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Protein B23 Is an Important Human Factor for the Nucleolar Localization of the Human Immunodeficiency Virus Protein Tat

YI-PING LI*

Departments of Medicine and Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

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Nucleolar shuttle protein B23 was found to bind to human immunodeficiency virus protein Tat, and this binding required the nucleolar localization motif of Tat. A fusion protein containing the B23 binding domain and β-galactosidase caused mislocalization of Tat to the cytoplasm and inhibited the transactivation activity of Tat. These data suggest that B23 is a human factor necessary for the nucleolar localization of Tat.

The nucleolus has long been established as the site where preribosomes are formed. Recent developments suggest that the nucleolus is also involved in poly(A)$^+$ RNA transport and processing (reviewed in reference 19). In addition, the nucleolus is implicated in viral replication (22). In our studies of human nucleolar proteins, B23 (nucleophosmin) was found to bind to the nucleolar localization signal (NoLS) of proteins p120 (21) and C23 (13). Although enriched in nucleoli, B23 has

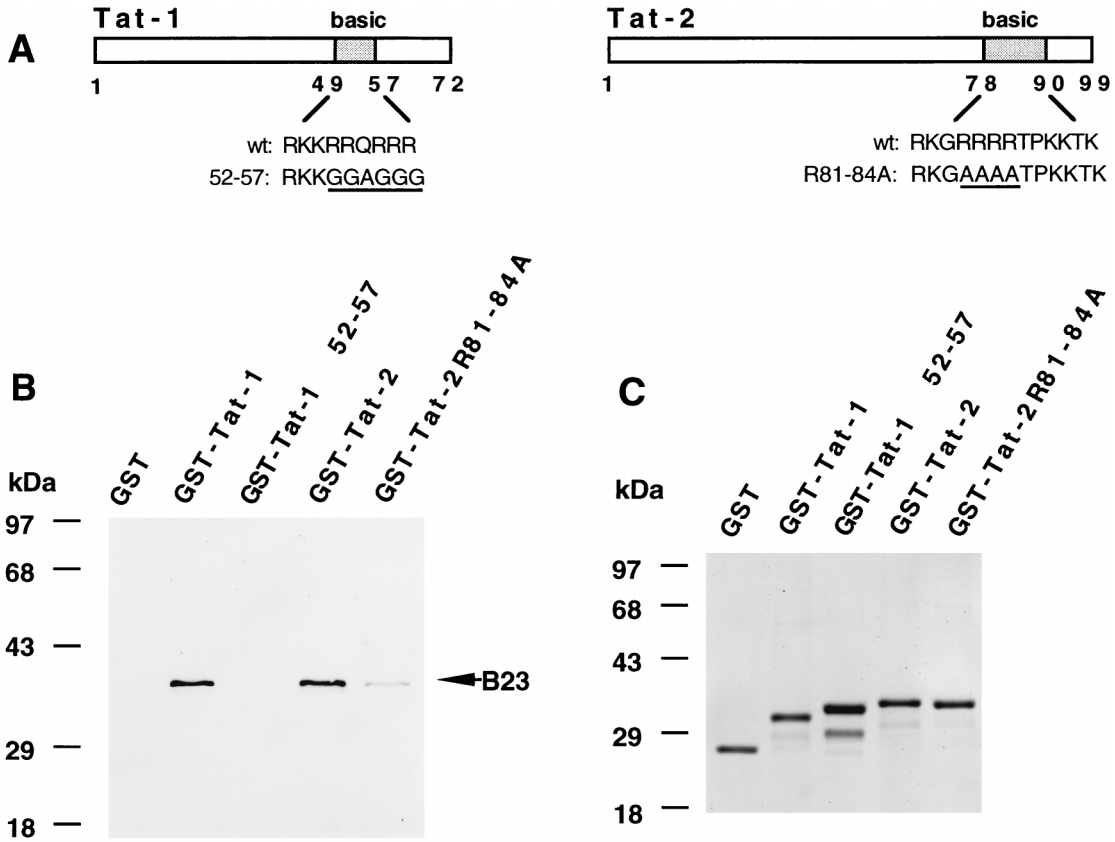

FIG. 1. Binding of B23 to the basic region of Tat. (A) Schematic diagram of the first coding exons of Tat-1 and Tat-2 used in the experiments and the mutations made. The numbers under the diagrams are amino acid positions. The basic regions are indicated by the filled boxes, and the corresponding amino acid sequences are shown. The mutations made in the mutants are underlined. wt, wild type. (B) GST-Tat fusion proteins bound to glutathione-Sepharose 4B beads were incubated with HeLa cell nuclear extract and then pelleted. Western blotting was done to visualize coprecipitated B23. (C) Coomassie blue staining of a second sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel showing the GST-Tat fusion proteins used to precipitate B23.

