Specific Interaction with Transcription Factor IIA and Localization of the Mammalian TATA-binding Protein-like Protein (TLP/TRF2/TLP)*

Received for publication, May 23, 2003, and in revised form, August 15, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M305412200

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TBP-like protein (TLP) is structurally similar to the TATA-binding protein (TBP) and is thought to have a transcriptional regulation function. Although TLP has been found to form a complex with transcription factors II A (TFIIA), the in vitro functions of TFIIA for TLP are not clear. In this study, we analyzed the interaction between TLP and TFIIA. We determined the biophysical properties for the interaction of TLP with TFIIA. Dissociation constants of TFIIA versus TLP and TFIIA versus TBP were 1.5 and 10 nM, respectively. Moreover, the dissociation rate constant of TLP and TFIIA (1.2 × 10⁻⁴ M⁻¹ s⁻¹) was significantly lower than that of TBP (2.1 × 10⁻³ M⁻¹ s⁻¹). These results indicate that TLP has a higher affinity to TFIIA than does TBP and that the TLP-TFIIA complex is much more stable than is the TBP-TFIIA complex. We found that TLP forms a dimer and a trimer and that these multimerizations are inhibited by TFIIA. Moreover, TLP multimers were more stable than a TBP dimer. We determined the amounts of TLPs in the nucleus and cytoplasm of NIH3T3 cells and found that the molecular number of TLP in the nucleus was only 4% of that in the cytoplasm. Immunostaining of cells also revealed cytoplasmic localization of TLP. We established cells that stably express mutant TLP lacking TFIIA binding ability and identified the amino acids of TLP required for TFIIA binding (Ala-32, Leu-33, Asn-37, Arg-52, Lys-53, Lys-78, and Arg-86). Interestingly, the level of TFIIA binding defective mutant TLPs in the nucleus was much higher than that of the wild-type TLP and TFIIA-interactable mutant TLPs. Immunostaining analyses showed consistent results. These results suggest that the TFIIA binding ability of TLP is required for characteristic cytoplasmic localization of TLP. TFIIA may regulate the intracellular molecular state and the function of TLP through its property of binding to TLP.

TATA-binding protein (TBP) plays a central role in all three classes of transcription (1) and is thought to be a unique universal transcription factor (2). In the case of RNA polymerase II genes, TBP binds to the TATA-box element found in genes transcribed by RNA polymerase II and then triggers the pre-initiation complex assembly (3). TBP directly binds to the general transcription factor II A (TFIIA). TFIIA facilitates binding of TBP to DNA via several distinct mechanisms and thus activates TBP-dependent transcription of class II genes. TFIIA promotes stabilization of TBP on the TATA box by inducing a conformational change of TBP (4, 5). Furthermore, TFIIA acts as an anti-repressor against co-repressors that inhibit DNA binding and TFIIIB binding of TBP (6, 7). TBP or TFIIID forms a transcription-inactive homodimer in solution, and dissociation of a TBP dimer is induced by direct interaction of TFIIA (8, 9, 11, 12). Those processes concerning TFIIA functions are further influenced by distinct classes of TFIIA-interactive regulatory factors. Because some transcriptional activations are dependent on TFIIA/TBP and TFIIA/activator interactions (6, 13–20), TFIIA is thought to be responsible for promoter selectivity for the RNA polymerase II transcription machinery via interactions with both regulatory factors and TBP.

Higher eukaryotes have TBP family genes. A TBP-related factor involved in neurogenesis, TBP-related factor 1 (TRF1), has been identified in Drosophila (21). TRF1 forms a multiprotein complex with novel proteins (22), binds to promoter DNA bearing a non-traditional TATA box, and directs embryogenesis-related expression of the tudor gene (23). A recent study has also demonstrated that TRF1 rather than TBP formed a complex with TAFs of TFIIIB and directs the expression of class III genes (24). Thus, TRF1 is thought to behave as a promoter-selective factor like bacterial σ factors (25), although it is still a mystery why TRF1 has been identified only in Drosophila.

At least one other TBP-like protein (TLP) exists in metazoa (26–28). It has been shown that defects in functional TLP in several animals resulted in developmental abnormalities accompanying decreased expression levels of particular genes (29–34). Recently, we reported that a promoter-bound TLP and free TLP functioned as transcriptional activators (35, 36) and that TLP affected levels of transcription of some cell cycle-related genes in chicken cells (37). The PCNA gene was identified as a native target of TRF2/TLP in Drosophila (38). These findings suggest that TLP plays a role as a positive regulatory factor for some genes in a different way from that of TBP. It has been demonstrated that TLP is associated with TFIIA and TFIIIB but does not bind to TATA-DNA in solution (39, 40) and that TLP purified from nuclear extracts of HeLa cells is associated with TFIIA (41). Although TLP is not able to be substituted with TBP in in vitro transcriptional activation, the addition of TLP into in vitro transcription and in vivo reporter gene assay systems inhibited TATA box-dependent RNA polymerase II transcription by a model of TLP-mediated titration out of TFIIA (41, 42). However, the molecular mechanism underlying the role of vertebrate TLP in transcriptional activation has not been elucidated.

