A detailed study of nuclear import mediated by the HIV-1 Tat peptide (YGRKKRRQRRR\textsuperscript{57}, Tat\textsubscript{RRR}) is reported. Fluorescence-based measurements, calibration of protein concentrations, and binding assays are exploited to address the physicochemical mechanisms of Tat peptide recognition by the classical Importin α (Impα) and Importin β (Impβ) receptors both \textit{in vitro} and in intact cells. We show that Tat\textsubscript{RRR} is an unconventional nuclear localization sequence (NLS) that binds directly to both Impα and Impβ carriers in the absence of competitors (\textit{in vitro}), while this property is silenced in the actual cellular environment. In the latter case, Impα/β-dependent nuclear import can be successfully restored by replacing the ‘RRR’ stretch with ‘GGG’. We apply a recently developed method to determine quantitatively Tat\textsubscript{GGG} affinity for each receptor. Based on these results we can rationalize previous controversial reports on Tat peptide and provide coherent guidelines for the design of novel intracellular targeting sequences.

Cellular compartments are the defining feature of eukaryotic cells. The nucleus is surrounded by a double membrane called the nuclear envelope (NE) which separates the genetic material and transcriptional activity from the translational and metabolic processes of the cytoplasm. Communication between nucleus and cytoplasm is mediated by nuclear pore complexes (NPCs) (1,2), large macromolecular assemblies that punctuate the NE. NPCs form a selective barrier that inhibits translocation of large cargo molecules (>40kD) (3), unless they possess specific targeting signals called nuclear localization sequences (NLSs). The best characterized NLSs consist of either one (monopartite) or two (bipartite) stretches of basic amino acids (4,5). Monopartite NLSs are exemplified by the SV40 large T antigen NLS (126PKKKRRV\textsubscript{132}) and bipartite NLSs are exemplified by the nucleoplasmin NLS (155KRPAATKKAGQAKKKK\textsubscript{170}). These sequences are specifically recognized by a heterodimer of proteins, named Importin α (Impα) and Importin β (Impβ) (6). Impα binds the NLS specifically (7), whereas Impβ both enhances the affinity of Impα for the NLS (8) and mediates the transfer of the cargo-Impα complex across the NPC (9). The cargo is then released in the nucleus upon RanGTP binding to Impβ (10). Because of the surfeit of known classical NLS-containing proteins, it is assumed that this pathway is the most prevalent in the cell; yet, to date no studies have empirically established the proportion of cargoes imported via this mechanism. Furthermore, there is evidence that NLS sequences with unconventional nuclear import properties may
exist, most of which are derived from viral proteins. Among these, we have been interested for a long time in the human immunodeficiency virus type 1 (HIV-1) Tat protein. Tat is an unusual transcripational transactivator that attaches to cell surface heparan sulfate proteoglycans (11), enters cells by endolysosomal pathways (12), reaches the nucleus (13), and dramatically enhances the processivity of transcription directed by the viral long terminal repeat (LTR) promoter element (14,15). Indeed, the NLS properties of Tat are commonly ascribed to the stretch YGRKKRRQRRR (16,17) (in the following: TatGGG). Notably, the same sequence was also shown to be responsible for the cell-penetrating properties of the full-length protein (for a review see (18)) and for its RNA-binding specificity (19-21). As mentioned above, several reports ascribe to Tat NLS (and homologous viral sequences) novel nuclear import properties, albeit with contrasting results. In particular, Efthymiadis et al. (16) reported that the Tat NLS is able to mediate nuclear import in vitro in the absence of both Impα and Impβ, through binding to nuclear components. In turn, Truant and Cullen (17) observed that Tat NLS directly interacts with Impβ but not Impα in vitro and showed that Impβ is both necessary and sufficient for the nuclear import of Tat into isolated nuclei. Contrary to these in vitro experiments, we recently demonstrated that passive diffusion is the dominant mechanism of Tat peptide-mediated nuclear transport in live cells (22). This apparent paradox is accounted for by the overwhelming binding affinity of the C-terminal ‘RRR’ stretch towards negatively charged biomolecules (e.g. RNAs) that hinders Tat-peptide interactions with the transport machinery. Indeed, the NLS properties of Tat can be recovered in engineered mutants where the ‘RRR’ stretch is substituted by other motifs (e.g. ‘GGG’; sequence: YGRKKRRQGGG, TatGGG in the following) (23). Overall, however, the molecular details of the nuclear import process mediated by wild-type and mutant Tat NLS remain elusive. We recently established a novel and straightforward method that combines fluorescence lifetime imaging microscopy (FLIM) and fluorescence recovery after photobleaching (FRAP) with in vivo calibration of protein concentrations, to gain access to both the thermodynamic (binding specificity and affinity) and kinetic (import rate) details of the nuclear transport process in intact cells (24). The broad applicability of the method was demonstrated for the interaction between NLS of SV40 and the transport receptor Impα (24). Here we extend this quantitative approach to the study of wild-type and mutant Tat NLS interactions with the classical import carriers Impα, Impβ, and their dual complex. We show that TatGGG is not able to establish interactions with either Impα or Impβ in the intact cellular environment, in keeping with our previous results (22,23). Conversely, we demonstrate that the TatGGG mutant binds directly to both Impα and Impβ. Note that the conventional NLS from SV40 can establish direct interactions solely with Impα (activated by Impβ). Finally, by a complementary in vitro binding assay, we find that in the absence of competitors (i.e. intracellular cytosolic and nuclear factors) TatGGG does bind to Impα and Impβ. Overall, these results indicate that TatGGG is characterized by a non-classical NLS that is silenced in the cellular environment but can be easily observed in vitro (in absence of competitors) or restored in vivo in engineered mutants (TatGGG). We believe that these findings rationalize the picture of previous controversial results on Tat peptide nuclear import properties, and can provide the basic knowledge for the rational design of localization sequences better tailored to the nucleus.