* Mailing address: Pulmonary Medicine, Suite 520B, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3126. Fax: (713) 798-3619. E-mail: yiping@tmc.bcm.edu.

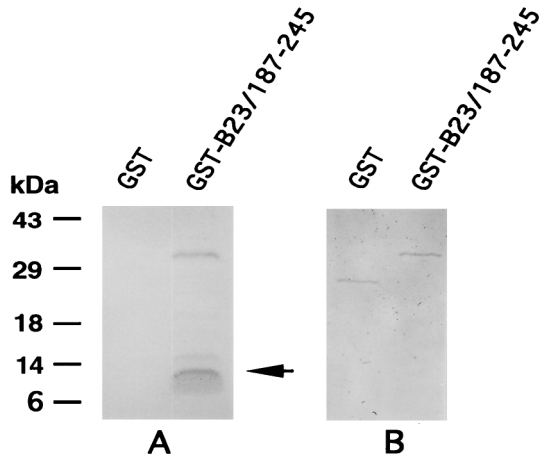

FIG. 2. Binding of the truncated B23 NoLS-binding domain to Tat. (A) Western blot showing that Tat-1 was coprecipitated with the B23 binding domain (aa 187 to 245) fused to GST. The arrow indicates Tat-1 (12 kDa). (B) Coomassie blue staining of a second sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel in which GST-B23/187-245 fusion protein and GST were used to precipitate Tat-1.

also been shown to shuttle between the nucleolus and the cytoplasm (4). These observations suggest that B23 may be a carrier protein which is involved in the transport of specific cellular proteins from the cytoplasm to the nucleolus. Interestingly, protein B23 has also been reported to bind the arginine-rich basic regions of human T-cell leukemia virus protein Rex (1) and human immunodeficiency virus (HIV) protein Rev (8), both of which localize to the nucleolus. The arginine-rich basic regions of Rex and Rev serve as the RNA-binding and nucleolar localization domain. When their basic regions are mutated, the two viral proteins do not localize to the nucleolus (3, 5, 16). Although McDonald et al. (15) reported that Rev function does not depend on its nucleolar localization, D'Agostino et al. (7) demonstrated that partial relocation of Rev to the cytoplasm induced by mycophenolic acid treatment reduces the Rev-dependent expression of Gag by 56%.

The fact that B23 binds to the NoLS of specific cellular and viral proteins raised the possibility that B23 is a transporter utilized by certain viral, as well as cellular, proteins for nucleolar localization. To examine this hypothesis, the interaction of B23 with the HIV Tat protein was investigated in the present study. Tat protein is required for efficient viral transcription by stimulating transcription directed by the viral long terminal repeat sequence (12). Similar to Rev (20), Tat has a basic region which binds to the transactivation region of the viral RNA and is required for its nucleolar localization (11).

