Novel human-derived cell-penetrating peptides for specific subcellular delivery of therapeutic biomolecules

Catherine DE COUPADE*[1,2], Antonio FITTIPALDI†[1], Vanessa CHAGNAS*, Matthieu MICHEL*, Sophie CARLIER*, Ennio TASCIOTTI†, Audrey DARMON*, Denis RAVEL*, Jonathan KEARSEY*, Mauro GIACCA† and Françoise CAILLER*

*Diatos S.A., 166 Boulevard du Montparnasse, 75014 Paris, France, and †Molecular Medicine Laboratory, International Center for Genetic Engineering and Biotechnology, Area Science Park, Padriciano 99, 34012 Trieste, Italy

Short peptide sequences that are able to transport molecules across the cell membrane have been developed as tools for intracellular delivery of therapeutic molecules. This work describes a novel family of cell-penetrating peptides named Vectocell® peptides [also termed DPVs (Diatos peptide vectors)]. These peptides, originating from human heparin binding proteins and/or anti-DNA antibodies, once conjugated to a therapeutic molecule, can deliver the molecule to either the cytoplasm or the nucleus of mammalian cells. Vectocell® peptides can drive intracellular delivery of molecules of varying molecular mass, including full-length active immunoglobulins, with efficiency often greater than that of the well-characterized cell-penetrating peptide Tat. The internalization of Vectocell® peptides has been demonstrated to occur in both adherent and suspension cell lines as well as in primary cells through an energy-dependent endocytosis process, involving cell-membrane lipid rafts. This endocytosis occurs after binding of the cell-penetrating peptides to extracellular heparan sulphate proteoglycans, except for one particular peptide (DPV1047) that partially originates from an anti-DNA antibody and is internalized in a caveolar independent manner. These new therapeutic tools are currently being developed for intracellular delivery of a number of active molecules and their potentiality for *in vivo* transduction investigated.

Key words: cell-penetrating peptide, delivery vector, membrane translocation, proteoglycan, Tat peptide, therapeutic drug delivery.

INTRODUCTION

The cell membrane is a highly selective barrier to hydrophilic macromolecules thereby presenting an obstacle to cellular internalization of molecules including peptides, proteins, DNA and oligonucleotides for use as therapeutic agents. A number of different approaches for overcoming low biomembrane permeability and allowing intracellular delivery of therapeutic macromolecules have been developed. One strategy is to use the cell's own transport mechanisms for example, receptor-mediated endocytosis. Several receptors, including the transferrin receptor, have been successfully exploited to allow the intracellular transport of hydrophilic therapeutic molecules. Normally, the membrane-embedded transferrin receptor is bound by the iron–transferrin complex, and the receptor–ligand complex then enters the cell through clathrin-dependent endocytosis. This pathway can be exploited to deliver a therapeutic molecule by conjugating it to transferrin or anti-transferrin receptor antibodies. This methodology has been successfully applied *in vivo* to deliver both protein and nucleic acid conjugates to a number of different tissue types, including malignant cells [1].

A further method for achieving the internalization of large therapeutic molecules is the use of specific carrier peptides that are capable of crossing the plasma membrane. Such carrier peptides were initially developed following the observation that certain proteins, including the HIV-1 protein Tat, could cross the cell membrane [2]. The HIV-1 transcriptional activator Tat is a multifunctional protein that, in addition to acting as a powerful inducer of viral gene expression, is transported in and out of the cells [3]. This cell penetration property relies on the integrity of a highly basic arginine-rich sequence (amino acids 49–58). Peptides containing this arginine-rich sequence have been developed, named Tat peptides, that after conjugation to a range of macromolecules can facilitate cellular entry of the conjugate. This method of intracellular delivery has been used successfully *in vitro* for a range of macromolecules including fluorochromes, enzymes, antibodies and liposomes [4–9]. The Tat peptide has also been shown to facilitate cellular entry of functional proteins such as β-galactosidase *in vivo* [10].

A number of other proteins and their peptide derivatives have been found to possess similar cell internalization properties including the herpes virus tegument protein VP22 [11], the homoeotic protein of *Drosophila melanogaster* antennapedia (the internalizing peptide is called penetratin) [12], the protegrin 1 (PG-1) antimicrobial peptide SynB [13] and the basic fibroblast growth factor [14]. The carrier peptides that have been derived from these proteins show little sequence homology with each other, but are all highly cationic and arginine- or lysine-rich. Indeed, synthetic polyarginine peptides have been shown to be internalized with a high level of efficiency [15,16]. Competitive inhibition of the internalization of a given peptide can be achieved using a different peptide from this group [17], suggesting that these peptides share a common internalization mechanism.

Abbreviations used: CHO-K1, Chinese-hamster ovary K1; CtxB, cholera toxin B subunit; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DPV, Diatos peptide vector; EGFP, enhanced green fluorescence protein; FCS, fetal calf serum; GAG, glycosaminoglycan; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK-293 cells, human embryonic kidney 293 cells; LDH, lactate dehydrogenase; MBCD, methyl-β-cyclodextrin; MTD, maximum tolerated dose; PFA, paraformaldehyde; PO, peroxidase; SLO, streptolysin O; TMR, tetramethylrhodamine; TRITC, tetramethylrhodamine β-isothiocyanate.

[1] These authors have contributed equally to this work.

[2] To whom correspondence should be addressed, at Diatos S.A. Laboratories, Research Division, 3-5 Impasse Reille, 75014 Paris, France (email cdecoupade@diatos.com).

Cell-penetrating peptides present a major opportunity for improving intracellular drug delivery. The aim of this study was to characterize novel human non-immunogenic cell-penetrating peptides with improved performances, specific subcellular localization and versatile delivery properties. Human Vectocell® penetrating peptides were selected for their ability to interact with aminoglycans, including heparin and chondroitin sulphates [18], and show a high level of basic amino acids. In addition, the properties of natural anti-DNA antibodies, present in patients with autoimmune disease such as lupus erythematosus, were exploited, as these autoantibodies can enter the cell and be transported to the nucleus. These autoantibodies contain sequences that allow penetration of both the cell and nuclear membranes in order to reach their nuclear DNA target [19–21]. Thus a collection of novel human-derived peptides denominated Vectocell® have been identified with enhanced cell penetration characteristics. These new peptidic sequences can internalize molecules of varying sizes with efficiency often greater than that of the Tat peptide. The present study describes the molecular mechanisms of internalization of these proprietary Vectocell® peptides and their capacities for delivery of a broad range of molecules in different cell types.

MATERIALS AND METHODS

Antibodies, fluorescent markers and reagents

Monoclonal antibody raised against horseradish peroxidase was generated in our laboratory and used at 25 μg/ml. Monoclonal mouse anti-human Ki-67 antigen was supplied by Dako (Trappes, France). Monoclonal mouse anti-human Hsp60 (heat-shock protein 60) antigen was supplied by BD Biosciences (San Diego, CA, U.S.A.). Rhodamine (TRITC, tetramethylrhodamine β-isothiocyanate)-conjugated affinipure donkey anti-mouse antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). Alexa Fluor 594-labelled CtxB (cholera toxin subunit B), TRITC-labelled transferrin and tetramethylrhodamine-5-maleimide were from Molecular Probes (Eugene, OR, U.S.A.). EZ-Link™ maleimide-activated horseradish peroxidase was from Pierce (Woburn, MA, U.S.A.). The protease inhibitor cocktail was from Roche (Strasbourg, France). SLO (streptolysin O) was supplied by Dr H.-G. W. Meyer (Institut fuer Medizinische Mikrobiologie und Hygiene, Johannes Gutenberg-Universitaet Mainz, Mainz, Germany). SLO was dissolved at 1 mg/ml in ICT/DTT buffer (78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 1 mM dithiothreitol and 50 mM Hepes, pH 7.2) and stored at -80 °C. The colorimetric assay for the determination of *N*-acetyl-β-D-glucosaminidase and the cytotoxicity detection kit used to quantify the LDH (lactate dehydrogenase) activity were from Roche (Basel, Switzerland) and were both used according to the manufacturer's instructions. All other reagents were from Sigma–Aldrich (Lyon, France). All cell-culture reagents were supplied by Invitrogen (Cergy Pontoise, France). All peptides were synthesized and purified by Neosystem (Strasbourg, France) with a terminal cysteine residue at their N- or C-terminal position to allow chemical conjugation. The Tat peptide sequence corresponding to residues 48–60 was from HIV-1 Tat.

Recombinant protein

Plasmids expressing the GST–DPV–EGFP (where GST stands for glutathione S-transferase, DPV for Diatos peptide vector and EGFP for enhanced green fluorescent protein) recombinant proteins (named DPV–EGFP) were produced by replacing the Tat-coding region in the GST-Tat-GFP plasmid (where GFP stands for green fluorescent protein) [22] with sequences encoding for Vectocell® peptides using the BamHI and HindIII restriction sites. These sequences were obtained by the annealing of the following oligos that present protruding single-strand DNA corresponding to BamHI and HindIII sites: DPV3 sense, 5′-*GATCC*CGTAAAAAGCGTCGTCGAGAAAGCCGTAAGAAACGTCGACGTGAAAGC*A*-3′; DPV3 antisense, 5′-*AGCTT*GCTTTCACGTCGACGTTTCTTACGGCTTTCTCGACGACGCTTTTTACG*G*-3′; DPV15b sense, 5′-*GATCC*GGTGCGTATGATCTGCGTCGTCGAGAACGTCAGAGCCGTCTGCGTCGACGTGAAAGACAGAGCAGA*A*-3′; DPV3 antisense, 5′-*AGCTT*TCTGCTCTGTCTTTCACGTCGACGCAGACGGCTCTGACGTTCTCGACGACGCAGATCATACGCACC*G*-3′; DPV1047 sense, 5′-*GATCC*GTTAAACGTGGACTGAAACTTCGTCATGTTCGTCCGCGTGTGACCCGTGATGTG*A*-3′; DPV1047 antisense, 5′-*AGCTT*CACATCACGGGTCACACGCGGACGAACATGACGAAGTTTCAGTCCACGTTTAAC*G*-3′.

