

# A novel peptide, THALWHT, for the targeting of human airway epithelia

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**Abstract** Targeting gene vectors to human airway epithelial cells may help to overcome the current inefficiency of gene transfer as the major problem confronting cystic fibrosis gene therapy. To elucidate novel ligands targeting abundant, apically located receptors on airway epithelial cells, a phage display library was screened for peptides binding with high affinity to such cells. This screening yielded a selectively enriched amino acid sequence, Thr-His-Ala-Leu-Trp-His-Thr (THALWHT). Subsequent binding studies confirmed that THALWHT-displaying phages bound much stronger than phages displaying control peptides to human airway epithelial cells. In contrast, no significant binding differences were observed on a variety of non-airway-derived human cell lines suggesting selective binding of the THALWHT motif to airway epithelia. Confocal microscopy of such cells after exposure to labelled synthetic THALWHT peptide indicated that its binding is followed by specific internalisation via endocytosis. A synthetic peptide comprising a cyclic CTHALWHTC domain and a DNA binding moiety enabled efficient targeted gene delivery into human airway epithelial cells. Competition assays with free THALWHT peptide confirmed the specificity of gene delivery. Thus, the THALWHT motif may prove a useful targeting moiety for both non-viral and viral gene therapy vectors. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Phage display; Airway epithelium; Gene delivery; Cystic fibrosis gene therapy

## 1. Introduction

Progress in the field of cystic fibrosis gene therapy has been hampered by low gene transfer efficacy [1,2], thus the development of vectors allowing more efficient gene delivery has become a predominant research strategy in this field. The lack of cellular receptors that may permit high-affinity binding to the apical surface of human airway epithelial cells appears to be the main reason for the inefficiency of adenoviral vectors used

in recent clinical trials [3–7]. To circumvent this problem lung-targeting from the vascular side has been suggested [2,8]. The construction of novel vectors to include targeting domains binding with high affinity to abundant, apically located receptors on airway epithelial cells is another promising approach to overcome this drawback. Non-viral vectors avoid some crucial problems inherent to viral vectors [9,10] and are usable in combination with any given plasmid DNA, thus allowing fast and flexible adaptation to a particular gene transfer requirement. However, they have not yet reached the gene transfer efficacy of the best viral vectors and targeted delivery is an important approach towards their improvement. Non-viral systems also provide an excellent means for the rapid testing of a particular targeting moiety with respect to its suitability for incorporation into viral vectors. Several candidate moieties for the targeting of human airway epithelia, including the surfactant protein A receptor [11] and monoclonal antibodies [12] have been investigated. Our group has been developing synthetic peptides comprising a cell surface receptor-targeting domain and a cationic DNA binding moiety as novel non-viral vectors [13–17]. Following our recent identification of a peptide which binds to  $\alpha_9\beta_1$ -integrin, a receptor abundant throughout the human airway epithelia [18,19], we have developed non-viral vectors targeting this integrin [17]. Here we report the isolation of a novel peptide from a phage display library screened for binding to a human airway epithelial cell line and describe its use for efficient and selective gene delivery into human airway epithelial cells. We anticipate that these peptides may be used as targeting moieties of different non-viral vectors and may also be linked to or incorporated into the coat of viral vectors [20–26] to allow selective infection of target cells.

## 2. Materials and methods

### 2.1. Cell lines and synthetic peptides

The human cell lines 16HBE14o<sup>−</sup> (bronchial epithelium-derived) and HTEo<sup>−</sup> (tracheal epithelium-derived) were maintained in Eagle's minimal essential medium (MEM) with L-glutamine (Sigma, Poole) containing 10% foetal calf serum (FCS), penicillin and streptomycin (100 U/ml of each). The human cell lines CaCo2 (colon adenocarcinoma), HeLa (cervix adenocarcinoma), SW480 (colorectal adenocarcinoma) and HUH-7 (hepatoma) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS (HeLa, SW480, HUH-7) or 20% FCS (CaCo2) and penicillin/streptomycin (100 U/ml of each). Mz-ChA1 cells (gall bladder adenocarcinoma) were maintained in CMRL-1066 medium (Life Technologies, Paisley, UK) containing

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**Abbreviations:** BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MEM, Eagle's minimal essential medium; PBS, Dulbecco's phosphate-buffered saline; TBS, Tris-buffered saline

10% FCS and penicillin/streptomycin (100 U/ml of each). Standard tissue culture reagents were purchased from Life Technologies, Paisley, UK.

