Introduction

The delivery of molecules through the plasma membrane into cells is a major technical obstacle for the development of diagnostic tools and therapeutic agents acting on intracellular targets. In many cases, molecules cannot penetrate cellular membranes due to their biophysical properties. This is especially true for peptides and proteins containing polar or even charged side chains. One way to achieve internalization is the fusion of cargos to ligands of cell-surface receptors. Following receptor–ligand interaction, the cargo is taken up into endosomal compartments by receptor-mediated endocytosis [1, 2]. After endosomal escape, the therapeutic agent, for instance, can inhibit a given target protein by blocking its active site. However, this method requires the covalent coupling of a ligand to the cargo which often leads to altered properties with respect to ligand affinity as well as to the activity of the cargo. Moreover, this targeted approach is highly restricted to certain cell types that express a certain cell-surface receptor and to the internalization properties of the targeted receptor.

An alternative way to induce cell membrane permeability of the cargo is its linkage to protein transduction domains (PTDs), also known as cell penetrating peptides. A PTD is a short peptide comprising an amino acid sequence, which enables it to induce its own internalization into a variety of eukaryotic cell types. Examples of well-studied cationic PTDs are the Tat13 peptide of the HIV-1 trans-activator protein (YGRKKRRQRRRPP) [3–7], penetratin, also known as Ant16 (RQIKIWFQNRRMKWKK) [7, 8] and Ant7 (RMRKWKK) [7], both derived from the homeodomain of the Drosophila melanogaster Antennapedia protein, or polyarginines like R9 [7, 9, 10]. Since their discovery in 1988 [3, 4], a broad range of bioactive molecules has been delivered by PTDs into cells both in vitro and in vivo [11]. This technology is thus considered to bear enormous potential to introduce macromolecular therapeutics into cells [12, 13]. Notably, PTDs can also penetrate intact...
epidermis and dermis, which enables the transdermal delivery of cargos upon topical application [14–16].

However, the genetic fusion of PTDs to cargos can result in reduced protein expression and purification levels [7]. Moreover, the genetic or chemical linkage of PTDs can impair the biophysical properties of the cargo [7]. Therefore, attempts have been made to generate universal cell permeable transporters that allow the concomitant internalization of cargos by non-covalent binding of ligand-fused cargos. Cargos fused to streptavidin (SA), an extracellular homotetrameric protein secreted by the bacterium *Streptomyces avidinii* [17], were delivered into cells by PTD-biotin [18, 19], on the basis of the high affinity of the SA/biotin interaction [20]. *Vice versa*, SA linked to the Tat-PTD was generated as a transporter for biotinylated proteins [21, 22]. However, biotinylation requires chemical linkage and may be inefficient for some cargos, leading to the biotinylation of only a small portion of the cargo. Furthermore, it cannot be excluded that biotinylation of a cargo or its fusion to a scaffold, like SA, may impair its activity. To overcome possible difficulties due to chemical modifications of the cargo, Futaki *et al.* synthesized a cell-permeable nickel-nitrilotriacetic acid (Rs-Ni-NTA) for polyhistidine tagged recombinant proteins [23].

As an alternative for polyhistidine/Ni-NTA, the *Strep*-tag II system has become very common for one-step purification of proteins or high-affinity detection [24]. The *Strep*-tag II peptide (WSHPQFEK) can be easily fused to recombinant proteins during DNA subcloning without the necessity of cross-linking. Moreover, *Strep*-tag II is regarded as biologically inert, proteolytically stable and does usually not interfere with membrane translocation or protein function [24]. Notably, *Strep*-tag II exhibits intrinsic affinity (Kd of 1 μM) towards *Strep*-Tactin (ST), a derivative of SA [25].

Here, we engineered and biochemically characterized a series of PTD-ST transporters which we named Transtactins (derived from transduction and *Strep*-Tactin) (schematic model in Fig. 1). We tested their ability to internalize several cargos into human cells which included (i) FITC-*Strep*-tag II, a cell-impermeable small molecule, (ii) His6-*Strep*-tag II complexed with Ni-NTA-fused horseradish peroxidase (HRP), a proteinaceous multicomponent complex and (iii) biotin-HRP, a biotin-linked model compound. Cellular uptake and intracellular distribution of Transtactins and Transtactin-linked cargos were analysed. HRP activities were determined to test whether cargo proteins remain functional upon intracellular delivery by Transtactins.

