Functional Analysis of Backbone Cyclic Peptides Bearing the Arm Domain of the HIV-1 Rev Protein: Characterization of the Karyophilic Properties and Inhibition of Rev-Induced Gene Expression†

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ABSTRACT: This work describes the synthesis and activity of a novel backbone cyclic (BC) peptide library based on the sequence of the HIV-1 Rev arginine-rich motif (ARM). All the peptides in the library possess the same sequence but differ in their ring-moiety properties. The BC peptides were synthesized using simultaneous multiple-peptide synthesis and were fully assembled using bis(trichloromethyl)carbonate as a coupling agent. All the peptides in the library had inhibitory effects on the binding of Rev-GFP to importin β in vitro. Studies performed with one of the BC Rev-ARM analogues, Rev-13, demonstrated that, like its parental linear peptide, it is karyophilic; i.e., it is able to mediate the nuclear import of conjugated bovine serum albumin (BSA) molecules. The cell penetrating properties of the BC peptides were assessed utilizing an ELISA-based system. This assay provides a quantitative evaluation of cell penetration. Most of the peptides from the library were able to penetrate intact Colo-205 cells to varying degrees. Furthermore, these BC peptides were able to carry BSA into intact Colo-205 cells. In addition to its cell penetrating and binding properties, the BC Rev-13 analogue inhibited Rev-induced gene expression in HeLa cells by 60−70% in the low micromolar range and exhibited no cell toxicity. The potential of BC peptides bearing ARM domains as lead compounds for the production of anti-HIV drugs is discussed.

Small molecules, such as peptides, which interact with specific domains within target proteins and thus modify their structure and biological function have been instrumental in elucidating numerous molecular and biochemical processes. Protein−protein interactions are also involved in host−pathogen interactions, such as viral replication and infection. Specific domains within viral proteins are responsible for the interaction with host-cell receptors and with other viral and cellular proteins, allowing the completion of the viral life cycle within the host cell (*1*−*3*). Thus, antagonists that interfere with virus−host or virus−viral protein interactions represent invaluable molecular tools for revealing and characterizing the biological functions of viral proteins, as well as for use as therapeutic agents. Although many small molecules and peptides that can modulate such interactions have been discovered, the design of these agents, based solely on specific knowledge of a target protein−protein interaction or by random screening, remains very difficult. The peptidomimetic approach utilizes peptides derived from the interacting target proteins and modifies them to improve binding affinity and physicochemical properties (*4*−*7*). This approach continues to be very attractive, as it has resulted in the identification of inhibitory peptides for use as research tools and drugs. However, due to their tendency to equilibrate between multiple conformations, adoption of the bioactive conformation by linear peptides comes at an entropic cost (*7*). Hence, conformationally constrained peptides such as backbone cyclic (BC)[1] peptides are more suitable than their linear counterparts for use in the peptidomimetic approach (*7*). Backbone cyclization also results in peptides with

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[1] Abbreviations: BC, backbone cyclic; NLS, nuclear localization signal; HIV, human immunodeficiency virus; PIC, preintegration complex; MA, matrix protein; ARM, arginine-rich motif; TAR, transactivation response; RRE, Rev response element; CPP, cell-penetrating peptide; TB, transport buffer; BSA, bovine serum albumin; Fmoc, 9-fluorenylmethoxycarbonyl; NMP, *N*-methylpyrrolidinone; BTC, bis(trichloromethyl)carbonate; Rho-BSA, lissamine rhodamine-labeled bovine serum albumin; Bb, biotinylated BSA; BU, building unit.

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improved metabolic stability, as reflected in their relative resistance to proteolytic digestion (*8*). In a previous work, we showed that BC peptides bearing the nuclear localization signals (NLSs) of the HIV-1 matrix (MA) and Tat proteins block nuclear import mediated by the NLSs of these karyophilic viral proteins (*8*, *9*).

Human immunodeficiency virus (HIV) is able to infect nondividing cells productively because the viral nucleoprotein complexes, namely, its preintegration complex (PIC), is actively imported into the nucleus (*3*, *10*). The MA protein, which has been reported to be karyophilic and bears a specific NLS domain, is associated with the HIV-1 PIC (*11*, *12*). Although it has been suggested that the MA protein is essential for promoting nuclear import of the viral PIC, its role is still controversial (*3*, *13*−*15*). Virus replication depends on integration of the viral DNA into the host cell genome. Expression of the viral genes is induced by two HIV regulatory factors, Tat and Rev, both of which are actively imported into the host-cell nucleus (*3*, *16*−*18*). Nuclear import of these HIV regulatory proteins is mediated by specific NLSs which are characterized by clusters of arginine residues and therefore have been designated arginine-rich motifs (ARMs) (*19*). Within the nucleus, Tat and Rev bind to HIV transcripts. Tat interacts with the transactivation response (TAR) element in the 5′ terminus of all HIV transcripts (*18*, *20*, *21*). Rev binds the Rev response element (RRE) within the unspliced and partially spliced HIV mRNA and promotes export of the RRE-containing mRNAs from the nucleus to the cytoplasm (*22*−*25*). Tat protein is also secreted from HIV-1-producing cells (*26*). Extracellular Tat can then be taken up by different types of uninfected cells, accumulate within their nuclei, transactivate cellular genes, and induce various biological activities (*27*, *28*). Binding of Tat and Rev to their specific target RNA domains, as well as translocation of Tat via the cells' plasma membranes, is ascribed to the same domain that mediates their nuclear import, namely, the ARM (*29*−*31*). The triple biological function of peptides bearing this domain makes them a very attractive experimental tool for studying protein−protein interactions involved in the biological activities of Tat and Rev gene regulation, as well as for developing anti-HIV-1 lead compounds and cell delivery systems (*29*, *32*, *33*).

The ability of peptides bearing the ARM sequence to directly cross culture cells' plasma membranes has been extensively studied in the past few years (*29*, *34*). Indeed, it has been shown that linear peptides bearing the ARM domains of Tat, Rev, or even a stretch of basic amino acids such as arginine-rich oligopeptides are able to penetrate biological membranes (*27*, *35*). Although their mode of action is still unknown and remains controversial (*36*), they have been defined as cell-penetrating peptides (CPPs) (*35*). Recently, CPPs have become an attractive and useful tool for the delivery of various cargos, such as drugs, genes, and proteins, into cells. The use of CPPs for the intracellular delivery of proteins may lead to the development of novel ways of controlling cell function and to elucidation of new cellular therapeutic targets (*37*). Although we have previously studied BC peptides derived from various domains, including the ARM (*8*, *9*, *38*), their cellular penetration abilities have never been investigated.

In this work, we demonstrate that a BC peptide selected from a BC library and bearing the ARM of the HIV-1 Rev protein is biologically active; i.e., it is able to mediate nuclear import and binding to importin β. Moreover, the BC ARM peptides were found to be cell-permeable and mediated the translocation of BSA molecules into cultured mammalian cells; hence, they are characterized by the same features that typify CPPs. A cell-permeable BC ARM peptide also successfully blocked Rev-induced gene expression at nontoxic concentrations.

