10^{-7}$ M after 4 h, and $5.9 \pm 0.5 \times 10^{-7}$ M after 20 h for Ec1-ETA(252–412)-NI$_2$C. We also attempted to determine the endosome:cytosol ratio of protein in the cell lysates using the anti-HA signals and directly loaded fusions toxins as a reference. Surprisingly, for a number of proteins, we detected higher amounts of biotinylated proteins than HA-tagged protein, suggesting the HA tag is lost, e.g. through proteolysis [37]. This notion was further supported by the detection of the bands with anti-DARPin, anti-HA and anti-avi-tag antibodies, which showed different ratios of cleaved versus non-cleaved protein (Fig. S3), suggesting loss of the HA tag.

So far, all uptake experiments were performed at 200 nM. To investigate whether higher amounts of proteins could be delivered via the translocation domain of ETA, we assessed the delivery of a phosphorylated ERK-binding N2C DARPin, pE59 [22] at 200 nM and at 2 μM after a 4-h incubation (Supplementary Fig. S2i–k). However, no increased cytosolic uptake or total internalization was observed, implying saturation of the system. Further investigations will have to clarify whether this saturation is at the level of receptor binding (EpCAM expression level), retro-transport to the ER (KDEL receptor expression level) or the presumed retrograde trans-membrane transport itself (Sec61 expression level) or at the level of any additional accessory factor.

We then compared uptake of 200 nM with 20 nM, also including the lower affinity EpCAM-targeting DARPin Ac2 and the negative control DARPin Off7 (binding to *E. coli* maltose-binding protein), which has no affinity for EpCAM and thus should not lead to internalization. With both Ec1 and Ac2, we saw less internalization at 20 nM than at 200 nM, whereas Off7-containing constructs were not detected in the cell at all (Fig. S3). While levels of furin-cleaved toxin were much higher for Ec1-ETA(252–412) at 200 nM than at 20 nM, or than for Ac2 fusions at either concentration, the amount of biotinylated protein differed much less, also suggesting the saturation of one or multiple steps in the cytosolic delivery process.

In order to compare results from our model system, where EpCAM was artificially overexpressed, with a system that naturally overexpresses EpCAM, we chose to study cellular uptake and cytosolic delivery (with transient overexpression of BirA) in the breast cancer cell line MCF7 as well (Supplementary Fig. S4). Also in MCF7 cells, only the cleaved part of two Ec1-ETA(252–412) fusions was found to reach the cytosol (Supplementary Fig. S4a), which is consistent with the proposed internalization process. We chose to detect the constructs with the anti-DARPin serum, because anti-HA signals were very low in MCF7 cells after 20 h (presumably since the tag is cleaved off). It should be noted, however, that band intensities cannot be used for reliably quantifying the degree of processing (see also Supplementary Fig. 3), because the processed (i.e. furin-cleaved) constructs miss the larger (N3C) receptor-targeting DARPin and contain only a smaller (N2C) consensus DARPin, yet both are detected with the anti-DARPin serum.

In a direct comparison between the model Flp-In 293 cells and the MCF7 cells, we found similar levels of cellular internalization in both cell lines after a 20-h incubation (Supplementary Fig. S4b). To assess cytosolic delivery, we chose a semi-quantitative approach where we transiently overexpressed biotin ligase in MCF7 cells, and estimated the transfection efficiency by transfection with a GFP-fusion protein (Supplementary Fig. S3c). As the BirA level required for efficient biotinylation of delivered cargo is not known, we can only roughly estimate the percentage of MCF7 cells that actually biotinylate delivered cargo. If we assume, from the GFP data, a transfection efficiency of 20 to 40%, cargo delivery to the cytosol would be about as efficient in MCF7 cells as in the model Flp-In 293 cells that stably overexpresses EpCAM (Supplementary Fig. S4c, d). Even though the processing (i.e. furin cleavage) was higher in Flp-In 293 cells, at 200 nM fusion construct added to the cells this difference did not translate into a markedly higher cytosolic delivery. As stated above, this is presumably due to the saturation of one or more processes involved in cytosolic delivery. Importantly, the data are consistent with the known high density of EpCAM on MCF7 cells (>200,000 receptors/cell), the very high affinity of DARPin Ec1 for EpCAM (k_D = 68 pM) and the fast EpCAM internalization rate [38].

