Visualization of in Vivo Direct Interaction between HIV-1 TAT and Human Cyclin T1 in Specific Subcellular Compartments by Fluorescence Resonance Energy Transfer*

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Human cyclin T1, a component of the P-TEFb kinase complex, was originally identified through its biochemical interaction with the Tat transactivator protein of human immunodeficiency virus type 1 (HIV-1). Current understanding suggests that binding of Tat to P-TEFb is required to promote efficient transcriptional elongation of viral RNAs. However, the dynamics and the subnuclear localization of this process are still largely unexplored in vivo. Here we exploit high resolution fluorescence resonance energy transfer (FRET) to visualize and quantitatively analyze the direct interaction between Tat and cyclin T1 inside the cells. We observed that cyclin T1 resides in specific subnuclear foci which are in close contact with nuclear speckles and that Tat determines its redistribution outside of these compartments. Consistent with this observation, strong FRET was observed between the two proteins both in the cytoplasm and in regions of the nucleolus outside of cyclin T1 foci and overlapping with Tat localization. These results are consistent with a model by which Tat recruits cyclin T1 outside of the nuclear compartments where the protein resides to promote transcriptional activation.

The human immunodeficiency virus type 1 (HIV-1) transactivator protein Tat is a small polypeptide (86–101 amino acids, according to the viral strains) essential for efficient transcription. The function of Tat is exerted through interaction with several cellular proteins raising some fundamental questions. Does Tat directly interact with its partners inside living cells? Which is the subcellular compartment of these interactions? Are they occurring simultaneously or consecutively? Some of these questions can be successfully addressed by taking advantage of fluorescence resonance energy transfer (FRET) measurements (17), allowing investigation of direct interaction of proteins labeled with optically matched fluorophores. FRET exploits radiationless energy transfer driven by dipole-dipole interaction occurring from a fluorophore (the donor) in the excited state to another fluorophore (the acceptor) when in close proximity; energy transfer is followed by acceptor fluorescence. The presence of FRET indicates actual protein-protein interaction at distances in the range of the FRET length scale, the Förster radius ($R_0$), defined as the distance at which FRET efficiency ($E_T$) is 50%. $E_T$ is defined as the ratio between the sixth power of $R_0$ and the sum of the sixth power of $R_0$ and the sixth power of $R$, $R$ is the actual distance among the donor and the acceptor fluorophores. $E_T$ dramatically decreases as $R$ increases by a fraction of the nanometer (nm) around $R_0$, which is commonly of the order of the nm for many pairs of matched fluorophores (18–20). In particular, $E_T$ reaches 98 and 1.5% for donor-acceptor separations lower than 0.5 $R_0$ and higher than 2 $R_0$, respectively. This implies that simple co-localization of two proteins is not sufficient to yield energy transfer; thus, the presence of FRET is a powerful indicator of physical protein-protein interaction.

Through this interaction, Tat activates HIV-1 transcription by promoting the assembly of transcriptionally active complexes at the LTR by multiple protein-protein interactions (for a recent review, see Ref. 2). Over the last few years a number of cellular proteins have been reported to interact with Tat and to mediate or modulate its activity. These include general transcription factors, among which TBP, TAFII250, TFIIIB, TFIIH (3–7); RNA polymerase II (8); transcription factor Sp1 (9); the transcriptional co-activators and histone acetyltransferases p300/CPB and P/CAF (10–12); and the cyclin subunit of the positive transcription elongation factor complex (P-TEFb), cyclin T1 (13–16).

