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Effect of Macromolecular-Translocation Inhibitor-III on Binding of Activated Glucocorticoid-Receptor Complex to Specific DNA[1]

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We previously purified macromolecular-translocation inhibitor-III (MTI-III), which inhibits the binding of the activated glucocorticoid-receptor complex (GR) to nuclei, to homogeneity from rat liver, and we found that the purified MTI-III bound to partially purified activated GR under low salt conditions at slightly acidic pH [Liu, G., Okamoto, K., and Isohashi, F. (1993) *Eur. J. Biochem.* 218, 679-687]. This was the first direct evidence that the inhibitor acts through a direct interaction with the activated GR. In this study, we examined whether the purified MTI-III could interfere with the binding of GR to a DNA fragment containing a specific glucocorticoid-response element (GRE). Under nearly isotonic salt conditions at neutral pH, the activated GR bound to the GRE but not to nonspecific DNA. Under similar conditions, the activated GR also bound to the purified MTI-III. The resulting GR/MTI-III complex did not bind to the GRE. We also found that addition of MTI-III to the GR/GRE complex resulted in time-dependent disruption of the GR/GRE complex and formation of the GR/MTI-III complex. The half-life of the GR/GRE complex in the presence of MTI-III was about 13 min. These results suggest that MTI-III enhances the release of GR from the GR/GRE complex and immediately forms a stable GR/MTI-III complex.

Key words: glucocorticoid-receptor, glucocorticoid-response element, translocation-inhibitor.

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Unliganded/unactivated glucocorticoid receptor (a 9 S form) is a cytosolic soluble protein which consists of a single steroid-binding protein (98 kDa) and several other proteins, such as heat-shock proteins (hsp 90, hsp 70, and hsp 56) (*1-4*). The 9 S heterocomplex binds glucocorticoid hormone with high affinity, and the glucocorticoid hormone-receptor complex (GR) subsequently undergoes a conformational change, which leads to its irreversible dissociation into a monomeric activated GR (a 4 S form) and other heat-shock proteins (*5, 6*). The 4 S activated GR can bind to nuclei, to chromosomal proteins and subsequently to a specific DNA sequence termed glucocorticoid-response element (GRE), where the GR effects the specific gene expression (*5, 6*).

The precise regulatory mechanisms of association of activated GR with the nuclear components are still unclear. However, many studies (see Ref. *1* for review) have demonstrated that the binding of activated GR to nuclei is enhanced by an ATP-stimulated translocation promoter (*7-9*) and inhibited by macromolecular-translocation inhibitors (MTI-I, -II, and -III) (*10-12*). We speculated that these intracellular components function in intact cells and are involved in intracellular regulation of hormone response. To clarify the regulatory mechanisms of these intracellular components, it is prerequisite to purify the factors and to reconstruct the physiological regulation system *in vitro*. Recently, we purified an MTI-III to apparent homogeneity and characterized it (*12*). The purified MTI-III has a molecular mass of approximately 69 kDa and a sedimentation coefficient of approximately 3.7 S, and it interacts with monomeric activated GR to form a 6.8 S GR/MTI-III complex under low salt conditions (25 mM KCl) at slightly acidic pH (pH 6.3 at 10°C). This was the first direct evidence that the translocation inhibitor acts through interaction with activated GR. In this paper, we examined the effect of the purified MTI-III on the binding of GR to specific GRE under nearly isotonic salt conditions (130 mM NaCl equivalent) at neutral pH (pH 7.2 at 4°C) and found that the purified MTI-III enhances the dissociation of the GR/GRE complex.

MATERIALS AND METHODS

Materials—[^{3}H]Triamcinolone acetonide {9α-fluoro-11β,21-dihydroxy-16α,17α-(1-methylethylidene-bis-[oxy])pregna-1,4-diene-3,20-dione; 30 Ci/mmol} and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from the Radio-

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Abbreviations: GR, glucocorticoid hormone-receptor complex; GRE, glucocorticoid-response element; MTI, macromolecular-translocation inhibitor of GR binding to nuclei; MMTV57, double-stranded 57-bp DNA fragment derived from mouse mammary tumor virus long-terminal repeat; NS38, double-stranded 38-bp DNA containing no GRE motif.

chemical Centre (Amersham, United Kingdom). Glycerol (ultra pure) was from GIBCO (Grand Island, NY). Bovine serum albumin (ultra pure, DNase-, RNase-free) was from Takara (Ohotsu). All other chemicals (reagent grade) were from Sigma (St. Louis, MO).

