Characterization of HIV-1 Tat proteins mutated in the transactivation domain for prophylactic and therapeutic application

Monica Betti [a], Rebecca Voltan [a], Marco Marchisio [b], Irene Mantovani [a], Chiara Boarini [a], Filomena Nappi [c], Barbara Ensoli [c], Antonella Caputo [a],*

[a] *Department of Experimental and Diagnostic Medicine, University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy*
[b] *Department of Morphology and Embryology, University of Ferrara, Via Fossato di Mortara 64, 44100 Ferrara, Italy*
[c] *Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

Abstract

Previous work from our group showed that genetic immunization of mice with HIV-1 *tat* genes (tat_{22} and $tat_{22/37}$), encoding Tat proteins mutated in the transactivation domain and lacking Tat-transactivating activity, evoke an immune response to wild-type Tat, both humoral and cellular. In the present work we report that the mutated Tat proteins localize within the cells, are released and taken up by the cells in a fashion similar to wild-type Tat. Moreover, the exogenous mutated Tat proteins interfere with the transactivating function of extracellular wild-type Tat. These results support the notion that tat_{22} and $tat_{22/37}$ genes may represent good candidates for the development of an anti-HIV-1 vaccine, especially for HIV-1 infected patients. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Tat protein of HIV-1 is a small molecule of 86–102 amino acids (aa) encoded by two exons. The first exon of 72 amino acids is necessary for the HIV-1 transactivating activity and contains four domains including the amino-terminal (aa 1–21), the cysteine-rich (aa 22–37), the core (aa 38–48) and the basic (aa 49–72) domain. The cysteine-rich region represents the transactivation domain and the basic region contains the nuclear and nucleolar localization signals and the binding site to TAR RNA. In addition, the basic region and the RGD sequence, present in the second exon, are required for the interaction of extracellular Tat with heparan-sulphate proteoglycans and with cell surface molecules of the integrin family, respectively, and mediate uptake of Tat by infected and uninfected target cells [1,2].

The *tat* gene of HIV-1 may represent a component of a prophylactic and/or therapeutic DNA vaccine against AIDS for several reasons. Tat exerts key functions for virus replication and spreading of infection and is involved in AIDS pathogenesis and in AIDS-associated malignancies [1,2]. Tat is released by HIV-1 infected cells [3–5] and is taken up by neighbor cells [3,6,7], including accessory cells, and presented with the MHC class I to CD8+ T lymphocytes [8], as also indicated by our studies in monkeys [9]. Tat is well conserved among different HIV-1 clades [10] and is immunogenic during natural infection. In this respect, several studies indicate that the presence of humoral and cellular immune responses against Tat inversely correlates with the progression to the symptomatic stage of infection [11–16]. The immunogenicity of the HIV-1 wild-type *tat* DNA has been shown in mice [17,18], in monkeys [19] and in humans [20]. In addition, recent experiments showed that immunization with a biologically active wild-type Tat protein of HIV-1 elicits a broad immune response and controls infection with the highly patho-

genic SHIV89.6P to undetectable levels in 5 out of 7 vaccinated monkeys, preventing CD4 + T cell decline and disease onset [9]. Finally, the efficacy of immunization against Tat was also shown in monkeys, vaccinated with live vectors producing SIV Tat in combination with Rev, in which plasma viremia was transient and significantly lower than in control animals, and no cell-associated virus could be detected [21]. Although in these studies no toxic effects nor activation of HIV replication were observed in seronegative or seropositive individuals, respectively, nor in naive [9,19] or in SHIV-infected monkeys [Ensoli et al., unpublished results], however, it cannot be excluded as yet that the constitutive expression of Tat following genetic immunization may determine, in seropositive patients, reactivation of HIV-1 infection or other pathogenic effects associated to Tat. For these reasons in a previous study we characterized in mice the immunogenicity of *tat* genes mutated in cysteine 22 (substituted with a glycine) and cysteine 37 (substituted with a serine) (tat_{22} and $tat_{22/37}$), which lack Tat transactivating activity. The results of those experiments demonstrated that these *tat* mutated genes elicit both humoral and cellular immune responses to wild-type Tat [22]. In addition, sera from mice vaccinated with the *tat* mutated genes neutralized the effect of extracellular wild-type Tat in activating HIV-1 replication [22], as found for sera from Tat-immunized monkeys [9], suggesting that these mutated genes may be considered as candidates for prophylactic and, most of all, for therapeutic intervention against AIDS. Since the cysteine domain of Tat contains seven cysteines that are critical for the formation of intramolecular disulphide bonds, for correct protein folding and for Tat-transactivation [23,24], here we have studied whether disruption of the structural integrity of Tat due to mutations in the cysteine-region affects protein conformation and properties of the *tat* mutated genes and proteins that may be relevant for their prophylactic and/or therapeutic use.

2. Material and methods

2.1. Eukaryotic expression plasmids

The full-length cDNA (258 bp) of the HIV-1 (BH10 clone) *tat* gene (wild-type or mutated in cysteine 22 to glycine and/or in cysteine 37 to serine) were derived from plasmids described previously [25] and cloned into SalI and BamHI sites of the pEGFP-N3 vector (Clontech, Palo Alto, CA) upstream to and in frame with the enhanced green fluorescent protein (EGFP) gene. The recombinant plasmids were sequenced with a commercial kit (Dye Terminator Cycle Sequencing, Perkin Elmer, Forster City, CA) on an ABI 373 DNA automated sequencer (Applied Biosystem, Perkin Elmer

Biosystem). Expression of the fusion proteins was tested in 293 cells (1.5×10^6) after transfection of plasmid DNA (10 μg) using the calcium-phosphate co-precipitation technique [26]. Forty-eight h later, cells were fixed with cold 4% paraphormaldehyde and colored with DAPI (0.5 μg/ml, Sigma, St. Louis, MO). The green signals were observed at a fluorescence microscope (Zeiss Axiophot, Zeiss, Oberkocken, Germany) with a FITC-fluorescence filter set (Zeiss, excitation 450–490 nm, beam splitter 510 nm, emission filter > 510 nm). The nuclear image was observed by DAPI fluorescence with a 397 nm filter. In addition, plasmids (25 or 250 ng) were transfected on to HL3T1 cells (6×10^5) [27], a HeLa derivative containing an integrated copy of the HIV-1 long terminal repeat-chloramphenicol acetyl transferase gene (LTR-CAT) construct, and CAT activity was determined 48 h later as described below.

