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Identification of targeting peptides for mucosal delivery in sheep and mice

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ABSTRACT

In this study we identified and characterized a novel cyclic peptide that facilitates the rapid transportation of conjugated molecules across the epithelial layer of the small intestine. The peptide was initially selected from phage display libraries using a large animal experimental model which employed consecutive in vitro and in vivo panning. The procedure was designed to enrich for peptides that facilitated transcytosis across the intestinal epithelium into the intestinal afferent lymphatic system. A small set of peptides was repeatedly isolated using this selection method,

however the cyclic nonamer CTANSSAQC, 13C dominated. The activity of the putative targeting peptide C13 was then verified using a mouse model.

These experiments showed that the 13C peptide as well as macromolecules conjugated to it were rapidly transported across the intestinal mucosa into distinct subsets of epithelial cells and CD11+ cells located in the lamina propria and Peyer's Patches. Significant amounts of intact protein could be delivered into the systemic circulation after rectal and nasal application. Thus, peptide 13C is regarded as an attractive carrier candidate for mucosal delivery of large molecules. The preferential targeting to distinct intestinal cells may be utilized to deliver active biological drugs for the effective control of diseases of the gut.

KEYWORDS

Phage display, transcytosis, lymphatic cannulation, sheep, mucosal uptake, protein delivery

INTRODUCTION

While most diseases including inflammatory diseases or tumors are localized and display tissue-specific features, drugs to combat these diseases are usually applied via systemic routes. This implies a potential loss of treatment selectivity and efficacy. Similarly, vaccines elicit quantitatively and qualitatively different immune responses depending on the route of application. For example, gastrointestinal administration of proteins, in the absence of adjuvants, preferentially induces immunological tolerance ("oral tolerance")[1], but adjuvant-including enteric vaccines are superior in inducing IgA responses which are important to prevent infections with

gastrointestinal pathogens. However, targeting delivery of effective vaccines into the immune-inductive sites of the intestine remains a significant challenge for the pharmaceutical industry.

Novel delivery of drugs via the gastrointestinal tract or airways is convenient for patients, although to date not efficient for non-permeating agents, notably protein based biologics or vaccines. The efficiency of mucosal delivery is compromised by potential degradation of proteins and peptides in the gastrointestinal tract, by the thickness of the mucus layer as well as by the low transport capacity of epithelial cells for uncleaved macromolecules. New treatments that enable tissue-specific delivery of bioactive agents would therefore offer significant benefits compared to systemic delivery. The aim of the present study was to develop a new delivery vehicle that would facilitate the rapid transportation of macromolecular agents upon mucosal administration.

The epithelial layer is composed of epithelial cells connected by tight junctions. It functions as a physical barrier that prevents environmental agents from penetrating into host tissue. The intestinal epithelium facilitates the directed transport of nutrients, ions, lipids and, to some degree, also macromolecules. In addition, specialized cells in the epithelial layer overlaying Peyer´s patches (PP) and isolated lymphoid follicles, the M (microfold) cells, are known for their high uptake capacity for luminal antigens and even particulate matter[2, 3]. Moreover, recent studies have shown that goblet cells, besides their known capacity to secret mucins, are able to sample peptides from the lumen of the small intestine[4].

Phage display technology has been widely used for in vitro and in vivo screening of tissue specific markers and peptides[5]. The method has been successfully employed to dissect biological processes, such as homing to vascular tissue in tumors[6-9], cancer metastasis[8, 10-12], synovial

targeting[13-15] and receptor identification[16, 17]. Extensive screening of phage display libraries has also been used to identify ligands that facilitate translocation across the mucosa of the gastrointestinal tract[18] or binding to and transport via M cells[19, 20] or goblet cells[21, 22].

The aim of this study was to identify and characterize novel peptides that are actively transcytosed via the intestinal mucosa. These peptides were selected from phage display libraries by consecutive in vitro and in vivo panning using a large animal experimental model. The selection method led to the identification of the cyclic nonamer CTANSSAQC (13C) peptide. The activity of this putative targeting peptide was then verified using a mouse model. We found that 13C as well as 13C-conjugated macromolecules were rapidly transcytosed via the intestinal mucosa in both sheep and mouse models. Peptide-protein conjugates were taken up by, and enriched in, both epithelial and dendritic cells located in the lamina propria and PP and additionally delivered into systemic circulation via the lymph. Thus, peptide 13C is regarded as an attractive carrier candidate for the mucosal delivery of proteins or other macromolecular agents in sheep, mice and possibly other animals.

EXPERIMENTAL SECTION

1.   Animals.

All sheep experiments were conducted under the approval of the Animal Ethics Committee of the Wallaceville Animal Research Centre, New Zealand.

All mice experiments were performed in accordance with the European directive on the protection of animals used for scientific purposes and the respective German animal welfare legislation. Female BALB/c mice were purchased from Charles River and used at 8 to 10 weeks of age. Mice were maintained under specific pathogen free conditions and randomly assigned to the different experimental groups.

2.   Phage libraries.

   The cyclic Ph.D.-C7C[TM] and linear Ph.D.-12[TM] libraries were purchased from New England Biolabs (Beverly, MD). They are based on a combinatorial library of random peptides fused to a minor coat protein (pIII) of M13 phage displaying either a cyclic or linear peptide at up to 5 copies per phage. Each library consists of 1.28×10^9 possible residue sequences, and yields approximately 200 copies of each sequence per 10 µl of phage.

3.   Panning procedures

3.1.   In vitro binding of phage to sheep intestine

For selection of phage binding in vitro to sheep tissue, both libraries were panned independently. A 10 cm segment of the jejunum, including a PP, was removed from sheep at necropsy, washed with PBS and tied off at both ends. The lumen was partly filled with 10 µl of an aliquot of a phage display library diluted in 5 ml of PBS supplemented with a complete protease inhibitor cocktail (Roche, Basel, Switzerland). The segment was incubated 30 min slowly rocking on ice. The content was then discarded and an approximately 3 cm segment containing a PP isolated. This segment was cut open on the minor curvature, repeatedly washed with cold PBS and transferred to an *E. coli* K91/Kan culture growing at the mid-log phase at an OD_{600} of 0.5 to

enable tissue associated phage to infect the bacteria. After 30 min, tissue was discarded and aliquot samples were taken for phage titering, while the remainder was amplified for 4.5 h at 37 °C. Amplified phage were purified by PEG/NaCl precipitation, washed in PBS and titered using standard protocols. The amplified phage were then subjected to a similar second selection procedure using freshly prepared tissue. PP tissue was isolated and washed >10 times in cold PBS / 5mM EDTA prior to recovery of phage.