To elucidate the function of TLP distinct from that of TBP,
molecules that have specific affinity to TLP and affect localization and interaction with the general transcription machinery of TLP must be identified. In this regard, the fact that TLP is transiently translocated to mouse spermatocytes and stressed vertebrate cells is interesting (37, 45). It is known that TLP, but not TBP, is stably associated with TFIIA in cells. Hence, it is mostly probable that, among the TLP-interactable proteins, TFIIA is one critical regulator that gives TLP unique properties distinct from those of TBP. In this study, we investigated in detail the biochemical properties of interaction between TLP and TFIIA in vivo and in vitro. We found that TLP has a high affinity to TFIIA and that the pre-formed TFIIA-TLP complex is more stable than is the TFIIA-TBP complex. We also found that TLP forms TFIIA-sensitive dimers and trimers. Last, we found that mutant TLPs lacking TFIIA binding ability do not exhibit a clear cytoplasmic distribution pattern. These results imply that TFIIA negatively regulates TLP function as a potential nuclear transcription factor by inducing cytoplasmic localization of TLP. We discuss in this paper how TFIIA works for dynamism and function of TLP in a cell.

EXPERIMENTAL PROCEDURES

Cell Culture and Establishment of Mouse TLP-expressing Cells—The open reading frame of mouse TLP (mTLP) with FLAG and oligohistidine tags at its amino terminus (F/H-mTLP) was subcloned into the pCI-neo vector (Promega). Mutant TLPs (Fig. 5A) were made by substitution of one or two residues of F/H-mTLP/pCI-neo by a standard procedure. NIH3T3 cells were maintained in a low-glucose Dulbecco’s modified Eagle’s medium (Sigma) and transfected with F/H-mTLP and mutant F/H-mTLPs in the pCI-neo vector by using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer’s instructions. The stable transformants were isolated by a standard procedure in a medium containing 400 μg/ml G418 (Amersham Biosciences). Preparation of Extracts and Immunoprecipitation—Nuclear extracts, cytoplasmic extracts, and whole cell extracts (WCE) were prepared as described previously (44). F/H-protein in WCE prepared by the method of Dignam (44) was precipitated by M2-agarose (Sigma). Bound materials were eluted by an addition of 0.25 mg/ml FLAG peptide.

Purification of Bacterially Expressed Recombinant Proteins—The open reading frames of F/H-mTLP and F/H-hTBP were subcloned into the pET3a vector (Novagen). The open reading frames of hTFIIAβ and histidine-tagged hTFIIAβ were subcloned into the pET16b vector (Novagen). The open reading frames of the human TFIIAβ β, mouse TLP, and human TBP were subcloned into the pEX4T-1 vector (Amerham Biosciences). The constructs were introduced into Escherichia coli BL21(DE3)pLysS strain (Stratagene). Expression of F/H-proteins in E. coli cells were induced according to the manufacturer’s instructions. Soluble F/H-proteins were purified by nickel-nitriotriacetic acid-agarose (Qiagen) (45). F/H-hTBP in eluates from nickel-agarose was further purified by Mono-Q HR5/5 column (Amerham Biosciences) followed by Mono-S-HR5/5 column (Amerham Biosciences). F/H-mTLP in eluates from nickel-agarose was further purified by Mono-S HR5/5 column (Amerham Biosciences). Recombinant hTFIIAβ and oligohistidine-tagged hTFIIAβ (His-hTFIIAβ) were purified under a denaturing condition and renatured as described previously (46). Expressions of recombinant GST-hTBP, GST-mTLP, and GST-hTFIIAβ were induced by the same procedure as that used for F/H fusion proteins. GST fusion proteins were purified by using glutathione-Sepharose beads (Amerham Biosciences) according to the manufacturer’s instructions. Eluates containing GST-hTFIIAβ were subjected to SDS-PAGE and renatured as previously described (46). Eluates of GST-hTBP and GST-mTLP were loaded onto a Superdex-200 column to remove glutathione. All recombinant proteins were finally dialyzed against a buffer used for each assay.

