A Protein Transduction Domain with Cell Uptake and Selectivity Profiles that Are Controlled by Multivalency Effects

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SUMMARY

Protein transduction domains (PTDs) are reagents that facilitate the delivery of diverse cargo to the interior of mammalian cells. We identified a PTD called “Ypep” (N-YTFGLKTSFNVO-C), with cell penetration selectivity and potency profiles that are tightly controlled by multivalency effects. Pentavalent display of Ypep on M13 bacteriophage enables selective uptake of this phage in PC-3 human prostate cancer cells at low picomolar concentration and in the presence of human blood. All Ypep-dependent delivery is nontoxic and proceeds through energy-dependent endocytosis. Collectively, our results establish Ypep-displaying phage as a cell-penetrating platform with selectivity and potency profiles that compare to, or exceed, antibodies and their fragments. Our findings may have broader implications on the design of PTD technologies generated from phage display, as well as the use of Ypep-displaying phage as a prostate cancer cell-selective delivery platform.

INTRODUCTION

Macromolecular biopolymers, nanometer-sized materials, and bacteriophage offer unique opportunities for advances in bio-imaging and medicine (Boisselier and Astruc, 2009; Ghosh et al., 2012; Leader et al., 2008; McGregor, 2008; Thorek et al., 2006). Perhaps the most important impediment to their broader use is the difficulties associated with the potent delivery of these reagents across the lipid bilayer membrane of mammalian cells (Torchilin and Lukyanov, 2003). Various technologies have been developed that address challenges in the delivery of large cargos to mammalian cells. These generally include liposomes (Gregoriadis, 1995), lipid-linked compounds (Zelphati et al., 2001), fusons to cell-surface receptor ligands (Gabel and Foster, 1986; Rizk et al., 2009), and supercharged proteins (Cronican et al., 2010, 2011; McNaughton et al., 2009).

One promising approach for the delivery of macroscopic cargo to mammalian cells is attachment with a protein transduction domain (PTD). PTDs are short peptides (typically <20 amino acids) that promote the internalization of cargo in mammalian cells (Deshayes et al., 2005; Ford et al., 2001; Måe and Langel, 2006). Although PTDs promote general transmembrane transport, virtually every strategy to identify and combat disease can be improved by selective delivery of cargo specifically to diseased cells. The most common method for targeting cargo to a specific cell is through fusion to a monoclonal antibody or its fragment antigen-binding region (Fab fragment), which binds a receptor present on the targeted cell. Although such immunoconjugate-targeted therapies are often cell-selective, they do not directly address the problem of cell internalization. Further, their production, distribution, and storage are very costly (Hughes, 2010; Wu and Senter, 2005). Unfortunately, commercially available and commonly used polycationic PTDs, such as the HIV-1 transactivator of transcription (Tat) peptide (Frankel and Pabo, 1988; Green and Loewenstein, 1988), the Drosophila Antennapedia-derived penetratin peptide (Derossi et al., 1994), and polyarginine (Dubkovskaya et al., 2008; Fuchs and Raines, 2005), although simple to use, do not selectively penetrate diseased cells, and their potency of uptake is relatively moderate to poor.

An ideal delivery platform would combine the cell selectivity of antibodies with the membrane transport capability and ease of production of PTDs. As part of a broader research program focused on new methods to identify (image) and treat prostate cancers, we set out to generate a prostate cancer cell-selective PTD. The prostate-specific membrane antigen (PSMA) is a membrane-bound glycoprotein that is restricted to the prostate. Previously, studies analyzing the expression of PSMA have found an upregulation in correlation with prostate cancer (Bostwick et al., 1998; Lapidus et al., 2000). Unsurprisingly, targeted delivery of cargo to prostate cancer cells often relies on antibodies, Fab fragments (Henry et al., 2004; Nanus et al., 2003; Patri et al., 2004), or other ligands, such as aptamers (Dassie et al., 2009), that bind PSMA. However, the expression level of PSMA varies drastically among prostate cancer cells, and many prostate cancer cells do not express PSMA (Murphy et al., 1998; Wright et al., 1995). Therefore, in addition to targeted delivery platforms, which extend beyond the antibody paradigm, reagents that selectively target PSMA-negative prostate cancer cells are required.

Bacteriophage (phage) evolution has been used to identify PTDs not found in nature (Gao et al., 2002; Ivanenkov et al., 2002; Koo et al., 2004; Gao et al., 2009; Lim et al., 2009).
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1999; Kim et al., 2006; Nomura et al., 2007). We began by performing consecutive rounds of positive selections to enrich phage that penetrate PC-3 cells, a human prostate cancer cell line with high metastatic potential (Pulukuri et al., 2005) that does not express significant levels of PSMA (PSMA-neg) (Ghosh et al., 2005), and remove phage that do not penetrate the four off-target cell lines described below. After three rounds of positive and negative selection, only phage with modest potency of uptake and very poor cell selectivity were enriched. Changing the order of the selection (positive to negative or negative to positive) did not change the outcome. One possible reason for this undesired outcome is the selection strategy itself. In this scheme, “winners” may be phage with poor uptake efficiencies in both targeted and untargeted cells. This phage would be expected to barely survive both the positive and negative selection.