The peptide [K]<sub>16</sub>-GGCRGDMFGCA was synthesised on a solid-phase batch peptide synthesiser (Applied Biosystems, Foster City, CA, USA) and disulphide-cyclised as described previously [14], the peptides [K]<sub>16</sub>-GGCTHALWHTC, [K]<sub>16</sub>-GGCHLTWTAHC and CTHALWHTC (all disulphide-cyclised) were synthesised by Affiniti Research Products, Exeter. The ability of each peptide to bind DNA was analysed by addition of increasing amounts of peptide to a constant amount of plasmid DNA, followed by electrophoresis on a 1% agarose gel.

## 2.2. Biopanning

The random peptide phage display library Ph.D.-7 (New England Biolabs, Beverly, MA, USA) was applied to 16HBE14o<sup>-</sup> cells grown in 6-well plates to confluency. In this library seven amino acid-polymers are displayed on the surface of M13-derived phages in fusion with the phage coat protein pIII. 3 h prior to the biopanning procedure the cell culture medium was replaced by serum-free MEM, followed after 2 h by application of 1% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (PBS), further incubation for 1 h and washing with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl).  $2 \times 10^{11}$  phages in 1 ml of TBS were applied to a single well and allowed to bind to the cells at 4°C for 60 min, followed by washing away unspecifically bound phages with TBS (10 washes) and elution of bound phages with 0.2 M glycine-HCl buffer, pH 2.2. The eluate was neutralised with 1 M Tris-HCl, pH 9.1 (150 µl/ml eluate), amplified in *Escherichia coli* ER2537 cells according to the protocol provided by the manufacturer of the library and then re-applied to the cells. In further panning rounds the phage binding time was reduced to 15 min (second round) and 10 min (third round) to increase the stringency of the procedure. Stringency was also increased by using washing buffers containing Tween-20 detergent (0.1% in the second round, 0.2% in the third round). The phages were quantified both after elution and amplification to enable the use of equal amounts ( $2 \times 10^{11}$  phages) in each round of biopanning. Phage eluates were stored at 4°C. After three rounds of phage selection 20 phages were characterised by DNA sequencing on an ABI PRISM 377 DNA sequencer using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

## 2.3. Phage binding assays

Binding studies were performed using enzyme-linked immunosorbent assays (ELISAs) on different human cells grown in 96-well plates to confluency. After blocking the plates with 1% BSA, 2-fold serial dilutions of the phages ( $6 \times 10^{10}$ – $4.9 \times 10^8$ ) were applied to the wells. Phages were allowed to bind to the cells for 60 min at 4°C. Unbound phages were removed by washing the plates six times with a wash buffer containing 0.2% Tween-20. An anti-M13 antibody conjugated with horseradish peroxidase was then added to each well, followed by addition of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)/H<sub>2</sub>O<sub>2</sub> solution after 1 h and measurement of the absorbance at 405 nm.

## 2.4. Confocal microscopy

Confluent 16HBE14o<sup>-</sup> cells grown on coverslips were transferred to a damp box and incubated with 200 µl of either FITC-labelled THALWHT peptide or FITC-labelled HLTWTAH peptide (both at concentrations of 50 µg/ml) in prewarmed DMEM containing 10% FCS for 60 min at 37°C, 5% CO<sub>2</sub>. Following incubation the cells were rinsed five times with PBS and fixed with 3% paraformaldehyde for 15 min. The coverslips were then rinsed and treated with 50 mM NH<sub>4</sub>Cl in PBS for 10 min prior to a quick rinse with water, mounted in Vectorshield (Vector, Burlingame, Canada) and sealed in paraffin wax. Cells were observed using a confocal microscope (Leica, Germany).

