In Vivo Phage Display To Identify M Cell–Targeting Ligands

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Received September 23, 2003; accepted December 5, 2003

Purpose. The purpose of this study was to use *in vivo* phage display screening technology to identify novel lead peptides that target delivery to M cells and to follicle-associated epithelium (FAE) of the intestine.

Methods. Phage display libraries were screened *in vivo* within the gastrointestinal tract of a rat model by successive screenings across four cycles of selection.

Results. Following four cycles of *in vivo* screening, we identified 30 unique peptide sequences that bound to Peyer's patch tissue, human Caco-2, and rat IEC-6 epithelial cells. Two of the lead targeting peptides, peptides P8 (LETTCASLCYPS) and P25 (VPPHPMTYSCQY), were shown to bind to receptors on the surface of human intestinal tissue. The L-form, D-form, retro-inverted D-form, and selective Cys-to-Ala site-directed mutants of peptides P8 and P25 were also shown to retain binding to Caco-2 cell membranes when immobilized on the surface of a model particulate. Finally, the D-peptide analog of peptide P8 (yqcsytmphppv) enhanced the delivery of polystyrene particles to M cells *in vivo* in a mouse model, and these particles were delivered into Peyer's patch tissue, as determined by confocal microscopy.

Conclusions. In summary, we have identified novel ligands that target M cells and Peyer's patch tissue, and thus may have utility in the targeted oral delivery of vaccines and vaccine carrier systems to the mucosal immune system within the gastrointestinal tract.

KEY WORDS: M cell–targeting ligands; vaccines.

INTRODUCTION

Oral delivery of vaccines giving rise to a mucosal immune response is highly desirable as many pathogens invade via mucosal surfaces (1,2). However, major challenges to oral delivery of vaccines and antigens are recognised (3,4). Key barriers to achieving effective oral absorption of vaccines, macromolecules, and proteins include their low permeability across the plasma membrane of intestinal epithelial cells, susceptibility to degradation by peptidases and proteases in the gastrointestinal tract (GIT), and hepatic and biliary clearance of absorbed drugs from the portal circulation. In the case of proteins, susceptibility to denaturation in the acidic environment of the stomach is also a barrier to oral delivery. Protection of such macromolecular drugs, proteins, and vaccines from acid and enzymatic attack through encapsulation in nano- and microparticulate dosage forms such as polylactide (PLA), polylactide-coglycolide (PLGA), or liposomal-based systems and use of enteric-coated capsules or tablets may offer some protection from enzymatic degradation and acidic attack. However, drug or vaccine encapsulation into particulates such as PLA, PLGA, liposomes, or other particulates in itself may not offer sufficient advantage given the relatively inefficient absorption of such systems into and across intestinal epithelial cells.

One approach to enhance drug and particulate delivery into and/or across the intestinal epithelial barrier is to target particulate formulations to receptor sites of the intestine. M-cells are professional antigen sampling cells that are found in the epithelium of the gut-associated lymphoid tissue or Peyer's patch and play an important role in sampling foreign materials, particulates, and antigens from the lumen of the GIT, resulting in downstream mucosal immune responses (5,6). The transcytotic capacity of M cells and the downstream processing of antigen sampled would suggest that targeting vaccines to M cells would enhance oral immunization. A targeted particulate system may prolong residence time in the GIT, produce a high local concentration of vaccine at the epithelial cell surface and promote absorption to the underlying lymphoid tissue. However, limited knowledge of human M-cell receptors means that there is considerable difficulty in the selection of suitable targets for delivery of vaccines and/or other drugs through the M-cell route. We have recently described the application of genomics technology for the identification of gene profiles expressed in M cells and Peyer's patch tissue relative to the corresponding gene profile expressed in normal epithelial cells found in the GIT (7,8). In addition, through the use of combinatorial chemistry, we recently identified small organic mimetics of the glycoprotein UEA-1 lectin that were shown to target particulates to M cells and the FAE (9).

The aim of the current study was to identify novel targeting peptide ligands for M cells of Peyer's patch using *in vivo* phage display library screening. Phage display technology has previously been used to identify peptide ligands that target normal tissue and tumor vasculature (10–12). The key advantage of this technique is that peptides are selected on the basis of interaction with target receptors *in situ*. Here we describe the *in vivo* phage display screening within the GIT of a rat model and the downstream selection and analysis of resulting peptides that target M cells *in vivo*. Such targeting ligands may have utility in the targeted oral delivery of vaccines to the mucosal immune system within the GIT.

MATERIALS AND METHODS

In vivo Phage Display

A library consisting of random dodecapeptides fused to the N-terminal of protein pIII of the filamentous phage

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ABBREVIATIONS: CLSM, confocal laser scanning microscope; GIT, gastrointestinal tract; PEG, polyethylene glycol; pfu, plaque-forming units; PLA, polylactide; PLGA, polylactide-coglycolide.

0724-8741/04/0400-0695/0 © 2004 Plenum Publishing Corporation

M13KE was used (New England Biolabs, Inc., Boston, MA, USA). For selection of phage that binds to rat Peyer's patch, the library [1.5×10^{11} plaque forming units (pfu)] was administered in a volume of 0.5 ml into a closed ileal loop (2–3-cm long) of rat intestine (n = 5 animals in each cycle) essentially as a modification of the mouse model previously described (13). Briefly, male Wistar rats weighing approximately 300 g were starved overnight and anesthetised with pentobarbitone sodium (60 mg/kg) prior to administration of the phage library into the closed loop. The abdomen was incised and gut loops of 2–3-cm length containing one or more Peyer's patches were created by application of appropriate ligatures. Care was taken to minimize surgical trauma and to maintain an adequate blood supply to the ligated tissues during these procedures. A baseline blood sample was taken from the tail artery prior to administration of the phage library into the gut loops and further samples were taken at 30 min prior to sacrificing animals. Blood samples (0.4 ml) were collected into heparinized tubes and kept at 4°C. After 30 min, the animals were sacrificed and the gut loops excised. From these loops, Peyer's patch and non-Peyer's patch intestinal epithelial tissues were isolated, washed, and homogenized. The bacteriophage present in the tissue samples were pooled, amplified in *Escherichia coli* (strain ER2537), and isolated by polyethylene glycol (PEG) precipitation, essentially as described elsewhere (14). Peyer's patch selected phage were titered and were used in subsequent screening cycles. Four *in situ* screening cycles in the GIT were completed in total, prior to selection of individual phage clones for downstream characterization. All experiments involving animals adhered to the "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, revised in 1985).