3.4. Destabilization of LF fusions

As shown above, LF-NI_2C and LF-NI_3C could not be delivered efficiently via anthrax toxin pores into the cytosol, and LF and LF-NI_1C were prone to degradation by the proteasome. Since the proteins differ by both size and stability, we designed a set of NI_2C and NI_3C variants aiming to progressively destabilize the DARPin framework. For this purpose, we introduced hydrophobic cavities first in NI_2C, away from the randomized surface that serves to bind interactions partners in DARPins (Fig. 4a), and thus to develop a potentially transferrable approach to other DARPins, since hydrophobic cavities typically lead to only modest structural rearrangements [39]. ΔΔG calculations with the Rosetta suite [40] were performed to provide estimates on the overall changes in stability. As intended, all alanine replacements were mildly destabilizing, whereas glycine replacements were calculated to be considerably more destabilizing (Supplementary Table ST2).

When uptake was assessed in a 4-hour uptake experiment in the presence of proteasome inhibitor, we observed that MBP–PA_m–Ac2 mediated translocation was highly comparable for all destabilized NI_2C variants, with already the smallest perturbation being sufficient, and that it was now as efficient as translocation of LF-NI_1C, and much better than translocation of the original LF-NI_2C or LF-NI_3C (Fig. 4b).

To confirm and extend this rational destabilization approach, we attempted to render NI_3C translocation-competent as well, which was virtually not translocated in its original form. To this end, we produced a set of progressively more destabilized NI_3C DARPins (Supplementary Table ST2) and tested whether they could be translocated by MBP–PA_m–Ac2 (Fig. 4c, d). As for the destabilized NI_2C variants, we indeed observed that efficient translocation could be restored. Consistent with this explanation, the least destabilized variant (NI_3C dest. 1), which has only a single leucine-to-alanine mutation per repeat, was still only poorly translocated, suggesting that N3C DARPins require somewhat harsher destabilization than N2C DARPins, as one would also expect from the respective T_m values of the full consensus proteins (90 °C for NI_2C vs >100 °C for NI_3C).

To further investigate determinants of the intracellular stability of the LF fusions, we generated all-arginine variants of the fused DARPins (NI_1C-all-R and NI_2C-all-R) in order to test whether a reduced number of available lysines on the surface would delay degradation by the proteasome. However, in the absence of proteasome inhibitor, the amount of LF-NI_1C-all-R in the cytosol was not higher than that of LF-NI_1C. Importantly, just like LF-NI_2C, LF-NI_2C-all-R was also not translocated efficiently (Fig. 4b), suggesting that too high a barrier against unfolding inhibits the transport of both constructs. An alternative approach, where the DARPin was fused behind an all-arginine (all-R) ubiquitin variant that would be cleaved by deubiquitinases in the cytosol and might release free DARPin from LF, an approach inspired by a recent publication from Bachran et al. [41], only led to minor amounts of free DARPins in the cytosol (arrow in Fig. 4c and d). At present, it is unclear whether the translocation step is less efficient for the all-R ubiquitin or whether its degradation in the cytosol is enhanced, despite the use of a ubiquitin (all-R) variant in which all seven lysine residues have been replaced by arginine residues [41].

4. Discussion

An efficient, cell-specific targeting of cytoplasmic proteins would obviously dramatically expand the number of potential drug targets. Two layers of specificity would be introduced, one for the cell (via its cell-surface marker), and one for the target. The existence of engineered binding proteins with shapes ranging from concave to convex [42] would furthermore make proteins accessible that are very difficult to target specifically with small-molecule drugs, such as mostly flat protein interaction surfaces, since small molecules normally need to bind to cavities. Individual members of large protein families (e.g. kinases) could be distinguished by its surface features. However, the absence of an efficient cytosolic delivery system has so far prevented proteins from being seriously considered as potential drug targets that would act in the cytoplasm or nucleus. Nevertheless, rapid progress is being made in this area, as illustrated by two very recent studies that