2.2. Immunodetection of released Tat proteins

293 cells (4×10^6) were transfected with 30 μg of plasmid DNA. A total of 48 h later, proteins were immunoprecipitated from cell supernatants with a rabbit anti-GFP polyclonal serum (5 μl; Clontech) pre-adsorbed to protein A-sepharose (Pharmacia, Uppsala, Sweden) in the presence of protease inhibitors [bestatine (20 μl/ml; Sigma), phosphoramidon (5 μg/ml) and Timp 2 (20 μg/ml) (Roche, Milan, Italy)]. Immunocomplexes were washed with 50 mM Tris pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS, separated onto 10% SDS-polyacrylamide gels and blotted onto nitrocellulose filters (Amersham Corp., Arlington Heights, IL). Filters were incubated in blocking buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% BSA, 0.05% Nonidet-P40) for 1 h at 4°C and with an anti-Tat polyclonal rabbit serum (raised in-house using the MBP-Tat protein as the antigen) for 12 h at 4°C. After extensive washing with 10 mM Tris pH 7.4, 150 NaCl, 0.1% BSA and 0.3% Tween 20, they were incubated with a goat horse-radish peroxidase-conjugated anti-rabbit IgG serum (Sigma) for 1 h at 4°C. The ECL western blotting detection reagent (Amersham) was used to reveal specific reactions.

2.3. Recombinant wild-type and mutated HIV-1 Tat proteins

Tat proteins were expressed in *Escherichia coli* using the pMal-c2 prokaryotic expression vector and purification system according to the manufacturer's instructions (New England Biolabs, Beverly, MA). The full-length cDNA of *tat* (wild-type or mutated) was cloned into EcoRI and BamHI sites of the pMal-c2 vector downstream from and in frame with the *malE* gene, which encodes a maltose-binding protein (MBP),

resulting in the expression of MBP-Tat fusion proteins. Vectors were sequenced as described above. For some experiments the wild-type Tat protein of HIV-1 was expressed and purified in *E. coli* and isolated by high pressure chromatography or heparin-affinity chromatography, as described previously [3,5]. The purified Tat protein was >95% pure as tested by SDS-polyacrylamide gel electrophoresis and had full biological activity as determined by several assays described previously [3,28,29]. To avoid oxidation and loss of biological activity, proteins were stored and handled as described [3,5].

2.4. Immunofluorescence

The dose of recombinant protein was determined in preliminary experiments using increasing amounts of MBP-Tat (5, 12.5 and 25 µg/ml corresponding to 1, 2.5 and 5 µg/ml of Tat, respectively). Since a more intense signal was observed with 25 µg/ml of MBP-Tat protein, this concentration was used in the following experiments. HL3T1 cells (1×10^5) were incubated the MBP-Tat proteins (wild-type or mutated) or the purified Tat (5 µg/ml), and chloroquine (100 µM; Sigma) to prevent lysosomal degradation. In some experiments, heparin (MW 6000; Sigma) was added at 1 µM simultaneously with the proteins. After incubation, cells were washed with phosphate buffered-saline (PBS), fixed with 4% paraphormaldehyde, and treated with 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% Nonidet-P40 and 0.25% gelatin type IV bloom 60 (Sigma) for 30 min at room temperature. Immunofluorescence was carried out with the anti-Tat monoclonal antibody mAb 4B4C4, directed against residues 49–86 of wild-type Tat [30], and with a goat Cy3-conjugated anti-mouse IgG secondary serum (Amersham). For immuno-colocalization studies, HL3T1 cells were incubated for 3 h with the recombinant proteins, fixed and simultaneously incubated with a rabbit anti-Tat polyclonal serum (Intracel Corporation, Issaquah, WA) and an anti-γ-adaptin (AP-1) monoclonal antibody (clone 100/3; Sigma). Immunocomplexes were detected with a goat FITC-conjugated anti-rabbit IgG (Sigma), and with a goat TRITC-conjugated anti-mouse IgG (Sigma). Cells were colored with DAPI and observed at a fluorescence microscope. The green and blue fluorescence were detected as described above. The red fluorescence was observed with 590-nm cut off filter.

2.5. CAT assay

CAT assay was carried out as described [26], using volumes of cell extracts corresponding to equal amounts of proteins. HL3T1 cells (7.5×10^6) were seeded in 60-mm Petri dishes and immediately lipofected with recombinant wild-type MBP-Tat, mutated MBP-Tat, or the MBP protein alone, using the lipofectin reagent (Gibco-BRL), as described previously [31]. CAT activity was determined 16 h later. Alternatively, MBP-Tat was added directly to HL3T1 (5×10^5) cells in the presence of chloroquine, and CAT activity was determined 48 h later. For competition experiments, wild-type MBP-Tat (500 ng/ml corresponding to 100 ng/ml of Tat) was simultaneously lipofected, or directly added to the cells, together with a 5–20-fold molar excess of each MBP-Tat mutated protein.

3. Results

Since it was previously shown that the Tat protein of HIV-1 is released from infected and transfected cells and that extracellular Tat is taken up by neighbor cells [3,6], and considering that mutations in the cysteine-rich region of Tat disrupt disulphide bonds and the structural integrity of the molecule [23,24], we analyzed whether Tat proteins mutated in the cysteine-domain are released from the cells, are internalized by the cells and localize within the cells in a fashion similar to wild-type Tat. Furthermore, the capability of these mutated Tat proteins, which lack Tat-transactivating activity [22], to interfere with functions of extracellular wild-type Tat was determined.

