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HIV-1 Tat Protein Is Poly(ADP-ribosyl)ated *in Vitro*

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Purified recombinant HIV-1 Tat protein stimulated acceptor-dependent reaction of poly(ADP-ribose) polymerase in a dose-dependent manner. Analysis of the reaction products by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-poly(ADP-ribose) antibody revealed that recombinant Tat proteins were covalently modified with poly(ADP-ribose) in the enzyme reaction. Eventhough no significant effect of the modification was detected in the activity of Tat to form a specific complex with TAR (a viral transactivation response element) RNA, the present results raise the possibility that poly(ADP-ribose) polymerase is involved in the regulation of HIV-1 through the modification of a virus-encoded transactivator, Tat protein. © 1999 Academic Press

Tat is an HIV-1-encoded transcriptional activator, which is essential for the virus replication (1, 2). Recent studies revealed that Tat is involved in the regulation of the transcription of the viral and cellular genes through the interaction with various protein factors involved in the regulation of RNA polymerase II-dependent transcription: The association of Tat with P-TEFb (3), CTD phosphatase (4), TAF-250 component of TFIID (5), CREB-binding protein (CBP) and a coactivator p300 (6), were shown to be important for Tat-mediated regulation of various genes.

On the other hand, increasing evidence suggests that poly(ADP-ribose) polymerase (PARP[1]), a nuclear enzyme which catalyzes a covalent modification of a wide variety of nuclear proteins and enzymes with pADPR (7–12), is involved in modulation of the expression of various genes (13–16), even though the enzyme is not necessarily essential for the fundamental mechanism of the gene expression considering that the gene-disrupted mice showed almost normal development (17, 18).

In this respect, we found that NF-κB, a transcription factor involved in the regulation of a wide variety of cellular and virus genes, was poly(ADP-ribosyl)ated by PARP (18a). In addition, we found also that PARP was involved in the transcriptional regulation of HIV-1 gene upon a PMA-dependent induction of the virus in U1 cells (18b).

In an attempt to find the role of PARP in HIV-1 replication, we examined whether PARP poly(ADP-ribosyl)ates Tat protein, which is considered to be one of the most important viral factors for development of AIDS (19–22) as well as for the virus replication (1, 2), utilizing a purified poly(ADP-ribosyl)ating enzyme system.

MATERIALS AND METHODS

Materials. Bovine thymus PARP (23), activated DNA (24), and poly (ADP-ribose) polymer (pADPR) (25) were prepared as described, respectively. Antiserum against pADPR (anti-pADPR) was prepared by immunizing a rabbit with a mixture of pADPR and methylated bovine serum albumin in 1:1 emulsion with Freund's complete adjuvant. Anti-RGS His antibody (anti-His) was purchased from Qiagen (Valencia, CA). [Adenine-2,8-^{3}H]NAD (^{3}H-NAD$^+$) was purchased from Moravek Biochemicals Inc. (Brea, CA). 3-AB was obtained from Sigma Chemical Co. (St. Louis, MO).

Plasmid constructions. A full length *tat* cDNA was amplified from ACH-2 (26) mRNA by using primers, 5′-CGGGATCCCATGGAGCCAGTAG-ATC-3′ and 5′-AACTGCAGCCTATTCCTTCGGGCCT-3′ (underlines indicate the *Bam* HI and *Pst* I restriction sites, respectively). Plasmid pQE-Histat, which expressed a 6 × His-tagged (N-terminal) full length Tat (HisTat), was constructed by cloning a *tat* cDNA into the *Bam* HI-*Pst* I site of pQE31, an expression vector for 6 × His-tagged protein (Qiagen, Valencia, CA). pET23-Stat, which expressed a full length Tat fused with a streptavidin binding peptide (3′-) (STat) (27), and pGEM7WT, pGEM7DB and pGEM7DL, which expressed wild-type-, bulge-deleted- and loop-deleted-TAR RNA, respectively (28), were generous gifts from Drs. K. Fujinaga and B. M. Peterlin (Howard Hughes Medical Institute, UCSF).

Preparation of recombinant proteins. Plasmid pQE-Histat was expressed in E. coli M15 [pREP4] and HisTat protein was purified using Ni-nitrilotriacetic acid resin under denaturing condition, as suggested in manufacturer's protocol (QIA*express* Type IV kit, Qiagen). pET23-Stat was expressed in E. coli BL21(DE31)pLysS and STat protein was purified according to the method described previ-

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Abbreviations used: PARP, poly (ADP-ribose) polymerase; pADPR, poly (ADP-ribose); HIV-1, human immunodeficiency virus type-1; TAR, transactivation response element; 3AB, 3-aminobenzamide; EMSA, electrophoretic mobility shift assay.

