Targeting of Nonkaryophilic Cell-Permeable Peptides into the Nuclei of Intact Cells by Covalently Attached Nuclear Localization Signals†

Elana Hariton-Gazal,‡ Rina Feder,§ Amram Mor,§ Adolf Graessmann,|| Ruth Brack-Werner,⊥ David Jans,# Chaim Gilon,‡ and Abraham Loyter*,§

Department of Organic Chemistry, Institute of Chemistry, and Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel, Institut fuer Molekularbiologie und Biochemie, Free University of Berlin, Berlin, Germany, Institut fuer Molekulare Virologie, GSF-Forschungszentrum fuer Umwelt und Gesundheit, Ingolstaedter Landstrasse, Neuherberg, Germany, and Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia

Received February 20, 2002; Revised Manuscript Received May 2, 2002

ABSTRACT: Dermaseptins are a family of antimicrobial peptides that lyse target bacterial cells by destabilization of their membranes. Here we present a novel application of a peptide derived from the dermaseptin S4, $S4_{13}$. At nontoxic concentrations, fluorescently labeled $S4_{13}$ was able to penetrate intact cultured HeLa cells but essentially failed to enter their nuclei despite its low molecular weight. Covalent attachment of nuclear localization signal (NLS) motifs of the SV40-T-antigen and of the HIV-1 Rev protein (ARM) conferred karyophilic properties upon the $S4_{13}$. The resulting peptides, which were designated as PV-$S4_{13}$ and RR-$S4_{13}$ penetrated into intact HeLa cells and were able to accumulate within the cells' nuclei. In studies with digitonin-permeabilized cells, nuclear uptake of the PV-$S4_{13}$ and the RR-$S4_{13}$ peptides showed the same features that characterize active nuclear import. Nuclear import was observed at 37 °C, was ATP-dependent, and was inhibited by the free peptides bearing the SV40 NLS and the Rev and Tat ARMs. Microinjected $S4_{13}$ remained in the cytoplasm while microinjected RR-$S4_{13}$ was translocated into the cells' nuclei. The new type of cell-permeable "karyophilic" peptides described here may be of potential application as a lead compound for therapeutic purposes, as a tool to study nucleocytoplasmic shuttling in intact cells, and for the delivery of peptides to the nucleus.

The introduction of macromolecules such as proteins (*1*) or nucleic acids (DNA or RNA) (*2*) into living cells by microinjection (*3*) or electoporation (*4*) has been used as a tool to study various aspects of intracellular processes. Evidently, these approaches are limited to in vitro systems and cultured mammalian cells. However, for clinical use especially in the fields of drug delivery and gene therapy, methods that allow the release of macromolecules into the intracellular or nuclear space of cells in the living organism are required. Liposomes or reconstituted viral envelopes loaded with proteins or nucleic acids have been employed as carriers that allow the delivery of their content into cells of specific tissues (*5*, *6*). However, as been demonstrated by numerous studies, such vesicular carriers are taken into the cells via a process of receptor-mediated endocytosis, subjecting their content to extensive degradation by endosomal or lysosomal enzymes. Evidently, this makes such loaded vesicles inefficient carriers characterized by low yield for cargo delivery.

During the past few years it has became apparent that certain small proteins and peptides are able to cross cell plasma membranes directly without being susceptible to degradation by the intraendosomal enzymes. Indeed, a number of natural proteins and peptides of various origins as well as synthetic peptides have been defined as cell penetrating proteins or peptides (CPPs),[1] due to their ability to penetrate cell plasma membranes independently of transporters or specific receptors (*7*). Mastoparan, the third α-helix from the Antennapedia homeodomain of *Drosophila* (penetratins) (*8*), transportan (*9*), and the HIV-1 Tat protein have been shown to penetrate into intact cells and even deliver protein molecules as well as nucleic acids that have been covalently attached to them (*10*). In fashion similar to the carrier protein, the conjugates penetrate into the recipient cells via a nonendocytic process, thus escaping hydrolysis

† This work was supported by grants from the GIF (German Israeli Foundation), I-590-105.09/98 (to A.L. and C.G.) and from the Israel Science foundation, 107/00 (to A.L.).

* Corresponding author. Tel: 972-2-6585422. Fax: 972-2-6586448. E-mail: loyter@vms.huji.ac.il.

‡ Department of Organic Chemistry, Institute of Chemistry, The Hebrew University of Jerusalem.

§ Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem.

|| Free University of Berlin.

⊥ GSF-Forschungszentrum fuer Umwelt und Gesundheit.

Monash University.

[1] Abbreviations: ARM, arginine-rich motif; BSA, bovine serum albumin; CPP, cell-penetrating peptide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; FCS, fetal calf serum; Fmoc, fluorenylmethoxycarbonyl; HBSS, Hank's balanced salt solution; HIV-1, human immunodeficiency virus type 1; LR, lissamine rhodamine; NLS, nuclear localization signal; NPC, nuclear pore complex; PBS, phosphate-buffered saline; PTD, protein translocation domain; SPPS, solid-phase peptide synthesis; TDW, triply distilled water; TFA, trifluoroacetic acid; TOF-MS, time-of-flight mass spectrometry.