3.2.   In vivo binding of phage to sheep intestine and PP

For in vivo panning, phage preselected by in vitro panning were amplified and further independently subjected to an in vivo panning procedure in sheep. For all in vivo panning experiments input phage were supplemented with complete protease inhibitor and chloroquine (500 mg) was injected i.m. to temporarily inhibit the processing of antigens within antigen presenting cells[23]. Three approximately 30 cm long intestinal segments containing at least one PP were accessed by laparotomy of an anaesthetized sheep. The ingesta were flushed out by injection of 20 ml of water and the ends clamped off. An aliquot of amplified in vitro pre-selected phage library diluted in 5 ml PBS was injected into loops for 15, 30 or 60 min. The animal was euthanized by barbiturate overdose (sodium pentobarbitone 125 mg/kg), intestinal segments removed, flushed with PBS / 5mM EDTA and rinsed several times with 0.2M glycine pH 2.2. Glycine-washes were used to remove any mucus, or mucus- and epithelium- bound phage from the tissue. PP tissue was isolated from each of the washed segments, washed again repeatedly in glycine, processed by dissection into small pieces and partly solubilized in PBS 1% CHAPS prior to incubation in *E. coli* and phage titering and amplification of phage as above.

3.3.   Recovery of phage from sheep intestinal lymph and PP

Amplified phage were pooled across sampling time points but pools derived from the cyclic or linear phage library were independently subjected to one modified in vivo selection procedure, where transcytosed phage were recovered from afferent lymph of sheep via lymphatic cannulation[24, 25]. The drainage of afferent lymph was visualized by injection of trypan blue into the sub-serosa of the intestine, and a pseudo-afferent lymph vessel was cannulated by insertion of a small catheter. Up to three approximately 30 cm segments of the intestine with at least one PP in the drainage region of the catheter were prepared followed by luminal challenge with an aliquot of pre-selected amplified phage as above. Lymph was collected continuously for up to 6 h after challenge, into ice-cooled collection tubes containing heparin as an anti-coagulant. The animals were then euthanized and intestinal segments removed, PP tissue isolated and processed as above. Cells were separated from lymph plasma by centrifugation, solubilized in PBS/1% CHAPS and individual samples, including a pooled lymph sample, used to infect the *E. coli* host strain. Elution samples were taken after 30 min for titering, while the remaining phage were amplified as above.

All samples derived from cyclic and linear phage libraries were then pooled and subjected to two additional in vivo selection procedures in sheep fitted with an afferent lymphatic cannula as above.

3.4. In vivo validation for specificity of the selected peptide phage

To test whether the peptides selected for the ability to promote phage transcytosis were indeed preferentially transcytosed from the intestinal lumen into the lymphatic system of sheep, six clones that were identified in previous experiments (2L, 4L, 13C, 26PP, 28PP and 38PP) were individually amplified and then combined in equal amounts. An aliquot of this phage pool

consisting of approximately $5x10^{10}$ pfu was mixed with approximately $1x10^{15}$ pfu wild type phage ($2x10^4$ fold excess) to assess non-specific uptake. The phage pool was injected into an intestinal segment and tested for recovery of transcytosed phage via afferent lymphatic cannulation as above. Phage were isolated from lymphatic cells and lymph plasma 30 min and 2 h post-injection. Peptide displaying phage were colorimetrically distinguished from wild type phage based on expression of β-galactosidase.

4.   Identification of individual phage clones.

Samples were titered on LB/IPTG/Xgal plates that enabled differentiation between wild type and library phage plaques using the *E. coli* strain K91/Kan/BK in the standard procedure recommended by New England Biolabs. Individual library phage plaques were picked from elution samples (details are given in Tables 1 and 3), re-amplified and subjected to M13 phage DNA extraction using a commercially available kit (Qiagen, Venlo, Netherlands). DNA was sequenced using the 96gIII sequencing primer as recommended by New England Biolabs and a bioinformatics analysis performed using a basic BLAST search (BLASTp).

5.   Synthetic peptides and coupling procedures.

For sheep experiments, a selection of the identified peptides (2L (CPTSHHRAC), 13C (CTANSSAQC), 28PP (CDPSPSRHC) and two versions of a scrambled 13C peptide (13Csc (CSNAQATSC or CASQNSTAC), which served as controls) were synthesized with a purity of >90%. Peptides were modified to enable radiolabelling and precipitation by C-terminal tyrosine and an N-terminal Biotin extension (AnaSpec, San Jose, CA). Spacers (LC and PEG4) were included to provide separation of the extensions from the peptide molecule. Radiolabelling with ^{125}I was performed by the chloramine T method[26]. To test for efficiency of radiolabelling of

peptides 5 µl of radioactively labelled peptide was diluted in 200 µl PBS, mixed with 50 µl of streptavidin agarose (SIGMA), incubated for 10 min on ice, pelleted and washed prior to measuring radioactivity with a gamma counter. Approximately 70% of radioactivity seen in the original sample was found in the precipitate, demonstrating that 70% of the radioactivity was peptide related.