MATERIALS AND METHODS

Cultured Cells. HeLa cells were grown in a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 0.3 g/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Beit Haemek). Colo-205 cells [human Colo-205 adenocarcinoma cells (ATCC CCL 222)] were maintained in RPMI 1640 medium, supplemented with 10% FCS, 0.3 g/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Chemicals. Protected amino acids, Rink amide methyl benzhydril amine resin (Rink amide MBHA resin), and coupling reagents were purchased from Novabiochem (San Diego, CA). Other chemicals were purchased from Sigma (St. Louis, MO) or Merck (Whitehouse Station, NJ). Solvents for peptide synthesis were purchased from Baker (Phillipsburg, NJ).

Buffers. Transport buffer (TB) consisted of 20 mM Hepes (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 0.1 mM PMSF. Phosphate-buffered saline (PBS) consisted of 140 mM sodium chloride buffered with 20 mM sodium phosphate (pH 7.3).

Synthesis of the BC Rev-ARM Library. The Rev-ARM library (Figure 1) was synthesized on Rink amide MBHA resin (loading 0.6 mmol/g). The resin (250 mg) was sealed in semipermanent polypropylene bags. The bags were preswollen in NMP (*N*-methylpyrrolidone for 2 h). The Fmoc (9-fluorenylmethoxycarbonyl) protective group was removed using 20% piperidine in NMP (2 × 30 min), and the resin was washed with NMP (5 × 2 min) and DCM (dichloromethane) (2 × 2 min). Reaction completion was monitored by a qualitative chloranil test. Coupling of Fmoc amino acids was performed using bis(trichloromethyl)carbonate (BTC) (*39*). A mixture of BTC (1.65 equiv) and Fmoc-Thr(OtBu)-OH (5 equiv) was dissolved in DCM. After the mixture had been cooled in an ice bath, 2,4,6-collidine (14 equiv) was introduced and the solution was added to the peptidyl resin. The reaction mixture was shaken for 1 h, and the peptidyl resin was washed with DCM (5 × 2 min). Reaction completion was monitored with the chloranil test. Synthesis of the building unit (functionalized *N*-alkyl Gly), cyclization, removal of the t-Boc (*tert*-butyloxycarbonyl protecting group), and peptide cleavage and purification were performed as described previously (*8*). The full synthetic procedure using BTC will be published elsewhere.

Expression and Purification of Importin β- and Rev-GFP. The pET28-hIMPb1 expression vector was obtained from V. Citovsky (State University of New York, Stony Brook, NY) and was expressed in *Escherichia coli* strain BL21-

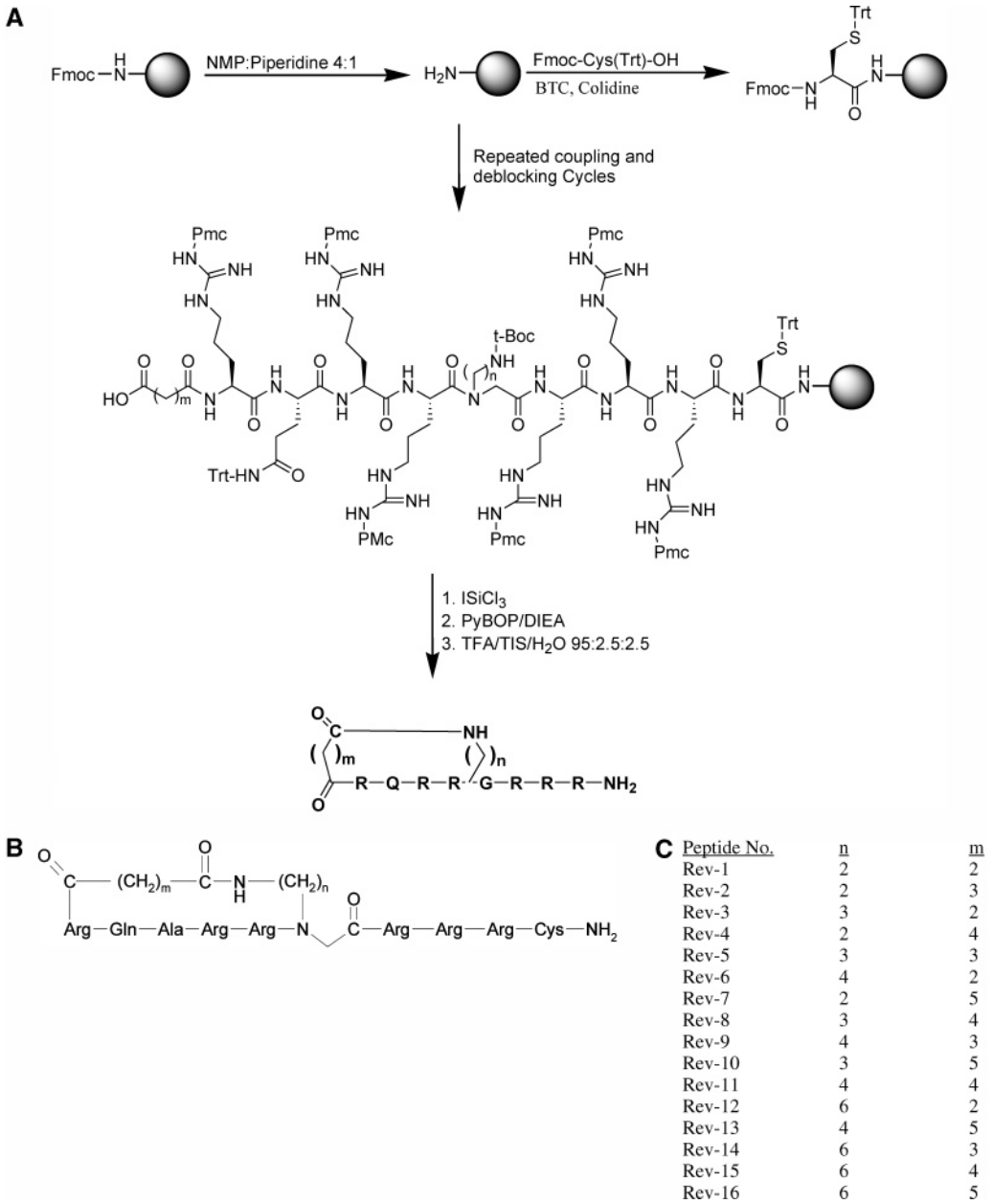

FIGURE 1: Structure of the BC Rev-ARM library. (A) A scheme describing the steps involved in the synthesis of the ARM mimetic library. (B) A general structure of the cyclic peptide library. (C) Detailed description of the peptides in the library. *n* and *m* depict the lengths of the alkyl chains. For experimental details, see Materials and Methods.

(DE3). Histidine-tagged (His-Tag; Qiagen, Valencia, CA) fusion proteins were expressed and purified by standard protocols following the growth at 37 °C and induction at 25 °C of the *E. coli* strains.