Partial Purification of Activated GR from Rat Liver—The activated receptor-[^{3}H]triamcinolone-acetonide complex (GR) was purified about 3,000-fold from the livers (60 g) of male albino Donryu rats (adrenalectomized 3 days before) as described previously (*13*). This partially purified activated GR preparation contains no detectable amount of translocation inhibitors (*13*) or unactivated GR (*13*).

Purification of MTI-III—MTI-III was purified about 4,500-fold from the livers of male albino Donryu rats (adrenalectomized 3 days before) as described previously (*12*). The purified sample gave a single protein band on 10-15% gradient SDS-PAGE (*12*). The purified sample was mixed with bovine serum albumin (4 mg/ml) and dialyzed at 4°C overnight with buffer A (20 mM Tris/HCl, 4 mM $MgCl_2$, 75 mM NaCl, 10% glycerol, and 1 mM dithiothreitol, pH 7.2 at 4°C). This MTI-III preparation was used in the following experiments.

Oligonucleotides—Oligonucleotides used for DNA binding assays were obtained by annealing the synthetic single-stranded oligonucleotides, and their sequences are as follows [underlined nucleotides correspond to the core sequence of the GRE (*22*)]: MMTV57, 5′-AGCTTGGCTGCAGGTCGACATGGTTACAAACTGTTCTTAAAACAAGGATGCTCGACG-3′ and 5′-GATCCGTCGAGCATCCTTGTTTTAAGAACAGTTTGTAACCATGTCGACCTGCGCCA-3′; NS38, 5′-GGATCCACCCTGTCTCATGAATATGCAAATCAGGTGAG-3′ and 5′-CTCACCTGATTTGCATATTCATGAGACAGGGTGGATCC-3′. The resulting double-stranded oligonucleotides were phosphorylated using T4 polynucleotide kinase (Takara) in the presence of [γ-^{32}P]ATP. The ^{32}P-labeled DNA was separated from unincorporated [γ-^{32}P]ATP by spin-column chromatography.

Preparation of Anti-(GR) Antibody—We immunized rabbits with synthetic peptide representing amino acids 366-387 of rat glucocorticoid-receptor, according to the method of Cidlowski *et al.* (*23*). An IgG fraction from sera of the immunized rabbits contained specific antibodies to GR (*17*). The anti-(GR) IgG was characterized with regard to titer, cross-reactivity and specificity (*17*). Immunoblot analysis using the anti-(GR) antibody revealed a specific band of approximately 100 kDa in rat liver cytosol and in the partially purified activated GR. The synthetic peptide blocked the interaction of the antibody with GR (*17*).

Glycerol-Density-Gradient Analysis—The binding of GR to DNA was performed in a total volume of 100 μl of buffer B containing 20 mM Tris/HCl, 96 mM NaCl, 4 mM $MgCl_2$, 1 mM Na_2EDTA, 10% glycerol, bovine serum albumin (2.5 mg/ml), and 1 mM dithiothreitol, pH 7.2 at 4°C. The conductivity of this buffer was equivalent to 130 mM NaCl. Partially purified activated GR (2 pmol) was mixed with ^{32}P-labeled DNA (1 pmol). The mixture was incubated for 15 min at 4°C. For glycerol-density-gradient analysis of the binding of GR/MTI-III complex to DNA, GR (2 pmol) was first mixed with purified MTI-III (2 pmol) in buffer B. The mixture was incubated for 15 min at 4°C, then ^{32}P-labeled MMTV57 (1 pmol) was added, and the mixture was incubated further for 15 min at 4°C. For glycerol-density-gradient analysis of the binding of GR/MMTV57 complex to MTI-III, GR (2 pmol) was first mixed with ^{32}P-labeled MMTV57 (1 pmol) in buffer B. The mixture was incubated for 15 min at 4°C, then purified MTI-III (2 pmol) was added, and the mixture was incubated for the time indicated in Fig. 3 at 4°C.

