Conformational Change in the Human Glucocorticoid Receptor Induced by Ligand Binding Is Altered by Mutation of Isoleucine 747 by a Threonine*

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Limited proteolysis experiments were performed to study conformation changes induced by ligand binding on in vitro produced wild-type and I747T mutant glucocorticoid receptors. Dexamethasone-induced conformational changes were characterized by two resistant proteolysis fragments of 30 and 27 kDa. Although dexamethasone binding affinity was only slightly altered by the I747T substitution (Roux, S., Térouanne, B., Balaguer, P., Loffreda-Jausons, N., Pons, M., Chambon, P., Gronemeyer, H., and Nicolas, J.-C. (1996) Mol. Endocrinol. 10, 1214–1226), higher dexamethasone concentrations were required to obtain the same proteolysis pattern. This difference was less marked when proteolysis experiments were conducted at 0 °C, indicating that a step of the conformational change after ligand binding was affected by the mutation. In contrast, RU486 binding to the wild-type receptor induced a different conformational change that was not affected by the mutation. Analysis of proteolysis fragments obtained in the presence of dexamethasone or RU486 indicated that the RU486-induced conformational change affected the C-terminal part of the ligand binding domain differently. These data suggest that the ligand-induced conformational change occurs via a multistep process. In the first step, characterized by compaction of the ligand binding domain, the mutation has no effect. The second step, which stabilizes the activated conformation and does not occur at 4 °C seems to be a key element in the activation process that can be altered by the mutation. This step could involve modification of the helix H12 position, explaining why the conformation induced by RU486 is not affected by the mutation.

In its inactivated form, the glucocorticoid receptor (GR), a member of the steroid receptor family (1–4), is part of a large heterooligomeric complex that includes hs90 and other heat shock proteins (5). This heterocomplex is required to maintain GR in a conformation appropriate for ligand binding and to prevent GR from binding to DNA (6, 7). Binding of the specific hormone induces a cascade of different molecular events, leading to an activated GR that can, after DNA binding, regulate gene transcription by interacting with the transcriptional machinery. Transcriptional activation is achieved through activation functions with the major transactivation function AF-1 located within the N-terminal domain and the weak ligand-dependent transactivation function AF-2 in the C-terminal hormone binding domain (for reviews and references see Refs. 8 and 9). The AF-2 activation domain, which is part of the larger AF-2 and conserved in the nuclear receptor superfamily, was shown to be essential for transactivation (10–14). Comparison of ligand binding domain crystal structures of the unliganded retinoid-X receptor α with liganded retinoic acid receptor γ, liganded thyroid hormone receptor α, and liganded estrogen receptor (ER) suggests that ligand binding triggers folding back of the H12 α-helix, which contains the AF-2 activation domain, whereas in aporeceptors this helix protrudes from the ligand binding domain (LBD) core (15–18).

Previous studies have shown that ligand-induced conformational change is involved in the activation of various nuclear receptors (19–22). This structural modification converts the entire LBD of various nuclear receptors to a more compact structure that is less accessible to proteases (19, 22–34). A different conformational change induced by antihormones that affects the C-terminal end of the receptor was previously reported (20, 22, 25, 26). Antagonist-induced proteolysis-resistant fragments obtained for various nuclear receptors are smaller than those obtained in the presence of agonists (19, 20, 22, 25, 29, 35) or less resistant to proteolytic degradation (23).

Most GR mutations described in the LBD involve a decrease in ligand binding affinity (8, 36–41). In contrast, we recently described a mutant (substitution of human GR isoleucine 747 by a threonine, hereafter referred to as I747T) with no significant alteration of ligand binding affinity but which required higher dexamethasone concentrations than the wild-type GR (wt GR) to induce reporter gene transactivation (42). This mutation is located just before the H12 helix. It has been proposed that modification of helix H12 positioning upon agonist binding generates a surface for interactions with various coactivators, whereas the conformation of the apo-receptor and the antagonist-receptor complex does not permit these interactions (for reviews and references see Refs. 43 and 44).