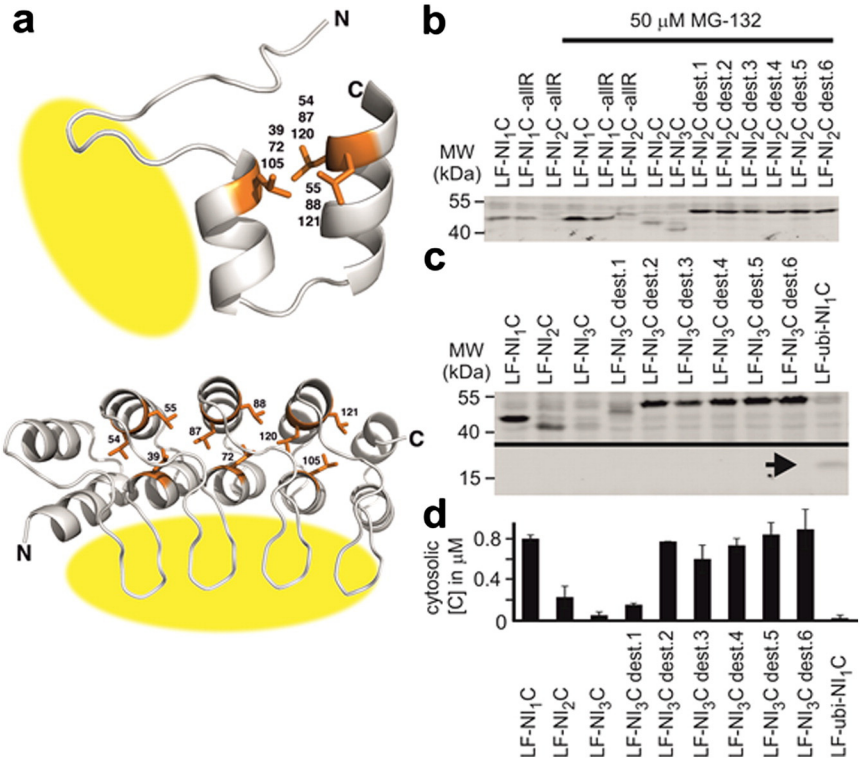

Fig. 4. DARPin destabilization for improved anthrax-mediated translocation. (a) Positions where mutations were introduced in the consensus DARPin NI_3C (2QYJ) (seen sideways, in one repeat, upper panel) or in the whole protein (lower panel). The yellow oval reflects the spatial position of a hypothetical DARPin target. Numbers indicate the positions where mutations were introduced (b) A 4-h-uptake experiment in Flp-In 293 cells stably expressing BirA and EpCAM with 20 nM MBP–PA_m–Ac2 and fusions of LF with destabilized NI_2C DARPins (see Supplementary Table ST2) and controls. (c) Identical uptake experiment with inhibitor MG-132, gradually destabilized NI_3C variants and LF-ubi-NI_1C. (d) Western blot quantification of 4-h-uptake experiments with MG-132 and indicated fusion toxins. Average and range are shown. N = 2. BirA, *E. coli* biotin ligase; LF, anthrax lethal factor; MBP, maltose-binding protein; PA, protective antigen.

investigate delivery and activity of distinct binding proteins delivered to the cytosol [43,44].

In this study, we have focused on characterizing the cytosolic delivery of designed ankyrin repeat proteins (DARPins) as model binding proteins and first developed a robust assay to measure it. This has permitted us to define molecular features required of the cargo. We show that we can efficiently deliver DARPins into the cytosol in a cell-specific manner via protein-based modular transport systems that exploit distinct bacterial translocation mechanisms. Since intracellular DARPins can be used as inhibitors after expression [15], this successful transport represents another important step towards the exploitation of binding proteins such as DARPins as intracellular drugs. Nonetheless, there is nothing in our transport systems that limits the cargo to being DARPins and they are used as a convenient model cargo here, simply because the molecular properties (size, stability, charge) of these robust proteins can be altered over a very wide range. Thus, the cell-specific transport systems we describe can also likely be harnessed for the delivery of other functional proteins such as missing, dysfunctional or transcriptionally repressed proteins.