Based on our initial results, we modified the positive and negative selection to enrich for phage that potently penetrate targeted PC-3 cells and remove polycationic PTD, which should not be cell selective. From this selection, we identified phage that display five copies of a linear 12-amino-acid peptide on the N terminus of phage minor coat protein 3 (p3). This peptide is referred to as “Ypep” (N-YTFGLKTSSFVNQ-C) throughout. Our data show that multivalency effects dramatically tune the potency and, perhaps more importantly, the cell selectivity of Ypep uptake. When cells are treated with a solution containing 5 μM GFP equipped with a single N-terminal Ypep peptide (Ypep-GFP), ~4×, ~5×, and ~8-fold more Ypep-GFP penetrate targeted PC-3 cells than off-target LNCaP (PSMA-positive prostate cancer [Israel et al., 1994]), Hs 697.Sp (human spleen) cells, and HEK293T (human embryonic kidney), respectively. We observe ~40% more GFP in targeted PC-3 cells following treatment with 5 μM Ypep-GFP, compared to off-target MRC-9 human lung cells. The N termini and C termini of folded GFP are proximal and are therefore spatially arranged in such a way as to facilitate multivalent interactions between the termini of GFP and a receptor, making this protein well suited to examine the role multivalency effects play in uptake. When Ypep is fused to the N termini and C termini of GFP (Ypep-GFP-Ypep), the potency of GFP delivery increased ~29-fold in targeted PC-3 cells. Perhaps more importantly, multivalency effects also control the cell selectivity of cell penetration. The cell selectivity profiles of Ypep-GFP and Ypep-GFP-Ypep for targeted PC-3 cells over off-target LNCaP, HEK293T, and Hs 697.Sp cells were similar. However, ~3.8-fold more Ypep-GFP-Ypep penetrated PC-3 cells than off-target MRC-9 cells. GFP bearing two that display five copies of Ypep on its N terminus ((Ypep)5-GFP) penetrates PC-3 cells with similar potency. M13 phage

RESULTS AND DISCUSSION

Phage Display In Vitro Evolution of a Protein Transduction Domain Platform Targeting PC-3 Prostate Cancer Cells

As summarized in Figure 1, the selection involved incubating the phage library with a monolayer of PC-3 prostate cancer cells. In order to assure that only internalized phage were selected and enriched, as opposed to phage that simply bound to the surface of PC-3 cells, an extensive washing protocol was developed from components of numerous literature reports (Gao et al., 2002; Ivanenkov et al., 1999; Kim et al., 2006). Media containing phage without affinity for PC-3 cells was removed, and the cells were washed five times with PBS and then three times with Tris-buffered saline (TBS)/0.1% Tween-20 solution. Cells were then treated with a solution containing 3 mg/ml subtilisin in TBS for 40 min at 4 °C, which removed any remaining cell-surface-bound phage by proteolytic degradation of the phage coat proteins and/or human cell surface proteins involved in the phage-cell interaction. Following the subtilisin treatment, cells were washed three times with PBS containing a protease inhibitor cocktail and pelleted by centrifugation. Titering from the final PBS wash solution after subtilisin treatment showed that no cell-surface-bound phage were present, indicating that the washing scheme removed all phage that did not penetrate the
cells (Supplemental Experimental Procedures). Cells were then lysed with a solution previously shown to lyse human cells, but not phage (Ivanenkov et al., 1999). Phage present in cell lysate was amplified in Escherichia coli.

In order to remove phage that contain polycationic PTDs, which would likely penetrate cells nonselectively, or phage that simply bind the tissue culture plate, phage enriched from the positive selection were incubated for 1 hr with a vacuum-gas plasma-treated tissue culture plate, which contains a monolayer of carboxylic acids on the tissue culture plate surface and is therefore negatively charged at physiological pH. Only phage remaining in the solution, and not bound to the culture plate surface, were enriched. Coupled rounds of positive and negative selections were performed three times. After the final negative selection round, enriched phage were amplified in E. coli and grown as single plaques on an agar plate. Single plaques were isolated and grown in Luria Broth (LB), and phage was isolated and precipitated using standard methods. The sequence of evolved PTDs was identified using standard DNA sequencing methods. The most abundant sequence enriched from this selection was “Ypep” (N-YTFGLKTSFNVQ-C); little sequence homology was observed among the evolved peptide sequences. Among these enriched peptides, Ypep was determined to be the most cell-selective and potent PTD.

Determining the Potency of Ypep-GFP Uptake
We prepared a fusion protein consisting of an N-terminal Ypep sequence, a (GGS)_4 linker, and super-folder GFP (sfGFP). This fusion protein is referred to as “Ypep-GFP” throughout. In many ways, GFP is an ideal protein to test the characteristics of a PTD. For example, GFP is simple to express, is stable, and is easy to image (Tsien, 1998). Although obviously not of human origin, the molecular weight (27 kDa) and net theoretical charge at physiological pH (~6) of GFP are within the average range among human proteins expressed in E. coli (Cronican et al., 2011). Like most proteins, wild-type GFP does not penetrate human cells (Supplemental Experimental Procedures). Finally, GFP and its variants, or proteins with similar biophysical characteristics, have been used for various in vitro and in vivo bioimaging applications (Filonov et al., 2011; Shaner et al., 2004).