3.1. Release of mutated Tat proteins

Eukaryotic vectors, expressing Tat-EGFP fusion proteins carrying mutation in cysteine 22 and/or 37 of Tat, or expressing the wild-type Tat-EGFP protein, were constructed and transfected in 293 cells. A clear nuclear and nucleolar localization of the mutated Tat-EGFP proteins was detected, similar to that of the wild-type Tat-EGFP, whereas the EGFP protein alone appeared uniformly distributed within the cells without a specific subcellular localization (Fig. 1), indicating that neither mutations in cysteines 22 and/or 37 nor fusion to EGFP affect translocation to the nucleus of the endogenously synthesized Tat proteins. In addition, plasmids were transfected onto HL3T1 cells containing an integrated copy of the reporter vector HIV-1 LTR-CAT. As expected, a lack of transactivation of the reporter gene was observed after transfection of the mutated p*tat*-EGFP plasmids, whereas a dose-dependent transactivation of the CAT gene was induced by the wild-type p*tat*-EGFP vector (not shown). The results of both experiments suggest that plasmids expressed functional Tat-EGFP proteins. Thus, vectors were used for the analysis of the release. After cell transfection, released proteins were searched in culture supernatants by immunoprecipitation with an anti-GFP polyclonal serum followed by western blot analysis of the precipitated proteins with an anti-Tat specific
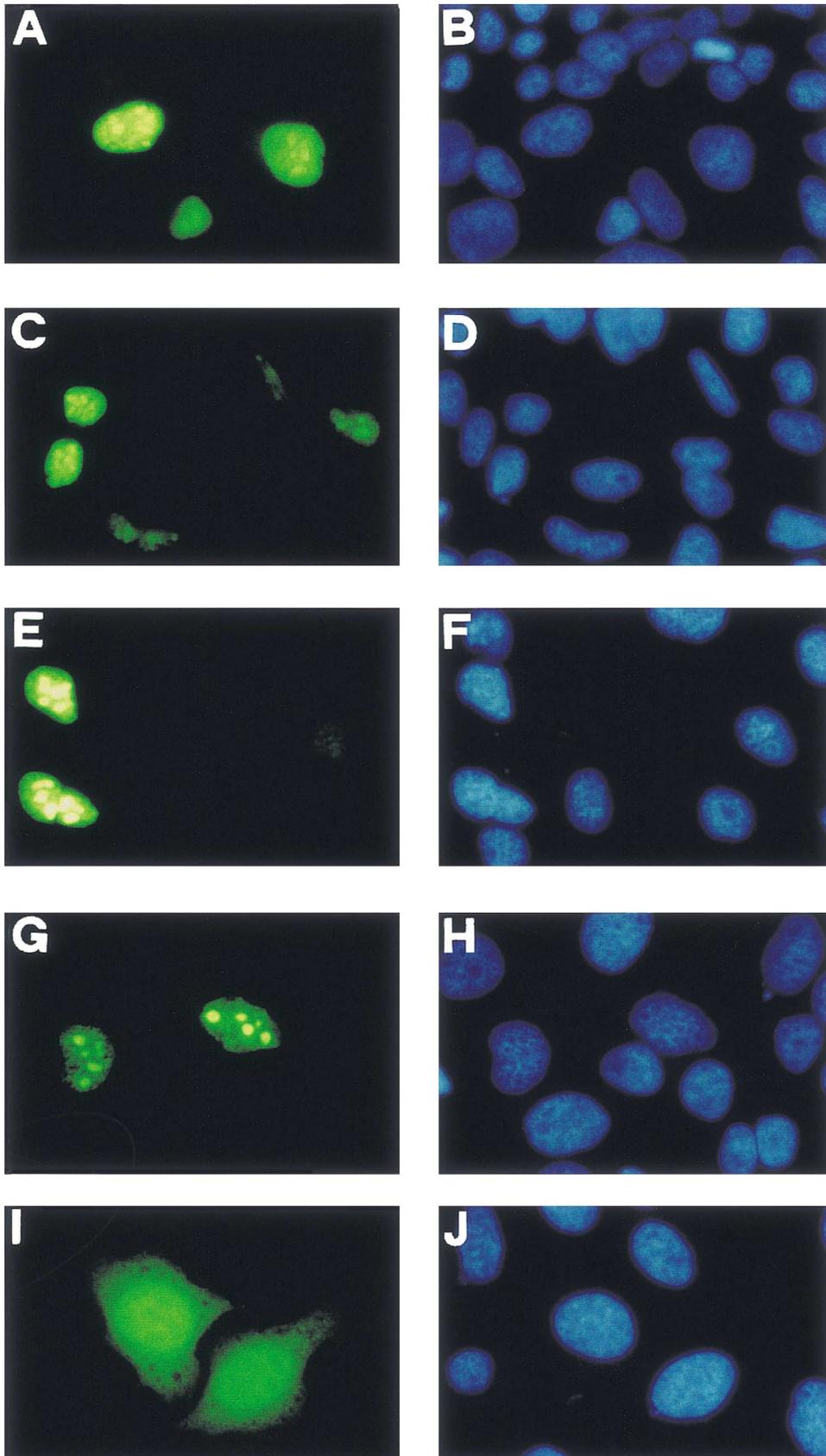
Fig. 1. Intracellular localization of mutated Tat proteins. 293 cells were transiently transfected with 10 μg of plasmid p*tat*-EGFP (A, B), p*tat*$_{22}$-EGFP (C, D), p*tat*$_{22/37}$-EGFP (E, F), p*tat*$_{37}$-EGFP (G, H) or the control vector pEGFP-N3 (I, J), respectively. The EGFP signal (green) was used to localize Tat-EGFP proteins (left panels), whereas the DAPI fluorescence (blue) stains the nuclei (right panels). The same microscopic field is shown in each left and right panel.

serum. A 41 kD band corresponding to the mutated Tat-EGFP fusion proteins was detected in the culture supernatants of cells transfected with each mutated vector (Fig. 2, lanes 1–3). A similar molecular weight band was present in the culture medium of cells transfected with the wild-type p*tat*-EGFP plasmid (Fig. 2, lane 4), whereas the specific band was not detected in the culture medium of cells transfected with the control vector pEGFP-N3 (Fig. 2, lane 5). These results indicate that mutations in the cysteine-domain of Tat do not affect the capability of the protein to be released from the cells.