Preparation of Transport Substrates. Peptides used in this work were covalently attached to either lissamine rhodamine-labeled bovine serum albumin (Rho-BSA) or biotinylated BSA (Bb) (Bio-BSA, Sigma) molecules using sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) as the cross-linker to give Rho-BSA−peptide and Bb−peptide conjugates, respectively, as described previously (*40*).

Peptides were biotinylated as follows. The various peptides (10 mM in PBS) were introduced into a solution of biotin maleimide (18 mg/mL, Sigma) in DMF (*N*,*N*-dimethylformamide) The mixture was allowed to stir for 4 h at room temperature, and the biotinylated peptides were then lyophilized and analyzed by high-pressure liquid chromatography−mass spectrometry (HPLC−MS).

Quantitative Analysis of Nuclear Import. Nuclear import was quantitatively determined with an ELISA-based system using Bb−peptide conjugates as transport substrates, essentially as described previously (*41*). Briefly, the Bb−Rev-13 conjugate was used as the transport substrate. A suspension of Colo-205 cells was permeabilized with digitonin, and transport was initiated by the addition of substrate (Bb-ARM); then the reaction mixture was transferred to 30 °C for 30 min. All other experimental conditions were as described previously (*41*). Reported results are an average of triplicate ELISA determinations, the standard deviation of which never exceeded ±20%.

Binding of Rev-GFP to Importin β. Maxisorp plates (96 wells, Nunc Inc., Rochester, NY) were coated by overnight incubation at 4 °C with 200 μL, per well, of a solution

containing recombinant importin β (25 μg/mL) in carbonate buffer ($NaHCO_3/Na_2CO_3$ buffer at pH 9.6) as described previously (*42*). Following removal of the solution, the plates were washed three times with PBS and blocked by incubation with 200 μL, per well, of PBS containing BSA (5%, w/v) for 2 h at 37 °C. Following three washes with PBS, 200 μL, per well, of a solution containing biotinylated Rev-GFP or Bb-Rev-ARM conjugate (12.5 ng) was added, with or without the various BC Rev-ARM peptides (Rev-GFP/Bb-Rev-ARM:BC Rev-ARM peptides, 1:10 molar ratio), in PBS and 5% BSA. Following incubation for 1 h at 37 °C and three washes with PBS, the amount of surface-bound biotinylated Rev-GFP was estimated following the use of the streptavidin−horseradish peroxidase (HRP) conjugate as described previously (*43*).The enzymatic activity of the HRP was estimated by monitoring the optical density (OD) of the product obtained at 490 nm using an ELISA plate reader (Tecan Sunrise, Durham, NC). Reported results are an average of triplicate ELISA determinations, with standard deviations never exceeding ±20%.

Penetration of Rho-BSA−Rev Peptide Conjugates into Cultured HeLa Cells: Microscopic Observations. Penetration was followed microscopically essentially as described previously (*44*). HeLa cells (3×10^4 cells/well) were cultured on 10 mm coverslips to subconfluent density. Following the removal of the culture medium, the cells were washed three times with TB and then exposed to various conditions in the presence of the Rho-BSA−Rev-13 conjugate at 37 °C in a final volume of 50 mL of TB. At the end of the incubation period (1 h), the cells were washed three times with TB, and in a few experiments, they were observed directly by fluorescence microscopy without fixation. For most of the experiments, however, the cells were fixed in 4% (v/v) formaldehyde dissolved in TB. Fixed and nonfixed cells were examined with a fluorescence microscope (Zeiss, Thornwood, NY) with a 40× objective.

Estimation of the Amount of BC ARM Peptides that Penetrated into the Cytosol and Nuclei of Intact Colo-205 Cells. For quantitative estimation of the intracellular distribution of peptides, the peptides were conjugated to biotin maleimide or covalently attached to biotinylated BSA (Bb) as described previously (*44*). Labeled BC−Rev-ARM peptides were added to a suspension of Colo-205 cells, and the extent of intracellular accumulation was estimated exactly as described previously for the cellular accumulation of histones (*44*). For an estimation of amounts of biotinylated peptides contained within the nuclear and cytosolic compartments, lysates of both compartments were obtained in two steps by using two different types of detergent, digitonin and Triton, as described previously (*44*). On the basis of previously reported results, demonstrating tight binding of polycationic peptides and basic proteins to glass or plastic surfaces (*45*), the various lysate fractions that were obtained were incubated with noncoated plates for attachment of BC Rev-ARM peptides. All the subsequent steps were performed exactly as described previously (*44*). The amount of biotinylated BC Rev-ARM peptide was then estimated by the enzymatic reaction of the streptavidin−HRP conjugate as described previously (*44*).

Rev-Induced Expression of Reporter Protein. ROD (red fluorescence on demand) reporter cells were generated by stable integration of the plasmid pLRed(2xINS)R (*46*, *47*) in HeLa-Tat cells that constitutively produce the HIV-1 Tat protein (*48*). A clonal population of cells was selected that exhibited red fluorescence in the presence of Rev and no background fluorescence in the absence of Rev.

ROD reporter cells (5×10^4) were seeded in six-well plates in 1 mL of DMEM with 10% FCS (Biochrom AG, Berlin, Germany) and 1% Pen/Strep (Invitrogen GmbH, Karlsruhe, Germany) and cultured for 24 h. Cells were incubated with the various BC peptides at 4−12 μM for 24 h, after which cells were washed with PBS. Subsequently, the cells were transfected with 100 ng of the Rev-GFP expression plasmid pCsRevsg143 (*49*), using FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Twenty-four hours after transfection, the cells were trypsinized and analyzed by flow cytometry. The percentage of reporter-positive cells in the transfected population was determined and the activity of the Rev-only sample without peptide set to 100% as previously described (*47*).

Evaluation of the Cytotoxic Potential of Peptides. The cytotoxic potential of BC peptides was investigated with a commercially available assay based on luminescent quantitation of intracellular ATP (CellTiter Glo Luminescent Cell viability assay, Promega, Mannheim, Germany). ROD reporter cells (5×10^3) were plated in each well of a 96-well plate and treated with BC peptides in the same manner as in experiments evaluating the influence on Rev-dependent reporter expression. Viabilities of cells incubated without peptide or with a detergent (0.1% Tween 20) were analyzed as positive or negative controls, respectively.

RESULTS

Design and Synthesis of the BC Rev-ARM Peptide Library. On the basis of the NMR structure of the Rev-ARM−RRE complex (*50*) and our previous work describing a BC peptide library bearing the Tat-ARM peptide (*9*), a BC library of Rev-ARM was designed and synthesized. As revealed by the structure of the Rev−RRE complex, the ARM sequence mediates binding of the Rev protein to the RRE domain within the viral RNA (*51*). In the BC library, the Arg residues remained unchanged while Asn 40 was replaced with Gly building units (BUs). Cyclization was performed by covalent attachment of the backbone amide nitrogen of the Gly BU to the N-terminus of the Rev-ARM peptide using dicarboxylic acid, resulting in "backbone-to-end" cyclization (see Figure 1 and ref *7*). In addition, a Cys residue was added to the C-terminus to allow chemical conjugation to BSA molecules. The library, which contained a total of 16 peptides (Figure 1), was prepared by the simultaneous multiple-peptide synthesis (SMPS) "tea bags" method (*7*) using BTC as the coupling agent. It should be mentioned that BTC has proven to be one of the most efficient coupling agents for the synthesis of single peptides (*39*).