10.1021/bi0201466 CCC: $22.00
Published on Web 06/27/2002

by lysosomal hydrolytic enzymes. The translocation process of the CPPs was attributed to the activity of a specific domain within the carrier proteins, which have been termed protein translocation domains (PTD) (*7*). The ability of the HIV-1 Tat protein, for example, to cross cell plasma is due to a cluster of positively charged amino acids containing mostly arginine residues (*11*). A peptide bearing this cluster, which has been defined as an ARM (arginine-rich motif), is able to cross plasma membranes of living mammalian cells.

The biological function of the Tat ARM domain (amino acids 48−60) is to serve as a nuclear localization signal (NLS) in order to promote nuclear import of the HIV-1 Tat protein. The intranuclear presence of the Tat protein in virus-infected cells is essential to activate transcription of the virus genome. Also, the NLS of the HIV-1 karyophilic Rev protein is characterized by a cluster of arginine residues and, similar to that of Tat, is termed ARM. Recently, it has been demonstrated that various arginine-rich peptides, including the Rev ARM, share the same property that they are able to cross cell plasma membrane (*12*). These arginine-rich peptides, similar to the ARM peptide, are able to serve as carriers and to deliver covalently attached macromolecules into intact cells. Recently, a somewhat different strategy for protein delivery was described (*13*). A short amphiphatic peptide, designated as Pep-1, has been shown to efficiently deliver proteins into mammalian cells, without the need for prior covalent attachment (*13*).

Here, we describe a new peptide with cell-penetrating properties, which was derived from the dermaseptin family (*14*). The dermaseptins are a large family of antimicrobial peptides, which appear on the skin of frogs and belong to the *Phyllomedusinae* genus (*15*). These peptides have been shown to be cytolytic in a broad spectrum of phatogenic microorganisms such as bacteria, protozoa, yeast, and filamentous fungi (*15, 16*). Among the natural dermaseptins, dermaseptin S4 is also highly toxic to erythrocytes (*17*). Although the exact mechanism of action of these antimicrobial peptides is not yet fully understood, it appears that they destabilize target cells membranes, thus causing cell death. The dermaseptins are linear polycationic peptides composed of 28−34 amino acids and possess an amhiphatic α-helix in apolar solvents (*18*). The α-helical peptide has been shown to interact with phospholipids in liposomes (*18*−*20*).

The present work shows that a peptide derived from dermaspetin S4, designated $S4_{13}$ (*19, 21*), efficiently penetrates plasma membranes of intact mammalian cultured cells. Since $S4_{13}$ is of low molecular weight, composed of only 13 amino acids, it was expected that such a small peptide would freely diffuse through the nuclear pore complex (NPC) (*22*, *23*) and accumulate within the intranuclear space of the recipient cells. However, our results show that the $S4_{13}$ peptide was largely nonkaryophilic, being retained within the cytoplasm and excluded from the cells' nuclei. Nuclear import could be conferred upon $S4_{13}$ by covalent attachment of peptides bearing NLSs such as those of the SV40-large tumor antigen (T-antigen) or the Rev ARM (*24*). Incubation of the $S4_{13}$-NLS "fusion peptides" with intact cultured HeLa cells resulted in the appearance of the $S4_{13}$-NLS fusion peptides within the intranuclear space. Nuclear import of this karyophilic peptide exhibited the same features that characterize active nuclear import. $S4_{13}$-NLS peptides thus have interesting properties for therapeutic purposes in cellular and nuclear drug delivery.

MATERIALS AND METHODS

Chemicals. Protected amino acids, Rink amide MBHA resin, and coupling reagents were purchased from NOVA Biochem (Laufelfingen, Switzerland). Other chemicals were purchased from Sigma (St. Louis, MO) or Merck, Darmstadt, Germany. Solvents for peptide synthesis were purchased from Baker, Phillipsburg, NJ.

Cultured Cells. HeLa cell monolayers were grown in DMEM growth medium supplemented with 10% FCS, 0.3g/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Beit Haemek, Israel). Cells were incubated at 37 °C in 5% CO_2 atmosphere and recultured every 4 days.

Peptide Synthesis: Fluorescent Labeling of the Synthetic Peptides. The peptides described in the present work were synthesized according to the SPPS method, using an Applied Biosystems peptide synthesizer model 433A on Rink amide resin (loading 0.65 mmol/g) by the standard Fmoc chemistry procedure.

The Fmoc protecting group was removed from the peptidyl-resin by treatment with 20% piperidine in DMF for 30 min. The peptides were labeled at the N terminus as follows: lissamine rhodamine sulfonyl chloride (10 mg/mL; Molecular Probes) and DIEA (7 equiv, 3.4 mmol) were dissolved in dry DMF and were added to the peptidyl-resin. The reaction mixture was stirred in the dark for 24 h. The peptidyl-resin was washed with DMF five times and with DCM twice. The peptides were deprotected and cleaved from the resin with trifluoroacetic acid (TFA) containing 1% anisole and 1% TDW as scavengers at 0 °C for 30 min and at room temperature for another 2 h. The TFA was evaporated under nitrogen, and the peptides were precipitated by cold ether, washed three times with ether, dissolved in TDW, and lyophilized. Following purification by reversed-phase HPLC using a C-18 column (acetonitrile/TDW containing 0.1% TFA, wavelengths 220 and 600 nm), the resulting peptides were characterized by TOF-MS.