After incubation, the mixture was layered onto a 5-ml gradient of 12-25% glycerol prepared in buffer A. The gradient was centrifuged in a vertical rotor (SRP 83VT, Hitachi) at 54,900 × *g* for 30 min at 10°C. After centrifugation, fractions (200 μl per fraction) were collected from the bottom of the gradient. Tritium and ^{32}P were measured in a WALLAC 1490 liquid scintillation counter (Pharmacia-LKB). Figures showing radioactive diagrams are corrected for ^{32}P spillover into the tritium channel. Correction was made for radioactive decay of the labeled DNA using a ^{32}P half-life of 14.3 days.

RESULTS

GR Binding to GRE—The binding of GR to GRE was performed in the nearly isotonic salt buffer (equivalent to 130 mM NaCl) and analyzed by sedimentation of GR and GRE through a 12-25% glycerol gradient in the same buffer. To simplify the study, we used synthetic 57-bp DNA (MMTV57) containing one GRE motif and a partially purified activated GR containing no detectable amounts of unactivated GR (a 9 S heteromeric form) or translocation inhibitors (*13*). As shown in Fig. 1A, the partially purified activated receptor-[^{3}H]triamcinolone-acetonide preparation contains only a homogenous population of the 4 S monomeric form. The tritium at the top of the gradient represents dissociated hormone ligand. Figure 1B shows that the ^{32}P-labeled MMTV57 alone formed a peak near the top of the gradient after centrifugation for 30 min. When the gradient was centrifuged for 60 min, the ^{32}P-labeled MMTV57 formed a single peak sedimenting at approximately 3.8 S (data not shown), suggesting that the ^{32}P-labeled MMTV57 contains no free ^{32}P-labeled nucleotides.

Several laboratories (*14-16*) have reported that two GR molecules bind to one GRE motif as a homodimer under nearly isotonic salt conditions (75-100 mM NaCl). Thus, we incubated the GR and MMTV57 in a molar ratio of 2:1 under nearly isotonic salt conditions (130 mM NaCl equivalent). Figure 1C shows the sedimentation profile of receptor-[^{3}H]triamcinolone-acetonide (2 pmol) after incubation with ^{32}P-labeled MMTV57 (1 pmol). The incubation resulted in decrease in the amount of the 4 S receptor-[^{3}H]triamcinolone-acetonide and the simultaneous formation of a new peak of tritium sedimenting at approximately 7.5 S. The ^{32}P-labeled MMTV57 also formed a peak co-sedimenting with the tritium peak at approximately 7.5 S. Incubation with ^{32}P-labeled DNA (NS38) containing no GRE site did not affect the sedimentation coefficient of the 4 S receptor-[^{3}H]triamcinolone-acetonide (Fig. 1D). These results indicate that the 4 S activated GR bind to the GRE site in MMTV57, and the resulting GR/MMTV57 complex sediments at approximately 7.5 S. To confirm that the 7.5 S tritium peak contains the receptor molecule, we used a mono-epitope specific anti-(GR) antibody (IgG fraction). This anti-(GR) IgG did not inhibit the binding of GR to DNA-cellulose (*17*). Figure 1E shows that incubation of GR with the anti-(GR) IgG increased the sedimentation coeffi-