The BC Rev-ARM Peptides Block Binding of Rev-GFP to Importin β and Mediate Nuclear Import. Screening of the BC Rev-ARM peptide library revealed that all the peptides inhibit in vitro binding of Rev-GFP or Bb−Rev-ARM conjugates to importin β by ~80−90% (Figure 2A,B). Inhibition could be due to competitive binding of the BC Rev-ARM peptides to importin β. To directly investigate the karyophilic properties of the BC Rev-ARM peptides, the

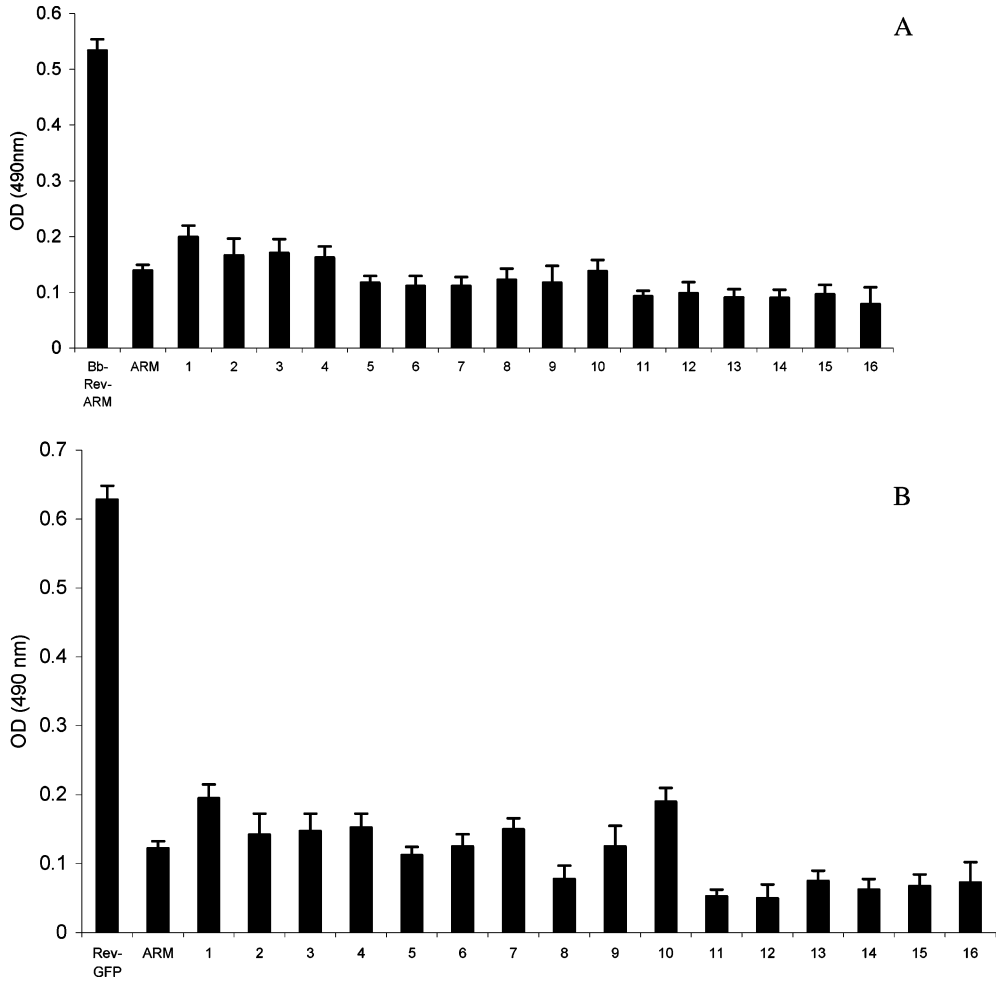

FIGURE 2: Inhibition of binding of Rev-GFP and the Bb−Rev-ARM conjugate to importin β by BC Rev-ARM peptides. The Bb−Rev-ARM conjugate (A) or purified recombinant biotinylated Rev-GFP (B) was added to importin β-coated plates as described in ref *41* and Materials and Methods. The binding was tested with and without the addition of BC Rev-ARM peptides. Numbers indicate the numbers of the peptides in the library (see Figure 1).

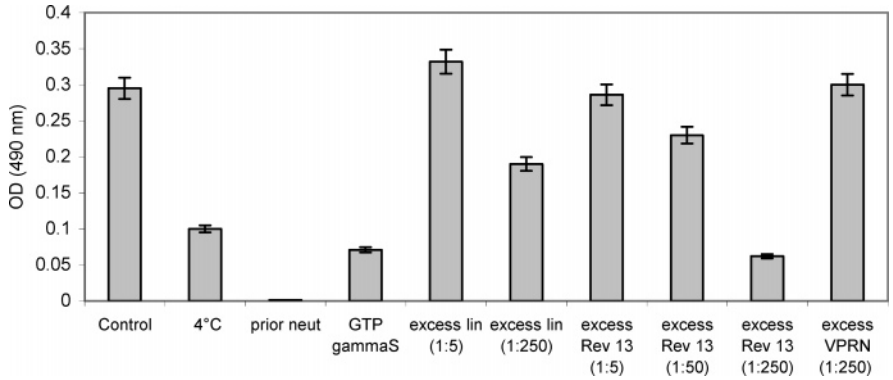

FIGURE 3: Nuclear import of the Bb−Rev-13 conjugate into permeabilized cells. Characterization and inhibition by Rev-ARM peptides. The extent of nuclear import was estimated by a quantitative ELISA-based system (*44*). For "prior neut" (prior neutralization), biotinylated molecules were neutralized with avidin and biocytin before being added to the Colo-205 cells. For "excess lin (1:5)" and "excess lin (1:250)", the process was like the 37 °C control but in the presence of excess unlabeled linear Rev-ARM peptide ($\times$5 and $\times$250 mol/mol, respectively). For "excess Rev 13 (1:5)", "(1:50)", and "(1:250)", the process was like the 37 °C control but in the presence of excess unlabeled Rev-13 ($\times$5, $\times$50, and $\times$250 mol/mol, respectively). For "excess VPRN (1:250)" (peptide bearing the NLS of the HIV-1 Vpr; see ref *52*), the process was like the 37 °C control but in the presence of excess unlabeled linear VPRN peptide ($\times$250 mol/mol).

ability of one analogue, Rev-13, to mediate nuclear import was studied (Figure 3), using peptide−BSA conjugates and a quantitative assay system involving permeabilized Colo-205 cells (Figure 3 and ref *40*). Active nuclear import of BC Rev-13−BSA conjugates was evident from the results showing that it does not occur at 4 °C, and that it is inhibited by GTPγS (see Figure 3) and wheat germ agglutinin (not shown), as well as by free unlabeled Rev-ARM peptides (both linear and cyclic). Furthermore, inhibition was not observed by a peptide bearing an NLS derived from the Vpr protein whose nuclear import is not mediated by importin β (Figure 3 and ref *52*), clearly indicating a specific effect.