## 2.5. Gene transfer experiments

The American firefly luciferase gene under the control of a simian virus 40 promoter and enhancer in the plasmid vector pGL3 (Promega, Madison, WI, USA) was used as reporter gene. Plasmid DNA was purified from overnight cultures of transformed *E. coli* JM109 using an endotoxin extraction kit (Qiagen, Hilden, Germany). Cells were seeded in tissue culture-treated 24-well plates at a density of

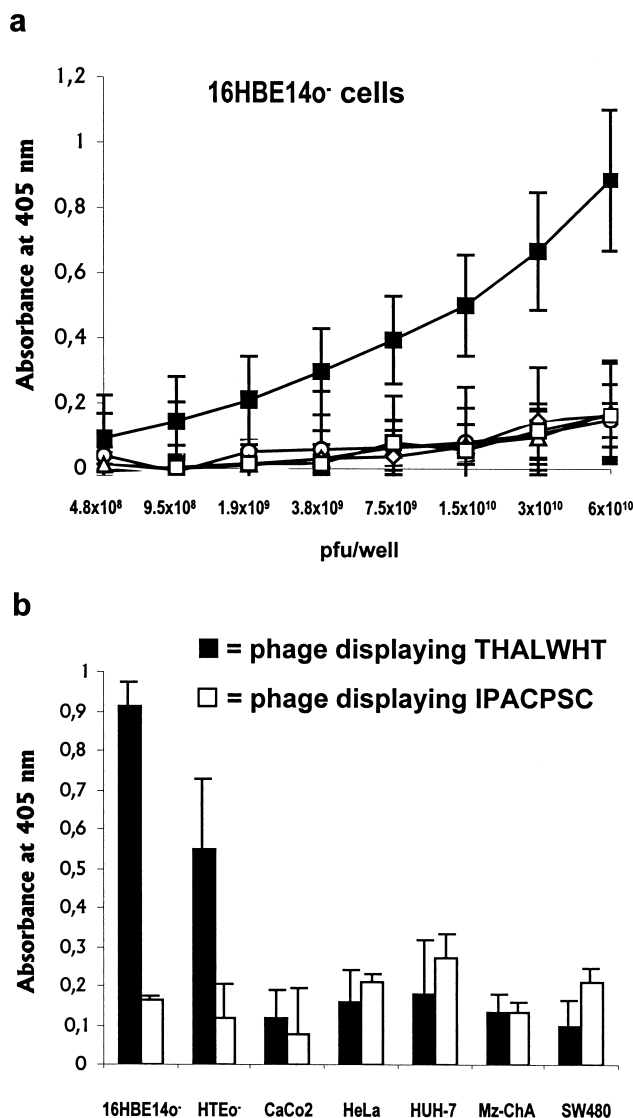


Fig. 1. Specific binding of THALWHT-displaying bacteriophages to the human bronchial epithelial cell line 16HBE14o<sup>-</sup>. a: The binding of bacteriophages displaying the heptamer THALWHT was compared with that of several control phages using ELISA. Two-fold serial dilutions ranging from  $6 \times 10^{10}$  to  $4.9 \times 10^8$  pfu were added to confluent 16HBE14o<sup>-</sup> cells, followed by incubation for 1 h. After careful washing cell-bound phages were quantified using an anti-M13 antibody-horseradish peroxidase conjugate and measurement of the absorbances at 405 nm. Each point represents the average from six individual experiments. Error bars indicate the standard deviations. ■ = phage displaying the heptamer THALWHT, △ = control phage displaying the heptamer VHPLPSF, □ = control phage displaying the heptamer IPACPSC, ◇ = control phage displaying the dodecamer WKPPAYLSPSPT, ○ = control phage displaying the dodecamer TPYFHSSLPARA. b:  $6 \times 10^{10}$  pfu of either THALWHT- or IPACPSC-displaying phages were added to 16HBE14o<sup>-</sup> (bronchial epithelium-derived), HTEo<sup>-</sup> (tracheal epithelium-derived), CaCo2 (colon carcinoma), HeLa (cervix epitheloid carcinoma), HUH-7 (hepatoma), Mz-ChA1 (gall bladder epithelium-derived) and SW480 (colon epithelium-derived) cells. The attached phages were quantified using anti-M13 antibody-horseradish peroxidase conjugate and measurement of the absorbances at 405 nm. The average of three independent experiments is shown (16HBE14o<sup>-</sup>:  $n = 8$ ). Error bars indicate the standard deviations.

$5 \times 10^4$ /well and incubated until confluent. Peptide–DNA and DNA–LipofectAMINE complexes (24  $\mu$ g of LipofectAMINE/ $\mu$ g of DNA) were prepared in 50  $\mu$ l of serum-free OptiMEM medium (Life Technologies, Paisley, UK). The cells were washed and incubated in OptiMEM for 30 min at 37°C prior to addition of the complexes and further incubation for 4 h. The medium was then replaced by DMEM containing 10% FCS supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) and the incubation was continued for an additional 44 h.