Western Blotting, Immunostaining, and Antibodies—SDS-PAGE and Western blotting were performed by standard procedures (45). Horseradish peroxidase-conjugated antibody against mouse IgG (Amerham Biosciences), horseradish peroxidase-conjugated anti-mouse IgG (New England Biolabs), or horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) and an ECL-Plus system (Amerham Biosciences) or alkaline phosphatase-conjugated anti-rabbit IgG and a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Promega) were used for Western blotting. Indirect immunofluorescent staining of NIH3T3 cells on coverslips was carried out as described previously (37). Anti-TLP polyclonal antibody was prepared and purified by an antigen-conjugated column (39). Anti-TBP polyclonal antibody (47) and anti-TFIIAβ polyclonal antibody (Upstate Biotechnology) were used. Rabbit polyclonal antibodies against anti-TFIIAβ and γ were also prepared (39). Anti-FLAG monoclonal antibodies M2 or M5 (Sigma), anti-β-tubulin monoclonal antibody (TUB2.1, Sigma), and anti-c-Fos polyclonal antibody (Carbiochem) were also used.

Surface Plasmon Resonance Analysis—Surface plasmon resonance measurements were performed using a Biacore 2000 (Biacore). All experiments were conducted in a running buffer (10 mM Hepes-KOH, 150 mM NaCl, 0.1 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% Tween 20, pH 7.9) at 25 °C. Recombinant proteins that were >95% pure were excessively dialyzed against the running buffer. At first, F/H-mTLP and F/H-hTBP were immobilized on a sensor chip and TFIIA was used as an analyte. However, a specific interaction was not observed because TFIIA non-specifically bound to the matrix of the sensor chip. Moreover, TFIIA could not be immobilized on a sensor chip. Thus, GST-TFIIAβ was indirectly immobilized on a sensor chip as described below.

To capture GST-TFIIAβ, anti-GST antibodies (Biacore) were immobilized on the surface of a CM5 sensor chip (Biacore) by the amine coupling procedure described in the manufacturer’s instructions. GST-hTFIIAβ (1000 RU) was bound to the anti-GST antibody on the sensor chip. An equal molar amount of GST (Biacore) (400 RU) against GST-hTFIIAβ was also bound to the anti-GST antibody on another sensor chip. His-hTFIIAγ was then injected and ~250 RU of His-hTFIIA was bound to the GST-hTFIIAβ-bound flow cell. Based on molecular mass of GST-hTFIIAβ (85 kDa) and His-hTFIIAγ (15 kDa), the amount (250 RU) of His-hTFIIAβ that had bound to GST-hTFIIAβ (1000 RU) indicated that most of the hTFIIAβ bound to hTFIIAγ. Then F/H-hTBP or F/H-mTLP was injected into a flow cell pre-bound with GST or GST-hTFIIAβ/hTFIIAγ complex and washed with running buffer to monitor the dissociation phase at a flow rate of 30 μl/min. The surfaces were regenerated with 15 μl of 2 mM NaCl-containing running buffer. For kinetic analysis, various concentrations of analytes were injected. Each sensorgram of the flow cell immobilized with GST-hTFIIAβ was corrected with that of GST, and kinetic parameters were calculated from hTFIIA-specific responses (49). It was necessary to reduce the concentrations of analytes, and the obtained response was relatively weak because the amount of indirectly immobilized TFIIA was a ligand only ~1300 RU.

Analysis of Multimerized Proteins and Chemical Cross-linking—The multimerized forms of proteins were analyzed by glyceral density gradient centrifugation (GDGC) as described previously (47) with slight modifications. In this case, the buffer was replaced with that used for BMH (1,6-bismaleimidohexane) cross-linking (BMH) buffer. The fractions including sedimented proteins were subjected to SDS-PAGE followed by silver staining and Western blotting. Relative band intensities of recombinant proteins were determined using ImageMaster one-dimensional software (Amerham Biosciences). Chemical cross-linking with BMH (Pierce) and GST pull-down were performed as described previously (12). Double-strand DNA containing a TATA sequence of the adenovirus major late promoter from 41 to 15 (50) was synthesized. Double-strand DNA containing a GAGA sequence instead of the TATA sequence was also prepared.