PC-3 cells were treated with 0.5, 1, 5, or 10 μM Ypep-GFP. Following treatment, this solution was removed, and the cells were washed with a solution previously shown to remove surface-bound protein (Cronican et al., 2010, 2011; McNaughton et al., 2009; Meade and Dowdy, 2008), trypsinized, and assayed for internalized Ypep-GFP by cytometry. As shown in Figure 2A, we observed a concentration-dependent increase in Ypep-GFP levels in PC-3 prostate cancer cells. Flow cytometry data were collected in three separate experiments; ∼42- and ∼86-fold more cell fluorescence is observed in cells following treatment with 5 or 10 μM Ypep-GFP, respectively. Fluorescence microscopy images show internalized Ypep-GFP in PC-3 cells following treatment with 10 μM Ypep-GFP (Figure 2B). In contrast, no appreciable fluorescence was observed in cells treated with 10 μM GFP, then washed, and imaged using the same conditions (Supplemental Experimental Procedures). Taken together, these data validate the PTD selection.

Determining the Effect Multivalency Has on the Potency of Ypep-Dependent Delivery
Although we were pleased to observe that a single copy of Ypep delivers GFP to PC-3 prostate cancer cells, we were somewhat surprised by the modest potency of cellular uptake. We reasoned that because selections involve phage, which present Ypep on the N terminus of five separate copies of phage minor coat protein 3, multivalent display might be needed in order to fully realize highly potent Ypep-dependent delivery. Multivalency effects play critical roles in numerous biological processes
(Mammen et al., 1998) and have been shown to contribute to the mechanism of uptake for a previously reported PTD (Moss et al., 2005). The N termini and C termini of GFP are in close proximity and have similar directionality (Supplemental Experimental Procedures). Therefore, GFP is well suited to serve as a protein scaffold and test the dependency of multivalent effects on uptake. Toward this end, we prepared a fusion protein with Ypep on the N termini and C termini of GFP (Ypep-GFP-Ypep).

PC-3 cells were treated with solutions containing 0.1–10 μM Ypep-GFP-Ypep, were washed with a solution shown to remove cell-surface-bound protein, were trypsinized from the tissue culture plate, and internalized GFP levels were measured in three separate experiments by flow cytometry. As shown in Figure 2C, we observed a concentration-dependent increase in Ypep-GFP-Ypep delivery in PC-3 cells, with a dramatic increase in the potency of uptake. This large difference in uptake potency was confirmed by comparing microscopy images of cells treated with 10 μM Ypep-GFP (Figure 2B) to cells treated with the same concentration of Ypep-GFP-Ypep (Figure 2D). A ~29-fold increase in GFP fluorescence was observed in PC-3 cells treated with 0.5 μM Ypep-GFP-Ypep, compared to PC-3 cells treated with the same concentration of Ypep-GFP. PC-3 cells treated with 0.5 μM Ypep-GFP-Ypep exhibited ~3-fold higher GFP fluorescence compared to cells treated with 5 μM Ypep-GFP. Precise spatial orientation of Ypep does not appear to play a prominent role in the potency of uptake, at least in the context of GFP. A GFP fusion protein containing two copies of Ypep on the N terminus (Ypep)2-GFP has similar potency of uptake (Supplemental Experimental Procedures). Taken together, these data suggest an important role for multivalency in the potency of Ypep-dependent delivery.

**Comparing the Effect Bivalent Display of Ypep, Tat, and Penetratin Has on the Potency of GFP Uptake**

Intrigued by the dramatic increase in the potency of GFP uptake in PC-3 cells, as a result of bivalent display of Ypep, we set out to measure if this observation is universal among commonly used and commercial PTDs. We prepared GFP fusion proteins that contain either a single N-terminal fusion with Tat or penetratin PTDs (referred to as Tat-GFP and Pen-GFP, respectively). In addition, we prepared GFP fusion proteins that contain N- and C-terminal Tat or penetratin (referred to as Tat-GFP-Tat and Pen-GFP-Pen, respectively). Like Ypep-GFP and Ypep-GFP-Ypep fusion proteins, in each case, Tat or penetratin is separated from GFP through a (GGSG)6 linker. Polyarginine is a commonly used PTD (Dubikovskaya et al., 2008); unfortunately, all attempts to express and purify (Arg)29-GFP and (Arg)29-GFP-(Arg)6 failed.

