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The fd phage and a peptide derived from its p8 coat protein interact with the HIV-1 Tat-NLS and inhibit its biological functions

A. Krichevsky [a,*], M. Rusnati [b], A. Bugatti [b], E. Waigmann [c], S. Shohat [a], A. Loyter [a]

[a] *Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[b] *Department of Biomedical Science and Biotechnology, School of Medicine, University of Brescia, Brescia 25123, Italy*
[c] *Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Institute of Medical Biochemistry, Dr. Bohrgasse 9A-1030 Vienna, Austria*

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Abstract

Filamentous fd bacteriophages are used to construct phage-display peptide libraries, which have been instrumental in selecting peptides that interact with specific domains within target molecules. Here we demonstrate that the fd bacteriophage itself, as well as NTP8 – a synthetic peptide derived from it and bearing amino acids 1–20 of the phage p8 protein – interact with the nuclear localization signal (NLS) of the HIV-1 Tat protein. Accordingly, fd bacteriophage and the NTP8 peptide inhibit binding mediated by the Tat-NLS to the nuclear-import receptor importin β and Tat-NLS-mediated translocation into cell nuclei. The NTP8 peptide, at 100 μM concentration, also caused about 50% inhibition of HIV-1 propagation in cultured cells. The fd bacteriophage prevents heparan sulfate proteoglycans-mediated uptake of extracellular Tat by target cells and consequently transactivation of a chloramphenicol acetyltransferase (CAT) reporter gene. A BSA-NTP8 conjugate inhibits Tat-NLS-mediated binding to heparin immobilized on a BIAcore surface. BLAST analysis of the NTP8 amino-acid sequence revealed similarity to sequences in several human proteins, including ADA2 and CD53.

Keywords: HIV-1; Tat; Fd bacteriophage; Inhibitory peptide; Nuclear-import

1. Introduction

The HIV-1 Tat protein is essential for viral replication because it is a potent transcriptional activator of the viral long terminal repeat (LTR) promoter (Cullen, 1995). Activation results from the Tat protein's interaction with the transactivation response (TAR) element at the 5′-terminus of all HIV-1 transcripts, a process which is mediated by the protein nuclear localization signal (NLS) domain, also termed arginine-rich motif (ARM). This leads to transcription stimulation by inducing chromatin remodelling of the viral promoter region and recruitment of factors that enhance the elongation activity of RNA polymerase II complex (reviewed in (Karn, 1999). To exert its biological function, Tat is actively imported into the nucleus (Cullen, 1995; Sherman and Greene, 2002) using its NLS domain, which binds directly to the cellular nuclear-import receptor importin β (Truant and Cullen, 1999).

Tat protein is also secreted from HIV-1-producing cells (Chang et al., 1997). Extracellular Tat is then taken up by different types of uninfected cells and transactivates cellular and latent viral genes. The mechanism of extracellular Tat uptake by target cells is dependent upon its interaction with cell-surface heparan sulfate proteoglycans (HSPGs) (Tyagi et al., 2001). The bindings of Tat to HSPGs, as well as to heparin, and the consequent internalization process, have been shown to be mediated by the NLS (ARM) of the Tat protein (Futaki et al., 2000). In addition, uptake of extracellular Tat has been suggested to promote several AIDS-associated pathologies (Rusnati and Presta, 2002). Thus, due to the central role of the Tat protein in the life cycle of the virus and HIV pathogenesis, interference with Tat activities, and specifically with its NLS function, has been suggested as a potential antiviral approach (for a review, see (Rusnati and Presta, 2002).

* Corresponding author. Present address: Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA. Tel.: +1 631 632 1016/1015; fax: +1 631 632 8575.
E-mail address: akrichevsky@notes.cc.sunysb.edu (A. Krichevsky).

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Combinatorial phage-display libraries provide an effective tool for the selection of peptides or single-chain antibodies (scFvs) that interact with – and thus block the function of – a specific domain within the target protein. Indeed, using a phage-display peptide library, peptides that specifically interact with the NES sequence of the HIV-1 Rev protein have been selected (Jensen et al., 1998). In addition, we recently used a phage-display scFv library to select antibody fragments that specifically interact with the N-terminal NLS sequence of the HIV-1 Vpr protein (VprN). Such anti-VprN scFvs have been shown to block the import of HIV-1-recombinant Vpr protein or of BSA-VprN conjugates into the nuclei of permeabilized and microinjected cells (Krichevsky et al., 2003). However, during our recent attempts to select anti-Tat-NLS peptides from a phage-display peptide library (Enshell-Seijffers et al., 2001), we observed that the fd bacteriophage itself binds the NLS sequence of the Tat protein. In the present study, we show that a peptide derived from the fd phage's major coat protein interacts with the NLS of the HIV-1 Tat protein and inhibits its biological functions including inhibition of HIV-1 replication in cultured cells. The possibility of the newly discovered anti-NLS peptide's serving as a lead peptide to the development of anti-HIV drugs is discussed.

2. Materials and methods

2.1. Preparation of fd bacteriophage-particles

Phage-particles were prepared by the same procedure used to amplify phages between biopanning rounds as described by Enshell-Seijffers et al. (2001). Briefly, 50 ml of DH5αF$^+$ bacterial cells were infected with 100–200 μl of fd bacteriophage (10^{10} cfu/ml). Following the addition of 950 ml of LB medium, the cells were grown overnight in the presence of 20 μg/ml of tetracycline. The phages were purified by precipitation with polyethylene glycol (PEG) or CsCl gradient and stored at −20 °C or 4 °C.

2.2. Synthesis of peptides

The following peptides were synthesized exactly as described by Karni et al. (1998): SV40 large T-antigen NLS (Kalderon et al., 1984), a peptide derived from the N-terminus of the Vpr protein – the VprN (Karni et al., 1998), the HIV-1 Tat-NLS (Friedler et al., 2000), VirE2-NLS (Citovsky et al., 1992), the HIV-1 Rev-NLS (Truant and Cullen, 1999), a peptide derived from the N-terminus of the fd bacteriophage p8 protein – NTP8 (Rasched and Oberer, 1986), and a peptide containing the same amino-acid composition as NTP8 but with a scrambled sequence – "scrambled" NTP8 (for peptide sequences see Table 1). Cysteine residues were added to the N- or C-terminus of the original peptide sequences. Biotinylated peptides were synthesized using the same procedure and a biotin molecule was added to the N-terminus during synthesis.

Table 1
Peptide sequences

Peptide	Sequence
SV40 large T-antigen NLS	PKKKRKV
VprN	NEWTLELLEELKNEAVRHF
Tat-NLS	GRKKRRQRRRAHQN
VirE2 NLS	KLRPEDRYIQTEKYGRR
Rev NLS	RRNRRRRW
NTP8	AEGDDPAKAAFDSLQASATE
Scrambled NTP8	EAGQAPDSLAKATEADADFS

2.3. Expression and purification of recombinant fusion proteins

A recombinant 86-amino-acid HIV-1 Tat protein, as well as an HIV-1 Vpr protein, were expressed in *Escherichia coli* and purified as glutathione-*S*-transferase fusion proteins [Tat-GST (Demarchi et al., 1996) and Vpr-GST (Krichevsky et al., 2003)]. The purity (>95%) and integrity of the proteins were routinely checked by SDS-PAGE and silver staining. GST and GFP moieties do not interfere with the heparin-binding capacity and LTR-transactivating activity of Tat protein (Tyagi et al., 2001). Recombinant Rev-GFP and importin β proteins possess a His-tag sequence and were expressed in *E. coli* strain BL21 (DE3) and purified using Ni-NTA resin, essentially as described by Fineberg et al. (2003).

