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On the mechanisms of the internalization of $S4_{13}$-PV cell-penetrating peptide

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Cell-penetrating peptides have been shown to translocate across eukaryotic cell membranes through a temperature-insensitive and energy-independent mechanism that does not involve membrane receptors or transporters. Although cell-penetrating peptides have been successfully used to mediate the intracellular delivery of a wide variety of molecules of pharmacological interest both *in vitro* and *in vivo*, the mechanisms by which cellular uptake occurs remain unclear. In the face of recent reports demonstrating that uptake of cell-penetrating peptides occurs through previously described endocytic pathways, or is a consequence of fixation artifacts, we conducted a critical re-evaluation of the mechanism responsible for the cellular uptake of the $S4_{13}$-PV karyophilic cell-penetrating peptide. We report that the $S4_{13}$-PV peptide is able to accumulate inside live cells very efficiently through a rapid, dose-dependent and non-toxic process, providing clear evidence that the cellular uptake of this peptide cannot be attributed to fixation artifacts. Comparative analysis of peptide uptake into mutant cells lacking heparan sulphate proteoglycans demonstrates that their presence at the cell surface facilitates the cellular uptake of the $S4_{13}$-PV peptide, particularly at low peptide concentrations. Most importantly, our results clearly demonstrate that, in addition to endocytosis, which is only evident at low peptide concentrations, the efficient cellular uptake of the $S4_{13}$-PV cell-penetrating peptide occurs mainly through an alternative, non-endocytic mechanism, most likely involving direct penetration across cell membranes.

Key words: cell-penetrating peptide, endocytosis, fixation artifact, heparan sulphate proteoglycan, protein transduction domain.

INTRODUCTION

Over the past years, the discovery of a number of small peptides, defined as cell-penetrating peptides or protein transduction domains, which are able to cross cell membranes very efficiently through an energy-independent mechanism, has been enthusiastically considered of key interest for the development of novel therapeutic approaches ([1]; for a comprehensive review, see [2]).

Despite great variability in the amino acid sequence, cell-penetrating peptides are usually short peptide sequences rich in basic amino acids, in some cases exhibiting the ability to be arranged in amphipathic α-helices. The peptides derived from the HIV-1 Tat protein and from the homoeodomain of the Antennapedia protein of *Drosophila* (Tat and Penetratin peptides respectively) [3,4], as well as the synthetic Pep-1 peptide [5], are among the best-characterized cell-penetrating peptides.

Cell-penetrating peptides have been successfully used for the intracellular delivery of different cargoes [6], including nanoparticles [7], full-length proteins [8–10], bacteriophages [11] and liposomes [12]. Moreover, efficient delivery was achieved *in vivo* upon intraperitoneal injection of fusion proteins, resulting in successful transduction in all animal tissues, including the brain [10].

Consistent with the capacity of cell-penetrating peptides and of their conjugates to enter into a wide variety of cell types from different tissues and organisms, the GAG (glycosaminoglycan) moieties of the ubiquitous cell surface heparan sulphate proteoglycans have been identified as playing an important role in mediating electrostatic interactions between cell-penetrating peptides and biological membranes [13,14].

Despite the extensive use of cell-penetrating peptides for delivery purposes, the exact mechanisms underlying their cellular uptake and that of peptide conjugates remain poorly understood, and are the object of some controversy. Contradicting initial observations, recent reports have demonstrated that the massive intracellular accumulation, and particularly the nuclear localization observed for some of these peptides and protein conjugates, is a consequence of artifactual observations caused by redistribution of surface-bound cell-penetrating peptides upon cell fixation [15–17]. On the other hand, results from critical re-evaluations of cellular uptake under experimental conditions that avoid artifactual observations have implicated the involvement of well-characterized endocytic pathways, such as clathrin-mediated endocytosis [14], caveolae-mediated endocytosis [18,19] or macropinocytosis [20,21], in the internalization of several peptides and peptide conjugates.

Given these conflicting results concerning the mechanisms of peptide internalization, the main goal of the present study was to critically re-evaluate the mechanism responsible for the uptake of the $S4_{13}$-PV karyophilic cell-penetrating peptide [22]. This peptide results from the combination of a 13-amino-acid cell-penetrating sequence, derived from the dermaseptin S4 peptide, with the SV40 (simian virus 40) large T antigen nuclear localization signal. Dermaseptin S4 peptide belongs to the large family of dermaseptins, which are antimicrobial, polycationic peptides that have been shown to have the capacity to be arranged in amphipathic α-helices in apolar solvents [23]. Table 1 compares the sequence of the $S4_{13}$-PV peptide with that of other cell-penetrating peptides.

The results presented here demonstrate that the $S4_{13}$-PV peptide is able to accumulate inside cells through a rapid and very efficient process, independently of cell fixation. In contrast with previous reports on the cellular uptake of the $S4_{13}$-PV peptide [22], we show that the intracellular accumulation of this peptide is

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; GFP, green fluorescent protein; HA, haemagglutinin; LacCer, lactosylceramide; MβCD, methyl-β-cyclodextrin.

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Table 1 Comparison of the $S4_{13}$-PV peptide sequence with those of cell-penetrating peptides commonly used for cargo delivery

The SV40 large T antigen nuclear localization signal is underlined in the Pep-1 and $S4_{13}$-PV peptides.

Peptide	Sequence
Penetratin	RQIKIWFQNRRMKWKK
Tat	YGRKKRRQRRR
Pep-1	KETWWETWWTEWSQPKKKRKVC-NH_2
$S4_{13}$-PV	ALWKTLLKKVLKAPKKKRKV-NH_2

temperature-sensitive and energy-dependent. Additionally, we demonstrate that, depending on peptide concentration, two alternative mechanisms are responsible for the cellular uptake of the $S4_{13}$-PV peptide: a GAG- and endocytosis-dependent mechanism, dominant at low peptide concentrations, and a GAG- and endocytosis-independent mechanism that occurs preferentially at high peptide concentrations. Moreover, we provide clear evidence that the main mechanism by which the $S4_{13}$-PV peptide is internalized into cells is the one distinct from endocytosis, which most likely occurs through direct penetration of the peptide across cell membranes.

EXPERIMENTAL

Cells

HeLa (human epithelial cervical carcinoma) cells were maintained at 37 °C in 5 % CO_2 in DMEM (Dulbecco's modified Eagle's medium)/high glucose (Sigma, St Louis, MO, U.S.A.) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (Biochrom KG, Berlin, Germany) and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma).

CHO-K1 and pgs A-745 Chinese hamster ovary cell lines were grown in F-12 (Ham's) nutrient mixture (Invitrogen, Paisley, Scotland, U.K.) supplemented with 10 % fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml), 2 mM L-glutamine (Sigma), 10 mM Hepes (Sigma) and 14 mM sodium bicarbonate (Sigma).

Peptides

High purity (> 95 %) $S4_{13}$-PV peptides were obtained from Thermo Electron (Thermo Electron GmbH, Karlsruhe, Germany). During peptide synthesis, peptides were either fluorescently labelled with TAMRA [5-(6)-tetramethylrhodamine], or modified with an acetyl group at the N-terminus. Both peptides were modified by introducing a cysteine and an amide group at the C-terminus.