As the I747T mutation alters the transactivation function of the receptor without modifying ligand-receptor interactions, we assumed that the dynamics of the transconformation process were modified. In this study, we used partial proteolysis to analyze the conformation of the wt GR and the mutant I747T before and after agonist and antagonist binding.
Ligand-induced Conformational Changes of wt and I747T Mutant GR

EXPERIMENTAL PROCEDURES

Materials—Dexamethasone and trypsin (type XIII) were purchased from Sigma. RU486 was a gift from Roussel Uclaf (Romainville, France). Rainbow™ 14C-methylated protein molecular weight markers were obtained from Amersham Pharmacia Biotech as well as the enhanced chemiluminescence (ECL) Western blotting detection reagents (Les Ulis, France). [35S]Methionine (>1000 mCi/mmol) was obtained from ICN; TNTT™ reticulocyte lysate system and RNasin ribonuclease inhibitor were from Promega (Charbonnieres, France), and ENTEN-SIF was from NEN Life Science Products. The GR polyclonal antibody P-20 was purchased from Santa Cruz Biotechnology Inc. (Tebu, Le Perray en Yvelines, France).

Plasmids—Plasmid wt human GR containing the open reading frame of the wild-type GR as well as the ΔA/B wt GR and the mutant I747T have been previously described (42). Plasmids containing coding sequences of mouse GR (pSV2Wrec) and three mutants (M758A/L759A, E761A, and I762A/I763A) were obtained from the laboratory of Professor M. G. Parker (11). To place them under the control of the T7 promoter, Xbal-BglIII fragments containing the coding sequences were excised, blunted, and subcloned into the blunt EcoRI site of pSG5 (45).

Coupled in Vitro Transcription and Translation—Expression plasmids (pSG5-GR) were transcribed and translated with the TNT™-coupled reticulocyte lysate system in the presence of [35S]Methionine (1000 Ci/mmol, Amersham Pharmacia Biotech) according to the manufacturer’s instructions for 1 h at 30 °C.

Limited Proteolysis—Pretreatment of labeled translation mixtures was performed for 10 min at room temperature with vehicle or ligand (conserved in ethanol, dried, and re-suspended in 50% (v/v) PEG300, 1% BSA serum albumin (see Fig. 4, 2 g/ml trypsin concentration (Fig. 5), 6 M dexamethasone for 10 min at 20 °C before digestion with increasing trypsin concentrations (0, 5, 10, 25, and 50 µg/ml; lanes 1, 2, 3, 4, and 5, respectively). Autoradiograms of each sample, denatured and analyzed on a 12.5% polyacrylamide gel, are shown. The migration of molecular weight marker is indicated on the left, and resistant proteolysis fragments are indicated by asterisks on the right.

Sucrose Gradient Centrifugation—In vitro synthesized 35S-wt GR or I747T were incubated for 10 min at 20 °C with 10–7 M dexamethasone followed by an additional incubation with ATP (10 mM) and NaCl (0.4 M) aliquots (50 µl) of SDS sample buffer was added, and the samples were boiled for 5 min. The proteolysis products were separated on a 7.5%–20% (w/v) sucrose gradient and quantified. Three-drop fractions were collected by piercing the bottom of each tube. The collected fractions were analyzed on a 12.5% polyacrylamide gel, and the radioactivity was quantified using a phosphoimager. The band corresponding to the 94-kDa fragment was excised, blunted, and subcloned into the blunt EcoRI site of pSG5 (45).

Immunoblotting—A limited proteolysis experiment was conducted with the in vitro synthesized 35S-wt GR proteins. After SDS/polyacrylamide gel electrophoresis (10% gel), the protein fragments were electroblotted onto nitrocellulose. The blotted membranes were probed with the rabbit polyclonal antibody P-20 directed against a peptide corresponding to amino acids 750–769 mapping at the C-terminus of the human GR (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

Bioimaging Analysis on Fujix Bas 1000PC—Quantitative analysis of proteolytic fragments was performed by gel scanning with a Fujix film imaging plate. Photo-stimulated photon for each band were determined densitometrically scanned by image analysis (Optilab, Graftek, France) and quantified.