RESULTS

Kinetics of Interactions between TBP and TFIIA and TLP and TFIIA—Results of previous studies have shown that TLP binds to TFIIA (41). However, because the specificity of the interaction between TLP and TFIIA had not been precisely determined, we investigated the kinetic parameters of TBP-TFIIA and TLP-TFIIA interactions using a Biacore system to find differences between TBP-TFIIA and TLP-TFIIA interactions. As stated under “Experimental Procedures,” we indirectly immobilized GST-hTFIIAβ to a sensor chip mediated by an anti-GST antibody. After binding of GST-hTFIIAβ to antibodies on the sensor chip, oligohistidine-tagged hTFIIAβ was incubated for 5 min and we found that hTFIIAβ, but not TBP, is stably associated with TFIIA in cells. Hence, it is mostly probable that, among the TLP-interactable proteins, TFIIA is one critical regulator that gives TLP unique properties distinct from those of TBP. In this study, we investigated in detail the biochemical properties of interaction between TLP and TFIIA in vivo and in vitro. We found that TLP has a high affinity to TFIIA and that the pre-formed TFIIA-TLP complex is more stable than is the TFIIA-TBP complex. We also found that TLP forms TFIIA-sensitive dimers and trimers. Last, we found that mutant TLPs lacking TFIIA binding ability do not exhibit a clear cytoplasmic distribution pattern. These results imply that TFIIA negatively regulates TLP function as a potential nuclear transcription factor by inducing cytoplasmic localization of TLP. We discuss in this paper how TFIIA works for dynamism and function of TLP in a cell.
(His-hTFIIAγ) was subsequently injected to form a TFIIAα/β/γ complex. This complex was not dissociated by a continuous flow under our experimental conditions (data not shown). F/H-hTBP or F/H-mTLP as a ligand was then injected. TLP of various concentrations was applied over a flow cell immobilized with GST-hTFIIAα/β/γ or control GST, and typical sensorgrams were obtained (Fig. 1A). The obtained data indicated a specific interaction between TLP and TFIIA. Kinetic parameters were calculated from appropriate parts of the sensorgrams. Residual plots of obtained sensorgrams against fitted curves (Fig. 1, B and C) were less than ±2 resonance response units, indicating that the sensorgrams of TLP-TFIIA interaction fit well to a 1:1 binding model. From the ideal curve, the association rate constant (kₐ) between TLP and TFIIA was calculated to be 7.66 ± 0.02 × 10⁹/sec and the dissociation rate constant (k₈) was calculated to be 1.16 ± 0.02 × 10⁻⁸/sec; thereby, the Kₐ value was calculated to be 1.52 × 10⁻⁸ M (Fig. 1G). The interaction between TBP and TFIIA was similarly analyzed (Fig. 1, D–F). The kₐ, k₈, and Kₐ values were calculated to be 2.00 ± 0.03 × 10⁷/sec, 2.07 ± 0.02 × 10⁻⁷/sec, and 1.04 × 10⁻⁹ M, respectively (Fig. 1G). These data demonstrated that TLP has a one-order higher affinity to TFIIA than does TBP. Notably, it was also found that the k₈ value of TLP from TFIIA was significantly lower (approximately one-seventeenth) than that of TBP from TFIIA, whereas k₈ of TBP was slightly higher (~3 times) than that of TLP. This means that a pre-formed TLP-TFIIA complex is much more stable than is a TBP-TFIIA complex.

TFIIA-sensitive Multimer of TLP—One of the unique properties of TBP is its formation of a transcriptionally negative dimer, and it has been shown that TFIIA induces dissociation of the dimer and promotes DNA binding of TBP (9, 10). Thus, we carried out experiments to determine whether TLP has the same nature as that of TBP and clarify the multimerization of TLP. We analyzed the molecular states of recombinant F/H-hTBP and F/H-mTLP by GDGC under a physiological condition. Because, as is well known, TBP is a sticky protein, TLP was also thought to be a sticky protein. Therefore, we avoided using solid supports for analysis of a high-molecular weight state of TLP. When F/H-hTBP was analyzed by GDGC, F/H-hTBP was mainly detected in fractions around 43 kDa, and a minor population was found in fractions lighter than 150 kDa (Fig. 2A), indicating that a certain population of TBP was polymerized (probably dimerized) as previously reported (8). On the other hand, a peak of F/H-mTLP was detected in a dimer position but not in a monomer position (Fig. 2B). Moreover, a considerable amount of TLP was also detected in 67–158-kDa fractions. These results suggested that TLP exists as a multimer (at least a dimer) like TBP in a physiological condition. Because monomeric TLP was minor (Fig. 2B) and a significant population of TBP was present in monomer fractions (Fig. 2A), it seems that a TLP dimer is more stable than is TBP.

