Macromolecular Translocation Inhibitor II (Zn$^{2+}$-binding Protein, Parathymosin) Interacts with the Glucocorticoid Receptor and Enhances Transcription in Vivo*

Kazuki Okamoto and Fumihide Isohashi

From the Department of Biochemistry, St. Marianna University School of Medicine, Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

Macromolecular translocation inhibitor II (MTI-II), which was first identified as an *in vitro* inhibitor of binding between the highly purified glucocorticoid receptor (GR) and isolated nuclei, is an 11.5-kDa Zn$^{2+}$-binding protein that is also known as ZnBP or parathymosin. MTI-II is a small nuclear acidic protein that is highly conserved in rats, cows, and humans and widely distributed in mammalian tissues, yet its physiological function is unknown. To elucidate its *in vivo* function in relation to GR, we transiently transfected mammalian cells with an expression plasmid encoding MTI-II. Unexpectedly, we found that the expression of MTI-II enhances the transcriptional activity of GR. The magnitude of the transcriptional enhancement induced by MTI-II is comparable with that induced by the steroid receptor coactivator SRC-1. In contrast, MTI-II had little effect on the transcriptional activity of estrogen receptor. Immunoprecipitation analysis showed that in the presence of glucocorticoid hormone, GR coprecipitates with MTI-II, and, vice versa, MTI-II coprecipitates with GR. The expression of various deletion mutants of MTI-II revealed that the central acidic domain is essential for the enhancement of GR-dependent transcription. Microscopic analysis of MTI-II fused to green fluorescent protein and GR fused to red fluorescent protein in living HeLa cells showed that MTI-II colocalizes with GR in discrete subnuclear domains in a hormone-dependent manner. Coexpression of MTI-II with the coactivator SRC-1 or p300 further enhances GR-dependent transcription. Immunoprecipitation analysis showed that in the presence of glucocorticoid hormone, p300 and CREB-binding protein are coprecipitated with MTI-II. Furthermore, the knockdown of endogenous MTI-II by RNAi reduces the transcriptional activity of GR in cells. Moreover, expression of MTI-II enhances the glucocorticoid-dependent transcription of the endogenous glucocorticoid-inducible enzyme in cells. Taken together, these results indicate that MTI-II enhances GR-dependent transcription via a direct interaction with GR *in vivo*. Thus, MTI-II is a new member of the GR-coactivator complex.

Without glucocorticoid hormone, the glucocorticoid receptor (GR), a member of the nuclear receptor family, is localized in the cytosol through its association with a variety of heat shock/chaperone proteins. Upon hormone binding, GR dissociates from the chaperone proteins and translocates from the cytosol to the nucleus (1, 2). In the nucleus, the ligand-bound GR binds to specific DNA sequences termed glucocorticoid response elements (GREs), where it recruits a coactivator complex containing the p160 steroid receptor coactivator (SRC), acetyltransferases (CBP, p300, and pCAF), and methyltransferases (CARM1 and PRMT1) (3–5). In association with this coactivator complex, GR directly activates gene transcription.

Previously, using an *in vitro* assay of binding between highly purified activated GR and isolated nuclei, we identified at least three macromolecular translocation inhibitors (MTI-I, MTI-II, and MTI-III) from the cytosol of rat hepatocytes (6) and other cells (7). We purified the most potent of these *in vitro* inhibitors (MTI-II) to apparent homogeneity and determined the N-terminal amino acid sequences of four enzyme-digested fragments, which showed that MTI-II is identical to the 11.5-kDa Zn$^{2+}$-binding protein ZnBP (also known as parathymosin) (8).

MTI-II is a small and very acidic protein consisting of 101 amino acid residues, of which 11 are aspartic acid and 36 are glutamic acid residues, yielding a pI value of about 4.0. This protein has been found to be highly conserved in rats, cows, and humans (9) and is widely distributed in mammalian tissues, with the highest concentrations in liver followed by kidney and lowest concentrations in thymus, spleen, and muscle (10–13).

Haritos et al. (10, 14) initially isolated MTI-II from rat thymus as a homolog of the protein prothymosin α (PTα). MTI-II/parathymosin shares 46% identity with PTα (14), but the two proteins show a reciprocal tissue distribution in rats (10, 12). MTI-II and PTα were thought to be the native proteins or the putative precursor peptides of thymosin α1, the immunomodulatory polypeptide (or "thymic hormone") that influences lymphocyte maturation. Although the N-terminal region of MTI-II consists of 28 amino acids that are homologous to thymosin α1 (corresponding to 46% identity), the proposed role of this protein as the precursor of thymosin α1 has been brought into question, because MTI-II was found to have no coding sequence for signal peptides, as would be expected for a secretory protein, but instead was found to have an active bipartite nuclear localization signal (NLS) in its C-terminal region (15). Thus, this protein is actively translocated into the nuclei of *Xenopus* oocytes (16), HeLa S3 cells (17), dedifferentiated rat primary hepatocytes (13), COS cells (15), Reuber H35 rat hepatoma cells (15), and T24 (human bladder carcinoma) cells (18). Furthermore, MTI-II has been reported to bind to the linker histone H1 *in vitro* (19)....
Recently, it has been reported that MTI-II affects the binding of histone H1 to the nucleosome and is likely to be involved in remodeling of the chromatin structure (20). Therefore, this protein is now thought to be a ubiquitous nuclear acidic protein that affects histone H1 function, rather than being the precursor of the thymosin α1 secreted peptide.