For competition experiments the cells were washed with OptiMEM and pre-incubated with the peptide CTHALWHTC for 30 min prior to the addition of peptide–DNA complexes. The peptide was added to the wells in 500  $\mu$ l of OptiMEM medium at a final concentration of 100  $\mu$ M. If chloroquine was used, this was added to the medium 20 min before the peptide–DNA complexes were applied. Luciferase activity was determined after harvesting the cells in 200  $\mu$ l of reporter lysis buffer (Promega, Madison, WI, USA) using the luciferase assay kit from Promega and a Berthold luminometer. The relative light units measured in 30 s were standardised per mg of total protein.

### 3. Results and discussion

Ligands of apically located receptors on human airway epithelial cells have been tested as targeting moieties of gene transfer systems by our group and others [11,17]. However, ligands which bind more selectively and with higher affinity to the target cells are still required for most gene therapy applications. In our continuing search for such ligands we have selected random peptides by screening a random heptamer library in M13 bacteriophages on the human bronchial epithelium-derived cell line 16HBE14o<sup>−</sup>. This cell line has been described to develop the morphologic features of differentiated airway epithelial cells in vitro [27]. On cells forming a confluent layer the presence of microvilli on the apical surface and of tight junctions separating apical and basolateral cell membranes was confirmed by electron microscopy (data not shown). Biopanning of the linear heptamer library Ph.D.-7 (New England Biolabs, Beverly, MA, USA) was performed by three rounds of phage binding, acid-elution and amplification using a confluent 16HBE14o<sup>−</sup> cell layer in each round of selection. During the biopanning steps the stringency of the procedure was increased both by reducing the phage binding time and by increasing the detergent concentration in the washing buffer. After the third round of phage selection 20 phage clones were characterised by DNA sequencing. Amongst those only the insert DNA sequence encoding the peptide Thr-His-Ala-Leu-Trp-His-Thr (THALWHT) was found repeatedly, in 15% of the phage clones analysed. Recovery of residual bound phages by lysis of the cells followed by three rounds of phage binding, elution from the cell lysate and amplification did not yield a consensus sequence, although amino acid motifs with some similarity or partial homology to THALWHT, for example the motif LWH, were found. This fraction, however, is unlikely to represent specifically internalised phages, because phage display via modified gene III proteins of the phage coat is limited by the fact that a maximum of five copies of a peptide can be displayed by any one phage, whereas binding of a larger number of identical peptides would be required for phagocytosis allowing internalisation of particles as large as a phage.

Using ELISA the binding of the selectively enriched phage to 16HBE14o<sup>−</sup> cells was compared with that of several control phages across a range of concentrations between  $4.9 \times 10^8$  and  $6.3 \times 10^{10}$  plaque forming units (pfu)/well. In this assay the THALWHT-displaying phage bound markedly better

than any of the control phages to 16HBE14o<sup>−</sup> cells, at all concentrations tested (Fig. 1a). When the same assay was performed on five other human cell lines derived from various other tissues the binding of the THALWHT-displaying phage, a control phage displaying the peptide IPACPSC (Fig. 1b) and various other control phages (not shown) did not differ significantly. This suggested a strong binding of the peptide THALWHT specifically to 16HBE14o<sup>−</sup> cells via a receptor abundant on these cells but not on non-airway derived cells. To investigate whether the THALWHT motif would also bind to airway epithelial cells different from 16HBE14o<sup>−</sup> we performed the same ELISA also on the human tracheal epithelial cell line HTEo<sup>−</sup> [28] and observed significantly better binding of the THALWHT-displaying phage compared with the IPACPSC-displaying phage (Fig. 1b) and various other control phages (not shown).

Confluent 16HBE14o<sup>−</sup> cells incubated with a synthetic fluorescently labelled THALWHT peptide showed fine vesicular staining at the cell surface (Fig. 2a) and slightly larger vesicles accumulated in a juxta-nuclear region of the cells in subsequent 1  $\mu$ m serial sections (Fig. 2b–d). In contrast, 16HBE14o<sup>−</sup> cells exposed to a fluorescently labelled peptide containing the same amino acids in a randomly generated order ('scrambled' peptide HLTWTAH) did not exhibit vesicular staining, but only a faint diffuse fluorescence signal assumed to be at the cell surface (Fig. 2f). The staining pattern of the THALWHT peptide is a typical feature of internalisation via endocytosis and suggests that binding of this peptide to 16HBE14o<sup>−</sup> cells is followed by its specific internalisation.