Experimental procedures

Plasmids and cell culture- Plasmids expressing the mCherry-tagged NLSsv40-TatGGG and TatGGG sequences were obtained by subcloning starting from their EGFP-tagged counterparts, previously described (22). The cDNA encoding for mCherry obtained from Roger Y. Tsien’s laboratory (25) was amplified by PCR introducing the HindIII and EcoRI restriction sites at the 5’ and 3’ extremities, respectively. These sites were used to replace EGFP with mCherry. TatGAAAARRR-mCherry and TatGAAAAGGG-mCherry mutants were obtained by site directed mutagenesis using a QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene). In both constructs, the first moiety of Tat sequence MYGRKKRRQ was substituted with MYGRAARRR. In order to introduce the two mutations the following primer (Invitrogen) was used: 5’ ATGTATGGCACGGCGGCGCGGAGACAG

[2]
3'. The antisense primer has the respective reverse complementary sequence. The plasmid encoding for the EGFP-tagged Importinα (mouse full-length mNPI2) was kindly provided by Yoshihiro Yoneda (Department of Frontier Biosciences, Osaka University, Japan) (26). The plasmid encoding for the EGFP-tagged human Importin β was kindly provided by Marilena Ciciarello (Institute of Molecular Biology and Pathology, National Research Council, Rome, Italy) (27). CHO-K1 were purchased from American Type Culture Collection (CCL-61 ATCC) and were grown in Ham’s F12K medium supplemented with 10% of fetal Bovine Serum at 37°C and in 5% CO₂. Transfections were carried out by using Lipofectamine Reagent (Invitrogen) according to the manufacturer’s instructions. For live imaging, ~10⁵ cells were plated 24h before experiments onto 35-mm glass bottom dishes (WillCo-dish GWSt-3522). For energy depletion measurements, cells were incubated in medium supplemented with sodium azide and 2-deoxy-D-glucose, as described elsewhere (22).

Cloning, extraction and purification of recombinant proteins- Importin β was amplified by using the polymerase chain reaction (PCR) and ligated to pGEX-6P1 vector into BamHI and NdeI sites. Importin α was subcloned into pGEX-6P1 vector into EcoRI and SalI sites. Expression of Importin α and Importin β recombinant proteins were induced in the E.Coli BL21 DE3 strain (Invitrogen) growing in the log-phase upon treatment with 1 mM isopropyl-B-D-galactoside for 14 h at 20°C. Bacteria expressing recombinant proteins were recovered by centrifugation, re-suspended in TE lysis buffer (50mM Tris-HCl pH 8.3, 1mM EDTA, 2mM DTT, 500 mM NaCl, 1 mM PMSF and protease inhibitors) and lysed on ice by sonication. The resulting supernatant was incubated with glutathione-agarose beads at 4°C with gentle rotation. cDNA encoding for TatRRR-EGFP, TatGGG-EGFP, NLS₅ᵥ₅₄-EGFP and EGFP (22) were cloned by PCR into pASK-IBA33plus HIS-Tag vectors (IBA vectors). Protein expression is induced upon addition of 200 μg anhydrotetracycline per 1 liter of E.Coli shaking culture (A₅₅₀ nm 0.5). Purification of 6xHistidine-tag proteins was performed according to standard protocols by using gravity flow Ni-NTA Superflow columns (IBA BiotagTechnology).