Determination of Penetration of Synthetic Peptides into Intact Cultured Cells by Fluorescence Microscopy. HeLa cells (3×10^4 per well) were cultured on eight-well Lab-Teck coverslips (Nunc Inc.) or on 10 mm coverslips to subconfluence. Following the removal of the culture medium, the cells were washed three times with PBS and then exposed to different concentrations of the lissamine rhodamine-labeled peptides at 37 or 4 °C. At the end of the incubation period the cells were washed three times in PBS and fixed in 4% (v/v) formaldehyde dissolved in PBS. Fixed cells were examined by fluorescence microscopy (Zeiss, Germany, a 40× objective; Apoplan) or by confocal microscopy using an MRC 1024 confocal imaging system (Bio-Rad). The microscope (Axiovert 135M; Zeiss, Germany, a 63× objective; Apoplan; NA 1.4) was equipped with an argon ion laser for rhodamine excitation at 514 nm (emission 580).

Determination of Nuclear Uptake in Permeabilized Cells by Fluorescence Microscopy. HeLa cells were cultivated on 10 mm coverslips to subconfluence density and then permeabilized with digitonin as described previously (*25*). The cells were incubated with the peptides, and nuclear import was followed by fluorescence microscopy as described (*25*).

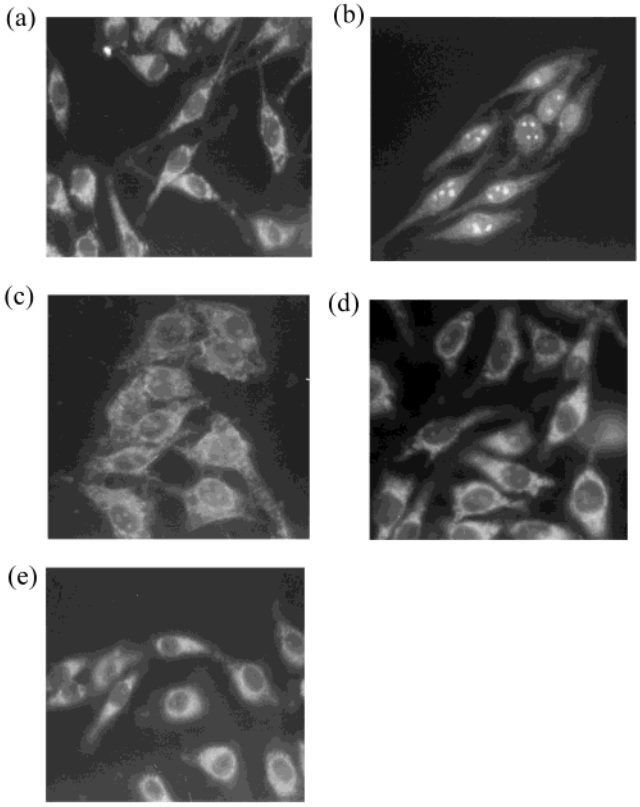

FIGURE 1: Cell penetration and nuclear import of the $S4_{13}$ and the $S4_{13}$-derived peptides as visualized by fluorescence microscopy. HeLa cells were incubated for 30 min with 5 μM (a) $S4_{13}$, (b) PV-$S4_{13}$, or (c) rPV-$S4_{13}$ peptides at 37 °C or (d) $S4_{13}$ or (e) PV-$S4_{13}$ at 4 °C. Note that only in (b) are the nuclei and mainly the nucleoli highly fluorescent while in other panels (a, c, d, e) cytoplasmic retention is observed.

Effect of the Various Peptides on Cell Viability. To study the effect of the peptides on cell viability, increasing concentrations (1−15 μM) of the peptides were added to cultured cells (96 wells, 3×10^4 cells per well in DMEM). Following incubation at 37 °C for 30 min, 100 μL tryphan blue solution was added (0.4% in HBSS buffer; Sigma) and HBSS buffer (5:3), and viable cells were counted after 5 min of continuous stirring. Cell death was not greater than ±20%.

Microinjection of Peptides into HeLa Cells. HeLa cells were microinjected with either $S4_{13}$ or RR-$S4_{13}$ using the CompInject AIS2 automated microinjection system (Cell Biology Trading, Hamburg, Germany) as described previously (*26*) using a microinjection method developed by Graessmann et al. (*27*).

RESULTS

$S4_{13}$ Is a Nonkaryophilic Peptide. The results shown in Figure 1a demonstrate that fluorescently labeled $S4_{13}$, a 13 amino acid peptide (Table 1) derived from the antimicrobial peptide dermaseptin S4, penetrated readily into intact cultured HeLa cells. The $S4_{13}$ peptide essentially accumulated within the cells cytoplasm and not in the cells' nuclei despite its low molecular weight. The same was observed for its parent peptide, S4 (data not shown). Penetration of $S4_{13}$ into intact cells occurred at 37 °C as well as at 4 °C (Figure 1d) and even in ATP-depleted cells, indicating a nonmetabolic process. Kinetic studies revealed that penetration of $S4_{13}$ into HeLa cultured cells was relatively fast, occurring within 5 min of incubation with 1 μM peptide either at 37 °C or at 4 °C. $S4_{13}$ retained its nonkaryophilic properties and remained in the cell cytoplasm with very little, if any, nuclear uptake even after 24 h at 37 °C (not shown). Examination of intact HeLa cells incubated with lissamine rhodamine (LR) labeled $S4_{13}$ revealed the appearance of clusters of large intracellular fluorescent dots, probably indicative of a process of self-aggregation resulting in the formation of aggregates, which may be too large for passive diffusion into the nuclei.