This is also supported by a number of studies that have provided proof of principle for the delivery of a number of different cargo proteins via ETA and anthrax toxin [25,26,43,45]. Nevertheless, some proteins could not be translocated. We believe that in the anthrax system, reducing the stability of proteins which cannot be unfolded, yet without hampering their (re)folding capabilities, may improve delivery. This was achieved here for DARPins via introducing hydrophobic cavities.

However, since there is presumably no reducing environment encountered in the anthrax intoxication pathway, the presence of disulfide bridges may well be incompatible with anthrax toxin-mediated delivery, as also suggested by a previous study where the introduction of an artificial disulfide bridge in the diphtheria toxin A chain blocked translocation [45]. In contrast, the ETA pathway actually includes a reduction step, so here it is less likely that additional disulfide bridges would be incompatible with translocation. For function the reduced cargo proteins need to refold well in the cytosol.

Since assays that accurately and objectively quantify cytosolic delivery of proteins in a generic fashion are lacking at present, we developed such an assay. It exploits the highly specific biotinylation of the avi tag through the prokaryotic enzyme BirA. In contrast to the western blot retardation assay published by Petris et al. [20], which also employs BirA for determining the intracellular localization of proteins but requires preincubation with streptavidin, detection in our assay format is achieved, after SDS-PAGE and blotting, through fluorescently labeled streptavidin, thereby circumventing mutual steric hindrance of two distinct reagents detecting the C-terminal HA and avi tags. Moreover, we found that the direct detection of biotinylated proteins via streptavidin was more sensitive than detection of the HA tag via an antibody, presumably due to differences in reagent affinities to the tag. Despite the robustness of the biotinylation activity of BirA, biological processes may still interfere with the accurate determination of the internalized cargo that reaches the cytosol, such as the observation by us and others that the HA tag may become undetectable inside cells over time [37], presumably through proteolysis.

We used consensus DARPins for comparison and found that Ec1-ETA(252–412) delivered all cargoes efficiently, and for Ec1-ETA(252–608) low efficiencies in general were observed. The low efficiency of ETA(252–608) in comparison to ETA(252–412) was somewhat surprising to us, particularly because low efficiencies were also observed in the absence of cargo for ETA(252–608), i.e., by using the (enzymatically inactive) domain translocated in the natural toxin in its natural context. The successful delivery NI_3C via the ETA translocation domain does suggest that high stability is not a limiting factor in this system, an observation which is in agreement with the previously observed delivery of GFP, also a stable molecule, via this domain [25].

In contrast, for the anthrax toxin, there was a clear cutoff of stability: MBP–PA_m–Ac2 in combination with LF delivered only NI_1C efficiently. The fact that this is not a size- but a stability-issue was shown by destabilizing NI_2C and NI_3C by design. The finding that LF could only translocate moderately stable cargoes is in agreement with previous studies that found that ligand-stabilization of dihydrofolate reductase and diphtheria toxin chain A inhibited their translocation [45]. Liao et al. very recently found that a DARPin with two internal repeats (an N2C DARPin) previously selected against c-jun N-terminal kinases [15] was apparently translocated efficiently via anthrax toxin [43]. It should be noted that the parental 'consensus' DARPins used in the present study have been optimized for stability [29,46], and thus library members with randomized residues are less stable, and N2C DARPins are less stable than N3C. Conversely, even though this is only a single data point, it may suggest that some N2C DARPins may not even need engineering to be transported with the anthrax toxin.

The direct comparison of delivery of the same cargo through multiple mechanisms, performed within this study, allowed us to discover distinct dependencies on cargo stability for the translocation domain of ETA as compared to the anthrax pores made from protective antigen. For both toxins, it has been proposed previously that cargo unfolding is required in order for translocation to occur: at the molecular level, this process is better understood for lethal factor translocation by anthrax toxin [47,48] than it is for ETA, where the evidence is more circumstantial. The requirement for unfolding of ETA is supported by the involvement of the endoplasmic reticulum-associated degradation (ERAD) machinery [49,50] and its physical association with the Sec61 translocon [51,52]. Our observation that the ETA-associated delivery mechanism has the capacity to deliver more stable cargoes than anthrax toxin leads to the conclusion that anthrax toxin's own unfolding machinery appears to have a more limited unfolding capacity, while ETA exploits the more forceful cellular unfolding and translocation machinery.