Next, we performed chemical cross-linking of TLP using BMH to detect stable multimers. As shown in Fig. 2D (lanes 2 and 3), TBP was cross-linked efficiently to form dimers but not larger multimers, as was previously reported (8). When F/H-mTLP was cross-linked in the same way, dimers as well as trimers were clearly detected (Fig. 2E, lanes 2 and 3). These results suggest that the high-molecular weight TLPs observed in GDGC fractions of 67–158 kDa (Fig. 2B) are mainly trimers. It is known that TBP dimerization is inhibited by TFIIA and TATA DNA and that the dimerization and TATA binding are

![Figure 1: Real-time kinetic analysis of interactions of TLP or TBP with TFIIA by using a Biacore system.](image-url)
**Fig. 2. TLP forms a dimer and a trimer.** A, GDGC to analyze the molecular state of recombinant F/H-hTBP. F/H-hTBP was subjected to GDGC. Top, relative band intensities of TBP calculated from Western blotting. Middle, Western blotting of F/H-hTBP. Bottom, silver staining. Peaks of marker proteins are indicated in the graph. B, GDGC analysis for F/H-mTLP as described in panel A. C, immunoprecipitation of F/H-mTLP and F/H-hTLP with hTFIIA in buffer used in experiments shown in panels D–F. F/H-proteins (2.5 pmol) with (lanes 6 and 8) or without (lanes 5 and 7) 5 pmol of TFIIA were immunoprecipitated with 5 μl of M2-agarose without F/H-protein (lane 4). Input proteins (20%) were loaded to lanes 1–3. Precipitated proteins were detected by Western blotting using anti-TFIIAαβ, anti-TBP, and anti-TLP antibodies. Positions of hTFIIAαβ, F/H-hTBP, and F/H-mTLP are indicated. D and E, F/H-hTBP (panel D) and F/H-mTLP (panel E) were cross-linked with BMH and subjected to Western blotting with antibodies against each protein. TFIIA, bovine serum albumin (BSA), TATA DNA, and GAGA DNA (lanes 4–6, 7–9, 13–15, and 10–12, respectively) were added to the reactions at the indicated concentrations. As a control, proteins were treated with only dimethyl sulfoxide (lane 2). Positions of monomers and multimers are indicated by arrows. Positions of molecular weight markers (lane 1) are also indicated. F, TLP dimer formation is inhibited by TFIIA. GST-hTBP and F/H-hTBP (lanes 4–6) or GST-mTLP and F/H-mTLP (lanes 10–12) were mixed with or without 5 (lanes 5 and 11) or 15 pmol (lanes 6 and 12) of TFIIA as indicated and precipitated with glutathione beads. F/H-proteins co-precipitated with GST-proteins were subjected to Western blotting with anti-FLAG antibody. Input F/H-hTBP and F/H-mTLP (10%) were loaded to lanes 1 and 7, respectively. Positions of F/H-proteins are indicated by arrows.

Molecular Numbers of TLP, TBP, and TFIIA in Nuclear and Cytoplasmic Fractions—Although TLP was originally identified as a protein similar to nuclear-concentrated TBP, vertebrate TLP has been demonstrated to be basically a cytoplasmic protein, the localization of which is dynamically regulated depending on the cell states (37, 43). Actually, vertebrate TLP has no apparent nuclear localization signal. These facts suggest that the considerably high level of TFIIA-TLP affinity is related to the TLP-specific localization pattern. Before we investigated the effects of TFIIA binding to TLP in vivo, we first precisely quantified the molecular numbers of TLP in subcellular fractions of NIH3T3 cells. The cell fractionation procedure used was sufficient for our purpose, because the marker nuclear protein c-Fox (51) and cytoplasmic protein β-tubulin (45) were mostly detected in nuclear and cytoplasmic fractions, respectively (Fig. 3A).

We determined the molecular numbers of three transcription factors in subcellular fractions of NIH3T3 cells by Western blotting (Fig. 3, B and C). The number of TBP molecules in the nuclear fraction was calculated to be 1.34 ± 0.04 × 10^5/cell and that in the cytoplasmic fraction was calculated to be 1.0 ± 0.2 × 10^5/cell, indicating that TBP is abundant in the nucleus, being consistent with the generally accepted notion (Fig. 3B). The sum of molecular numbers of TBP in the nucleus and cytoplasm is comparable with that reported previously (48). Molecular numbers of TFIIAα were 4.4 ± 0.7 × 10^5/cell in the nucleus and 1.7 ± 0.4 × 10^5/cell in the cytoplasm (Fig. 3B). Molecular numbers of TFIIAαβ precursor were 5.8 ± 1.3 × 10^5/cell in the nucleus and 1.9 ± 0.2 × 10^5/cell in the cytoplasm (Fig. 3B). Moreover, TFIIAαβ and -γ were also enriched in the cytoplasm.