Interestingly, the peptide $S4_{13}$ promoted the copenetration of small molecules into intact cells such as the fluorophore LR. Incubation of HeLa cells with a mixture of unlabeled $S4_{13}$ and free LR resulted in the appearance of a few intracellular fluorescent dots, which were absent from cells incubated with the fluorophore alone (Table 2). $S4_{13}$ was nontoxic at the concentrations used as was indicated by the tryphan blue test (not shown; see Materials and Methods).

The NLS of SV40-T-Antigen Confers Karyophilic Properties upon $S4_{13}$. Composite peptides bearing the sequences of both the $S4_{13}$ peptide and the NLS motif of the SV40-T-antigen were obtained by the synthesis of fused peptides containing both sequences (Table 1; see Materials and Methods). This was done to test whether the addition of an

Table 1: Amino Acid Sequences[a] of the Various Peptides Used in the Present Work

peptide	peptide sequence[b]
dermaseptin S4	ALWMTLLKKVLKAAAKAALNAVLVGANA-COOH
$S4_{13}$	**ALWKTLLKKVLKA**-NH_2
$S4_{13}$-PV	**ALWKTLLKKVLKA**PKKKRKV-NH_2
PV-$S4_{13}$	PKKKRKV**ALWKTLLKKVLKA**-NH_2
rPV-$S4_{13}$	VKRKKKP**ALWKTLLKKVLKA**-NH_2
RR-$S4_{13}$	RQARRNRRR**ALWKTLLKKVLKA**-NH_2
Rev ARM	RQARRNRRRC-NH_2
Tat ARM	GRKKRRQRRRPPQC-NH_2

[a] Single-letter code. [b] The $S4_{13}$ sequence is highlighted in boldface.

Table 2: Cell Penetration and Nuclear Import of Various Dermaseptin-Derived Peptides[a]

peptides	incubation temp (°C)	penetration into[b]	
		cytoplasm	nuclei
S4	37	+	−
$S4_{13}$	37	+	−
PV-$S4_{13}$	37	+	+
$S4_{13}$-PV	37	+	+
rPV-$S4_{13}$	37	+	−
$S4_{13}$	4	+	−
PV-$S4_{13}$	4	+	−
PV-$S4_{13}$ − ATP-depleted cells[c]	37	+	−
$S4_{13}$ + unlabeled PV-$S4_{13}$ (1:100)	37	+	−
PV-$S4_{13}$ + unlabeled PV-$S4_{13}$ (1:100)	37	+	−
L-rhodamine	37	−	−
$S4_{13}$ + L-rhodamine	37	+	−
PV-$S4_{13}$ + L-rhodamine	37	+	−

[a] The labeled peptides were incubated for 5 min (1 μM) at the indicated temperature with a monolayer of cultured HeLa cells. Following fixation the various samples were examined by fluorescence microscopy. All other experimental procedures were as described in Materials and Methods. [b] (+) Most of the nuclei in the microscopic fields are highly fluorescent; (−) no fluorescence in the nuclei. [c] The cells were preincubated with 0.25 mM 2,4-dinitrophenol for 30 min prior to the addition of the peptides.

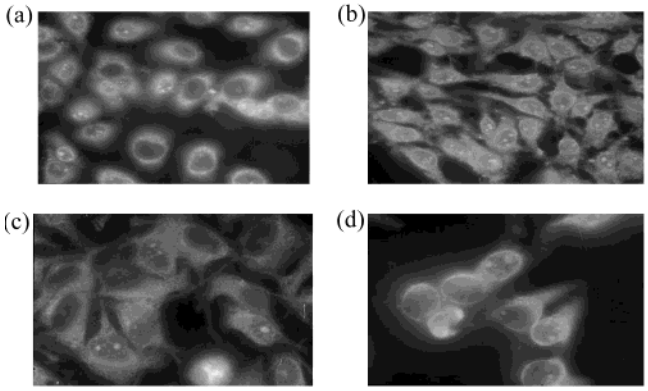

FIGURE 2: Nuclear import of PV-$S4_{13}$ in permeabilized HeLa cells as visualized by fluorescence microscopy. All experimental conditions were as described in Materials and Methods and in the legend to Table 3. Digitonin-permeabilized HeLa cells were incubated in the presence of reticulocyte extract at 30 °C for 1 h with 1 μM (a) $S4_{13}$, (b) PV-$S4_{13}$, or (c) rPV-$S4_{13}$. In (d) PV-$S4_{13}$ incubation was as in (b) but in the absence of reticulocyte extract.

Table 3: Nuclear Import of the PV-$S4_{13}$ Peptide in Permeabilized HeLa Cells: Requirement and Characterization[a]

exptl conditions	nuclear import of PV-$S4_{13}$[b]
(i) complete system	+
(ii) hexokinase (ATP-depleted system)	−
(iii) as in (i) but without reticulocyte extract	−
(iv) as in (i) at 4 °C	−
(v) as in (i) + GTPγS	−
(vi) as in (i) + unlabeled SV40 NLS	+/−
(vii) as in (i) + unlabeled $S4_{13}$	+
(viii) as in (i) + unlabeled PV-$S4_{13}$	+/−

[a] Experimental conditions of nuclear import in digitonin-permeabilized cells (complete system) were as described in Materials and Methods. PV-$S4_{13}$ was used at 1 μM, and all incubations were performed at 37 °C in the presence of reticulocyte lysate, unless otherwise indicated. Additions: GTPγ*S* (20 μM) and the following unlabeled peptides, SV40-T-antigen NLS, $S4_{13}$, and PV-$S4_{13}$, at a ratio of 1:100 (mol/mol). [b] (+) Most of the nuclei in the microscopic fields are highly fluorescent; (−) no fluorescence in the nuclei; (+/−) most of the nuclei are very weakly fluorescent.