Although the limited translocation ability of the anthrax pores means that very stable DARPins cannot be translocated, a rational destabilization rescues their ability to be translocated to the cytoplasm. We found that lower thermodynamic stability of the cargo leads to an improved translocation only up to a certain point, suggesting a cutoff stability point beyond which a further destabilization of the protein does not increase translocation efficiency anymore. This limit probably indicates the stability below which the protein can be unfolded for translocation. For example, for NI_3C, a leucine-to-alanine mutation in each repeat was not sufficient to restore translocation, while a leucine-to-glycine mutation in each repeat was sufficient, and further destabilization beyond three leucine-to-glycine mutations did not prove more helpful for cytosolic delivery as determined by our assay. Importantly, through the rational destabilization of the DARPin framework by the introduction of hydrophobic cavities, which are expected to lead to only modest structural arrangements, it is highly probable that selected DARPins can be rendered anthrax -translocation-competent through the introduction of one or more of the destabilizing mutations, and would show improved delivery. Alternatively, one might start selections already with libraries of mildly destabilized DARPins, thereby obviating the need to reconfirm binding activity after introducing framework mutations.

Regarding the cytosolic delivery efficiency of bacterial toxins in comparison with other protein transport systems, two independent comparisons between an ETA-based systems or an anthrax-based systems with prototypical cell-penetrating peptides (CPPs) were recently reported. In one of these studies, Mohammed et al. [25] found that the delivery of eGFP to the cytosol was much better in a construct containing the translocation domain ETA and R10 as compared to a fusion of eGFP with only R10. Similarly, Liao et al. [43] recently reported that, whereas anthrax toxin efficiently delivered various LF fusion proteins to the cytosol of CHO-K1 cells, no cytosolic delivery via tat could be measured as determined via digitonin extraction. Although this indicates that cytosolic delivery via bacterial toxins is more efficient than via classical CPPs, it still remains to be tested how the bacterial toxins compare to the use of CPPs that have been combined with endosomal escape functionalities [53].

With respect to cell-type specificity, prototypical CPPs such as tat, polyarginine and penetratin enter virtually all cells tested, presumably through non-specific charge-based interactions [54], though novel variants may target certain receptors specifically [55]. In contrast, the design of our systems is inherently cell-specific, though the degree of cell-specificity that can be obtained with our transport system is a function of the specificity of the cell-targeting moiety and the lack of extracellular interactions of the cargo protein.

A potential disadvantage of using (parts of) protein toxins in delivery vehicles is their immunogenicity. However, major strides have been made in the deimmunization of recombinant immunotoxins, particularly those based on *Pseudomonas* exotoxin A [56,57]. Strikingly, most of the translocation domain can be removed while still retaining high toxicity of the catalytic domain when added externally [56,57]. Currently, studies are ongoing that aim to determine whether shorter, potentially less immunogenic fragments of the ETA translocation domain retain similar abilities to translocate cargo into the cytosol.

To conclude, in this study we have established an approach for the easy comparison of cytosolic delivery of binding proteins and applied this method for assessing the value of various bacterial toxin-derived translocation mechanisms for delivering heterologous cargo into the cytosol. We identified two mechanisms, derived from ETA and anthrax toxin, that lead to high nanomolar intracellular concentrations of binding proteins with distinct thermodynamic requirements for the cargo that can be accommodated. In addition, we show that DARPins can be rationally and gently destabilized in order to restore efficient translocation via anthrax toxin.

Acknowledgments

W.P.R.V. was supported by a Rubicon Grant (NWO 825.12.002) from the Netherlands Organisation of Scientific Research. This research was also supported by the EU Innovative Medicines Initiative COMPACT (grant 115363), and an ERC Advanced Grant NEXTBINDERS (both to A.P.). The funders had no role in study design, execution of the experiments or interpretation of the results.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2014.12.019.

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