In vitro protein-protein binding assays- First, 6XHis-tagged fusion proteins were incubated with glutathione-agarose beads for 1 hour at 4°C to avoid aspecific binding of fusion proteins to the matrix. GST-tagged Importin α and GST-tagged Importin β were incubated with stoichiometric amount of 6XHIS-tagged fusion proteins (EGFP-HIS tag, NLS₅ᵥ₅₄-EGFP-HIS tag, TatRRR-EGFP-HIS tag, TatGGG-EGFP-HIS tag) in IP buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 20 mM NaF, 10% glycerol, 1% Nonidet P-40, and protease inhibitors) at 4°C for 2 hours. Then beads were washed four times with IP buffer and incubated with 100 μl of 4× SDS gel loading buffer at 95°C for 5 min. Proteins were analyzed by SDS-PAGE and Western blotting. Filter was incubated with anti-GFP monoclonal antibody (JL-8, Clontech, Mountain,View, Ca). Purified proteins were also analyzed by Comassie blue staining.

Fluorescence microscopy and concentration analysis- Cell fluorescence was measured using a Leica TCS SP2 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany) interfaced with an Ar laser for excitation at 488 nm, and with a HeNe laser for excitation at 561 nm. Glass bottom Petri dishes containing transfected cells were mounted in a temperature-controlled chamber at 37°C (Leica Microsystems) and viewed with a 40x 1.25 NA oil-immersion objective (Leica Microsystems). Images were collected at low excitation power and monitoring emission by means of the acousto-optical beam splitter (AOBS) detection system of the confocal microscope. The following collection ranges were adopted: 500-550 nm (EGFP) and 580-650 nm (mCherry). The global concentrations of intracellular EGFP- and mCherry-linked proteins were determined by using the synthetic adduct Fluorescein-Glycine, as thoroughly described in a previous publication (24).

FRAP experiments- Each FRAP experiment started with a four-time line-averaged image (pre-bleach) of the cell followed by a single-point bleach (non-scanning) near the center of the nucleus with laser pulse at full power to photobleach most of the nuclear fluorescence.

[3]
Fluorescence recovery was measured by starting a time-lapse acquisition within few milliseconds after bleaching, with the imaging settings described above. Hence, under the assumption of fluorescence proportionality to concentration, the collected FRAP curves in both compartments were fitted to a mono-exponential equation:

$$F(t) = F^\infty + (F^0 - F^\infty) \cdot e^{-t/\tau} \quad [1],$$

where superscripts 0 and $\infty$ label the fluorescence intensity collected at time zero and asymptotically after bleaching, respectively. Fluorescence values were normalized by the signal of the entire cell at the same time to correct for bleaching caused by imaging and by pre-bleach fluorescence, in order to verify the presence of an immobile fraction (IF) of fluorescent molecules within the nucleus. As described in (24), the excess flux of cargo towards the nucleus solely due to active transport ($\Phi_{C\rightarrow N}$), the concentration of cargo molecules bound to the import carrier ([NLS:Imp]) in the cytoplasm, and the nuclear envelope permeability ($P_X$) are linked by the equation:

$$\Phi_{C\rightarrow N} = [\text{NLS:Imp}] \cdot (v_{C\rightarrow N} - P_X), \quad [2]$$

where $v_{C\rightarrow N}$ ($\mu^3/s$) is the maximum rate for active transport towards the nucleus (i.e. the rate achievable when all the cargo molecules are bound to import carriers). Thus, the calculated $\Phi_{C\rightarrow N}$ was plotted against the cytoplasmic cargo concentration ($C_{\text{NLS/Tat}}$) for each cell. Finally, if we assume a single binding equilibrium between the NLS/Tat-cargo and the import carrier, [NLS:Imp] can be expressed as a function of $C_{\text{NLS/Tat}}$, the global cytoplasmic concentration of import carriers ($C_{\text{Imp}}$), and the binding dissociation constant ($K_D$), according to:

$$[\text{NLS:Imp}] = \frac{1}{2}(C_{\text{Imp}} + C_{\text{NLS/Tat}} + K_D^*) \left[ 1 - \frac{1}{2}((C_{\text{Imp}} + C_{\text{NLS/Tat}} + K_D^*)^2 - 4C_{\text{Imp}}C_{\text{NLS/Tat}})^{1/2} \right] \quad [3]$$

In order to recover the biochemically relevant parameter $K_D^*$, we fitted the $\Phi_{C\rightarrow N} \text{ vs } C_{\text{NLS/Tat}}$ curve with Eqs. 2 and 3, setting $C_{\text{Imp}}=1\mu M$ (see (24) for more details).