### Materials and methods

#### Construction, expression and purification of PTD-*Strep*-Tactin

*Escherichia coli* strain TG2 was used as host for cloning. The pET-21a plasmid (Novagen, Darmstadt, Germany) encoding core SA was kindly provided by P.S. Stayton. ST was expressed from the same vector backbone after QuikChange mutagenesis (Stratagene, Heidelberg, Germany) of the SA portion with primers 5'-G ACC GCT TAC ATG GCC AGG GCT AAG GCT GAA GCT GGT ATC ACC GGC ACC-3' for Ant16-ST, 5'-GGT GAA TTC CAT ATG CTT GCT GGT ATC ACC GCC ACC-3' for Ant17-ST, 5'-G GAA TTC CAT ATG CAG ATT AAG ATT TGG TTC CAG AAC CGC CGC ATG AAG TGG AAG AAG for R9-ST (bold letters denote the respective PTD sequences), and reverse primer 5'-GGC CAT ATT GG TTT CAG ATG TGA ACC AAG AAG AAG AAG AGA AGA AAG CCG AGA CAA AGA CAA CAA AGA AGA CCG AGA AGA AGA AGA CCG AGA AGA AGA AGA GCC AGA GCC GCC GCC GCC GCC GCC GCC ACC ACC ACC ACC-3' for Rs-ST (bold letters indicate mutations to induce amino acid substitutions E41I, S52G and V62R which convert SA into ST [25]). PTD-ST fusions were generated by PCR-amplification using *Tag* DNA polymerase (Invitrogen, Karlsruhe, Germany), primers 5'-G GAA TTC CAT ATG CTC CAG ATT AAG ATT TGG TTC AAG AAG AAG GGT GCT GAA GCT GGT ATC ACC GCC ACC-3' for Ant16-ST, 5'-GGT GAA TTC CAT ATG CTT GCT GGT ATC ACC GCC ACC-3' for Ant17-ST, 5'-G GAA TTC CAT ATG CAG ATT AAG ATT TGG TTC CAG AAC CGC CGC ATG AAG TGG AAG AAG GCT GAA GCT GGT ATC ACC GCC ACC-3' for Rs-ST (bold letters denote the respective PTD sequences), and reverse primer 5'-GAG AAT TGA ATT TGA ATT TGG TTT CAG ATG TGA ACC AAG AAG AAG AAG AAG AAG AGA AGA AAG AAG CCG AGA CAA AGA CAA CAA AGA AGA CCG AGA AGA AGA AGA CCG AGA GCC GCC GCC GCC GCC GCC GCC GCC ACC ACC ACC ACC-3' for Rs-ST. Crude peptides were purified by C18 reversed phase high-performance liquid chromatography and analysed by MS. Peptide stocks in DMSO (Merck, Darmstadt, Germany) were stored at –80°C and freshly diluted in H2O.

#### Peptide synthesis

FITC-*Strep*-tag II and His6-*Strep*-tag II peptides were chemically synthesized at the Peptide Synthesis Core Facility of the German Cancer Research Center (Heidelberg, Germany) using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. Crude peptides were purified by C18 reverse phase high-performance liquid chromatography and analysed by MS. Peptide stocks in DMSO (Merck, Darmstadt, Germany) were stored at –80°C and freshly diluted in H2O.

#### Cell culture

HeLa, SiHa and U-2 OS cells were cultured in Dulbecco’s minimal essential medium (DMEM, Gibco, Eggenstein, Germany), supplemented with 10% foetal bovine serum (FBS, PAA Laboratories, Pasching, Austria), 1% penicillin streptomycin sulphate and 1% of 200 mM L-glutamine (Sigma-Aldrich, Taufkirchen, Germany), at 37°C in 5% CO2 atmosphere.