NLS could confer karyophilic properties upon $S4_{13}$ without affecting its cell-permeability properties. The SV40-T-antigen NLS was covalently attached either to the N terminus or to the C terminus of $S4_{13}$, resulting in the composite peptides PV-$S4_{13}$ and $S4_{13}$-PV, respectively. Also, the reverse (nonfunctional) sequence of the SV40-T-antigen NLS was attached to $S4_{13}$, yielding peptide rPV-$S4_{13}$ (Table 1).

The results in Figure 1 and those summarized in Table 2 show that incubation of PV-$S4_{13}$ (Figure 1b) and $S4_{13}$-PV (not shown) with intact cultured HeLa cells resulted in their cell penetration and accumulation within the cells' nuclei. Clearly, both peptides were cell permeable like $S4_{13}$ but, unlike $S4_{13}$, possessed karyophilic properties. NLS-specific nuclear import was suggested by the results showing that the peptide rPV-$S4_{13}$, containing a nonfunctional NLS, was much less karyophilic than the PV-$S4_{13}$ and showed very little, if any, accumulation within the cells' nuclei/nucleolei (Figure 1c and Table 2).

Similar to $S4_{13}$, the composite PV-$S4_{13}$ and $S4_{13}$-PV peptides penetrated intact HeLa cells at 37 °C as well as at 4 °C (Figure 1). However, at 4 °C the PV-$S4_{13}$ and $S4_{13}$-PV peptides were retained in the cytoplasm, strengthening the view that the nuclear import observed at 37 °C was an active process.

The cell viability test revealed that, like $S4_{13}$, also the PV-$S4_{13}$ and $S4_{13}$-PV peptides were not toxic at the concentrations used (not shown).

Import of PV-$S4_{13}$ and $S4_{13}$-PV into Nuclei of Permeabilized HeLa Cells. The results in Figure 2 and in Table 3 show that the peptide $S4_{13}$ was excluded from the nuclei of even digitonin-permeabilized HeLa cells. On the other hand, accumulation of PV-$S4_{13}$ in permeabilized cells did occur and was absolutely dependent on the addition of a cytosolic extract, indicating that its nuclear translocation shows the same features that characterize SV40-T-antigen NLS: mediated nuclear import (*28*, *29*) in terms of the requirement for exogenously added cytosolic factors (Figure 2 and Table 3). In contrast, the peptide rPV-$S4_{13}$ either did not accumulate or showed poor nuclear accumulation whether cytosolic extract was present or not. This indicates that the nuclear import observed of PV-$S4_{13}$ and $S4_{13}$-PV was directly dependent on the attached NLS and likely to be mediated by importin. Nuclear import of PV-$S4_{13}$ and $S4_{13}$-PV was ATP dependent and inhibited by GTPγS, WGA, and an excess of free unlabeled SV40-T-antigen NLS peptide, but it did not occur at 4 °C (Table 3). It was also inhibited by excess unlabeled PV-$S4_{13}$ but not by $S4_{13}$ itself. All these results clearly show that nuclear import of PV-$S4_{13}$ is characterized by the same features that characterize active import of karyophilic proteins. Results identical to those observed with PV-$S4_{13}$ were obtained when $S4_{13}$-PV ($S4_{13}$ bearing an NLS sequence at its C terminus) was used as a transport substrate (not shown).

Rev ARM-Mediated Nuclear Import of $S4_{13}$ (RR-$S4_{13}$) in Intact and Permeabilized HeLa Cells. The results in Figure 3 and in Table 4 show that a peptide bearing the Rev ARM RR-$S4_{13}$ (see Table 1 and Materials and Methods) was also able to penetrate into intact HeLa cells and accumulate, within a short period of time, within the nuclei of these cells. Nuclear import was also observed for RR-$S4_{13}$ in digitonin-permeabilized HeLa cells but did not require cytosolic extract (*30*). Active and specific nuclear import is evident from the results, showing that in intact cells, as well as in permeabilized cells, nuclear import was ATP dependent and inhibited by excess unlabeled RR-$S4_{13}$ as well as by other ARM peptides such as those bearing the Rev and Tat ARM (Tables 4 and 5). The inhibition observed by the externally added ARM peptides clearly indicates that these peptides are cell permeable. This indeed can be inferred from results in Figure 3, which demonstrate that the Rev ARM, similar to what has been demonstrated for the Tat ARM peptide (*11*), is cell permeable (see also ref *12*). Excess unlabeled RR-$S4_{13}$ also blocked nuclear import of the Rev ARM−BSA conjugate into the nuclei of permeabilized cells as well as of RR-$S4_{13}$ in intact cells. Such inhibition was not observed for an excess of SV40-T-antigen NLS (Tables 4 and 5).

Determination of cell viability (data not shown) revealed that also the ARM-derived peptides Tat, Rev, and RR-$S4_{13}$ were not toxic to the cells at concentrations up to 15 μM.