PC-3 cells were treated with solutions containing 5 μM Ypep-GFP, Tat-GFP, or Pen-GFP. This concentration was chosen because it was the lowest concentration that resulted in relatively high levels of Ypep-GFP delivery. After such time, cells were washed three times with a PBS solution containing 20 U/ml heparin sulfate to remove any cell-surface-bound protein. Cells were trypsinized from the tissue culture plate, and GFP fluorescence was measured by flow cytometry. As seen in Figure 3A, treatment with this concentration of fusion protein resulted in the delivery of comparatively high levels of Ypep-GFP and Pen-GFP to PC-3 cells. In contrast, appreciable levels of Tat-GFP did not penetrate PC-3 cells at the concentration tested. Next, we incubated PC-3 cells with solutions containing 100 nM Ypep-GFP, Tat-GFP-Tat, or Pen-GFP-Pen. This concentration was chosen because it was the lowest concentration that resulted in relatively high levels of Ypep-GFP-Ypep delivery. As shown in Figure 3B, although bivalent display of Ypep resulted in a ~20-fold increase in GFP delivery, the same effect was not observed for Pen-GFP-Pen and Tat-GFP-Tat fusions. In contrast to Ypep-GFP-Ypep, bivalent display of penetratin does not increase uptake. Previous reports have shown that 4–5 copies of Tat and as many as 10–50 copies of penetratin are required for a significant increase in the potency of uptake, compared to the potency of delivery observed for a monomeric fusion (Tseng et al., 2002). In addition, multivalent dendrimer-PTD conjugates have been reported to increase potency of uptake compared to a monovalent species (Li et al., 2009; Sung et al., 2006). However, these species typically contain >20 copies of the PTD per dendrimer. In contrast, we observe a dramatic increase in the potency of delivery for GFP fusions containing two copies of
Ypep-GFP, we observed ciable selectivity of delivery. Following treatment with 5 μM Ypep-GFP, Ypep-GFP-Ypep, (Ypep)2-GFP, and (Ypep) 5-additive delivery, we hypothesized that those same effects may contribute to the cell selectivity of uptake. We compared the delivery to the potency of Ypep-GFP. Given the role multivalency plays in the potency of Ypep-dependent delivery of GFP.

**Determining the Cell Selectivity of Ypep-Dependent Delivery**

Given the role multivalency plays in the potency of Ypep-dependent delivery, we hypothesized that those same effects may contribute to the cell selectivity of uptake. We compared the delivery of Ypep-GFP, Ypep-GFP-Ypep, (Ypep)2-GFP, and (Ypep)5-phage in PC-3 human prostate cancer cells (PSMA-neg), LNCaP human prostate cancer cells (PSMA-pos), HEK293T human embryonic kidney cells, MRC-9 human lung fibroblast cells, and Hs 697.Sp human spleen fibroblast cells. The potency and cell selectivity of Ypep-GFP and Ypep-GFP-Ypep delivery was measured by flow cytometry. Phage titering from cell lysate was used to compare the amount of internalized phase in each cell line.

**Cell Selectivity of Ypep-GFP Delivery**

Cells were treated with solutions containing 0.5–5 μM Ypep-GFP and then washed three times with a PBS solution containing 20 U/ml heparin sulfate to remove any cell-surface-bound protein. After removal from the tissue culture plate, internalized GFP levels were measured by flow cytometry.

As shown in Figure 4, a single copy of Ypep endows appreciable selectivity of delivery. Following treatment with 5 μM Ypep-GFP, we observed ~4-, ~8-, and ~5-fold more internalized GFP in targeted PC-3 cells compared to off-target LNCaP, HEK293T, and Hs 697.Sp cells. High levels of internalized GFP were found in off-target MRC-9 cells; only ~1.4-fold more fluorescence was observed in targeted PC-3 cells. Taken together, these data demonstrate that monomeric Ypep is moderately selective for PC-3 cells.

**Cell Selectivity of Ypep-GFP-Ypep Delivery**

We compared cell penetration of Ypep-GFP-Ypep in PC-3, LNCaP, HEK293T, MRC-9, and Hs 697.Sp cells. Cells were treated with solutions containing 0.1–5 μM Ypep-GFP-Ypep and then washed three times with a PBS solution containing 20 U/ml heparin sulfate to remove any cell-surface-bound protein. Cells were trypsinized from the tissue culture plate, and cell fluorescence was measured by flow cytometry.

As shown in Figure 5, we observed higher levels of cell selectivity for Ypep-GFP-Ypep delivery in targeted PC-3 cells, as compared to monomeric Ypep-GFP. Like Ypep-GFP, Ypep-GFP-Ypep was taken up by targeted PC-3 cells, and much lower levels of internalized GFP were observed in off-target LNCaP, HEK293T, and Hs 697.Sp cells. However, unlike Ypep-GFP, which was taken up well in both targeted PC-3 and off-target MRC-9 cells, Ypep-GFP-Ypep showed a ~4-fold preference for PC-3 cells over MRC-9 cells. Taken together, these data demonstrate an important role for multivalency in the cell selectivity of delivery.