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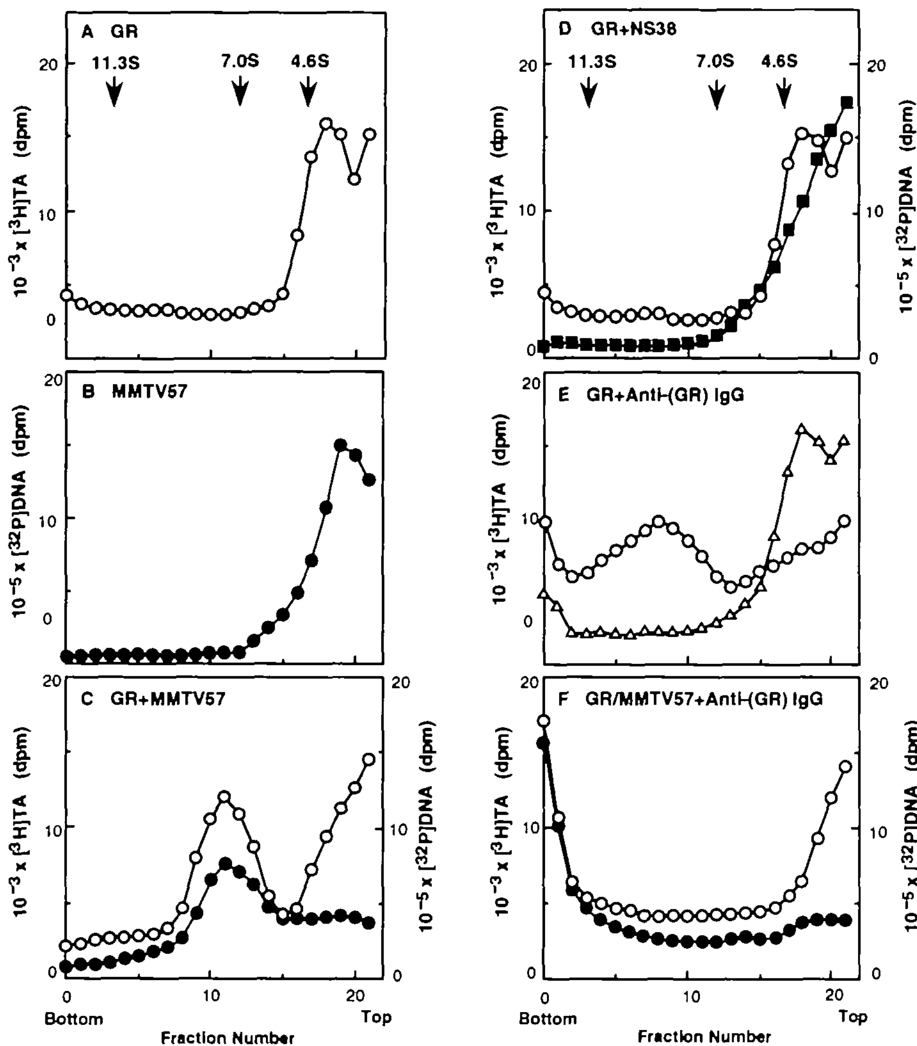

Fig. 1. **Glycerol-density-gradient analysis of GR and MMTV57.** (A) GR (partially purified activated receptor-[^{3}H]triamcinolone acetonide, 2 pmol) was incubated in buffer B at 4°C for 15 min. (B) ^{32}P-labeled MMTV57 (1 pmol) was incubated in buffer B at 4°C for 15 min. (C) GR (○) was incubated with ^{32}P-labeled MMTV57 (●) in buffer B at 4°C for 15 min. (D) GR was incubated with ^{32}P-labeled NS38 (■) in buffer B at 4°C for 15 min. (E) GR was incubated with anti-(GR) IgG (○) or with preimmune-IgG (△) in buffer B at 4°C for 15 min. (F) GR (○) was incubated with ^{32}P-labeled MMTV57 (●) in buffer B at 4°C for 15 min, then the mixture was incubated with anti-(GR) IgG for a further 15 min. The mixture was layered onto a gradient of 12-25% glycerol, and the gradient was centrifuged in a vertical rotor as described in "MATERIALS AND METHODS." Fractions (200 μl each) were collected from the bottom of the tube, and the radioactivity remaining in the tube was counted as the "zero" fraction. The external standards were catalase (11.3 S), γ-globulin (7.0 S), and bovine serum albumin (4.6 S). Similar results were obtained from three separate experiments. TA, triamcinolone acetonide.

cient of the GR from 4 S to 8-9 S, while the preimmune IgG affected it little. Figure 1F shows the sedimentation profiles of the mixture of GR and MMTV57 in the presence of anti-(GR) IgG. The anti-(GR) IgG increased the sedimentations of both the 7.5 S tritium peak and the 7.5 S ^{32}P-labeled MMTV57 peak to more than 12 S. Thus, the activated GR (4 S) and MMTV57 (3.7 S) formed a stable complex (7.5 S) under nearly isotonic salt conditions (130 mM NaCl equivalent).