2.4. Chemical conjugation of the synthetic peptides to BSA, rhodamine-labelled BSA (Rho-BSA) and to biotinylated BSA (Bb)

BSA was labelled with rhodamine (lissamine rhodamine B sulfonyl chloride, mixed isomers, from Molecular Probes) according to manufacturer's instructions and as described by Karni et al. (1998) to give Rho-BSA. Biotinylated BSA (Bb) molecules were purchased from Sigma. The aforementioned synthetic peptides were conjugated to the Rho-BSA or Bb molecules, to give Rho-BSA-NLS or Bb-NLS conjugates, as well as to unlabelled BSA molecules, as described by Broder et al. (1997).

2.5. Determination of ligand-NLS binding

2.5.1. ELISA

MaxiSorb plates (NUNC) were coated as follows: each well was incubated overnight at 4 °C with 200 μl of a solution containing the corresponding "antigen", namely BSA-peptide conjugates or recombinant proteins (25–30 μg/ml), or with the fd bacteriophage (10^8 cfu/ml) in carbonate buffer (0.05 M Na_2CO_3/0.05 M $NaHCO_3$, pH 9.6). Following removal of the solutions, the plates were washed three times with PBS and then blocked by incubation with 200 μl/well of PBS containing 4% BSA for 2 h at 37 °C. Following three washes with PBS, the appropriate ligand (see legends to figures) in a total volume of 200 μl of a blocking solution (4% BSA in PBS) was added and the plates

were incubated for another 2 h at 37 °C. The plates were rewashed three times with PBS and a volume of 200 μl/well of the followings reagents – all dissolved in the blocking solution (4% BSA in PBS) – were added to detect the antigen-ligand complexes: avidin–POD (Roche Diagnostics) for biotin-labelled molecules (0.25–0.1 unit/ml) or of anti-M13 monoclonal mouse antibody (Serotech, 1:5000) mixed with anti-mouse-HRP antibody (Jackson, 1:5000) for the fd bacteriophages. Following incubation for 1 h at 37 °C, the plates were washed three times with PBS and the binding of the ligand was detected using appropriate substrates (according to manufacturer's instructions). Each of the binding experiments was repeated at least three times. Data in the figures represent the results obtained from one of those repetitions.

2.5.2. BIAcore binding-assay

BIAcore technology (Johnsson et al., 1991) was used to determine the changes in refractive index caused by the capacity of free BSA-NTP8 to bind GST-Tat and prevent its interaction with immobilized heparin. It was also used to measure the interaction of surface-immobilized BSA-NTP8 with various BSA-NLS conjugates.

2.5.2.1. Inhibition of the interaction between Tat-GST and heparin by BSA-NTP8 conjugate. Heparin (13.6 kDa) was biotinylated at its reducing end. Following streptavidin activation of an F1 sensorchip flow-cell, the biotinylated heparin was allowed to react with the streptavidin-coated sensorchip. GST-Tat alone (125 nM) or in the presence of increasing concentrations of BSA-NTP8 or BSA-VirE2 conjugates was then injected over the heparin surface for 5 min and washed until dissociation was observed. The SPR signal was expressed in terms of resonance units (RU) (Rusnati et al., 2001).

2.5.2.2. The interaction of various BSA-NLS conjugates with surface-immobilized BSA-NTP8 conjugate. BSA-NTP8 conjugates were immobilized on the CM5 sensorchip through their primary amino groups according to Johnsson et al. (1991). Briefly, the flow-cell matrix was activated using a solution (70 μl) containing 0.4 M *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) in DDW. Then, 100 μg/ml BSA-NTP8 conjugate in 10 mM sodium acetate buffer (pH 3.5) was injected at a flow rate of 10 μl/min until the desired surface density was obtained (usually 5000–6000 RU). The remaining carboxyl groups were blocked by injecting a 70 μl volume of 1 M ethanolamine hydrochloride. BSA molecules were immobilized in the reference flow-cell using the same procedure. For the binding measurements, the various BSA-NLS conjugates [in PBS, pH 7.4 with 1 mg/ml of CMD (carboxymethyl dextran, Fluka)] were injected at a flow rate of 20 μl/min through the two flow-cells (with immobilized BSA-NTP8 and the immobilized BSA) for 3 min. The injection was then continued for an additional 3 min with buffer only. Flow-cells were regenerated by a 10-μl pulse of 10 mM glycine, pH 2.0. The binding curves, namely binding of the BSA-NLS conjugates to the immobilized BSA-NTP8, were obtained using BIA evaluation software and subtracting the values obtained using the reference cell (with immobilized BSA).

2.6. Cell cultures

HeLa and HL3T1 cells were maintained in DMEM, supplemented with 10% FCS, 0.3 g/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, as previously described (Broder et al., 1997). HL3T1 cells were derived from HeLa cells and contained integrated copies of chloramphenicol acetyltransferase (CAT) under HIV-1 LTR regulation (Wright et al., 1986). HeLa–$CD4^+$ β-gal cells were obtained through the NIH Reagent Program (Division of AIDS, NIAID, NIH, USA) and were maintained in and 100 μg/ml Hygromycin B (Calbiochem, La Jolla, CA, USA) RPMI 1640 and DMEM media supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine (Biological Industries, Israel).

2.7. Internalization of GST-Tat-GFP in HL3T1 cells and computerized image analysis

HL3T1 cells adhered to glass coverslips were incubated for 6 h at 37 °C in DMEM containing 10% FCS, 100 μM chloroquine and GST-Tat-GFP (400 ng/ml) in the presence or absence of increasing concentrations of fd bacteriophage. At the end of the incubation period, the cells were washed with 2.0 M NaCl in PBS to remove cell-surface-bound fluorescent labelled molecules, and fixed. Observations were carried out under a Nikon photomicroscope equipped for epifluorescence, and GST-Tat-GFP internalization was quantified by the computerized Image Pro-Plus analysis system (Media Cybernetics, Silver Spring, MD). Briefly, three to six fields were randomly chosen for each experimental condition and were input via a TV camera (Sensicam, CCD Imaging, Kelheim, Germany) mounted on the microscope, digitalized on a high-resolution monitor, and stored in the Pro-Plus analysis system's memory. The amount of GST-Tat-GFP internalized by HL3T1 cells was quantified by counting the intracellular fluorescent granules corresponding to cell lysosomes loaded with the GFP-fusion protein.

2.8. HIV-1 LTR transactivation assay

The transactivation assay was performed as described in Rusnati et al. (1997). Briefly, HL3T1 cells were incubated for 24 h in DMEM containing 10% FCS, 100 μM chloroquine, and GST-Tat at two different concentrations (200 and 400 ng/ml) was added in the presence or absence of increasing concentrations of fd bacteriophage. Heparin, a well known extracellular inhibitor of Tat, as described by Rusnati et al. (1997), was used as a positive control. The conditioned