Cell culture

Cell lines were obtained from A.T.C.C. (Manassas, VA, U.S.A.). HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FCS (fetal calf serum), 2 mM L-glutamine and 1 mM sodium pyruvate. HCT116 were cultivated in McCoy's 5a medium supplemented with 10 % FCS and 1.5 mM L-glutamine. CHO-K1 (Chinese-hamster ovary K1) and PgsA-745 cells were maintained in Kaighn's modification of Ham's F12 medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10 % FCS. K562 cells were maintained in Iscove's modified DMEM containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 20 % FCS. For all qualitative and quantitative experiments, cells were seeded at day 0 on glass slides from Labtech for DPV–PO (where PO stands for peroxidase) and on permanox Labtech for DPV–TMR (where TMR stands for tetramethylrhodamine) (Dominique Dutcher, France). The internalization experiments were performed on day 1 with cells at 60–80 % confluence.

Peptide conjugation

A cysteine residue had been added to either the C- or N-terminus of all peptides to allow conjugation of the peptide to various macromolecules. To prepare PO–peptide conjugates, 1 mg of EZ-Link™ maleimide-activated horseradish peroxidase (Pierce, Rockford, IL, U.S.A.) at a concentration of 5 mg/ml in 0.5 M NaCl, 50 mM sodium phosphate, 5 mM EDTA (pH 7) was conjugated with 125 μmol of peptide (solution at 5 mM in 0.5 M NaCl, 50 mM sodium phosphate and 5 mM EDTA, pH 7), for 45 min at room temperature (20–25 °C). Free peptide was then eliminated by ultrafiltration through a membrane with a molecular-mass cut-off of 10 kDa (Vivascience, Chartres, France). PO activity was determined by a classical ELISA test using *O*-phenylenediamine dihydrochloride (Sigma–Aldrich) as a substrate of the enzyme. By comparing the activity of the conjugates to a standard curve of free PO activity, concentrations of the conjugate solutions were determined, and adjusted to 1 mg/ml in 0.15 M NaCl. Peptide–PO conjugates were stored at -20 °C. To prepare TMR–peptide conjugates, 200 μl of a 50 mM solution of tetramethylrhodamine-maleimide in DMF (dimethylformamide) were mixed with 700 μl of a peptide solution (10 mM in DMF). After a brief vortex-mix and incubation for 2 h at room temperature in the dark followed by dichloromethane extraction, the aqueous phase was freeze-dried and stored in the frozen condition. To prepare maleimide–peptide conjugates, 500 μl of a 100 mM solution of maleimide in DMF were mixed with 500 μl of a peptide solution (10 mM in DMF). After a brief vortex-mix and incubation for 3 h at room temperature in the dark, followed by dichloromethane extraction,

the aqueous phase was freeze-dried and stored in the frozen condition.

Quantitative analysis of the internalized material

Peptide–PO conjugates were diluted in the appropriate culture medium and treatment of the cells with conjugate was as described in the legend to Figure 1. Cells were then rinsed three times in PBS and detached from the culture slides using trypsin-EDTA. Cells were counted, washed in PBS and then lysed in lysis buffer (0.1 M Tris, pH 8.0, and 0.5 %, v/v, Nonidet P40, at 4 °C) in the presence of protease inhibitors (protease inhibitor cocktail; Roche). The PO present in the cells was quantified using *O*-phenylenediamine dihydrochloride. Peptide–TMR conjugates were prepared as follows: after dilution of the conjugates in culture medium and incubation with the cells, cells were rinsed in PBS three times and detached from the culture slides by trypsin–EDTA. Cells were counted, washed in PBS and then lysed in RIPA buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1 % Nonidet P40, 1 %, v/v, deoxycorticosterone and 2 mM EDTA, at 4 °C) in the presence of protease inhibitors. Quantification of the fluorescence emitted by the cell lysates allowed the determination of the quantities of internalized TMR in comparison with a standard curve.

Quantification of peptide–EGFP internalization

Quantification of internalized DPV–EGFP recombinant proteins was performed in HeLa cells as described already [3]. Briefly, 2×10^5 cells/ml were incubated at 37 °C with 2 μg/ml DPV–EGFP conjugate in the presence of 100 μM chloroquine for the indicated periods of time. Cells were rinsed twice in PBS and trypsinized for 10 min to remove any membrane-bound peptides. Cells were fixed in 4 % (v/v) PFA (paraformaldehyde) at 4 °C for 10 min and washed in ice-cold PBS [22]. Cells were resuspended in ice-cold PBS solution before flow cytometry analysis using a Coulter Epics® XL flow cytometer (Beckman Coulter, Cassina De'Pecchi, Italy); 5000 cells/sample were analysed. Data acquisition and analysis were performed using the Expo 32 software (Beckman Coulter) and data were expressed as RFI (relative fluorescence intensity).

Peptide–EGFP co-localization experiments

For the uptake experiments, 1.5×10^5 HeLa cells/ml were plated on Labtech glass slides one day before the experiment. Cells were rinsed for 30 min in serum-free DMEM, before the addition of fresh serum-free medium containing the GST–DPV–EGFP fusion proteins (2 μg/ml) and transferrin–TRITC or TRITC-conjugated CtxB (10 μg/ml). After incubation (1 h), cells were fixed with 2 % PFA in PBS for 10 min, washed in PBS, incubated with 100 mM glycine in PBS for 5 min and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). For the experiments with Triton X-100 permeabilization, HeLa cells were incubated with DPV–EGFP together with transferrin–TRITC for 1 h, washed and incubated in ice-cold 1 % (v/v) Triton X-100 in PBS for 20 min. Cells were washed with 2 % PFA in PBS and fixed as above. Treatment with MBCD (methyl-β-cyclodextrin) was performed by incubating the cells for 30 min with the drug (5 mM) before the addition of the fluorescent proteins in fresh medium containing the drug. Cells were fixed after incubation (1 h). All images were obtained using a TCS-SL laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

Qualitative analysis of the internalized material

Visualization of the internalized peptide–PO conjugates was performed using PO substrate. Briefly, after the internalization experiment, cells were rinsed three times in PBS before fixation in cold ethanol for 15 min at − 20 °C. Cells were then rinsed in PBS at room temperature before the addition of PO substrate (diaminobenzidine, one tablet in 10 ml of water + 330 μl of 3 % H_2O_2). Cells were rinsed three times in PBS to stop the reaction and kept in water; observations were performed using a Leica DMR-HC microscope (Type 020-525-024) and photographed with a Nikon DXM 1200 camera. Images were further analysed using the Lucia 4.8 software. For visualization of DPV–PO conjugates using anti-PO monoclonal antibodies, cells were incubated with the primary antibody for 30 min at room temperature. Cells were then rinsed once with the medium and twice with PBS. Incubation with the secondary antibody diluted according to the manufacturer's instructions took place in PBS + 0.15 % (v/v) glycine + 0.5 % (w/v) BSA for 30 min at room temperature in the dark. Cells were then rinsed twice in the same solution at room temperature, then twice in PBS, before the slides were mounted in Slow Fade Light Antifade kit containing DAPI (4,6-diamidino-2-phenylindole). Images were obtained with a confocal laser scanning system (MRC-1000; Bio-Rad, Hercules, CA, U.S.A.) mounted on a Nikon Optiphot microscope. Dual detection was performed with separate photomultiplier tubes and the resultant images were merged. After the internalization of peptides-TMR, cells were rinsed extensively with PBS before being fixed for 20 min at room temperature in a 4 % PFA solution. After another series of washings in PBS, cells were then mounted in PBS + 50 % (v/v) glycerol.

Determination of the peptide conjugate released into the cytosol

HeLa cells were seeded on two-well glass Labtech slides at 1.5×10^5 cells/ml. Penetration studies were undertaken 24 h after seeding. All internalization experiments were performed in three independent experiments, each in duplicate. Briefly, internalization of the conjugates took place at 37 °C in 5 % CO_2 atmosphere, in complete culture medium for 2 h. DPV–IgG, DPV–PO and DPV–TMR were incubated at an initial concentration of 75, 25 and 60 μg/ml respectively. Cells were then rinsed twice in PBS, trypsinized and then counted. Cells were then incubated with 1 μg/ml SLO in PBS for 10 min at 37 °C, briefly centrifuged and the supernatant (fraction A) was kept on ice. PBS (400 μl) was then added to the cells followed by incubation for 30 min at 37 °C. Cells were then centrifuged, and the supernatant (fraction B) was kept on ice. The pellet was then lysed in RIPA buffer containing protease inhibitors. All three fractions (A + B + pellet) were tested for the presence of the following enzymes: *N*-acetyl-β-D-glucosaminidase (NabGase), a lysosomal enzyme that should not be present in the cytosolic fractions if the lysosomes have not been damaged by the SLO treatment, and LDH, a cytosolic enzyme that should only be present in fractions A and B, and allows to quantify the purity of cytosol that was recovered during the experiment. The reported molecules were then quantified in the three fractions and the proportion of each conjugate in the cytosol was calculated.