**Cell Selectivity of (Ypep)5-Phage Delivery**

We tested the selectivity of (Ypep)5-phage delivery in PC-3, LNCaP, HEK293, MRC-9, and Hs 697.Sp cells by comparing the titer of phage from lysate of each cell after incubation and washing steps. Cells were treated with 5 ml of F12K/10% FBS containing 1.0 × 10^9 plaque-forming units (pfu)/ml of (Ypep)5-phage. This equates to a solution that is 1.7 μM in phage. Thus, cell penetration at this concentration indicates very high potency. After incubating the phage with cells, cell-surface-bound phage was removed, and cells were lysed as described previously. In addition to titering cell lysate, aliquots from each final washing solution were titrated to ensure that all surface-bound phage was removed before cell lysis. No phage was found in any of the final washing solution (Supplemental Experimental Procedures). In contrast, (Ypep)5-phage potently penetrate PC-3 prostate cancer cells (PSMA-neg); high levels of (Ypep)5-phage plaque-forming units per milliliter (pfu/ml) were found from...
PC-3 cell lysate. However, appreciable levels of (Ypep)_5-phage plaques were not observed from the lysate of LNCaP (PSMA-pos), HEK293T (kidney), Hs 697.Sp (spleen), and MRC-9 (lung) cells (Figure 6). Experiments were independently performed in triplicate. Appreciable levels (>25 pfu/ml) of phage were never observed in E. coli treated with cell lysate from LNCaP (PSMA-pos), HEK293T (kidney), Hs 697.Sp (spleen), or MRC-9 (lung) cells. In contrast, in each experiment >1,500 pfu/ml were observed in E. coli treated with PC-3 cell lysate. Although appreciable, but significantly lower, levels of Ypep-GFP and Ypep-GFP-Ypep were found in off-target LNCaP and MRC-9 cells, and much lower levels of (Ypep)_5-phage were not found in the lysates of these off-target cells, compared to targeted PC-3 cells. This represents a dramatic change in the cell selectivity of Ypep-dependent delivery. Although these data suggest that multivalency effects likely play a role in the cell selectivity of uptake, the architecture of Ypep display in the context of fusion to GFP and fusion to the N terminus of phage coat protein p3 differ greatly. We cannot rule out the possibility that these architectural changes may play an important role in the cell selectivity profiles we observe. Nonetheless, the cell selectivity and potency profiles displayed by bivalent Ypep-GFP fusions and (Ypep)_5-phage make Ypep well suited for targeted bioimaging applications, as well as phage-based approaches to biomedical science.

**Determining the Mechanism of Ypep-GFP, Ypep-GFP-Ypep, and (Ypep)_5-Phage Internalization**

The majority of known PTDs have high positive net charge. In contrast, Ypep has a theoretical net charge of +1. In addition, we have shown that Ypep displays interesting multivalency-dependent behavior. Given the characteristics of Ypep, we were very interested in establishing the mechanism of internalization for Ypep-GFP, Ypep-GFP-Ypep, and (Ypep)_5-phage.

Unlike experiments conducted at 37°C, cell penetration was not observed when PC-3 cells were cooled to 4°C before and during treatment with either 15 μM of Ypep-GFP or 10 μM Ypep-GFP-Ypep (Figures 7B and 7H, respectively). These results suggest that cell penetration of Ypep-GFP and Ypep-GFP-Ypep requires an energy-dependent process, consistent with endocytosis (Meade and Dowdy, 2008). We next evaluated the cell penetration of each Ypep variant under conditions that block a particular component of an endocytotic pathway. PC-3 cells were incubated with relatively high concentrations of Ypep-GFP or Ypep-GFP-Ypep to assure that changes in cell fluorescence were due to inhibition of cell penetration as opposed to relatively small changes in uptake of low concentrations of GFP fusions that may or may not be associated with inhibition of endocytosis. When PC-3 cells were pretreated with PBS containing 5 μg/ml filipin, a small molecule known to inhibit lipid-raft/caveolae-dependent endocytosis (Schnitzer et al., 1994), and then incubated with a solution containing 5 μg/ml filipin and either Ypep-GFP or Ypep-GFP-Ypep, much lower cell fluorescence was observed compared to cells that were not treated with the inhibitor molecule (Figures 7C and 7I, respectively). In addition, pretreating cells with 5 μg/ml cytochalasin D, an actin polymerization inhibitor (May et al., 1998), followed by incubating those cells with 5 μg/ml cytochalasin D and either Ypep-GFP or Ypep-GFP-Ypep significantly decreased cell penetration (Figures 7D and 7J, respectively). Internalization of caveolae requires disruption of the local actin cytoskeleton (Head et al., 2006; Shen and Turner, 2005). Therefore, inhibition of cell penetration as a result of lipid-raft/caveolae-dependent endocytosis and actin polymerization supports the conclusion that Ypep-GFP and Ypep-GFP-Ypep penetration of PC-3 cells is mediated by lipid-raft/caveolae-dependent endocytosis. Internalization of Ypep-GFP and Ypep-GFP-Ypep is inhibited by 400 μg/ml heparin sulfate (Figures 7F and 7L, respectively). Based on previous studies (De Coupade et al., 2005; Mano et al., 2005), this suggests that internalization requires interaction(s) with one or more glycosaminoglycans on the surface of PC-3 cells.

We next applied the conditions shown in Figure 7, and described above, to PC-3 cells treated with (Ypep)_5-phage. Unlike Ypep-GFP and Ypep-GFP fusions, which can be removed using a relatively simple washing procedure, and for which internalized protein can be immediately imaged, experiments with phage require a relatively substantial and lengthy washing procedure in order to assure that all phage are removed from the cell surface and only internalized phage are isolated. Unfortunately, following treatment with filipin, cytochalasin D, and chlorpromazine, PC-3 cells did not withstand the washing protocol. We observed significant loss of cells throughout the experiment due to cytotoxicity. Therefore, we were unable to obtain meaningful data from those experiments. Because of this, studies on the mechanism of (Ypep)_5-phage penetration in PC-3 cells were limited to conditions that were not cytotoxic to the cells during the course of the experiments.