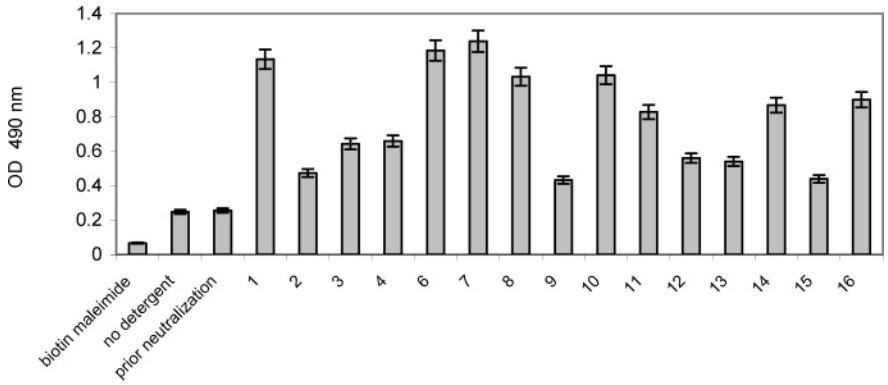

FIGURE 4: Quantitative estimation of the cellular accumulation of biotinylated BC Rev peptides in intact cultured Colo-205 cells. The biotinylated BC peptides (2 mg/mL) as well as biotin maleimide (4 mg/mL) were incubated with Colo-205 cells at 37 °C for 1 h, and then the level of cellular accumulation of the biotinylated peptides was estimated. For "prior neutralization", biotinylated molecules were neutralized with avidin and biocytin before being added to the Colo-205 cells. For "no detergent", biotinylated Rev-13 (2 mg/mL) was incubated with Colo-205 cells, and following neutralization of surface-bound biotin, the amount of remaining biotin was estimated on nonlysed cells (cells were not treated with detergent). Numbers indicate number of peptide in the library.

Thus, it appears that nuclear import mediated by the BC Rev-13 peptide is characterized by the same features that characterize active import of karyophilic proteins.

The BC Rev-ARM Peptides Penetrate Intact Colo-205 Cultured Cells. The ability of the BC Rev-ARM peptides to penetrate cultured mammalian cells was studied using biotinylated BC peptides and a recently developed ELISA-based quantitative assay system (*44*, *53*). This assay system allowed us to compare the amounts of peptides accumulated in the cytosol to those imported into the cell nuclei (see Materials and Methods). In this assay, following the addition of biotinylated peptides to the cultured cells, any surface-bound biotin molecules are neutralized by the addition of excess avidin; after treatment of the cells with detergent, the number of intracellular biotin molecules is estimated (see Materials and Methods). Indeed, after neutralization of surface-bound biotinylated Rev, very few, if any, biotin molecules were detected (no detergent, Figure 4) until the cells were lysed with Triton. Only those biotin molecules identified after neutralization of surface-bound biotin and treatment of cells with Triton were considered to be present in the intracellular space. It is also evident from our results that most of the biotin molecules in the biotin−BC peptide conjugates are available and can be neutralized since very little biotin was detected when these conjugates were incubated with avidin before they were introduced into the cells.

It appears that all the peptides from the library were able to penetrate intact cultured Colo-205 cells (Figure 4). It is clear that the translocation process is mediated by the BC peptide and not by the biotin moiety since free biotin (maleimide) molecules failed to penetrate the Colo-205 cells, even when added at a relatively high concentration (Figure 4). The externally added BC peptides could already be detected in the cell's cytoplasm after incubation for 1 h at 37 °C with peptides BC Rev-1, BC Rev-6, and BC Rev-7 exhibiting the best ability to penetrate. These three peptides contain an $n = 2$ alkyl chain in the structure of the backbone ring, suggesting that such a structure may enhance the ability to penetrate.

BC Rev-ARM Peptides Mediate Penetration of Covalently Attached BSA Molecules into Cultured Cells. Next we studied the ability of the BC ARM peptides to mediate the penetration of covalently attached BSA molecules into cultured intact Colo-205 cells. This was performed by using BC Rev−BSA conjugates, in which the BSA molecules, and not the Rev peptides, were biotinylated. The results (Figure 5A) show that following incubation of Bb−Rev-13 conjugates with the Colo-205 cells, most of the surface-bound biotin molecules were neutralized (without detergent), reinforcing the view that only intracellular BSA molecules were being estimated (compare "prior neutralization" and "no detergent" to "control" in Figure 5A). As can be seen, the amount of biotin found in neutralized nonlysed cells never exceeded 20% of the total amount of intracellular biotinylated conjugates.

To study the mode of penetration, the Bb−Rev-13 conjugates were incubated with Colo-205 cells in the presence of endocytic inhibitors and their intracellular amount was estimated (Figure 5A). A variety of inhibitors, such as colchicine (*54*), chloroquine (*55*), cytochalasin D (*56*), and nocodazole (*57*), which are known to affect internalization via endocytosis did not cause any inhibition of Bb−Rev-13 penetration. Even incubation of cells with a mixture of 0.5 M sucrose and nystatin did not block cell penetration (*55*). The inhibitory activity of the various inhibitors was confirmed by demonstrating their ability to block cellular uptake of low-density lipoprotein (LDL) and lucifer yellow (LY) as described before by us (not shown and ref *44*). Moreover, incubation of the cells with the Bb−Rev-13 conjugate at 4 °C did not inhibit the penetration ability of the peptide conjugate (Figure 5A), indicating that the penetration process is temperature-independent. ATP depletion by treatment of the cells with NaI, DNP, and iodoacetic acid (*58*) caused an only 20% decrease in the level of intracellular conjugates. Taken together, these results indicate penetration via a nonendocytic pathway.