For mouse experiments, 13C and 13Csc peptide conjugates were either purchased from JPT Peptide Technologies (Berlin, Germany) or synthesized in house (for details see Supplementary Table 1). Briefly, solid phase peptide synthesis (SPPS) was performed on a 50 µM scale using a peptide synthesizer (Intavis, MultiPep RS) and a standard Fmoc/*t*-Bu protocol, comprising double coupling at every cycle. The synthesis of the peptides was performed in plastic syringes at room temperature on a TentaGel S Ram resin (RappPolymere, Tübingen, Germany). Couplings were achieved by reacting four equivalents Fmoc-AA-OH with four equivalents PyBOP and eight equivalents N-Methylmorpholin (NMM) in Dimethylformamid (DMF). Biotin was coupled using the same conditions. A solution of 20% Piperidine in DMF was used to remove the Fmoc protection group. Peptides were deprotected and cleaved from the resin using a mixture of 10 mL Trifluoroacetic acid (TFA), 0.75 g phenol, 0.5 mL water, 0.5 mL methylphenylsulfide, and 0.25 mL 1,2-ethandithiol. After 3 h at room temperature, the cleavage solution was collected and the crude peptides were precipitated from ice-cold tert-Butyl-methyl ether. Crude peptides were washed five times with dry diethyl ether. Final Reverse Phase High Performance Liquid Chromatography (RP-HPLC; Waters, Eschborn, Germany) purification and analysis were achieved using a linear solvent gradient (eluent A: 0.05% TFA in water; eluent B: 0.05% TFA in acetonitrile; linear gradient from 5 to 60% B over 30 min at a flow rate of 20 ml•min^{-1}, RT) over a Vydac C18 column (Hesperia, CA, US) and detection at 214 nm. Identities

of peptides were validated by MALDI-TOF mass spectrometry (VoyagerLT, Applied Biosystems, Weiterstadt, Germany). For immunohistochemical analysis of peptide uptake, the peptides were labeled with fluoresceinisothiocyanate (FITC). Full analysis and characterization of the peptides is provided in the supporting information (Supplementary Table 1; Supplementary Figures 2-6).

For analysis of protein uptake in mice, several different streptavidin conjugates were prepared. Biotinylated peptide was added in a 10-fold excess to streptavidin solution and incubated at room temperature for one hour. The peptide-streptavidin conjugate was then purified by size exclusion using a PD Spintrap G25 column (GE Healthcare, Chalfont St Giles, UK). Conjugates with Alexa Fluor®488 and Alexa Fluor®546 coupled streptavidin (Jackson Immuno Research Laboratories, Inc., West Grove, PA) were used for in vivo visualization of protein uptake in mouse intestine and radiolabeled [I-125]-streptavidin (PerkinElmer, Inc., Waltham, MA) was used for in vivo analysis of transcytosis of peptide-protein constructs in mice.

6.   Analysis of peptide transcytosis in sheep.

Afferent intestinal lymphatic cannulation was performed in sheep as described previously [24]. Sheep were additionally fitted with a catheter (inner diameter 1.2 mm) that reached into the lumen of the jejunum and was anchored into the intestinal wall and exteriorised through the same abdominal opening as the lymphatic cannula. The intestinal catheter was placed in a part of the jejunum from which lymph was likely to drain into the cannulated lymphatic vessel, although lymph draining from other sites could not be excluded. After full recovery from surgery, 50 µg of radiolabelled peptide in 5 ml PBS supplemented with complete protease inhibitor was administered directly into the intestinal lumen. Peptides 13C, 28PP, 2L and 13Csc were

administered consecutively, every third or fourth day once radioactivity had declined to near background levels depending on how long the lymphatic cannula remained patent. Each peptide was given to 3 – 4 different animals (details are given in Supplementary Figure 1). Lymph was collected continuously and sampled for 30min prior to application, then at time points 30min, 1h, 2h, 3h, 4h and 6h after administration. Radioactivity was measured at all collection time points in 100 µl whole lymph, cells from 1 ml of lymph washed once in PBS, 100 µl of lymph plasma and streptavidin precipitate of 1 ml lymph plasma. During the experiment it became obvious that radioactivity was lost during cell washing and consequently measuring the activity of cells separated from 1 ml lymph was included.

Intact biotin-tagged radioactive peptide was precipitated from lymph plasma using streptavidin agarose (SIGMA, St. Louis, MO). Lymph plasma was obtained by centrifugation of whole lymph for 5 min at 400 g. Streptavidin agarose was washed in PBS immediately prior to use, then 100 µl mixed with 1ml of lymph plasma, incubated for 30 min on ice, pelleted and washed before measuring radioactivity with a gamma counter.

7. Visualization of peptide and protein uptake in mouse intestine.

For in vivo visualization, BALB/c mice were anesthetized by i.p. injection of Ketamine/Xylazine (10 mg/kg / 200 mg/kg). The abdominal cavity was then opened by a 1cm midline incision and a segment of the intestine (approximately 2cm) containing at least one PP was located and tied off with surgical nylon. 150 µl peptide-construct solution was injected into the isolated segment, the intestine was placed back into the abdominal cavity and the wound closed. To analyze receptor saturation, unlabeled 13C was first injected into the isolated segment in a 10-fold excess and incubated for 10 min before subsequent injection of 13C-FITC. Mice

were sacrificed by cervical dislocation 5-45 minutes later, the isolated segment removed and flushed twice with PBS. The tissue was fixed in 2% PFA solution for approximately 16 h. Tissue was dehydrated by incubation in solutions of increasing sucrose concentrations starting with 10%, 20% and finally 30%, each for 8-16 h at 4°C. After embedding the tissue in Tissue-Tec OTC cryo embedding medium and freezing, cryostat sections of 7 µm were cut using the HM 560 Cryotome (Thermo Fischer Scientific, Waltham, MO). Slides were frozen at -20°C until further use.

For immunohistochemical staining, the sections were warmed up to room temperature and rehydrated in PBS. All washing steps were performed using PBS containing 1% BSA and 0.1% Tween20 (SIGMA). Blocking buffer additionally contained 10% rat serum. After 20 min incubation with blocking buffer, the sections were stained with anti-CD11c mAb (N418; produced in-house) conjugated Alexa to Fluor®647 for 1 h at room temperature and washed twice. After staining, the slices were mounted with DAKO mounting medium containing DAPI (SIGMA). To examine the staining, a LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) was used and photomicrographs were taken using the software Zen 2011 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

8.   Analysis of transcytosis of peptide-protein constructs.

Balb/c mice were dosed orally, rectally or nasally with radiolabelled peptide-streptavidin-[I-125] constructs. For oral dosing, mice were gavaged with 0.5 mg in 200 µl PBS using a feeding needle. For rectal dosing, mice were injected rectally with 0.5 mg in 200 µl PBS using an umbilical catheter with a diameter of 0.5 x 0.8 mm that was introduced into the rectum. For nasal dosing mice were anesthetized with dexdomitor (0.4 mg/ml, Pfizer, New York City, NY) and

isoflurane (Forene®, Abbott, Chicago, IL) inhalation. After intra-nasal application of 0.05 mg construct in 20 μl PBS, mice were injected with anti-mepazole (2 mg/ml, Pfizer). After 4 h incubation with the peptide-streptavidin construct the mice were sacrificed, blood, urine and various organs (thymus, stomach, small intestine, large intestine, lung, liver, kidneys, heart and skin including the fur) were sampled. Blood cells were obtained by centrifugation, blood plasma was treated with Trichloroacetic acid (TCA) to separate degraded protein or free radioactivity from intact labeled protein (TCA-soluble plasma fraction). Radioactivity in the blood compartments was computed for a total blood volume of 1.5 ml per mouse (60 ml/kg). All samples were analyzed using a gamma counter (Wallac, Turku, Finland).