Freeze-dried peptides were reconstituted in high-purity water, and peptide quantification was performed by using the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.) and by measuring light absorption at 280 nm.

Peptide uptake and cytotoxicity studies

For experiments on peptide uptake, 0.8×10^5 cells/well were seeded on to 12-well plates (flow cytometry experiments) or 12-well plates containing 16 mm glass coverslips (confocal microscopy experiments) 24 h before incubation with the peptide. The cells were then washed with PBS and incubated with $S4_{13}$-PV peptide in DMEM supplemented, or not, with 10 % fetal bovine serum.

To study the effect of low temperature on the cellular uptake of the $S4_{13}$-PV peptide, HeLa cells were washed with PBS and pre-incubated in serum-free DMEM for 1 h at 4 °C. The cells were then incubated with the peptide in serum-free medium for 1 h at 4 °C.

For energy-depletion studies, the cells were washed with PBS and pre-incubated for 1 h at 37 °C in energy-depletion medium [glucose-free DMEM (Invitrogen)/6 mM 2-deoxy-D-glucose (Sigma)/10 mM sodium azide (Sigma)] supplemented with 10 % fetal bovine serum [24,25], followed by incubation with the peptide in the same medium for 1 h at 37 °C.

To address the effect of different drugs on the uptake of the $S4_{13}$-PV peptide, cells were washed with PBS and then pre-treated for 30 min at 37 °C in serum-free DMEM with: (i) 30 μM chlorpromazine; (ii) 5 mM MβCD (methyl-β-cyclodextrin); (iii) 25 μg/ml nystatin; (iv) 5 μg/ml filipin; (v) 5 mM amiloride; (vi) 5 μM cytochalasin D; or (vii) 0–25 μg/ml heparin (sodium salt from bovine intestinal mucosa; all drugs were obtained from Sigma). The cells were then incubated with the peptide in the presence of each drug for 1 h at 37 °C in serum-free medium.

Parallel experiments were performed to address the effect of these drugs (with the exception of heparin) on the cellular uptake of transferrin, a known marker of clathrin-mediated endocytosis (25 μg/ml; 30 min incubation with Alexa Fluor 546 transferrin conjugate; Molecular Probes Europe BV, Leiden, The Netherlands), and of LacCer (lactosylceramide), a marker of raft/caveolae-dependent endocytosis (500 nM; 10 min incubation with BODIPY®-LacCer conjugate; Molecular Probes).

Analysis of peptide internalization was performed by confocal laser-scanning microscopy and/or flow cytometry. Cellular uptake of fluorescently labelled transferrin and BODIPY®-LacCer was evaluated by flow cytometry.

Cell toxicity caused by the $S4_{13}$-PV peptide under all experimental conditions was evaluated by flow cytometry.

Dynamin-K44A experiments

HeLa cells were co-transfected with plasmids encoding HA (haemagglutinin)-tagged dynamin-K44A [pcDNA3.1(−)HA-Dyn1-K44A; American Type Culture Collection, Manassas, VA, U.S.A.] and GFP (green fluorescent protein) (pEGFP-C1; Clontech, Palo Alto, CA, U.S.A.) at a ratio of 10:1. Co-transfection of these two plasmids allows the easy identification of transfected cells by analysing the expression of the GFP reporter plasmid.

To address the effect of overexpression of the dynamin-K44A dominant-negative mutant on the cellular uptake of the $S4_{13}$-PV peptide, 24 h post-transfection, peptide uptake was analysed in live cells expressing GFP by confocal microscopy.

As a control, 24 h post-transfection, the internalization of fluorescently labelled transferrin was evaluated by confocal microscopy in cells expressing the dynamin-K44A mutant.

All transfection experiments were performed using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Penzberg, Germany) according to the manufacturer's instructions, and 1 μg of plasmid DNA/well.

For immunofluorescence analysis of HA-tagged dynamin-K44A expression, cells were washed with PBS, fixed with 4 % paraformaldehyde for 15 min and rinsed with PBS. Cells were then permeabilized with 0.2 % (v/v) Triton X-100 for 2 min at room temperature ($\approx$ 20 °C), blocked with 3 % (v/v) bovine serum albumin in PBS at room temperature for 1 h, and incubated for 1 h at room temperature with anti-HA antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) diluted 1:100 in blocking solution. After an extensive wash with PBS, cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG antibody (Molecular

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Probes) for 1 h. Finally, coverslips were washed with PBS and mounted on glass slides for fluorescence microscopy analysis.

Confocal microscopy

For analysis of the subcellular localization of the $S4_{13}$-PV peptide in live cells, following peptide–cell incubation the cells were washed and mounted in PBS, and immediately visualized by confocal microscopy.

Alternatively, the subcellular localization of the peptide was evaluated in fixed cells. For this purpose, following peptide–cell incubation the cells were washed with PBS, fixed with 4 % paraformaldehyde for 15 min at room temperature, rinsed again with PBS and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for subsequent analysis.

All observations were made using a Bio-Rad MRC 600 fluorescence confocal microscope equipped with an argon/krypton laser (Bio-Rad Laboratories, Inc.).

Flow cytometry

Flow cytometry analysis under the different experimental conditions was performed in live cells using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences). Data were obtained and analysed using CellQuest software (BD Biosciences).

After incubation with $S4_{13}$-PV peptide, transferrin or BODIPY®-LacCer for the indicated times, the cells were washed once with PBS and treated with trypsin (10 min, 37 °C) to remove extracellular, surface-bound peptide and transferrin. The cells were then washed further, resuspended in PBS and immediately analysed. In the case of BODIPY®-LacCer, before trypsin treatment an additional washing step was performed to remove fluorescent lipid present at the cell surface (six washes for 10 min; 5 % defatted bovine serum albumin), as described previously [26].

Live cells were gated by forward/side scattering from a total of 10 000 events.

To evaluate the toxicity of the $S4_{13}$-PV peptide under the different experimental conditions, parallel flow cytometry experiments were performed using a non-labelled peptide, and cytotoxicity was assessed on the basis of propidium iodide exclusion (1 μg/ml for 30 min at room temperature).

RESULTS

Subcellular localization of $S4_{13}$-PV peptide is not affected by cell fixation

Reports that peptide penetration is a consequence of artifactual observations caused by an intracellular redistribution of surface-bound peptides upon cell fixation have motivated the extensive re-evaluation of the mechanisms responsible for the uptake of cell-penetrating peptides.

To address the effect of cell fixation on the internalization of the $S4_{13}$-PV peptide, its cellular uptake was examined both in live and fixed cells by confocal laser-scanning microscopy. As shown in Figure 1, no significant differences in the cellular uptake or subcellular localization of the $S4_{13}$-PV peptide were observed between live and fixed HeLa cells. Under both experimental conditions, the peptide exhibited a diffuse rather than punctate distribution throughout the cytoplasm and nucleus. Interestingly, nucleolar accumulation of the $S4_{13}$-PV peptide was observed, similarly to what has been described for Tat peptide [16,27].