GR Binding to the Purified MTI-III—In the previous paper, we showed that the purified MTI-III binds to the monomeric activated GR under low salt conditions (25 mM KCl) at slightly acidic pH (pH 6.3 at 10°C) (*12*). In this work, we tried to demonstrate the association of MTI-III with the activated GR under nearly isotonic salt conditions (130 mM NaCl equivalent) at neutral pH (pH 7.2 at 4°C). As shown in Fig. 2A, incubation of 2 pmol of GR with 2 pmol of the purified MTI-III shifted most of the 4 S GR to a new peak sedimenting at approximately 6.8 S. This sedimentation profile of GR is similar to that observed under the low salt conditions (*12*). Thus, under the nearly isotonic salt conditions at neutral pH, purified MTI-III binds to activated GR. Next, we added the anti-(GR) IgG to the GR/MTI-III complex. As shown in Fig. 2B, incubation of the anti-(GR) IgG with the GR/MTI-III complex shifted the 6.8 S tritium peak to more than 12 S, while pre-immune IgG failed to shift the 6.8 S tritium peak to a larger form. Moreover, the anti-(GR) IgG-Sepharose did not absorb the purified MTI-III (data not shown), indicating that the anti-(GR) IgG did not cross-react with MTI-III. Thus, anti-(GR) IgG/GR/MTI-III complex (over 12 S) was formed and precipitated significantly faster than GR/MTI-III complex (6.8 S). These results also indicate that the anti-(GR) IgG can bind to the GR/MTI-III complex as well as to the GR/MMTV57 complex.

Effect of MTI-III on the GR Binding to MMTV57—Under the nearly isotonic salt condition, we examined the interactions of GR, MTI-III, and GRE. First, we incubated the GR with MTI-III for 15 min to form GR/MTI-III complex, then added MMTV57 to the complex. As shown in Fig. 2C, receptor-[^{3}H]triamcinolone-acetonide formed a peak sedimenting at approximately 6.8 S, coinciding with the GR/MTI-III complex, and the ^{32}P-labeled MMTV57 precipitated near the top of the gradient, coinciding with the free MMTV57. Addition of the anti-(GR) antibody to the mixture resulted in precipitation of tritium to the bottom, whereas the ^{32}P-labeled MMTV57 remained near the top of the gradient (Fig. 2D). Thus, the preformed GR/

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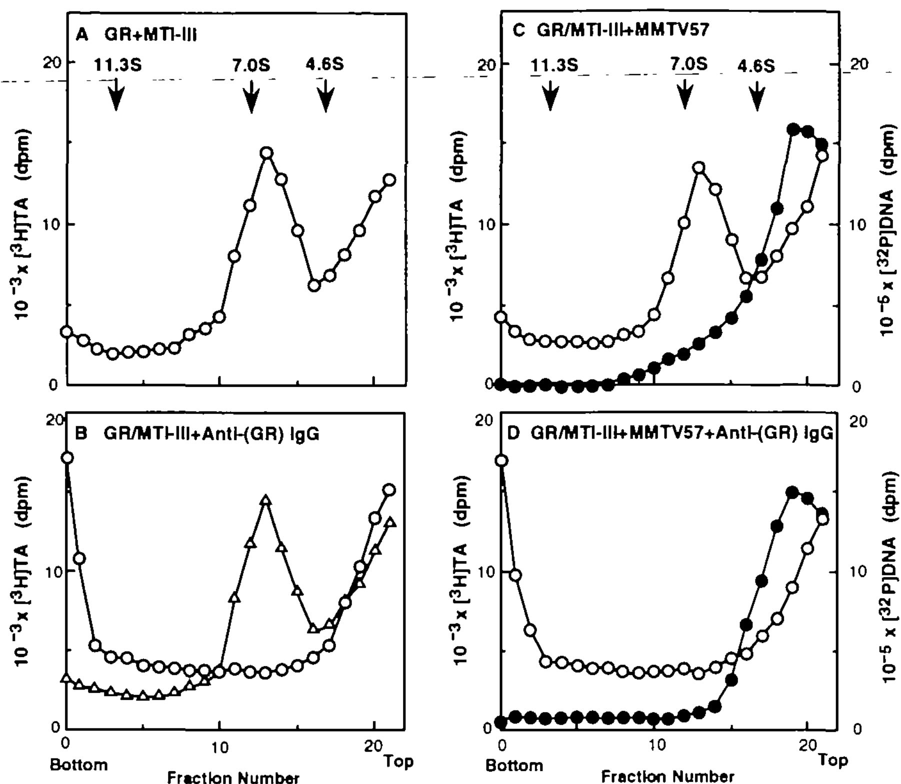