Generation of the LoxP-EGFP reporter cell line

A DNA fragment comprising the 5′-loxP1 site (5′-ATGATA-ACTTCGTATAATGTA-TGCTATACTAATGGAG-NdeI-3′), the HSV-1 thymidine kinase (TK) gene (obtained by PCR amplification), the 3′-loxP2 site (5′-NdeI-TAGTCGACGCGTAT-AACTTCGTATAAT-GCTACGAAGTTATCAGGCCTGCACC-CG-3′-HindIII) and the *GFP* cDNA (obtained by PCR amplification) was obtained by PCR amplification and inserted into the pcDNA3 eukaryotic expression vector, also expressing the neoR

Table 1 Sequence of Vectocell® penetrating peptides

The intracellular localization of DPV–PO and DPV–IgG conjugates were assessed as described in the text. The asterisk indicates the position of the cysteine residue that has been added for conjugation to the various molecules.

Vectocell® peptide	Sequence	No. of amino acids	% of basic amino acids	Source (NCB accession no.)	Intracellular localization of DPV–PO/IgG (HeLa cells)
DPV3	RKKRRRESRKKRRRES*	16	70	Superoxide dismutase (AAH14418)	Cytoplasm
DPV6	GRPRESGKKRKRKRLKP*	17	50	PDGF (platelet-derived growth factor) (AAA60045)	Cytoplasm
DPV7	GKRKKKGKLGKKRDP*	15	56	Epidermal-like growth factor (AAC15470)	Cytoplasm
DPV7b	GKRKKKGKLGKKRPRSR*	17	61	Epidermal-like growth factor (AAC15470)	Cytoplasm
DPV3/10	RKKRRRESRRARRSPRHL*	18	58	Superoxide dismutase and intestinal mucin	Cytoplasm
DPV10/6	SRRARRSPRESGKKRKRKR*	19	60	Intestinal mucin and PDGF	Cytoplasm
DPV1047	*VKRGLKLRHVRPRVTRMDV	19	35	Apolipoprotein B (P04114) and anti-DNA antibody (AAB26429)	Nucleus
DPV10	SRRARRSPRHLGSG*	14	33	Intestinal mucin (AAA59164)	Nucleus
DPV15	LRRERQSRLRRERQSR*	16	47	CAP37 (1617124A)	Nucleus
DPV15b	*GAYDLRRRERQSRLRRRERQSR	22	43	CAP37 (1617124A)	Nucleus

gene. The resulting vector (named LTLG) was transfected into HEK-293 cells (human embryonic kidney 293 cells) by the calcium phosphate procedure. The transfected cells were selected by resistance to G418, and tested for Cre-mediated LoxP recombination by transfection of a Cre-expression vector (results not shown).

Recombinant Cre fusion proteins

To obtain plasmids expressing the Cre recombinase carrying an N-terminal extension corresponding to the different Vectocell® peptides and to the HIV-1 Tat transduction domain, the Cre gene was PCR-amplified using primer pairs carrying HindIII and EcoRI tails and cloned into the GST-DPVs-EGFP or the GST-Tat-EGFP plasmids to replace the EGFP portion. The fusion proteins were expressed in BL21 *Escherichia coli*; when bacterial cultures reached the absorbance *A* of 0.6–0.8, protein expression was induced using 500 μM IPTG (isopropyl β-D-thiogalactoside; Sigma–Aldrich) and protein synthesis was allowed to proceed for 4 h at 30 °C. Cells were then lysed, sonicated and the GST–Tat–Cre and GST–DPVs–Cre proteins were affinity-purified using glutathione–Sepharose beads (Amersham Biosciences, Cologno Monzese, Italy) according to established procedures. Protein concentration was determined by the Bradford protein assay and by Coomassie Blue gel staining. Protein aliquots were stored at −80 °C. For the Cre protein transduction experiments, the purified recombinant proteins were added to the LTLG cell-culture supernatant. After incubation at 37 °C (4 h), the medium was removed and the cells were extensively washed with PBS and then grown for 3 days under standard conditions. Cell fluorescence was then analysed by fluorescence microscopy (results not shown) and flow cytometry.

In vitro toxicity determination

Cells were incubated in the presence of increasing concentrations of DPV–maleimide conjugates up to 1 mM, for 48 h at 37 °C in a medium containing the serum, and a viability assay (WST-1; Roche) was performed according to the manufacturer's instructions.

In vivo toxicity and MTD (maximum tolerated dose) determination

The MTD determination was performed on 9-week-old female OF1 mice by bolus injection. DPV–maleimides were first diluted in water [Cooper (Coopération Pharmaceutique Française), Place Lucien Auvert, Melun, France]; after complete dissolution, the solutions were diluted in 0.9 % (w/v) NaCl (Cooper). The solutions were prepared on the day of injection. After filtering through 0.2 μM DPV–maleimide solution, vehicles were administered with bolus intravenous injection in the lateral vein. Body weight and mortality were evaluated every two or three days for at least 7 days after the injection.

Haemolytic activity

After isolation of erythrocytes by centrifugation of total human blood, cells were counted and 10^6 cells were incubated with decreasing concentrations of DPV–maleimide conjugates for 1 h at 37 °C, with starting dilution at 1 mM. Maximum release of haemoglobin was evaluated by incubation of the erythrocytes in water under the same conditions. Optic deviation at 414 nm allowed evaluation of the percentage of haemolysis by comparison with the values obtained in water, after correction of the absorbance values by the blank value.

RESULTS

Vectocell® penetrating peptide family

Vectocell® peptides were developed and assessed for their capacity to transport a range of macromolecules across mammalian cell membranes. These Vectocell® peptides were derived either from known heparin-binding proteins or from anti-DNA autoantibodies or a chimaera of both (Table 1).

The toxicity of the Vectocell® peptides was tested *in vitro* (IC_{50}) and *in vivo* (MTD); as shown in Table 2, the *in vitro* IC_{50} of the majority of the peptides was close to or higher than the highest concentration tested (1 mM). The MTD was determined for each DPV after intravenous bolus injection. DPV1047, DPV10, DPV15b and DPV7 were 2–3-fold less toxic than the Tat peptide *in vivo*. Since the ultimate use of these peptides would be by intravenous injection of therapeutic conjugates, the haemolytic activity of Vectocell® peptides was assessed. None of them caused haemolysis of human erythrocytes (1 h incubation, 37 °C at concentrations up to 1 mM) with the exception of DPV15b, for which the IC_{50} (concentration at which 50 % haemolysis occurred) was approx. 500 μM (results not shown). Overall, these data suggest that the cytotoxic and haemolytic effects of these peptides occur at concentrations that are significantly higher than that required for *in vivo* applications.

Table 2 Toxicity of DPV–maleimide conjugates

Cells were incubated for 48 h in the presence of peptide–maleimide conjugates before cell viability was estimated by a WST-1 test. IC_{50} values were estimated by regression from the curves and are the means for three independent experiments. For *in vivo* experiments, Vectocell® peptides were administered by bolus intravenous injection in the lateral tail vein for each group (three mice/group). Body weight and mortality were evaluated every 2 or 3 days for at least 7 days after the injection.

	IC_{50} (μM)		
	In vitro		*In vivo*
Vectocell® conjugate name	CHO-K1 48 h incubation	HeLa 48 h incubation	MTD (μmol/kg)
DPV3–maleimide	~616	~900	9–12
DPV6–maleimide	>1000	>1000	12–15
DPV7–maleimide	>1000	~1000	⩾18
DPV7b–maleimide	~650	~850	<9
DPV3/10-maleimide	~600	~675	<9
DPV10/6–maleimide	>1000	>1000	<9
DPV1047–maleimide	>1000	>1000	>21
DPV10–maleimide	>1000	>1000	>21
DPV15b–maleimide	>1000	>1000	>21
DPV15–maleimide	~516	~833	<9
Tat–maleimide	>1000	>1000	7–9

Vectocell® peptide internalization

In an initial set of screening experiments, Vectocell® peptides, also termed DPVs, were chemically conjugated to two different reporter proteins, an enzymatically active PO protein (40 kDa) or a full-size immunoglobulin (anti-PO antibody, anti-PO IgG, 150 kDa). Two subgroups of peptides could be distinguished (Table 1). DPV1047, DPV10, DPV15 and DPV15b transported reporter proteins to the nucleus, whereas the remaining DPVs resulted in cytoplasmic localization of the reporter proteins. The DPV3 and DPV15b peptides were selected for subsequent studies, being representative of peptides driving cytoplasmic and nuclear localization respectively. DPV1047, a peptide partially derived from an anti-DNA autoantibody was also selected for further study because of both its distinct origin and its low basic amino acid content. In addition to PO and anti-PO IgG, these three peptides were conjugated to two other reporter molecules of dissimilar sizes and properties: the small fluorochrome TMR (386 Da) and the autofluorescent protein EGFP (26 kDa).