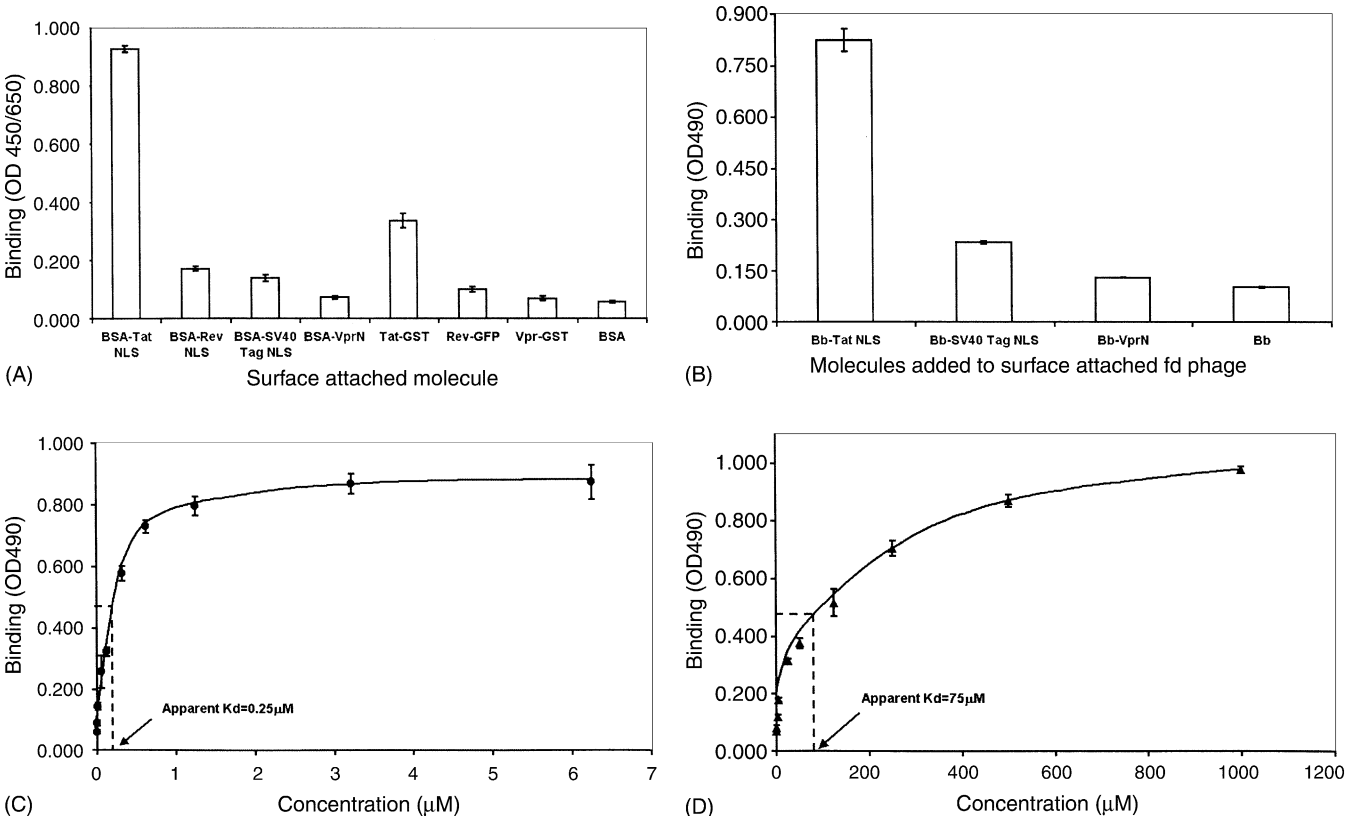

Fig. 1. Specific interaction between the fd bacteriophage and the NLS of the HIV-1 Tat protein, as determined by an ELISA assay. (A) The fd bacteriophage (10^7–10^8 cfu/ml) was incubated on plates coated with the specified BSA-NLS conjugates or karyophilic proteins; (B) soluble biotinyliated BSA-NLS conjugates (65 nM) were incubated on plates coated with bacteriophage-particles; (C) saturation curve of Bb-Tat-NLS binding to plates coated with fd bacteriophage; (D) saturation curve of biotinylated Tat peptide binding to plates coated with fd bacteriophage.

medium was then removed and the cells were further incubated for 24 h in DMEM/FCS. At the end of the incubation period, the amount of CAT protein present in the cell extracts was determined using the CAT ELISA kit (Boehringer, Mannheim, Germany) according to manufacturer's instructions.

2.9. Nuclear-import assays

2.9.1. Microscopic observations

Nuclear-import was studied in digitonin-permeabilized cells exactly as described previously (Broder et al., 1997), and analyzed by confocal microscopy using an MRC 1024 confocal imaging system (Bio-Rad). The microscope (Axiovert 135M; Zeiss, Germany, with a 63× objective; Apoplan; NA 1.4) was equipped with an argon ion laser for rhodamine excitation at 514 nm (emission 580 nm).

2.9.2. Quantitative estimation of nuclear-import

Nuclear-import was quantitatively determined by an ELISA-based method using biotinylated BSA-NLS conjugates as transport substrates essentially as described previously (Melchior et al., 1993); with the following modifications: a suspension of Colo-205 cells was permeabilized with 75 μg/ml digitonin (0.5–1 ml); the reaction was terminated by 100-fold dilution with transport buffer (20 mM Hepes pH 7.3, 110 mM potassium acetate, 20 mM magnesium acetate, 0.5 mM EDTA, 2 mM DTT, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin and 0.1 mM PMSF). Transport substrates were added and the process was terminated following 1 h incubation at 30 °C. Four samples were taken for quantitative determination from each experimental system and each experiment was repeated at least three times.

2.10. Estimation of HIV-1 replication

Estimation of HIV-1 replication was carried out by the multinuclear activation of a galactosidase indicator (MAGI) assay, as described by Kimpton and Emerman (1992) and in Hutoran et al. (2004). Briefly, HeLa–CD4$^+$ β-gal cells were cultivated in 96-well plates at 12×10^3 cells per well. The NTP8 peptide was added (from a stock solution of 10 mM in DDW) at the indicated concentrations; each concentration to three plates. On the following day, the cells were infected with 50 μl of serially diluted virus HIV-1IIIB (at a multiplicity of infection of 0.1) in the presence 20 μg/ml of DEAE-dextran (Pharmacia, Sweden). Two days post-infection, cultured cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde in PBS. Following intensive wash with PBS, cells were stained with a solution of 4 mM potassium ferrocyanide, 4 mM potas-