As these data indicated that the peptide THALWHT may be a high-affinity ligand of receptors on human airway epithelia we synthesised a peptide comprising disulphide-cyclised CTHALWHTC as receptor-targeting domain and a DNA binding moiety consisting of 16 lysine residues, together with an appropriate control peptide containing the scrambled sequence CHLTWTAHC. A cyclic head group was chosen, because all our previous studies had shown that similar peptides containing cyclic head groups were more effective in mediating gene transfer into cells than their linear equivalents [14,17]. With the synthetic peptide [K]<sub>16</sub>-GGCTHALWHTC specific gene delivery into 16HBE14o<sup>−</sup> cells could be achieved. Peptide–DNA complexes formed with one or two retardation units of this peptide were found to deliver a luciferase reporter gene more than one order of magnitude more efficiently into 16HBE14o<sup>−</sup> cells than complexes formed with identical amounts of [K]<sub>16</sub>-GGCHLTWTAHC (Fig. 3). The relative gene transfer efficiency (standardised as percentage of LipofectAMINE-mediated transfection on these cells) was significantly higher than that achieved with any of the systems for integrin-mediated gene transfer developed in our laboratory [14,17]. In different experiments the relative efficiencies of [K]<sub>16</sub>-GGCTHALWHTC-mediated gene delivery varied between 6 and 12% of the respective LipofectAMINE control, in comparison with about 1% following gene transfer by the RGD-containing peptide [K]<sub>16</sub>-GGCRGDMFGCA. Gene transfer assays performed in parallel on 16HBE14o<sup>−</sup> cells and on the non-airway-derived epithelial cell lines CaCo2 and HeLa confirmed that the peptide [K]<sub>16</sub>-GGCTHALWHTC enables selective gene transfer to human airway epithelial cells (Fig. 3, insert), whereas for [K]<sub>16</sub>-GGCRGDMFGCA which was used as a control peptide in these experiments no significant differences between the three