3.2. Characterization of mutated Tat proteins and analysis of their effect on wild-type Tat-transactivating activity

To investigate whether extracellular Tat$_{22}$, Tat$_{22/37}$ and Tat$_{37}$ proteins are internalized by the cells in a

fashion similar to Tat, recombinant MBP-Tat fusion proteins (MBP-Tat$_{22}$, MBP-Tat$_{22/37}$, MBP-Tat$_{37}$ and MBP-Tat) were produced. As concerned the use of recombinant Tat proteins, it is important to point out that Tat is very labile when exposed to air and light, because of its high content in cysteine residues (seven cysteines from aminoacid 22 to 37), and loses very rapidly its activity in different type of assays [3,5,29]. To this respect, it was shown that even partial oxidation of wild-type Tat induces conformational changes that abolish the capability of the protein to be internalized by the cells [30], likely because the basic and RGD domains of Tat, that mediate Tat uptake [3,5,29,32–36], become hindered and not available for interaction with the cellular receptor. The use of biologically active proteins was thus essential for the internalization studies, and for this reason we first characterized the activity of wild-type and mutated Tat proteins. The activity was evaluated as their capability to trans-activate the
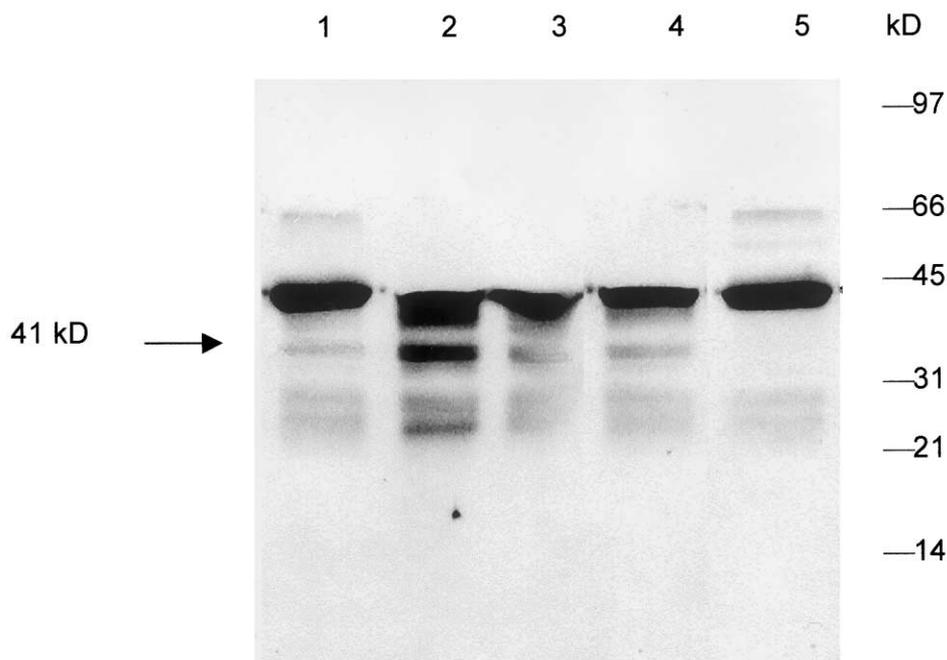

Fig. 2. Release of mutated Tat-EGFP proteins. Culture media of 293 cells transfected with 30 μg of plasmid p*tat*$_{22}$-EGFP (lane 1), p*tat*$_{22/37}$-EGFP (lane 2), p*tat*$_{37}$-EGFP (lane 3), p*tat*-EGFP (lane 4) or the control vector pEGFP-N3 (lane 5) were immunoprecipitated with a rabbit anti-GFP polyclonal serum and analyzed by western blot using a rabbit anti-Tat polyclonal serum. The arrow points to the 41 kD wild-type or mutated Tat-EGFP hybrid proteins; molecular weight markers are indicated on the right.