The finding that Tat biochemically and functionally interacts with several cellular proteins raises some fundamental questions. Does Tat directly interact with its partners inside living cells? Which is the subcellular compartment of these interactions? Are they occurring simultaneously or consecutively? Some of these questions can be successfully addressed by taking advantage of fluorescence resonance energy transfer (FRET) measurements (17), allowing investigation of direct interaction of proteins labeled with optically matched fluorophores. FRET exploits radiationless energy transfer driven by dipole-dipole interaction occurring from a fluorophore (the donor) in the excited state to another fluorophore (the acceptor) when in close proximity; energy transfer is followed by acceptor fluorescence. The presence of FRET indicates actual protein-protein interaction at distances in the range of the FRET length scale, the Förster radius ($R_0$), defined as the distance at which FRET efficiency ($E_T$) is 50%. $E_T$ is defined as the ratio between the sixth power of $R_0$ and the sum of the sixth power of $R_0$ and the sixth power of $R$, $R$ is the actual distance among the donor and the acceptor fluorophores. $E_T$ dramatically decreases as $R$ increases by a fraction of the nanometer (nm) around $R_0$, which is commonly of the order of the nm for many pairs of matched fluorophores (18–20). In particular, $E_T$ reaches 98 and 1.5% for donor-acceptor separations lower than 0.5 $R_0$ and higher than 2 $R_0$, respectively. This implies that simple co-localization of two proteins is not sufficient to yield energy transfer; thus, the presence of FRET is a powerful indicator of physical protein-protein interaction.

Once integrated in the host cell chromosomes, transcription of HIV-1 genes reflects the complex processes that characterize endogenous mammalian gene expression. A critical event that regulates processivity of transcription from the HIV-1 LTR promoter is the phosphorylation of the carboxyl-terminal domain of cellular RNA polymerase II (RNP), an enzymatic modification carried out by different kinase complexes that regulate promoter clearance and counteract the effect of negative transcription elongation.
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EXPERIMENTAL PROCEDURES

Cell Culture—HL3T1 cells, a HeLa derivative containing an integrated HIV-1 LTR driving the expression of a CAT reporter gene, were a kind gift of G. Pavlakis (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD). All other cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle’s medium with glutamax (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, and gentamicin (100 μg/ml) at 37°C in a humidified 95% air, 5% CO2 incubator.

Plasmids—pCDNA3-Tat-BFP and pCDNA3-Tat-EGFP plasmids were generated by polymerase chain reaction cloning of the Tat gene from HIV-1 HXB2c (86 amino acids) fused at its 3′-end to BFP (Quantum, pQB50-FC1) or EGFP (CLONTECH, pEGFP-N1) in pCDNA3 (Invitrogen). Similarly, pCDNA3-BFP-cyclin T1 and pCDNA3-EGFP-cyclin T1 were constructed by polymerase chain reaction amplification of the human cyclin T1 gene and contain BFP and EGFP fused at its 5′-end. Plasmid pCMV-HA-Cyclin T1 contains the epitope for an anti-CA mono- clonal antibody fused at the NH2 terminus of the cyclin T1 cDNA. It was a kind gift of K. A. Jones (Salk Institute, La Jolla, CA). Plasmid p35R-III, containing the HIV-1 LTR upstream of the CAT reporter gene, was a kind gift of J. Sodroski (Dana Farber Cancer Institute, Boston, MA). EGFP-SF2/ASF and Fibrillarin-EGFP were generously provided by T. Misteli (National Institutes of Health, Bethesda, MD).

FRET—Cells were transiently transfected with expression plasmids for Tat and cyclin T1 fused to the different fluorescent proteins by the calcium phosphate method in LabTek II four-chamber glass slides (Nal- gen), p35R-III, containing the HIV-1 LTR upstream of the CAT reporter gene, was a kind gift of J. Sodroski (Dana Farber Cancer Institute, Boston, MA). EGFP-SF2/ASF and Fibrillarin-EGFP were generously provided by T. Misteli (National Institutes of Health, Bethesda, MD).