RESULTS

Higher Dexamethasone Concentrations Are Required to Protect I747T GR against Proteolysis Than for the Wild-type GR—To analyze changes in wt and I747T GR conformation induced by dexamethasone binding, in vitro produced receptors were incubated with 10–5 M dexamethasone, then limited trypsinization was performed, and the resulting proteolysis fragments were analyzed (Fig. 1). At low dexamethasone concentrations (5 and 10 µg/ml; lanes 2–3, respectively), similar proteolysis patterns were obtained for both receptors (Fig. 1A and I747T GR (Fig. 1B) with a main 30-kDa fragment. At 25 µg/ml dexamethasone concentration (lane 4), 2 fragments, 30- and 27-kDa, were obtained for both receptors. At 50 µg/ml dexamethasone concentration (lane 5), the abundance of the 30-kDa species decreased to the benefit of 27-kDa species. The 27-kDa fragment became predominant for both receptors. The 30- and 27-kDa fragments contained all or part of the LBD, as previously reported for various nuclear receptors because they were also obtained with a ΔA/B-GRwt and were recognized by a specific antibody directed against the C-terminal region of this domain (data not shown).

Further proteolysis experiments were performed using various dexamethasone concentrations (10–9 to 10–5 M), and the intensities of the proteolytic fragments were quantified. Values obtained for both fragments were added and presented as a percentage of the intensity of the full-length receptors (Fig. 2). At 5 µg/ml dexamethasone concentration (Fig. 2A), fragments generated from wt GR had the same intensity as fragments generated from I747T GR, indicating that at this trypsin concentration both receptors were similarly protected. Because more than half of the methionine residues were located in the C-terminal part of the receptor, we assumed that at 10–7–10–6 M dexamethasone concentration 100% of both GRs would be converted into a more compact structure. These results are consistent with the similar dexamethasone affinities reported for wt GR and I747T (42). At higher trypsin concentrations (10, 25, and 50 µg/ml; Fig. 2, B, C, and D, respectively), the maximal level of protection for the wt GR was within the same order of magnitude and was reached regardless of the trypsin concentration used at the same dexamethasone concentrations (10–7–10–6 M). In contrast, for the mutant at these trypsin concentrations (10, 25, and 50 µg/ml; Fig. 2, B, C, and D, respectively), there was a lower protection level for both fragments. I747T required higher dexamethasone concentrations to achieve the same extent of protection as noted for the wt GR. Because the affinity for dexamethasone was not drastically affected by the mutation, these results suggested that this mutation could affect the conformational process induced by dexamethasone binding.

A Temperature-dependent Step in the Transconformation Process Is Altered by the I747T Mutation—In vitro activation of GR is a temperature- and ligand-dependent process (46). Nev-
Nevertheless, in nonactivating conditions, ligand binding to the receptor appears sufficient to induce a structural modification detectable by proteolysis (22, 47). We thus compared the proteolytic pattern of the wt and I747T GR after incubation with 10^{-2} or 10^{-5} M dexamethasone concentrations at 0 °C, a temperature that inhibits the activation process, or at room temperature. This incubation was followed by trypsin treatment performed for 1 h at 0 °C, conditions in which the unliganded wt or I747T GR were totally digested. The percentage of 30- and 27-resisting fragments obtained for vehicle alone, dexamethasone 3 \times 10^{-2} M, or dexamethasone 3 \times 10^{-5} M are given in Fig. 3. The maximum level of protection (i.e., maximal intensity of the 30- and 27-kDa fragments) was obtained for the wt GR after incubation with a 10^{-5} M dexamethasone concentration at 0 °C or with a 10^{-7} M concentration at 20 °C (Fig. 3A). In contrast, the maximum level of protection for I747T, similar to that obtained with wt GR, was reached only after incubation with a 10^{-5} M dexamethasone concentration at 20 °C (Fig. 3B) (45 ± 5% versus 50 ± 4%, respectively). These results suggest that a step in the ligand-induced conformational change occurring at room temperature was drastically altered. The similar level of protection obtained for both receptors at 10^{-5} M suggests that higher dexamethasone concentrations could maintain a protected conformation even in the absence of any activation process.