#### Preparation of PTD-ST complexes

(PTD-)ST proteins were complexed with FITC-*Strep*-tag II, biotin-HRP (Pierce, Rockford, IL, USA), or His6-*Strep*-tag II and Ni-NTA-HRP (Qiagen, Hagen, Germany) by incubation for 15 min. at room temperature. The complexes were directly injected into FBS-free DMEM media of cultured cells.

#### Edman sequencing

Aliquots of protein samples were spotted on trifluoroacetic acid (TFA)-treated filters (Applied Biosystems, Darmstadt, Germany), dried under
nitrogen stream and introduced into a cartridge of an ABI Procise 494 Sequencer (Applied Biosystems) followed by N-terminal Edman sequencing performed with a pulsed-liquid program.

MALDI-TOF mass spectrometry

Prior to MS, protein samples were desalted using C4 ZipTip pipette tips (Millipore, Bedford, MA, USA). Briefly, ZipTips were prewashed with 0.1% TFA (Pierce)/50% acetonitrile (Roth, Karlsruhe, Germany) and equilibrated with 0.1% TFA by repetitive pipetting steps. After loading of the protein samples, ZipTips were washed three times with 0.1% TFA to remove salts. For MALDI-TOF MS 0.3 μl of a saturated solution of sinapinic acid (Bruker-Daltonics, Bremen, Germany) in ethanol were deposited as thin film onto individual spots of a MALDI target plate. Subsequently, proteins were eluted from the ZipTips with 1–2 μl of a saturated solution of sinapinic acid in 0.1% TFA/50% acetonitrile, directly loaded on top of the thin film spots and allowed to co-crystallize slowly at ambient temperature. MALDI mass spectra were recorded in the positive ion linear mode with delayed extraction on a Reflex II TOF instrument (Bruker-Daltonics) equipped with a SCOUT-26 probe and a 337 nm nitrogen laser. Ion acceleration voltage was set to 20.0 kV and the first extraction plate to 17.1 kV. Mass spectra were obtained by averaging up to 200 individual laser shots. Spectra were calibrated externally by a quadratic fit using the singly protonated average masses of ubiquitin I at m/z 8565.89, cytochrome c at m/z 12,361.55 and myoglobin at m/z 16,952.55 (Protein Calibration Standard I, Bruker Daltonics).

Thermal tetramer stability

Three micrograms of purified proteins were combined with SDS-containing sample buffer and heated at selected temperatures for 5 min., then chilled on ice and subsequently analysed by SDS-PAGE. Proteins were stained with Coomassie brilliant blue (Serva, Heidelberg, Germany).

CD spectroscopy

CD spectroscopy was carried out using a J-710 spectropolarimeter (JASCO, Gross-Umstadt, Germany) calibrated with a solution of 0.05% β-androsterone dissolved in dioxane. Sample temperature control during measurement was achieved through use of a JASCO PFD-350S Peltier thermostat. Samples at a concentration of ~100 μg/ml protein in distilled water were scanned in a 1 mm quartz cuvette from 190 to 240 nm for secondary structure determination. Final spectra were the result of four-fold signal averaging. Thermal denaturation spectra were run from 40°C to 95°C at a gradient of 1.0°C/min. Care was taken to ensure that all relevant parameters were identical for the several proteins measured, since properties such as the melting temperature (Tm) are not absolute and vary with measurement conditions. Due to the unusual spectral characteristics of SA and related proteins, the observational wavelength for temperature denaturation was chosen to be 215 nm rather than the standard 222 nm. Secondary structure content was calculated from the far UV CD spectra after subtracting an identically scanned and signal-averaged solvent baseline.

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After their conversion to mean residue ellipticity (θ_{mean}) and removal of residual noise through a fast Fourier transform program, the program PEP-FIT [27] (specifically designed for the secondary structure analysis of peptides rather than globular proteins) was employed for fitting of the processed spectra. The relative Tm of SA and its derivatives was obtained from the denaturation curves using the JASCO Denatured Protein software.