Binding of B23 to the basic region of Tat with its NoLS-binding domain. An in vitro binding assay (13) utilizing bacterially expressed wild-type and mutant Tat-1 or Tat-2 (Fig. 1A) fused to glutathione *S*-transferase (GST; the plasmids were gifts from Richard Gaynor [University of Texas Southwestern Medical Center] and Andrew Rice [Baylor College of Medicine]) was done to evaluate their binding to B23 in HeLa cell nuclear extract (13). Both wild-type Tat-1 and Tat-2 fusion proteins bound to B23 present in HeLa cell nuclear extract, while GST alone did not (Fig. 1B). However, mutations made within the Tat-1 basic region abolished the B23 binding, and similar mutations within the Tat-2 basic region greatly reduced the binding (Fig. 1B), indicating that Tat proteins bind to B23 and such binding requires native basic regions. By using the same technique, the binding of the GST–Tat-1 fusion protein to various deletion mutant forms of B23 in *Escherichia coli* lysate (13) was analyzed. Similar to the binding of B23 to p120 (21) and C23 (13) described previously, the NoLS-binding domain including amino acids (aa) 194 to 239 was found to be required for the binding of B23 to Tat-1 (data not shown). In addition, the truncated B23 binding domain (aa 187 to 245) fused to GST bound to purified Tat-1 mixed in bacterial lysate, while GST alone did not (Fig. 2). The higher-molecular-weight band also seen above Tat in the blot is likely oligomers of Tat which were formed as a result of oxidation of the purified cysteine-rich Tat (9). Another possibility is that this band represents Tat bound to another protein.

Although the arginine-rich basic region of Tat is critical for binding, its interaction with B23 does not seem to be purely ionic. The binding domain of B23 (aa 194 to 239; pI 11.20) is much less acidic than the highly acidic cluster present in the molecule (aa 161 to 188; pI 2.55), yet only aa 194 to 239 showed binding activity. In addition, as demonstrated previously, B23 does not bind to the nuclear localization signals in p120 and C23, which also contain arginines (13, 21). It is likely that the spatial conformation of this domain is the major factor contributing to its binding ability.

The above results suggest that there is an interaction between B23 and Tat, and this interaction may be related to the nucleolar localization of Tat.

Sequestration of Tat in the cytoplasm by a fusion protein containing the NoLS-binding domain of B23. To obtain in vivo evidence that B23 interacts with Tat and that this interaction is necessary for Tat localization to the nucleolus, an in vivo competition experiment was performed. A fusion protein construct (pCMV-βgal-B23/187-255; Fig. 3A) was made by linking the cDNA of the B23 binding domain (aa 187 to 255) to the cDNA of β-galactosidase in the pBC12/CMV vector (6) and was cotransfected into COS-7 cells with a Tat construct (pCMV-

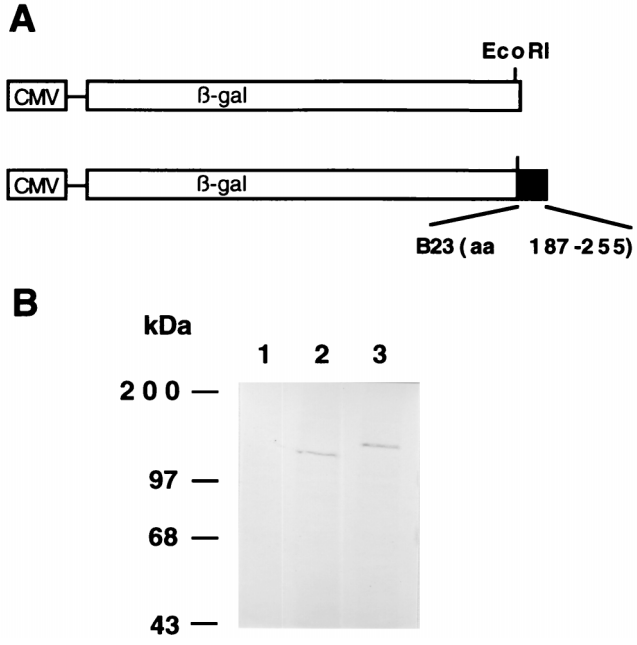

FIG. 3. Construction and expression of fusion protein βgal-B23/187-255. (A) Schematic representation of the constructs for β-galactosidase (β-gal) and fusion protein βgal-B23/187-255. CMV, cytomegalovirus. (B) Western blotting with anti-β-galactosidase antibody to show expression of β-galactosidase and fusion protein βgal-B23/187-255 in transfected COS-7 cells. Lanes: 1, cells transfected with vector pBC12/CMV; 2, cells transfected with pCMV-βgal; 3, cells transfected with pCMV-βgal-B23/187-255.