**FLIM measurements** - FLIM measurements were performed illuminating the sample with a 468-nm pulsed laser diode at 40 MHz repetition rate. Fluorescence emission was detected by means of fast photon-counting heads (H7422P-40, Hamamatsu) and Time-Correlated Single Photon Counting (TCSPC) electronics (SPC-830, Becker & Hickl, Berlin, Germany) at 500-540 nm (bandpass filter 510AF23, Omega Optical, Brattleboro, VT). Measurements were performed in living cells with the confocal system previously described with a 40x oil immersion objective (Leica Microsystems). Additional measurements were carried out using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany) coupled to a PicoQuant single-molecule detection module. Laser power was adjusted to yield photon-counting rates of about $10^5$ cps. Fluorescence decay was analyzed by the SP-Image-Software package. TCSPC-detection was used to generate a lifetime map by fitting the fluorescence-decay curve in each pixel of the image. We used EGFP as the donor fluorophore (high brightness and photostability and monoeponential lifetime) fused to transport carriers (Imp$\alpha$ and Imp$\beta$) and mCherry as the acceptor (fast maturation, large absorption, and high photostability) fused to the candidate localization sequences (NLS, Tat$_{\text{GGG}}$, and Tat$_{\text{RRR}}$). Fluorescence decay curves of biological samples containing only isolated donor molecules (i.e. donor alone or in the presence of a non-interacting acceptor) were fitted within a monoeponential decay model. The result of the fitting procedure is thus a single fluorescence lifetime ($\tau_D$), characteristic of that donor form. When a mix of unbound and bound donor molecules was present (i.e. donor in presence of an interacting acceptor), lifetime data were fitted to a bi-exponential decay law:

$$F(t) = X_B \cdot e^{-t/\tau_D} + X_F \cdot e^{-t/\tau_F}, [4]$$

where $X_B$ and $X_F$ ($X_B+X_F=1$) are the amplitude coefficients corresponding to the individual lifetime components of bound ($\tau_B$) and free donor molecules ($\tau_F$). We set $\tau_F$ to the calculated value for isolated donor (see above), and analyzed the distribution of the average lifetime $\tau_m$ according to the relation: $\tau_m = (X_B \cdot \tau_B + X_F \cdot \tau_F) / (X_B + X_F)$. A decrease in the $\tau_m$ value highlights the appearance of a fraction of donor molecules bound to acceptor molecules, and capable of FRET. To quantitatively address the equilibrium constant


Subcellular localization of fusion proteins. First, the fluorescent localization chimera constructs were used in this study were individually transfected in CHO-K1 cells and analyzed by confocal microscopy (Fig. 1). EGFP-tagged Impα and Impβ were detected in both the nucleus and the cytoplasm, with a local enrichment on the nuclear envelope (Fig. 1A and 1B). This localization is consistent with their ability to shuttle across the nuclear envelope (28) and bind protein components of the NPC (29). As expected, NLS<sub>SV40</sub>-mCherry protein was predominantly localized in the nucleus (Fig. 1C), owing to the contribution of carrier-mediated active transport, while passively diffusing Tat<sub>RRR</sub>-mCherry is uniformly distributed across NE, with a slight enrichment in nucleolar fluorescence (Fig. 1D). Mutation of the last three arginines into glycines conferred to the Tat peptide the ability to perform active transport: accordingly, Tat<sub>GGG</sub>-mCherry was predominantly localized in the nucleus (Fig. 1E). The behavior of mCherry-tagged localization signals is in keeping with previous results obtained on their EGFP-tagged counterparts (22,23).