There is a possibility that TFIIA-mediated interference of TLP dimerization is just because of inhibition of the cross-linking reaction by TFIIA. We performed a pull-down assay to detect direct interaction between TLP molecules in the presence of TFIIA (Fig. 2F). GST proteins and F/H-proteins were mixed and subjected to pull-down experiments with glutathione beads. In the absence of TFIIA, F/H-proteins were precipitated with GST-mTLP (Fig. 2F, lane 10) and GST-hTLP (lane 4) but not with GST (lanes 2 and 8). Co-precipitations of F/H-proteins with GST fusion proteins were drastically decreased by TFIIA (Fig. 2F, lanes 5, 6, 11, and 12). These results suggested that TLP is dimerized and trimerized in solutions and that this multimerization is inhibited by binding of TFIIA like TBP.
fraction like TFIIAα (Fig. 3D, top and bottom panels, respectively). We concluded that holo-TFIIA is concentrated in the cytoplasm.

The molecular number of TLP was also determined (Fig. 3, B and C). Molecular numbers of TLP in the nucleus and cytoplasm were calculated to be 3.2 ± 1.1 × 10⁻⁶ cell and 8.9 ± 0.3 × 10⁻⁶ cell, respectively (Fig. 3B). TLP in the nuclear fraction was only 3.5% of that in the cytoplasmic fraction and only 2% of TBP in the nuclear fraction (Fig. 3B). These results demonstrated that TLP molecules are highly concentrated in the cytoplasmic fraction, as was confirmed by results of cytochemical analysis (Fig. 3E). Consistent results were also obtained using HeLa cell extracts (data not shown). Based on these results, it was concluded that TLP is a cytoplasmic protein in normally dividing NIH3T3 cells in contrast to TBP even though TLP and TBP are structurally similar.

**TLP-TFIIA Association and Localization of TLP—**We examined the relationship between TFIIA association ability of TLP and its localization. First, we established cell lines that expressed different mutant TLPs to find TFIIA-binding defective mutant TLPs (Fig. 4A). These mutants were designed on the basis of results of crystallographic analysis of the TBP-TFIIA complex and results of radical mutation analysis of TBP (6, 52, 53). Eight mutants (AL32/33EA, N37E, R52E, K53E, R55E, EE74/75RA, K78E, and R86S) had substitutions of one or two residues in the NH₂-terminal tandem repeat in which some of these corresponding residues of TBP were involved in TFIIA contact. Two other mutants (E133R and L134E) had substituted residues in the COOH-terminal tandem repeat. We investigated the TFIIA binding of each mutant TLP by immunoprecipitation of WCE from established cell lines that express exogenous F/H-mTLP at a level comparable with that of endogenous TLP (50–100% of endogenous TLP, Fig. 5, A and C). F/H-mTLP-R55E, -L134E, and -E133R associated with comparable amounts of TFIIA like F/H-mTLP (Fig. 4B, lanes 2, 7, 11, and 12). F/H-mTLP-EE74/75RA bound a 2-fold greater amount of TFIIA than F/H-mTLP did (Fig. 4B, lane 8). On the other hand, F/H-mTLP-AL32/33EA, -N37E, -R52E, and -R86S lost their TFIIA binding ability (Fig. 4B, lanes 3–5 and 10). F/H-mTLP-K53E and -K78E showed slight impairment in TFIIA binding ability (Fig. 4B, lanes 6 and 9). These results are consistent with those obtained for TFIIA/TBP interaction.