The three peptides analysed (DPV3, DPV15b and DPV1047) internalized all four reporter molecules in a variety of fixed mammalian cell lines (both adherent and non-adherent) including epithelial (HeLa, HCT116), myeloid (HL-60), erythroid (K562), lymphoid (Molt4), fibroblast (NIH3T3) cells and primary hepatocyte cultures. The uptake of Vectocell® peptides was quantified after cell trypsinization to remove cell surface-bound DPV conjugates [23]. A representative set of data is shown in Figure 1, in which the internalization of DPV3, DPV15b and DPV1047 in HeLa and HCT116 cells is compared with that of the Tat peptide conjugated to the same reporter molecules. DPV3 always showed the greatest internalization efficiency of the three DPV peptides, with uptake being greater than that of Tat. The relative internalization efficiencies of the three different DPVs were similar when fused to PO, IgG or EGFP (Figures 1A, 1B or 1C respectively). DPV3–TMR, DPV15b–TMR and Tat–TMR conjugates were internalized in comparable quantities, whereas DPV1047–TMR was internalized at levels only slightly higher than the negative control (cysteine-TMR; Figure 1D). The simple mixing of the DPVs with any of the reporter molecules without conjugation did not result in internalization (results not shown),

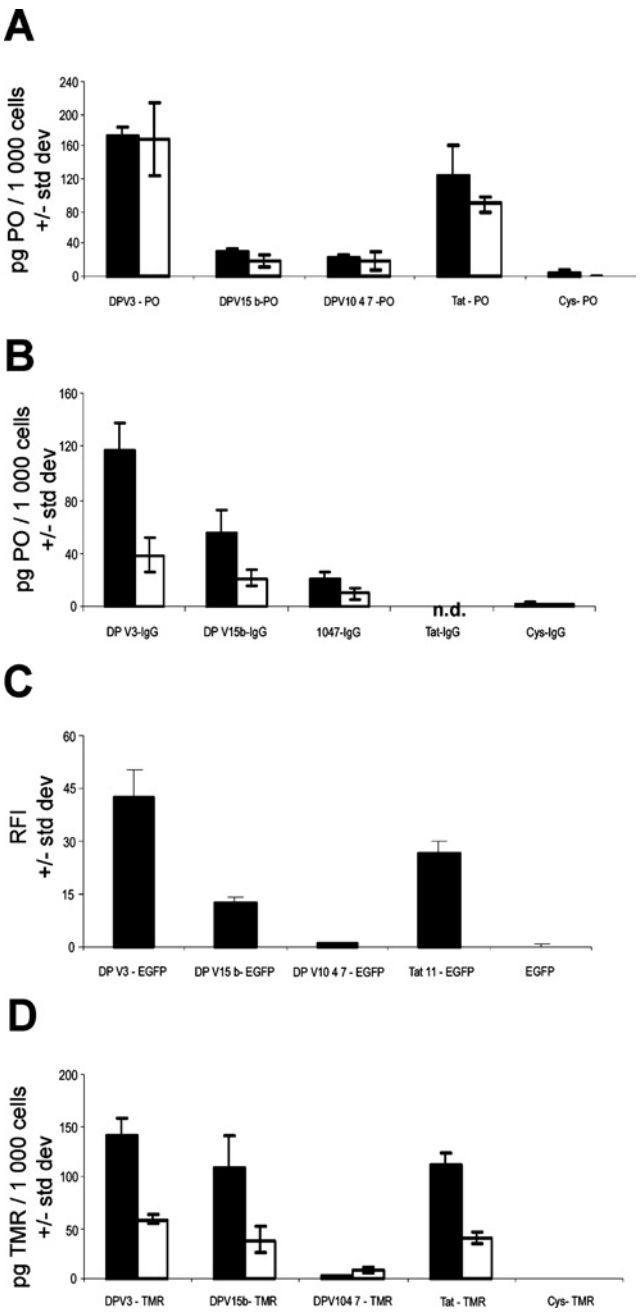

Figure 1 Vectocell® peptide internalization

HeLa cells (black bars) or HCT116 cells (white bars) were incubated at 37 °C with DPV conjugates for 4 h [PO (**A**) and IgG (**B**) conjugates], 8 h [EGFP recombinant conjugates (**C**)] or 2 h [TMR conjugates (**D**)]. All the results reported in the graphs are means ± S.E.M. for three independent experiments each performed in duplicate; n.d., not determined.

thus confirming previous reports that covalent conjugation is required for peptide-driven internalization [24].

Fixation artifacts can arise when studying the cellular localization of highly charged peptides [25–27]; for this reason, the intracellular localization of Vectocell® peptides was assessed in both fixed and live cells. As shown in Figure 2, subcellular localization was dependent on the choice of the peptide conjugate. DPV3–PO was detected in the cytoplasm, whereas both DPV15b–PO and DPV1047–PO were principally located in the nuclei. These experiments were confirmed using an anti-PO antibody (Figure 2, column B). Vectocell® conjugated to anti-PO

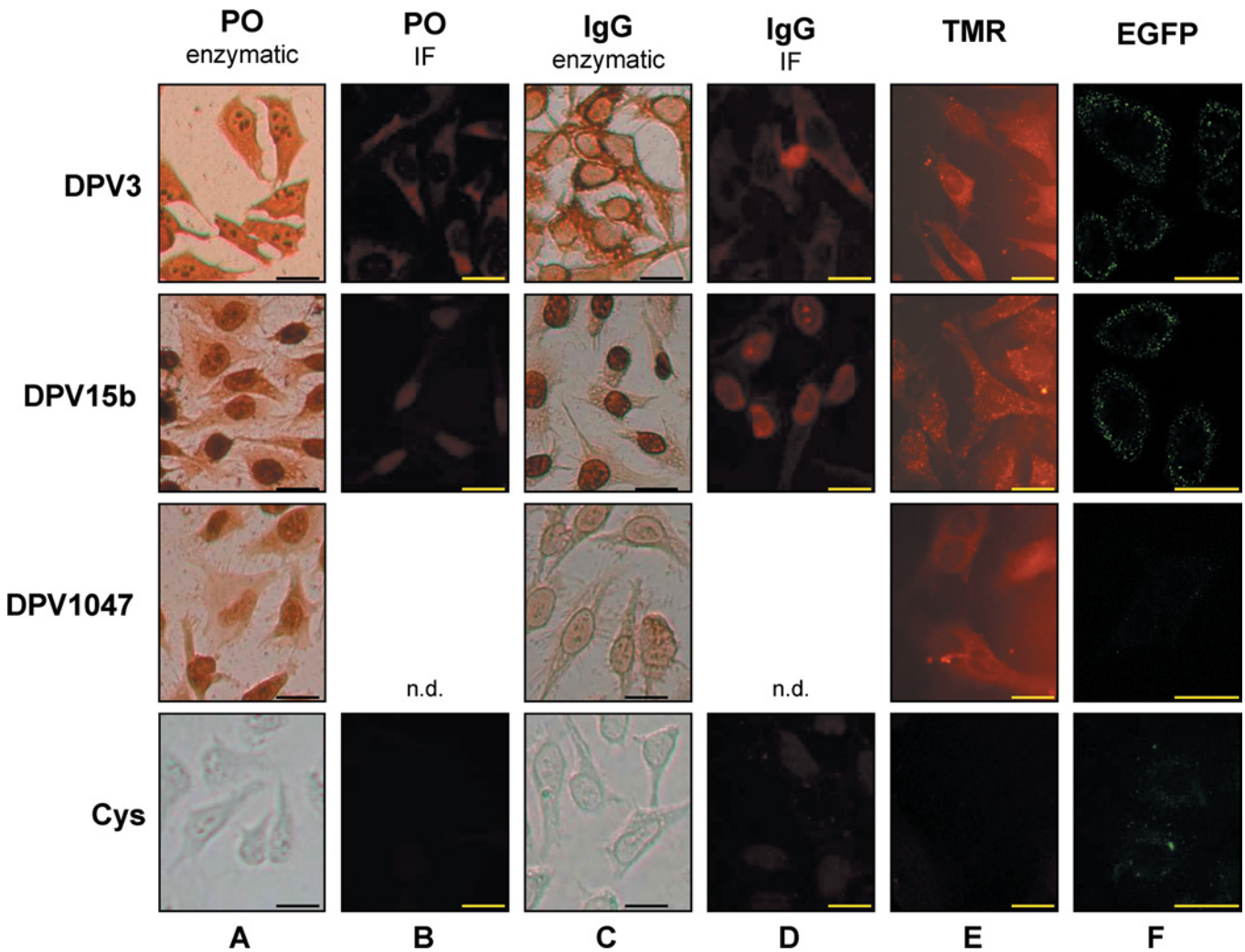

Figure 2 Intracellular localization of Vectocell® peptides

HeLa cells were incubated with the different conjugates for 2–4 h, before fixation (**A**–**E**) or direct observation of live cells (**F**). (**A**) Cells were incubated with diaminobenzidine for the visualization of active PO. (**B**, **D**) PO or anti-PO IgG respectively were detected by indirect immunofluorescence. (**C**) Cells were incubated with PO, then with diaminobenzidine for the visualization of anti-PO immunoglobulin. (**E**, **F**) TMR and EGFP were visualized directly; n.d., not determined. Scale bar = 10 μm.

immunoglobulin were internalized to the same intracellular locations as the DPV–PO conjugates as shown in Figure 2 (columns C and D). When Vectocell® peptides were conjugated to the small TMR molecule, fluorescence was observed to be cytoplasmic (Figure 2, column E). DPV3–EGFP and DPV15b–EGFP fusion proteins, led to a strong punctuate pattern in the cytoplasm of HeLa cells (Figure 2, column F), whereas no nuclear staining was visible for DPV15b–EGFP. This observation confirms that, due to its folding characteristics [22], EGFP is not a suitable reporter to assess the final nuclear destination of internalized proteins.

For many therapeutic applications, the internalized conjugate is required to be in the cytoplasm. This was assessed using SLO, which allows the permeabilization of the plasma membrane without disruption of intracellular organelles [28,29]. After incubation of HeLa cells with DPV3 conjugated to PO, IgG and TMR, followed by elimination of surface-bound material, the proportion of material free in the cytoplasm was quantified. As shown in Figure 3, 11–35 % of the internalized conjugates were freely available in the cytosol.