Phage Clone and Peptide Enzyme-Linked Immunosorbent Assay

The phage pools obtained at the end of cycle 4 were plated out on LB agar plates with top agar, and phage clones were individually selected, amplified in *E. coli,* and isolated by PEG precipitation according to standard procedures. Phage clones were subsequently characterized for binding to homogenates isolated from rat intestinal tissue as well as binding to plasma membrane fractions isolated from human (Caco-2) and rat (IEC-6) epithelial cell lines by ELISA. Intestinal tissue with or without Peyer's patch was harvested and homogenized in buffer containing 250 mM sucrose, 12 mM Tris, and 16 mM EDTA. Caco-2 cell (ATCC, Rockville, MD, USA) membrane fractions were prepared from confluent cell monolayers grown in 75 cm^2 flasks for up to 1 week at 37°C and 5% CO_2, as previously described (15,16), and were routinely characterized for the expression of a peptide transporter by ELISA and Western blotting (data not shown). The membrane fractions were coated onto 96-well microtiter plates at 10μg/ml in 0.05 M carbonate buffer (pH 9.6).

After 2 h incubation at room temperature, phage clones were serially diluted onto ELISA plates coated with 10 μg/ml of cell plasma membranes or tissue homogenates and blocked with 1% BSA-TBS. Plates were washed with TBS-Tween (0.05%). Bound phage was detected using biotinylated-anti-M13-phage antibody (Research Diagnostics, Flanders, NJ, USA) at a 1:5000 dilution for 30 min at room temperature. Plates were washed as before, and anti-M13 antibody was detected using extrAvidin Alkaline Phosphatase (Sigma, Dublin, Ireland) at a 1:5000 dilution and activity detected using 4-nitrophenyl-phosphate substrate (Sigma).

For the biotinylated-peptide ELISA, plates were prepared as above, and bound peptides were detected using streptavidin-peroxidase (Calbiochem, 1:500). Enzyme activity was measured using K-blue substrate (Neogens Life Sciences, Toronto, Ontario, Canada).

Sequencing of Selected Phage Clone Inserts

One hundred phage clones were selected following ELISA analysis. Phage DNA was isolated using M13 purification kits (Qiagen, Crawley, UK). The isolated DNA was precipitated, and DNA inserts were sequenced by MWG Biotech (Cork, Ireland).

Synthesis and Analysis of Peptides

Selected 12mer peptides were synthesized by standard peptide chemistry methods (Anaspec, San Jose, CA, USA) with biotin tags at either the amino terminal or the carboxyl terminal. All synthetic peptides were characterized by reverse-phase HPLC and mass spectroscopy to confirm identity and purity prior to characterization in binding assays. All peptides were greater than 95% pure as determined by RP-HPLC and mass spectroscopy. Retro-inverted peptides and stabilized D-form peptides were also synthesised (Anaspec). A 12mer D-form peptide (naagngnstg) shown previously to have no activity *in vitro* in binding and uptake assays was synthesized as a negative control (data not shown)

Immunohistochemistry

Biotinylated synthetic peptides derived from isolated clones were tested for binding to human Peyer's patch tissue sections. In addition, the known negative binding peptide (the D-peptide naagngnstg) shown previously to have no binding activity *in vitro* was used as a negative control (data not shown). Paraffin sections of human Peyer's patch were deparaffinized and dehydrated. The sections were rinsed in PBS, pH 7.4. Endogenous peroxidase was blocked using 1% hydrogen peroxide in methanol for 10 min followed by blocking with 2% BSA in PBS for 20 min at room temperature. The sections were incubated with peptide (50 μg/ml in 2% BSA in PBS) for 1 h at room temperature. Control tissue was treated with BSA alone. The sections were rinsed with 0.05% Tween in PBS. Streptavidin-HRP (Calbiochem) at 1:500 dilution in 2% BSA was added for 30 min at room temperature. Following washes in PBS/Tween, DAB substrate (DAKO) was added for up to 5 min, and the reaction stopped by immersing the slides in water. The sections were counterstained using hematoxylin for 50 s and then rinsed in water. The slides were differentiated in 1% acid alcohol for 5–10 s and then rinsed in water. The slides were mounted using aqueous mounting medium and a cover slip.

Particle–Peptide Conjugates

Biotinylated peptides were adsorbed to fluorescein isothiocyanate-loaded streptavidin polystyrene particles with a diameter of 0.289 μm (Estapor, Merck Eurolab, Fantenay/bois, France). Biotinylated peptides were adsorbed using routine methodologies at room temperature; following adsorp-

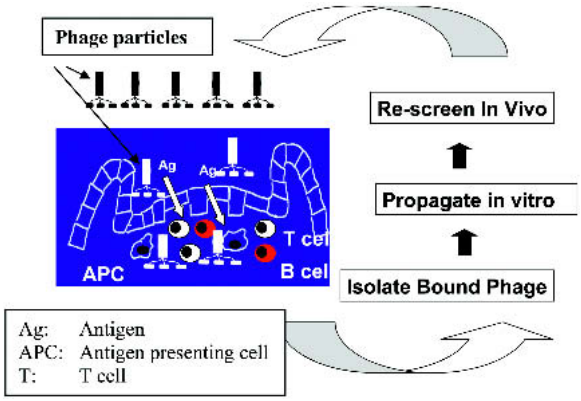

Fig. 1. Schematic diagram showing *in vivo* phage display procedure on Peyer's Patch tissue.

tion, the particles were repeatedly washed to remove any free, nonabsorbed peptides, and the particles were recovered by centrifugation and resuspended in PBS. Peptide-coated particles were tested in an ELISA-based assay for binding to Caco-2 cell membrane fractions. Briefly, particles coated with peptides were incubated in ELISA plates coated with cell plasma membrane fractions, and binding was detected using a peroxidase-labeled anti-biotin antibody (1:1000; Sigma).

Targeted particles were also tested for uptake and binding *in situ* using a mouse intestinal loop model as described elsewhere (13). Briefly, mice were fasted overnight and anesthetized by intraperitoneal administration of 0.1 ml/10 g hypnorm/midazolam. The abdomens were incised, and gut loops containing one or more Peyer's patches were ligated. Gut loops were innoculated with microparticle suspensions at 5.0×10^{10} particles per milliliter in PBS, warmed to 37°C, and were incubated for 30 min. After incubation, tissues were repeatedly washed in PBS to remove any free or loosely bound particles, mounted on cork mounts, and fixed in 2% paraformaldehyde in PBS. Fixed tissue was permeabilized with 0.1% triton/PBS for 20 min at room temperature. Tissue was rinsed in PBS and stained with UEA-1 lectin labeled with tetramethyl rhodamine isothiocyanate (Vector Labs, Peterborough, UK)/PBS (50 μg/ml) for 1 h at room temperature. Following incubation, tissue was rinsed in PBS and mounted in Vectashield (Vector Labs, Burlingame, CA, USA) and analyzed using a Zeiss LSM 510 confocal laser scanning microscope (CLSM).