These results demonstrate that the $S4_{13}$-PV peptide is able to efficiently penetrate into cells and accumulate inside the nucleus,

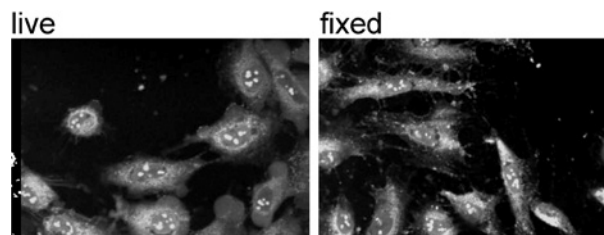

Figure 1 Effect of cell fixation on the cellular uptake and subcellular distribution of the $S4_{13}$-PV peptide

HeLa cells were incubated with 1.0 μM rhodamine-labelled $S4_{13}$-PV peptide for 1 h at 37 °C. The cells were then either immediately observed by confocal fluorescence microscopy (live cells; left panel), or fixed with paraformaldehyde for subsequent analysis (right panel).

providing clear evidence that its cellular uptake and nuclear accumulation cannot be attributed to fixation artifacts.

Cellular uptake of $S4_{13}$-PV peptide is concentration-dependent and non-toxic

In order to assess the extent of peptide internalization, flow cytometry analysis of cells exposed for 1 h to increasing concentrations of rhodamine-labelled $S4_{13}$-PV peptide was performed. Since flow cytometry analysis does not discriminate between membrane-associated and internalized peptide, before analysis the cells were treated with trypsin to remove any surface-bound, non-internalized peptide, which could result in an overestimation of cellular uptake [16,17,25].

Flow cytometry analysis revealed that the number of cells containing the peptide markedly increased with peptide concentration, and also that the amount of peptide internalized by cells increased with peptide dose, within the concentration range examined (Figure 2a).

Additional studies conducted to evaluate the toxicity of the $S4_{13}$-PV peptide demonstrated that its incubation with HeLa cells up to a concentration of 2 μM does not result in any cytotoxicity (Figure 2b).

$S4_{13}$-PV peptide cellular uptake is a very rapid and efficient process

To characterize further the uptake process of the $S4_{13}$-PV peptide, the time course of peptide internalization was investigated. Flow cytometry analysis of cells exposed to the peptide for different times revealed that peptide uptake is a rapid and very efficient process (Figure 3). Uptake of the peptide starts immediately upon its addition to cells, reaching equilibrium after 30 min incubation.

The presence of 10 % serum in the medium slightly retards the cellular uptake of the peptide, most likely due to non-specific electrostatic interactions of peptide molecules with serum components.

Cellular uptake of $S4_{13}$-PV peptide requires energy

As a first approach to elucidate the mechanisms involved in the cellular uptake of the $S4_{13}$-PV peptide, the temperature-sensitivity and energy-dependence of the uptake process were evaluated.

Results obtained by confocal microscopy and flow cytometry demonstrated that both low temperature and depletion of cellular ATP dramatically decrease the number of cells containing the peptide (Figure 4), strongly suggesting that the cellular uptake of $S4_{13}$-PV peptide is mediated by an energy-dependent process. Nonetheless, it is interesting to note that approx. 25 % of the ATP-depleted cells contained the peptide at levels similar to untreated cells. Based on this observation, the additional contribution of

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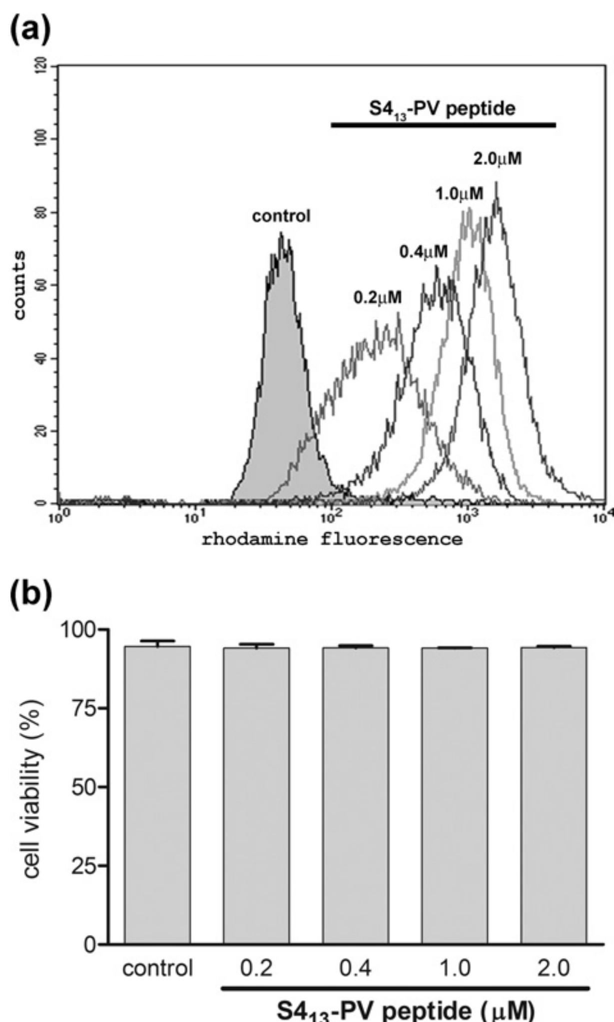

Figure 2 Effect of the S4$_{13}$-PV peptide dose on its cellular uptake and cytotoxicity

(**a**) Flow cytometry quantification of peptide uptake by HeLa cells incubated for 1 h at 37 °C with increasing concentrations of rhodamine-labelled S4$_{13}$-PV peptide. (**b**) Viability of HeLa cells exposed to increasing concentrations of S4$_{13}$-PV peptide. Cell viability was evaluated by flow cytometry, based on propidium iodide exclusion by viable cells. Data are shown as means ± S.D., based on triplicates of at least two independent experiments.

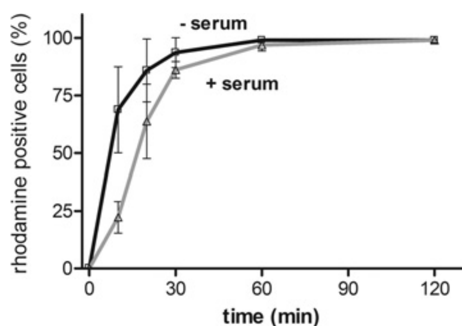

Figure 3 Time course of the cellular uptake of S4$_{13}$-PV peptide

HeLa cells were incubated with 1.0 μM rhodamine-labelled S4$_{13}$-PV peptide at 37 °C for different time periods. The extent of peptide uptake was evaluated by flow cytometry in the absence (black line) or presence (grey line) of 10 % fetal bovine serum in the medium. Data are shown as means ± S.D., based on triplicates of at least two independent experiments.

an energy-independent mechanism to the cellular uptake of the S4$_{13}$-PV peptide cannot be excluded.