DPV–Cre driven LoxP recombination

In order to obtain an independent conformation of DPV-mediated internalization of functional macromolecules in living cells, and to verify their different subcellular localizations, Vectocell® peptides were fused to Cre recombinase and tested for their efficiency to drive genetic recombination of a LoxP cassette. For this purpose a cell line was obtained containing an integrated construct in which the EGFP gene was separated from its promoter by an intervening cassette flanked by two LoxP sites (Figure 4A). After Cre-mediated recombination (driven by the internalized DPV–Cre conjugate), the cassette is excised and EGFP is expressed. Thus the quantitative analysis of cell fluorescence provides a tool to assess cellular internalization, protein functionality and nuclear targeting of the DPV–Cre conjugates in living cells.

DPVs and Tat peptide were conjugated to the N-terminus of the recombinant Cre protein and were purified from bacterial lysates as fusions to GST (> 80 % purification, as estimated by Coomassie Blue gel staining; Figure 4B) and added to the supernatant of HEK-293 cells carrying the integrated LoxP–EGFP

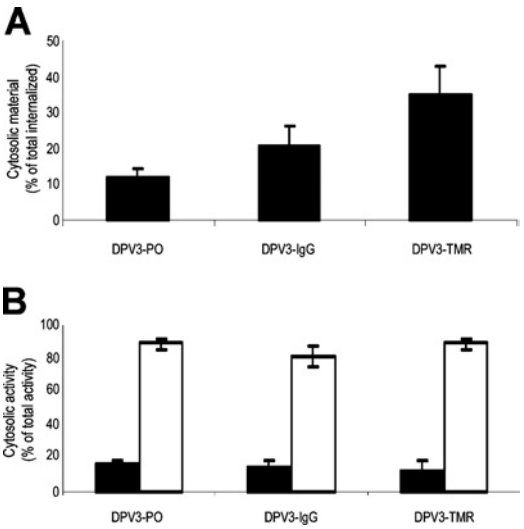

Figure 3 Delivery of Vectocell® peptides to the cytosol

After internalization of PO, IgG or TMR conjugated to DPV3, cells were lysed using SLO, allowing separation of the cytosolic material from the membranes and intracellular organelles. (**A**) Percentage of internalized material that was recovered in the cytosolic fraction. (**B**) The cytosolic enzyme LDH (white bars) and the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (black bars) were quantified in each experiment in order to verify the integrity of the fractions. More than 85 % of the total LDH was recovered in the cytosol for each experiment, and this value was used to adjust the percentage of cytosolic PO (see the Materials and methods section). All the reported values are the results of three to six independent experiments. The means $\pm$ S.E.M. were determined from three independent experiments performed in duplicate.

reporter construct. Using flow cytometry analysis, DPV3–Cre and DPV15b–Cre, as well as Tat–Cre, were shown (72 h after treatment) to induce LoxP recombination in a dose-dependent manner (as shown for DPV15b–Cre in Figure 4C). However, the levels of recombination induced by treatment with DPV1047–Cre were very low, most probably due to the modest internalization of this Vectocell® peptide in HEK-293 cells. Figure 4(D) shows the efficiency of LoxP recombination induced by treatment with the three proteins after normalization according to their relative efficiency of cell penetration. Interestingly, DPV15b was found to be the most active in driving the recombination process, a result that is consistent with the preferential nuclear localization of this peptide.

The kinetics of Vectocell® peptide internalization is dependent on the reporter molecule

The kinetics of cellular internalization of various reporter molecules conjugated to DPV3 is shown in Figure 5. Large molecules such as the DPV3–PO conjugate and the GST—DPV3–EGFP recombinant protein both displayed maximum internalization levels after several hours of incubation with HeLa cells. In contrast DPV3–TMR conjugate reached maximum internalization after only 15–30 min of incubation. This observation clearly suggests that the kinetics of cellular uptake depends upon the size of the conjugated molecules, as has been reported for Tat peptide conjugates [6].

Mechanisms of internalization of DPV3 and DPV15b

To obtain further insight into the mechanism of cell entry, the energy and temperature-dependent nature of the internalization process was assessed. Internalization of PO by both Vectocell® peptides and Tat in HeLa cells is completely inhibited at 4 °C

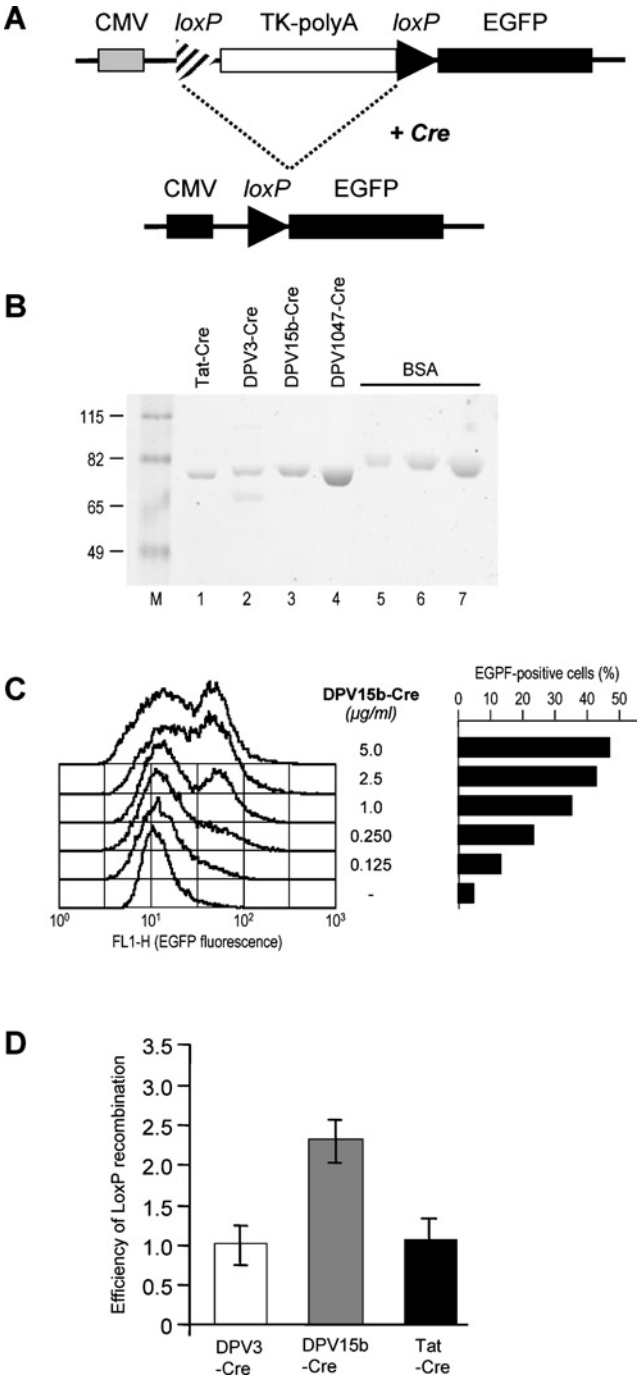

Figure 4 Intracellular localization of Vectocell® peptides in live cells

(**A**) Schematic representation of the integrated LoxP-EGFP construct, before and after Cre-mediated recombination. (**B**) Coomassie Blue-stained gel showing the recombinant proteins containing Cre and the indicated DPV peptides or the Tat peptide, as indicated, after purification from BL21 *E. coli* cells as fusions with GST (lanes 1–4; 10 μl of each eluate per lane). Lane M, protein ladder marker; lanes 5–7, 1.0, 2.5 and 5.0 μg of BSA respectively. (**C**) Flow cytometry profiles showing the EGFP fluorescence of cells stably transfected with a Lox-TK-Lox-GFP construct after treatment with recombinant DPV15b–Cre. The indicated amounts of protein were added to the cell-culture supernatant; after 4 h, the cells were extensively washed, and fluorescence was analysed after 72 h. The graph on the right-hand side shows the percentage of EGFP-positive cells at each protein dose. (**D**) Efficiency of Cre delivery by DPV3, DPV15b and Tat (1.5 μg/ml). The histogram shows the efficiency of LoxP recombination after DPV or Tat treatment, obtained by dividing the number of EGFP-positive cells (after subtraction of the background) by the efficiency of internalization of each peptide relative to DPV3. The latter parameter was measured by treating the same cells with recombinant EGFP fused to the different DPVs or to Tat. Data represent means $\pm$ S.E.M. for at least three independent experiments.

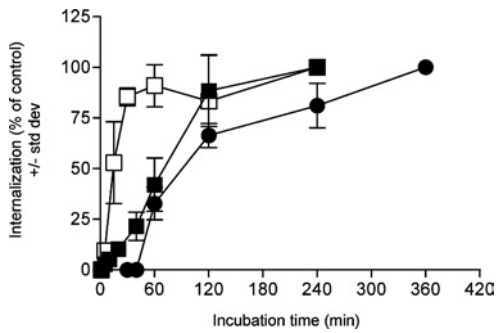

Figure 5 Kinetics of internalization depends on the conjugated molecule

HeLa cells were incubated in the presence of DPV3–PO (25 μg/ml; ■), DPV3–TMR (20 μM; □) or the recombinant protein DPV3–EGFP (2 μg/ml; ●) for the indicated period of time, before trypsin treatment, lysis of the cells, and quantification of the internalized material using the appropriate measurement unit. The reported values are means ± S.E.M. for three independent experiments.