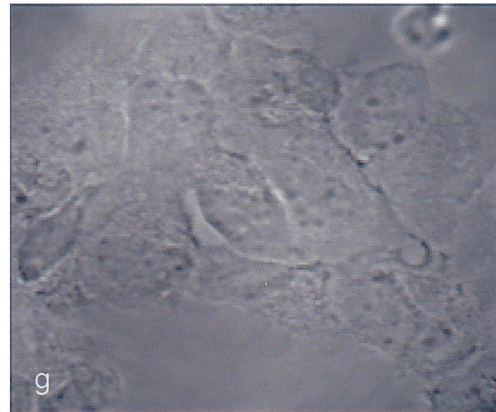
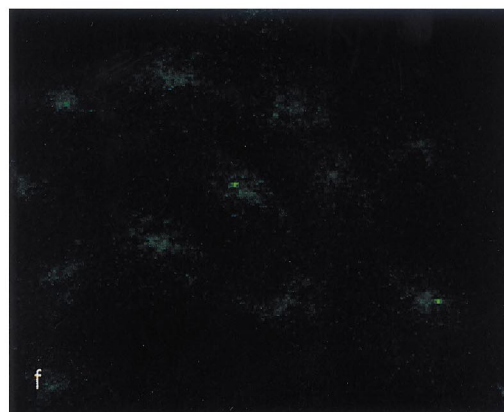
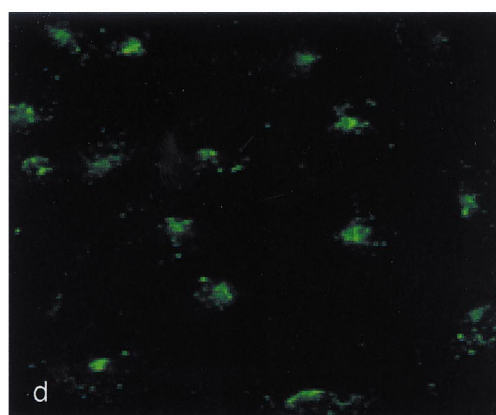
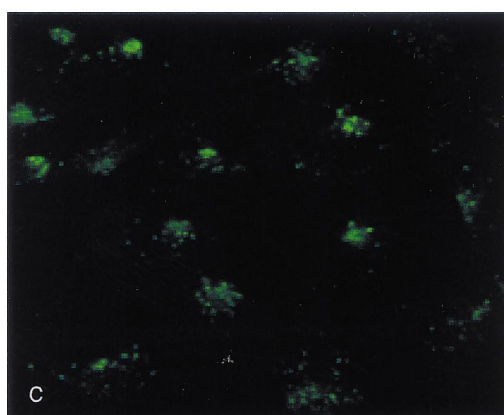
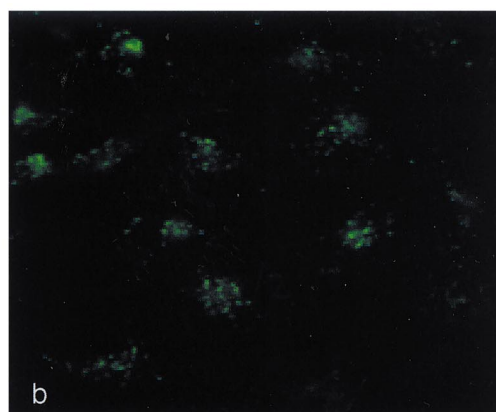
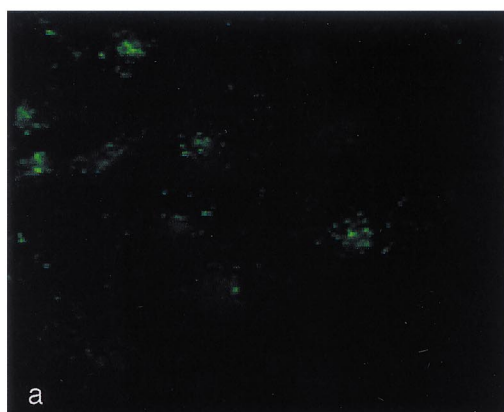


Fig. 2. Specific internalisation of THALWHT peptides into 16HBE14o<sup>-</sup> cells. Confluent 16HBE14o<sup>-</sup> cells were incubated with either FITC-labelled THALWHT (a–e) or FITC-labelled ‘scrambled’ peptide HLTWTAH (f and g) at equal concentrations for 60 min. Representative 1  $\mu$ m confocal optical serial sections starting near the cell surface (a) down through the cells (b–d) are shown. Apart from a slight signal believed to be at the cell surface no cell-associated scrambled peptide was detectable (f). The same fields are also shown under phase contrast (e and g).

cell lines were seen (not shown). The specificity of gene delivery by [K]<sub>16</sub>-GGCTHALWHTC was further demonstrated in competition assays using an excess of the peptide CTHALWHTC. Reporter gene expression following [K]<sub>16</sub>-GGCTHALWHTC-mediated gene delivery was 5-fold reduced when the gene transfer was performed in the presence of free CTHALWHTC, whereas no significant effect of this peptide on [K]<sub>16</sub>-GGCRGDMFGCA- or [K]<sub>16</sub>-GGCHLTWTAHC-mediated gene transfer could be observed (Fig. 4). Competition with free CTHALWHTC thus reduced the binding of the corresponding peptide–DNA complexes to the target cells, thereby decreasing the number of complexes which are internalised unspecifically due to the charge of the peptide. In summary, the gene transfer experiments demonstrate that THALWHT is the targeting motif which can direct the transfection of the corresponding peptide–DNA complex.

As our assay standard, the cationic liposome LipofectAMINE, gave on average 12-fold higher reporter gene expression values, we tried to enhance gene delivery by addition of the lysosomolytic agent chloroquine. This approach allowed us to increase the gene transfer efficacy by one order of magnitude (Fig. 5). Together with the confocal microscopy data this result strongly indicates that the peptide–DNA complexes

are taken up by endocytosis after binding to the cells and that their release from the endosomes is an important factor determining the gene expression efficiency.