9.   Statistical analysis.

Mixed modelling was used for data analysis of peptide transcytosis in sheep, with individual animals specified as random effects and peptides as fixed effects, using the REML (Residual Maximum Likelihood) criterion. The data consisted of LOGe AUC (area underneath curves over time) corrected for the value at hour 0 as a covariate, testing for differences between the four peptides. Values for streptavidin precipitated lymph samples were included in the analysis. Data were described as LOGeMeans (the mean of the LOGe AUC for each peptide, SE, minimum and maximum. Statistically significant differences were observed at p<0.05.

Data of protein transport in mice are presented as means ± SEM. Significance was determined with unpaired student's t test after confirming normality distribution by D'Agostino & Pearson omnibus normality test. Differences were considered as statistically significant with $p \leq 0.05$ (*), very significant with $p \leq 0.01$ (**) and extremely significant with $p \leq 0.001$ (***).

RESULTS

1. Selection for peptides promoting intestinal transcytosis of phage

For the in vitro selection procedures of Ph.D.-C7C^{TM} and Ph.D.-12^{TM} peptide display phage libraries, the numbers of input and recovered phage typically were $>10^{12}$ pfu and $>10^6$ pfu/ml, respectively. The in vivo selection of peptides that promote binding of phage to PP, with amplified in vitro preselected phage ($\sim10^{13}$ pfu) resulted in the recovery of $>10^6$ pfu/ml phage from PP tissue following a stringent low pH washing procedure.

The first selection procedure for peptides that facilitate transcytosis from the lumen of intestinal segments into the draining lymph with amplified preselected phage ($\sim10^{13}$ pfu) resulted in the recovery of typically $>10^4$ and occasionally $>10^6$ pfu/ml from PP tissue, lymph plasma and leukocytes recirculating in lymph.

Two additional panning procedures involving afferent lymphatic cannulation with amplified preselected phage pooled across compartment origin and the collection time points resulted in an increase in recovered phage. The titer of phage recovered from PP was 10^6-10^7 pfu/ml, from the lymph pool 10^5–10^6 pfu/ml, and from the lymphatic cells 10^4–10^6 pfu/ml over the first two hours of sampling, with little recovery at the later sampling time points.

Table 1. Identification of phage clones recovered via afferent lymphatic cannulation after the final two rounds of in vivo selection. Library origin, peptide sequence of individual phage clones and frequency of isolations in the respective tissue are shown. Over-represented amino acids H, P and S are highlighted in bold.

			1st round			2nd round		
ID	Ph.D Library Origin	Peptide Sequence	Frequency of isolations (n=33)			Frequency of isolations (n=31)		
			Lymph	cells	PP	Lymph	cells	PP
2L	C7C[TM]	**CP**TS**HH**RAC	3	0	0	8	0	0
4L	C7C[TM]	CKQ**PSH**SAC	3	0	0	2	0	0
13C	C7C[TM]	CTAN**S**SAQC	0	2	6	2	12	0
26PP	12[TM]	**S**TVMD**S**AVIVK**S**	0	0	0	0	0	8
28PP	C7C[TM]	CD**PSPS**RHC	0	0	0	0	0	1
38PP	12[TM]	CKSAIT**P**GS**PS**I	0	16	0	0	0	0
70PP	C7C[TM]	**CLH**T**HS**LHC	0	0	1	0	0	0
71PP	12[TM]	VTKQGV**H**VGL**P**R	0	0	1	0	0	0
73PP	C7C[TM]	CM**QP**GNILC	0	0	1	0	0	0

More than 30 individual phage clones recovered from the three different compartments were subjected to sequencing following each of the final two in vivo transcytosis enrichment cycles. As shown in Table 1, only nine different peptides were found to be displayed on the selected phage because several peptides were represented in multiple clones. Three of the peptides, 2L, 4L and 13C were found following both rounds of transcytosis enrichment, usually in the same compartment. Bioinformatics analysis of the nine peptides for homologues found no compelling matches in protein databases containing non-redundant protein sequences. Analysis of the frequency of amino acids in different positions revealed that their distribution was not random. Constraint peptides tended to have an over-representation of H, S and P but E, F, W, Y and V were not present.

2. In vivo validation for specificity of the selected peptide phage

Preferential transcytosis of selected library phage was assessed in the presence of a 2×10^4 fold excess of wild type phage in an afferent cannulation experiment. Phage were isolated from lymphatic cells ($\sim 10^5$ pfu) and lymph plasma ($\sim 10^6$ pfu). Between 1% and 40% of phage recovered from the three compartments at 30 min and 2 h post-injection were non-wild type, demonstrating an approximately 10^2-10^4 fold enrichment of putative transcytosis peptide-displaying phage compared to wild type phage (Table 2). Much greater enrichment was observed at the 30 minute as compared to the 2 hour time point, further suggesting that the peptide-displaying phage were entering by a facilitated transcytosis process.

Table 2. Recovery of phage from intestinal lymph after challenge with selected library phage clones in the presence of 2×10^4 fold excess of wild type phage. An approximately 10^2-10^4 fold enrichment of putative transcytosis peptide-displaying phage compared to wild type phage was achieved.

Elution sample		Library phage pfu/ml	Wild type phage pfu/ml	Library phage enrichment
30min	lymph plasma	30×10^6	200×10^6	3×10^3 fold
	lymphatic cells	40×10^5	100×10^5	8×10^3 fold
2h	lymph plasma	4×10^6	200×10^6	4×10^2 fold
	lymphatic cells	1×10^5	100×10^5	2×10^2 fold

Sequence analysis was performed to determine the relative abundance of phage displaying the six different putative transcytosis peptides obtained from the three compartments following in

vivo selection (Table 3). Two cyclic displayed peptides dominated the selection, 13C and 28PP, representing 36 of 40 clones sequenced.