(Figure 6A), an observation that is consistent with an energy-dependent endocytosis process. In sharp contrast, when the experiments were performed with DPV–TMR conjugates (~2.5 kDa), internalization still occurred at 4 °C although the efficiency was decreased (45 %, 27 % and 10 % internalization for DPV3–TMR, Tat–TMR and DPV15b–TMR respectively) indicating an energy-independent process (Figure 6B). Internalization of the DPV–TMR conjugates at 4 °C most probably occurs by diffusion through the plasma membrane as has been suggested for both Tat and several other cell-penetrating peptides [30–32]. This conclusion is also in agreement with the rapid kinetics of internalization of the small TMR conjugates shown in Figure 5.

The importance of the binding to GAGs (glycosaminoglycans) for Vectocell® internalization was demonstrated using CHO-K1 cells and the daughter cell line PgsA-745 that is devoid of the xylosyltransferase enzyme and consequently does not produce detectable levels of GAGs [33]. As shown in Figures 6(C) and 6(D), the internalization of the DPV3 and DPV15b conjugates (PO and TMR) was impaired by approx. 80 % in PgsA-745 cells as compared with their wild-type counterpart. The observation that the internalization of all conjugates was inhibited by heparin (results not shown) is also consistent with this conclusion.

The pathways of DPV3 and DPV15b internalization were further characterized using recombinant GST–DPV–EGFP fusion proteins. Although these proteins are not suitable to determine the final subcellular DPV destination (see Figure 2), they are excellent tools for visualizing the early steps of the internalization process. Recombinant DPV3–EGFP fusion proteins were mixed with either labelled transferrin or CtxB (a protein that is internalized through caveolar endocytosis) before treatment of HeLa cells. Transferrin and CtxB are commonly used as markers of clathrin-coated pit endocytosis and caveolar endocytosis respectively [22]. As shown in Figure 7(A), the recombinant DPV3–EGFP protein was found to co-localize with CtxB but not with transferrin in the intracellular vesicles, suggesting that internalization is dependent on the caveolar pathway. The endocytic pathway leading to CtxB internalization originates from cell-membrane lipid rafts, membrane regions that are defined by their high content of cholesterol and sphingolipids and their insolubility in non-ionic detergents such as Triton X-100 [34,35]. The sensitivity of the endosomes (containing DPV conjugates) to Triton X-100 treatment was therefore tested. As shown in Figure 7(B), treatment with Triton X-100 completely solubilized the endosomes containing transferrin–TRITC but left the DPV3–EGFP and CtxB containing vesicles unaffected. The specific

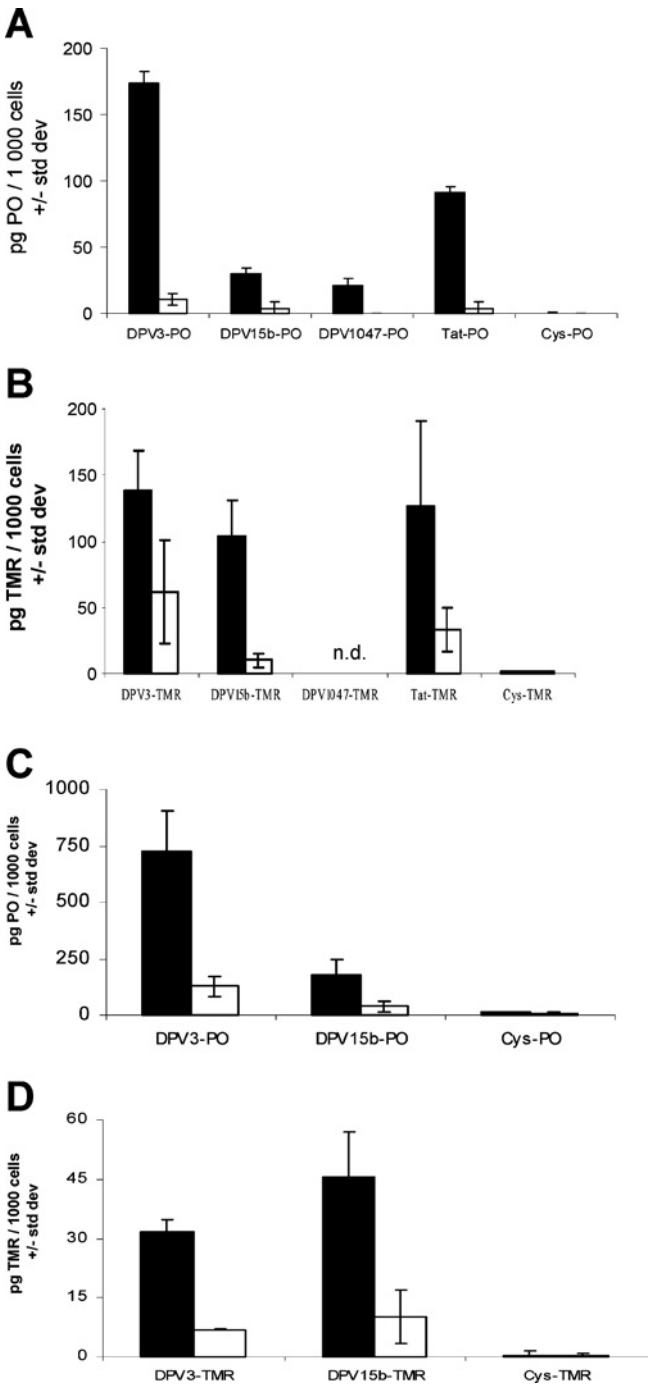

Figure 6 Internalization of Vectocell® peptides is temperature- and GAGs-dependent

(**A**) HeLa cells were incubated with DPV/Tat–PO conjugates for 4 h at a concentration of 25 μg/ml or DPV/Tat–TMR conjugates for 2 h at a concentration of 20 μM, at either 37 °C (black bars) or 4 °C (white bars). Intracellular PO or TMR was then quantified after trypsin treatment and lysis of the cells. (**B**) Internalization of the DPV/Tat–TMR conjugates was significantly decreased but still occurred at 4 °C (white bars). (**C**, **D**) CHO-K1 cells (black bars) or PgsA-745 cells (white bars) were incubated for 4 h in the presence of the indicated conjugates at the same concentrations, before trypsin treatment and lysis of the cells. PO or TMR was then quantified as described. All graphs display means ± S.E.M. for three independent experiments each performed in duplicate.

inhibitor MBCD is a cholesterol-binding agent that disrupts the lipid rafts at the plasma membrane and impairs internalization via this pathway. As shown in Figure 7(C), after cell treatment with