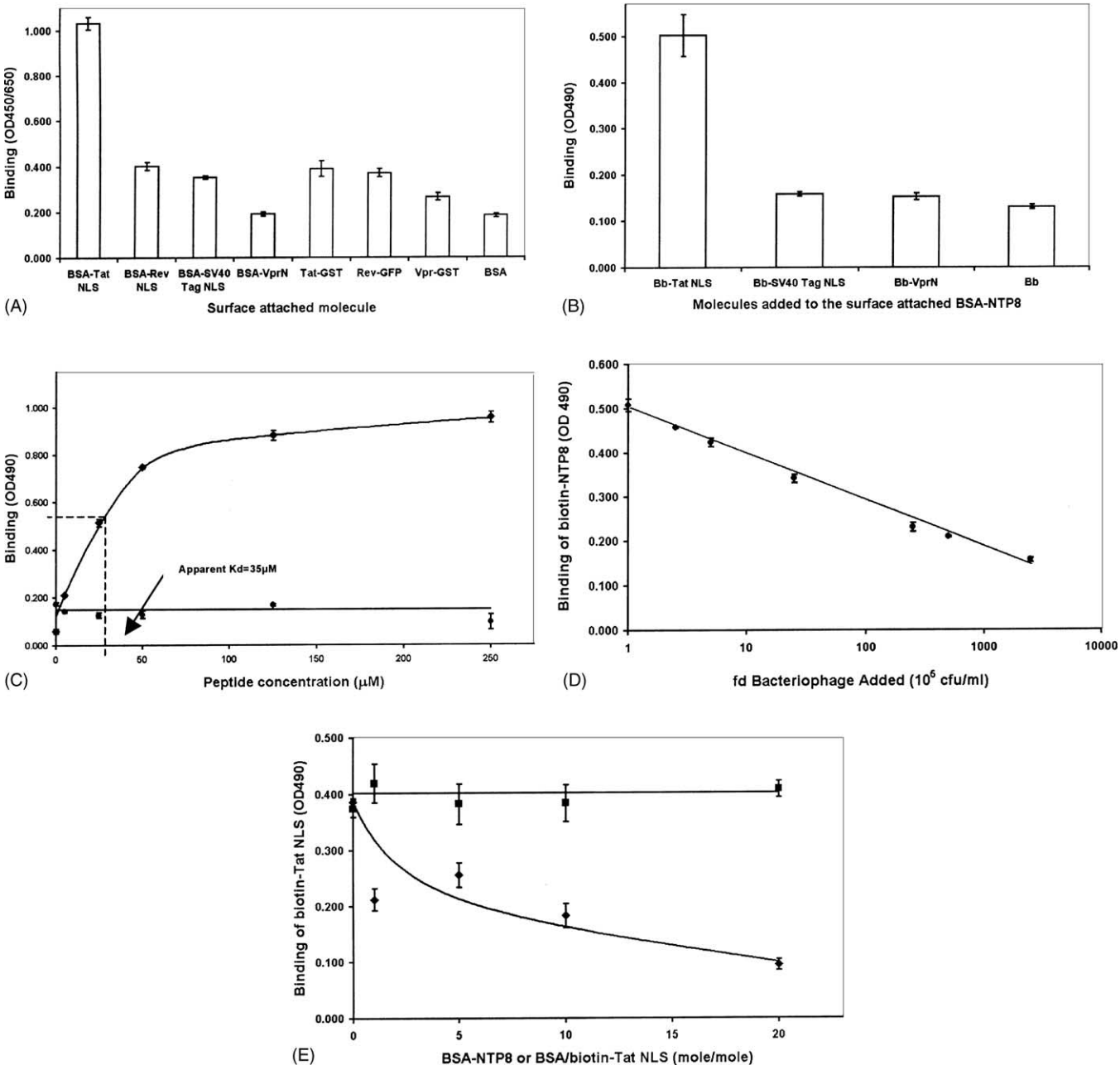

Fig. 2. Specific interaction between NTP8 peptide and Tat-NLS, as determined by an ELISA assay. (A) Biotinylated NTP8 peptide (250 μM) was incubated on immunoplates coated with the specified BSA-NLS conjugates or karyophilic proteins; (B) the specified biotinylated BSA-NLS conjugates (65 nM) were incubated on immunoplates coated with BSA-NTP8 conjugate; (C) binding of the NTP8 peptide (♦) but not of a "scrambled" NTP8 peptide (●) to surface-immobilized BSA-Tat-NLS; (D) binding of the NTP8 peptide to surface-immobilized BSA-Tat-NLS conjugate: inhibition by the fd bacteriophage. A biotinylated NTP8 peptide (250 μM) was incubated on plates coated with BSA-Tat-NLS conjugate in the presence of increasing concentrations of fd bacteriophage; (E) competitive inhibition of the binding between Tat-NLS peptide and surface-immobilized fd phage. Biotinyiated Tat-NLS (25 μM) was incubated on plates coated with fd bacteriophage in the presence of increasing amounts of BSA-NTP8 (♦) or BSA (■) molecules.

sium ferricyanide, 2 mM MgCl2 and 0.4 mg/ml of X-Gal (Ornat, Israel). Blue cells as well as total number of cells were counted under a light microscope at a magnification of 200×. The total number of cells obtained, at the different systems (virus infected in the absence or presence of the NTP8) after 72 h of growth showed a difference of ±10%. Each experiment was repeated three times.

3. Results

3.1. The fd bacteriophage recognizes the NLS sequence of the HIV-1 Tat protein

The fd phage-particles attached strongly to the immunoplates' wells coated with BSA-Tat-NLS conjugates (Fig. 1A).

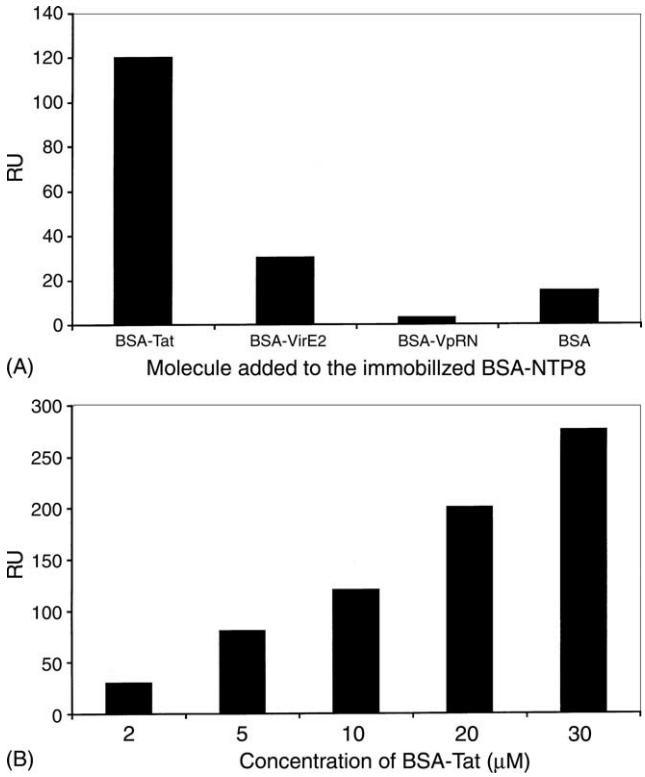

Fig. 3. The interaction between Tat-NLS and NTP8 peptide as determined by BIAcore technology. (A) Specific binding of BSA-Tat-NLS (10 μM) conjugate to BSA-NTP8 immobilized on a CM5 chip using the BIAcore 3000 system; (B) concentration dependence of BSA-Tat-NLS binding to BSA-NTP8 immobilized on a CMS chip. Each experiment was repeated at least three times histograms show the results obtained from one typical experiment.

The specificity of the fd bacteriophage-Tat-NLS interaction is proven by the fact that very little, if any, binding was obtained to conjugates bearing the NLSs of the HIV-1 Vpr or the Rev proteins, or to that of the SV40 T-antigen (Kalderon et al., 1984). The fd bacteriophage bound to the Tat-GST fusion protein as well but not to Vpr-GST or Rev-GFP-fusion proteins (Fig. 1A). These results were confirmed by the reverse experimental approach, whereby labelled (biotinylated) BSA-Tat-NLS conjugates (Bb-Tat-NLS), as well as labelled SV40 T-antigen-NLS and VprN conjugates, were added to plates coated with the bacteriophage (Fig. 1B); much higher degrees of binding were obtained with a conjugate bearing Tat-NLS than with conjugates bearing other NLSs or with the Bb carrier itself.

In most of the binding experiments, as well as in the nuclear-import assays, BSA-NLS conjugates were used, rather than the free NLS peptides. This is due mainly to the fact that binding of most of the synthetic peptides to their specific target proteins is of low affinity, with Kd values in the micromolar range (Hodel et al., 2001). On the other hand, the binding affinities of the parent proteins, i.e. those from which the sequence of the synthetic peptides were derived, are, in most cases, relatively high, with Kd values in the nanomolar range. Indeed, conjugation of synthetic peptides to an intact protein such as BSA increased their binding affinities by almost 1000-fold (Hu and Jans, 1999). It is also worth noting that BSA-NLS conjugates have been used extensively as transport substrates in nuclear-import studies (Goldfarb et al., 1986).

The binding of the Tat-NLS sequence to the fd particles was dose-dependent and saturable (Fig. 1C and D), clearly indicating a specific interaction. The affinity of BSA-Tat-NLS conjugates to the phage-particles reached an apparent Kd of about 0.25 μM (Fig. 1C), while that of the Tat-NLS peptide was about 75 μM (Fig. 1D).

Binding of labelled BSA-Tat-NLS conjugates (Bb-Tat-NLS) to the fd bacteriophage was competitively inhibited, in an ELISA-based assay, by unlabelled BSA-Tat-NLS, but not by other BSA-peptide conjugates such as unlabelled BSA-VirE2-NLS (not shown). VirE2 is a karyophilic Agrobacterium protein, the NLS of which has been shown to mediate nuclear entry of the VirE2 protein into nuclei of agrobacterium-infected cells (Citovsky et al., 1992). Similar to the Bb-Tat-NLS conjugates, the Tat-NLS peptide was also able to bind the fd bacteriophage; this binding was inhibited by unlabelled Tat-NLS peptide, but not by the VirE2 peptide (data not shown).