Analysis of Impα/β direct binding to Tat peptides in intact cells. As a first test we performed FLIM analysis of cells expressing the donor molecule alone (EGFP-Impα or Impβ-EGFP, as shown in Fig. 2A and 2B). As expected, the obtained lifetime decays are well fitted to a monoexponential function (Experimental Procedures) yielding the characteristic decay constant of the unquenched donor (τ<sub>D</sub> = 2.56 ± 0.03 ns for EGFP-Impα, 2.57 ± 0.03 ns for Impβ-EGFP, mean ± SD for N=12 cells). Subsequently, we measured EGFP lifetime in cells co-expressing EGFP-Impα and NLS<sub>SV40</sub>-mCherry (Fig. 2C). We quantitatively addressed this interaction in a previous publication (24): in this context, it is displayed as a control for the classical import pathway through the adaptor carrier Impα. As expected, two exponential components (Eq. 4) are necessary for a satisfactory fit of the EGFP lifetime decays (Fig. S1), revealing the appearance of a fraction of NLS<sub>SV40</sub>-bound Impα undergoing FRET (Fig. 2C). The average lifetime values measured show FRET occurrence in all analyzed cells (τ<sub>m</sub>=2.37± 0.13 ns, N=45). An analogous set of measurements on cells co-expressing Impβ-EGFP and NLS<sub>SV40</sub>-mCherry (Fig. 2D), yielded no detectable FRET signal in N=24 analyzed cells (accordingly, EGFP lifetime was adequately fitted by a monoexponential function, τ<sub>D</sub>=2.55 ± 0.13 ns, see also example in Fig. S1). This result confirms that this classical monopartite NLS does not bind directly to Impβ: in this case, in fact, the transfected Impβ-EGFP and NLS<sub>SV40</sub>-mCherry can form a ternary complex with the endogenous Impα. According to the molecular model, Impα binds directly to NLS<sub>SV40</sub>-mCherry while Impβ-EGFP in turn binds to the IBB of Impα. Note that FRET efficiency varies nonlinearly with the distance between fluorophores: thus we can argue that the adaptor protein Impα leads to a distance between tagged NLS<sub>SV40</sub> and Impβ in the ternary complex that strongly reduces FRET efficiency. The same FLIM-based approach was used to test the capability of Tat-based sequences to bind importins. Consistently with all the results we obtained so far, we found no detectable interaction of Tat<sub>RRR</sub>-sequence with either Impα or Impβ in intact cells (examples are reported in Fig. 2E and 2F), as EGFP decays were adequately fitted by a monoexponential function yielding lifetime values close to that of the donor alone (τ<sub>D</sub>=2.55 ± 0.03 ns, N=12 for Impα and τ<sub>D</sub>=2.55 ± 0.02 ns, N=12 for Impβ). On the other hand, we observed direct interaction of Tat<sub>GGG</sub>-mutant with both Impα and Impβ (Fig. 2G and 2H): FLIM measurements yielded shorter average lifetimes compared to the donor-only sample (τ<sub>m</sub>=2.37 ± 0.11 ns, N=21 for Impα and τ<sub>m</sub>=2.32 ± 0.13 ns, N=26 for Impβ), revealing the presence of a fraction of Tat<sub>GGG</sub>-bound import carriers.
Calculation of TatGGG-Impα and TatGGG-Impβ effective dissociation constant (K_D) from FLIM data. As recently showed for the case of NLS_{SV40}-Impα interaction, affinity values can be extracted from FLIM data (24). Briefly, the K_D value can be calculated if the characteristic lifetime of the complex (τ_B) is known (τ_F is easily derived by measuring the unquenched donor, as shown above). The τ_B values for TatGGG-Impα and TatGGG-Impβ complexes were derived by FLIM measurements in energy-depleted cells (Fig. S2) and combined to the corresponding τ_F values to calculate the molar fraction of bound (X_B) and unbound (X_F) import carriers (Eq. 4, Experimental Procedures). These X_B and X_F values can be used together with intracellular protein concentrations to derive the effective K_D through Eq. 5. For what concerns TatGGG binding to Impα, we found two characteristic ranges of affinity depending on Impα cytoplasmic concentration (Tab. 1 and Fig. 3A). At an EGFP-Impα concentration close to the endogenous value (≤1 µM (30)), we obtained K_D = 26±5 µM, while at high EGFP-Impα expression levels (>10 µM), we found K_D = 175±35 µM. As recently discussed for NLS_{SV40}-Impα (24), the two ranges of TatGGG affinity for Impα can be interpreted as the result of endogenous Impβ intervention in altering the binding affinity. This hypothesis was further strengthened by an in vitro binding assay using purified importins and the recombinant fusion protein NLS_{SV40}-EGFP as a model substrate (Fig. S3). Remarkably, in the case of TatGGG binding to Impβ we found only one characteristic equilibrium constant (K_D = 320±75 µM, Tab. 1). This K_D value shows no significant correlation with the cytoplasmic carrier concentration (Fig. 3B) and confirms the absence of additional partners modulating Impβ affinity towards NLS-endowed molecules.