Selective inhibition of different endocytic pathways does not impair cellular uptake of S4$_{13}$-PV peptide

Given the results demonstrating that the cellular uptake of the S4$_{13}$-PV peptide is an energy-dependent process, the potential role of endocytosis in the uptake process was carefully evaluated. Endocytosis may occur through several distinct mechanisms, usually divided into two broad categories: phagocytosis, a process restricted to specialized mammalian cells, and pinocytosis, which occurs in all mammalian cells and encompasses macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, as well as other less characterized clathrin- and caveolae-independent endocytic pathways [28,29].

As a first approach to investigate the possible involvement of the different endocytic pathways in the cellular uptake of the S4$_{13}$-PV peptide, the effect of chlorpromazine (a known inhibitor of clathrin-mediated endocytosis), of MβCD, nystatin or filipin (which deplete or sequester membrane cholesterol, inhibiting endocytic pathways dependent on lipid rafts, such as macropinocytosis, caveolae- and clathrin-mediated endocytosis [29]), of amiloride (which specifically blocks the Na^+/H^+ exchange required for macropinocytosis [30]), and of cytochalasin D (an inhibitor of F-actin elongation required for phagocytosis, macropinocytosis and caveolar endocytosis [31]) was evaluated.

The results obtained by both confocal microscopy and flow cytometry demonstrated that, among all the tested drugs, only cytochalasin D had an inhibitory effect on the cellular uptake of the S4$_{13}$-PV peptide (Figure 5).

Control experiments were performed to address the effect of these drugs on the cellular uptake of transferrin, a 'classical' marker of clathrin-mediated endocytosis, as well as of LacCer, a marker of raft/caveolae-mediated endocytosis. As shown, transferrin uptake was only inhibited by chlorpromazine (Figure 5b). Although HeLa cells express low levels of caveolin-1, efficient cellular uptake of LacCer was observed, which was inhibited to different extents by the drugs that interfere with lipid rafts (MβCD, filipin and nystatin), as well as by amiloride and cytochalasin D (Figure 5b).

Taken together, the results from this systematic analysis strongly suggest that endocytosis is not involved in the cellular uptake of the S4$_{13}$-PV peptide, at the tested peptide concentration.

No significant cytotoxicity was associated with the treatment of cells with the different drugs used in this study (results not shown).

Cellular uptake of S4$_{13}$-PV peptide is not affected by overexpression of dynamin-K44A dominant-negative mutant

To investigate further the role of endocytosis in the cellular uptake of the S4$_{13}$-PV peptide, additional studies were performed by addressing the effect of the overexpression of a dominant-negative mutant of dynamin (dynamin-K44A) on peptide uptake.

Dynamin is a key regulator of membrane trafficking at the cell surface, its GTPase activity being required for several forms of endocytosis, including clathrin- and caveolae-mediated endocytosis, phagocytosis and other less well-characterized clathrin- and caveolae-independent endocytic pathways [28,32]. It has been demonstrated previously [28,33–35] that overexpression of dominant-negative mutants of dynamin that do not exhibit GTPase activity, such as the dynamin-K44A mutant, can lead to impairment of several endocytic pathways.

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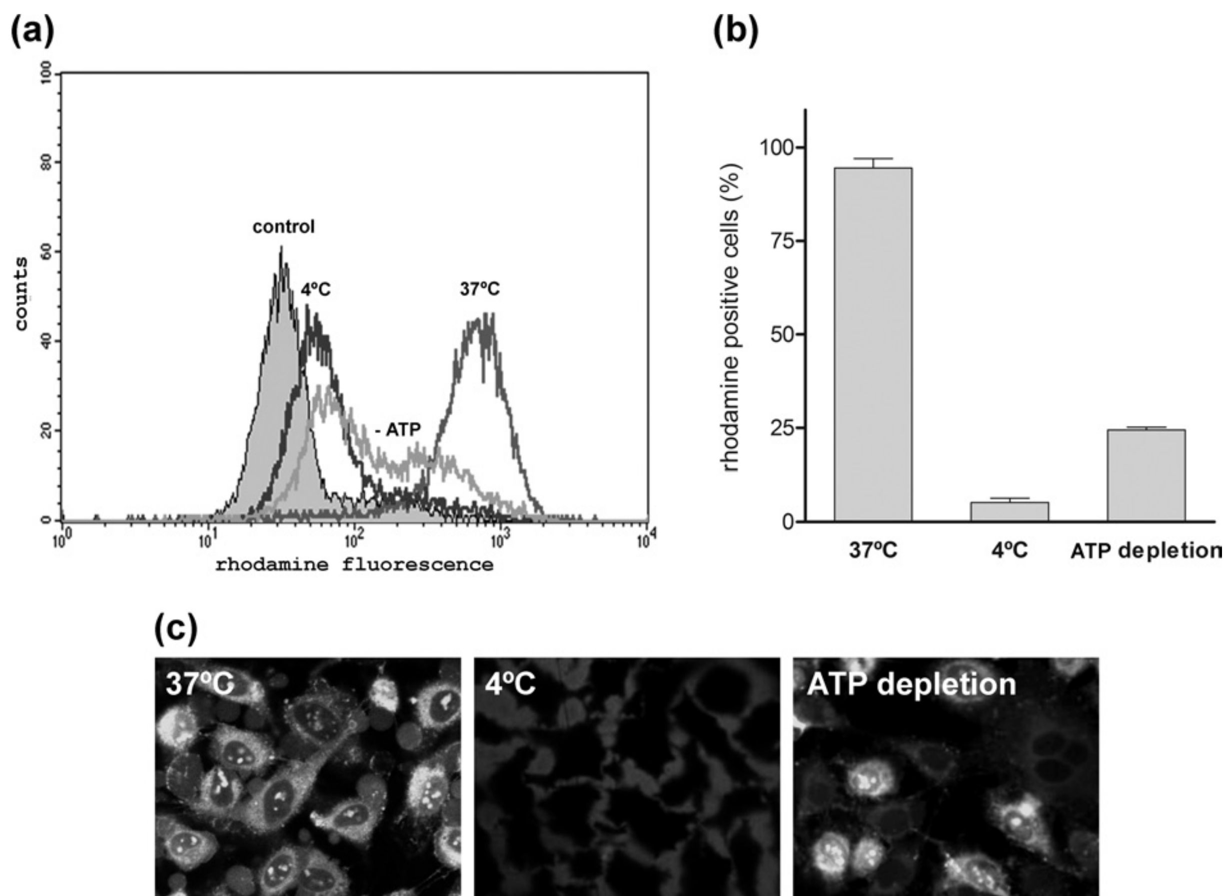

Figure 4 Effect of low temperature and energy depletion on the uptake of S4$_{13}$-PV peptide by HeLa cells

(**a**, **b**) Flow cytometry and (**c**) confocal microscopy analysis of S4$_{13}$-PV peptide cellular uptake at low temperature and upon depletion of cellular ATP. HeLa cells were either pre-incubated at 4 °C or pre-treated with energy-depletion medium for 1 h, and then incubated with 1.0 μM S4$_{13}$-PV peptide for 1 h under the same conditions, as described in the Experimental section. Confocal microscopy analysis was performed in live cells. In (**b**), data are shown as means ± S.D., based on triplicates of at least two independent experiments.