Independently, MTI-II was isolated as ZnBP, a Zn$^{2+}$-dependent reversible inactivator of phosphofructokinase-1 from rat liver cytosol (21), and thereafter was found to be identical to parathymosin (22). In the presence of Zn$^{2+}$ in vitro, MTI-II/ZnBP has been reported to bind directly to both phosphofructokinase-1 and a large variety of cytosolic proteins, including several glycolytic enzymes (23). This indicates that MTI-II has functions not only in the nucleus but also in the cytoplasm.

Brand et al. (13) showed that MTI-II is actively translocated into the nuclei of undifferentiated cells, whereas it stays in the cytoplasm of differentiated and nonproliferated cells of rat tissues. Further detailed studies have suggested that the nuclear/cyttoplasmic distribution of the protein is regulated by cell density and is related to the differentiation state of the cell (15, 24). Thus, in the context of its high conservation and wide distribution, this interesting protein is expected to play essential functions and precise molecular mechanisms remain unknown.

By contrast, the nuclear functions of PTα are known in more detail (reviewed in Ref. 25). PTα consists of 111 amino acid residues, of which 18 are aspartic acid and 35 are glutamic acid residues (26, 27). The N-terminal region of PTα consists of 28 amino acids that are identical to thymosin α, the C-terminal regions contain two NLSs, and the protein is promptly translocated into nuclei (16–18, 28). PTα is also widely distributed in mammalian tissues, with highest concentrations in the spleen and thymus and lowest concentrations in the liver and kidney (12). It has been reported that PTα is involved in chromatin remodeling (29) and that overexpression of PTα accelerates the proliferation and retards the differentiation of the HL-60 cell (30). PTα is transcriptionally up-regulated on expression of c-Myc (31, 32) and that overexpression of PTα accelerates the proliferation and retards the differentiation of the HL-60 cell (30). PTα may function as an oncogene when stably expressed in Rat-1 cells (33). Thus, PTα plays an important role in cell proliferation. Furthermore, PTα binds to histones (both core histones and histone H1) and shows a nucleosome assembly activity in vitro (34, 35). PTα also modulates the interaction of histone H1 with chromatin (36). Recently, it has been reported that PTα interacts with CBP and enhances the transcriptional potential of CBP (37). In addition, PTα interacts with the Epstein-Barr virus nuclear antigen 3C and p300, and cooperatively regulates the acetylation (38, 39) and deacetylation (40) of histones. Moreover, PTα selectively enhances the transcriptional activity of the estrogen receptor (ER) but not the transcriptional activity of other nuclear receptors (41). These recent results indicate that PTα might function as a coactivator of cellular and viral transcription.

In previous in vitro experiments (8), we found that highly purified GR binds to an MTI-II affinity matrix. In the context of the findings on PTα, we speculated that MTI-II might be involved in regulating the transcriptional activity of GR in vivo. In this study, we therefore transiently transfected an expression plasmid encoding MTI-II into mammalian cells and examined the in vivo effects of MTI-II expression on GR-dependent transcription. We report that overexpression of MTI-II enhances GR-dependent transcription in a dose-dependent manner. Vice versa, the RNAi-mediated knockdown of endogenous MTI-II reduces GR-dependent transcription. Immunoprecipitation analysis shows that MTI-II interacts with GR in a glucocorticoid hormone-dependent manner. The expression of MTI-II fused to green fluorescence protein (GFP) and GR fused to red fluorescence protein (RFP) in living HeLa cells shows that MTI-II colocalizes with GR in discrete subnuclear domains. Taken together, our findings identify a new physiological role for MTI-II as a coactivator of GR-dependent transcription.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The coding regions of the human MTI-II and SRC-1 cDNAs were amplified by reverse transcription-PCR from HeLa cDNA. The coding region of the human ERα cDNA was amplified by PCR from human breast tumor cDNA library (Novagen). To construct the pTriEx-MTI plasmid expressing MTI-II with no His tag, we subcloned the amplified MTI-II cDNA into the Ncol-HindIII sites of the pTriEx-4 vector (Novagen). For pTriEx-SRC-1, we subcloned the SRC-1 cDNA into the Smal-PstI sites of pTriEx-4. For pTriEx-ERα, we subcloned the ERα cDNA into the Smal-Sacl sites of pTriEx-4. For pTriEx-GR, we digested the recombinant transfer vector pVL1393-GR (42) with BamHI and NotI, and subcloned the GR cDNA into the PstI-HindIII sites of the pTriEx-4 vector. The three plasmids expressing deletion mutants of MTI-II (the thymosin homolog domain plus one NLS motif, the acidic domain plus the whole C-terminal NLS domain and the C-terminal NLS domain) were constructed by digesting pTriEx-MTI with Cas911/BamHI, PstI/Cas911, and PstI/EcoO109I, respectively. For the plasmids expressing GFP-fused MTI-II and RFP-fused GR, we subcloned the MTI-II cDNA and the GR cDNA into the EcoRV-NotI sites of pQBl25-FC1 (Qiagen) and the HindIII-Sall sites of pDsRed2-C1 (Clontech), respectively. The p300 expression plasmid (pCMVβ-p300) was obtained commercially (Upstate Biotechnology, Inc., Lake Placid, NY). All constructs were verified by sequencing.