Western blotting

Three micrograms of protein extracts were combined with SDS-containing sample buffer and boiled at 95°C for 5 min., separated by 15% SDS-PAGE, transferred in a semi-dry blotter system (cti, Idstein, Germany) to an Immobilon-P membrane (Millipore) and analysed by enhanced chemiluminescence (GE Healthcare, Munich, Germany). The following antibodies were used: rabbit anti-SA antibody (Sigma-Aldrich, 1:2000), mouse anti-tubulin antibody CP06 (Calbiochem, Schwalbach, Germany, 1:5000) and antimouse and anti-rabbit HRP-labelled secondary antibodies (Promega, Mannheim, Germany, 1:3000).

Fluorescence microscopy

HeLa cells were plated on 35 mm dishes (Greiner Bio-one, Frickenhausen, Germany) at 40% to 60% confluency. 10 μM of (PTD-)ST proteins were complexed with 10 μM FITC-Strep-tag II and directly injected into FBS-free media. After a 120-min. incubation at 37°C, 5% CO₂, the cells were washed, trypsinized, plated on glass cover-slips and fixed with 4% paraformaldehyde (Merck) after 4 hrs. Cover slips were stored at −20°C in 70% ethanol. ST was detected using a rabbit anti-SA antibody (1:500) and an anti-rabbit Cy3-labeled secondary antibody (Dianova, Hamburg, Germany, 1:400). Endosomes were visualized with the fluid-phase marker TRITC-dextran (Invitrogen), as described [22]. Cy3-, FITC- and TRITC-signals were detected using a Vanox-T fluorescence microscope (Olympus, Hamburg, Germany) and an F-View camera (Olympus).

HRP assay

HeLa cells were plated on 35 mm dishes at 80% to 90% confluency and treated with 1 μM (PTD-)ST complexes with 1.5 μM His6-Strep-tag II and 2 μM Ni-NTA-HRP or with 2 μM biotin-HRP. Cells were incubated at 37°C, 5% CO₂ for 2 hrs, then trypsinized, washed with 1× PBS and lysed with 400 μl 1× reporter lysis buffer (RLB, Promega). Cell lysates were incubated at room temperature for 15 min. After vortexing and pelleting, the supernatants were stored at −80°C. Extracts were diluted and 40 μl thereof were mixed with 10 μl 1× RLB. At the same time, and under identical conditions, calibration curves were generated using a series of dilutions of ST complexed with His6-Strep-tag II and Ni-NTA-HRP, or biotin-HRP. Complexes were diluted and 10 μl thereof were supplemented with 40 μl of dilutions of untreated HeLa lysates. Colorimetric reactions were initiated by adding 50 μl of 1-Step Ultra TMB-ELISA substrate solution (Pierce). The reaction was stopped by adding 50 μl of 2 M sulphuric acid (Merck) and the absorbance was measured at 450 nm. Calibration curves were fitted using the four parameter logistic (4PL) equation in SigmaPlot 10.0 (Systat Software, Erkrath, Germany). The absorbance was recalculated to the amount of internalized HRP and normalized to the amount of total protein determined by Bradford protein assay as described [28].

Results

Production, purification and characterization of PTD-ST fusion proteins

For generating different Transtactin variants, Ant16, Ant7, Tat13 and R9 PTDs were fused in frame to the 5’ end of the ST coding sequence and subcloned into pET-21a. Individual Transtactins, as well as unfused SA and ST, were expressed as cytosolic inclusion bodies in E. coli. SDS-PAGE analyses of bacterial whole cell extracts indicated that all recombinant proteins were expressed in full length (data not shown). Apart from Ant16-ST which remained denatured, all proteins could be refolded and purified in high yields of over 20 mg/l expression volume by fractionated ammonium sulphate precipitation (data not shown). Proteins were >95% pure as determined by SDS-PAGE analysis (Coomassie-staining, data not shown).

MALDI-TOF MS analyses of purified proteins detected molecular weights as expected for their theoretical masses (MH⁺, Table 1). All PTD-ST fusion proteins started with a methionine. In the case of unfused SA or ST, the N-terminal methionines were removed (Table 1), as verified by MALDI-TOF MS analysis and Edman sequencing (data not shown), most likely due to the activity of E. coli methionine aminopeptidase [29].