Table 3. Identification and frequency of library phage clones from intestinal lymph of sheep given a selection of library phage clones in the presence of 2×10^4 fold excess of wild type phage. A strong selection for clones 13C and 28PP was found.

compartment	Frequency of isolations					
	lymph			cells		
time of isolation	0.5 h	2h	4h	0.5 h	2h	4h
2L	0	1	1	0	0	0
4L	0	0	0	0	0	0
13C	9	2	2	10	0	1
26PP	0	0	1	0	0	1
28PP	1	2	2	7	0	0
38PP	0	0	0	0	0	0

3. Verification of peptide facilitated transcytosis from the intestine into lymph

Peptides 2L, 13C, 28PP and a scrambled version of 13C (13Csc) were tested for translocation from the intestinal lumen into the draining lymph. Overall, the highest level of peptide was found in the lymph at either 30 min or 1 h after administration, followed by a decline to around base line levels by 6 to 24 h. REML variance components analysis of LOGe AUC (area underneath curve as defined by cpm vs. time) revealed significant differences between levels of peptides transcytosed into lymph plasma (p=0.019). Peptide 13C and to a lesser extent peptide 28PP, but little or no 2L and 13Csc peptide (control peptide), were transcytosed into the lymphatic system

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of the intestine (LOGeMeans for peptides; 13C: 8.255; 2L: 5.677; 28PP: 6.717; and 13Csc: 5.237; SE: 0.683 (max 0.7429; min 0.6241)) (Figure 1, and Supplementary Figure 1). Transcytosis of intact peptide 13C was higher than for 2L and 13Csc ($p \leq 0.001$), and 28PP was higher than for scrambled 13C ($p = 0.027$) as determined by REML analysis. In summary, 13C peptide and, to a lesser extent, 28PP peptide display enhanced transcytosis from the lumen of the gut into the lymphatic system in a sheep model.
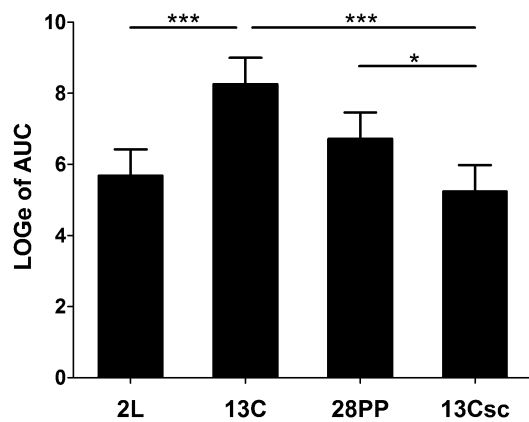

Figure 1. Increased transcytosis of peptides 13C and to a lesser extent of 28PP into intestinal lymph plasma. Data represent the AUC (area underneath curve) of recovered radioactivity (cmp/ml lymph) (n=4 for peptides 2L, 13C and 28PP; n=3 for scrambled peptide 13C). $p < 0.05$ between treatments.

4.   Visualization of intestinal 13C peptide uptake in vivo

Transcytosis of the 13C peptide was further verified in a mouse model by confocal microscopy. Uptake of 13C-FITC into small intestinal epithelial cells as well as cells within the villi was detected as early as 5 min after luminal delivery of the construct (Figure 2 A, B). In contrast, very little or no uptake of the control peptide 13Csc-FITC was observed with most of the

material being trapped in the mucus of the gut lumen (Figure 2 C, D). Furthermore, CD11c

positive cells, most likely dendritic cells, located in the villi in close proximity to the epithelium
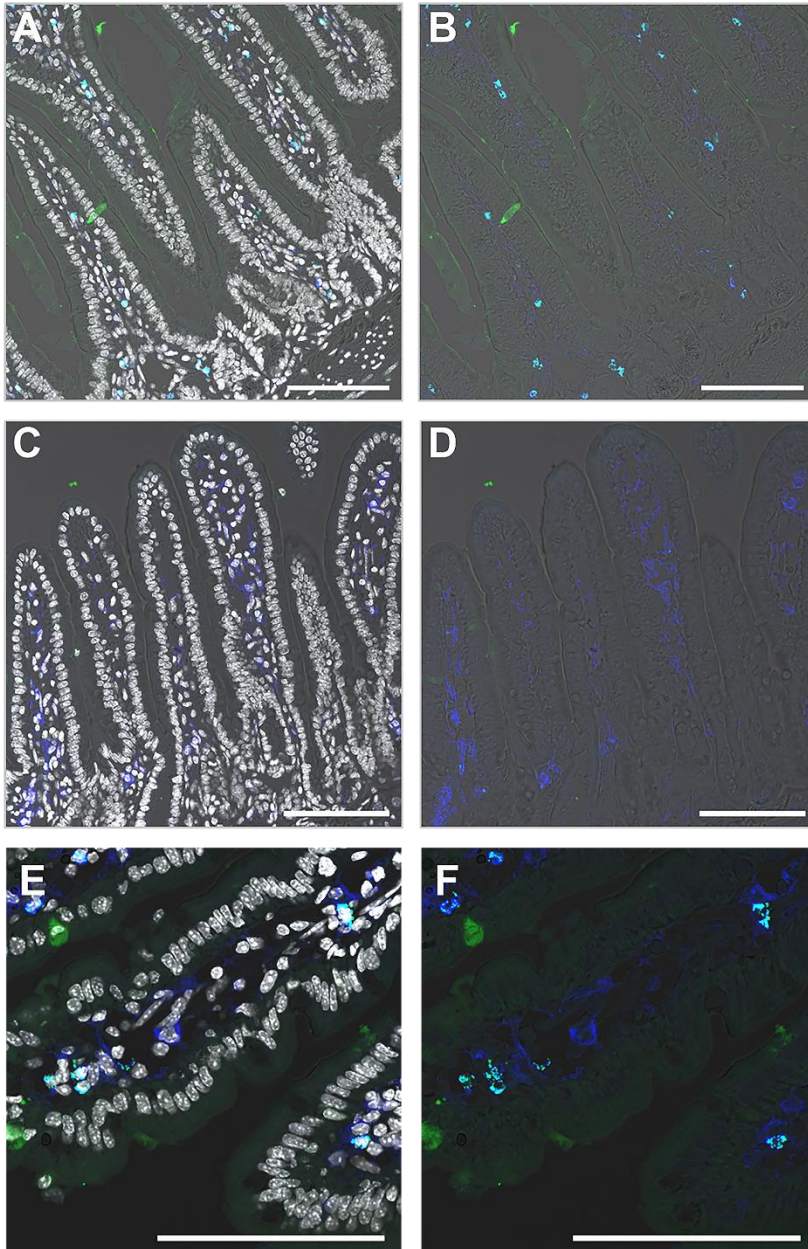
were found to be positive for 13C.