This view is further strengthened by the results in Figure 5B which show the cytoplasmic versus nuclear distribution of the Bb−Rev-13 conjugate. ATP depletion resulted in very little (∼20%) reduction in the number of accumulated intracellular biotin molecules, but the ratio between the intranuclear and cytoplasmic amounts was reversed (Figure 5B). Nuclear import is an ATP-driven process (*59*, *60*), and therefore, a reduced proportion of molecules in the nucleus is expected. Peptide-mediated translocation is evident from

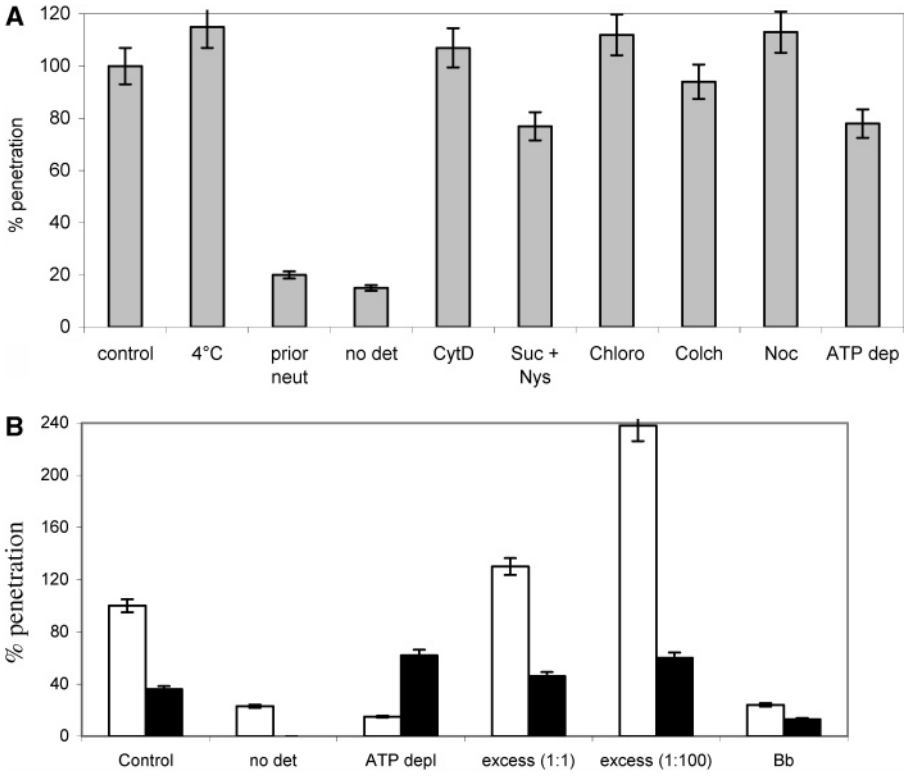

FIGURE 5: (A) Effect of endocytic inhibitors on the intracellular accumulation of the Bb−Rev-13 conjugate. The Bb−Rev-13 conjugate was incubated with Colo-205 cells at 37 or 4 °C, and after neutralization with avidin and incubation with biocytin, the cells were treated with detergents. The labels prior neut and no det are as described in the legend of Figure 4. The following endocytic inhibitors were preincubated with the cells for 30 min at 37 °C: cytochalasin D (CytD, 5 μM), sucrose (Suc, 0.5 M) with nystatin (Nys, 50 mg/mL), chloroquine (Chloro, 50 mM), colchicine (Colch, 20 μM), and nocodazole (Noc, 20 μM). For "ATP dep", cells were incubated, before the addition of Bb-Rev-7, with DNP (1 mM), NaF (2 mM), and iodoacetic acid (1 mM). (B) Nuclear and cytoplasmic accumulation of the Bb−Rev-13 conjugate. All experimental conditions of incubation with the Bb−Rev-13 conjugate are as described for panel A. Following the incubation period, nuclei and cytosolic fraction were obtained and analyzed as described in Materials and Methods: clear, nuclei; gray, cytosol. The amount of Bb−Rev-13 conjugate present in the nuclei of cells incubated at 37 °C was taken to be 100%.

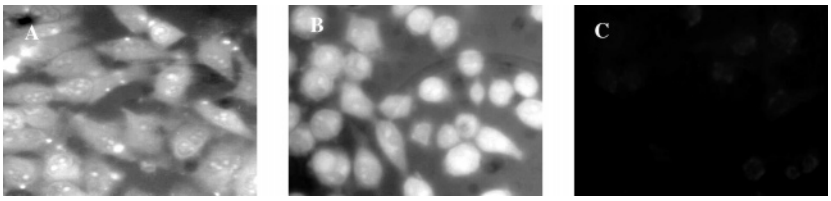

FIGURE 6: Fluorescence microscopy observations of intracellular accumulation of the Rho-BSA−Rev-13 conjugate in intact HeLa cells. (A) HeLa cells were incubated for 1 h in the presence of the Rho-BSA−Rev-13 conjugate (5 mg/mL) at 37 °C, and cells were observed with a fluorescence microscope. (B) Like panel A, but incubation performed in the presence of excess unlabeled Rev-13 (100 mol/mol). (C) Like panel A, but incubation performed with the Rho-BSA−SV40 peptide.

the results showing very little biotin detected in cells incubated with Bb alone. Surprisingly, the addition of nonlabeled Rev peptide not only did not inhibit but also actually stimulated the penetration of Bb−Rev-13 conjugates, particularly into nuclei (Figure 5B) (*44*, *53*). Fluorescence microscopic observation, using Rho-BSA−Rev-13 conjugates, further confirmed the view that BC Rev-13 mediates direct penetration of BSA molecules into cultured cells and again showed that the degree of penetration increases in the presence of free Rev peptides (Figure 6A,B). This figure shows results obtained with unfixed cells, but essentially the same pattern of cell penetration was observed with fixed cells (not shown). Labeled BSA molecules conjugated to peptides bearing the NLS of the large SV40 T antigen (PKKKRKV-NH_2) (*61*) failed to accumulate within these cells (Figure 6C), demonstrating the specific penetration activity of the Rev peptides. The results in Figure 7 show that the BC−Rev peptides were able to serve as carriers, namely, to mediate penetration of BSA molecules into cultured cells. As can be seen, in the entire library, Rev-1 and Rev-7, as was observed with the free unconjugated BC peptides (Figure 4), are the most active carriers. Free biotinylated BSA molecules failed to penetrate the Colo-205 cells, clearly indicating peptide-mediated translocation. However, as can be seen (compare Figure 4 to Figure 7), the penetration abilities of several BC−BSA conjugates, particularly Bb−Rev-6 and Bb−Rev-10 conjugates, were lower than those of the BC peptides.

BC Peptides Inhibit Rev-Induced Expression of the Reporter Protein. Following our results demonstrating the karyophilic and cell penetration properties of the BC Rev-ARM peptides, it was of interest to study whether the BC peptides, particularly Rev-13, are capable of inhibiting Rev-dependent activation of gene expression in intact cultured

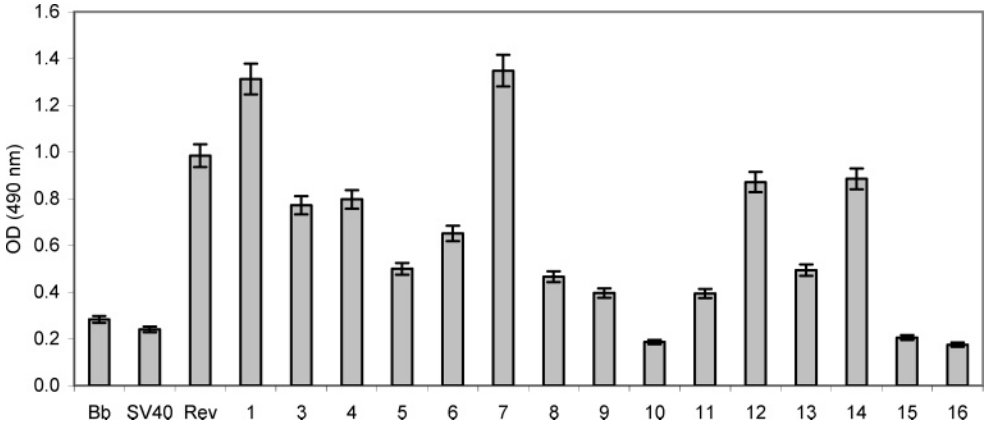

FIGURE 7: Quantitative accumulation of Bb−BC Rev-ARM peptide conjugates in Colo-205 cells. The Bb−peptide conjugates (BC Rev peptides, SV40) and Bb (5 mg/mL) were incubated with Colo-205 cells at 37 °C for 3 h as described in Materials and Methods. Numbers indicate the numbers of the peptides in the library (see Figure 1).