Tracking of Peptides *in Vivo*

Four high-binding phage clones were instilled into rat intestinal loops (n = 3) as described earlier. Blood samples were taken after 30 min. Animals were then sacrificed and the loops excised. One hundred microliters of each blood sample was serially diluted in LB growth media and plated out onto *E. coli* ER2537 in top agar plates containing IPTG/Xgal (14). Phage plaques were counted after incubation at 37°C overnight. Controls included a PBS control and an empty phage vector control.

RESULTS

Selection of Phage Clones

Figure 1 summarizes in schematic format the approach used to isolate phage that preferentially bind to Peyer's patch tissue regions in the GIT. A 12mer phage display library was instilled into the closed loop of rat GIT tissue that contained Peyer's patch tissue. Tissue was excised, washed, and gently homogenized in order to release phage particles that had either adsorbed to or been taken up into the GIT/Peyer's patch tissue. The bound phage were amplified in *E. coli* and this cycle of selection, recovery, and amplification was repeated in

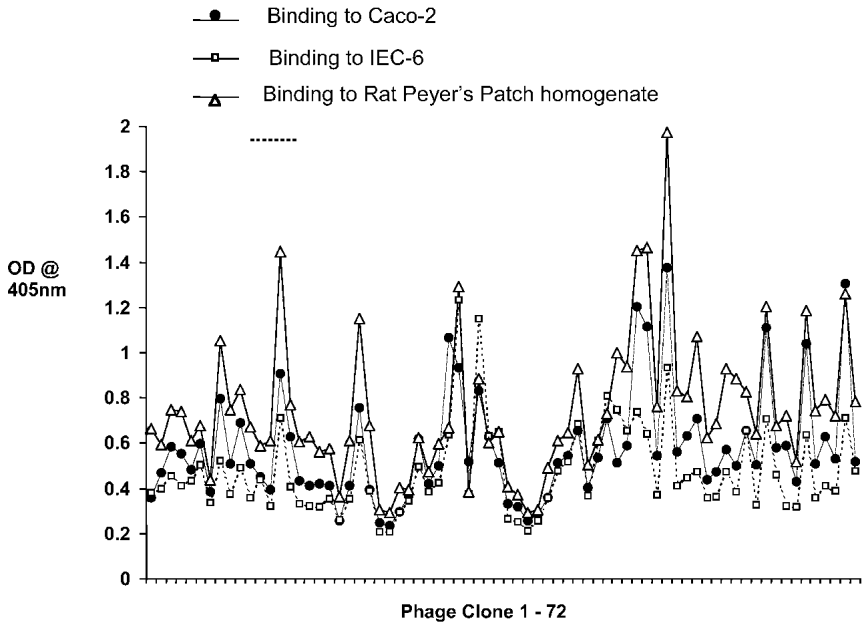

Fig. 2. Binding of 72 phage clones to homogenates of rat Peyer's patch tissue or to membrane fractions prepared from human Caco-2 and rat IEC-6 cells. Binding of phage to the tissue homogenates or cell membranes was determined by ELISA assay by measuring the optical density (O.D) at 450 nm, as outlined in "Materials and Methods."

Table I. Homologues to M Cell–Targeting Peptides

Peptide (P) name	Sequence	Homolog of interest	Alignment
P1	A T P P P W L L R T A P	None identified	Not applicable
P2	D G S I H K R N I M P L	None identified	Not applicable
P3	D Y D S L S W R S T L H	None identified	Not applicable
P4	G E P T T D M R W R N P	Mouse keratinocyte growth factor receptor	G E P T T D M R W G P T + M R W G N P T S T M R W
P5	G L W P W N P V T V L P	Human urokinase-type plasminogen activator precursor (u-pa)	P W N P V T V L P W N T V L P W N S A T V L
P6	H M L N D P T P P P Y W	Xenla epithelial-cadherin precursor (E-cadherin)	N D P T P P P Y N D P T P P Y N D P T A P P Y
P7	K P A Y T H E Y R W L A	Precursor (IgE Fc receptor, alpha-subunit)	Y T H E Y R W L Y T E Y R W L Y T I E Y R W L
P8	L E T T C A S L C Y P S	Vlalu lectin-related protein precursor	E T T C A S L C Y P S E T A S L Y P S E T L I A S L T Y P S
		Cavpo casein a precursor	E T T C A S L C E T C A S L C E T I C A S L C
P9	L G T D W H S V S Y T L	Pig zonadhesin precursor	L G T D W H S V S Y T L G T D W S + T L G T D W F S P N C T
P10	L G T L N A G V P G F P	Mouse elastin precursor (tropoelastin) (agvpgf motif is found multiple times in elastin precursor sequence	L N A G V P G F L A G V P G F L G A G V P G F
P11	L T H S K N P V F L S T	None identified	Not applicable
P12	L V P T T H R H W P V T	None identified	Not applicable
P13	L V S N A R G F N N L S	None identified	Not applicable
P14	N T R I P E P I R F Y M	None identified	Not applicable
P15	N V Y T F H S M S P M P	Rat sucrase-isomaltase, intestinal	N V Y T F H S M S P M P N Y T S + P + P N P Y T L T S I Q P L P
P16	Q H T T L T S H P R Q Y	Human placental-cadherin precursor (P-cadherin)	H T T L T S H P H T + T + H P H F T I T T H P
P17	S D F S D T M P H R P S	None identified	Not applicable
P18	S I D T I Q I L S L R S	None identified	Not applicable
P19	S I S W A S Q P P Y S L	Caphi beta casein precursor	S W A S Q P P Y S L S W Q P P L S W M H Q P P Q P L
P20	S M V K F P R P L D S R	ZO1 mouse tight junction protein zo-1 (tight junction protein 1)	P R P L D S R P R L D S R P R D L D S R
P21	S P T L G A S V A Q T N	None identified	Not applicable
P22	T M S P N V Y Y T A F G	None identified	Not applicable
P23	T Q I P S R P Q T P S Q	Mouse versican core protein precursor (large fibroblast proteoglycan) (chondroitin sulfate proteoglycan core protein 2) (pg-m)	T Q I P S R P Q T P S T + + P P T P S T E L P K F P S T P S T Q I P S R P Q T P S T I P S P Q P + T G I P S T P Q K P T
P24	V C S N M Y F S C R L S	None identified	Not applicable
P25	V P P H P M T Y S C Q Y	Papcy metalloproteinase inhibitor 1 precursor (TIPM-1)	V P P H P M T Y S C V P P H P T C V P P H P Q T A F C
P26	V P R L E A T M V P D I	Human versican core protein precursor (large fibroblast proteoglycan) (chondroitin sulfate proteoglycan core protein 2) (glial hyaluronate-binding protein) (ghap)	V P R L E A T M V P D I + P R A T + + P + I I P R K S A T V I P E I

(Table continues)

Table I. Continued

Peptide (P) name	Sequence	Homolog of interest	Alignment
P27	V P T K P E L P V N F T	Human collagen alpha 1 (vii) chain precursor (long-chain collagen	V P T K P E L P V N V P T P E L P V + V P T G P E L P V S
P28	W S S D L P Q P A S T Y	None identified	Not applicable
P29	Y I T P Y A H L R G G N	Rat insulin-like growth factor receptor	P Y A H L R G G P Y A H + G G P Y A H M N G G
P30	N V Y T D N T L S P T P	None identified	Not applicable

total four times in order to enrich for phage that preferentially bind to the GIT/Peyer's patch tissue *in situ*.