Fig. 3. Biological activity of mutated MBP-Tat fusion proteins and analysis of their effect on wild-type MBP-Tat transactivating function in HL3T1 cells, containing an integrated copy of the HIV-1 LTR-CAT reporter vector. In A and B proteins were introduced into the cells by lipofection (endogenous system), whereas in C and D proteins were added to the culture medium in the presence of chloroquine (exogenous system). In (A,C), cells were treated with MBP as the negative control (1 μg/ml), each MBP-Tat mutated protein (0.5 μg/ml corresponding to 100 ng/ml of mutated Tat) and increasing amounts of wild-type MBP-Tat (from 0.05 to 2.5 μg/ml corresponding to 10, 50, 100, 250 and 500 ng/ml of Tat, respectively). In (B) and (D) cells were treated with MBP, with wild-type MBP-Tat alone or with wild-type MBP-Tat together with MBP-Tat$_{22}$, MBP-Tat$_{22/37}$, or MBP-Tat$_{37}$. In (B), a 5-fold molar excess of each mutated MBP-Tat protein was used; in (D) a 20-fold molar excess of each mutated MBP-Tat protein was added. The percentage (%) of CAT activity was calculated as [cpm of the acetylated ^{14}C-chloramphenicol/ total cpm of acetylated and unacetylated ^{14}C-chloramphenicol] × 100. Results are the mean of two independent experiments performed with two different batches of each protein. In A-D, HL3T1 cell background corresponds to lane indicated as C of each panel.
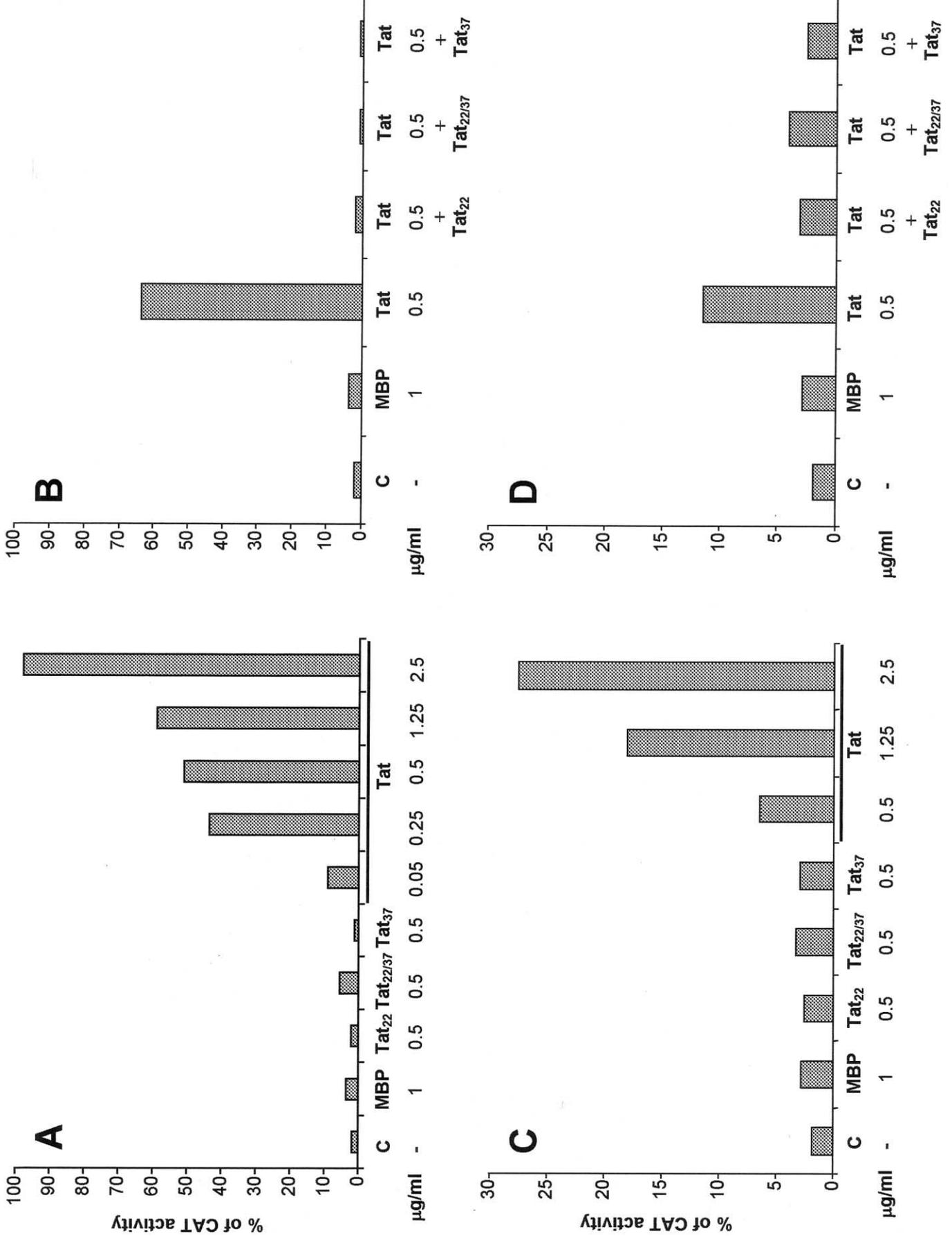
Fig. 3.

HIV-1 LTR promoter in HL3T1 cells by means of two different approaches, lipofection (endogenous system) and direct addition to the culture medium (exogenous system).

The results indicate that, after lipofection (Fig. 3A) or addition to the culture medium (Fig. 3C), wild-type MBP-Tat transactivated in a dose-dependent manner the expression of the HIV-1 LTR CAT-linked gene, indicating that the wild-type protein was not oxidized and was in its active conformation. The discrepancy in values of CAT activity between endogenous and exogenous systems likely depends on the different experimental procedure. Liposomes protect the proteins from proteolytic degradation and, in addition, they force the protein into the cell, allowing all molecules to reach the intracellular compartment and a larger number of those to reach the nucleus. In contrast, after direct addition to the culture medium, Tat may undergo in the extracellular environment some proteolytic cleavage and/or even light oxidation causing a decrease in the number of molecules that interact with Tat cell surface receptors, and accordingly a decrease in the number of Tat molecules that can be taken up by the cells and reach the nucleus. In addition, in the exogenous system, Tat enter the cell in endocytic vescicles [3,7,30], some of which may undergo intracellular proteolytic degradation. To this respect, results show that 0.5 µg/ml of MBP-Tat induced ~ 10–12% of CAT activity in the exogenous system (Fig. 3C), whereas, after lipofection, similar values of CAT activity were induced by a 10-fold lower (0.05 µg/ml) dose of MBP-Tat (Fig. 3A).