cells. To analyze the effects of peptides on Rev activity, ROD Rev reporter cells were incubated with several of the BC peptide analogues (BC Rev-1, BC Rev-10, BC Rev-11, BC Rev-13, and BC Rev-14) for 24 h.

ROD Rev reporter cells constitutively transcribe a reporter RNA with sequences encoding the red fluorescent protein and noncoding sequences mediating Rev dependence, including RRE. Expression of the RFP reporter protein requires Rev for the export of the reporter mRNA from the nucleus into the cytoplasm. After incubation with the peptides for 24 h, the cells were transfected with a Rev-GFP expression plasmid. Rev-dependent activation of reporter production was assessed by quantifying the number of RFP reporter-positive cells in the transfected population by flow cytometry. We have previously shown this reporter assay to be a very reliable tool for studying the efficiency of Rev-dependent gene expression (*46*, *47*), yielding results comparable to those obtained by measuring the level of Rev-dependent activation of HIV Gag protein synthesis by an integrated Rev-deficient provirus (R. Brack-Werner and H. Wolff, unpublished results).

As demonstrated in Figure 8A, BC peptide Rev-13 clearly inhibits Rev activity in the low micromolar range. The other peptides had only modest effects on Rev activity, with peptide Rev-14 even exhibiting slight stimulation of reporter expression. In subsequent experiments, we found inhibitory activity of BC Rev-13, starting at a concentration of approximately 1.5 μM and reaching a maximum level of inhibition of 65% at approximately 4.6 μM. The inhibitory effect was not increased further by increasing peptide concentrations up to 18.5 μM (Figure 8B). As can be seen in Figure 8C, all peptides analyzed in this manner were found to be nontoxic at the concentrations that were used, as determined by quantifying intracellular ATP levels.

DISCUSSION

The diverse biological functions of the two HIV-1 basic regulatory proteins, Tat and Rev, are essential for viral replication in infected cells (*18*, *62*, *63*). Both proteins have a similar ARM, which displays a triple function: mediating nuclear import, binding to specific viral RNA sequences, and mediating translocation through biological membranes. Indeed, this domain has attracted particular attention as a target for the development of anti-HIV-1 drugs and as a vehicle for the delivery of various cargos into living cells (*64*, *65*). Peptides bearing the ARM sequence have been used, in the past decade, as an experimental tool for studying the mechanisms mediating the various actions of these two proteins, as well as for inhibiting HIV-1 replication in cultured cells (*66*−*68*).

Previously, in our laboratory, we used the BC peptidomimetic approach to develop peptides that mimic the ARM of the HIV-1 Tat protein (*9*, *69*). Because of the similarity between the Tat and Rev ARM sequences, the Tat-ARM-derived cyclic peptides inhibited binding of the HIV-1 Rev-ARM to its corresponding RNA element (RRE). However, those studies were performed using in vitro assay systems. To assess the possibility of exploiting the BC ARM peptides as anti-HIV drugs and CPPs, it is essential to evaluate the behavior of biologically active BC peptides in living cells. It is our view that the use of cyclic peptides, due to their relative resistance to proteolysis (*7*), is preferable to the use of linear peptides. Therefore, in the work presented here, we screened a new BC peptide library bearing the ARM domain of the HIV-1 Rev protein and studied whether karyophilic BC ARM peptides retain the cell penetrating abilities of the ARM domain while blocking the biological function of the Rev protein in living cultured cells.

To evaluate the karyophilic properties of the peptide library, interaction of the BC peptides with the nuclear receptor of the Rev protein, importin β, was studied using two different assay systems. First, we tested whether the BC peptides were able to inhibit the interaction of Rev-GFP with the importin β receptor, and second, we studied the characteristics of the peptides' nuclear import in permeabilized HeLa cells. These peptides bear a relatively large ring size. Rev-13, which efficiently blocked binding of Rev-GFP to importin β, was covalently conjugated to Bb and was tested for nuclear import in permeabilized Colo-205 cells. Nuclear import of Rev-13−BSA conjugates was subjected to the same features that characterize active nuclear import (*60*, *70*). It did not take place at 4 °C and was inhibited by GTPγS and WGA. Moreover, nuclear uptake was inhibited by the presence of excess unlabeled Rev-ARM-bearing peptides.

The ability of the BC ARM peptide to penetrate cells' plasma membranes was studied by fluorescence microscopy in fixed (not shown) and nonfixed cells and by an ELISA-based system previously developed by us (*44*, *53*). In this