The results in Figure 4 show that, following microinjection into intact cells, the peptides $S4_{13}$ were retained in the cytoplasm while RR-$S4_{13}$ accumulated within the intranuclear space of these cells, again proving their nonkaryophilic and karyophilic properties, respectively. Thus in three different

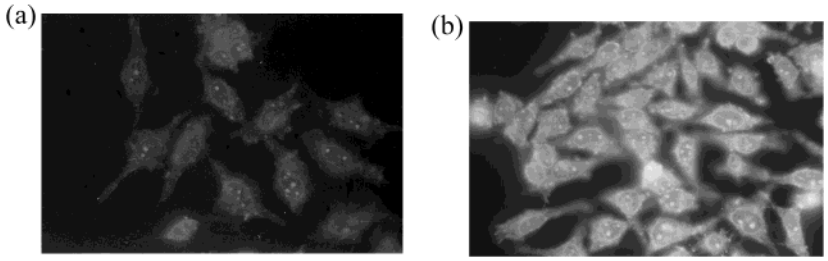

FIGURE 3: Cell penetration of the RR-$S4_{13}$ and the Rev-ARM peptides. Experimental conditions of peptide synthesis, labeling, and incubation with cultured intact HeLa cells were as described in Materials and Methods. Cells were incubated for 30 min with (a) 10 μM Rev-ARM at 37 °C or with (b) 2 μM RR-$S4_{13}$.

Table 4: Cell Penetration and Nuclear Import of the ARM Synthetic Peptides[a]

peptides	incubation temp (°C)	penetration into cytoplasm	penetration into nuclei
(i) Tat ARM[b]	37	+	+
(ii) Rev ARM[c]	37	+	+
(iii) RR-$S4_{13}$[d]	37	+	+
(iv) Tat ARM	4	+	+
(v) Rev ARM	4	+	+
(vi) RR-$S4_{13}$	4	+	−
(vii) as in (vi) + unlabeled RR-$S4_{13}$	37	+	−
(viii) as in (vi) + unlabeled Rev ARM	37	+	−
(ix) as in (vi) + unlabeled Tat ARM	37	+	−

[a] Synthetic peptides bearing the Tat and the Rev ARM sequences were synthesized and fluorescently labeled as described in Materials and Methods. Peptides were added in a molar ratio of 1:400 RR-$S4_{13}$ unlabeled peptide. [b] 1 μM. [c] 10 μM. [d] 2 μM.

Table 5: Nuclear Import of RR-$S4_{13}$ into the Nuclei of Permeabilized HeLa Cells[a]

exptl conditions	nuclear uptake[b]
(i) in the absence of cytosolic factors	+
(ii) in the presence of cytosolic factors	+/−
(iii) ATP-depleted cells	−
(iv) incubation at 4 °C	−
(v) in the presence of unlabeled RR-$S4_{13}$	+/−
(vi) in the presence of unlabeled Rev ARM	−
(vii) in the presence of unlabeled Tat ARM	+/−
(viii) in the presence of unlabeled SV40-T-antigen NLS	+
(ix) LR-BSA-Rev + unlabeled RR-$S4_{13}$	−

[a] The unlabeled peptides (RR-$S4_{13}$, SV40-T-antigen NLS, Rev and Tat ARM) were added at a peptide ratio of 1:100 (mol/mol). [b] (+) Most of the nuclei in the microscopic fields are highly fluorescent; (−) no fluorescence in the nuclei; (+/−) most of the nuclei are very weakly fluorescent.

systems, those of intact, permeabilized, and microinjected cells, the $S4_{13}$ peptide exhibited cytoplasmic retention/nuclear exclusion, which could overcome by the addition of a functional NLS.

DISCUSSION

The results of the present work demonstrate that nontoxic concentrations of the antimicrobial dermaseptin S4 as well as its derivative $S4_{13}$ readily penetrate into intact HeLa cells and are retained within the cytoplasm of these cells. This is based mainly on our microscopic studies showing that, following incubation with HeLa cells, fluorescently labeled S4 and S4 derivative peptides readily accumulate within the cytoplasm of these cells. Kinetic studies revealed that penetration was fast, and most of the cells in the field were labeled within 5 min of incubation at either 37 or 4 °C. Peptide penetration into the cultured cells was not blocked by the addition of excess unlabeled peptides, low temperature, or ATP depletion. All these results strongly indicate that penetration of the dermaseptin peptides was a receptor-independent process and probably did not occur via the endocytic pathway. On the basis of these results we suggest that the peptides described in the present work should be considered as novel CPPs. Being relatively small (13−28 amino acid residues), the S4 and $S4_{13}$ dermaspetin-derived peptides would be expected to passively diffuse into the intranuclear space following their translocation into the cell cytosol (*31*). However, both peptides appear to be nonkaryophilic on the basis of our microscopic observations with intact as well as with digitonin-permeabilized HeLa cells. The failure of these peptides to diffuse into the cells' nuclei is not as yet fully understood but may relate to their tendency to undergo self-aggregation which may lead to the formation of high molecular weight aggregates that may be too large to enter the nucleus by passive diffusion.