Figure 2. Verification of peptide uptake in murine intestinal cells in vivo. Peptides were injected

into the lumen of the small intestine and incubated for 5 minutes. (A+B) injection of 13C-FITC;

(B+C) injection of scrambled control peptide 13Csc-FITC; (E+F) 10 min preincubation with 13C-peptide before injection of 13C-FITC and 5 min incubation (grey: nuclear stain DAPI; green: peptide-FITC construct; blue: CD11c-Alexa Fluor® 647; cyan: overlay of FITC peptide and CD11c signal; scale bar: 100 μm; A, C and E show an overlay of all channels with a bright field image; B, D and F show an overlay of FITC and Alexa Fluor® 647 signal).

We conducted 13C uptake studies to reveal if a saturating receptor-ligand interaction is involved. Pre-expose of the intestinal mucosa with unlabeled peptide did not block the uptake of labeled peptide. The 13C-FITC construct was found inside the villi of the small intestine with a cellular distribution and visual fluorescence intensity similar to the previous experiments in which pre-incubation with unlabeled 13C peptide was omitted (Figure 2 E, F).

5.   Peptide 13C facilitates protein uptake

Peptide 13C facilitated mucosal transcytosis both as a peptide and when expressed on M13 phage (size: 7 x 900nm; equivalent to a MW $>1 \times 10^7$). We next tested whether the peptide would promote the delivery of protein across the mucosal barrier. We used fluorescent streptavidin as a model protein which was coupled to biotinylated peptide 13C. In order to visualize the transcytosis of 13C-protein and control 13Csc-protein, the biotinylated peptides were coupled to fluorescently labeled streptavidin and injected into the intestinal lumen simultaneously (Figure 3).
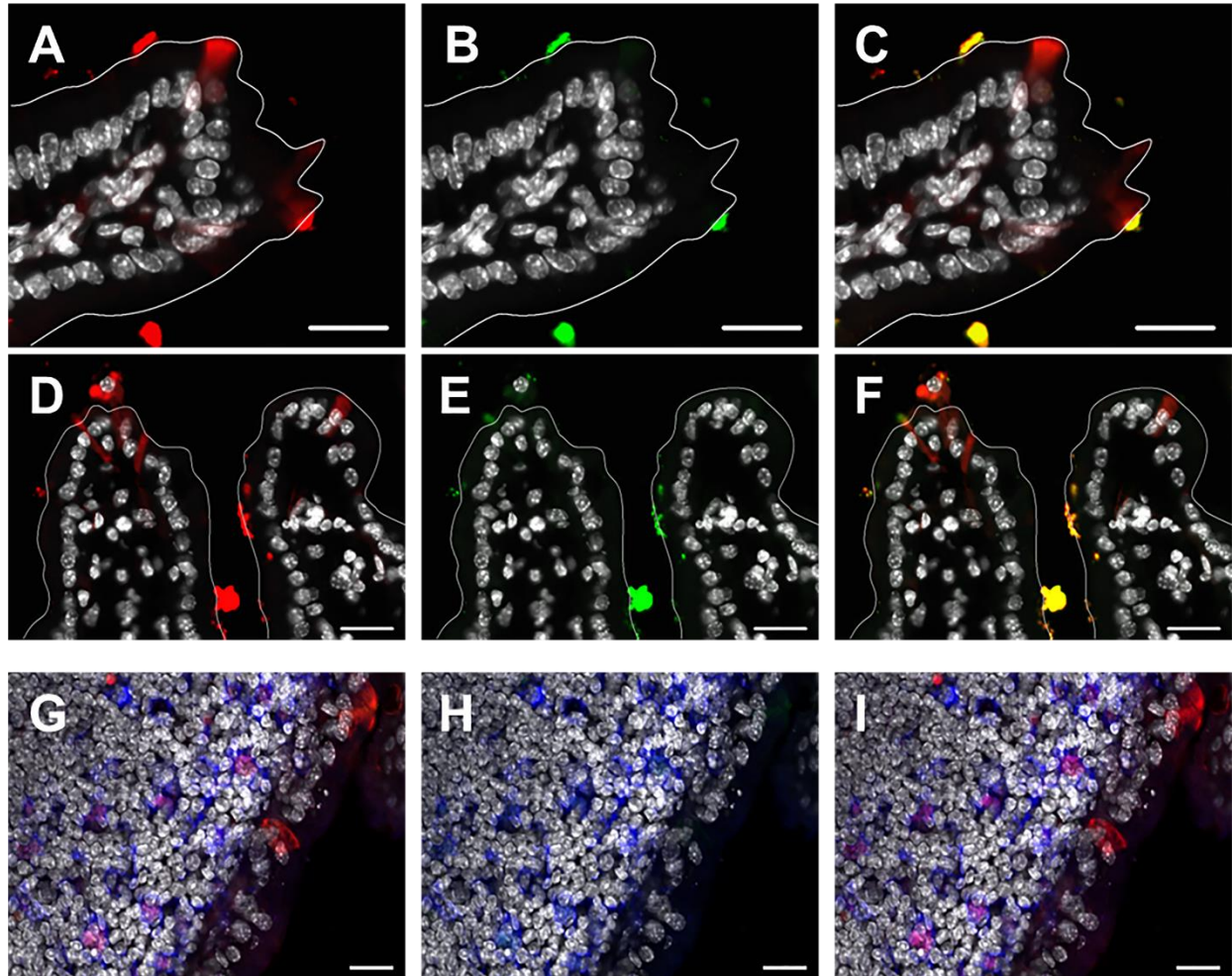
Figure 3. Determination of peptide-protein-construct binding and uptake in murine intestinal cells in vivo. 13C-streptavidin and control peptide construct (13Csc-streptavidin) were simultaneously injected into the intestinal lumen and incubated for 45 minutes. The right panel (C,F and I) show the merged images of the 13C-streptavidin (A,D and G) and 13Csc-streptavidin (B, E and H) images. (A-F) villous region of the small intestine; (G-I) PP region. (grey: nuclear stain DAPI; blue: CD11c-Alexa Fluor® 647; red: 13C-streptavidin-Alexa Fluor® 694; green: 13Cscrambled-streptavidin-Alexa Fluor® 488; scale bar: 20 μm).