FRAP analysis of TatGGG nuclear import kinetics and binding specificity. To this point we showed that TatGGG is a functional NLS capable of direct binding to both Impα and Impβ carriers in live cells, albeit with different relative affinities. Here we validate our conclusions by investigating the kinetics of TatGGG nuclear import by FRAP and relating it to the thermodynamics of binding to Importins. Quantitative FRAP analysis of TatGGG-GFP nucleocytoplasmic shuttling was performed as described in the Experimental Procedures section (example in Fig. 4A). By means of our mathematical model we could derive the excess flux of cargo toward the nucleus solely due to active transport (ϕ_{C→N}, mol/s), and plot it in Fig. 4B against the cytoplasmic cargo concentration (C_{cargo}) for each cell. We used the variability of protein expression levels to examine the relationship between cargo concentration and import fluxes. As previously demonstrated (24) and showed here by the green dots in Fig. 4B, NLS_{SV40}-GFP import fluxes follow a simple linear relationship with respect to the available cytoplasmic cargo concentrations up to 10-15 µM of NLS_{SV40}-GFP, where they begin to level off, reaching a plateau for high cargo concentrations. Fitting the ϕ_{C→N} plot to Eqs 2-3 (Experimental Procedures) yields an estimate of the maximum rate for active transport toward the nucleus (ϕ_{C→N}~300 µm/s) and, in the case of NLS_{SV40}, the binding dissociation constant to Impα (K_D ~16 µM). Remarkably, replacement of NLS_{SV40} with TatGGG leads to a similar ‘saturation-like’ behavior but to much higher maximum rates of cargo delivery to the nucleus (ϕ_{C→N}~1000 µm/s; compare plateau levels of red and green dots in Fig. 4B). This evidence points at the presence of a different molecular mechanism for TatGGG-driven import into the nucleus, relying on two Importins rather than just one. Note that the difference between TatGGG and NLS_{SV40} becomes particularly evident for cargo concentrations above ~50 µM. On the basis of the affinities for import carriers calculated by FLIM, we know that this behavior can be linked to TatGGG direct binding to Impβ: above ~50 µM cargo concentration, in fact, we can assume that TatGGG binding to Impα (K_D ~26 µM, by FLIM) almost reached saturation, while its binding to Impβ (K_D ~320 µM, by FLIM) starts to play a role in the nuclear import process. We confirmed this hypothesis by fitting TatGGG import fluxes above ~50 µM cargo concentration: we obtained K_D = 285 ± 45 µM (Fig. S4), in keeping with FLIM. In Fig. 4C we show that the purported additional interaction of TatGGG with Impβ is effectively leading to functional transport: TatGGG-GFP nuclear accumulation above ~50 µM cargo concentrations is still sustained (K_{eq} close to 2), while NLS_{SV40}-GFP distribution is almost uniform in the cells (K_{eq} drops to ~1 above 50 µM cargo concentration). Furthermore, in Fig. 4D we show that sequential addition of arginine residues to TatGGG decreases the relative affinity
DISCUSSION