Fig. 2. **Glycerol-density-gradient analysis of GR incubated with MTI-III.** (A) GR (2 pmol) was incubated with the purified MTI-III (2 pmol) in buffer B at 4°C for 15 min. (B) GR was incubated with the purified MTI-III in buffer B at 4°C for 15 min, then the mixture was incubated with anti-(GR) antibody (○) or with pre-immune-IgG (△) for a further 15 min. (C) GR (○) was incubated with the purified MTI-III in buffer B at 4°C for 15 min, then the mixture was incubated with ^{32}P-labeled MMTV57 (●) for a further 15 min. (D) GR (○) was incubated with the purified MTI-III in buffer B at 4°C for 15 min, then the mixture was incubated with ^{32}P-labeled MMTV57 (●) and anti-(GR) IgG for a further 15 min. The mixture was layered onto a gradient of 12-25% glycerol, and the gradient was centrifuged in a vertical rotor as described in "MATERIALS AND METHODS." Fractions (200 μl each) were collected from the bottom of the tube, and the radioactivity remaining in the tube was counted as the "zero" fraction. The external standards were catalase (11.3 S), γ-globulin (7.0 S), and bovine serum albumin (4.6 S). Similar results were obtained from three separate experiments. TA, triamcinolone acetonide.

MTI-III complex did not bind to MMTV57. To analyze the possible interaction of MTI-III with MMTV57, we incubated the labeled MMTV57 with the purified MTI-III and determined the sedimentation coefficient of the incubated MMTV57. The incubation little affected the S value of MMTV57 (data not shown). Thus, the purified MTI-III does not bind to MMTV57 and does not have DNase activity.

Next, we incubated GR first with MMTV57 to form the GR/MMTV57 complex. Then, we added purified MTI-III to the complex, and incubated them for the time indicated in Fig. 3. In the presence of bovine serum albumin instead of MTI-III (control experiment, Fig. 3A), both tritium and ^{32}P-labeled MMTV57 formed a peak sedimenting at approximately 7.5 S, coinciding with the expected sedimentation position of the GR/MMTV57 complex. In the presence of the purified MTI-III (Fig. 3, B-E), the 7.5 S tritium and ^{32}P-labeled MMTV57 peak decreased in a time-dependent fashion. Simultaneously, the tritium peak sedimenting at approximately 6.8 S, coinciding with the GR/MTI-III complex, and ^{32}P-labeled MMTV57 peak sedimenting near the top of the gradient increased. The total amount of the receptor-[^{3}H]triamcinolone-acetonide sedimenting at 7.5 S plus that sedimenting at 6.8 S was nearly constant. The decrease of the GR/MMTV57 complex and the increase of the GR/MTI-III complex showed an inverse correlation. From these data, we roughly estimated the amounts of GR/MMTV57 complex and GR/MTI-III complex at various times after addition of MTI-III. After incubation with the MTI-III for 5, 10, 15, and 30 min, the amount of GR/MMTV57 complex decreased to about 90, 70, 40, and 5% of that in the absence of MTI-III, respectively. The estimated half-life of the GR/MMTV57 complex in the presence of purified MTI-III was about 13 min. These results suggest that purified MTI-III not only inhibits the binding of GR to MMTV57, but also dissociates the GR from the GR/MMTV57 complex, and then forms a stable GR/MTI-III complex.