3.2. The N-terminus of the phage p8 protein promotes interaction between the fd bacteriophage and the Tat-NLS

The fd bacteriophage is composed of five structural proteins, of which the coat protein, p8, is present in approximately 2700 copies per phage; the other four are present in only 5 copies per phage. Only the N-terminal domains of the p8 and p3 proteins are exposed on the surface of the phage-particles (Rasched and Oberer, 1986). Preliminary experiments (not shown) have clearly shown that BSA-Tat-NLS does not affect p3-mediated phage infectivity (Rasched and Oberer, 1986) and thus it is unlikely that Tat-NLS interacts with the p3 protein. The p8 protein remained as a possible ligand for Tat-NLS. A peptide bearing the first 20 amino acids of the p8 protein was therefore synthesized and designated NTP8 peptide (N-terminus of p8; for amino-acid sequence, see Table 1).

The NTP8 peptide was able to interact with immobilized BSA-Tat-NLS conjugates at a binding degree that appeared to be higher than that observed with any other immobilized molecule, including immobilized BSA (Fig. 2A). A specific NTP8-Tat interaction was also observed when immobilized BSA-NTP8 was used as a ligand (Fig. 2B), using the reverse of the experimental approach described in Fig. 2A. The binding of the NTP8 peptide to immobilized BSA-Tat-NLS conjugates was concentration-dependent, reaching a saturation value at about 200–250 μM (Fig. 2C) with an apparent Kd of about 35 μM. The view that the NTP8-Tat interaction is sequence-specific is supported by the results in Fig. 2C, which shows that a

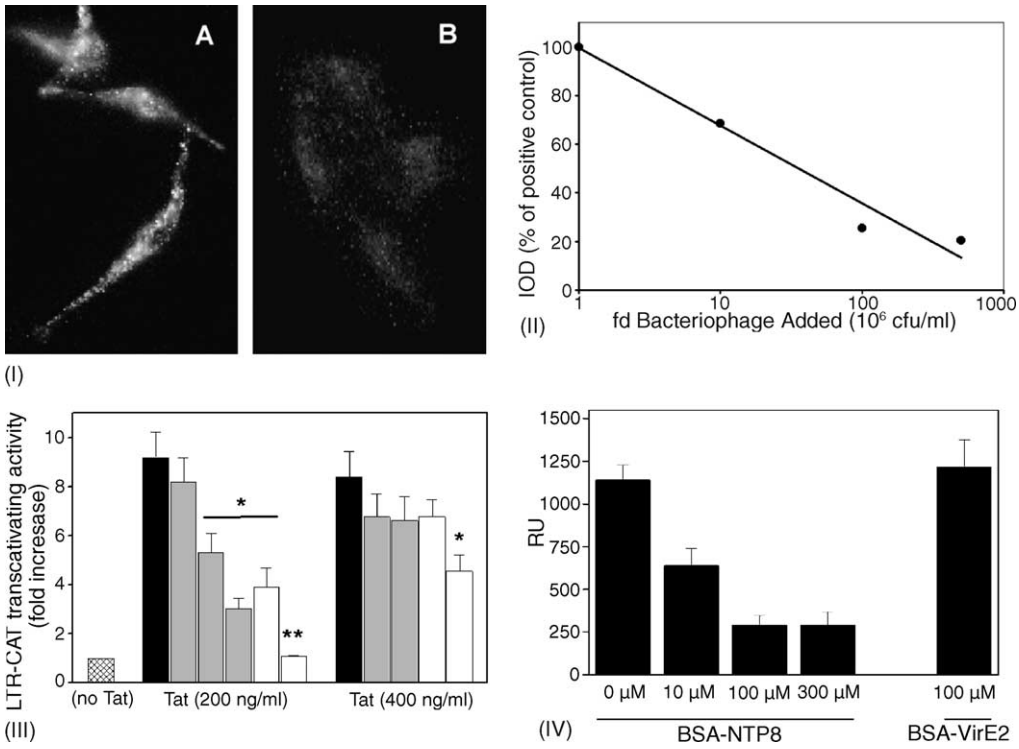

Fig. 4. (I) Inhibition of GST-Tat-GFP internalizatioh into HL3T1 cultured cells by the fd bacteriophage: GST-Tat-GFP (400 ng/ml) incubated with HL3T1 cells in the absence (A) or presence; (B) of fd bacteriophage (10^8 cfu/ml); (II) quantitative analysis of the internalization of GST-Tat-GFP by HL3T1. Observations were performed with a Nikon photomicroscope and the extent of internalization was quantified by a computerized Image Pro-Plus analysis system as described in Section 2; (III) the transactivation assay was performed exactly as described in Section 2 following the additions of culture medium alone (crossed bar) or of the indicated concentrations of the full length recombinant Tat protein to the HL3T1 cells in the absence (black bars) or in the presence of phage-particles (grey bars, 10, 100 and 500 cfu $\times$ 10^6/ml, from left to right) or of heparin (white bars, 0, 1 and 100 ng/ml, from left to right). Each point is the mean $\pm$ S.E.M. of three independent experiments assayed in duplicate ($^{*}p < 0.01$, $^{**}p < 0.001$, Student's *t*-test); (IV) inhibition of recombinant Tat-GST protein binding to surface-immobilized heparin by BSA-NTP8 conjugates as determined by the BIAcore technology.

peptide bearing the same amino acids as NTP8 but in a different order, a scrambled NTP8, failed to interact with the immobilized BSA-Tat-NLS conjugates. Binding of the NTP8 peptide to the immobilized BSA-Tat-NLS was competitively inhibited by the addition of increasing concentrations of the phage-particles (Fig. 2D). Moreover, specific binding of the Tat-NLS peptide to surface-immobilized fd bacteriophage was only competitively inhibited by BSA-NTP8 conjugates, and not by the BSA molecules (Fig. 2E).

Binding of Tat-NLS to the NTP8 peptide was further studied using BIAcore technology. BSA-NTP8 conjugates were immobilized in the flow-cells of a CM5 sensor chip. Flow-cells with immobilized BSA were used as a reference. A solution containing BSA conjugates bearing the Tat-NLS, VirE2-NLS and VprN peptides, as well as a solution containing only BSA molecules, were passed through the two flow-cells and the difference between the binding to each of them was monitored. Of the various BSA conjugates, only BSA-Tat-NLS showed significant binding, indicating a specific Tat-NTP8 interaction (Fig. 3A). No significant binding of the other BSA conjugates, or of BSA itself, to the immobilized BSA-NTP8 was observed. Binding of the BSA-Tat-NLS conjugates to the immobilized BSA-NTP8 was concentration-dependent (Fig. 3B). It was impossible to determine the binding parameters (K_{on} and K_{off}) in this assay, since each of the BSA molecule bears, on average, four to five molecules of either Tat-NLS or NTP8 (see Section 2), thus promoting multivalent interactions.

3.3. Inhibition of Tat protein penetration into cultured HL3T1 cells and of its binding to heparin by the fd phage and by the NTP8 sequence

As evidenced by microscopic observation (Fig. 4I) and quantitative estimation (Fig. 4II), the penetration of recombinant GST-Tat-GFP into cultured HL3T1 cells was inhibited by the fd bacteriophage. The phage-particles were also able to block Tat-dependent expression of a reporter gene (CAT) in intact HL3T1 cells (Fig. 4III). The inhibitory effect of the phage-particle, as well as heparin, a well known extracellular inhibitor of Tat, as described by Rusnati et al. (1997), was dose-dependent and reversible by increasing the concentration of Tat protein, thus indicating a specific interaction (Fig. 4III).

BSA-NTP8 conjugates were able to block NLS-mediated binding of recombinant Tat protein to surface-immobilized heparin, as evidenced by BIAcore technology (Fig. 4IV).