Five hundred clones from five different rats were selected following four rounds of screening in the rat intestinal loop model. These clones were analyzed for binding to intestinal tissue homogenates that contained or did not contain Peyer's patches. In addition, binding to cell membrane fractions isolated from epithelial cells (human Caco-2 and rat IEC-6) was examined. Figure 2 summarizes the binding of 72 clones, as representative data, to homogenates of rat Peyer's patch tissue and plasma membrane fractions of Caco-2 cells and IEC-6 cells. The negative control phage M13mp18, lacking a targeting peptide, showed minimal binding to the tissues and cells tested (OD < 0.15 units).

Peptide Synthesis and Analysis

Following ELISA analysis of the 500 phage isolates, the top 100 high-binding clones were selected, and the DNA sequences of the peptide coding inserts were determined. Of the 100 sequences, 30 unique DNA sequences, coding for 30 unique putative peptide sequences, were identified. Table I summarizes the amino acid sequences of peptides (P)1 to P30 encoded by DNA inserts in the isolated phage clones. A number of DNA sequences and corresponding peptide sequences were found multiple times in the sequenced clones; this included five different clones coding for peptides P5 and P29, three different clones coding for peptides P7 and P18, and two copies each of a number of other clones. Bioinformatic analysis of the 30 novel targeting peptides by SWISS-Prot analysis also identified potential homologies with known protein sequences as summarized in Table I. In addition, analysis of the peptide sequences revealed that several peptides contained common tripeptide motifs including LGT, PVT, PPY, NVY, and SXS motifs; the tetrapeptide motifs TPPP and LEAT, a pentapeptide motif LSWRS, and a heptapeptide motif NVYTXXXXSPXR (Table II). Analysis of the peptide sequences also indicated that a number of the peptides were either amphipathic or hydrophobic in nature (Table III).

Synthetic Peptide Characterization

Using the 30 unique sequences identified, peptides were synthesized with biotin tags, at either their amino or carboxyl terminus, for use in binding and uptake studies. Although all of these peptides were analyzed for binding activity *in vitro*, two peptides, peptides P8 (LETTCASLCYPS) and P25 (VPPHPMTYSCQY), were selected for further characterization in binding studies both *in vitro* and *in vivo* on the basis of their high binding activity *in vitro*.

To ascertain whether these peptides also bound to human intestinal tissue, immunohistochemisty was carried out to test for binding to human epithelium tissue. Positive binding to human Peyer's patch was observed with both peptides (Fig. 3). In addition, point mutants and stabilized versions (D-forms and retro-inverted D-forms) of these two peptides were synthesized (summarized in Table IV) and used in binding studies to Caco-2 membrane fractions. Ala-scan mutagenesis indicated a specific requirement for retention of the single cysteine in peptide P25 (compare binding of P25 vs. P25a; Fig. 4c) and either of the two cysteines in peptide P8 (compare binding of P8 vs. P8a, P8b, and P8c; Fig. 4a) in order to retain binding to Caco-2 cell membranes in the ELISA-based assay (Fig. 4a and 4c, respectively). D-form and retro-inverted D-form of peptides P8 (i.e., P8e and P8f, respectively) and P25 (i.e., P25c and P25d, respectively) also showed binding to Caco-2 membrane fractions (Fig. 4b and 4c, respectively).

Phage Trafficking *in Vivo*

To investigate whether intra-intestinal readministration of selected phage clones could translocate across the intesti-

Table II. M Cell–Targeting Peptide Motifs*

Motif	Peptide (P) name	Sequence
LGT	P9	**L G T** D W H S V S Y T L
	P10	**L G T** L N A G V P G F P
PVT	P5	G L W P W N **P V T** V L P
	P12	L V P T T H R H W **P V T**
PPY	P19	S I S W A S Q **P P Y** S L
	P6	H M L N D P T P **P P Y** W
TPPP	P1	A **T P P P** W L L R T A P
	P6	H M L N D P **T P P P** Y W
NVYTXXXXSPXP	P15	**N V Y T** F H S M **S P** M **P**
or	P30	**N V Y T** D N T L **S P** T **P**
NVY	P22	T M S P **N V Y** Y T A F G
LEAT	P8	**L E T T** C A S L C Y P S
	P26	V P R **L E A T** M V P D I
SXS	P3	D Y D **S L S** W R S T L H
	P9	L G T D W H **S V S** Y T L
	P30	N V Y T F H **S M S** P M P
	P19	**S I S** W A S Q P P Y S L
LSWRS	P3	D Y D S **L S W R S** T L H
	P18	S I D T I Q I **L S L R S**
	P19	S **I S W A S** Q P P Y S L

* Motifs highlighted in bold.

Table III. Amphiphilic and Hydrophobic Categorization of M Cell–Targeting Ligands

Amphiphilic peptides*												
Peptide (P) name	Sequence											
P4	*G*	**E**	**P**	**T**	**T**	**D**	**M**	**R**	*W*	*R*	*N*	*P*
P17	**S**	**D**	**F**	**S**	**D**	*T*	*M*	*P*	*H*	*R*	*P*	*S*
P18	**S**	**I**	**D**	**T**	*I*	*Q*	*I*	*L*	*S*	*L*	*R*	*S*
P7	*K*	*P*	*A*	**Y**	**T**	**H**	**E**	**Y**	**R**	**W**	*L*	*A*
P9	*L*	*G*	**T**	**D**	**W**	**H**	**S**	*V*	*S*	*Y*	*T*	*L*
P11	**L**	**T**	**H**	**S**	**K**	*N*	*P*	*V*	*F*	*L*	*S*	*T*
P16	**Q**	**H**	**T**	**T**	**L**	**T**	**S**	**H**	**P**	*R*	*Q*	*Y*
P19	*S*	*I*	*S*	*W*	*A*	*S*	*Q*	*P*	*P*	**Y**	**S**	**L**
P28	*W*	*S*	*S*	*D*	*L*	*P*	*Q*	*P*	**A**	**S**	**T**	**Y**