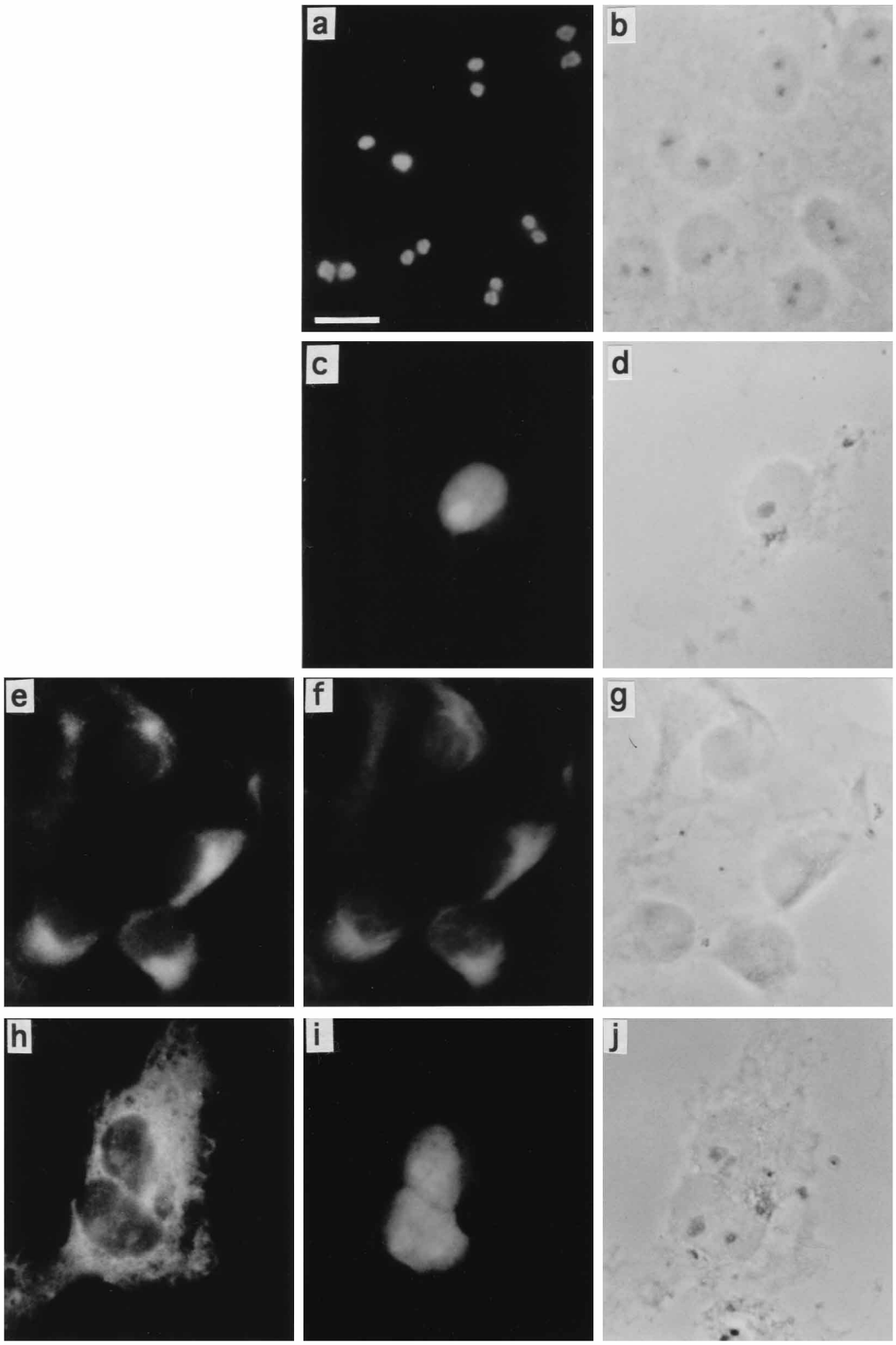

FIG. 4. COS-7 cells stained by indirect immunofluorescence demonstrating the effect of transiently expressed fusion protein βgal-B23/187-255 on the localization of coexpressed Tat-1. The photos in each horizontal row were taken from the same field with the rightmost as the phase. (a) Endogenous B23 localized to nucleoli. (c) Tat-1 localized to the nucleus and highly enriched in the nucleolus. (e) Fusion protein βgal-B23/187-255 localized to the cytoplasm. (f) In cells highly expressing βgal-B23/187-255, Tat-1 localized to the cytoplasm. (h) Wild-type β-galactosidase localized to the cytoplasm. (i) Unaltered localization of Tat-1 coexpressed with wild-type β-galactosidase. B23, β-galactosidase, and the fusion protein were stained with a fluorescein-conjugated goat anti-mouse second antibody. Tat-1 was stained with a rhodamine-conjugated goat anti-rabbit second antibody. Bar, 10 μm.