The thorough understanding of any signal-dependent nuclear import mechanism requires a quantitative analysis of both the thermodynamic and kinetic aspects of the phenomenon. To this end we recently presented a method that combines FLIM and FRAP measurements with protein concentration calibration, and showed its application to the analysis of the well-known NLS\textsubscript{SV40}:Imp\textalpha interaction (24). Here we apply the same approach to the study of Tat-peptide-mediated nuclear transport. Motivation for this study is our demonstration that the mechanism driving Tat\textsubscript{RRR} nuclear permeation in live cells is passive diffusion (22), a result contrasting previous \textit{in vitro} studies that suggest that active processes are involved (16,17). We recently linked this discrepancy to the observation that the first eight residues of Tat peptide (YGRKKRRQ) can indeed operate as a NLS in engineered mutants (e.g. Tat\textsubscript{GGG}), but the remaining three arginine residues (RRR) hinder active transport by promoting binding to intracellular moieties, including RNAs (23). Accordingly here we show that mutation of the purported NLS stretch of Tat leads to inhibition of active import (i.e. inhibition of Importin-binding capabilities) (Fig. S5). Which specific nuclear import carriers are potentially involved if that stretch is not altered remains, however, an open question. In this article we combine FLIM microscopy and protein concentration calibration in order to directly monitor Tat peptide-importin interactions and measure the corresponding effective dissociation constant (K\textsubscript{D}) in the actual cellular environment. In keeping with all our previous results in live cells, the wild-type Tat\textsubscript{RRR} sequence shows no detectable interaction with importins. On the contrary, we find that the mutated Tat\textsubscript{GGG} sequence is a direct target of both Imp\textalpha and Imp\textbeta. It is worth noting that the K\textsubscript{D} of Tat\textsubscript{GGG}:Imp\textalpha binding is dependent on Imp\textalpha expression level, analogously to what we observed for the NLS of SV40 (24). This effect is a consequence of the fact that endogenous Imp\textbeta can modulate this affinity through direct binding to the auto-inhibitory IBB domain of Imp\textalpha (8,24). On the contrary, the K\textsubscript{D} value of Tat\textsubscript{GGG}:Imp\textbeta binding is not dependent on Imp\textbeta expression level, as expected for a non-mediated interaction. Thanks to FRAP experiments we obtained independent proof of Tat\textsubscript{GGG} transport mechanism. By measuring Tat\textsubscript{GGG} import rate as
a function of cargo concentration, in fact, we recovered a saturation behavior markedly different from that of the classical NLS of SV40. In particular, the much higher Tat\(_{GGG}\) import rates suggest the presence of a different molecular mechanism of transport, that we argued relies on two importins rather than just one. Accordingly, fitting FRAP data to our model of nucleocytoplasmic shuttling revealed an additional (low-affinity) interaction of Tat\(_{GGG}\) with the import machinery (i.e. with Imp\(\beta\), as suggested by FLIM). This interaction proved to be functional, as showed by the sustained nuclear accumulation of Tat\(_{GGG}\) at high cargo concentrations (above 50\(\mu\)M), compared to the NLS\(_{SV40}\) case. Furthermore, the FRAP assay was used to test the effect of addition of arginines to Tat\(_{GGG}\); besides showing the expected decrease of affinity for the import machinery, our results suggest that all the Tat mutants tested share the same import mechanism. We thus speculate that Tat\(_{GGG}\) shares Tat\(_{RRR}\) properties and that these are merely progressively unveiled by arginine substitution by restoring its capability to bind importins. Finally, we emphasize that the FRAP assay validates FLIM results in the absence of Imp\(\alpha/\beta\) overexpression. This in turn discounts the possibility that many other cellular importins bind Tat peptides (perhaps with high affinity) and thereby mediate transport under conditions when Imp\(\alpha/\beta\) are not overexpressed, since this would produce a detectable effect on the slope of \(\phi_{C-N} vs C_{cargo}\). Our hypothesis of a dual functionality of Tat peptide sequence implies that the importin-binding capability observed for Tat\(_{GGG}\) in living cells be fully recovered for Tat\(_{RRR}\) in vitro. In this latter case the absence of cellular components would make the ‘RRR’ stretch irrelevant and let the ‘YGRKKRRQ’ domain operate as a NLS. This prediction was tested and confirmed based on an in vitro binding assay. We observed that the wild-type Tat peptide can function as a NLS with unconventional properties, since it is direct target of both Imp\(\alpha\) and Imp\(\beta\). Interestingly, we found that Tat\(_{RRR}\) binds Imp\(\alpha\) and Imp\(\beta\) with comparable affinity, while Tat\(_{GGG}\) shows a clear preference for Imp\(\alpha\). While the latter result is consistent with the data reported in living cells (dissociation constants calculated by FLIM), the former reveals that the ‘YGRKKRRQ’ and ‘RRR’ domains act cooperatively in determining importin-binding specificity and affinity (as we already demonstrated for the complementary binding to intracellular moieties (23)). The observation that Imp\(\alpha\) directly contributes to Tat peptide transport to the nucleus is new but somewhat expected, as it was recently showed that the ‘KKRR’ domain is widely conserved as an optimal target of Imp\(\alpha\) (31). We believe that these findings complement previous reports on the Tat peptide properties and lead to a coherent picture on the molecular details of its nuclear import process. More importantly they provide useful knowledge for the rational design and the accurate in vivo testing of a new generation of localization sequences.

REFERENCES

FIGURE LEGENDS

Fig. 1. Subcellular localization of fusion proteins. Confocal images of transfected EGFP-Impα (A), Impβ-EGFP (B), NLS$_{SV40}$-mCherry (C), Tat$_{RRR}$-mCherry (D), Tat$_{GGG}$-mCherry (E). Scale bar: 10μm.