Visualization of Intracellular Tat-Cyclin T1 Interaction Using FRET—To explore the interaction of Tat with cyclin T1 using FRET, we obtained fusion constructs of the two proteins with either the blue fluorescent protein (BFP) or the enhanced green fluorescent protein (EGFP) (34) (Fig. 1A). This fluorescent protein pair has excitation and emission properties favorable for FRET, since the emission wavelength of BFP partially overlaps with the excitation wavelength of EGFP (35, 36). To ascertain that fusion of the fluorescent proteins did not interfere with the transcriptional functions of Tat or cyclin T1, we studied HIV-1 LTR transactivation in co-transfection experiments. As shown in Fig. 1B, both Tat-EGFP and Tat-BFP were able to transactivate the HIV-1 LTR indistinguishably from wt Tat. Similarly, both EGFP-Cyclin T1 and BFP-Cyclin T1, while inactive per se on LTR transcription (not shown), were able to synergize with wt Tat for transactivation. More important for the application to FRET, both the Tat-EGFP:BFP-Cyclin T1 and the Tat-BFP:EGFP-Cyclin T1 pairs were equally efficient in their synergistic activation of transcription as the corresponding wt proteins.

A first set of FRET experiments was performed by transfection of human HL3T1 cells, a HeLa derivative cell line carrying an integrated LTR-CAT cassette (37), with the constructs expressing the Tat-EGFP:BFP-Cyclin T1 protein pair. FRET image analysis of cells transfected with Tat-EGFP and BFP-Cyclin T1 and controls are shown in Fig. 2A, panels a1 to e4. Panel in row b show the intracellular distribution of fluorescence around 520 nm (these represents the emission wavelength for EGFP detection) under excitation at 480 nm. In these conditions, most cells transfected with Tat-EGFP (panels b1 to b4) showed the characteristic pattern of overexpressed Tat, consisting of diffuse nucleoplasmic fluorescence with intense nuclear staining (38, 39). FRET analysis was performed by illuminating the same cells at 350 nm (to excite BFP) and recording at 520 nm (panels in row c), thus allowing compari-
Fig. 1. Transactivation of HIV-1 LTR by Tat and cyclin T1 fused to EGFP and BFP. A, schematic representation of Tat and cyclin T1 fusion constructs to BFP and EGFP. CMVp, cytomegalovirus immediate early promoter. Positions of the Tat-binding domain and of the PEST sequence in cyclin T1 are indicated. B, transcriptional activity of EGFP and BFP fusion constructs. The experiments were performed by calcium-phosphate transfection of the indicated constructs (Tat plasmids: 100 ng; cyclin T1 plasmids: 1 μg) together with 0.5 μg of a Tat-responsive CAT reporter (plasmid pU3R-III) in Chinese hamster ovary cells. In these cells, Tat transactivation is poorer than in human cells, since the endogenous cyclin T1 gene lacks a critical cystein residue required for high affinity Tat interaction (26, 49, 50). As a consequence, co-transfection of human cyclin T1 greatly enhances Tat activity. CAT activity was measured after 24 h as previously described (51).

Fig. 2. In vivo analysis of the interaction between Tat-EGFP and BFP-Cyclin T1 measured by FRET. A, visualization of FRET in human HL3T1 cells. The plasmid constructs indicated on top of each column were transfected in HL3T1 cells; transfected cells were visualized by transmitted light in Nomarski configuration (panels in row a), by excitation at 480 nm and collection at 520 nm, showing EGFP fluorescence after direct EGFP excitation (panels in row b), and by excitation at 350 nm and collection at 520 nm, showing BFP fluorescence after BFP excitation, indicating FRET (panels in row c). B, quantification of FRET between Tat-EGFP and BFP-Cyclin T1. Fluorescent emission at 520 nm from individual cells transfected with the indicated constructs was recorded after excitation at 350 or 480 nm, and integrated intensities over the whole cell were evaluated. Plotted values (indicated by dots) represent the ratio between these two measurements: higher values indicate more efficient resonant energy transfer between BFP and EGFP. Ten consecutively analyzed cells were considered for each transfection; both their individual fluorescence ratios and their percentile box plot distribution are shown. Horizontal lines from top to bottom mark the 10th, 25th, 50th, 75th, and 90th percentiles, respectively.
As shown in Fig. 4, SC35, the latter visualized by immunofluorescence with a specific antibody. As reported above, a fraction of cells transfected with pcDNA3-EGFP-cyclin T1 (panel a) or with pCMV-HA-Cyclin T1 (panel b); in the latter case, cyclin T1 was visualized with a fluorescein isothiocyanate-labeled anti-HA antibody. B and C, cyclin T1 domains are juxtaposed to SC35 (splicing) nuclear speckles. Human U2OS cells (panels B, a-c) and HL3T1 cells (panels C, a and b) were transfected with pcDNA3-EGFP-cyclin T1; after 24 h, cells were probed with an anti-SC35 antibody and a tetramethylrhodamine isothiocyanate-labeled secondary antibody, followed by visualization of EGFP and tetramethylrhodamine isothiocyanate fluorescence by laser scanning confocal microscopy. Panels in B show both individual EGFP-Cyclin T1 and SC35 fluorescence as well as their merged image; panels in C show only merged images. Green color indicates EGFP, red color, SC35, and yellow color, co-localization of the two proteins. In most cases, cyclin T1 foci juxtapose to SC35 nuclear speckles, with only partial overlap.