Hydrophobic peptides												
Peptide (P) No.	Sequence											
P3	D	Y	D	S	L	S	W	R	S	T	L	H
P5	G	L	W	P	W	N	P	V	T	V	L	P
P6	H	M	L	N	D	P	T	P	P	P	Y	W
P29	Y	I	T	P	Y	A	H	L	R	G	G	N
P26	V	P	R	L	E	A	T	M	V	P	D	I
P14	N	T	R	I	P	E	P	I	R	F	Y	M
P20	S	M	V	K	F	R	R	P	L	D	S	R
P10	L	G	T	L	N	A	G	V	P	G	F	P
P24	V	C	S	N	M	Y	F	S	C	R	L	S
P25	V	P	P	H	P	M	T	Y	S	C	Q	Y
P15	N	V	Y	T	F	H	S	M	S	P	M	P
P8	L	E	T	T	C	A	S	L	C	Y	P	S

* >60% hydrophobic domain highlighted in italics; >60% polar domain highlighted in bold.

nal epithelium, phage clones were selected for instillation into rat intestinal ileal loops. Four lead phage clones, coding for peptides P5, P8, P9, and P30 and the negative control phage vector M13mp18 were administered via intra-intestinal administration, and the presence of phage in peripheral blood was measured after 30 min. Of the four clones examined, one clone, coding for peptide P8, was detected at a high concentration in the peripheral blood (i.e., 4.5×10^7 phage particles per 100 μl of blood) (Table V).

Model Targeted Delivery Systems

To investigate whether the targeting peptides retain functionality when presented on the surface of a model particulate system, in initial studies we investigated whether the addition of targeting ligands to the surface of inert polystyrene particles would enhance binding to Caco-2 membranes. Fluorescent polystyrene particles conjugated with streptavidin were used to immobilize several biotinylated ligands (i.e., peptides P25, P8, P8a, P8c; see Table IV) along with the negative control biotinylated peptide (naagngnstg). Binding of these targeted delivery systems to Caco-2 plasma membrane fractions was investigated in an ELISA-based assay. Particles modified with peptides P25, P8, and P8a showed comparable binding to Caco-2 membrane fractions (Fig. 5). Particles modified with peptide P8c, containing a double Cys to Ala substitution, showed reduced binding to the Caco-2 membrane fractions (Fig. 5). In contrast, the nonimmobilized peptide P8c showed no binding to Caco-2 membrane fractions (Fig. 4a).

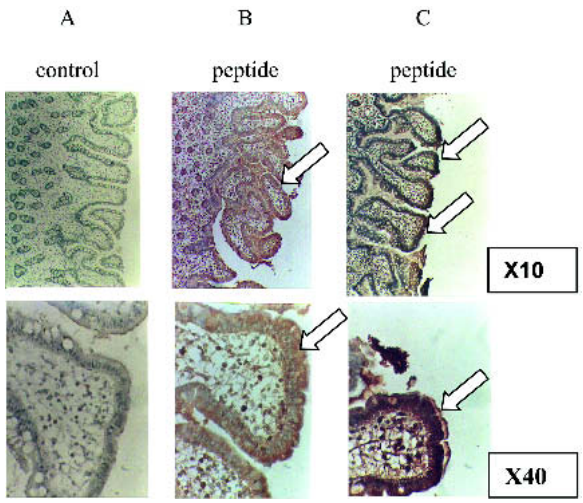

Fig. 3. Immunohistochemistry showing binding of biotinylated peptides P8 and P25 to human intestinal tissue sections. Panel A: binding of a negative control peptide (sequence: NAAGNGNSTG); panel B: peptide P8; panel C: peptide P25. Images were taken at 10× and 40× magnification. The arrows highlight the binding of peptide P8 (panel B) and P25 (panel C) to the human intestinal epithelium.

Binding of Particulate Systems *in Vivo*

We next investigated binding and uptake of the peptide-coated polystyrene particles *in vivo.* The targeting peptide used was a stabilized derivative of peptide P25, that is, peptide P25d (the D-form peptide), which was adsorbed onto the surface of the fluorescent polystyrene particle. Following particle administration *in vivo*, the M cells in the Peyer's patches

Table IV. Derivatives of Peptides (P) 8 and P 25

Peptide (P) name	Sequence*												Modification
P8	L	E	T	T	C	A	S	L	C	Y	P	S	Wild type
P8a	L	E	T	T	A	A	S	L	C	Y	P	S	cys to ala
P8b	L	E	T	T	C	A	S	L	A	Y	P	S	cys to ala
P8c	L	E	T	T	A	A	S	L	A	Y	P	S	Double cys to ala
P8d	L	E	T	T	S	A	S	L	S	Y	P	S	Double cys to ser
P8e	l	e	t	t	c	a	s	l	c	y	p	s	D-form
P8f	s	p	y	c	l	s	a	c	t	t	e	l	Retro-inverted
P25	V	P	P	H	P	M	T	Y	S	C	Q	Y	Wild type
P25a	V	P	P	H	P	M	T	Y	S	A	Q	Y	cys to ala
P25b	V	P	P	H	P	M	T	Y	S	S	Q	Y	cys to ser
P25c	v	p	p	h	p	m	t	y	s	c	q	y	D-form
P25d	y	q	c	s	y	t	m	p	h	p	p	v	Retro-inverted

* Shaded residues depict site of site-directed mutagenesis; capital letters depict L-amino acids; lowercase letters depict D-amino acid residues.