As shown in Figure 6(a), co-transfection of HeLa cells with the plasmids encoding dynamin-K44A and GFP (10:1 ratio) resulted in the simultaneous expression of these two proteins in transfected cells, thus allowing the easy and unambiguous identification of cells expressing the dominant-negative mutant of dynamin by analysis of GFP fluorescence.

Accordingly, cells expressing GFP, which also express the dynamin-K44A dominant-negative mutant, were analysed for their ability to internalize either the S4$_{13}$-PV peptide or fluorescently labelled transferrin, the latter being used as a control, since its cellular uptake is known to occur through clathrin-mediated endocytosis.

Our results demonstrated that the internalization of transferrin is significantly reduced in cells overexpressing the dynamin-K44A dominant-negative mutant, whereas the cellular uptake of the S4$_{13}$-PV peptide is not affected by the expression of the dynamin-K44A mutant (Figure 6b).

Since dynamin has been described to be required for several pathways of endocytosis, these results clearly suggest that endocytosis is not the mechanism responsible for the cellular uptake of the S4$_{13}$-PV peptide at the tested concentration.

Cell surface proteoglycans facilitate S4$_{13}$-PV peptide uptake

Based on reports that cell surface heparan sulphate proteoglycans are involved in the uptake of cell-penetrating peptides/fusion proteins [13,27,36], experiments were performed to investigate the role of these membrane components in the initial steps of interaction of the S4$_{13}$-PV peptide with cell membranes.

As a first approach to address this question, the competitive effect of soluble heparin on the uptake of the S4$_{13}$-PV peptide by HeLa cells was evaluated. Heparin mimics the GAG moieties of some proteoglycans present at the cell surface, and has been shown to bind Tat peptide very efficiently [37] and to impair the cellular uptake of Tat fusion proteins [13,19,21,38].

Cellular uptake studies performed in the presence of heparin demonstrated that concentrations of heparin as low as 2.5 μg/ml are sufficient to completely block S4$_{13}$-PV peptide uptake (Figure 7), supporting the involvement of cell surface proteoglycans as mediators of peptide–cell interaction.

To accurately examine whether the interaction of the S4$_{13}$-PV peptide with biological membranes is dependent on the presence of cell surface heparan sulphate proteoglycans, comparative uptake studies were performed in CHO-K1 and pgs A-745 cell lines. pgs A-745 is a mutant cell line derived from CHO-K1 that is unable to synthesize proteoglycans, since it is defective in xylosyltransferase I, an enzyme involved in the early steps of GAG biosynthesis [39,40].

As shown in Figure 8, flow cytometry and confocal microscopy analysis of peptide uptake by these two cell lines demonstrated that cellular uptake of the S4$_{13}$-PV peptide is more extensive in cells containing heparan sulphate proteoglycans (CHO-K1) when

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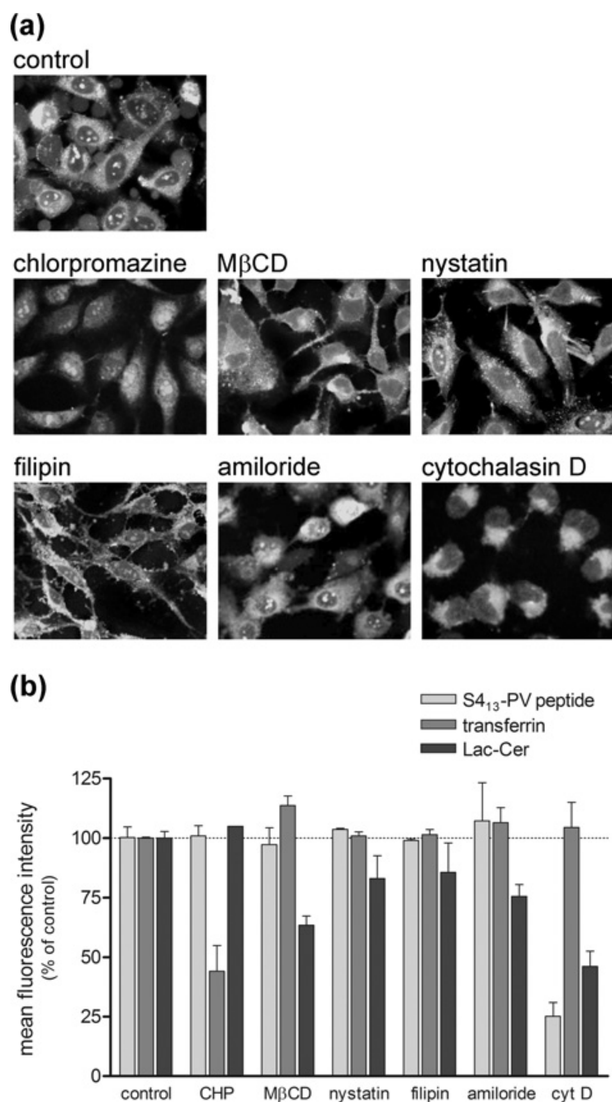

Figure 5 Cellular uptake of $S4_{13}$-PV peptide, transferrin and lactosylceramide in the presence of drugs that affect endocytosis

(**a**) Confocal microscopy analysis of $S4_{13}$-PV peptide cellular uptake and (**b**) quantification by flow cytometry of the extent of cellular uptake of $S4_{13}$-PV peptide, transferrin and LacCer in the presence of drugs that inhibit different endocytic pathways. HeLa cells, pre-treated with the different drugs for 30 min at 37 °C, were incubated in the presence of the drugs with fluorescently labelled $S4_{13}$-PV peptide (1.0 μM for 1 h), transferrin (25 μg/ml for 30 min) or BODIPY®-LacCer (500 nM for 10 min) at the same temperature, as described in the Experimental section. Confocal microscopy ($S4_{13}$-PV peptide only) and flow cytometry analysis was performed in live cells. Chlorpromazine (CHP) inhibits clathrin-mediated endocytosis, MβCD, nystatin and filipin inhibit endocytic pathways dependent on lipid rafts by depleting or sequestering membrane cholesterol, amiloride blocks the Na^+/H^+ exchange required for macropinocytosis, and cytochalasin D (cyt D) inhibits F-actin elongation required for phagocytosis, macropinocytosis, caveolar endocytosis and other cellular processes. In (**b**), data are shown as means ± S.D., based on triplicates of at least two independent experiments.

compared with mutant cells lacking proteoglycans (pgs A-745). Nonetheless, it is important to note that the effect of proteoglycans on facilitating the cellular uptake of $S4_{13}$-PV peptide was highly dependent on peptide concentration. At low peptide concentrations, peptide uptake by cells lacking proteoglycans (pgs A-745) was approx. 10-fold lower than that by cells containing proteoglycans (CHO-K1), whereas, for higher peptide concentrations, smaller differences were observed (Figure 9).