These observations prompted us to analyze in more detail the subnuclear localization of cyclin T1. Both EGFP-Cyclin T1 (Fig. 4A, panel a) and a HA-tagged cyclin T1 construct probed with anti-HA antibody (Fig. 4A, panel b) were visualized in a nuclear dotted pattern. Recently reported evidence suggest that these sites of cyclin T1 accumulation inside the nucleus coincide with nuclear speckles (33), which contain pre-mRNA splicing factors and are commonly defined by the localization of the non-RNP protein SC35 (40). To confirm that cyclin T1 was localized in nuclear speckles, we studied localization of EGFP-Cyclin T1 and SC35, the latter visualized by immunofluorescence with a specific antibody. As shown in Fig. 4B for U2OS cells and Fig. 4C for HL3T1 cells, we found that most of the cyclin T1 nuclear foci were actually associated with SC35 speckles, but that only in few cases was their distribution exactly overlapped. These results suggest that cyclin T1 foci are possibly distinct from nuclear speckles, but that in most cases these two domains are spatially juxtaposed.

Tat-Cyclin T1 Interaction in Different Subcellular Compartments—As reported above, a fraction of cells transfected with Tat-EGFP and BFP-Cyclin T1 showed Tat fluorescence distributed in discrete foci instead than diffuse and nucleolar (Fig. 2A, panel b2). Conversely, EGFP-Cyclin T1 in cells co-transfected with Tat-BFP showed a less pronounced dotted pattern and an unusual staining of the nucleolus (compare Fig. 3A, panels b1 and b5, with Fig. 3A, panel b5). These observations indicate a reciprocal influence of the two proteins on their subcellular localization. The issue of Tat relocalization by cyclin T1 was also experimentally tested in a Chinese hamster ovary derivative cell line which stably expresses Tat-EGFP (41). In this cell line, in which Tat is constitutively expressed at low levels, EGFP fluorescence is found diffuse in the nucleoplasm (Fig. 5, panel a). Transfection of these cells with an expression vector for cyclin T1 determined a remarkable re-distribution of Tat-EGFP to a dotted pattern resembling that of cyclin T1 bodies (Fig. 5, panels b-d). These considerations prompted us to measure the interaction between cyclin T1 and Tat in the different subcellular compartments. FRET results for 10 HL3T1 cells transfected with EGFP-Cyclin T1 and Tat-BFP are shown in Fig. 6A. In all cells, FRET highly above average of controls was observed in all analyzed subcellular compartments, including cytoplasm, nucleus, nucleolus, and cyclin T1 foci. Thus, high FRET intensities were observed also in compartments where cyclin T1 does not normally reside such as the nucleolus. This clearly results from its direct interaction with Tat, since fibrillarin, another protein that physiologically accumulates in the nucleolus, scored negative for FRET when tested together with Tat (see Fig. 3B). Interestingly, the highest FRET values were observed in the cytoplasm, suggesting that Tat-cyclin T1 interaction also occurs in this compartment. Inside the nucleus, FRET values were higher in the nucleolus, significantly lower inside the cyclin T1 bodies, and intermediate in the remaining regions (summarized by percentile distribution in Fig. 6B). This is consistent with the recruitment of cyclin T1 out of its nuclear bodies when Tat is expressed inside the cell.
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**DISCUSSION**