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ously (27). These proteins were dialyzed extensively against 25 mM Hepes-KOH buffer, pH 7.8, 20 mM KCl, 0.1 mM EDTA, 5 mM DTT, 0.5 mM sodium metabisulfite, 5% glycerol and 0.2 mM phenylmethylsulfonyl fluoride before subjecting to poly(ADP-ribosyl)ation reaction. The purity of recombinant protein was examined by SDS-PAGE as shown in Fig. 1. Green fluorescent protein with a (His)6-Tag at COOH-terminal (GFP-His) was a generous gift from Drs. Matsuura, T. and Urabe, I. (Osaka University).

Poly(ADP-ribosyl)ation reaction. Two reaction mixtures were used: (I) the mixture for the "acceptor-dependent reaction" contained 25 mM Tris-HCl buffer, pH 8.0, 1 mM DTT, 0.05% Triton x-100, appropriate concentrations of PARP, ^{3}H-NAD^{+} and acceptor proteins examined; under the reaction condition, automodification reaction of PARP was minimum and the synthesis of pADPR became dependent on the concentration of acceptor proteins added (29), (II) the mixture for the "Mg^{2+}-dependent reaction" contained 10 mM $MgCl_2$ in addition to all of the components as above; Mg^{2+} stimulates extensive chain elongation of oligo-ADP-ribose bound to an acceptor protein as well as that bound to PARP itself (automodification product) (30, 31). The reaction was carried out at 25°C for 30 min and terminated by the addition of a final concentration of 2.5 mM 3-AB. Acid-insoluble radioactivity was determined as described previously (23).

Electrophoretic mobility shift assay (EMSA). ^{32}P-labeled wild-type and mutant TAR RNAs were prepared by *in vitro* transcription of linearized pGEM7 plasmids with T7 polymerase in the presence of [α-^{32}P]UTP (NEN Life Science Products Inc., Boston, MA), as described previously (28). EMSA was carried out essentially as described previously (32). TAR RNA (50,000 cpm) was incubated in the absence or presence of recombinant Tat protein in 16 μl-binding medium containing 30 mM Tris-HCl buffer, pH 8.0, 70 mM KCl, 0.01% NP-40, 5.5 mM $MgCl_2$, 1 mM DTT, 12% glycerol, 53 μg/ml poly (dI-dC) and 31 μg/ml poly (I-C), at 25°C for 20 min. RNA-protein complex was separated in a 6% nondenaturing polyacrylamide gel (electrophoresis was carried out at 4 watts for 2 h at 4°C). The gel was dried and radioactive bands were analyzed by autoradiography.

Immunoblot. The transblot of samples on a gel to PVDF membrane was carried out as described previously (33). After blocking with 2.5% non-fat milk in PBS, the blots were immuno-stained by a successive treatment with an appropriately diluted primary antibody and peroxidase-labeled secondary antibody, and then the immuno-complex was located using ECL Western blotting detection reagents (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England).

RESULTS

Several reports from other laboratories (34, 35) and ours (18b) suggested that PARP is involved in the expression of HIV-1 genes. In an attempt to approach this subject, we tried to examine whether Tat protein is modified by PARP or not. For this purpose, we constructed an expression plasmid for His-tagged-Tat protein (HisTat) and prepared the recombinant protein from E. coli which was transfected with the plasmid. Another recombinant Tat protein, STat was also prepared as described in Materials and Methods. The purities of prepared HisTat and STat were approximately 95 and 90%, respectively, as shown in Fig. 1.

As previously reported (31), a most convenient method for the screening of an acceptor protein for PARP reaction is to measure the effect of a protein on "acceptor-dependent reaction" of PARP, since, under the reaction condition, automodification of PARP was minimum and pADPR synthesis became almost completely dependent on an acceptor protein added (29–31).

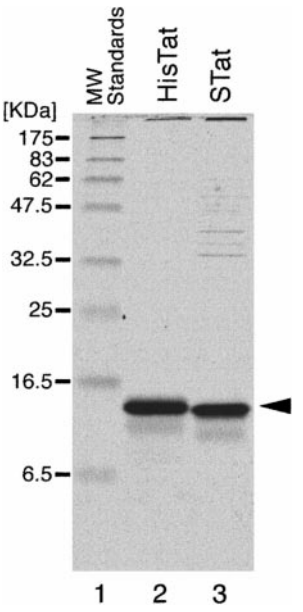

FIG. 1. SDS-PAGE of purified, recombinant Tat proteins. Purity of the recombinant proteins, HisTat (lane 2) and STat (lane 3) used in the present study were analyzed by SDS-PAGE as described under Materials and Methods. Prestained protein markers (New England Biolabs, Beverly, MA) were used as molecular weight-standards (lane 1). Proteins were stained with Coomassie brilliant blue.