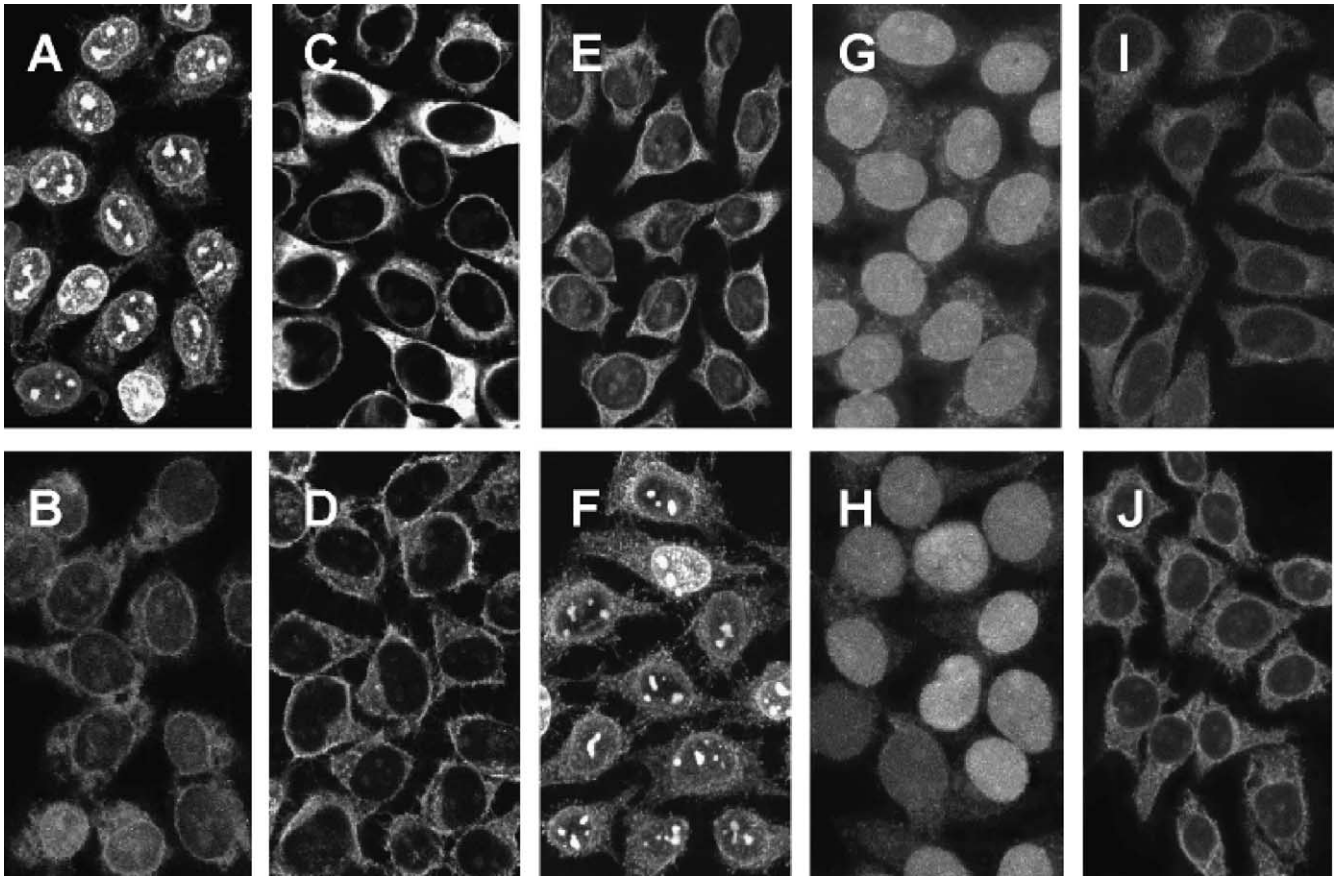

Fig. 5. Specific inhibition of Tat-NLS mediated nuclear-import by the fd bacteriophage. Import of fluorescently labelled BSA-Tat-NLS conjugates (Rho-BSA-Tat-NLS; 40 μg/system) was performed as described in Section 2 and previously (Broder et al., 1997). Fluorescently labelled BSA-VprN conjugates (Rho-BSA-VprN 25 μg/system) were used as a control system. (A) Nuclear-import of Rho-BSA-Tat-NLS at 37 °C. As in (A), but with the following modifications: (B) in the presence of 10^9–10^8 cfu/ml of phage-particles; (C) at 4 °C; (D) in the presence of 25 μM WGA; (E) with free Tat-NLS peptide added (at a Tat-NLS peptide to Rho-BSA-Tat-NLS molar ratio of 20); (F) with the free SV40 T-antigen NLS peptide added (at an SV40 T-antigen NLS peptide to Rho-BSA-Tat-NLS molar ratio of 20). Nuclear-import of Rho-BSA-VprN: (G) at 37 °C; (H) with 10^9–10^8 cfu/ml of phage-particles; (I) at 4 °C; (J) with 25 μM WGA. Experiments were repeated at least three times; micrographs show the results obtained in a single typical experiment.

3.4. Inhibition of the Tat-NLS-mediated nuclear-import and binding to importin β by the fd bacteriophage and the NTP8 peptide

The fd bacteriophage strongly blocked Tat-NLS biological function, namely, its ability to mediate import into the nuclei of permeabilized cells (compare Fig. 5A and B). Under the experimental conditions used, nuclear-import of the BSA-Tat-NLS conjugates was a specific and active process. It was temperature-dependent (Fig. 5C), and was inhibited by wheat germ agglutinin (WGA) (Fig. 5D) (Mattaj and Englmeier, 1998), as well as by the addition of excess free Tat-NLS peptide (Fig. 5E), but not by the SV40 T-antigen NLS peptide (Fig. 5F). In contrast to the inhibition of the Tat-BSA conjugates' nuclear-import (Fig. 5B), import of the BSA-VprN conjugates (Fig. 5G–J and Karni et al., 1998; Krichevsky et al., 2003) was not inhibited by the phage-particles (compare Fig. 5G and H), indicating their specific anti-Tat activity. Note that nuclear-import of BSA-Tat-NLS (Fig. 5A and F), but not of BSA-VprN (Fig. 5G and H), resulted in a high degree of nucleolar fluorescence staining. A very similar observation was recently reported for the import of core histones into nuclei of permeabilized cells (Baake et al., 2001). Like the histones, the positively charged Tat-NLS, but not the negatively charged VprN, probably interacts with the nucleolus' negatively charged ribosomal RNA (Baake et al., 2001).

The Tat-NLS domain is well known to specifically interact with the cellular receptor importin β, the presence of which is required to promote Tat translocation into the cell nucleus (Truant and Cullen, 1999). As can be seen in Fig. 6A, the fd bacteriophage inhibited the binding of Bb-Tat-NLS conjugates to importin β in a dose-dependent manner. The Bb-Tat-NLS-importin β interaction was inhibited by the addition of Tat-NLS peptide, but not by SV40 T-Ag NLS peptide, whose nuclear receptor is importin α (Fig. 6B).