Tat-1) by the calcium phosphate precipitation method (10). COS-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Twenty hours before transfection, 10^5 cells were seeded on a glass coverslip placed in a six-well plate (35-mm well diameter). A 0.5-μg sample of pCMV-Tat-1 plasmid DNA and 2 μg of pCMV-βgal or pCMV-βgal-B23/187-255 plasmid DNA were transfected into the cells. The cells were allowed to grow under normal conditions for 40 h before staining by indirect immunofluorescence as described previously (18).

The proper expression of β-galactosidase and the fusion protein βgal-B23/187-255 in transfected COS-7 cells was verified by Western blotting with a monoclonal anti-β-galactosidase antibody (Boehringer Mannheim). The two proteins were recognized by the same antibody, but the fusion protein had a greater apparent molecular weight than wild-type β-galactosidase (Fig. 3B).

B23 is a ubiquitous protein, and its presence in COS-7 cells was verified by indirect immunofluorescence with a monoclonal anti-B23 antibody (17) as shown in Fig. 4a. Tat protein stained with a polyclonal antiserum (obtained from Bryan Cullen through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) was seen in the nucleus and was highly enriched in the nucleolus (Fig. 4c), consistent with a previous report (11). Fusion protein βgal-B23/187-255, stained with the monoclonal anti-β-galactosidase antibody, localized to the cytoplasm (Fig. 4e). By using the double-staining technique, coexpressed Tat protein in these cells which highly expressed βgal-B23/187-255 was seen to colocalize with βgal-B23/187-255 to the cytoplasm (Fig. 4f). The cytoplasmic localization of Tat protein depended on the expression level of βgal-B23/187-255. In cells which expressed βgal-B23/187-255 at lower levels, Tat protein was seen in both the cytoplasm and the nucleus. Wild-type β-galactosidase also localized to the cytoplasm (Fig. 4h), but it did not affect the localization of the coexpressed Tat protein (Fig. 4i). These results strongly suggest that the overexpressed fusion protein containing the B23 NoLS-binding domain binds to and sequesters Tat protein in the cytoplasm and that binding to the B23 present in the cytoplasm is necessary for Tat nucleolar localization. The cells overexpressing the fusion protein appeared to be rounder and less flat (Fig. 4e) than cells overexpressing wild-type β-galactosidase (Fig. 4h). This difference in cell appearance may reflect an adverse effect of the fusion protein on the cells, possibly due to the mislocalization of certain cellular proteins, such as p120 and C23, caused by this fusion protein.

Inhibition of Tat activity by the fusion protein. Further investigation was done to determine if the mislocalization of Tat caused by fusion protein βgal-B23/187-255 would result in a reduction of Tat transactivation activity in T lymphocytes. A Tat reporter cell line, 1G5 (2), derived from Jurkat T cells which were stably transfected with a luciferase reporter gene driven by an HIV type 1 long terminal repeat, was used to test Tat activity. The cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. Transient transfections were done by using the DMRIE-C Reagent (GIBCO Bethesda Research Laboratories) in duplicate or triplicate. In addition to the plasmids being tested, 0.3 μg of pRSV-CAT was included as a control. The medium also contained phytohemagglutinin and phorbol myristate acetate at final concentrations of 1 μg/ml and 50 ng/ml, respectively. Cells were harvested and assayed at 24 h posttransfection by using the luciferase assay kit from Promega. Chloramphenicol acetyltransferase (CAT) levels in the lysis supernatant were assayed with the CAT enzyme-linked immunosorbent assay kit from Boehringer Mannheim. As shown in Fig. 5, when 2 μg of pCMV-βgal-B23/187-255 (expressing the fusion protein) was cotransfected with pCMV-Tat-1 (0.1 μg), Tat-activated luciferase transcription was reduced by 49% compared with the control, in which 2 μg of pCMV-βgal (expressing wild-type β-galactosidase) was cotransfected with pCMV-Tat-1 (0.1 μg). Although the inhibitory effect is less than striking, it is not unexpected for the following reasons. Due to the nature of double transient transfection, not all cells that expressed Tat protein also expressed the βgal-B23/187-255 fusion protein. In addition, there is a competition for Tat binding between the fusion protein and endogenous B23 so that the block of Tat localization by the fusion protein in a single cell is likely not 100%. Since Tat is a potent transactivator, a few molecules of Tat that enter the nucleus can be enough to activate transcription. Nevertheless, these data support the observation that the fusion protein containing the B23 NoLS-binding domain altered the localization of Tat.