As shown in Fig. 2, purified HisTat markedly stimulated the PARP reaction in a dose-dependent manner: The amounts of acid-insoluble product synthesized paralleled the concentration of HisTat in the reaction mixture in a range 0 to 2 μg/0.1 ml. In this range, the efficiency of HisTat to stimulate pADPR synthesis was about 30%, on a weight basis, of that of histone H1, one of the most potent acceptors for ADP-ribose in PARP reaction (7, 29), whereas GFP-His protein, a negative control, was totally ineffective (data not shown). 3AB, an inhibitor of PARP, markedly suppressed the Tat-dependent pADPR synthesis (Fig. 2). The results described above suggested that the HIV-1 Tat protein functioned as an acceptor protein in PARP reaction and were covalently modified with pADPR. In order to prove this, two independently constructed recombinant Tat proteins, HisTat and STat, were incubated in a poly(ADP-ribosyl)ating reaction mixture under the "acceptor-dependent reaction" condition as described in Materials and Methods except that the indicated concentration of cold NAD^{+} was used in place of labeled NAD^{+} and then, the reaction products were analyzed by SDS-PAGE followed by immunoblotting with anti-pADPR antibody. As shown in Fig. 3A, lanes 3 and 10, a major part of the Tat-dependent reaction product synthesized at a limited concentration of NAD^{+} (10

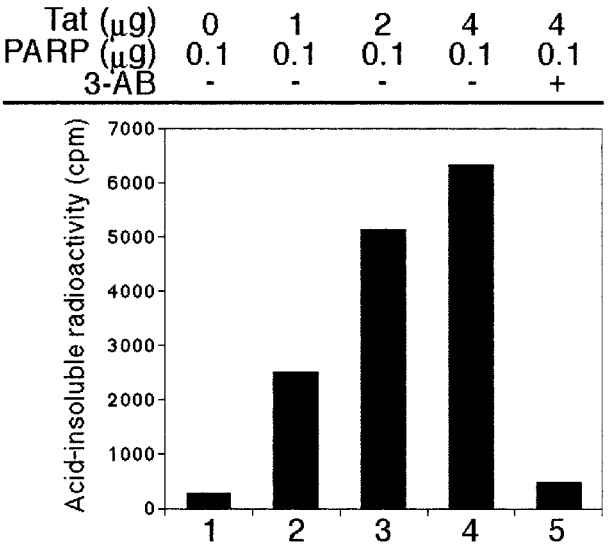

FIG. 2. Tat protein markedly stimulates acceptor-dependent poly(ADP-ribosyl)ation reaction. The indicated amount of HisTat protein at the top panel of columns 1 to 5, respectively, was incubated in a poly(ADP-ribosyl)ating reaction mixture containing 0.1 μCi (4 nmol) of ^{3}H-NAD$^+$, 0.1 μg activated DNA, 0.1 μg PARP in a total volume of 100 μl under "acceptor-dependent" reaction condition as described under Materials and Methods. A control sample of column 5 contained a PARP inhibitor, 3AB (2.5 mM final). After the reaction for 30 min, acid-insoluble radioactivity was determined.

μM) was located at a position close to the position of unmodified Tat proteins, indicating that synthesized pADPR (or rather oligomer) is covalently bound to recombinant Tat proteins. Upon increasing concentration of NAD$^+$ (100 μM, lanes 4 and 11) the ADP-ribosylated Tat proteins shifted toward the position of large molecules, indicating that the chain length and/or the chain number of the polymer covalently bound to one molecule of Tat increased significantly. Also, the results clearly showed that the synthesis of the products located at an area between 16.5 and 62 kDa molecular markers was completely dependent on both Tat and PARP and was markedly inhibited by 3AB. Aparallel experiments to detect poly- or oligo(ADP-ribosyl)ated HisTat protein with the use of anti-His antibody, shown in Fig. 3B, demonstrated that more than 50% of HisTat protein shifted from the position of unmodified protein after poly(ADP-ribosyl)ation (lanes 3 and 4 in Fig. 3B) and that a major part of the shifted protein was detected at positions coincided with the respective major product bands shown in lanes 3 and 4 in Fig. 3A.