Peptide libraries in bacteriophages consisting of fully randomised peptides have proven useful for the identification of several interesting targeting ligands [8,29–32], however, a peptide identified in a particular biopanning experiment does not necessarily resemble a natural ligand of the proposed receptor. The biopanning procedure iteratively selects those peptides which bind best to the target under the panning conditions in vitro, which may differ significantly from the in vivo situation. For peptides binding to large surfaces of a protein or to discontinuous regions of the primary sequence, the selected motifs are therefore not likely to correspond exactly to the natural binding site. The symmetry of the amino acid motif identified here may suggest that the tripeptide Ala-Leu-Trp (ALW) represents the active binding site, whereas the threonine and histidine residues may be necessary to fulfil conformational requirements for binding to the receptor. In any natural ligand of the proposed receptor, however, the binding site may be surrounded by different amino acids. We have been unable to find a protein containing a THALWHT motif in the accessible databases, thus the proposed receptor and its natural ligands still remain to be identified.

To answer the question whether the THALWHT motif

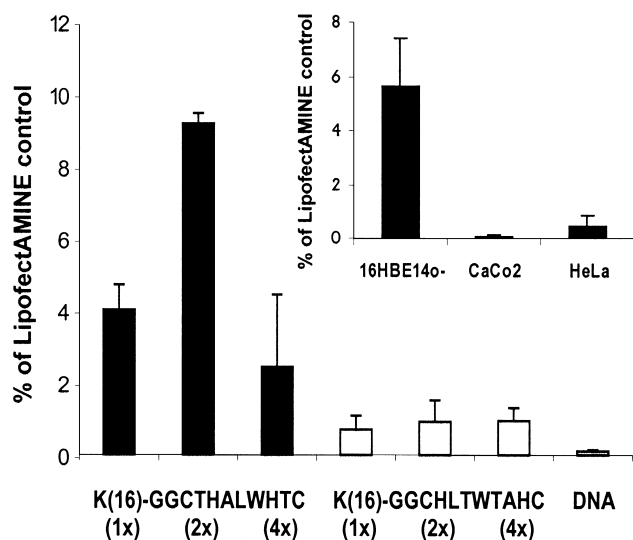


Fig. 3. Reporter gene delivery using a cyclic [K]<sub>16</sub>-GGCTHALWHTC peptide. Peptide–DNA complexes containing one, two or four retardation units of [K]<sub>16</sub>-GGCTHALWHTC (black bars) or the ‘scrambled’ peptide [K]<sub>16</sub>-GGCHLTWTAHC (white bars) (one retardation unit corresponding to the amount of peptide required for complete retardation of the electrophoretic mobility of 1  $\mu$ g of DNA) or pGL3 DNA without peptide (grey bar) were incubated with 16HBE14o<sup>-</sup> cells on 24-well dishes for 4 h at 37°C. Following further incubation in fresh medium for an additional 44 h, luciferase activity in the cell lysates was determined. Data are presented as percentages of those obtained with LipofectAMINE in the same experiment. Each column represents the average result of three experiments each performed in sextuplets. Error bars indicate the standard deviations. The insert shows an independent experiment using two retardation units of [K]<sub>16</sub>-GGCTHALWHTC on 16HBE14o<sup>-</sup>, CaCo2 and HeLa cells.