As concerned the recombinant mutated proteins, lipofection (Fig. 3A) or direct addition (Fig. 3C) of the MBP-Tat mutated proteins did not induce transactivation of HIV-1 LTR transcription, in a fashion similar to the MBP control protein. Indeed, the percentage of CAT activity was comparable to that of untreated HL3T1 cells (Fig. 3A,B, lane C). Considering that mutations in cysteine 22 and 37 abrogate Tat-transactivating function, this result was expected. However, Tat_{22}, Tat_{37} and $Tat_{22/37}$ contain 6 or 5 cysteine residues, respectively. Therefore, to exclude that we were using oxidized inactive mutated molecules, Tat-competition experiments were carried out. Wild-type MBP-Tat was lipofected or added to the culture medium together with a 5–20 fold molar excess of each mutated Tat protein. These experiments showed that, in both the endogenous (Fig. 3B) and exogenous (Fig. 3D) systems, the mutated proteins interfered with wild-type Tat-transactivating function and inhibited the expression of the reporter CAT gene. However, a higher fold molar excess of mutated Tat proteins was required for Tat 'neutralization' in the exogenous system (20-fold) (Fig. 3D), as compared to the endogenous system (5-fold) (Fig. 3B), suggesting

that mutations in the cysteine-region induces conformational changes of Tat that decrease its binding affinity for the receptor. These results confirm previous data of gene transfection competition experiments [22,25], and accordingly they indicate that the recombinant mutated proteins were biologically active and not oxidized, since they behaved in a fashion similar to the mutated Tat proteins encoded in vivo after DNA transfection. In addition, the results show that, not only intracellular, but also extracellular Tat mutated proteins modulate wild-type Tat transactivation activity. It reasonable to think that modulation of wild-type Tat function in the exogenous system may depend on intracellular interference (as in the endogenous suystem) and /or on extracellular competition of wild-type and mutated Tat proteins for binding to the receptor.