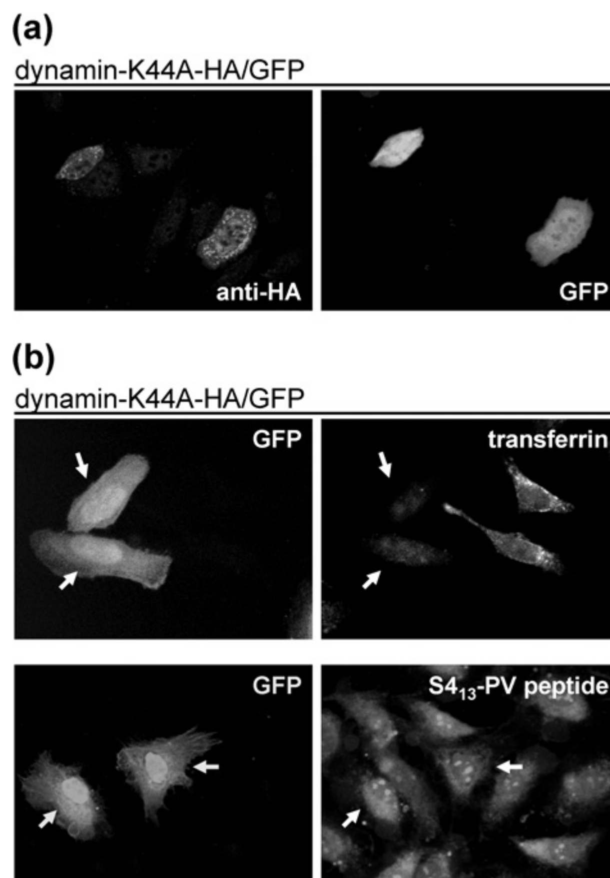

Figure 6 Effect of dynamin-K44A dominant-negative mutant expression on the cellular uptake of $S4_{13}$-PV peptide

(**a**) Analysis of the expression of HA-tagged dynamin-K44A (immunofluorescence, left panel) and GFP (right panel) in HeLa cells co-transfected with plasmids encoding dynamin-K44A and GFP (at a 10:1 ratio). (**b**) Uptake of fluorescently labelled transferrin (25 μg/ml, 15 min incubation; control) or $S4_{13}$-PV peptide (1.0 μM, 1 h incubation) by HeLa cells co-transfected with plasmids encoding dynamin-K44A mutant and GFP. Confocal microscopy analysis of transferrin and peptide uptake was performed using live cells. The white arrows show cells that express GFP and the dynamin-K44A dominant-negative mutant.

Although the presence of heparan sulphate proteoglycans at the cell surface was not mandatory, the results from this comparative analysis reinforce the importance of these membrane components in the cellular uptake of the $S4_{13}$-PV peptide.

At low peptide concentrations, cellular uptake of the $S4_{13}$-PV peptide is partially mediated by clathrin-dependent endocytosis

Results demonstrating that cell surface heparan sulphate proteoglycans play an important role in the cellular uptake of the $S4_{13}$-PV peptide at low peptide concentrations, but not at high peptide concentrations, suggested the possible involvement of an additional mechanism in the cellular uptake of the $S4_{13}$-PV peptide, which would be dominant at low peptide concentrations.

To evaluate whether, at low $S4_{13}$-PV peptide concentrations, endocytosis would also be involved in peptide uptake, the effect of chlorpromazine and nystatin was assessed in CHO-K1 cells at different peptide concentrations. Interestingly, at the lower peptide concentrations tested (0.2 and 0.4 μM), a significant inhibition of peptide uptake was observed upon cell treatment with chlorpromazine (Figure 9), showing that, at these peptide concentrations, clathrin-mediated endocytosis is also involved in the

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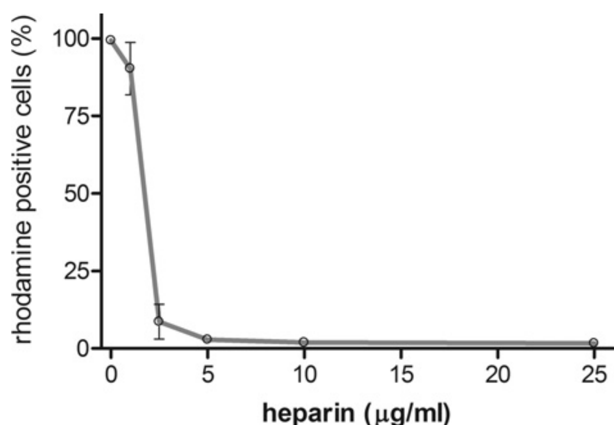

Figure 7 Competitive inhibition of S4$_{13}$-PV peptide cellular uptake by soluble heparin

Flow cytometry evaluation of the extent of peptide uptake in the presence of heparin, a soluble glycosaminoglycan that mimics proteoglycan moieties ubiquitously present at cell surfaces. HeLa cells were pre-treated with increasing concentrations of heparin for 30 min at 37 °C, and then incubated for 1 h with 1.0 μM rhodamine-labelled S4$_{13}$-PV peptide in the presence of heparin. Data are shown as means $\pm$ S.D., based on triplicates of at least two independent experiments.

cellular uptake of the S4$_{13}$-PV peptide. At higher peptide concentrations, chlorpromazine had no effect on the cellular uptake of the S4$_{13}$-PV peptide by CHO-K1 cells (Figure 9), similarly to what was demonstrated previously in HeLa cells. Treatment of CHO-K1 cells with nystatin, which was used to evaluate the involvement of raft/caveolae-mediated endocytosis in the cellular uptake of the S4$_{13}$-PV peptide, did not compromise peptide uptake, regardless of the peptide concentration tested.

As controls, the effect of chlorpromazine and nystatin on the cellular uptake of transferrin and LacCer was also evaluated in CHO-K1 cells. Results were similar to those described previously for HeLa cells, although a greater inhibition of LacCer uptake was observed upon treatment of cells with nystatin (30 % inhibition; results not shown), most likely due to a higher caveolae-mediated endocytic activity in CHO-K1 cells.

Overall, the results described herein support the existence of two alternative mechanisms mediating the cellular uptake of the S4$_{13}$-PV peptide: a GAG- and endocytosis-dependent mechanism that is dominant at low peptide concentrations, and a GAG- and endocytosis-independent mechanism that preferentially occurs at high peptide concentrations.

DISCUSSION

Accumulated studies that fixation procedures are on the basis of artifactual observations of intracellular and nuclear accumulation of some cell-penetrating peptides have prompted a critical re-evaluation of the mechanisms responsible for the cellular uptake of these peptides. Despite the fact that some controversy still exists concerning certain mechanistic aspects of their cellular uptake, cell-penetrating peptides have been extensively used for biotechnological purposes, particularly for the *in vitro* and *in vivo* delivery of proteins, with unquestionable success.

The studies described in the present paper were aimed at clarifying the mechanisms involved in the cellular uptake of the dermaseptin-derived S4$_{13}$-PV cell-penetrating peptide. Comparative analysis of the cellular uptake and subcellular localization of the S4$_{13}$-PV peptide in live and fixed cells demonstrated that the S4$_{13}$-PV peptide is able to accumulate inside cells through a rapid and very efficient process, irrespectively of cell fixation. Moreover, the diffuse cytoplasmic distribution of the S4$_{13}$-PV peptide was shown to be independent of fixation artifacts, unlike that reported previously for other cell-penetrating peptides [3,16].