DISCUSSION

Previously, we showed that the purified MTI-III binds to the monomeric activated GR under low salt conditions (25 mM KCl) at slightly acidic pH (pH 6.3 at 10°C) (*12*). In this work, we showed that the purified MTI-III could bind to the activated GR under nearly isotonic salt conditions (130 mM NaCl equivalent) at neutral pH (pH 7.2 at 4°C). Several laboratories (*14-16*) reported that, under nearly isotonic salt conditions, the activated GR specifically binds to DNA with a GRE motif, but not to non-specific DNA fragments without a GRE motif. We also observed that under these salt conditions, the partially purified activated GR binds to MMTV57 (Fig. 1C) but not to a non-specific DNA fragment (Fig. 1D). However, under low salt conditions (25 mM NaCl), GR binds both to MMTV57 and to the non-specific DNA fragment (data not shown). Thus, the binding of GR to MMTV57 under the nearly isotonic salt conditions is specific for DNA containing the GRE motif.

Incubation of GR/MMTV57 complex with purified MTI-III resulted in a decrease in the amount of GR/MMTV57 complex sedimenting at 7.5 S in a time-dependent fashion, and in the simultaneous formation of a peak of tritium sedimenting at 6.8 S, coinciding with the GR/MTI-III complex (Fig. 3, B-E). The estimated half-life of the GR/MMTV57 complex in the presence of MTI-III was about 13 min. In the absence of MTI-III, incubation of the GR/MMTV57 complex for 30 min did not increase the

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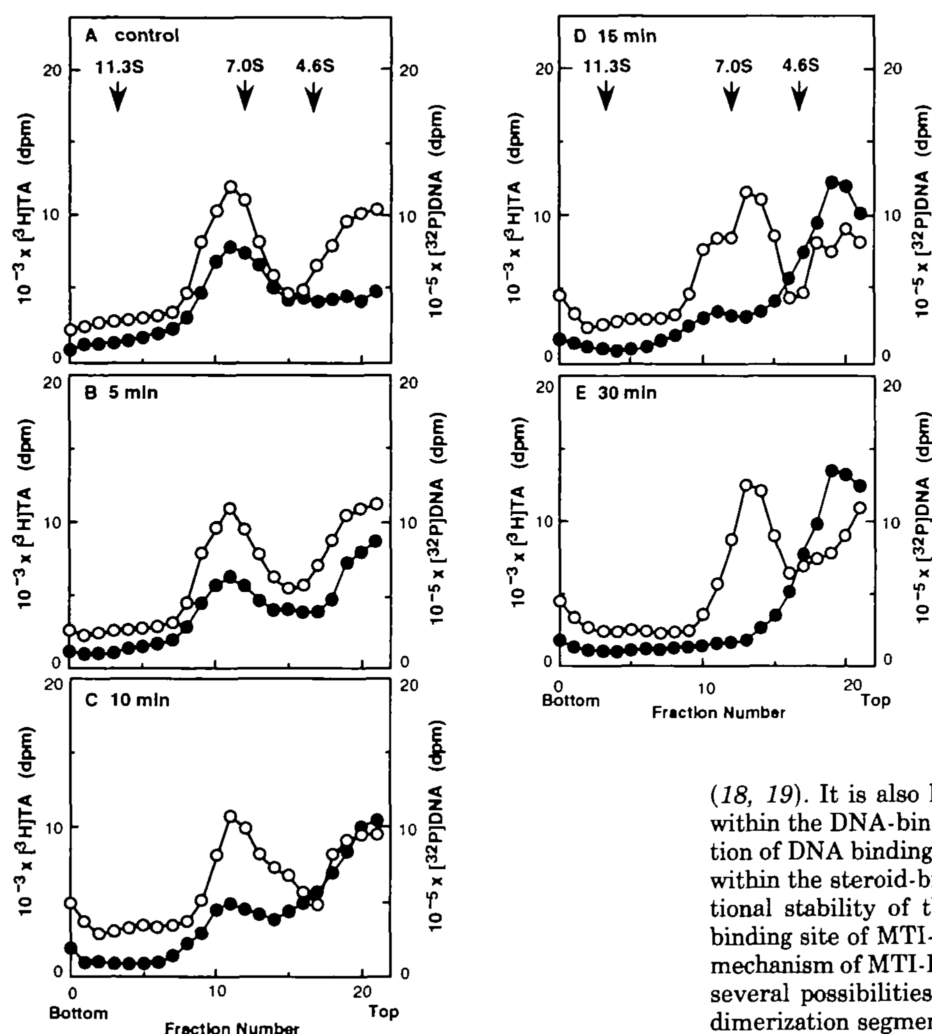