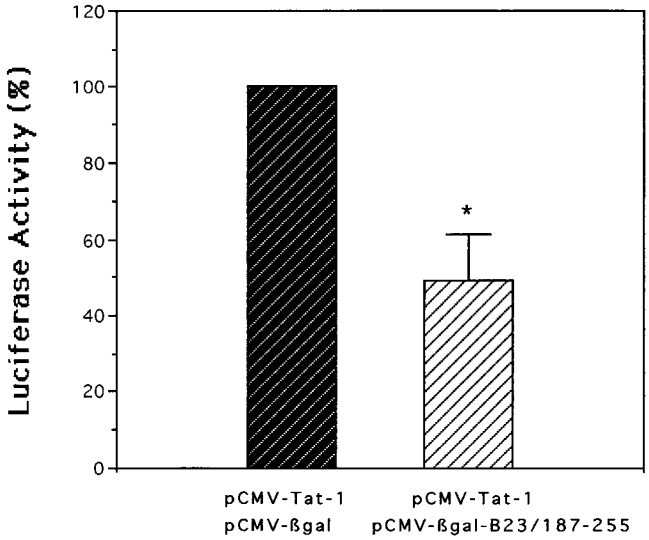

FIG. 5. Effect of coexpressed fusion protein βgal-B23/187-255 on the transactivation activity of Tat in 1G5 cells. Cells were transiently transfected by the DMRIE-C reagent and harvested 24 h posttransfection for luciferase assay. A 100-ng sample of pCMV-Tat-1 and 2 μg of pCMV-βgal or 2 μg of pCMV-βgal-B23/187-255 were transfected as indicated. Plasmid pRSV/CAT (0.3 μg) was cotransfected, and luciferase activities are normalized to coexpressed CAT levels. The data shown are means ± the standard errors of three experiments, and the asterisk indicates a significance level of $P < 0.05$ analyzed by the paired Student t test.

It has been reported that Tat is colocalized with nucleolar protein B23 in stably transfected Jurkat T cells (14). The present study strongly suggests a direct interaction between Tat and B23. This interaction requires the NoLS of Tat and the NoLS-binding domain of B23. In addition, the interaction between Tat and B23 is necessary for its proper localization to the nucleolus.