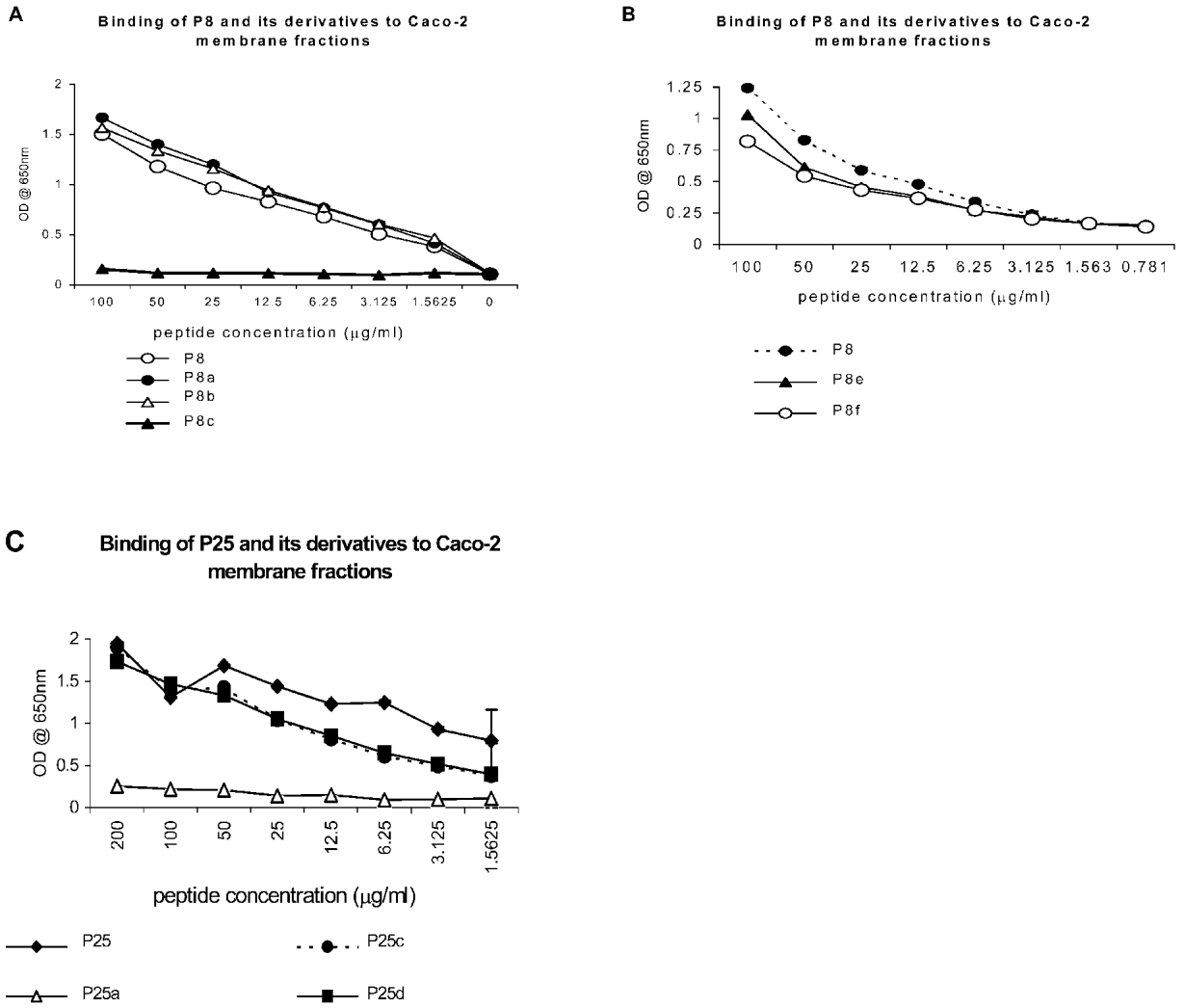

Fig. 4. Binding of biotinylated peptides and their derivatives to Caco-2 cell membrane fractions in an ELISA-based assay. Panels A and B: Binding of peptides P8 and its derivatives P8a–P8c, P8e, and P8f. Panel C: Binding of peptide P25 and its derivatives P25a–P25d to membrane fractions isolated from Caco-2 cells. Binding of the peptides to the cell membranes was determined by ELISA assay by measuring the optical density (O.D) at 650 nm, as outlined in "Materials and Methods."

were stained for the UEA-1 receptor, a known marker for M cells in mice (8,9). In Fig. 6, M cells are thus stained in red (i.e., stained for the UEA-1 receptor). The modified particulate system was administered to mouse intestinal ileal loops, essentially as previously outlined (9). As shown in Fig. 6, there was an enhancement in the binding of the P25d-modified particulates to M cells compared to negative control particles (coated with biocytin) that showed little or no binding to M cells or mouse FAE. Not only were particles observed bound to the surface of M cells, but they were also observed below M cells in the Peyer's patch following confocal microscopy analysis in the XZ plane (Fig. 6c).

Table V. Titration of Blood Samples for Translocation of Phage from the Gut into the Blood

Peptide expressed on phage surface		Total phage per 100 μl of blood
Peptide (P) name	Sequence	
P30	N V Y T D N T L S P T P	19
P5	G L W P W N P V T V L P	0
P8	L E T T C A S L C Y P S	4.5×10^7
P9	L G T D W H S V S Y T L	34
M13control	No peptide	180
PBScontrol	—	0

DISCUSSION

Using *in vivo* phage display screening technology, we have identified several lead peptides for targeted delivery to M cells and FAE of the intestine. The clones exhibited a broad range of binding activity across the Peyer's patch and cell types tested (Fig. 2) with concentration-dependent binding detectable for all high binding clones (data not shown). Binding profiles for each clone were similar, although not identical, on rat Peyer's patch tissue, human Caco-2 cells, and rat IEC-6 cells (Fig. 2). Bioinformatic analysis of the targeting peptides identified potential tripeptide, tetrapeptide, and/or pentapeptide motifs found in a number of the peptides (Table II). In addition, bioinformatic analysis identified possible sequence homologies for a number of the targeting peptides to

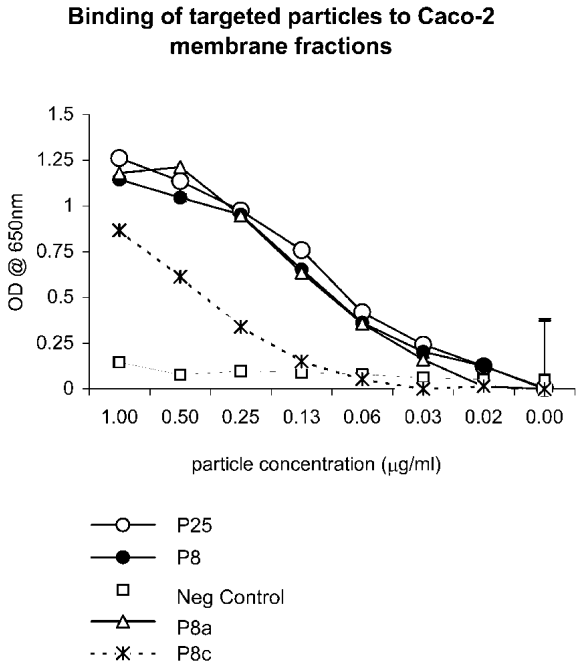

Fig. 5. Binding of polystyrene particles coated with biotinylated peptides P8 and P25, with the P8 derivatives P8a and P8c or, as a control, with the negative control peptide (sequence: NAAGNGNSTG) to the membrane fractions isolated from Caco-2 cells. Binding of the particle (μg/ml) to the cell membranes was determined by ELISA assay by measuring the optical density (OD) at 650 nm, as outlined in "Materials and Methods."

naturally occurring receptors or proteins, which may provide some insight as to how the targeting peptides function *in vivo* (Table I). Of note is the possible homology of peptide sequences to the mouse keratinocyte growth factor receptor (peptide P4), the human epithelial E-cadherin precursor (peptide P6), the IgE Fc receptor α-subunit precursor (peptide P7), the Vlalu lectin-related protein precursor (peptide P8), the zona-adhesion precursor (peptide P9), the sucrase–isomaltase intestinal enzyme (peptide P15), the human placental P-cadherin precursor (peptide P16), the ZO1 mouse tight junction protein (peptide P20), and the rat insulin-like growth factor receptor (peptide P29). Each of these proteins are membrane-bound proteins. In the case of the sucrase-isomaltase protein, this stalked protein is expressed primarily by epithelial cells of the jejunum, representing one of the most abundantly expressed proteins by jejunum epithelial cells and is highly conserved across species. Cadherin proteins are primarily involved in cell–cell contacts and cell–cell interactions. ZO-1 proteins are primarily expressed at tight junctions between epithelial cells, including epithelial cells along with other tight junction proteins such as occludin.