In line with our previous observations, we detected 13C-streptavidin in distinct epithelial cells and in cells within the villi, while the 13Csc-streptavidin control construct could only be

observed in the lumen of the gut (Figure 3 G-I, representative images). Strikingly, a distinct

uptake of 13C-streptavidin construct was also observed in CD11c-positive cells, most likely

dendritic cells, located in the lamina propria or in PP. To exclude artefacts due to properties of

the different Alexa dyes, we switched labelling of 13C and 13Csc, using streptavidin Alexa

Fluor® 488 and Alexa Fluor® 546, respectively, and repeated the experiment. Once again, only

the 13C coupled protein was detectable inside epithelial cells (data not shown). Therefore,

peptide 13C facilitated uptake and transportation of a specific protein across the intestinal wall in

a mouse model.

6. Determination of systemic delivery of a 13C - protein construct

We next tested if peptide 13C would improve the delivery of proteins into the systemic

circulation. Radiolabelled 13C and 13Csc constructs were given either orally, rectally, or nasally.

Four hours after administration, the fraction of radioactivity in blood cells and blood plasma was

measured. The background level of total radioactivity in the TCA-soluble plasma fraction ranged
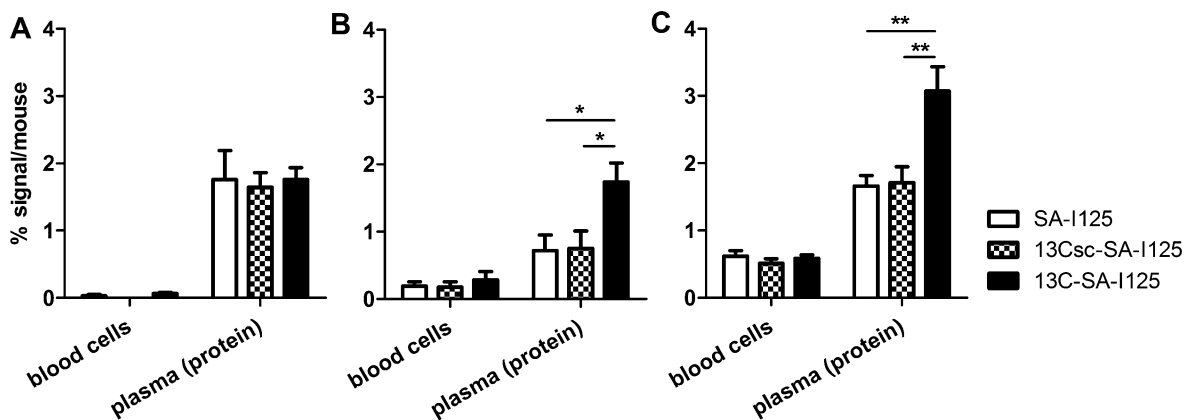
between 0.4-1.8% (data not shown).

Figure 4. Mucosal uptake of peptide-protein-construct and distribution into blood plasma. Mice were treated orally (A), rectally (B) or nasally (C) with peptide conjugated to radiolabeled (125I)-streptavidin (SA-I125). After four hours, mice were dissected and radioactivity was analyzed in various compartments. Graphs show the radioactivity in the blood compartments as % of total radioactivity recovered from the mouse. (Mean ± SEM; oral: n=5; rectal: n=9; nasal: n=13-15; D'Agostino & Pearson omnibus normality test α=0.05, unpaired t test; * p< 0.05; ** p< 0.01)

While a major part of the radioactivity remained at the site of administration (data not shown), a small but significant enrichment of intact 13C-conjugate as compared to 13Csc- or streptavidin control could be detected in the systemic circulation after rectal and nasal, but not after oral administration of the construct (Figure 4 A-C). 13C peptide increased the systemic levels of the model protein approximately 2.5- and 1.9-fold after rectal and nasal delivery, respectively. In none of the application routes were significant amounts of 13C-streptavidin detected within the cellular fraction of the blood, showing that 13C protein is not transported by blood leukocytes. In conclusion, peptide 13C facilitated the transportation of the 13C-protein construct into the systemic circulation after mucosal administration.

DISCUSSION

In this study we identified a cyclic peptide that facilitates the rapid transportation of intact macromolecules across the mucosal surface into distinct cells of the submucosa as well as into the systemic circulation. The peptide was identified in a large animal model by using a combination of in vitro and in vivo panning procedures of phage display libraries. The panning

was designed to specifically enrich for peptides that facilitated transcytosis across the mammalian intestinal epithelium into the intestinal afferent lymphatic system. The selection resulted in a small set of peptides that were repeatedly isolated, dominated by the cyclic nonamer CTANSSAQC, 13C. 13C was isolated from lymph plasma as well as from cells that traffic in afferent lymph.

Our panning strategy involved the independent selection of both a conformationally constrained and a linear phage display library, initially in vitro and then in vivo in a sheep model. These preselected libraries were then combined and further selected in vivo in an effort to select for phage that were transported into the lymphatic system directly draining from the small intestine. Interestingly, these additional panning steps resulted only in the selection of several cyclic peptides, suggesting that disulphide-bonded cyclic peptides form superior transcytosis ligands. It may be that the disulphide bond stabilizes the peptide and / or makes it more resistant to proteolytic degradation and thus is superior in a harsh environment such as the intestine of animals.