Thermal tetramer stability

SA and ST tetramerization [25] is required for building the binding pocket for biotin and Strep-tag II [30]. Both SA and ST tetramers are extremely stable, even in the presence of SDS, and therefore can be detected on SDS-PAGE [31, 32]. Since instability of transporters can restrict their use, the influence of different PTD fusions on the temperature-dependent ST tetramer breakup was studied, as exemplified for Tat13-ST in Fig. 2(A). Up to a temperature of 60°C, all proteins were detectable in a tetrameric state (Fig. 2B). Unfused ST exhibited a slightly reduced tetrameric stability when compared to unfused SA (60°C versus 65°C). Notably, all PTD fusions slightly increased the tetramer stability of ST: Ant7-ST showed a tetramer stability of 65°C, Tat13-ST and R9-ST tetramers were stable up to 70°C (Fig. 2B). At further increased temperatures, the amount of tetramers started to decrease for all proteins, with concomitant appearance of monomeric forms (Fig. 2).

Secondary structure analysis

Since PTD fusions can influence the biophysical properties of cargos [7], far UV CD spectroscopy was performed to assess possible alterations in the secondary structure of ST due to the N-terminal PTD-fusions. The spectra of all Transtactins showed an almost identical curve progression as unfused ST (Fig. 3A), similar to the CD spectrum of SA. CD spectra were interpreted using
The fractions of secondary structure were compared with the crystal structures of SA [33] and ST [34] (Fig. 3B). Both the line shapes of the CD spectra and the PEPFIT data indicated that all proteins share similar \( \beta \)-sheet content of approximately 44.5% to 54% and \( \alpha \)-helix rates up to 7% (Fig. 3B). These minor differences can be explained by the N-terminal PTD-fusions or slight skewing of the analyses of CD spectra due to the need to compensate for the unusual pronounced positive peaks at 230 nm [35] (most likely one lobe of an exciton interaction between aromatic side chains [36, 37]). Thermal unfolding analyses also revealed that the conformational stabilities of individual Transtactins were comparable, exhibiting high melting temperatures (Tms) which ranged from 74.63°C for R9-ST to 76.46°C for Tat13-ST (Table 1).

### Internalization of PTD-ST

To investigate the ability of Transtactin proteins to internalize into mammalian cells, HeLa cells were incubated with different...
concentrations of Tat13-ST for 2 hrs. Titration experiments revealed that internalized Transtactins could be detected by immunoblotting upon external application of doses as low as 100 nM (data not shown). In order to test whether PTD-mediated internalization is cell-type restricted, cultured HeLa and SiHa cervical carcinoma as well as U-2 OS osteosarcoma cells were incubated for 2 hrs with 1 μM of different Transtactin variants or unfused ST. Subsequently, whole cell protein extracts were prepared and analysed by immunoblotting. In contrast to unfused ST, all Transtactins were internalized at readily detectable levels. Tat13-ST and R9-ST reproducibly exhibited a more efficient internalization than Ant7-ST, under identical experimental conditions (Fig. 4).

**Intracellular distribution of internalized Transtactins**

Next, we analysed the intracellular distribution of internalized Transtactins. HeLa cells were treated with 10 μM of Tat13-ST or R9-ST for 2 hrs. Tat13-ST and R9-ST proteins were detected in almost 100% of the cells (data not shown), either in a punctuated pattern around the cell nucleus or cytosolically solubilized with nuclear exclusion (Fig. 5A). This pattern is consistent with previous reports investigating internalization of PTD-fused cargos which accumulated in the cytosol and in distinct perinuclear inclusions which were subsequently identified as endosomal compartments [21, 22].
Transtactin-mediated co-internalization of FITC-Strep-tag II