Attachment of NLS sequences to the peptide $S4_{13}$ converted it into a karyophilic CPP, a peptide that accumulated within the nuclei of the recipient cells while retaining its cell penetration properties. The SV40-T-antigen NLS, whose nuclear import is known to be mediated by the importin α-β heterodimer of the cellular nuclear import machinery, was covalently attached to the $S4_{13}$ peptide, resulting in obtaining the PV-$S4_{13}$ and the $S4_{13}$-PV peptides. Experiments using the digitonin-permeabilized cell system clearly demonstrated that the nuclear import of both peptides is subjected to the same features that characterize active nuclear import. Nuclear import of the PV-$S4_{13}$ was absolutely dependent on the addition of reticulocyte extract (providing the importins and other cytosolic components such as guanine nucleotide binding protein Ran) and was inhibited by GTPγS. No nuclear uptake was observed when rPV-$S4_{13}$, the peptide bearing the SV40-T-antigen NLS in a reverse order, was used. Interestingly, the covalent attachment of the SV40-T-antigen NLS, in its correct or reverse order, to $S4_{13}$ had no effect on its cell permeability properties, and all three peptides, PV-$S4_{13}$, $S4_{13}$-PV, and rPV-$S4_{13}$ in a fashion similar to that of $S4_{13}$, readily penetrated into HeLa cells. Thus, the CPP $S4_{13}$ may be used as an efficient carrier for the delivery of short peptides and possibly other small molecules (see results for LR) into intact cultured cells. In the absence of an NLS, it may deliver its cargo to the cell cytoplasm while with the NLS it should carry it into the cell nucleus. The advantage of using $S4_{13}$ as a carrier to deliver molecules into

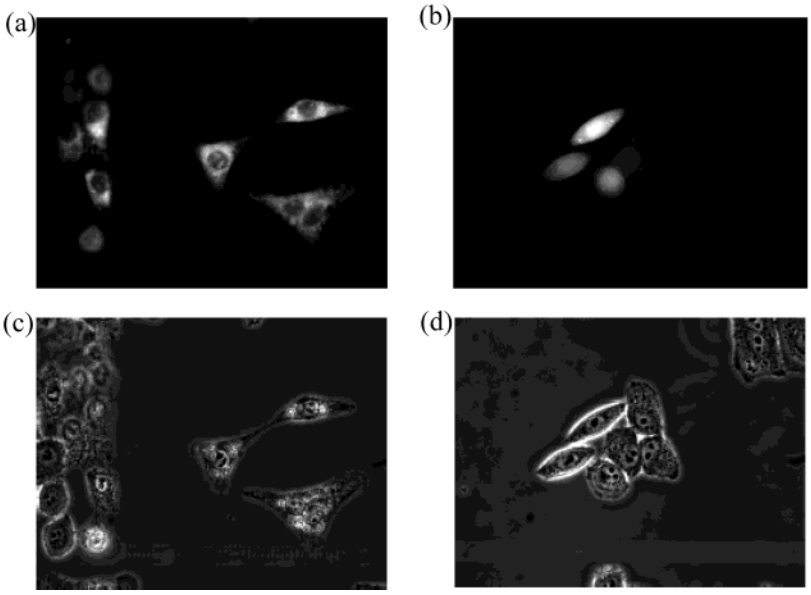

FIGURE 4: Nuclear import of $S4_{13}$ and RR-$S4_{13}$ in microinjected cells. Microinjection of the $S4_{13}$ and the RR-$S4_{13}$. HeLa cells were microinjected with the fluorescently labeled peptides $S4_{13}$ and RR-$S4_{13}$ (1 μM). The injection mixture also included fluorescently labeled BSA as an injection control (data not shown). The pictures were taken after 2 h of incubation at 37 °C. RR-$S4_{13}$ accumulated in the cells' nuclei (b and d) as opposed to $S4_{13}$ which remained in the cytoplasm (a and c). Phase contrast images of injected cells are shown in (c) and (d).

living cells over other CPPs such as the HIV-1 Tat NLS (*11*, *12*) is that $S4_{13}$ is an "inert peptide" lacking any known intracellular function.

Similar to the SV40-T-antigen NLS, the Rev ARM also conferred peptide karyophilic properties upon the $S4_{13}$ without affecting its cell penetration abilities. Thus the Rev ARM remained biologically active following its attachment to $S4_{13}$. The fact that the Rev ARM peptides were biologically active within the RR-$S4_{13}$ can also be inferred from our experiments using the in vitro nuclear assay system.

Import of RR-$S4_{13}$ into the nuclei of the permeabilized HeLa cells did not require the addition of exogenous cytosolic factors as opposed to nuclear import mediated by the SV40-T-antigen NLS (*32*). Similar results, namely, nuclear import in the absence of cytosolic factors and its inhibition by the reticulocyte extract, have been observed by us before for nuclear import mediated by the Tat ARM (*32*, *33*). Our recent experiments (manuscript in preparation) indicate that such inhibition appears to be due to nonspecific electrostatic interactions between the positively charged Rev ARM and negatively charged molecules present in the cytosolic extracts. However, inhibition observed in the presence of GTPγS and the free Rev peptide of the Rev ARM-mediated nuclear import implies the involvement of Ran and indicates that transport is saturable, consistent with the role of a specific cellular receptor, presumably importin β (*34*).

On the basis of our in vitro nuclear experiments with RR-$S4_{13}$, we assume that also its nuclear import in intact cells is through an active process. Our results showing that nuclear import of RR-$S4_{13}$ in intact cells could be inhibited by externally added RR-$S4_{13}$, Rev, and Tat ARM peptides demonstrate that the nuclear import of $S4_{13}$ NLS composite peptides in intact cells could be used as a system to screen for cell-permeable nuclear import inhibitors.