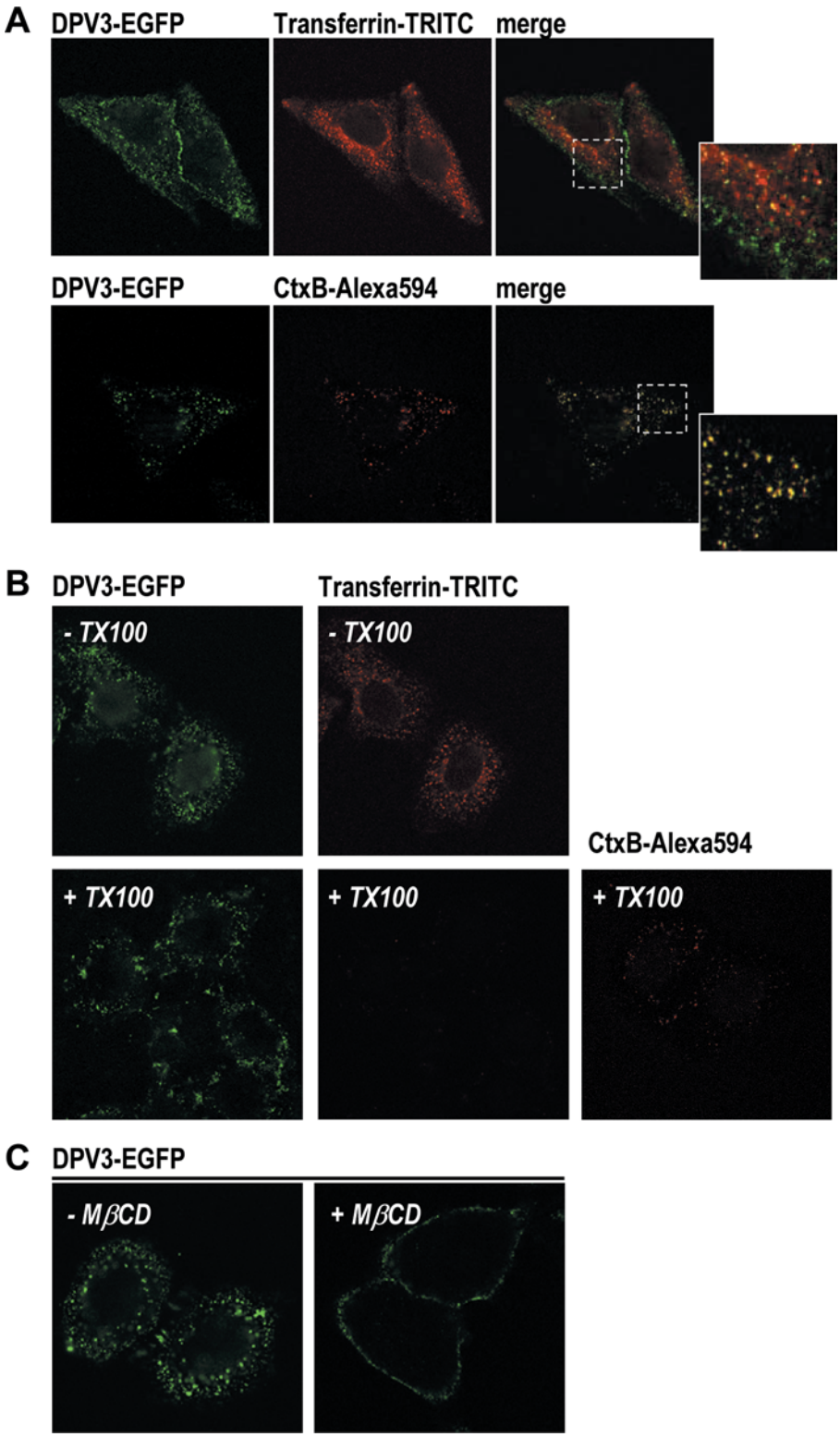

Figure 7 Co-localization and internalization of DPV3

(**A**) Co-localization of DPV3 with different endocytosis markers. Upper panel: HeLa cells were incubated with DPV3–EGFP and transferrin–TRITC for 1 h, followed by fixation and visualization of the internalized proteins by confocal microscopy. Transferrin and the DPVs are detected in distinct vesicles inside the cells. Lower panel: HeLa cells were incubated for 1 h with DPV3–EGFP and CtxB–Alexa 594. DPV3 was found to co-localize in the same endocytic vesicles as CtxB. (**B**) Internalization of DPV3 from cell membrane lipid rafts. HeLa cells were incubated with DPV3–EGFP and transferrin–TRITC. After 1 h incubation, cells were treated with Triton X-100, which was found to selectively solubilize transferrin-containing endosomes but not the endosomes containing the Vectocell® peptides. Endosomes containing the CtxB protein (bottom right panel) were equally insensitive to Triton X-100 solubilization. (**C**) HeLa cells were treated with MBCD for 30 min before treatment with DPV3–EGFP. Cholesterol depletion by the drug blocked DPV internalization.

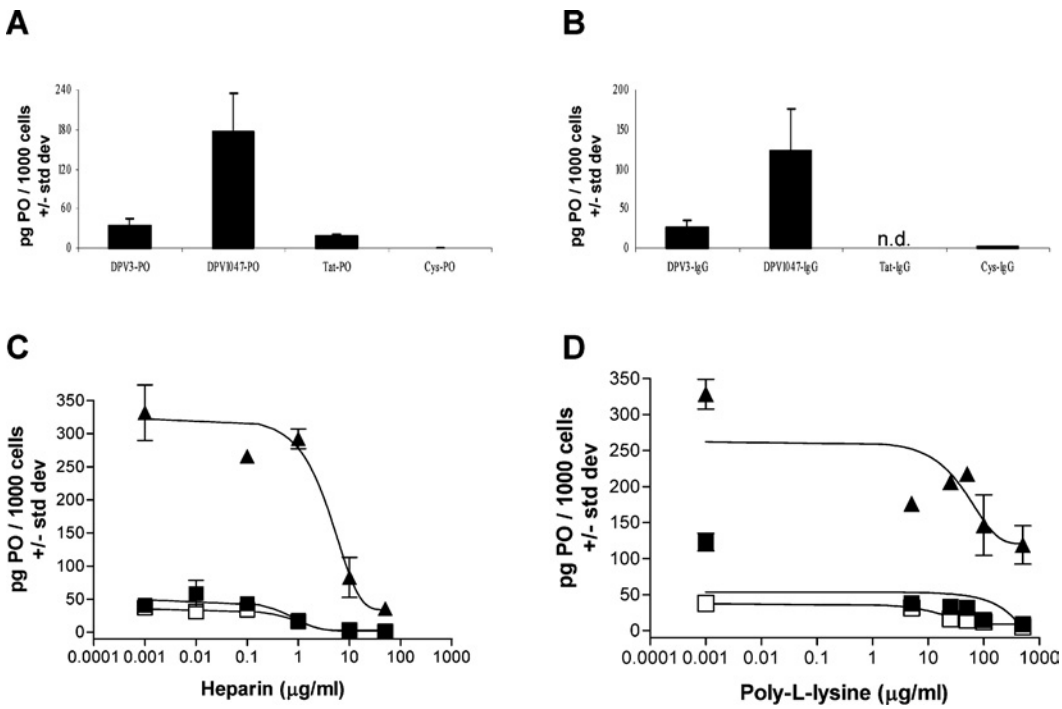

Figure 8 Internalization of DPV1047 conjugates in K562 cells

(**A**, **B**) K562 cells were incubated for 4 h with DPV/Tat–PO (**A**) or DPV–IgG (**B**) conjugates. Intracellular material was quantified following trypsin treatment and lysis of the cells. Data are means ± S.E.M. for three independent experiments each performed in duplicate. (**C**, **D**) DPV3–PO (■), DPV1047–PO (▲) and Tat–PO (▼) were incubated at 25 μg/ml in the presence of increasing concentrations of heparin (**C**) or poly(L-lysine) (**D**). Values are from one representative experiment out of three performed in duplicate.

MBCD, the cellular internalization of DPV3–EGFP-containing endosomes was blocked at the level of the cell surface, in a similar manner to that observed for CtxB and Tat, after MBCD treatment [22]. DPV15b showed similar results (results not shown).

Mechanism of internalization of DPV1047

DPV1047 originates from the fusion of the heparin-binding domain of apolipoprotein B to the complementary determining region 3 (CDR3) of an anti-DNA antibody. As shown in Figures 1 and 2, low levels of internalization were observed in adherent cells for this Vectocell® peptide compared with the other internalizing peptides. In contrast, internalization was high in suspension cells (K562), in which both DPV3 and Tat were largely ineffective (Figures 8A and 8B). The internalization process in K562 cells was found to be temperature-dependent (results not shown) and to be inhibited by heparin with a dose–response curve not dissimilar to that of Tat entry in other cell types (the concentration of heparin at which 50 % of entry occurred was 6.4 μg/ml; Figure 8C) [3]. This result most probably indicates that the positive charges of DPV1047 are required for internalization. However, one peculiarity of K562 cells is that they display only very low levels of GAGs at their cell surface [36] thus rendering the possibility that internalization involves HSPG (heparan sulphate proteoglycan) binding unlikely (this observation also accounts for the low efficiency of both DPV3 and Tat in these cells). Finally, the effect of cell incubation with poly(L-lysine), which masks the negative charges on the cell surface, showed that the uptake of DPV1047 was only partially inhibited even when using high concentrations of the polymer (Figure 8D). Taken together, these results indicate that DPV1047 has a distinct mechanism of internalization from other commonly known cell-penetrating peptides since it is internalized by an energy-dependent mechanism that is independent of GAGs expressed at the cell surface.

DISCUSSION

One of the challenges in drug development is to overcome the unfavourable physicochemical nature of molecules such as proteins and oligonucleotides that are unable to penetrate the cell membrane in order to reach their intracellular targets. Cell-penetrating peptides have been developed as a solution to this challenge; once conjugated to a therapeutic molecule these peptidic sequences facilitate intracellular delivery.

A group of novel proprietary peptidic sequences, named Vectocell® [18] or DPVs, have the ability to efficiently mediate the internalization of molecules from as little as a few Daltons, up to 200 kDa (M. Michel, unpublished work). Moreover, the use of different Vectocell® peptides allows delivery of molecules to either the cell nucleus or cytoplasm. Previous studies of the intracellular localization of peptide-transported molecules have been misleading due to artifactual effects of the fixation methods used [26,27,37]. In the present study erroneous artifacts were eliminated by assessing the cellular location of peptide conjugates in living cells using either the enhanced green fluorescent protein (EGFP) or by quantification of a nuclear-specific recombination event (Cre-Lox). These novel human-derived peptides have low *in vivo* toxicity profiles consistent with their use as therapeutic delivery systems, unlike existing carrier peptides [38].

Internalization of Vectocell® peptides (with the noticeable exception of DPV1047) requires their interaction with cell-surface GAGs. As is the case for receptor-mediated internalization, the mechanism of uptake of the cell-surface Vectocell® peptide–GAG complexes requires an active caveolar endocytosis, a pathway that

is involved in signal transduction and intracellular transportation of lipid raft-associated molecules [22,39]. However, the internalization of small molecules bound to Tat has been recently reported to occur by passive entry through the cell membrane in an energy-independent manner [30,32,40]. It is important to note that Vectocell® peptide internalization is independent of the choice of the optical isomer (either D or L; results not shown) and that internalization is inhibited by masking the negative charges of the cell membrane. These observations reinforce the notion that binding of these Vectocell® peptides to GAGs is due to an electrostatic interaction rather than specific receptor recognition, as has been shown for other cell-penetrating peptides [41,42].

Our work confirms that the two early steps of cellular internalization (tight binding to the cell surface and endocytosis) occur efficiently with Vectocell® peptides. Protein transfer from endosomes to the cytosol (DPV3) or the nucleus (DPV15b) is more variable and cargo-dependent. The route by which these Vectocell® peptides escape the endosomal compartment is still obscure. One hypothesis is that these peptides bind to GAGs on the cell surface and once inside the vesicles, heparan sulphate is degraded by heparanase, first in vesicles of neutral pH and then in acidic endosomes [43], releasing the peptides. Some unbound peptides may escape from the endosomes before acidification by promoting vesicular leakage due to the charged nature of the peptides [44]. The second hypothesis is that peptides could form an amphiphatic α-helix that facilitates their insertion into the bilayer [45]. The internalization of large and small molecules was shown to differ in terms of their kinetics of internalization and temperature dependence. The data obtained are consistent with the notion that highly charged peptides (such as Tat together with DPV3 and DPV15b) have the capacity to translocate directly through the plasma membrane when conjugated to small conjugates, whereas larger conjugates can only enter cells via an energy-dependent endocytic process. These issues have to be considered when developing an effective strategy for the delivery of a given therapeutic molecule.