Nonetheless, the present study demonstrates that cellular uptake of the S4$_{13}$-PV peptide occurs through a temperature-sensitive and energy-dependent process, in contrast with what has been recently reported for this peptide [22]. These conflicting results stem from the fact that initial reports on the cellular uptake of this peptide, as well as of other cell-penetrating peptides, relied on fluorescence microscopic analysis of fixed cells, recurrently reflecting artifactual observations caused by an intracellular redistribution of membrane-bound peptide molecules upon cell fixation. Artifactual observations are particularly misleading under experimental conditions in which cellular uptake is blocked or severely compromised, such as low temperature and energy depletion, as was the case in the initial study on the cellular uptake of the S4$_{13}$-PV peptide [22]. In fact, similar observations of a temperature-insensitive and energy-independent cellular uptake of the S4$_{13}$-PV peptide were made by us, when working with fixed cells (results not shown).

These results underline the importance of this and other studies, in which the mechanisms involved in the uptake of cell-penetrating peptides (and/or fusion proteins containing these peptides) are carefully re-evaluated using improved procedures that minimize artifacts, either by avoiding cell fixation and/or by enzymatically removing membrane-bound, but non-internalized, peptide/fusion protein [16,17,25]. Accordingly, it is interesting to observe that several studies performed by using these improved methods revealed that some of the so-called cell-penetrating peptides are in fact internalized into cells by endocytosis [14,15,19–21,41].

In this context, the possible involvement of endocytosis in the cellular uptake of the S4$_{13}$-PV peptide was thoroughly investigated by analysing peptide uptake in the presence of drugs that selectively compromise different cellular processes, as well as that in cells overexpressing a dominant-negative mutant of dynamin.

Taking into consideration that the cellular uptake of the S4$_{13}$-PV peptide occurs through a temperature-sensitive and energy-dependent mechanism, inhibition of peptide uptake by cytochalasin D might support a role for endocytosis in the internalization process. However, the lack of inhibition observed for all the other tested drugs, which also compromise the endocytic pathways that are affected by cytochalasin D, is not consistent with this hypothesis. Most likely, the inhibition of peptide uptake by cytochalasin D is an indirect consequence of its effect on other cellular processes in which actin is also involved. The additional observation that uptake of the S4$_{13}$-PV peptide is not inhibited upon cell treatment with metabolic inhibitors that non-specifically block all endocytic pathways (sodium azide, sodium fluoride, antimycin A; results not shown), as well as the diffuse, rather than punctate, intracellular distribution of the peptide, reinforce further that, under these experimental conditions, endocytosis is not involved in the cellular internalization of the S4$_{13}$-PV peptide.

The overexpression of dominant-negative mutants of proteins has been successfully used to study different cellular processes, including endocytosis. In the present study, a dominant-negative mutant of dynamin (dynamin-K44A) was used as an alternative approach to investigate further the role of endocytosis in the cellular uptake of the S4$_{13}$-PV peptide. Although dynamin was initially described to be required for clathrin-mediated endocytosis, additional evidence has implicated dynamin in several other endocytic pathways, including caveolae-mediated endocytosis,

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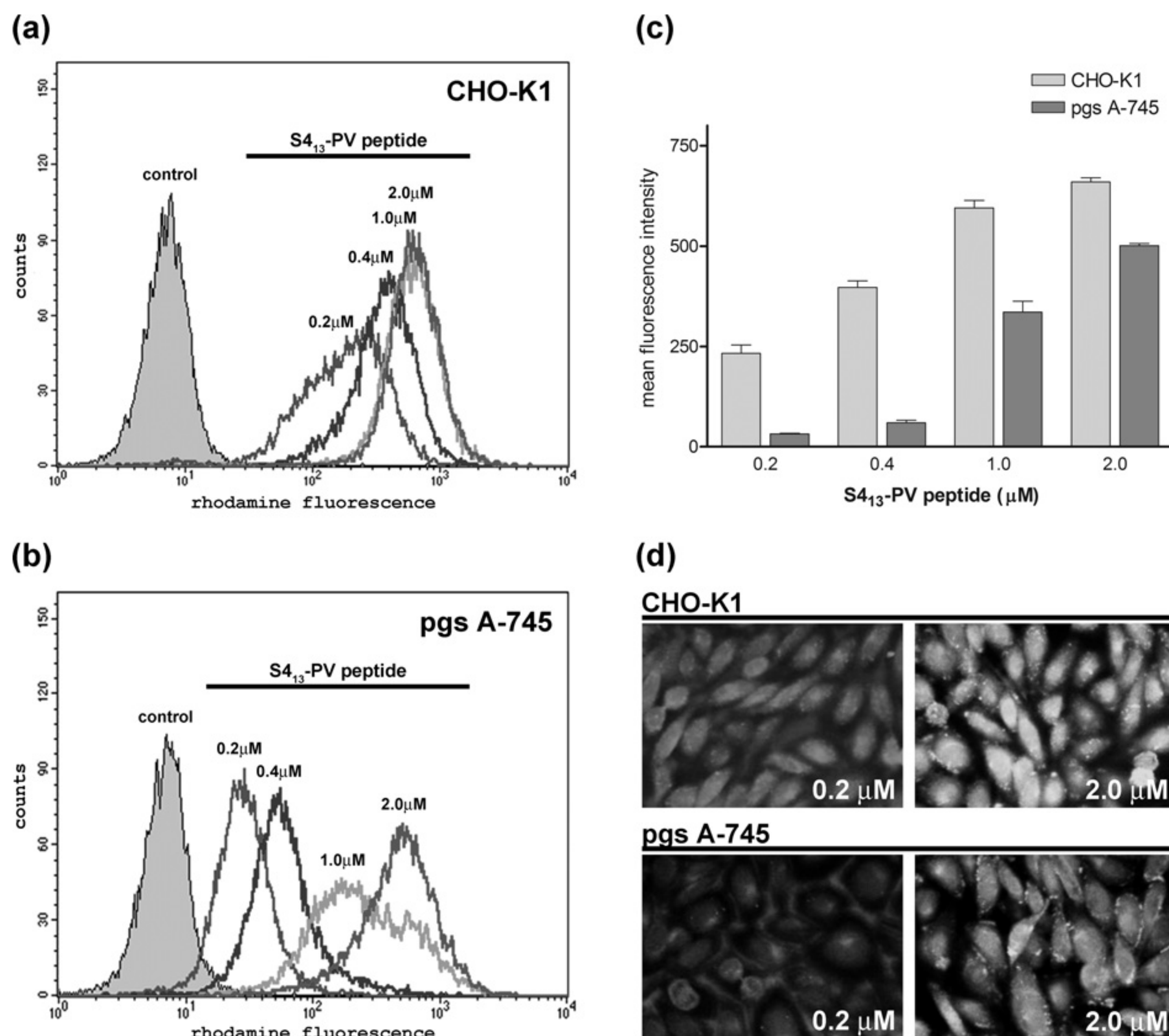

Figure 8 Uptake of $S4_{13}$-PV peptide by cells genetically deficient in proteoglycan biosynthesis

Wild-type CHO-K1 cells and pgs A-745 mutant cells, deficient in proteoglycan biosynthesis, were incubated for 1 h at 37 °C with increasing concentrations of rhodamine-labelled $S4_{13}$-PV peptide. (**a**, **b**) Flow cytometry analysis of $S4_{13}$-PV peptide uptake by CHO-K1 and pgs A-745 cell lines. (**c**) Quantification of the uptake of fluorescently labelled $S4_{13}$-PV peptide by CHO-K1 and pgs A-745 cells. Data are shown as means ± S.D., based on triplicates of at least two independent experiments. (**d**) Confocal microscopy analysis of $S4_{13}$-PV peptide uptake by CHO-K1 and pgs A-745 cells. Microscopy analysis of peptide uptake was performed in live cells.

phagocytosis and clathrin- and caveolae-independent pathways [28,32].