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REFERENCES

1. **Adachi, Y., T. D. Copeland, M. Hatanaka, and S. Oroszlan.** 1993. Nucleolar targeting signal of Rex protein of human T-cell leukemia virus type I specifically binds to nucleolar shuttle protein B-23. J. Biol. Chem. **268:**13930–13934.
2. **Aguilar-Cordova, E., J. Chinen, L. A. Donehower, L. A. Lewis, and J. W. Belmont.** 1994. A sensitive reporter cell line for HIV-1 tat activity, HIV-1 inhibitors, and T cell activation effects. AIDS Res. Hum. Retroviruses **10:** 295–301.
3. **Berger, J., C. Aepinus, M. Dobrovnik, B. Fleckenstein, J. Hauber, and E. Bohnlein.** 1991. Mutational analysis of functional domains in the HIV-1 Rev trans-regulatory protein. Virology **183:**630–635.
4. **Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg.** 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell **56:**379–390.
5. **Cochrane, A. W., A. Perkins, and C. A. Rosen.** 1990. Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. J. Virol. **64:**881–885.
6. **Cullen, B. R.** 1986. Transactivation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46:**973–982.
7. **D'Agostino, D. M., V. Ciminale, G. N. Pavlakis, and L. Chieco-Bianchi.** 1995. Intracellular trafficking of the human immunodeficiency virus type 1 Rev protein: involvement of continued rRNA synthesis in nuclear retention. AIDS Res. Hum. Retroviruses **11:**1063–1071.
8. **Fankhauser, C., E. Izaurralde, Y. Adachi, P. Wingfield, and U. K. Laemmli.** 1991. Specific complex of human immunodeficiency virus type 1 Rev and nucleolar B23 proteins: dissociation by the Rev response element. Mol. Cell. Biol. **11:**2567–2575.
9. **Frankel, A. D., D. S. Bredt, and C. O. Pabo.** 1988. Tat protein from human immunodeficiency virus forms a metal-linked dimer. Science **240:**70–73.
10. **Graham, F. L., and A. J. Van Der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
11. **Hauber, J., M. H. Malim, and B. R. Cullen.** 1989. Mutational analysis of the conserved basic domain of human immunodeficiency virus *tat* protein. J. Virol. **63:**1181–1187.
12. **Jones, K. A.** 1993. Tat and the HIV-1 promoter. Curr. Opin. Cell Biol. **5:** 461–468.
13. **Li, Y.-P., R. K. Busch, B. C. Valdez, and H. Busch.** 1996. C23 interacts with B23, a putative nucleolar-localization-signal-binding protein. Eur. J. Biochem. **237:**153–158.
14. **Marasco, W. A., A. M. Szilvay, K. H. Kalland, D. G. Helland, H. M. Reyes, and R. J. Walter.** 1994. Spatial association of HIV-1 Tat protein and nucleolar transport protein B23 in stably transfected Jurkat T-cells. Arch. Virol. **139:**133–154.
15. **McDonald, D., T. J. Hope, and T. G. Parslow.** 1992. Posttranscriptional regulation by the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex proteins through a heterologous RNA binding site. J. Virol. **66:**7232–7238.
16. **Nosaka, T., H. Siomi, Y. Adachi, M. Ishibashi, S. Kubota, M. Maki, and M. Hatanaka.** 1989. Nucleolar targeting signal of human T-cell leukemia virus type I rex-encoded protein is essential for cytoplasmic accumulation of unspliced viral mRNA. Proc. Natl. Acad. Sci. USA **86:**9798–9802.
17. **Ochs, R., M. Lischwe, P. O'Leary, and H. Busch.** 1983. Localization of nucleolar phosphoproteins B23 and C23 during mitosis. Exp. Cell Res. **146:**139–149.
18. **Ren, Y., R. Busch, E. Durban, C. Taylor, W. C. Gustafson, B. Valdez, Y.-P. Li, K. Smetana, and H. Busch.** 1996. Overexpression of human nucleolar proteins in insect cells: characterization of nucleolar protein p120. Protein Exp. Purif. **7:**212–219.
19. **Schneiter, R., T. Kadowakiand, and A. M. Tartakoff.** 1995. mRNA transport in yeast: time to reinvestigate the functions of the nucleolus. Mol. Biol. Cell **6:**357–370.
20. **Subramanian, T. M., M. Kuppuswamy, L. Venkatesh, A. Srinivasan, and G. Chinnadurai.** 1990. Functional substitution of the basic domain of the HIV-1 transactivator, Tat, with the basic domain of the functionally heterologous Rev. Virology **176:**178–183.
21. **Valdez, B. C., L. Perlaky, D. Henning, Y. Saijo, P.-K. Chan, and H. Busch.** 1994. Identification of the nuclear and nucleolar localization signals of the protein p120: interaction with translocation protein B23. J. Biol. Chem. **269:** 23776–23783.
22. **Walton, T. H., P. T. Moen, Jr., E. Fox, and J. W. Bodnar.** 1989. Interactions of minute virus of mice and adenovirus with host nucleoli. J. Virol. **63:**3651–3660.