**Cell Culture, Transient Transfection, and Luciferase Assay**— COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) in the presence of penicillin/streptomycin. Cells were plated at a density of 3.5 × 10^4 cells/well in 6-well plates. For the GRE-luciferase assay, in order to reduce the level of the glucocorticoid hormone in the cells, we changed the medium to DMEM plus 10% FCS treated with dextran-charcoal (Hyclone) at 18 h after the cell plating. At 24 h after the medium change, the cells were transfected with 2.0 μg of GRE-firefly luciferase (Clontech) plus 40 ng of Renilla luciferase control reporter plasmid pRL-TK (Promega); 10 ng of pTriEx-GR; various amounts of pTriEx-MTI, pTriEx-SRC-1, or pCMVβ-p300; and control vector to a total of 5.0 μg of DNA/well, by using a cationic lipid reagent (TransFast; Promega). At 24 h after transfection, the cells were treated with 120 nM triamcinolone acetonide (TA) or control vehicle. The cells were harvested 24 h after hormone treatment, and the luciferase activities in the cell extracts were detected by using a dual luciferase assay system (Promega). For the estrogen response element-luciferase assay, in order to reduce the level of the estrogen hormone in the cells, we changed the medium to DMEM (without phenol red) plus 10% FCS treated with dextran-charcoal at 18 h after the cell plating. At 24 h after the medium change, the cells were transfected with 2.0 μg of estrogen response element-firefly luciferase (Clontech) plus 40 ng of Renilla luciferase control reporter plasmid pRL-TK; 20 ng of pTriEx-ERα; various amounts of pTriEx-MTI or pTriEx-SRC-1, and control vector to a total of 5.0 μg of DNA/well. At 24 h after transfection, the cells were treated with 12 nM 17β-estradiol or control vehicle. The cells were harvested 24 h after hormone treatment.

**Immunoprecipitation and Western Blotting**—HeLa cells were grown in DMEM plus 10% FCS in the presence of penicillin/streptomycin. Cells were plated at a density of 1.5 × 10^5 cells/100-mm diameter plate. After 18 h, the medium was changed to DMEM plus 10% FCS treated...
MTI-II Enhances GR-dependent transcription

with dextran-charcoal. At 24 h after the medium change, the cells were transfected with 7.25 μg of pTriEx-MTI (+H) per 100-mm plate by using a cationic lipid reagent (Tfx-20; Promega). At 24 h after transfection, the cells were treated with 120 nM TA or control vehicle. The cells were harvested 24 h after hormone treatment. Whole cell extracts were prepared from about 2 × 10^7 cells (four 100-mm plates), which were lysed in 270 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 5 mM Na₂EDTA, 135 mM NaCl, 1.0% Nonidet P-40, and 1 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Roche Applied Science). For cells treated with control vehicle, 20 mM Na₃MoO₄ was added to the lysis buffer in order to stabilize the ligand-free unactivated GR (i.e. heteromeric GR associated with Hsp90). The extracts were centrifuged at 16,000 × g for 30 min at 4 °C and preincubated with 20 μl of Protein G-agarose (Invitrogen) for 2 h at 4 °C in a rotator. The preincubated lysates were centrifuged at 16,000 × g for 10 min at 4 °C. The resulting supernatants were incubated with antibody (50 μg)-conjugated Protein G-agarose beads (10 μl) overnight at 4 °C in a rotator. After incubation, the beads were washed three times with lysis buffer and then resuspended in 40 μl of SDS gel loading buffer (10 mM Tris-HCl, pH 8.0, 40 mM dithiothreitol, 1 mM Na₂EDTA, and 10% glycerol). After boiling for 10 min, the samples were applied to a 4–20% acrylamide gradient gel (for the analysis of GR, SRC-1, p300, and CBP) or a 15% acrylamide gel (for the analysis of MTI-II). The proteins were electroblotted from the gel onto a polyvinylidene difluoride membrane and detected with ECL Plus Western blotting detection reagents (Amersham Biosciences). We used the following antibodies: anti-GR polyclonal antibody (43) and anti-SRC-1 polyclonal antibody (M–341) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), both for immunoprecipitation only; anti-His tag monoclonal antibody (Novagen) and antibodies against p300 (N–15) and CBP (A–22) (both from Santa Cruz Biotechnology) for immunoprecipitation and detection; and anti-GRα (P–20) polyclonal antibody (Santa Cruz Biotechnology) and anti-SRC-1 monoclonal antibody (MS–1130) (NeoMarkers) for immunodetection.

Fluorescence Microscopy—HeLa cells were plated at a density of 2.0 × 10^5 cells/35-mm coverslip bottom dishes (Becton Dickinson). After 18 h, the medium was changed to DMEM plus 10% FCS treated with dextran-charcoal. At 24 h after the medium change, the cells were cotransfected with the pQBI-MTI-II and the pDsRed2-GR plasmids. At 24 h after transfection, cells were treated with 120 nM TA or control vehicle for 18 h, the medium was changed to DMEM plus 10% FCS treated with dextran-charcoal. At 24 h after the medium change, the cells were transfected with 20 μg of pTriEx-MTI or mock vector (pTriEx–4) per 100-mm plate by using a cationic lipid reagent (Tfx–20). At 24 h after transfection, the cells were treated with 120 nM TA or control vehicle. The cells were harvested 24 h after hormone treatment. Whole cell extracts were prepared from about 8 × 10^6 cells (one 100-mm plate), which were lysed in 180 μl of the lysis buffer. The extracts were prepared as described above. Tyrosine aminotransferase (TAT) activity was assayed as described previously (44), except in a total assay volume of 0.1 ml. Protein concentrations were determined by the absorbance at 280 nm using bovine serum albumin as a standard.