In order to investigate whether Transtactins were able to co-internalize a small model molecule linked to Strep-tag II, HeLa cells were treated for 2 hrs with Tat13-ST or R9-ST complexed with FITC-Strep-tag II. We observed that FITC-Strep-tag II was clearly internalized by both Transtactins (Fig. 5B). In contrast, cells control-treated with FITC-Strep-tag II alone or FITC-Strep-tag II complexed with unfused ST exhibited only background FITC fluorescent signals (Fig. 5B). Staining pattern for FITC-Strep-tag II inside cells was punctual, indicating enrichment in distinct cellular compartments. These signals co-stained with the fluid-phase endosomal marker TRITC-dextran [22] (Fig. 5B), indicating that FITC-Strep-tag II is enriched in endosomal compartments, consistent with previous findings for PTD-linked cargos [22, 38].

Internalization of peptides, proteins and complexes by Transtactins

Next, we tested whether Transtactins were also able to deliver Strep-tag II-fused peptides, proteins and multicomponent complexes into cells. To this end, Tat13-ST or R9-ST Transtactins were complexed with His6-Strep-tag II and Ni-NTA-HRP. In this scenario, the Ni-NTA-HRP portion should be non-covalently linked to the Transtactins via His6-Strep-tag II. Two hours following treatment of HeLa cells with these three-component complexes, high enzymatic HRP activities were measured in cellular lysates (Fig. 6A). Cells incubated with Ni-NTA-HRP alone (data not shown), or with a three-component complex of unfused ST, His6-Strep-tag II and Ni-NTA-HRP (Fig. 6A), showed only background enzymatic HRP activities.

The capacity of Transtactins to internalize proteins in a functional form was further confirmed by using biotin-HRP as a cargo under identical experimental conditions as described for the internalization of Ni-NTA-HRP. Again, high enzymatic HRP activities were measured in HeLa cells treated with complexes of Tat13-ST or R9-ST, and biotin-HRP (Fig. 6A). In contrast, cells incubated with biotin-HRP alone (data not shown) or with a complex of unfused ST with biotin-HRP (Fig. 6A) showed only background HRP activities. The concentrations of internalized HRP ranged from 8 to 15 pmol/mg (Fig. 6B).

Discussion

Cell permeable ST derivatives, termed Transtactins, were developed as a universal internalization system for Strep-tag II- or biotin-linked compounds. Cargos successfully internalized included FITC-Strep-tag II as a model compound for a small cell-impermeable cargo, His6-Strep-tag II complexed with Ni-NTA-HRP as a model for a multicomponent proteinaceous complex, and biotin-HRP as a model biotinylated cargo. The introduction of Strep-tag II-fused cargos revealed that a Kd of 1 μM is sufficient for Transtactin-mediated internalization. Transtactin-introduced HRPs were enzymatically active, showing that the system allows the internalization of functionally active proteins.

Transtactin proteins were produced as insoluble inclusion bodies in E. coli. Apart from Ant16-ST which remained denatured and could not be refolded, all proteins could be purified by fractionated ammonium sulphate precipitation [26]. The expression of Transtactins as inclusion bodies thus seems to prevent the common problems of both reduced expression levels and purification yields for PTD-fused cargos [7] since high amounts of over 20 mg/l purified proteins for the Transtactins were obtained.

The fusion of Tat13, Ant7 and R9 to ST only slightly altered its secondary structure, indicating that ST is an extremely rigid protein scaffold. This is an important requirement to retain the Strep-tag II-binding property of ST, following PTD fusion. SA and ST tetramerization [25] is essential to correctly form the biotin- and Strep-tag II-binding pocket which lies in the interface between SA and ST subunits [30]. High thermal tetramer stabilities were measured for all engineered Transtactin variants, as reported for SA [31, 32], indicating that Transtactins are stable within a wide temperature range and that no cooling at the bench or during storage and shipping is required.
All Transtactins were able to efficiently induce their own internalization into human cell lines derived from epithelial (HeLa, SiHa) or connective tissues (U-2 OS), in line with reports showing that PTD-linked compounds penetrate into a wide range of cell types [11]. Titration experiments revealed that internalization of Transtactins could be detected upon external application of doses as low as 100 nM. The precise uptake mechanism of PTD-linked cargos is currently a topic of lively discussion. However, in the case of cationic PTDs, it is most likely that the positively charged side chains interact with the anionic structure at the cell surface leading to an increased local concentration of PTDs, subsequently allowing cellular entry by a fluid-phase endocytotic mechanism via endosomal compartments [38]. Consistent with this proposed mechanism, Transtactins were found either diffusely distributed...
in the cytoplasm or in endosomal compartments as previously shown for Tat-SA [21, 22]. Notably, Transtactins were even able to internalize a proteinaceous complex including Ni-NTA-HPR or biotinylated HRP with a total MW of ~220 kD. The internalization efficiencies of HPR ranged between 8 and 15 pmol/mg total protein which is within the same range as reported for Tat-biotin-internalized FITC-SA (~23 pmol/mg) [28] with a total MW of only ~60 kD. As previously reported [9, 10], we found that internalization efficiencies increased with rising numbers of positively charged residues within the PTD portion (Ant7/H11021/Tat13/H11021/R9) of the different Transtactin variants.