Current experiments in our laboratory are focused on determining whether the CPP dermaseptin can serve as a delivery system for macromolecules such as proteins or nucleic acids, which could have important application in drug delivery. Indeed, recently (*35*), it has been demonstrated that derivatives of the dermaseptin S4 are still active and preserve their antibacterial properties in vivo, namely, following administration into infected mice.

ACKNOWLEDGMENT

We thank Stefan Schlicht for microinjection assays and Markus Neumann for valuable discussions. Special thanks are due to Mrs. Josefine Silfen from the Interdepartmental Core Laboratories of the Life Science Institute for linear peptide synthesis and amino acid analysis.

REFERENCES

1. Rojas, M., Donahue, J. P., Tan, Z., and Lin, Y. Z. (1998) *Nat. Biotechnol. 16*, 370−375.
2. Prochiantz, A. (1996) *Curr. Opin. Neurobiol. 6*, 629−634.
3. Capecchi, M. R. (1980) *Cell 22*, 479−488.
4. Neumann, E. (1982) *EMBO J. 7*, 841−845.
5. Felgner, P. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7413−7417.
6. Cepko, C. L. (1984) *Cell 37*, 1053−1062.
7. Lindgren, M., Hallbrink, M., Prochiantz, A., and Langel, U. (2000) *Trends Pharmacol. Sci. 21*, 99−103.
8. Higashhijima, T., Burnier, J., and Ross, E. M. (1990) *J. Biol. Chem. 265*, 14176−14186.
9. Pooga, M., Hallbrink, M., Zorko, M., and Langel, U. (1998) *FASEB J. 12*, 67−77.
10. Vives, E., Charneau, P., VanRietschoten, J., Rochart, H., and Bahraoui, E. (1994) *J. Virol. 68*, 3343−3353.
11. Vives, E., Brodin, P., and Lableu, B. (1997) *J. Biol. Chem. 272*, 16010−16017.
12. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) *J. Biol. Chem. 276*, 5836−5840.
13. Morris, M. C., Deppollier, J., Mery, J., Heitz, F., and Divita, G. (2001) *Nat. Biotechnol. 19*, 1173−1176.
14. Nicolas, P., and Mor, A. (1995) *Annu. Rev. Microbial. 4*, 277−304.
15. Mor, A., Nguyen, V. H., Delfour, A., Migloire, D., and Nicolas, P. (1991) *Biochemistry 30*, 8824−8830.

16. Pouny, Y., Rapaport, D., Mor, A., Nicolas, P., and Shai, Y. (1992) *Biochemistry 31*, 12416−12423.
17. Feder, R., Nehushtai, R., and Mor, A. (2001) *Peptides 22*, 1683−1690.
18. Mor, A., Amiche, M., and Nicolas, P. (1994) *Biochemistry 33*, 6642−6650.
19. Mor, A., and Nicolas, P. (1994) *J. Biol. Chem. 269*, 1934−1939.
20. Mor, A., Hani, K., and Nicolas, P. (1994) *J. Biol. Chem. 269*, 16.
21. Feder, R., Dagan, A., and Mor, A. (2000) *J. Biol. Chem. 275*, 4230−4238.
22. Gorlich, D., and Mattaj, I. W. (1996) *Science 271*, 1513−1518.
23. Gorlich, D. (1998) *EMBO J. 17*, 2721−2727.
24. Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989) *Nature 338*, 254−257.
25. Friedler, A., Zakai, N., Karni, O., Broder, Y. C., Baraz, L., Kotler, M., Loyter, A., and Gilon, C. (1998) *Biochemistry 37*, 5616−5622.
26. Neumann, M., Afonina, E., Ceccherini-Silberstein, F., Schlicht, S., Erfle, V., Pavlakis, G., and Brack-Werner, R. (2001) *J. Cell Sci. 114*, 1717−1729.
27. Graessmann, M., and Graessmann, A. (1983) *Methods Enzymol. 101*, 482−492.
28. Gorlich, D. (1997) *Curr. Opin. Cell Biol. 9*, 412−419.
29. Broder, Y. C., Stanhill, A., Zakai, N., Friedler, A., Gilon, C., and Loyter, A. (1997) *FEBS Lett. 412*, 535−539.
30. Friedler, A., Zakai, N., Karni, O., Friedler, D., Gilon, C., and Loyter, A. (1999) *J. Mol. Biol. 289*, 431−437.
31. Feldherr, C. M., and Akin, D. (1994) *Int. Rev. Cytol. 151*, 183−228.
32. Friedler, A., Luedtke, N., Friedler, D., Loyter, A., Tor, Y., and Gilon, C. (1999) in *16th American Peptide Symposium* (Fields, G. B., and G. B., Eds.) Kluwer Academic Publishers, Minneapolis, MN.
33. Efthymiadis, A., Briggs, L. J., and Jans, D. A. (1998) *J. Biol. Chem. 273*, 1623−1628.
34. Adam, S. A., and Gerace, L. (1991) *Cell 66*, 837−847.
35. Navon Venezia, S., Feder, R., Gaidukov, L., Carmeli, Y., and Mor, A. (2002) *Antimicrob. Agents Chemother. 46*, 689−694.

BI0201466