RESULTS

MTI-II Enhances the Transcriptional Activity of GR in Vivo but Not the Transcriptional Activity of ER—To define the in vivo function of MTI-II, we examined whether overexpression of MTI-II would affect the transcriptional activity of GR in mammalian cells. COS-7 cells were cotransfected with an MTI-II-expressing plasmid and the GRE-luciferase reporter gene, and were treated with glucocorticoid hormone or vehicle 24 h after transfection. To our surprise, expression of MTI-II enhanced GR-dependent transcription in a dose-dependent fashion (Fig. 1A, filled circles). In the absence of glucocorticoid hormone, expression of MTI-II did not affect transcription of the luciferase gene (Fig. 1A, open circles). As a positive control, we transfected a plasmid expressing the steroid-receptor coactivator SRC-1 instead of the MTI-II expression plasmid. As expected, the increasing expression of SRC-1 enhanced the GR-dependent transcrip-
expression of MTI-II had little effect on the transcriptional activity of ER. COS-7 cells were cotransfected with an MTI-II-expressing plasmid, and the estrogen response element–luciferase reporter gene. As shown in Fig. 1A, the expression of MTI-II had little effect on the transcriptional activity of ERα, whereas SRC-1 enhances the transcriptional activity of ERα. This result indicates that the enhancement of the transcription of GRE-driven luciferase gene by MTI-II is not caused by an artificial nonspecific action of the acidic protein on the transiently transfected synthetic reporter genes that do not form phased nucleosomal structures.

**MTI-II Interacts with GR in Vivo in the Presence of Glucocorticoid Hormone**—We previously showed that highly purified GR binds to an MTI-II-affinity matrix in vitro and is eluted from the matrix with 200 mM NaCl (8). To elucidate whether MTI-II interacts with GR in vivo, we examined whether MTI-II could be co-immunoprecipitated with the intrinsic GR and vice versa. Because MTI-II is poorly immunogenic and we could not raise an antibody effective in immunoprecipitation, we prepared a plasmid expressing His-tagged MTI-II and used an anti-His tag monoclonal antibody to immunoprecipitate and to detect MTI-II.

HeLa cells expressing the intrinsic GR were transfected with the His-tagged MTI-II expression plasmid. The cleared lysates from the cells were immunoprecipitated with either anti-GR or anti-His tag antibodies. The anti-GR antibody was used both as a positive control and to verify that the amount of intrinsic GR did not change during hormone treatment (Fig. 2A, lanes 1 and 2). As shown in Fig. 2A (lanes 5 and 6), GR was co-precipitated with MTI-II in the presence of glucocorticoid hormone but not in the absence of hormone. Vice versa, MTI-II was co-precipitated with GR in the presence but not in the absence of hormone (Fig. 2B, lanes 3 and 4). This result indicates that MTI-II directly interacts with the ligand-bound activated GR (monomeric GR) in vivo, but not with ligand-free unactivated GR (heteromeric GR). Therefore, these findings indicate that the direct interaction of MTI-II with ligand-bound activated GR may enhance the transcriptional activity of GR in vivo.

**MTI-II Enhances GR-dependent Transcription through Its Acidic Domain**—Next, we examined which region of MTI-II is responsible for the enhancement of GR-dependent transcription. As shown in Fig. 3A, MTI-II is roughly divided into three portions: namely the N-terminal thymosin homolog domain (amino acids 1–37), the central acidic domain (amino acids 38–75), and the C-terminal NLS domain (amino acids 76–101), which contains a bipartite NLS (amino acids 78–80 and 90–95) separated by a short acidic domain (amino acids 83–89). We constructed three deletion mutants (Fig. 3A). The first contained the thymosin homolog domain plus one NLS PKRQKT motif (amino acids 1–27 + 89–101) and no acidic regions; the second contained the central acidic domain plus the C-terminal NLS domain (amino acids 28–101) but no thymosin homolog domain; and the third contained only the C-terminal NLS domain (amino acids 76–101) and neither the thymosin homolog domain nor the central acidic domain.

COS-7 cells were transiently transfected with the increasing amounts of the deletion mutant expression plasmids. As shown in Fig. 3B, only the mutant containing the acidic domain plus the C-terminal NLS enhanced the transcriptional activity of GR. Neither the thymosin homolog domain nor the C-terminal NLS domain had any effects on the transcriptional activity of GR. Thus, the central acidic domain is responsible for the enhancement of GR-dependent transcription by MTI-II.

**MTI-II Colocalizes with GR in the Nucleus in the Presence of Glucocorticoid Hormone**—The intracellular localization of GR is well characterized. Ligand-free GR is localized in the cytoplasm, whereas ligand-bound GR is found in the nucleus. The localization of MTI-II, however, remains somewhat complicated. As described above, MTI-II contains a bipartite NLS and is actively translocated into the nuclei of undifferentiated cells (13, 15–18), but it remains in the cytoplasm of differentiated and nonproliferated rat cells (13, 15). Here, we wanted to identify the localization of MTI-II in glucocorticoid hormone-depleted cells to determine whether MTI-II can colocalize with GR in the nucleus in the presence of hormone. We therefore transiently transfected HeLa cells with expression plasmids encoding GFP-fused MTI-II and RFP-fused GR.