We established that the transcytosis of the ligands is not due to non-specific transportation of phage across the mucosa[27], as the selected transport of library phage was several orders of magnitude greater than that of wild-type phage. Furthermore, in contrast to a study in rats where single peptides were identified[18], we found that several of the transcytosed peptides were identified repeatedly, which provides further evidence for specific enrichment. This is supported by the fact that we found transcytosis into lymph occurring within 30 min after intestinal delivery of selected library phage. Rapid transportation of library phage was previously observed in a study in rats in which phage were recovered from tissues such as spleen, liver or kidney soon

after injection into the jejunum[21]. Taken together these results provide strong evidence that our panning procedure resulted in the selection of specific transcytosis reagents.

We next conducted experiments to establish which of the selected peptides would be the most effective transcytosis ligand. Three peptides identified in the phage panning were synthesized and then inoculated directly into the otherwise unmodified small intestine of sheep. Inoculation into the intestine was used to prevent potential degradation in the rumen and abomasum. To enable identification in the lymph in ex vivo samples, each of the synthetic peptides was tagged with biotin on the amino terminus and radioactively-labelled tyrosine at the carboxyl terminus. Detection of intact peptide involved binding to a streptavidin matrix via the biotin tag, followed by measurement of the radioactive label. To our knowledge this is the first report of an intact peptide that is selectively transcytosed into intestinal lymph from a fully functional small intestine. The recovery of significant amounts of synthetic peptides from lymph soon after inoculation (peak levels were detected from 30 min to 1 h followed by a constant decline) compared to a scrambled control peptide confirmed that preferential transcytosis has occurred. We found that peptide 13C outperformed the other two peptides tested. Consequently we used 13C in follow up mouse studies to confirm the efficacy of the trans-mucosal delivery of proteins in a second animal model.

Initially, we used confocal microscopy to determine if 13C facilitates the delivery of molecules into specific cellular compartments of the small intestine. Peptide 13C, when directly labeled to a fluorescent dye or when bound to streptavidin, could be detected in a subset of epithelial cells as well as specific cells that underlie the epithelium as early as 5 min after delivery into the small intestine. These findings demonstrate that peptide 13C facilitates transcytosis and enrichment in mouse epithelial cells and cells of the intestinal villi. A subset of the 13C-positive epithelial cells

showed goblet cell like morphology and location which indicates that goblet cells might be involved in the recognition and uptake of the peptide conjugate. While antigen transport by M cells is well established, a recent report showed that goblet cells can perform a similar function by taking up antigens from the gut lumen[4]. We also found that CD11c positive dendritic cells located in the intestinal villi and in jejunal PP take up 13C peptide conjugates. It remains to be determined if DCs directly sample the material via dendrites that protrude into the lumen of the intestine[28] or whether the 13C conjugate is presented by epithelial cells. However, a recent report that describes the capacity of goblet cells for antigen uptake from the intestinal lumen followed by antigen transfer to CD103+ dendritic cells[4], in conjunction with our observation, provides support that goblet cells present a 13C conjugate to DC. 13C positive cells were also found in cells that traffic in afferent lymph. It is therefore likely that uptake by DCs is followed by signals that initiate migration.

Interestingly a phage display study in rats identified a nine amino acid long peptide (CSK; CSKSSDYQC) which increased uptake via the intestinal mucosa[21]. Our peptide 13C and CSK share some sequence similarities, notably the presence of a ser-ser motif. The CSK peptide was reported to be taken up primarily by goblet cells. This suggests that the two peptides share some features, but uptake is likely to involve different mechanisms. In vitro competition experiments with CSK peptide suggested a receptor-mediated uptake[21, 22], whereas uptake of 13C constructs, as far as could be seen using fluorescence imaging, was not blocked by a 20-fold excess of free 13C peptide. As the scrambled version of 13C peptide is not transcytosed, non-specific uptake mechanisms such as pinocytosis are unlikely. One possible explanation is that 13C binds to a low affinity, but abundant, receptor which is not saturable and is only present on a subset of epithelial cells.

We also were able to re-isolate labeled 13C peptide from lymph plasma and consequently wanted to establish if 13C could be used to deliver molecules into the systemic circulation. Such an enhanced mucosal delivery of molecules into the blood system would be of significant interest for treatment with protein-based drugs. We have shown that 13C enhances uptake of radiolabeled streptavidin, and the labeled 13C-strepavidin complex could also be found in blood plasma. Thus, we investigated if 13C would facilitate the transportation of therapeutic proteins into systemic circulation. We found that 13C facilitated transportation of a protein into the systemic circulation after intra-rectal as well as intra-nasal delivery but not after oral delivery. It is possible that oral delivery of the non-protected target protein has resulted in gastric or intestinal degradation. It remains to be determined if the use of acid-resistant capsules or nanoparticles would prevent degradation and improve efficient delivery of 13C-coupled materials by the oral route.

In conclusion, this study describes a novel cyclic peptide that has functional characteristics of a transport vehicle for biomolecules. It enhances the uptake of proteins through the gut wall, promotes systemic delivery as well as accumulation in intestinal epithelial cells and dendritic cells. It therefore might find applications for oral, nasal or rectal delivery of biomolecular drugs making intravenous application procedures dispensable. Moreover, preferential targeting to intestinal cells might be useful therapeutically to deliver immunomodulatory or other agents so that they achieve more effective treatment of chronic inflammatory diseases of the gut. Whether or not 13C facilitated intestinal transportation enables the development of novel approaches for the induction of oral tolerance induction[29], or if it could be used to develop tolerogenic vaccination procedures, is currently under investigation.

ASSOCIATED CONTENT

Supporting Information. Detailed analysis and characterization of peptides and of peptide transcytosis into intestinal lymph plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AUC, area underneath curve; CHAPS, (3-((3-cholamidopropyl) dimethylammino)-1-propanesulfonate; cmp, counts per minute; DMF, Dimethylformamid; EDTA, ethylenediaminetetraacetic acid; i.m., intra musculary; i.p., intra peritoneal; NMM, N-Methylmorpholin; PBS, Phosphate buffered saline; PEG, polyethylenglycol; PP, Peyer's Patch; REML, Residual Maximum Likelihood; RP-HPLC, Reverse Phase High Performance Liquid Chromatography; SE, standard error; SPPS, Solid Phase Peptide Synthesis; TCA, trichloroacetic acid; TFA, Trifluoracetic acid

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