Using established cell lines expressing F/H-mTLP (L2-8 and L2-9 cells) and F/H-mTLP-AL32/33EA (M2-19 and M2-41 cells), we determined the ratio of nuclear TLPs to total TLP in WCE. From Western blotting of the nuclear extract (Fig. 5A, lanes 5–8), nuclear localizing endogenous TLPs were calculated to be 1.5–2.2% of the total number of TLPs in WCE of each cell line (Fig. 5B). These values are similar to those in normal NIH3T3 cells (Fig. 3). The amounts of F/H-mTLPs were 1.6 and 1.8% of the total F/H-mTLPs in WCE of L2-8 and L2-9 cells, respectively, which are comparable with those of endogenous TLPs (Fig. 5B). On the other hand, the amounts of F/H-mTLP-AL32/33EA were 9.8 and 7.3% of the total TLPs in M2-19 and M2-41 cells, respectively (Fig. 5B). We further investigated localization of ectopically expressed F/H-mTLP in other cell lines. In cells expressing TFIIA-interactable F/H-mTLPs (R55E, EE74/75RA, E133R, and L134E), ratios of F/H-mTLPs to endogenous TLP in WCE were comparable with those in nuclear extracts (Fig. 5C, f, g, j, and k, and D) like F/H-mTLP expressing L2-8 and L2-9 cells (Fig. 5B, C, a, and D). However, ratios of TFIIA binding defective F/H-mTLPs (N37E, R52E, and R86S) to endogenous TLPs in nuclear extract were clearly higher (2–5-fold) than those of TLPs in WCE (Fig. 5C, c, d, and i, and D) like F/H-mTLP-AL32/33EA in M2-19 and M2-41 cells (Fig. 5, B, C, b, and D). F/H-mTLP-K53E (S3-6, +; and S2-4, −/+ ) and -K78E (V2-4, −; V2-19, −/+ ) exhibited intermediate profiles of the former two groups (Fig. 5D). These results for mutant TLPs agree well with their TFIIA binding abilities (Fig. 4B). These results were also confirmed by immu-
nostaining, because L2-8 cells exhibited a cytoplasmically preferential staining pattern for exogenous TLPs (Fig. 5 E, d and f) as did normal NIH3T3 cells (Fig. 5 E, b), whereas M2-19 cells showed a homogenous staining pattern (Fig. 5 E, h and j).

Taken together, the results suggest that TFIIA binding activity of TLP is required for its cytoplasmic localization.

**DISCUSSION**

**High Affinity of TLP to TFIIA** — TFIIA was identified as the main TLP-interacting partner (41). The KD of TLP to TFIIA was 1.5 nM and that of TBP to TFIIA was 10 nM (Fig. 1). In this study, TLP was shown to have a higher affinity to TFIIA than that of TBP. Moreover, we found that the dissociation rate constant (k_d) of TLP from TFIIA was significantly lower than that of TBP (1/20-fold), indicating that a TLP-TFIIA complex is more stable than is a TBP-TFIIA complex. In an *in vitro* transcription assay, the addition of a three times molar excess of TLP than TBP inhibited TATA-dependent transcription (41). A TFIIA interception model has been proposed to explain this phenomenon. Our present data are consistent with the proposed model. TFIIA interacts with various activators that stabilize TBP/DNA interaction synergistically with TFIIA (4, 5, 54). If there is a potent TFIIA-interacting activator on a promoter, TFIIA acts as a mediator for TBP recruitment even though the promoter lacks a TATA box. This situation is equivalent to that of *Drosophila* TRF2, which is recruited to the PCNA promoter via DREF (38). On a TATA-less promoter, to which a powerful TFIIA-interacting activator is recruited, TLP may initiate transcription more efficiently through TFIIA binding than does TBP. Some genes whose expression levels were drastically decreased in functional TLP-deficient mice did not have a TATA box (31, 34). If the promoter has a TATA box, increasing amounts of nuclear TLP may inhibit such transcriptional activation.

**Formation of TFIIA-sensitive Dimers and Trimers of TLP** — Although we clarified that TLP forms a TFIIA-sensitive dimer as does TBP, several differences were found between a TLP dimer (or multimers) and a TBP dimer. First, TLP formed both dimers and trimers, whereas TBP predominantly formed dimers (Fig. 5 E, h and j). Taken together, the results suggest that TFIIA binding activity of TLP is required for its cytoplasmic localization.

**Interaction between TLP and TFIIA**

![Diagram](image-url)
containing fractions in GDGC (Fig. 2, A and B). Third, the TLP dimer and TLP trimer were not dissociated by TATA-DNA (Fig. 2E). This finding is consistent with the finding that TLP does not bind to traditional TATA-DNA (39, 40). The rate of dissociation of TBP dimers is significantly low, whereas TBP monomers generated by a dynamic equilibrium can rapidly bind to TATA-DNA (9). The finding that TLP forms a dimer/trimer complex rather than a TBP dimer implies that a TLP dimer acts as a potent rate-limiting element for TLP-dependent transcription if TLP functions as a monomer and is more dependent on TATA-DNA than is a TBP dimer.