Confocal microscopy analysis of living cells (Fig. 4A) showed that in the absence of hormone, RFP-fused GR was distributed in the cytosol, whereas GFP-fused MTI-II exhibited a mainly nuclear localization, with some expression in the nucleoli but no detectable expression in the cytoplasm. When we added glucocorticoid hormone to the culture medium (Fig. 4B), RFP-fused GR translocated into the nuclei, and GFP-fused MTI-II remained in the nuclei. These observations suggest that the interaction between MTI-II and GR is facilitated not by the nuclear...
MTI-II Enhances GR-dependent transcription

**FIGURE 3.** MTI-II enhances GR-dependent transcription via its central acidic domain. A, schematic representation of full-length MTI-II and the deletion mutants used in this study. B, expression plasmids encoding the various MTI-II proteins were transfected into COS-7 cells. At 24 h after transfection, the cells were treated with glucocorticoid hormone (closed symbols) or control vehicle (open symbols). At 24 h after hormone treatment, luciferase activities were detected as described under “Experimental Procedures.” ●, full-length MTI-II; ▲, the thymosin homolog domain plus one NLS motif (amino acids 1–27 + 89–101); ■, the central acidic domain plus the C-terminal NLS domain (amino acids 28–101); ○, the C-terminal NLS domain (amino acids 76–101).

**FIGURE 4.** Subcellular and subnuclear localization of GFP-fused MTI-II and RFP-fused GR in HeLa cells. Cells were cultured in dextran-charcoal-treated FCS medium and transiently transfected with plasmids expressing GFP-fused MTI-II and RFP-fused GR. At 24 h after transfection, the cells were treated with control vehicle (A) or glucocorticoid hormone (B and C). At 6 h after hormone treatment, the cells were viewed under a confocal microscope. The arrows in C indicate typical points of colocalization of the two proteins. Bar, 5 μm.

MTI-II Enhances GR-dependent Transcription in Cooperation with SRC-1 and p300 in Vivo—It is well known that in the nucleus, GR recruits a coactivator complex containing SRC-1, CBP/p300, and p300/CBP-associated factor and that its interaction with this coactivator complex mediates GR-dependent transcription (1–5). We therefore examined whether MTI-II cooperates with components of this coactivator complex in GR-dependent transcription.

COS-7 cells were transiently transfected with the increasing amounts of expression plasmids encoding the coactivators SRC-1 and p300 and a constant amount of the MTI-II expression plasmid (0.5 or 1.0 μg). As expected, expression of the SRC-1 and p300 enhanced GR-dependent transcription in a dose-dependent manner (Fig. 5). The coexpression of MTI-II with SRC-1 or p300 further enhanced GR-dependent transcription. The effects of MTI-II expression and SRC-1 or p300 expression appeared to be additive. By contrast, in the absence of hormone, cotransfection of MTI-II had no effect on transcription of the luciferase gene.

CBP and p300 Coprecipitate with MTI-II in a Glucocorticoid Hormone-dependent Manner—Next, we examined whether MTI-II is a component of the GR-coactivator complex in vivo by carrying out a co-immunoprecipitation analysis of the intrinsic coactivators p300, CBP, and SRC-1 and MTI-II. HeLa cells were transfected with an expression plasmid encoding His-tagged MTI-II, and the lysates were immunoprecipitated with anti-His tag antibody. As a positive control and to check whether the amounts of the intrinsic coactivators were unchanged by glucocorticoid hormone treatment, the lysates (Fig. 6, lanes 1 and 2) and the immunoprecipitates obtained with anti-coactivator antibodies (Fig. 6, lanes 3 and 4) were analyzed on the same gel.

As shown in Fig. 6, A and B (lanes 5 and 6), p300 and CBP coprecipitated with MTI-II in the presence of glucocorticoid hormone. However, we did not detect the co-immunoprecipitation of SRC-1 with MTI-II (Fig. 6C). This may be caused by the poor detection ability of the anti-SRC-1 antibody. Alternatively, another intrinsic p160 coactivator, such as GRIP1 or SRC-3 rather than SRC-1, may be used in GR-dependent transcription in HeLa cells. In the absence of the hormone, p300 was undetectable, but two weak CBP bands of similar size were present in the MTI-II immunoprecipitates. The nature of these weak CBP bands is unknown; nevertheless, it is clear that the intrinsic p300 and CBP are co-immunoprecipitated with MTI-II in a hormone-dependent manner.

co-translocation of cytosolic MTI-II and GR but by the recruitment of MTI-II by GR after it translocates to the nucleus. Furthermore, viewing the nucleus under a higher power of the microscope (Fig. 4C), we identified many points where GFP-fused MTI-II and RFP-fused GR were colocalized in discrete subnuclear domains. This observation further confirms that MTI-II and GR interact *in vivo.*
MTI-II Enhances GR-dependent transcription

RNAi-mediated Reduction of Endogenous MTI-II Reduces the Transcriptional Activity of GR—In order to confirm the in vivo function of MTI-II as a coactivator, we examined whether the knockdown of endogenous MTI-II would reduce the transcriptional activity of GR. We used RNAi to reduce the endogenous level of MTI-II and measured the luciferase activity induced by glucocorticoid hormone in the RNAi-treated cells.

To generate small interfering RNAs, we used a 27-nt hairpin siRNA expression vector system and selected three sequences from the MTI-II mRNA located at 35–56 nt (1-hp), 72–98 nt (3-hp), and 99–125 nt (4-hp). The cDNAs for the selected mRNAs, comprising two identical 27-nt sequence motifs arranged in an inverted orientation and separated by a 9-nt spacer (loop region) of nonhomologous sequence, were synthesized and inserted into the siRNA expression vector. HeLa cells were transiently transfected with the hairpin siRNA expression vectors, together with the GRE-luciferase reporter vector. To determine the level of endogenous MTI-II, we generated an antisense to the N-terminal domain (amino acid residues 6–31) of MTI-II in rabbits and affinity-purified the antibody from the antiserum with a resin conjugated with the peptide used for immunization. This antibody was effective in immunoblots. The immunoblot of MTI-II from HeLa cells appears as a doublet of bands (Fig. 7A, upper left lane). The doublet appearance of MTI-II has been previously observed in rat, rabbit, mouse, cow, and human tissues by Brand et al. (13). The nature of the doublet is unknown; however, a stable post-translational protein modification has been proposed (13).