The properties of DPV1047 are distinct from the others. DPV1047 partially originates from a heparin-binding protein but also from an anti-DNA autoantibody. Unlike the other Vectocell® peptides it does not significantly internalize the reporter molecules assessed (PO, IgG, TMR or EGFP) in HeLa, HCT116 or CHO-K1 adherent cells, but it is the only peptide that is internalized in K562 suspension cells. However, DPV1047 conjugates are able to internalize therapeutic molecules *in vivo* (results not shown). The fact that K562 cells express only low levels of GAGs and that poly(L-lysine) is only able to partially block DPV1047 internalization, suggests that it is not just the cationic nature of this peptide that is responsible for its cell-surface interaction and internalization. The K562 cell line might possess a mechanism of internalization that is caveolar endocytosis independent, which is consistent with the observation that the internalization of SynB1 and SynB3 cell-penetrating peptides have been described to occur in these cells [41,46].

In conclusion, novel human-derived Vectocell® peptides have been identified that show both enhanced and safe cell penetration characteristics. These new peptidic sequences could deliver both small and large active molecules inside cells that would otherwise have limited or no bioavailability. Indeed, *in vivo* therapeutic validation of Vectocell®-therapeutic molecule conjugates is currently being undertaken and confirms the therapeutic potential of these peptides.

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REFERENCES

1 Li, H. and Qian, Z. M. (2002) Transferrin/transferrin receptor-mediated drug delivery. Med. Res. Rev. **22**, 225–250
2 Ruben, S., Perkins, A., Purcell, R., Joung, K., Sia, R., Burghoff, R., Haseltine, W. A. and Rosen, C. A. (1989) Structural and functional characterization of human immunodeficiency virus tat protein. J. Virol. **63**, 1–8
3 Tyagi, M., Rusnati, M., Presta, M. and Giacca, M. (2001) Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. J. Biol. Chem. **276**, 3254–3261
4 Tasciotti, E., Zoppe, M. and Giacca, M. (2003) Transcellular transfer of active HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-1 Tat. Cancer Gene Ther. **10**, 64–74
5 Mie, M., Takahashi, F., Funabashi, H., Yanagida, Y., Aizawa, M. and Kobatake, E. (2003) Intracellular delivery of antibodies using TAT fusion protein A. Biochem. Biophys. Res. Commun. **310**, 730–734
6 Silhol, M., Tyagi, M., Giacca, M., Lebleu, B. and Vives, E. (2002) Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat. Eur. J. Biochem. **269**, 494–501
7 Vives, E., Brodin, P. and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. **272**, 16010–16017
8 Stein, S., Weiss, A., Adermann, K., Lazarovici, P., Hochman, J. and Wellhoner, H. (1999) A disulfide conjugate between anti-tetanus antibodies and HIV (37-72)Tat neutralizes tetanus toxin inside chromaffin cells. FEBS Lett. **458**, 383–386
9 Torchilin, V. P., Rammohan, R., Weissig, V. and Levchenko, T. S. (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc. Natl. Acad. Sci. U.S.A. **98**, 8786–8791
10 Schwarze, S. R., Ho, A., Vocero-Akbani, A. and Dowdy, S. F. (1999) *In vivo* protein transduction: delivery of a biologically active protein into the mouse. Science **285**, 1569–1572
11 Elliott, G. and O'Hare, P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell (Cambridge, Mass.) **88**, 223–233
12 Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. and Prochiantz, A. (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. J. Biol. Chem. **271**, 18188–18193
13 Kokryakov, V. N., Harwig, S. S., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., Korneva, H. A. and Lehrer, R. I. (1993) Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. **327**, 231–236
14 Jans, D. A. (1994) Nuclear signaling pathways for polypeptide ligands and their membrane receptors? FASEB J. **8**, 841–847
15 Futaki, S., Goto, S. and Sugiura, Y. (2003) Membrane permeability commonly shared among arginine-rich peptides. J. Mol. Recognit. **16**, 260–264
16 Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K. and Sugiura, Y. (2001) Possible existence of common internalization mechanisms among arginine-rich peptides. J. Biol. Chem. **277**, 2437–2443
17 Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K. and Sugiura, Y. (2002) Possible existence of common internalization mechanisms among arginine-rich peptides. J. Biol. Chem. **277**, 2437–2443
18 Avrameas, E. and Ternynck, T. (2001) Amino acid sequences facilitating penetration of a substance of interest into cells and/or cell nuclei. PCT Patent Application no. WO 01/64738.
19 Ternynck, T., Avrameas, A., Ragimbeau, J., Buttin, G. and Avrameas, S. (1998) Immunochemical, structural and translocating properties of anti-DNA antibodies from (NZB × NZW)F1 mice. J. Autoimmun. **11**, 511–521
20 Avrameas, A., Ternynck, T., Nato, F., Buttin, G. and Avrameas, S. (1998) Polyreactive anti-DNA monoclonal antibodies and a derived peptide as vectors for the intracytoplasmic and intranuclear translocation of macromolecules. Proc. Natl. Acad. Sci. U.S.A. **95**, 5601–5606
21 Avrameas, A., Ternynck, T., Gasmi, L. and Buttin, G. (1999) Efficient gene delivery by a peptide derived from a monoclonal anti-DNA antibody. Bioconjug. Chem. **10**, 87–93

22 Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F. and Giacca, M. (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J. Biol. Chem. **278**, 34141–34149
23 Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V. and Lebleu, B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J. Biol. Chem. **278**, 585–590
24 Morris, M. C., Depollier, J., Mery, J., Heitz, F. and Divita, G. (2001) A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat. Biotechnol. **19**, 1173–1176
25 Pichon, C., Monsigny, M. and Roche, A. C. (1999) Intracellular localization of oligonucleotides: influence of fixative protocols. Antisense Nucleic Acid Drug Dev. **9**, 89–93
26 Lundberg, M. and Johansson, M. (2002) Positively charged DNA-binding proteins cause apparent cell membrane translocation. Biochem. Biophys. Res. Commun. **291**, 367–371
27 Lundberg, M., Wikström, S. and Johansson, M. (2003) Cell surface adherence and endocytosis of protein transduction domains. Mol. Ther. **8**, 143–150
28 Walev, I., Hombach, M., Bobkiewicz, W., Fenske, D., Bhakdi, S. and Husmann, M. (2002) Resealing of large transmembrane pores produced by streptolysin O in nucleated cells is accompanied by NF-kappaB activation and downstream events. FASEB J. **16**, 237–239
29 Palmer, M. (2001) The family of thiol-activated, cholesterol-binding cytolysins. Toxicon **39**, 1681–1689
30 Lindgren, M., Hallbrink, M., Prochiantz, A. and Langel, U. (2000) Cell-penetrating peptides. Trends Pharmacol. Sci. **21**, 99–103
31 Pooga, M., Hallbrink, M., Zorko, M. and Langel, U. (1998) Cell penetration by transportan. FASEB J. **12**, 67–77
32 Hallbrink, M., Floren, A., Elmquist, A., Pooga, M., Bartfai, T. and Langel, U. (2001) Cargo delivery kinetics of cell-penetrating peptides. Biochim. Biophys. Acta **1515**, 101–109
33 Rostand, K. S. and Esko, J. D. (1997) Microbial adherence to and invasion through proteoglycans. Infect. Immun. **65**, 1–8
34 Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998) Dynamin-mediated internalization of caveolae. J. Cell Biol. **141**, 85–99
35 Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature (London) **387**, 569–572
36 Zhang, Z., Coomans, C. and David, G. (2001) Membrane heparan sulfate proteoglycan-supported FGF2-FGFR1 signaling. J. Biol. Chem. **276**, 41921–41929
37 Lundberg, M. and Johansson, M. (2001) Is VP22 nuclear homing an artifact? Nat. Biotechnol. **19**, 713–714
38 Trehin, R. and Merkle, H. P. (2004) Chances and pitfalls of cell penetrating peptides for cellular drug delivery. Eur. J. Pharm. Biopharm. **58**, 209–223
39 Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M. et al. (2001) Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. J. Biol. Chem. **276**, 26204–26210
40 Vives, E. (2003) Cellular uptake of the Tat peptide: an endocytosis mechanism following ionic interactions. J. Mol. Recognit. **16**, 265–271
41 Drin, G., Cottin, S., Blanc, E., Rees, A. R. and Temsamani, J. (2003) Studies on the internalization mechanism of cationic cell-penetrating peptides. J. Biol. Chem. **278**, 31192–31201
42 Ziegler, A., Blatter, X. L., Seelig, A. and Seelig, J. (2003) Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. Biochemistry **42**, 9185–9194
43 Belting, M. (2003) Heparan sulfate proteoglycan as a plasma membrane carrier. Trends Biochem. Sci. **28**, 145–151
44 Fuchs, S. M. and Raines, R. T. (2004) Pathway for polyarginine entry into mammalian cells. Biochemistry **43**, 2438–2444
45 Thoren, P. E., Persson, D., Karlsson, M. and Norden, B. (2000) The antennapedia peptide penetratin translocates across lipid bilayers – the first direct observation. FEBS Lett. **482**, 265–268
46 Mazel, M., Clair, P., Rousselle, C., Vidal, P., Scherrmann, J. M., Mathieu, D. and Temsamani, J. (2001) Doxorubicin-peptide conjugates overcome multidrug resistance. Anticancer Drugs **12**, 107–116

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