Increasing evidence indicates that transcriptional regulation by Tat is mediated by a specific association of Tat with a viral transactivation response element (TAR), a stem-loop structured RNA (36, 37). In order to assess the effect of poly(ADP-ribosyl)ation on the biological function of Tat, we examined the RNA-binding activity of modified and unmodified Tat proteins by EMSA using TAR RNA as a specific probe as described in Materials and Methods. As shown in Fig. 4A, the recombinant Tat protein (HisTat) could form a complex with wild type TAR RNA, as seen in a dose-dependent increase in a shift band in the lanes 2 to 6, whereas it did not form any complex with the bulge-deleted mutant TAR RNA (lanes 7–12). Similar results were obtained with STat also (data not shown). The deletion of central loop of TAR RNA did not affect its binding with the recombinant Tat proteins (data not shown). Considering that Tat binds to a U-rich bulge structure of TAR (37), the observed binding of the recombinant Tat proteins with the RNA probe may be taken as a specific one. Thus, we examined whether poly(ADP-ribosyl)ation affects the specific RNA-binding activity of HisTat or not. As shown in Fig. 4B, poly(ADP-ribosyl)ation did not affect so significantly the TAR-binding activity of HisTat protein, even though a larger fraction of the protein was found to be modified under the reaction condition (Fig. 3B). Since it is shown that chain elongation of poly(ADP-ribose) is limited in the

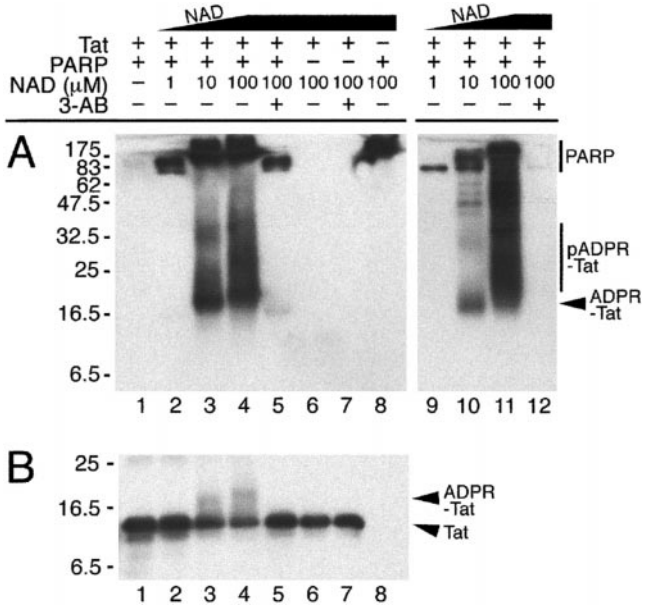

FIG. 3. Immunoblot analysis of poly(ADP-ribosyl)ated Tat with anti-PARP and anti-His antibodies. 1 μg each of HisTat (lanes 1–7) and STat (lanes 9–12) was incubated in a poly(ADP-ribosyl)ating reaction mixture (50 μl) containing the indicated concentration of cold NAD$^+$ (μM), activated DNA (0.1 μg), and PARP (0.1 μg) under "acceptor-dependent reaction" condition as described under Materials and Methods, except that some components were omitted from, or 3-AB (2.5 mM) was added to, some samples prior to the start of the enzyme reaction as indicated in the upper panel. After termination of the reaction with 3-AB (2.5 mM final), a 20-μl aliquot of the sample was subjected to SDS-PAGE in duplicate. Then, immunoblot analysis was performed using appropriately diluted (1/1,000) anti-pADPR (A) or anti-His (B) antibodies, as described under Materials and Methods. Arrowheads indicate the positions of unmodified and oligo(ADP-ribosyl)ated- (ADPR-) Tat proteins. The vertical lines indicate the range of widely distributing poly ADP-ribosylated (pADPR-) PARP and Tat.