Similar to the fd bacteriophage, the NTP8 peptide also inhibited Tat-NLS-mediated nuclear-import, as revealed by a quantitative nuclear-import assay system (Melchior et al., 1993). Active Tat-NLS-mediated nuclear-import is evident from the results, showing a low degree of import following

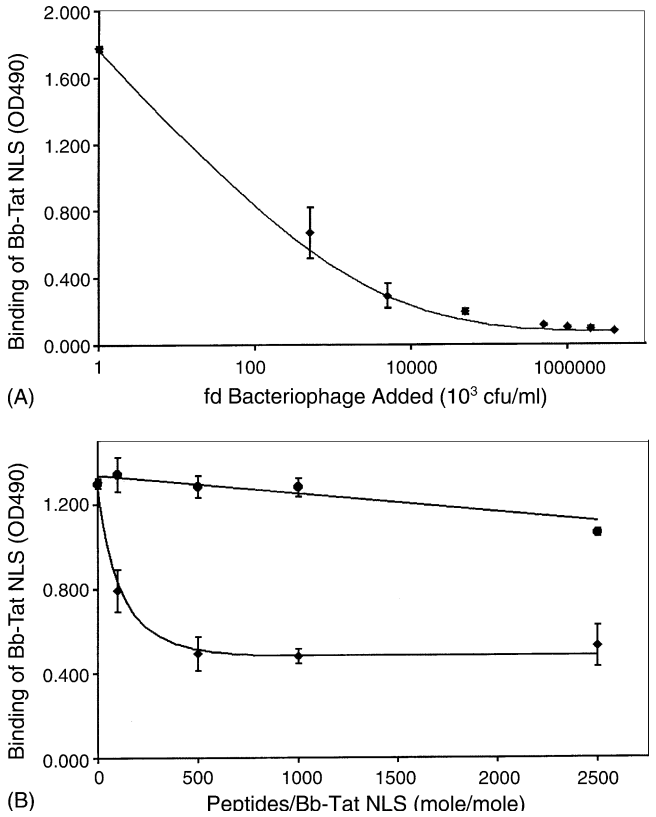

Fig. 6. Inhibition of the Bb-Tat-NLS interaction with surface-immobilized importin β. Bb-Tat-NLS (50 nM) was incubated with immobilized importin β as described in Section 2 in the presence of (A) fd bacteriophage; (B) Tat-NLS peptide (♦) or SV40 T-antigen NLS peptide (●).

incubation with Bb (Fig. 7Ab) or in the presence of GTPγS (Fig. 7Ac) (Mattaj and Englmeier, 1998). When nuclear-import was assessed in the presence of excess NTP8 peptide (Bb-Tat-NLS/NTP8, 1/100 ratio, mol/mol), an inhibition degree of about 40% was observed (Fig. 7Ae). The observed inhibition appears to be specific, since VirE2 peptide (Citovsky et al., 1992) in the same ratio was not inhibitory (Fig. 7Ad) (due to the use of anti-BSA-coated plates in the present assay system (see Section 2) the inhibitory effect of BSA-NTP8 conjugate could not be assessed). The addition of NTP8 peptide did not have any effect on nuclear-import mediated by VprN (Karni et al., 1998) (Fig. 7Bd). Active nuclear-import of Bb-VprN was evident from the inhibition observed following incubation at 4 °C (Fig. 7Bb) or in the presence of WGA (Fig. 7Bc). Inhibition of Tat-NLS-mediated nuclear-import by the NTP8 peptide was dose-dependent (Fig. 7C). The addition of excess Bb-Tat-NLS could partially overcome NTP8's inhibitory effect, further sustaining the view that the observed inhibition is due to a NTP8-Tat interaction (Fig. 7D).

3.5. Inhibition of HIV-1 propagation by the NTP8 peptide

The effect of the NTP8 peptide on HIV-1 propagation was studied using HeLa MAGI cells, which express β galactosidase gene under TAR regulation. The inhibitory effect of the NTP8 peptide on virus replication was assessed by estimation of the percentage of blue cells following infection with HIV-1 (for details see Section 2). As can be seen, at concentrations between 50 and 150 μM the NTP8 peptide caused between 40 and 60% inhibition of virus propagation. This inhibition appears to be specific, since at concentrations of 50 and 150 μM the "scrambled" peptide did not show any inhibitory effect (Fig. 8).

4. Discussion

The results of the present work clearly shows that fd phage-particles interact with the NLS domain of the HIV-1 Tat protein. The specificity of this interaction can be inferred from the observations that, of the battery of BSA-NLS conjugates studied, only the BSA-Tat-NLS conjugate showed binding activity. The number of fd particles used in our binding-assay systems (about 10^9–10^{10} cfu/ml) is very close, if not identical, to that used routinely to detect phage-particle attachment to a specific target molecule (Itoh et al., 2003). In addition, the fd phage-particles blocked various biological functions of Tat-NLS, such as binding to importin β, as well as nuclear-import. Cell internalization of Tat-GFP and Tat-dependent transactivation of the LTR-CAT reporter gene were also inhibited by addition of the phage-particles. A peptide bearing the first 20 N-terminal amino acids of the phage p8 coat protein (NTP8) was shown to specifically interact with the Tat-NLS domain and appears to mediate binding of the phage-particles to this domain. Furthermore the NTP8 peptide inhibited Tat mediated nuclear-import, as well as HIV-replication in cultured cells. Due to the fact that the binding affinity of the NTP8 peptide to its target Tat-NLS appears to be relatively low, exhibiting an apparent Kd of about 35 μM, relatively high concentrations were required to obtain inhibition of HIV-replication. Inhibition was demonstrated using the MGI assay, namely, trans-activation of the β-galactosidase gene under the control of the viral LTR (Hutoran et al., 2004). It should also be mentioned, that preliminary experiments have shown that the at 100 μM the NTP8 almost completely blocked (90–95%) HIV-replication in stimulated-infected peripheral human blood lymphocytes (PBL), as was estimated by reduction in the production of the viral p24 (not shown) (for estimating of the quantity of p24 see Friedler et al., 1998). However at these concentration the NTP8, as well as the scrambled peptide, caused between 50 and 60% cell toxicity. In this regard it should be mentioned that previous work in our laboratory have shown that a cyclic peptide bearing the NLS domain of the HIV-1 Rev protein, the BCvir peptide, also was able to inhibit HIV-1 infection of cultured cells as was estimated by reduction in the viral p24 (Friedler et al., 1998). The concentrations needed to exert an inhibitory effect by BCvir were almost the same as those required in the present work by NTP8, namely, around 100 μM. It is conceivable to assume that this may result not only from