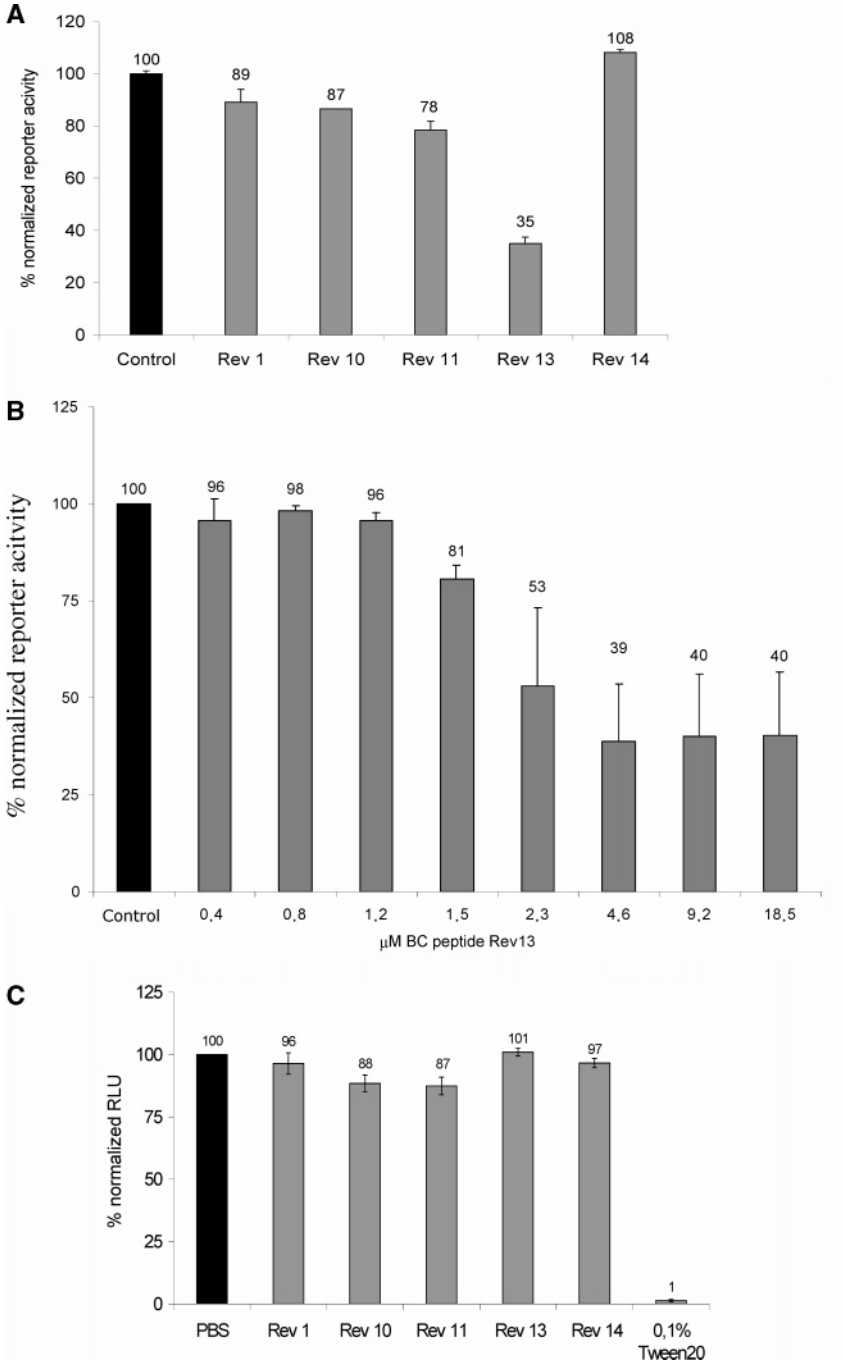

FIGURE 8: (A) Rev-induced expression of reporter protein. ROD reporter cells were either left untreated (control, black bar) or pretreated for 24 h with five different BC peptides (gray bars) at final concentrations of 16 (Rev-1), 6 (Rev-10), 7.6 (Rev-11), 4.6 (Rev-13), and 13.8 μM (Rev-14). After 24 h, cells were transfected with a Rev-GFP expression plasmid and Rev-dependent reporter protein expression analyzed by flow cytometry after an additional 24 h as described in Materials and Methods. Reporter activity of the control was set at 100%. Numbers above columns indicate mean values of three independent experiments, and error bars show the standard deviation. (B) Dose dependency of the inhibitory activity of Rev-13. Dose dependency experiments for BC peptide Rev-13 were performed as described for panel A. The BC Rev-13 peptide was added at concentrations of 0.4−18.5 μM for 24 h to ROD reporter cells, and Rev-dependent reporter activity was determined relative to the untreated control (black bar). Results give mean values obtained in three independent experiments, and error bars show the standard deviation. (C) Cytotoxicity of peptides. ROD reporter cells were treated with the same concentrations of BC peptides indicated for panel A, and intracellular ATP was quantified after 24 h using a luminescence assay. As a nontoxic control, PBS was used (black bar on the left), and as a toxic agent, 0.1% Tween 20 was added to the cells (right bar). Data represent mean values and standard deviations of four parallel samples in one experiment. Relative light units (RLU) represent the amount of intracellular ATP and were normalized to the PBS control (100%).

study, we used unfixed cells, since recently it was shown that the fixation of cells induces artificial penetration of CPPs and promotes their redistribution, which may appear as cell penetration and translocation into the cell nucleus (*71*). Penetration of the BC Rev peptides and BSA conjugates occurred under various conditions: it was observed at 4 °C, and it was not inhibited by endocytosis inhibitors or by the addition of a molar excess of unlabeled Rev-ARM. Moreover, ATP depletion of the cells inhibited the cellular import of the intracellular Rev−BSA conjugate by only 20%; the observed internalization can therefore not be explained by a typical endocytotic mechanism. Our results also show that

of five BC ARM peptides studied, only BC Rev-13 inhibited Rev-induced gene expression by ~60−70% in cultured HeLa cells at concentrations between 2 and 5 μM. The BC peptides, at the concentrations that were used, exhibited very little effect on cell toxicity.

Linear peptides that block the biological functions of the Tat and Rev proteins have been described previously (*42*, *68*). Indeed, phage-display peptide libraries as well as the yeast two-hybrid system have been used to evaluate linear peptides that can interact with Tat or Rev (*70*, *71*). Several of these linear peptides have displayed antiviral activity. In addition, in our laboratory, we have shown that a synthetic peptide bearing amino acids 1−20 of the bacteriophage fd p8 protein, designated NTP8, specifically interacts with the NLS of the HIV-1 Tat protein (*42*). Accordingly, the NTP8 peptide inhibited binding mediated by the Tat-NLS to importin β and Tat-NLS-mediated translocation into cell nuclei (*42*). The NTP8 peptide, at 100 μM, also caused ~50% inhibition of HIV-1 propagation in cultured cells. However, the peptide described in this work displays different properties. These are Rev−NLS−mimetic BC peptides, i.e., peptides derived from the Rev-ARM domain itself, and they are therefore not expected to interact with the Rev protein.

These peptides bear an ARM sequence and therefore should exert their effect by interacting either with the cellular nuclear import receptor importin β (*19*) or with the RNA target, namely, RRE. Indeed, all the BC peptides of the library blocked binding to importin β using an in vitro assay system. It should be noted that competitive inhibition of the nuclear import machinery in vivo, by blocking the function of importin β, should result in cell death. However, the five BC peptides tested in this work have been found to be nontoxic. Of these five peptides, BC Rev-13 inhibited Rev-induced gene expression. However, our results clearly show that this peptide did not block Rev-ARM-mediated nuclear import in vivo, contrary to its inhibitory effect in vitro. Thus, it is our assumption that the inhibitory effect of BC Rev-13 is due to its interaction with the RRE.

Our previous studies clearly showed that BC peptides bearing the ARM domain interact in vitro with RRE molecules, showing high binding affinity (*9*). In addition, recent studies using molecular modeling and dynamics have suggested specific binding of these peptides to the Rev target RNA domain, RRE (C. Devaux, unpublished results). These results raise the interesting possibility that, as opposed to the findings using an in vitro assay system, uptake of small BC ARM peptides by intact cells allows these peptides to enter the nucleus independent of nuclear import pathways. Once within the nucleus, BC ARM peptides may bind to the target RNA sequence, disrupting Rev function. If valid, this would certainly open up the unique possibility of using NLS−mimetic BC peptides as lead compounds for obtaining effective anti-HIV drugs without the need to look for peptides that will specifically interact with the corresponding proteins, and as a delivery system with no toxicity.

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