The application of Transtactins is straightforward. Only a short incubation step of the transporter with the cargo in buffer conditions is necessary for complex formation. Then, the complex is injected into serum- and biotin-free medium of cultured cells. Free biotin in the cell culture medium can be removed with avidin, a biotin-binding protein that does not interfere with Strep-tag II [24]. No further treatment or expensive laboratory equipment is required for internalization.

Transtactin transporters possess several advantages over the existing cargo delivering system Tat-SA [21, 22]. Firstly, the Strep-tag II/ST system has become very common for purification of various cargos. Strep-tag II can be easily fused to the N- or C-terminus of any kind of recombinant protein during subcloning [24] using Strep-tag II-expression vectors which are available for various host organisms [24]. Thus, no elaborate chemical linkage, as in the case of biotin, is necessary. Secondly, biotinylation often requires spacer arms between biotin and the cargo to reduce sterical hindrance which can interfere with binding to SA. Typical spacers, such as a 6 carbon spacer [39], often reduce the solubility.
of the cargo in aqueous media which is problematic for in vivo applications where organic solvents cannot be used. In contrast to biotin, Strept-tag II requires, if at all, only a short two amino acid spacer to ensure accessibility [24]. Thirdly, unlike Strept-tag II [24], biotinylation may disturb cargo functions due to the chemical linkage.

Transtactins also should have advantages over the introduction of cargos which are directly linked to a PTD. Firstly, PTD-fused cargos often suffer from reduced expression and purification levels [7] in contrast to Strept-tag II cargos prepared for internalization by Transtactins. Secondly, PTD-fused agents have been shown to penetrate intact skin [14–16] indicating that they could pose a health risk for experimentators working with potentially hazardous compounds. The fact that Transtactins and the cargo can be produced separately increases the bio-safety during preparation since ST has no reported biological functions apart from biotin-binding properties and the cargo cannot penetrate into cells without the transporter. Thirdly, by employing suitable linkers, the internalization of alternatively tagged cargos is also conceivable, e.g. using biotin-Ni-NTA linkers [40–42] for His6-fused proteins.

In summary, Transtactins meet the requirements for universal delivery systems for Strept-tag II-fused cargos. They thus fill the remaining gap between unspecific traditional transfection methods, like lipofection and cell-surface receptor-mediated endocytosis of ligand-fused cargos. Possible applications of Transtactins range from basic proteomics research to high-throughput testing of intracellularly active diagnostics [7, 43] and non-cell permeable therapeutic peptides [44, 45] and proteins [46–48]. Moreover, it could be envisioned to develop Transtactins as transporters for therapeutically useful cargos in human beings. However, at present, this latter issue still faces technical hurdles associated with protein therapeutics in general which include proteolytic instability of PTDs [49] and antigenicity of SA [50]. Possible solutions to reduce the potential antigenicity of Transtactins include site-directed mutagenesis of solvent-exposed side chains, as shown for SA [51], or replacement of ST by non-immunogenic scaffolds, like human-derived lipocalins [52, 53], engineered to bind protein tags, such as Strept-tag II or His6.

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