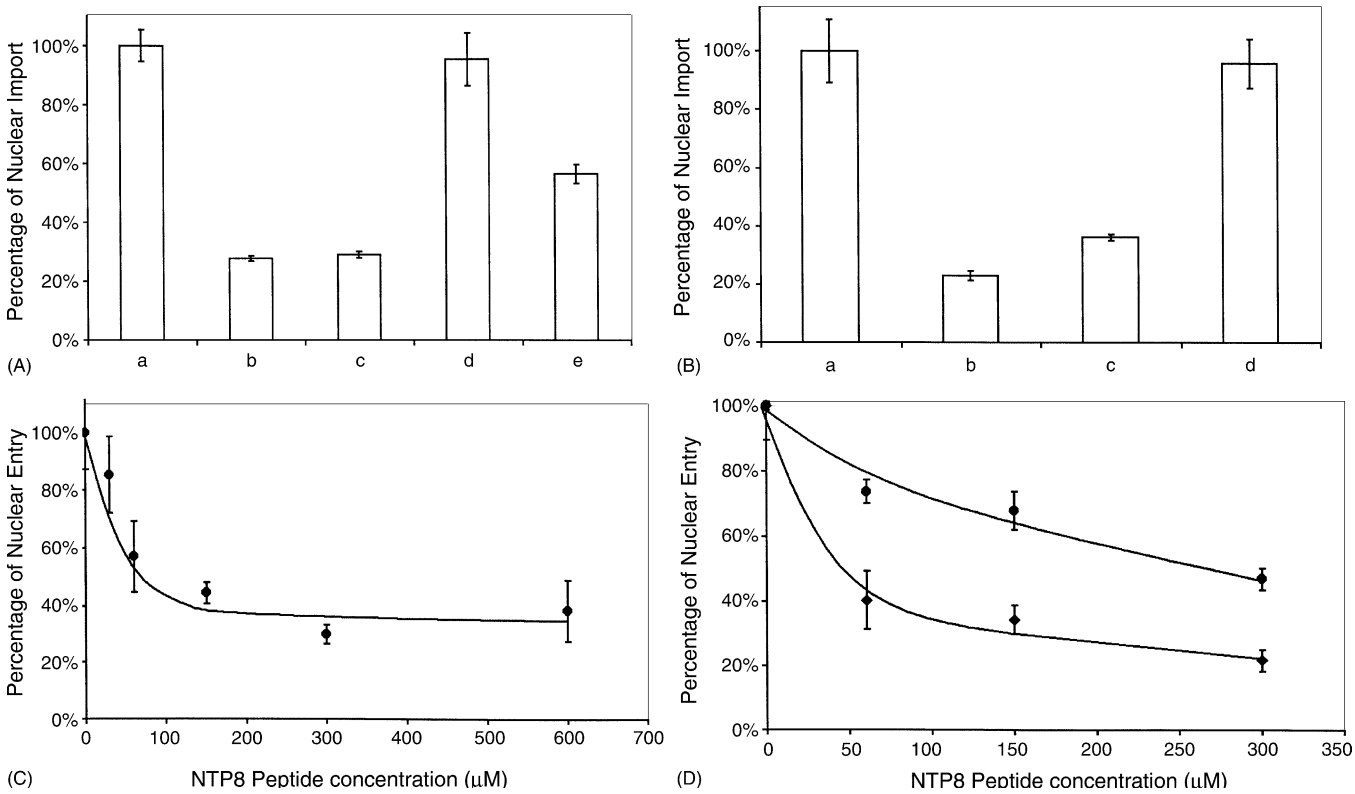

Fig. 7. Quantitative estimation of Tat-NLS-mediated nuclear-import's-inhibition by the NTP8 peptide. Nuclear-import of Bb-Tat-NLS (A, C and D) and of Bb-VprN (B) was estimated as described in Section 2 and previously (Melchior et al.,1993; Friedler et al., 1998). Bb-Tat-NLS (2.5 μg/system in (A) and (C), or 2.5 μg/system (♦) and 5.0 μg/system (●) in (D), Bb (2.5 μg/system) or Bb-VprN in (B) (2.5 μg/system) were incubated with permeabilized cells. (A): (a) Nuclear-import of Bb-Tat-NLS at 30 °C; (b) nuclear-import using Bb as a transport substrate at 30 °C; (c) in the presence of 2 mM GTPγS; (d) with VirE2 peptide (at a VirE2 peptide to Bb-Tat-NLS molar ratio of 100); (e) with NTP8 peptide (at an NTP8 peptide to Bb-Tat-NLS molar ratio of 100). B: (a) Nuclear-import of Bb-VprN at 30 °C; (b) nuclear-import of Bb-VprN at 4 °C; (c) in the presence of 25 μM WGA; (d) with NTP8 peptide (at an NTP8 peptide to Bb-VprN molar ratio of 100).

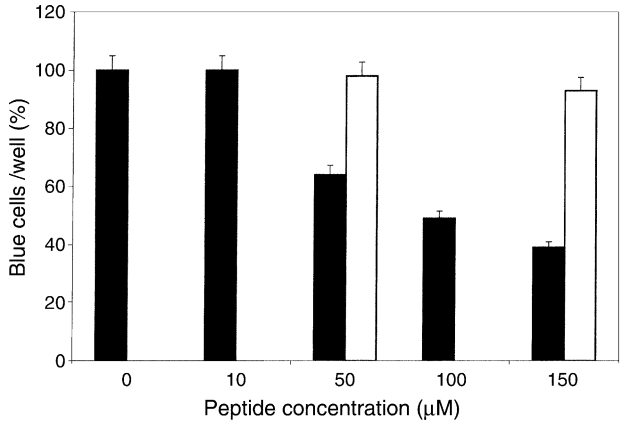

Fig. 8. The effect of the NTP8 peptide on HIV propagation as estimated by MAGI cells. The NTP8 peptide, as well as the "scrambled" peptide, were added at the indicated concentrations as described in Section 2. All other experimental conditions, as well as obtaining cells' staining, as described in Section 2 (■ NTP8 peptide, □ "scrambled" peptide).

the low binding affinities of these peptides, but also to their low cell permeability. However the NTP8, as opposed to the BCvir, which contains an NLS sequence is expected to block specifically and selectively the functions of the viral Tat proteins without interfering with the biological functions of any of cellular proteins.

Peptide sequences that bind to a large range of ligands, such as membrane receptors, intracellular proteins and monoclonal antibodies, are usually identified following the screening of either phage-display peptide or chemical-combinatorial libraries. In most cases, the selected peptides possess a low binding affinity to their target, which is very close to that observed in the present work for NTP8. For example, peptides that bind specifically to human serum albumin (HSA) were identified using a chemical-combinatorial cyclic peptide phage-display library. Most of the corresponding synthetic peptides obtained bound to the HSA with micromolar affinity, with some possessing a Kd of up to 300 μM (Sato et al.). In addition, several peptides that bind specifically to human P-selectin protein have been recently identified following the use of a phage-display library. Most of the obtained peptides displayed low (micromolar) binding affinity (Appeldoorn et al., 2003). Similarly, anti-monoclonal an-

Table 2
Human proteins with significant sequence similarities to NTP8

Protein Name	Sequence Similarity
Transcriptional adapter 2 (ADA-2)	NTP8: ^{4}DDPAKAAFDSL^{14} **DDP + FDSL** ADA-2: ^{150}DDPPRPTFDSL^{160}
Leukocyte surface antigen CD53 (Cell surface glycoprotein CD53)	NTP8: ^{4}DDPAKAAFDSLQA^{16} **D+ KAA+DS+Q+** CD53: ^{128}DNSTKAAWDSIQS^{140}
Ras-related protein (Rab-26)	NTP8: ^{8}KAAFDSLQASA TE^{20} **KA+FD++QA TE** Rab-26: ^{83}KASFDNIQAWLTE^{95}
SmcX protein (Xe169)	NTP8: ^{2}EGDDPAK^{8} **EGDDPA+** Xe169: ^{1468}EGDDPAR^{1474}

The NTP8 sequence is given at the top, and the corresponding protein at the bottom of the alignment. Identical amino acids between the two sequences are marked in bold.

tibody (3-E7) peptides, identified by phage-display library, exhibit binding affinities with a Kd between 0.35 and 8.3 μM (Cwirla et al., 1990). For therapeutic purposes, an antagonist peptide, in addition to being metabolically stable, should display a binding affinity in the nanomolar range.

In our laboratory, peptide cyclization was used to obtain peptides with improved selectivity, better binding affinity and enhanced metabolic stability. This was demonstrated, for instance, with cyclic peptides that functionally mimic the NLS domains of the HIV-1 matrix and Tat proteins (Friedler et al., 1998). Currently, we are attempting to obtain a library of cyclic peptides containing the NTP8 sequence. It is our contention that cyclic peptides showing high binding affinity to the Tat-NLS sequence will be identified and selected from such a library (Gilon et al., 1991). In addition, attempts are currently made in our laboratory to construct the chemical-combinatorial peptide libraries, based on the sequence of the NTP8 peptide. Potentially the use of this library should allow the identification and selection of new group of peptides that will interact with target Tat-NLS domain with higher affinities than the NTP8 peptide.

The NTP8 sequence may also be instrumental in revealing cellular proteins with similar sequences that could potentially interact with HIV-1 Tat and thus regulate its activity. Initial screening of the NTP8 sequence using InterPro (EMBL-EBI) software showed that motifs present in this sequence are not abundant in known eukaryotic proteins. Using BLAST software (NCBI), several similar sequences were found in four human proteins (for sequence alignment, refer to Table 2): leukocyte surface antigen CD53 (cell-surface glycoprotein CD53), transcriptional adapter 2-like (ADA2-like) protein, SmcX protein (Xe169 protein) and the Ras-related protein, Rab-26. Although we could not find any connection between SmcX and Rab-26 proteins and HIV-1 infection, the other two proteins with similarities to NTP8 seem to be linked to HIV-1: CD53 displayed on the surface of lymphocytes (Tomlinson et al., 1995), one of the major HIV-1 targets in the human body, as well as ADA-2, a transcriptional activator expressed in dendritic cells (Tsuboi et al., 1995).

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