The association of HIV-1 Tat with P-TEFb through the interaction with cyclin T1 represents a key event for transcriptional activation of the viral LTR. Although a great deal of information is available on the molecular details of this interaction in **vitro**, little is known on its spatial and temporal correlates in **vivo**. To explore binding between Tat and cyclin T1 inside the cells we exploited fluorescence resonance energy transfer using BFP and EGFP proteins. This fluorescent protein pair has excitation and emission properties favorable for transfer using BFP and EGFP proteins. This fluorescent protein pair has excitation and emission properties favorable for transfer using BFP and EGFP proteins. This fluorescent protein pair has excitation and emission properties favorable for transfer using BFP and EGFP proteins.

FRET in other biological settings (35, 36). To perform the analysis we fused EGFP or BFP to COOH terminus of Tat and to the NH2 terminus of cyclin T1. As shown in Fig. 1A, the fusion proteins performed as well their wild type counterparts in promoting HIV-1 LTR transcriptional activation. FRET experiments were conducted by co-transfecting cells with different pairs of plasmids expressing EGFP and BFP fusion constructs.

FIG. 6. **Subcellular localization of FRET.** **A,** subcellular localization of FRET between Tat-BFP and EGFP-Cyclin T1. Cells were transfected with pcDNA3-Tat-BFP and pcDNA3-EGFP-cyclin T1; after 48 h FRET measurements were carried out in 10 cells by comparing FRET ratios in four different subcellular compartments (C, cytoplasm; N, nucleus; Nu, nucleolus; CF, cyclin T1 foci). Each bar reports the ratio between emissions at 520 nm after cell excitation at 350 and 480 nm; higher values indicate resonant energy transfer between EGFP and BFP. The **horizontal line** indicates average values obtained in 20 control cells expressing Tat-BFP and fibrillarin-EGFP or EGFP-SF2/ASF (average emission ratio: 0.038 ± 0.016); n denotes cells in which nuclear foci were not visible. **B,** box-plot representation of values in panel A. For each subcellular compartment, **horizontal lines** from top to bottom graphically show the 10th, 25th, 50th, 75th, and 90th percentile distribution of values. The **horizontal line at the bottom** shows the average emission of controls, as in panel A. All analyzed cellular compartments showed FRET. This was statistically higher in cytoplasm that in nucleus (p = 0.036); inside the nucleus, FRET was more evident in nucleoli that in cyclin T1 foci (p = 0.009).

αD,350 P,350/αA,480 P,480: In particular, with our experimental settings, P,350/P,480 ~ 0.3 and αD,350/αA,480 ~ 0.5 (42), resulting in a FRET ratio of about 0.15 nA E T. The interaction between Tat and cyclin T1 was quantitatively analyzed by observing fluorescence of a series of individual cells. As shown in Fig. 3B, cells transfected with Tat-BFP plus EGFP-cyclin T1 showed average FRET values of 0.20 ± 0.08, which were about 5 times higher than those expressing Tat-BFP and fibrillarin-EGFP (0.040 ± 0.007) or Tat-BFP and EGFP-SF2/ASF (0.036 ± 0.023; p < 0.001 in both cases). These high FRET signal values imply that the product nA E T between the fraction of EGFPs coupled to a BFP and the FRET efficiency is very close to

FIG. 5. **Overexpression of cyclin T1 relocates Tat.** Chinese hamster ovary cells stably expressing Tat-EGFP (41) were transfected with pcCMV-HA-CycT1. All panels show Tat-EGFP fluorescence. Before transfection, in these cells Tat shows a nucleoplasmic distribution, with exclusion of nucleoli (panel a); after transfection, Tat is redistributed in a dotted pattern (shown for three individual cells in panels b-d).
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