Synthetic forms of a number of the targeting peptides retain binding activity to homogenates or plasma membranes isolated from rat Peyer's patch tissue, human Caco-2, and rat IEC-6 epithelial cells (Figs. 2 and 4). The synthetic peptides P8 and P25 were also shown to bind to human intestinal epithelium in immunocytochemistry studies (Fig. 3). In contrast, a number of the synthetic peptides did not display binding to Caco-2 membranes suggesting that the conformation and/or dimerization of the peptide when presented as a conjugate to a protein such as the pIII protein of the bacteriophage M13 may differ to the conformation or presentation of a free peptide in solution. Thus, conformation constraint or dimerization may be important for full retention of receptor binding activity. This is illustrated, in part, by peptide P8c (the double Cys-to-Ala mutant of peptide P8), which showed no binding to Caco-2 membranes as a free peptide (Fig. 4a), but does demonstrate binding to Caco-2 membranes when presented on the surface of the polystyrene nanoparticulates. Presentation of peptides on the surface of a particulate system may either (a) facilitate peptide:peptide dimerization or (b) constrain the possible conformation of a peptide when laid down on the surface of a particulate system.

We have also shown that stabilized versions of the peptides P8 and P25 retain activity to plasma membrane fractions isolated from human Caco-2 cells (Fig. 4) and that binding activity is also retained when these peptides are immobilized on the surface of model polystyrene nanoparticulate (Fig. 5). Among the stabilized peptides studied this included D-peptides as opposed to the naturally occurring L-peptides and retro-inverted D-peptides. Proteases cleave peptide bonds between adjacent native L-amino acids, rendering these peptides susceptible to proteolytic degradation in the GI tract. Artificial proteins or peptides composed of D-amino acids are largely resistant to proteolytic cleavage. Retro-inverted peptides are peptides having all D-amino acids but are synthesised in the reverse order compared to the original L-peptide. The net result of combining D-enantiomers and reverse synthesis is that the positions of carbonyl and amine groups in each amide bond are exchanged while the position of side-chain groups is preserved (17,18). In an ELISA-based assay, we showed that binding activity of stabilized and retro-inverted D-form peptides of P8 and P25 was retained (Fig. 4). This retention of binding activity of the D-peptide and/or retro-inverted D-peptides may be important for the design of oral delivery systems where stability of the targeting ligand in the gastrointestinal tract is of paramount importance.

Finally, we have shown enhanced delivery of targeted nanoparticulate, which are surface-coated with the stabilized peptide P25d *in vivo* in a mouse model, and that this targeting is directed at M cells as evidenced by co-localization of the delivered nanoparticulates to M cells that are stained for UEA-1 binding (Fig. 6). Furthermore, *in vivo* screening with a subpopulation of the phage clones demonstrated proficient translocation of the phage particles coding for peptide P8 across the GIT tissue to the underlying systemic circulation. The targeting peptides for the most part are either hydrophobic or amphipathic in nature (Table III), which may also facilitate their translocation both into and across biological membranes following initial binding to their target receptors.

Though a variety of inert particles efficiently target to and are transcytosed by M cells, such targeting is highly variable and depends on both the physical properties of the inert particle and the host species. It has previously been shown that delivery of both polystyrene particles and liposomes to M cells is enhanced when these delivery vehicles are coated with UEA-1 lectin that targets its receptor on M cells (19,20). A key disadvantage in the use of the UEA-1 glycoprotein itself as a natural targeting ligand includes its large size and inherent cytotoxicity. The large molecular weight of the UEA-1 lectin is likely to lead to an immune response to such a targeting ligand when presented on the surface of a nanoparticulate and thus limit its use in repeat immunizations. To