The RNAi-treated HeLa cells were harvested 24, 48, and 72 h after transfection of the siRNA expression vectors. An immunoblot for endogenous MTI-II (Fig. 7A) showed that at 48 h, MTI-II protein in the cells transfected with 3-hp and 4-hp was decreased to ~20% and 30%, respectively, of that in the control cells transfected with mock vector. This decrease in the level of MTI-II was still present at 72 h after transfection. In the cells transfected with 1-hp, a moderate decrease in MTI-II protein (~80%) was observed. Fig. 7B shows that there was no marked decrease in actin protein in the cells transfected with the siRNA expression vectors.

Since the level of endogenous MTI-II protein was maintained at a low level between 48 and 72 h after transfection of the siRNA expression vectors, we measured the luciferase activity induced by GR between 48 and 72 h after transfection. The HeLa cells were treated with glucocorticoid hormone at 48 h after transfection of the siRNA expression vectors and harvested at 72 h after transfection. The GRE-luciferase activities of the cells treated with 1-hp, 3-hp, and 4-hp were decreased to ~80, 40, and 50%, respectively, of that in the cells transfected with mock vector (Fig. 7C). To exclude the possibility that this decrease in luciferase activity was caused by a reduction in GR protein in the RNAi-treated cells between 48 and 72 h, we measured the GR protein level in the cells. Fig. 7D shows that there was no marked change in the GR protein level at 48 and 72 h. Thus, this RNAi analysis indicates that endogenous MTI-II is an important component of GR-dependent transcription in vivo.

MTI-II Enhances the Glucocorticoid-dependent Transcription of the Endogenous Tyrosine Aminotransferase Gene—It would be very important to check whether MTI-II can alter the activity of endogenous GR on the ligand-dependent activation of endogenous GR-dependent genes. TAT is a well known glucocorticoid-inducible enzyme (44). We measured TAT activity in HeLa cells transfected with the MTI-II expression vector. TAT activity in HeLa cells without transfection and without glucocorticoid hormone treatment is ~0.09 units/mg of protein. TAT activity after transfection with mock vector in the absence of glucocorticoid hormone is ~0.09 units/mg of protein (Fig. 8). Thus, the transfection procedure did not affect the basal TAT activity in cells. In the absence of the hormone, overexpression of MTI-II had no effect on the basal TAT activity (Fig. 8). In the presence of the hormone, TAT activity in the mock vector-transfected cells increased to ~150% of that in the absence of the hormone. Overexpression of MTI-II further increased the TAT activity to ~190% of that in the absence of the hormone. Thus, MTI-II enhances the transcription of the endogenous hormone-regul
**MTI-II Enhances GR-dependent transcription**

![Graph showing TAT activity](image)

**DISCUSSION**

MTI-II was first identified as the most potent inhibitor of binding between the highly purified GR and isolated nuclei or synthetic DNA in *vitro* (6–8). Here, we unexpectedly found that MTI-II enhances the *in vivo* transcriptional activity of GR via its central acidic domain. The reason why MTI-II inhibits the binding of purified GR to nuclei *in vitro* is unresolved. An unknown factor or factors, which are removed by the purification of GR and/or by the isolation of nuclei, may be required to enhance the *in vitro* nuclear binding of GR in the presence of MTI-II. Alternatively, an excess amount of MTI-II in the *in vitro* assay may cause nonspecific binding of MTI-II to GR, and this nonspecific binding might block access of GR to the nucleus or to DNA. A third possibility is that the temperature and/or the pH of the *in vitro* nuclear binding assay may cause the apparent inhibition in the presence of MTI-II. Further elucidation of the mechanism underlying the *in vitro* inhibition mediated by MTI-II is required.

Although the factors responsible for the apparent *in vitro* inhibition of nuclear binding by GR are obscure, our previous *in vitro* work clearly showed that the highly purified GR binds to MTI-II-Sepharose (8). In the current *in vivo* experiments, we have shown that MTI-II is co-immunoprecipitated with the intrinsic GR and *vice versa* and that MTI-II colocalizes with GR in discrete subnuclear domains in the presence of glucocorticoid hormone. We therefore conclude that MTI-II binds directly to ligand-bound activated GR *in vivo*. Furthermore, the co-precipitation of p300 and CBP with MTI-II in a hormone-dependent manner indicates that MTI-II is a member of the coactivator complex that is recruited by the nuclear-localized GR and to enhance GR-dependent gene transcription.

We found that an MTI-II mutant containing the acidic domains but not the N-terminal thymosin homolog domain could enhance GR-dependent transcription, which suggests that the GR-binding site in MTI-II is located in the central acidic domain and/or in the C-terminal short acidic domain. From its amino acid sequence, it can be seen that MTI-II has no LXXLL motif. Therefore, the nature of the interaction of MTI-II with GR is likely to be electrostatic. It should be noted here that GR has a unique bipolar nature (42, 45, 46). The net pI of GR is calculated to be 6.2; however, the pI of the C-terminal half (containing the DNA-binding domain and the steroid-binding domain) is 8.5, and the pI of the N-terminal half is 4.6. Indeed, GR can bind both to an anion exchanger (Mono Q) and to a cation exchanger (Mono S) at the same pH (pH 8.4). By making use of this bipolar nature of GR, we have purified GR to 97% purity in two common ion exchange steps (42, 45, 46). Therefore, the acidic regions of MTI-II may bind to the basic C-terminal half of GR.