TFIIA-binding Region of TLP—From studies on the relationship between localization and TFIIA binding of TLP, we identified TLP mutations that impair TFIIA binding (Fig. 4, A and B). Results of crystallographic and mutant analyses of TBP revealed that Ala-184/Leu-185 of mTLP and exogenous F/H-mTLP (or F/H-mTLP-AL32/33EA) are indicated. B, summary of the localization of nuclear TLPs. Molarity in each extract was calculated from Western blotting (panel A) as described in the legend to Fig. 3, and the proportion of TLP in the nucleus to that in WCE was determined. C, detection of endogenous and exogenous TLPs in extracts of NIH3T3 cells that stably express mutant TLP proteins. WCEs (10 μg, indicated with W) and nuclear extracts (20 μg, indicated with N) were subjected to Western blotting using anti-TLP antibody. Names of mutant mTLPs and cell lines are indicated below and above the panels, respectively. Positions of endogenous mTLP and exogenous F/H-mTLP are indicated by asterisks and arrowheads, respectively. D, summary of localization phenotype of endogenous exogenous F/H-mTLPs. Molarity in each extract was calculated from Western blotting (panel C) as described in the legend to Fig. 3, and the ratio of exogenous F/H-mTLP to endogenous TLP in WCEs was calculated and compared with that in nuclear extracts. – means that the ratio was not different between WCEs and nuclear extracts and that exogenous F/H-mTLP was localized in the cytoplasm as was endogenous TLP, + means that the ratio in nuclear extracts was higher (<2-fold, 2–3-fold, and >3-fold, respectively) than that in WCEs, E, detection of intracellular TLPs by immunostaining. Normal cells (WT), panels a and b, L2-8 cells, panels c–f, M2-19 cells, panels g–j. Endogenous TLPs in each cell were detected using anti-TLP antibody (b, d, and h). F/H-mTLP in L2-8 (j) and F/H-mTLP-AL32/33EA in M2-19 (j) cells were stained with anti-FLAG (M5) antibody. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) (a, c, e, g, i).
should homogeneously exist throughout a cell by simple diffusion because the molecular mass of a TLP monomer is small enough for it to pass through the nuclear pore (57). Therefore, the cytoplasmic localization of TLP implies the existence of an active mechanism for TLP localization. The localization profile of TLP was basically correlated with its TFIIA binding ability (Fig. 5). The diffused localization pattern of TLP was not related to its TFIIH binding ability (data not shown). This ability was thought to have no effect on the multimerization of TLP, because the AL32/33EA mutant exhibited a degree of dimerization kinetics comparable with that of F/H-mTLP (data not shown). Therefore, the results of the present study suggest that TFIIA is involved in the regulation of TLP localization.

There are two possible mechanisms by which TFIIA regulates cytoplasmic localization of TLP. First, TLP may be carried to the cytoplasm by TFIIA. Vertebrate TFIIA has not been demonstrated to have a obvious nuclear localization signal, and nuclear localization of TFIIA in NIH3T3 cells was not so evident (Fig. 3). Because it is known that the nuclear import/export pathway of some transcription factors is modified by intracellular signaling (57, 58), the nuclear import/export of TFIIA in mammalian cells would be regulated depending on the cell state and thus influence TLP localization. Second, TLP multimerization may be related to the intracellular localization of TLP. It is known that a stress-induced p53 tetramer is retained in the nucleus and that its dissociation results in export of p53 (59). Inactivated monomeric/dimeric HSF3, which is normally localized in the cytoplasm, is converted into a transcriptional active trimer depending on the degree of nuclear localization upon stress (60). These observations indicate that the dynamic change in molecular states, including multimerization and dissociation, is one of the regulation pathways for intracellular localization of proteins. Because TLP forms a TFIIA-sensitive dimer/trimer (Fig. 2) and the ratio of TLPs to TFIIAs in the nucleus is much smaller than that in the cytoplasm (Fig. 3), it is thought that the proportion of TLP monomers in the nucleus is less than that in the cytoplasm. Thus, a multimer-to-monomer transition of TLP induced by TFIIA could be related to the cytoplasmic localization of TLP.

The idea that TFIIA regulates the cytoplasmic localization of TLP seems to be inconsistent with another speculation that TFIIA potentiates transcriptional regulatory functions of TLP as described above. However, these apparent inconsistent actions of TFIIA on TLP are thought to be related to a unique role of TLP in transcriptional regulation that is different from that of TBP. When nuclear TLP is increased in a particular situation (37, 43) by an unknown TFIIA-independent mechanism, tight association between TLP and TFIIA generates the potential role of TLP in transcriptional regulation as described above and inhibits TBP-dependent transcription. When cells do not need TLP for their activity, TLP is exported from the nucleus by TFIIA association so that TLP does not accumulate in the nucleus and affect transcription of target genes.

Acknowledgments—We thank Dr. T. Aoki for valuable discussion throughout this study, and Y. Tanaka, T. Wakamatsu, and T. Mabuchi for technical assistance. The Biacore analysis was performed at the Radiosotope Research Center, Chiba University.
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