Unlike experiments conducted at 37°C, which yielded high levels of phage from cell lysate, no phage were isolated when PC-3 cells were cooled to 4°C before and during treatment with (Ypep)_5-phage. This result suggests that cell penetration of (Ypep)_5-phage requires an energy-dependent process, consistent with endocytosis (McNaughton et al., 2009; Meade and Dowdy, 2008). In addition, internalization of (Ypep)_5-phage was inhibited by heparin sulfate (Supplemental Experimental Procedures), suggesting that internalization requires interaction(s) with one or more glycosaminoglycans on the surface of PC-3 cells. The ability to significantly decrease or completely abrogate the levels of Ypep-GFP, Ypep-GFP-Ypep, and (Ypep)_5-phage by suppressing particular endocytotic pathways further supports
the conclusion that these protein fusions are internalized under normal conditions, which lack modulators of endocytosis.

**Determining the Cytotoxicity and Robustness of Ypep-Dependent Delivery**

To assess the cytotoxicity of Ypep variants under conditions required for appreciable uptake, we performed (3-(4,5-Dime- medicine)-2,5-diphenyltetrazolium bromide assay (MTT) on PC-3 cells after treatment with 0.5, 1, or 5 μM Ypep-GFP or Ypep-GFP-Ypep, or 1.7 pM (Ypep)5-phage. These assays revealed no apparent cytotoxicity to PC-3 cells for any of the Ypep variants (Supplemental Experimental Procedures).

Phage selections were performed in a complex solution consisting of F12K cell culture media and 10% fetal bovine serum (FBS). In order for a PTD to be used in vivo, it must penetrate the target cell in the presence of a complex solution, such as blood. We treated PC-3 cells with either 10 μM Ypep-GFP or Ypep-GFP-Ypep in F12K/10% FBS solution containing 50% human blood. Cells were then washed as described, and red blood cells were removed using standard methods. Cell fluorescence was measured by flow cytometry. Ypep-GFP-Ypep, but not Ypep-GFP, penetrates PC-3 cells in a solution containing human blood. In addition, when PC-3 cells were treated with 1.7 pM (Ypep)5-phage in a F12K/10% FBS solution containing 50% human blood, appreciable levels of phage were found in cell lysate (Supplemental Experimental Procedures). Taken together, these data suggest that multivalent Ypep-dependent

potency and selectivity for targeted PC-3 prostate cancer cells is tightly controlled through multivalency. When a single copy of Ypep is attached to the N terminus of GFP (Ypep-GFP), this fusion protein penetrates a diverse set of human cells with moderate potency and poor cell selectivity. When Ypep is attached to the N termini and C termini of GFP (Ypep-GFP-Ypep), potency increases ~9- to 29-fold over the same set of human cells, and cell selectivity increases. Although the same approximate level of Ypep-GFP is found in PC-3 (PSMA-neg) and MRC-9, ~4-fold more Ypep-GFP-Ypep is found in PC-3 cells over a range of concentrations. However, phage that display five copies of Ypep ((Ypep)5-phage) very potently and selectively penetrate PC-3 cells. When a diverse set of human cells are treated with media containing 10% FBS solution that is 1.7 pM in (Ypep)5-phage, these phage penetrate PC-3 cells, but not LNCaP (prostate cancer, PSMA-pos), HEK293T (kidney), Hs 697.Sp (spleen), and MRC-9 (lung) cells. Collectively, our results reveal Ypep as a PTD capable of delivering protein (GFP) or nanometer-sized cargo (phage), with cell-penetrating properties that are tightly controlled through multivalency. Both Ypep-GFP-Ypep and (Ypep)5-phage penetrate cells in the presence of human blood. All Ypep-dependent cell penetration is non-cytotoxic at conditions required for uptake and proceeds via endocytosis. Both Ypep-GFP and Ypep-GFP-Ypep penetrate PC-3 cells through caveolae/lipid-raft-mediated endocytosis.

We anticipate that (Ypep)5-phage may be useful as a potent and robust platform for the targeted delivery of cargo to prostate...
cancer tumors bearing PC-3 cells. (Ypep)₅-phage can be genetically engineered to display biopolymer imaging reagents and/or therapies on various phage coat proteins (p7, p8, and/or p9). Although functional therapeutic delivery will require release of phage from endosomes, phage can also be engineered to orthogonally display endosomolytic peptides, which have been shown to facilitate the disruption of endosomes from within various cells (Han et al., 2011; Lundberg et al., 2007; Meyer et al., 2008; Oishi et al., 2007). In addition, given that we observed no obvious change in the mechanism of internalization for each Ypep variant, identifying the cell surface receptor(s) for Ypep-GFP, Ypep-GFP-Ypep, and (Ypep)₅-phage on PC-3 cells, as well as the sequence dependence on cellular uptake, will be important for understanding the basis of changes in cell selectivity and uptake potencies. Progress toward this end will be reported in due course.