3.3. Cellular uptake of mutated Tat proteins

Internalization and intracellular distribution of the mutated Tat proteins was then analyzed by indirect immunofluorescence. As shown in Fig. 4, when the mutated MBP-Tat proteins were added to the culture medium they entered the cells in a fashion similar to wild-type MBP-Tat and purified Tat, the latter included as the control of the fusion proteins. No difference was observed in the internalization kinetic and in the intracellular distribution of the MBP-Tat mutated proteins compared to wild-type MBP-Tat or purified Tat (Fig. 4). Mutated MBP-Tat proteins localized either in the cytoplasm and/or in the nucleus of the cells, as wild-type MBP-Tat and purified Tat, after 15 min (not shown), 3 (not shown) and 24 h (Fig. 4). An intense fluorescence of punctuate structure resembling endosomes or lysosomes was observed around the nuclear membrane that colocalized with the AP-1 protein (Fig. 5), the major adaptor protein of clathrin-coated vescicles of the trans-Golgi network [37], suggesting that the Tat mutated proteins entered the cells by adsorptive endocytosis as described for Tat [3,7,30]. Furthermore, the uptake of MBP-Tat mutated proteins was blocked by heparin in a fashion similar to wild-type MBP-Tat and purified Tat (not shown). To this respect, it was previously reported for wild-type Tat that heparin interacts with the basic region of Tat and interferes with binding of Tat to the heparan-sulphate proteoglycans of the cell surface blocking its uptake [3,5]. These results, therefore, indicate that conformational changes due to mutations in the cysteine-region do not affect uptake, and that Tat_{22}, $Tat_{22/37}$ and Tat_{37} undergo the same internalization pathway and show identical intracellular distribution as Tat.
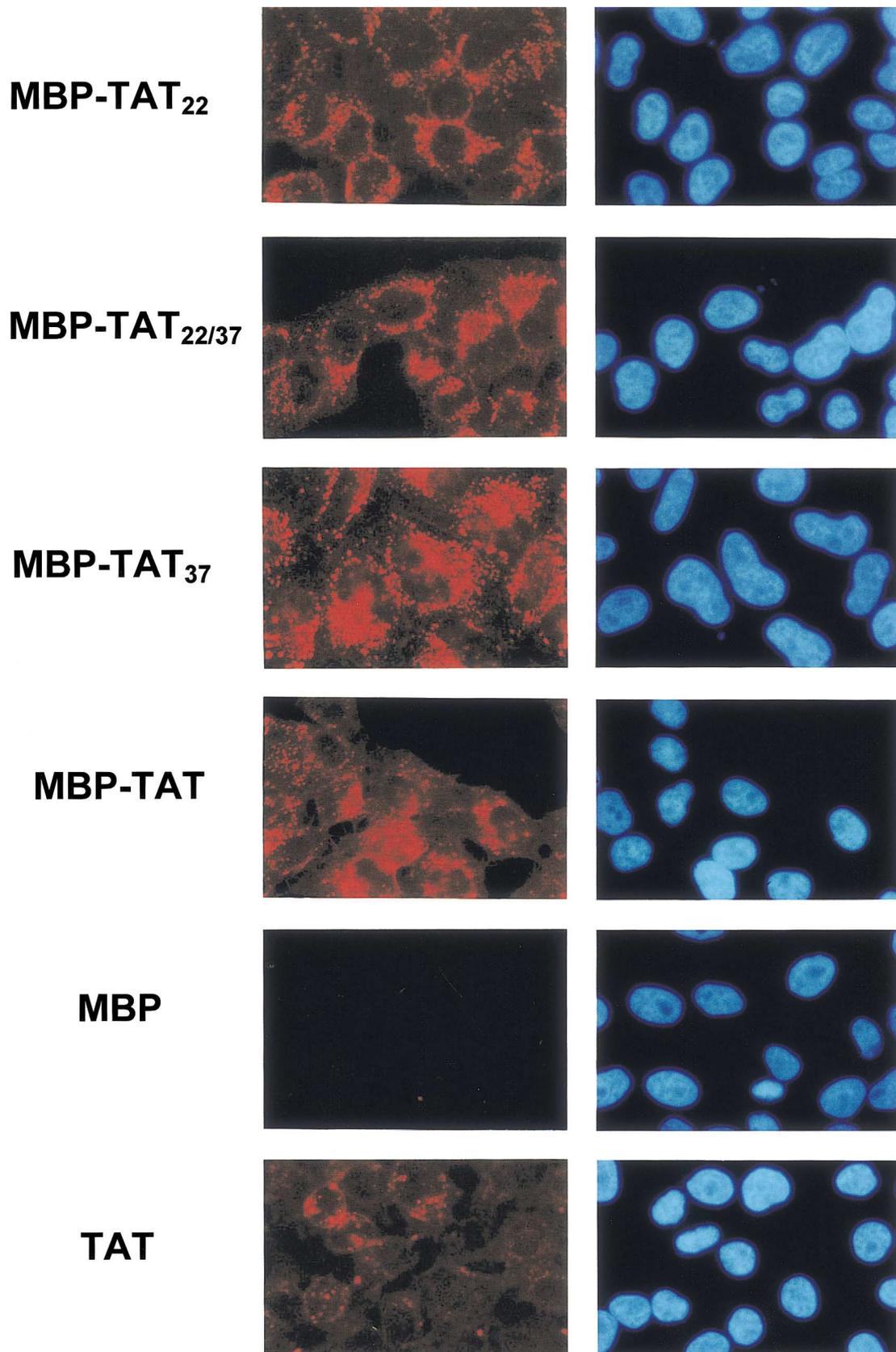
Fig. 4. Cellular uptake of mutated Tat proteins. MBP-Tat proteins (25 µg/ml, corresponding to 5 µg/ml of Tat), purified Tat (5 µg/ml) or MBP alone (5 µg/ml) were added directly to HL3T1 cells in the presence of chloroquine. Cells were incubated for 24 h, fixed and analyzed by immunofluorescence with the anti-Tat mAb 4B4C4 followed by staining with a Cy-3 conjugated secondary antibody. The red fluorescence localizes the Tat proteins (left panels), whereas the DAPI fluorescence (blue) stains the nuclei (right panels). The same microscopic field is shown in each left and right panel.
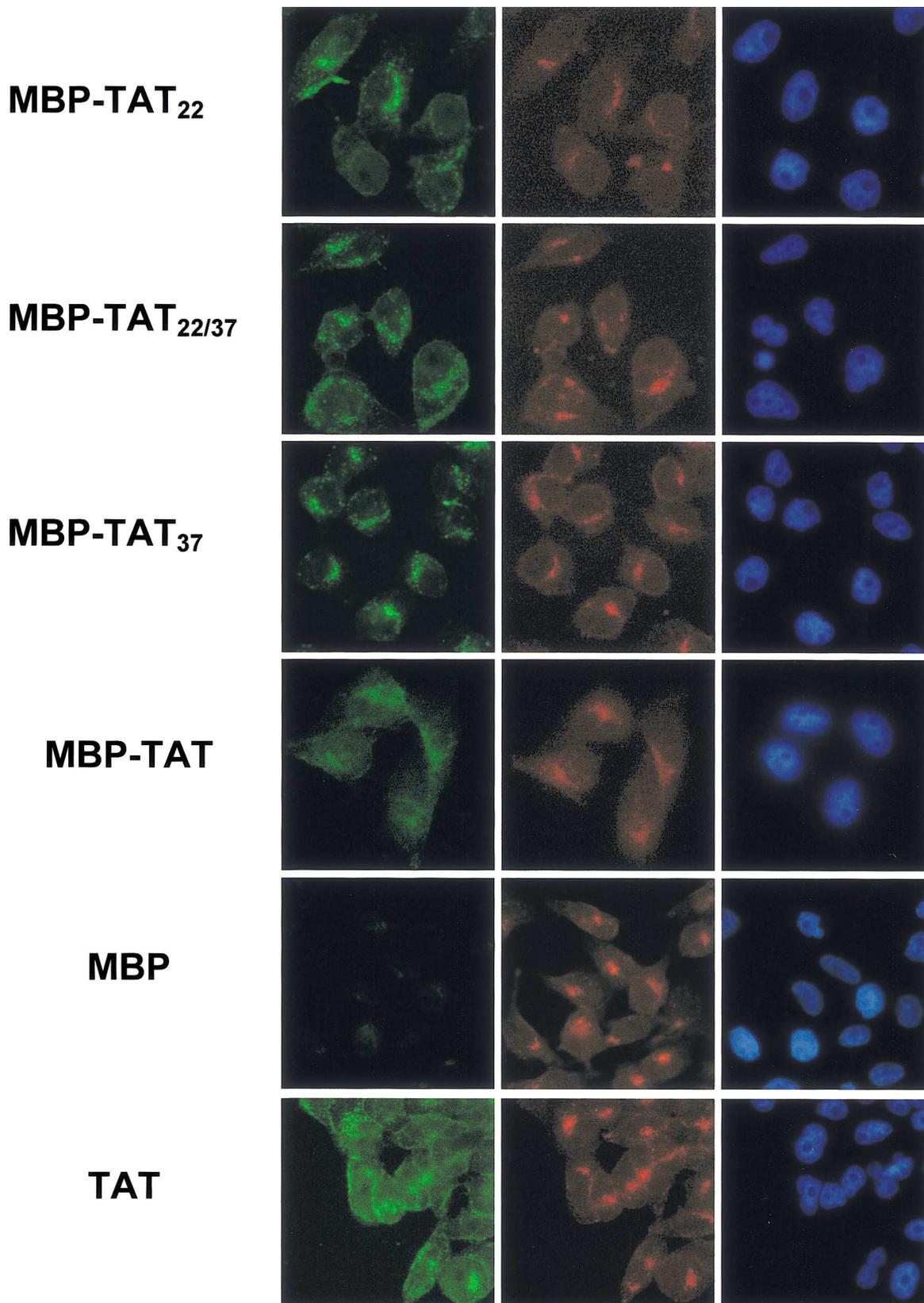
Fig. 5. Tat colocalizes with AP-1 in the trans-Golgi apparatus. Mutated and wild-type MBP-Tat proteins (25 µg/ml, corresponding to 5 µg/ml of Tat), MBP alone (5 µg/ml) or purified Tat (5 µg/ml) were added directly to HL3T1 cells in the presence of chloroquine. Cells were incubated for 3 h, fixed and reacted with both an anti-Tat polyclonal serum and an anti-AP-1 mAb. Staining for Tat was performed with a FITC-conjugated secondary antibody (green fluorescence, left panels), and for AP-1 with a TRITC-conjugated antibody (red fluorescence, middle panels). The DAPI fluorescence (blue, right panels) colors the nuclei. The same microscopic field is shown in each left, middle and right panel.