Fig. 3. **Glycerol-density-gradient analysis of GR/MMTV57 complex after incubation with MTI-III.** GR (○, 2 pmol) was incubated with ^{32}P-labeled MMTV57 (●, 1 pmol) in buffer B at 4°C for 15 min, then the mixture was incubated with bovine serum albumin for 30 min (A), or with the purified MTI-III (2 pmol) for the time indicated in the figure (B-E). The mixture was layered onto a gradient of 12-25% glycerol, and the gradient was centrifuged in a vertical rotor as described in "MATERIALS AND METHODS." Fractions (200 μl each) were collected from the bottom of the tube, and the radioactivity remaining in the tube was counted as the "zero" fraction. The external standards were catalase (11.3 S), γ-globulin (7.0 S), and bovine serum albumin (4.6 S). Similar results were obtained from three separate experiments. TA, triamcinolone acetonide.

amounts of free GR and free MMTV57 (Fig. 3A). It is reported that the dissociation constant of the GR/GRE complex is 1.1×10^{-4} s^{-1} and the half-life of the complex is 108 min at 25°C (*16*). These results suggest that MTI-III enhances the release of GR from the GR/MMTV57 complex and immediately forms a stable complex with GR. In this process, MTI-III should bind to the GR/MMTV57 complex. However, a peak corresponding to GR alone or a peak corresponding to a complex with higher sedimentation coefficient, such as the GR/MMTV57/MTI-III complex, was not observed (Fig. 3). Therefore, the MMTV57/GR/MTI-III complex appears to be an unstable complex that is immediately disrupted into the GR/MTI-III complex and free MMTV57.

As shown in Fig. 1F and Fig. 2D, the epitope-specific anti-(GR) IgG, which recognizes the amino-acids 366-387 within the N-terminal half of GR, binds to the GR/MMTV57 complex and to the GR/MTI-III complex. Furthermore, a GR/anti-(GR) IgG complex could bind to MMTV57 and to MTI-III (data not shown). These results suggest that the binding site of GR to MTI-III resides within the C-terminal half of the GR. The C-terminal half of the GR is known to consist of the steroid-binding domain and the DNA-binding domain (*18, 19*). Each domain contains a segment involved in the dimerization of the GR (*18, 19*). It is also known that the dimerization segment within the DNA-binding domain is involved in the restriction of DNA binding to binding sites, and that the segment within the steroid-binding domain is involved in the additional stability of the receptor dimer (*19*). The precise binding site of MTI-III on the GR molecule and the action mechanism of MTI-III are still unclear. However, there are several possibilities. 1) MTI-III may bind to one of the dimerization segments and disrupt the homodimer formation, thereby decreasing the stability of the GR/GRE complex. 2) MTI-III may bind near the DNA-binding site of the activated GR and cause a conformational change of GR to a form that cannot interact with DNA.

As shown in the previous paper (*12*), the purified MTI-III inhibited the binding of GR to histone-agarose. Thus, MTI-III appears to be involved both in the association of the activated GR with nuclei, such as histone and GRE, and in the dissociation of the activated GR from the GRE. MTI-III thus appears to play an important role in the regulation of the gene transcription by glucocorticoid hormone, especially in the "switch-off" mechanism.

Munck and colleagues suggested the operation of a receptor cycle system from their original observations with ATP-depleted cells (*20*). The receptor cycle system involves both a hormone-dependent phosphorylation-dephosphorylation and an ATP-dependent cycle reaction which provides the reassociation of activated, unliganded receptor with hsp 90 and other components to reconstitute the hyperphosphorylated, nonactivated, unliganded receptor. Pratt and colleagues (*21*) suggested that the translocation inhibitor(s) may also play an important role in the receptor cycle system that regulates the state of the glucocorticoid receptor in the intact cell, although the action of MTI-III is ATP-independent and MTI-III does not promote the dissociation of the hormone from the activated GR. Further studies such as molecular cloning of MTI-III

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and the preparation of a mono-epitope specific antibody against MTI-III will elucidate the physiological function and cellular distribution of MTI-III. The molecular cloning of MTI-III is now in progress.

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