**SIGNIFICANCE**

Protein transduction domains (PTDs) offer a straightforward method for the delivery of biopolymer and nanoscale reagents to the interior of mammalian cells. Although functionally simple, most PTDs promote general transmembrane transport and are not cell selective. Cell-selective recognition is typically achieved through the use of antibody-based immunon conjugates; however, these reagents are relatively difficult and expensive to prepare and do not directly address the problem of internalization. In this work, we identified a PTD with uptake, cell selectivity, and potency profiles that are tightly controlled through multivalency effects. We have shown that single attachment of Ypep to GFP promotes uptake of that fusion protein, with relatively modest cell selectivity and potency. Double fusion of Ypep to the N termini and C termini of GFP increased the potency of uptake for that fusion protein to levels beyond those explained by simple additive effects, suggesting a role for multivalency in cell uptake of Ypep variants. These bivalent display-dependent changes in potency and cell selectivity demonstrated by Ypep were not observed with Tat or penetratin fusion protein to levels beyond those explained by simple additive effects, suggesting a role for multivalency in the mechanism of internalization for each Ypep variant, identifying the cell surface receptor(s) for Ypep-GFP, Ypep-GFP-Ypep, and (Ypep)₅-phage on PC-3 cells, as well as the sequence dependence on cellular uptake, will be important for understanding the basis of changes in cell selectivity and uptake potencies. Progress toward this end will be reported in due course.

**Experimental Procedures**

**Materials**

- PBS, 0.25% trypsin, Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 medium, B-PER Bacterial Protein Extraction Reagent, a Modified Lowry Protein Assay Kit, and a Pierce Firefly Luciferase Glow Assay Kit were purchased from Thermo Scientific (Waltham, MA, USA). Cellgro F-12K medium was purchased from MediaTech (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Westborough, MA, USA). cComplete Mini Protease Inhibitor Cocktail Tablets were purchased from Roche (Indianapolis, IN, USA). TACS MTT reagent was purchased from Trevigen (Gaithersburg, MD, USA). The Ph.D.-12 Phage Display Library, Nco I HF, and KpnI HF were purchased from New England Biolabs (NEB) (Ipswich, MA, USA). Sodium deoxycholate, heparin sulfate, and imidazole were purchased from Sigma-Aldrich (St. Louis).

Mammalian Cell Culture

Human prostate adenocarcinoma cells (PC-3) cells were cultured in F12K with 10% fetal bovine serum (FBS). Human prostate carcinoma cells (LNCaP) and human embryonic lung fibroblasts (MRC-9) cells were cultured in RPMI-1640 media with 10% FBS; human spleen fibroblasts (Hs 697.Sp) and HEK293ST cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), PC-3, LNCaP, and MRC-9 cells were incubated at 37°C with 5% CO₂ environment. Hs 697.Sp cells were incubated at 37°C with 10% CO₂ environment. All cells were obtained from the American Type Culture Collection.

**Phage Selection Positive Selection**

A 10 ml solution of F12K/10% FBS containing 5 x 10ⁱ² phage library members (Ph.D.-12 Phage Display Library, NEB) was added to ~80% confluent PC-3 cells grown as a monolayer in a T25 culture flask and incubated at 37°C under 5% CO₂ environment for 3 hr. After incubation, cells were then placed on ice for 5 min and washed with 4°C PBS five times while on ice. Cells were then washed three times with 4°C Tris-buffered saline (TBS)/0.1% v/v Tween-20 for 3 min each while on ice. The remaining surface-bound phage was proteolyzed by addition of a 5 ml TBS/subtilisin (3 mg/ml) for 45 min at 37°C. After lysis, internalized phage was amplified in E. coli and 0.15 ml of tetracycline, 250 mg/ml tetracycline, and 0.15 ml of E. coli (ER2837) that had been grown to optical density (OD) = 0.5. The final wash solution (200 µl) or the cell lysate was then added, and this solution was incubated at 37°C, 250 rpm for 5 hr. E. coli was pelleted for 5 min at 10,000 rpm and 4°C. Supernatant containing phage was transferred to another tube, and E. coli cell debris was pelleted at 10,000 rpm for 10 min at 4°C. Phage from the supernatant was precipitated by addition of 5 ml PBS/subtilisin (3 mg/ml) for 45 min at 37°C. Cells were then transferred into a 15 ml plastic tube and pelleted for 5 min at 3,000 rpm and 4°C. Supernatant was removed, and cells were resuspended in 1 ml of PBS and pelleted for 5 min at 3,000 rpm and 4°C. Supernatant was removed and saved as the last wash solution for subsequent titering. Cells were lysed with 0.5 ml of lysis buffer (2% sodium deoxycholate, 10 mM Tris-HCl, and 2 ml EDTA) and 0.5 ml of TBS for 1 hr at room temperature. After cell lysis, internalized phage was amplified in a 150 ml flask containing 30 ml LB, 300 µl 0.1 M CaCl₂, 20 µg/ml tetracycline, and a Modified Lowry Protein Assay Kit, and a Pierce Firefly Luciferase Glow Assay Kit were purchased from Thermo Scientific (Waltham, MA, USA). Cellgro F-12K medium was purchased from MediaTech (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Westborough, MA, USA). cComplete Mini Protease Inhibitor Cocktail Tablets were purchased from Roche (Indianapolis, IN, USA). TACS MTT reagent was purchased from Trevigen (Gaithersburg, MD, USA). The Ph.D.-12 Phage Display Library, Nco I HF, and KpnI HF were purchased from New England Biolabs (NEB) (Ipswich, MA, USA). Sodium deoxycholate, heparin sulfate, and imidazole were purchased from Sigma-Aldrich (St. Louis).

Ni-NTA agarose resin was purchased from QIAGEN (Hilden, Germany). The Ph.D.-12 Phage Display Library, Nco I HF, and KpnI HF were purchased from New England Biolabs (NEB) (Ipswich, MA, USA). Sodium deoxycholate, heparin sulfate, and imidazole were purchased from Sigma-Aldrich (St. Louis).