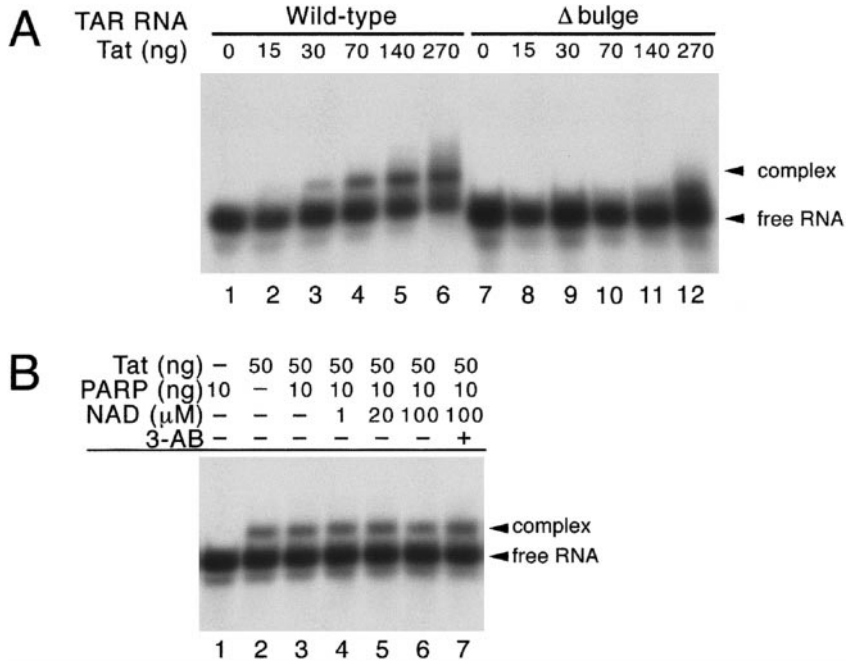

FIG. 4. Electrophoretic mobility shift assay (EMSA) of Tat/TAR complex. (A) The indicated amount of HisTat in the upper panel was subjected to EMSA using wild-type (lanes 1–6)- and bulge-deleted (Δbulge) (lanes 7–12) ^{32}P-labeled TAR RNAs as the probes as described under Materials and Methods. The RNA-protein complex formed was separated in a 6% nondenaturing polyacrylamide gel. (B) 0.5 μg of HisTat was incubated in a poly(ADP-ribosyl)ating reaction mixture (50 μl) containing the indicated concentration of NAD^+, activated DNA (0.1 μg), and PARP (0.1 μg). Some components were omitted from, or 3-AB (2.5 mM final, before start of the enzyme reaction) was added to, some samples as indicated in the upper panel. After termination of the reaction with 3-AB (2.5 mM final), a 5-μl aliquot of the sample containing 50 ng of HisTat was subjected to EMSA using wild-type TAR as a probe. Arrowheads indicate the position of RNA-protein complex and free RNA.

absence of Mg^{2+} (31) and association of a relatively large amounts of poly(ADP-ribose) was required for suppressing the activity of a modified protein (9, 31), we carried out the modification reaction on HisTat protein also in the presence of Mg^{2+} and at a higher concentration of NAD^+ (1 mM): Unexpectedly, however, even the poly(ADP-ribosyl)ation under the reaction condition could not cause any significant suppression of the TAR-binding activity (data not shown).

DISCUSSION

We demonstrated here that both recombinant Tat proteins, HisTat and STat functioned as good acceptor proteins in PARP reaction and were covalently modified by PARP *in vitro* (Fig. 3). GFP-His, used as a negative control, was totally ineffective as an acceptor protein in PARP reaction. Thus, Tat-domain itself seems to function as ADP-ribose acceptor in the enzyme reaction. As reviewed by Burzio (38), the modification sites catalyzed by PARP were either γ-COOH group of glutamic acid residues or α-COOH of terminal residue of acceptor proteins. Histone H1, which is known to be one of the most effective acceptors in PARP reaction (7, 29) and was used as a positive control in the present study, possessed 4 modification sites (38) and was approximately 3-fold more effective than HisTat in stimulating acceptor-dependent PARP-reaction (data not shown). Thus, the lower activity of Tat, compared to histone H1, may be due to the limited number of the modification site in this protein although it contains three glutamic acid residues at the positions 2, 9, and 86 (39).

The present results raised an important problem that PARP could be involved either in a mechanism for the progression of HIV-1 infection or in a defense mechanism of the host through the modification of Tat proteins. Considering that Tat is very important for progression of AIDS (19–22) as well as it is essential for the virus replication (1, 2), the studies for the biological significance of the modification reaction and its occurrence *in vivo* may be urgently required.

In respect to the effect of poly(ADP-ribosyl)ation on the function of Tat, we examined whether the modification affects the TAR-binding activity of this protein. Unexpectedly, however, the modification did not affect so significantly the TAR-binding activity of Tat under the presently used condition at least (Fig. 4). However, considering that Tat has another functional domain interacting with various protein factors involved in a transcriptional regulation besides TAR-binding domain (40, 41), another possibility that the modification

might affect the interaction of Tat with various protein factors remains to be examined urgently.

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