The precise molecular mechanism underlying how MTI-II enhances the transactivation of GR is still unknown. MTI-II has a short and simple acidic amino acid repeat structure, which suggests that it has neither enzymatic activity, such as histone acetyltransferase or histone methyltransferase activity, nor DNA binding activity. A high local concentration of acidic amino acid residues, in the form of monotonous runs of either glutamic or aspartic acidic residues, has been found in several chromatin and chromosomal proteins (reviewed in Ref. 47). The acidic regions of these proteins are thought to play important roles in transcription and replication in addition to their putative role in anchoring the proteins to chromatin through electrostatic interactions with the basic histones (48). Much evidence indicates that the acidic activation domain of herpes virus VP16 can function in various steps of the transcription reaction, probably via its many interactions with components of the basal transcriptional machinery (49–52) and ATP-dependent chromatin-remodeling complexes (53). Since GR has no such acidic domain, MTI-II may function to supply GR with an acidic transactivation domain. An alternative but not mutually exclusive possibility is that MTI-II plays the role of electrostatic “glue” that bonds GR and the other coactivators. As shown in Fig. 6, the p300 and CBP coactivators are co-immunoprecipitated with MTI-II in a glucocorticoid hormone-dependent manner, and the effect of MTI-II and the other coactivators on GR-dependent transcription appears to be additive (Fig. 5). Moreover, MTI-II possibly binds to the C-terminal side of GR, which is the same region that GR uses to interact with the other coactivators. It has been reported that the lengths of the functional acidic regions in the nuclear proteins lie within the range of the 13 amino acids of c-Myc to the 42 amino acids of HMG-1, although CENP-B, the 80-kDa human centromere autoantigen, has a larger acidic domain of 61 amino acids (47). Compared with the average length (23 amino acids) of the functional acidic regions, MTI-II has an acidic amino acid sequence (40 + 7 amino acids) that is long enough to bind both GR and the coactivators and has a weight (Mw = 11,427) that is small enough to slide into the GR-coactivator complex. Thus, MTI-II may stabilize the GR-coactivator complex.

As mentioned above, PTα has been reported to interact with CBP, Epstein-Barr virus nuclear antigen 3C, and p300 (37–40). These results indicate that PTα is a coactivator of transcriptional activation. We have shown here that the homologous acidic protein MTI-II is also a coactivator of GR-dependent transcription. Although it is unknown whether the interaction of PTα with CBP/p300 is hormone-dependent, the apparent interaction of MTI-II with CBP/p300 is clearly glucocorticoid-dependent. It should be noted here that PTα selectively enhances the transcriptional activity of ER but not that of GR or the progesterone receptor (41). This enhancement is mediated not by the interaction of PTα with ER but by its interaction with a repressor of ER (41). By contrast, we have shown here that MTI-II enhances the transcriptional activity of GR but not that of ER and that the enhancement is mediated by the direct interaction of MTI-II with GR. Therefore, these two homologous proteins seem to have distinct actions on the different nuclear receptors. In the context of the reciprocal tissue distribution of PTα and MTI-II, each protein will play a distinct role in specific tissues. Further elucidation of the functions of MTI-II will reveal the precise
mechanism underlying the transcriptional activation mediated by nuclear receptors.

Acknowledgments—We thank Dr. Naoya Suematsu for helpful discussion, Dr. Kiyotaka Shibata for helpful advice, and Junko Asano for technical assistance.