Fig. 2. FLIM analysis of Impα/β direct binding to nuclear localization signals. (A, B) Intensity image (grey), lifetime image (color), and lifetime distribution histogram (graphs) of EGFP-Impα and Impβ-EGFP, respectively. (C, D) Same FLIM analysis shown in (A, B) applied to NLS$_{SV40}$-mCherry co-transfected with either EGFP-Impα or Impβ-EGFP. (E, F) FLIM analysis of Tat$_{RRR}$-mCherry co-transfected with EGFP-Impα and Impβ-EGFP, respectively. (G, H) Tat$_{GGG}$-mCherry co-transfected with either EGFP-Impα or Impβ-EGFP, respectively. Scale bar in all images: 10μm. Monoexponential fit of decay curves is applied in A, B, D, E, and F (τ$_F$ is displayed). Biexponential fit is applied elsewhere (τ$_m$ is displayed).

Fig. 3. Affinity values for Tat$_{GGG}$ interaction with import carriers. (A) The K$_D$* value for Tat$_{GGG}$ interaction with Impα is plotted against the calculated Impα cytoplasmic concentration. Two average values of affinity are clearly distinguishable: K$_D$* ~ 26 μM for low Impα levels (around 1μM), where the endogenous Impβ may play a role in modulating Impα autoinhibition (schematic drawing); K$_D$* ~ 175μM for Impα levels >10μM, where the contribution of endogenous Impβ can be considered negligible. (B) In the case of Tat$_{GGG}$ interaction with Impβ we find a broad distribution of affinity values (plot), but with no clear dependence on Impβ expression levels. This can be explained by the lack of any possible modulation for the direct interaction to Impβ carrier (schematic drawing).

Fig. 4. FRAP analysis of Tat$_{GGG}$-driven nuclear import kinetics. A) Example FRAP measurement conducted on Tat$_{GGG}$-EGFP; representative images are depicted. Scale bar: 10μM. B) Excess active fluxes (φ$_{C\rightarrow N}$, mol/s) are calculated cell-by-cell and plotted against the corresponding cytoplasmic cargo concentration, obtaining the whole population data plot for Tat$_{GGG}$-EGFP (red dots) (here compared to NLS$_{SV40}$-EGFP, green dots). C) Plot of calculated K$_{eq}$ against cargo cytoplasmic concentration. D) φ$_{C\rightarrow N}$ plot for Tat$_{GGG}$, Tat$_{RGG}$, and Tat$_{RRG}$ mutants, showing the decrease in overall affinity (slope of the curve) and the conservation of the maximal import rate allowed (plateau).

Fig. 5. In vitro binding assay. (A) Purified His-tagged proteins composed by Tat$_{RRR}$, Tat$_{GGG}$, NLS$_{SV40}$ sequences fused to EGFP, and purified recombinant Impα and Impβ fused to glutathione-S-transferase. (B) WB filter showing the direct interaction of Tat$_{RRR}$, Tat$_{GGG}$ with Impα and Impβ. The NLS of SV40 was used as a control for the interaction with Impα and not Impβ, while His-tagged EGFP protein was used as a control for the absence of interaction with import carriers.

Tab. 1. Affinity constants derived by FLIM. For what concerns Tat$_{GGG}$ binding to Impα, we found two main ranges of affinities (K$_D$*) depending on the Impα cytoplasmic concentration (≤1μM or >10μM). In the case of Tat$_{GGG}$ binding to Impβ, only one affinity constant was extracted from FLIM data, independently of the carrier cytoplasmic concentration.
<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP-Impα</td>
<td></td>
</tr>
<tr>
<td>(≤1µM)</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>(&gt;10µM)</td>
<td>175 ± 35</td>
</tr>
<tr>
<td>Impβ-EGFP</td>
<td></td>
</tr>
<tr>
<td>(≥1µM)</td>
<td>320 ± 75</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 4

A) Pre-bleach, post-bleach, 40 s, 240 s images.

B) Plot showing $\dot{\phi}_{C-N}$ (mol/s) vs. $C_{cargo}$ (µM) with data points for NLSvivo-EGFP and Tatooo-EGFP.

C) Plot showing $K_{eq}$ vs. $C_{cargo}$ (µM) with data points for Tatooo-GFP and NLSvivo-GFP.

D) Graph showing $\dot{\phi}_{C-N}$ (mol/s) vs. $C_{cargo}$ (µM) with data points for Tatooo-EGFP and other variants.
Fig. 5
Quantitative analysis of tat peptide binding to import carriers reveals unconventional nuclear transport properties
Francesco Cardarelli, Michela Serresi, Alberto Albanese, Ranieri Bizzarri and Fabio Beltram

J. Biol. Chem. published online February 14, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.203083

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/02/14/M110.203083.DC1