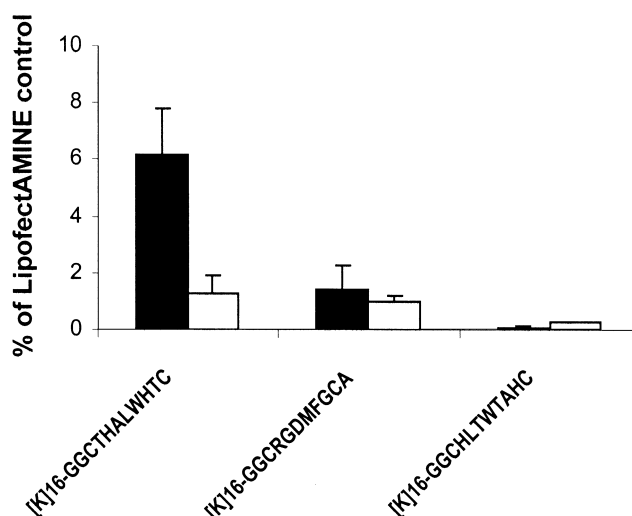


Fig. 4. Competitive inhibition of [K]<sub>16</sub>-GGCTHALWHTC-mediated gene delivery by free CTHALWHTC. Transfection of pGL3 DNA into 16HBE14o<sup>-</sup> cells using different synthetic peptides was performed as described above, either in the presence or in the absence of the peptide CTHALWHTC. The cells were pre-incubated with the free peptide at a concentration of 100  $\mu$ M for 30 min prior to the addition of peptide–DNA complexes. The graph shows luciferase reporter gene expression standardised against protein concentration in the absence (black bars) or presence (white bars) of the peptide CTHALWHTC. Data are presented as percentages of those obtained with LipofectAMINE in the same experiment. Each column represents the average result from sextuplet wells. Error bars indicate the standard deviations.

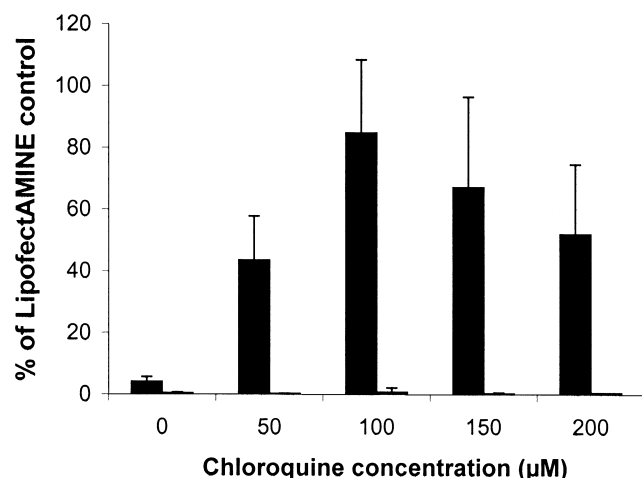


Fig. 5. Effect of chloroquine on [K]<sub>16</sub>-GGCTHALWHTC-mediated gene transfer. [K]<sub>16</sub>-GGCTHALWHTC-pGL3 complexes were applied to 16HBE14o<sup>-</sup> or CaCo2 cells together with increasing amounts of the lysosomolytic agent chloroquine (0–200 µM) followed by incubation for 4 h at 37°C. 48 h after gene delivery the luciferase activity in the cell lysates was determined. Each column shows the average result from sextuplet wells. Gene expression is represented as percentage of that obtained with LipofectAMINE in the same experiment (black bars: 16HBE14o<sup>-</sup> cells; white bars: CaCo2 cells). Error bars indicate the standard deviations.

would allow selective gene delivery into human airway epithelial cells and thus be suitable for incorporation into various gene therapy vectors, we primarily synthesised a synthetic peptide comprising this motif and a cationic DNA binding moiety. This peptide vector enabled efficient targeted gene delivery into 16HBE14o<sup>-</sup> cells suggesting that the THALWHT motif may allow selective gene transfer into human airway epithelia in vivo. As it is well known that differentiated airway epithelial cells are much less susceptible to gene transfer by any non-viral system [33], we have begun to generate a re-targeted adenoviral vector by incorporating THALWHT into the coat of the virus.

Recent work by Romanczuk et al. [25] identified a number of non-related peptides by screening a dodecamer phage display library on other human airway epithelial cells and demonstrated that coupling of one of these peptides to adenoviral vectors improved adenoviral infection of such cells significantly. Any epithelial cell line only partially reproduces the repertoire of receptors displayed on the surface of epithelia in vivo and the use of detergent in our panning procedure may have solubilised part of the receptors displayed on 16HBE14o<sup>-</sup> cells. This, in addition to the use of different peptide libraries, may explain the differences between the peptides found. Thus, direct comparison of the binding of the peptides identified by Romanczuk et al. and the THALWHT peptide to the cells of interest will be required to determine the usefulness of each peptide for a particular gene therapy application. We will continue this study by evaluating the THALWHT peptide on primary airway cells and by investigating whether its putative receptor would be located apically on airway epithelial cells in vivo and thus be accessible for gene therapy vectors delivered into the airways. Furthermore, we will explore the possibilities to improve the release of our peptide from the endosomes by addition of endosomolytic peptides [34] instead of chloroquine. The suitability of the

THALWHT motif demonstrated so far may be exploitable broadly, e.g. for the targeting of liposomes [35,36] or the re-targeting of adenoviral and retroviral vectors [20,24–26].

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