Therefore the observation that overexpression of the dynamin-K44A dominant-negative mutant significantly inhibits the extent of transferrin internalization, whereas it has no effect on the cellular entry of the $S4_{13}$-PV peptide, clearly indicates that endocytosis is not involved in its cellular uptake. These results are in agreement with those described above regarding the effect of several drugs that selectively inhibit different endocytic pathways on peptide uptake, which collectively exclude endocytosis as being the mechanism responsible for the efficient cellular uptake of the $S4_{13}$-PV peptide.

Previous reports have shown that heparan sulphate proteoglycans are absolutely required for the cellular uptake of full-length Tat and Tat-fusion proteins [13,14,27]. In view of these findings, the dramatic inhibition of cellular uptake of the $S4_{13}$-PV peptide by low heparin concentrations reported in the present study strongly suggests that the interactions between the positively charged peptide and highly negatively charged membrane components, such as the GAG moieties of cell surface proteoglycans, play an important role in the overall process of cellular uptake of this peptide [36]. Although this observation may also reflect non-specific electrostatic interactions between the $S4_{13}$-PV peptide and heparin, it has been described that heparin blocks uptake of cell-penetrating peptides and peptide conjugates more efficiently than other negatively charged soluble GAGs, such as chondroitin sulphates [13,21], most likely indicating that the strong interaction between cell-penetrating peptides, including the $S4_{13}$-PV peptide, and heparin involves some structural requirements.

More interestingly, results from the comparative analysis of cellular uptake of the $S4_{13}$-PV peptide by normal and genetically modified cells that are deficient in proteoglycan biosynthesis clearly demonstrate that heparan sulphate proteoglycans potentiate the cellular uptake of the $S4_{13}$-PV peptide, although their presence at the cell surface is not mandatory. In fact, the effect of proteoglycans on the cellular uptake of the $S4_{13}$-PV peptide was

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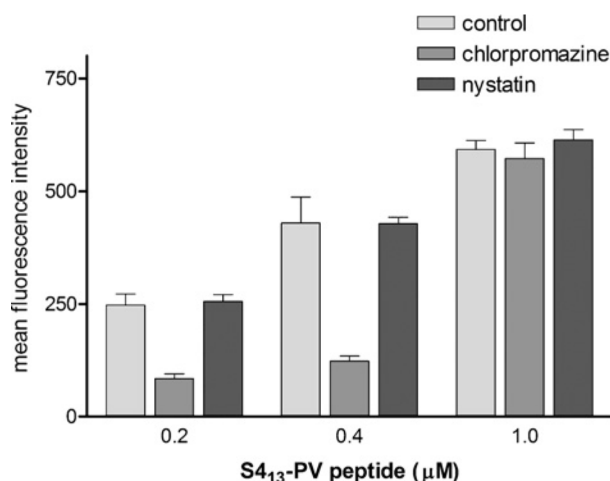

Figure 9 Effect of S4$_{13}$-PV peptide concentration on the mechanisms of cellular uptake

Flow cytometry quantification of the effect of chlorpromazine and nystatin on the extent of S4$_{13}$-PV peptide uptake by CHO-K1 cells. Cells were incubated for 1 h at 37 °C, with increasing concentrations of rhodamine-labelled S4$_{13}$-PV peptide. Data are shown as means ± S.D., based on triplicates of at least two independent experiments.

shown to be relevant only at low peptide concentrations, whereas at high peptide concentrations almost no differences were observed between cells containing, or not, proteoglycans.

Although these results may also reflect the role of proteoglycans in promoting peptide binding to cell membranes, which presumably is not a limiting step at high peptide concentrations, studies performed to address the involvement of endocytosis in the cellular uptake of the S4$_{13}$-PV peptide at low concentrations have demonstrated that two mechanisms are responsible for the cellular uptake of this peptide: a GAG- and endocytosis-dependent mechanism, which is dominant at low peptide concentrations, and a GAG- and endocytosis-independent mechanism that occurs preferentially at high peptide concentrations.

Despite the important role that the GAG- and endocytosis-dependent mechanism plays in peptide uptake at low concentrations, it should be highlighted that, under experimental conditions leading to efficient uptake of the S4$_{13}$-PV peptide by the majority of cells, the contribution of this mechanism is negligible, and the GAG- and endocytosis-independent mechanism of peptide uptake is clearly prevalent.

A possible explanation for the observations of a dominant endocytosis-independent mechanism only at high peptide concentrations is that a critical concentration of peptide at the membrane has to be achieved in order to elicit the events that lead to the endocytosis-independent translocation of the S4$_{13}$-PV peptide across biological membranes. Work by Hällbrink et al. [42], demonstrating that the extent of cellular uptake of the Penetratin peptide is dependent on the peptide-to-cell ratio rather than on the molar concentration of the peptide, argues in favour of this hypothesis.

In addition, it is interesting to note that the S4$_{13}$-PV peptide undergoes significant conformational changes in the presence of lipid vesicles of various compositions, which are consistent with the formation of helical structures (M. Mano, F. Gavilanes, S. Simões and M. C. Pedroso de Lima, unpublished work). Similar results have also been reported for other peptides, such as Penetratin and Pep-1 [43,44], which have been shown to penetrate cells independently of endocytosis. Moreover, we have observed recently that such conformational changes occur concomitantly with the penetration of the S4$_{13}$-PV peptide into the lipid bilayer, strongly suggesting that the resulting helical structures may be of critical importance for the non-endocytic cellular uptake of the peptide (M. Mano, F. Gavilanes, S. Simões and M. C. Pedroso de Lima, unpublished work).

To our knowledge, the findings in the present study provide the first systematic report that the peptide concentration in itself may determine the mechanism by which uptake of cell-penetrating peptides occurs and, consequently, their intracellular fate.

Although the main mechanism responsible for the efficient uptake of the S4$_{13}$-PV cell-penetrating peptide observed at high peptide concentrations remains elusive, the results presented in this study demonstrate that this mechanism is distinct from endocytosis. Studies are currently in progress in our laboratory aimed at investigating whether this mechanism will correlate with an increased capacity of the S4$_{13}$-PV peptide to mediate the intracellular delivery of molecules of pharmacological interest, including drugs, proteins and nucleic acids.

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