4. Discussion

The data demonstrate that Tat_{22}, $Tat_{22/37}$ and Tat_{37} synthesized within the cells, after gene transfection, translocate to the nucleus and are released from the cells in a fashion similar to wild-type Tat [3,5]. In addition, the results show that Tat_{22}, $Tat_{22/37}$ and Tat_{37} proteins when present in the extracellular environment are taken up by the cells and localize within the cells in a fashion similar to wild-type Tat [3,6,7]. These results are consistent with previous studies indicating that the basic and the RGD regions of wild-type Tat are key for those activities, and indeed, those regions are not involved in the mutated Tat proteins. However, since the cysteine-domain of Tat is important for correct protein folding and activity, it was reasonable to think that mutations in this region can affect protein conformation and consequently at least some of its properties. The results show for the first time to our knowledge that mutations in the cysteine-domain of Tat do not modify significantly the conformation of the protein and its properties such as the capability to be released and to be internalized by the cells, nor its internalization pathway. The results of 'Tat-competition' studies in the exogenous system suggest, however, that mutation in the cysteine-region may induce modification of Tat conformation that lowers its binding affinity to the receptor.

Furthermore, in addition to confirm previous studies indicating that endogenous expression of tat_{22} and $tat_{22/37}$ interferes with Tat-transactivating activity and with Tat-dependent HIV-1 replication [38], these results show for the first time that also the exogenous mutated Tat proteins interfere with the transactivating function of Tat. Since competition was observed in two different experimental conditions, when proteins were added directly to the culture medium and when they were forced into the cells by lipofection, it is reasonable to think that interference occurred mostly in the intracellular compartment. However, although mutated Tat proteins may have a lower receptor binding affinity as compared to wild-type Tat, the results do not rule out that competition for uptake may also play a role. The mechanism by which these Tat mutant proteins modulate wild-type Tat-transactivation remains to be fully elucidated. A competition of the mutant and wild-type Tat for binding to TAR RNA is unlikely, considering the recent results by Wei et al. [39]. Formation of hetero-dimers which compete with Tat for binding to TAR RNA is also unlikely, since a recent study showed that Tat exists predominantly as a monomer in living human cells [40]. Thus, a possible mechanism may depend on an indirect effect of Tat on HIV-1 gene expression, since Tat transactivates the LTR promoter through various and distinct pathways [1,2]. In this respect, intracellular and extracellular Tat enhances production of *bcl-2* and *c-fos* [41,42] which may represent important intermediate steps for Tat-dependent HIV-1 gene expression [41–47]. Since previous work showed that mutation of cysteine 22, in addition to abrogate the transactivating function of Tat, completely abolished Tat-mediated *c-fos* and *bcl-2* activation [41,42], and the anti-apoptotic effects induced by Tat [41], the Tat proteins mutated in the cysteine domain may interfere with these indirect functions of wild-type Tat.

The data also demonstrate that fusion of *tat* genes with EGFP and MBP did not compromise the activity of the recombinant proteins, since the Tat mutated proteins were incapable of transactivating the expression of the HIV-1 promoter, whereas the wild-type control proteins, Tat-EGFP and MBP-Tat, transactivated in a dose-dependent manner the expression of the CAT gene driven by the HIV-1 LTR, as expected [1,2]. In addition, the results indicate that recombinant proteins used in this study were not oxidized and were in their active conformation. This was a fundamental aspect of the study, since Tat is very labile because of its high content in cysteine residues and loses very rapidly its activity in different type of assays [3,5,29]. To this respect, loss of activity of oxidized Tat protein was shown to depend on lack of internalization and correlates with loss of Tat reactivity to several monoclonal antibodies directed against the basic and RGD regions, including mAb 4B4C4 used in our study [30]. In our experiments mAb 4B4C4 reacted well with both wild-type and mutated Tat proteins and, in addition, heparin blocked the uptake of all Tat proteins. Therefore, we believe that the results of this study likely reflect the behavior of the mutated Tat proteins encoded in vivo after gene immunization.

In conclusion, the data strengthen the notion that *tat* genes mutated in the transactivation domain may represent good candidates for a prophylactic and/or therapeutic vaccine against HIV infection. Following DNA vaccination, Tat_{22} and $Tat_{22/37}$ proteins are released in the extracellular milieu, taken up by cells and supposedly processed by antigen presenting cells as Tat thus evoking an immune response to wild-type Tat [22]. In addition, the observation that the mutated protein modulate wild-type Tat function may represent an additional advantage, if local concentrations of wild-type and mutated Tat proteins in vivo are such that competition can occur. The mutated proteins may enter infected or uninfected cells where Tat is present, localize in the same intracellular compartment as Tat and interfere with Tat functions that modulate viral gene expression. Moreover, since these *tat* mutant genes lack Tat transactivating activity required for virus replication and for modulation of cellular gene expression (at least of *bcl-2* and *c-fos*), these results suggest that they represent an alternative approach to the use of the wild-type type *tat* DNA for vaccine application.

Acknowledgements

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