Human blood was purchased from Innovative Research (Novi, MI, USA).

**Instrumentation**

Fluorescence microscopy images were taken on an EVOS fluorescence inverted microscope from the Advanced Microscopy Group (AMG). MTT assay readings were taken on a Synergy Mx microplate reader from BioTek Instruments (Winooksi, VT, USA). Flow cytometry experiments were performed on a MoFlo (Dako Colorado, Fort Collins, CO, USA) flow cytometer using a solid-state iCyt 488 nm (blue) laser to measure GFP fluorescence.
Plasmid Construction
All constructs were cloned into pET plasmids. DNAs encoding cell-penetrating peptide fusions with stGFP were assembled using oligonucleotide overlap gene construction and PCR.

Protein Purification
BL21-DE3 E. coli were typically grown in 500 ml LB cultures at 37°C to OD600
= 0.6 and induced with 1 mM IPTG at 30°C overnight. Cells were then pelleted by centrifugation and lysed with 25 ml B-PER. Cell lysate was cleared by centrifugation (17,000 rpm, 30 min), and supernatant was mixed with 1 ml of Ni-NTA agarose resin for 1 hr at 4°C under agitation. Resin was collected by centrifugation (4,950 rpm, 10 min). Ni-NTA agarose resin was washed with 50 ml of PBS containing 300 mM NaCl and then 50 ml of PBS containing 20 mM imidazole. Protein was then eluted with 5 ml PBS containing 300 mM NaCl and 500 mM imidazole. Eluted protein was dialyzed against PBS and analyzed for purity by SDS-PAGE followed by staining with Coomassie Blue. Protein concentrations were measured using a modified Lowry Protein Assay Kit.

Mechanism of Cell Penetration
PC-3 cells were grown to ~80% confluence in a 12-well tissue culture plate. Cells were then washed once with PBS and incubated with the small molecule inhibitor in PBS for 10 min at 37°C under 5% CO2 environment. The PBS-small-molecule solution was then removed, and a PBS solution containing 15 μM Ypep-GFP or 10 μM Ypep-GFP-Ypep was added. Cells were incubated with each PBS/protein solution for 3 hr at 37°C under 5% CO2 environment and then washed twice with PBS and three times with PBS-HS (heparin sulfate 20 U/ml) for 10 min each at 37°C. Cells were then removed from the tissue culture plate by addition of 0.5 ml of 0.25% trypsin and pelleted by centrifugation. Cell pellet was resuspended in PBS/10% FBS, and cell fluorescence was analyzed by flow cytometry.

Cell Selectivity Experiments
For experiments involving Ypep-GFP and Ypep-GFP-Ypep, cell penetration was measured using flow cytometry, as described above (see Flow Cytometry). For cell selectivity experiments involving (Ypep)_5-phage, cells were grown to ~80% confluence and then treated with 5 ml of media supplemented with 10% PBS and 1 x 10^9 pfu/ml (Ypep)_5-phage for 3 hr at 37°C and 5% CO2 environment. Cells were washed and lysed as previously described (see Phage Selection). An aliquot of the final wash solution was kept for titration. Following cell lysis (see Phage Selection), aliquots from cell lysate and the last wash before lysis were titrated. Titration was carried out as described above (see Phage Selection). After this time, the entire E. coli mixture was plated on IPTG/X-gal LBagar plates and incubated at 37°C for 18 hr.

MTT Cell Viability Assay
Assays were performed following the provided instructions. Briefly, PC-3 cells were grown to ~80% confluence in a 12-well tissue culture plate. Cells were then washed once with PBS and incubated with 0.5–5 μM Ypep-GFP or Ypep-GFP-Ypep in PBS for 3 hr at 37°C under a 5% CO2 environment. Cells were washed three times with PBS-HS (200 μl heparin sulfate) and then incubated with 0.5 ml media containing 25 μl of MTT reagent for 4.5 hr. After such time, a 250 μl detergent reagent was added to the cells, and they were incubated for an additional 30 min at 37°C under a 5% CO2 environment. Absorbance was measured at 570 nm on a Synergy Mx microplate reader. Cell viability of cells treated with (Ypep)_5-phage was determined after a 3 hr incubation with 5 ml of 1 x 10^9 pfu/ml Ypep-phage (1.67 pfu) in F2K medium supplemented with 10% PBS at 37°C under a 5% CO2 environment. PC-3 cells were washed twice with PBS, and the MTT assay was performed as described above.

Ypep-GFP and Ypep-GFP-Ypep Internalization in the Presence of Human Blood
PC-3 cells were grown to ~80% confluence in a 6-well plate. Whole blood was diluted in half with F12K/10% FBS. To this solution either Ypep-GFP or Ypep-GFP-Ypep was added to final concentrations of 10 μM. Cells were incubated with these solutions for 1 hr, washed once with PBS, and then washed twice with a red blood cell lysis buffer (0.15 M NaCl, 0.01 M KHCO3, and 0.0001 M EDTA [pH = 7.7]) to remove all red blood cells. Cells were washed three times with PBS-HS (20 mM heparin sulfate) and imaged on an EVOS FL fluorescence microscope.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.01.015.

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Multivalency Effects Control Cell Uptake