REFERENCES

1. Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. (1996) Endocr. Rev. 17, 245–261
2. Aranda, A., and Pascual, A. (2001) Physiol. Rev. 81, 1269–1304
3. McKenna, N. J., Lanz, R. B., and O’Malley, B. W. (1999) Endocr. Rev. 20, 321–344
4. Molella, H. A., Kilts, C. P., Allen, R. L., and Tetel, M. J. (2003) Biol. Reprod. 69, 1449–1457
5. Xu, L., and Qi, L. (2003) Mol. Endocrinol. 17, 1681–1692
6. Okamoto, K., Isohashi, F., Horiuchi, M., and Sakamoto, Y. (1982) Biochem. Biophys. Res. Commun. 108, 1655–1660
7. Isohashi, F., Horiuchi, M., Okamoto, K., and Sakamoto, Y. (1984) J. Steroid Biochem. 20, 1117–1122
8. Okamoto, K., and Isohashi, F. (2000) Exr. J. Biochem. 267, 155–162
9. Clinton, M., Frangou-Lazaridis, M., Panneerselvam, C., and Horecker, B. L. (1989) Biochem. Biophys. Res. Commun. 158, 856–862
10. Haritos, A. A., Salvin, S. B., Blacher, R., Stein, S., and Horecker, B. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1050–1053
11. Panneerselvam, C., Caldarella, J., and Horecker, B. L. (1987) J. Immunol. Methods 104, 131–136
12. Clinton, M., Frangou-Lazaridis, M., Panneerselvam, C., and Horecker, B. L. (1989) Arch. Biochem. Biophys. 269, 256–263
13. Brand, I. A., Heinickel, A., and Söling, H.-D. (1991) Eur. J. Cell Biol. 54, 157–165
14. Komiyama, T., Pan, L.-X., Haritos, A. A., Wideman, J. W., Pan, Y.-C. E., Chang, M., Rogers, I., and Horecker, B. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1242–1245
15. Trompeter, H. I., Blankenburg, G., Brügger, B., Menne, J., Schiernmeyer, A., Scholz, M., and Söling, H. D. (1996) J. Biol. Chem. 271, 1187–1193
16. Watts, J. D., Cary, P. D., Sauzier, P., and Crane-Robinson, C. (1990) Eur. J. Biochem. 192, 643–651
17. Clinton, M., Graeve, L., El-Dorry, H., Rodriguez-Boulan, E., and Horecker, B. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6608–6612
18. Vareli, K., Frangou-Lazaridis, M., van der Kraan, I., Tsolas, O., and van Driel, R. (2000) Exp. Cell Res. 257, 152–161
19. Kondili, K., Tsolas, O., and Papamarcaki, T. (1996) Eur. J. Biochem. 242, 67–74
20. Martic, G., Karetsoy, Z., Kefala, K., Politou, A. S., Clapier, C. R., Straub, T., and Papamarcaki, T. (2005) J. Biol. Chem. 280, 16143–16150
21. Brand, I. A., and Söling, H.-D. (1986) J. Biol. Chem. 261, 5892–5900
22. Trompeter, H. I., Brand, I. A., and Söling, H.-D. (1988) FEBS Lett. 253, 63–66
23. Brand, I. A., and Heinickel, A. (1991) J. Biol. Chem. 266, 20984–20989
24. Trompeter, H. I., Schiernmeyer, A., Blankenburg, G., Hennig, E., and Söling, H.-D. (1999) J. Cell Sci. 112, 4113–4122
25. Piñeiro, A., Cordero, O. J., and Nogueira, M. (2000) Peptides 21, 1433–1446
26. Haritos, A. A., Goodall, G. J., and Horecker, B. L. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1008–1011
27. Haritos, A. A., Blacher, R., Stein, S., Caldarella, J., and Horecker, B. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 343–346
28. Castro, J. M., and Barcia, M. G. (1996) Biochem. Biophys. Res. Commun. 224, 140–146
29. Gómez-Márquez, J., and Rodríguez, P. (1998) Biochem. J. 333, 1–3
30. Rodríguez, P., Viñuela, J. E., Álvarez-Fernández, L., Buceta, M., Vidal, A., Domínguez, F., and Gómez-Márquez, J. (1998) Biochem. J. 331, 753–761
31. Gauthier, S., Meichle, A., and Eilers, M. (1994) Mol. Cell Biol. 14, 3853–3862
32. Vareli, K., Frangou-Lazaridis, M., and Tsolas, O. (1995) FEBS Lett. 371, 337–340
33. Orre, R. S., Cotter, M. A., II, Subramanian, C., and Robertson, E. S. (2001) J. Biol. Chem. 276, 1794–1799
34. Papamarcaki, T., and Tsolas, O. (1994) FEBS Lett. 345, 71–75
35. Díaz-Juilli, C., Pérez-Estévez, A., Covel, G., and Freire, M. (1996) Biochim. Biophys. Acta 1296, 219–227
36. Karetsov, Z., Sandaltzopoulos, R., Frangou-Lazaridis, M., Lai, C. Y., Tsolas, O., Becker, P. B., and Papamarcaki, T. (1998) Nucleic Acids Res. 26, 3111–3118
37. Karetsov, Z., Kretsovali, A., Murphy, C., Tsolas, O., and Papamarcaki, T. (2002) EMBO Rep. 3, 361–366
38. Cotter, M. A., II, and Robertson, E. S. (2000) Mol. Cell Biol. 20, 5722–5735
39. Subramanian, C., Hasan, S., Rowe, M., Hottiger, M., Orre, R., and Robertson, E. S. (2002) J. Virol. 76, 4699–4708
40. Knight, J. S., Lan, K., Subramanian, C., and Robertson, E. S. (2003) J. Virol. 77, 4261–4272
41. Martini, P. G. V., Delage-Mouroux, R., Kraichely, D. M., and Katzenellenbogen, B. S. (2000) Mol. Cell Biol. 20, 6224–6232
42. Hyodo, M., Okamoto, K., Shibata, K., Suematsu, N., and Isohashi, F. (2001) J. Chromatogr. B 765, 89–97
43. Okamoto, K., Liu, G., Yu, W. G., Ochiai, T., and Isohashi, F. (1996) J. Biochem. (Tokyo) 119, 920–925
44. Okamoto, K., Isohashi, F., Tsukanaka, K., Horiuchi, M., and Sakamoto, Y. (1983) Endocrinology 112, 336–340
45. Okamoto, K., Suematsu, N., and Isohashi, F. (2003) J. Chromatogr. B 790, 349–353
46. Okamoto, K., and Isohashi, F. (2003) J. Chromatogr. B 797, 367–371
47. Earnshaw, W. C. (1987) J. Cell Biol. 105, 1479–1482
48. Hall, S. (1993) Cell 72, 481–483
49. Lin, Y.-S., Ha, I., Maldonado, E., Reinberg, D., and Green, M. R. (1991) Nature 353, 569–571
50. Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X., and Berk, A. J. (1991) Cell 67, 365–376
51. Hall, D. B., and Struhl, K. (2002) J. Biol. Chem. 277, 46403–46405
52. Dion, V., and Coulombe, B. (2003) J. Biol. Chem. 278, 11495–11501
53. Viglione, M., Hassan, A. H., Neely, K. E., and Workman, J. L. (2000) Mol. Cell Biol. 20, 1899–1910