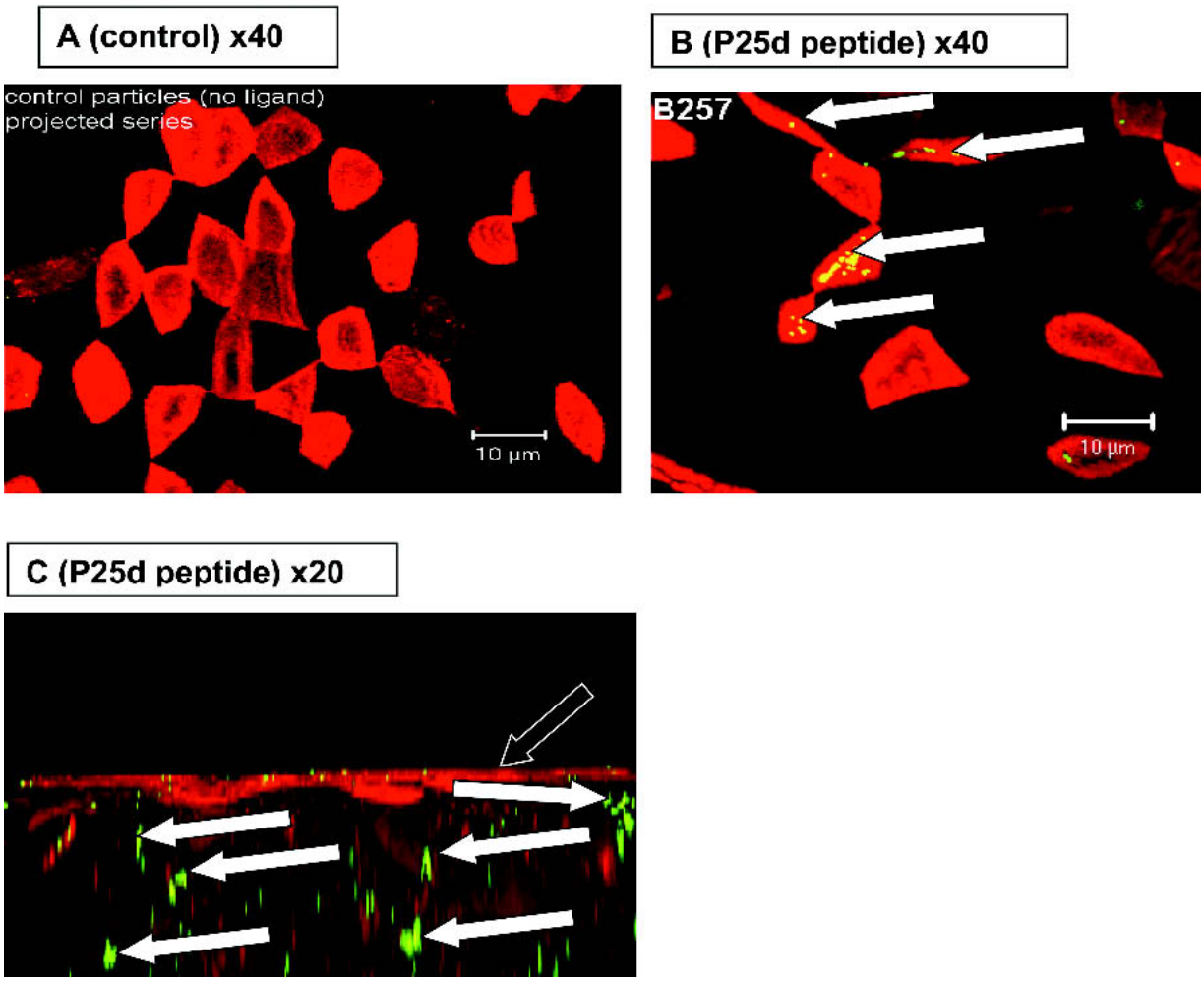

Fig. 6. Confocal scanning microscopy images showing association of FITC-loaded polystyrene particles with mouse Peyer's patch following instillation into mouse intestine (indicated by white arrows). Control particles were coated with biocytin (panel A) and targeted particles with biotinylated retro-inverted D-form peptide P25d (panels B and C). Images shown in panels A and B are projected Z-series showing particles associated predominately with M cells or projected XZ series (panel C) showing particles below the surface of the Peyer's patch. M cells are stained in red with UEA-tetramethylrhodamine isothiocyanate. The polystyrene particles are labeled with fluorescein and stain green. Particles were associated predominantly with M cells.

address these issues, we have recently identified small organic molecule mimetics of the UEA-1 lectin, following combinatorial library screening, using a competition-based screening assay. The UEA-1 mimetics were shown to target the delivery of model nanoparticulates to M cells *in vivo* in mice (9). The advantages of these UEA-1 mimetics over a large glycoprotein such as the lectin UEA-1 itself include their small size (typically less than 1500 Da), stability to simulated intestinal fluids, ease of synthesis, and low cost, as well as their suitability for incorporation into delivery systems using routine chemical procedures. Among the key advantages of the targeting peptides identified in the studies reported here vs. the UEA-1 mimetics include easy conjugation to protein-based antigens through recombinant DNA technology, the ease of expression of these targeting peptides on the surface of biological carriers for vaccine delivery, including bacterial carriers, noncolonizing microbial carriers of vaccines, viral and bacteriophage carriers that co-express vaccines and antigens.

A major hurdle to the design of targeting ligands to M cells is the dearth of knowledge on human M cell receptors. As well as the UEA-1 ligand, other receptors of potential interest in targeting to human M cells include GM1, the receptor for the B subunit of cholera toxin (21), sialyl Lewis A antigen (22), reovirus antigens (23), and IgA receptor (24). Most recently, we have established that the peptidoglycan recognition protein (PGRP)-S expression almost exclusively co-localized with UEA-1 + M cells (8). Studies aiming to target GM1 have shown that accessibility to the receptor may be limited to nanoparticles. Expression of the other receptors listed has been shown in man, although heterogeneity of expression would put into doubt their use as targets for vaccine design. As a consequence, we aimed to identify novel targeting ligands for M cells of Peyer's patch using both *in vivo* and *in vitro* random or open system phage display library screening. The key advantage of this technique is that peptides are selected on the basis of interaction with recombinant receptors *in vivo* and does not rely on prior knowledge of receptor expression by, for example, M cells and the FAE. By using random phage display screening in *in situ,* intestinal loop

model prior knowledge of known receptor expression at target cells or tissue is, thus, not required. Furthermore, the peptides or targeting ligands are selected following their presentation on a particulate system *in vivo,* which may mimic particulate-based delivery systems for vaccine delivery such as liposomes, PLA, and PLGA particulates.

Applications of vaccine targeting technologies are diverse. Peptides could be displayed through conjugation chemistries on the surface of liposomes (20,25), bilosomes (26,27), virosomes (28,29), or polymer formulations (30–33). Though a certain degree of success has been achieved using these approaches, a major disadvantage is the difficulty in inducing a CTL and Th1 T-cell response. These types of response are needed for protection of the host against intracellular pathogens and viruses (34). An alternative to the particulate and adjuvant approach to mucosal vaccine delivery is the use of attenuated bacterial and viral vectors to deliver foreign antigens to the host to elicit a humoral and cell mediated response (35,36). In certain cases, an immune response to the bacterial or viral vector itself can be induced. Live attenuated or killed bacterial vectors that have been used include the pathogens *Vibrio cholera* (37) and *Salmonella typhi* (38), nonharmful commensals *Lactobacillus* spp. (39) and *Streptococcus gordonii* (40), and viral vectors including adenovirus (41), poliovirus (42), vaccinia virus (43), rabies virus (44) and bacteriophage (45). *Saccharomyces cervisiae* or yeast (46) can also be engineered to express vaccine antigens, and their immunomodulatory properties have been shown to give rise to protective CTL responses to tumor or human immunodeficiency virus-1 antigens in mice. These bacterial, yeast, and viral systems could be engineered to express on their surface targeting peptides identified in our studies, thus enhancing delivery of these carrier systems and associated antigens to immune activating sites. Although certain of these bacterial and viral strains target Peyer's patches during natural infection, it has been reported that impaired colonization in attenuated vaccine strains expressing heterologous antigens can be a hurdle in the design of vaccine vectors and that optimization of recombinant strains to enhance immune outcome is needed (47). Constitutive expression of the ligands that target M cells, as outlined in these studies, might help overcome this problem. More in-depth studies are required to examine the effectiveness of M cell targeting on enhancing immune outcome following targeting of antigen to M cells. Ultimately, this will be the true test of oral targeted vaccines. In summary, we have used a novel approach to identify new peptide ligands that show potential as targeting ligands for design of a wide range of oral vaccine delivery platforms.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Barry. H. Hirst and Dr. M. Ann Clark at the University of Newcastle-Upon-Tyne (UK) for their invaluable assistance during studies in the *in situ* model. We are grateful to Therese Kinsella for critical review and comments on the manuscript. This research was funded by Elan Corporation, plc.

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