**Hsp Release Is Not Altered by the I747T Mutation**—It was

![Graphs](image-url)

**Fig. 2.** Effects of dexamethasone (Dex) concentrations on limited trypsin digestion of wt GR and I747T. In vitro translated wt GR and I747T were preincubated with various dexamethasone concentrations (0, 10^{-2}–10^{-5} M) for 10 min at 20 °C before digestion with 5 μg/ml (A), 10 μg/ml (B), 25 μg/ml (C), and 50 μg/ml (D) of trypsin. Autoradiograms of each samples, denatured and analyzed on a 12.5% polyacrylamide gel, are shown at the top of each graph. wt GR (lanes 1-6) and I747T (lanes 7-12) were incubated with 0 (lanes 1 and 7), 10^{-5} M (lanes 2 and 8), 10^{-4} M (lanes 3 and 9), 10^{-3} M (lanes 4 and 10), 10^{-2} M (lanes 5 and 11), and 10^{-5} M (lanes 6 and 12) dexamethasone. Each band was scanned and analyzed on a phosphoimaging analyzer (Fujix Bas 1000FC). The relative photo-stimulated luminescence of wt GR and I747T-resisting fragments are expressed as the percentage of photo-stimulated photon value of the full-length GRs (PSL) and plotted as a function of dexamethasone concentration. 30- and 27-kDa fragments are represented as associated wt GR-resisting fragments (□) or I747T-resisting fragments (●).
previously reported that transformation of GR by ligand binding into a transcriptionally activated form is accompanied by the release of Hsp from the receptor (48, 49). We postulated that alteration of Hsp release from I747T could account for the shifted ligand-induced conformational change. To investigate this hypothesis, we performed a ligand-independent removal of Hsp using high NaCl and ATP concentrations, a treatment known to remove Hsp from receptors (50), and analyzed the sedimentation profile of the mutant and wt GR on sucrose gradient. NaCl and ATP treatment was efficient to remove Hsp from the wild type and mutant GR as evidenced by the decrease in the sedimentation coefficient for both receptors (9–10 S versus 6–7 S) (Fig. 4).

When proteolysis experiments of liganded wt and mutant GR were performed after ATP/NaCl treatment, protection of the fragments was not markedly affected (Fig. 5, A and B, respectively; compare lanes 2–3 with lanes 5–6). Taken together, these results showed that hsp release was not altered by the mutation and that hsp dissociation was not involved in the shifted ligand-induced conformational change.

Conformational Change Induced by RU486 Binding Is Not Affected by the I747T Mutation—Previous experiments showed that RU486 presents a similar antagonistic activity with wt and I747T GR (42). To explore the conformational change of I747T after RU486 binding, we performed proteolysis experiments on translated wt or I747T receptors treated with 10^{-7} M RU486 concentration. A similar proteolysis pattern was obtained for both receptors (Fig. 6).

At 10 \mu g/ml trypsin concentration, a 30-kDa fragment was obtained (Fig. 6, lanes 2 and 7). Proteolysis with 25 \mu g/ml trypsin (lanes 3 and 8) decreased the amount of the 30-kDa fragment and increased the amount of the 27-kDa fragment. These two main species were split off into two slightly different additional fragments (29.5 and 27.5 kDa). At 50 \mu g/ml trypsin concentration (lanes 4 and 9), these resistant fragments were obtained as well as an additional 25-kDa fragment, with the smallest species (27 and 25 kDa) being the most abundant. At higher trypsin concentration (100 \mu g/ml, lanes 5 and 10), the 25-kDa fragment was the main fragment. These proteolysis fragments, except for the 25-kDa species, were recognized by the antibody directed against the 750–769 amino acid sequence (data not shown). After treatment with various RU486 concentrations (10^{-2}–10^{-6} M), quantification of main fragments (30-, 27-, and 25-kDa) led to indistinguishable protection curves for wt GR and I747T (data not shown), regardless of the trypsin concentrations applied. RU486 concentrations required to obtain half-maximal protection of each fragment are reported in Table I. The same RU486 concentration (~10^{-5} M) was sufficient to convert wt GR and I747T to a trypsin-resistant form. These results led us to conclude that the RU486-induced conformational change was not affected by the mutation and differed from that induced by an agonist.

**DISCUSSION**

In this study, we investigated the involvement of ligand-induced conformational changes in the previously described shifted transcriptional activity of the GR mutant I747T. This mutation drastically alters the ligand specificity for transactivation but not for binding (42).

Conformational changes observed after ligand binding are assumed to induce a decrease in the accessibility of cleavage sites within the receptor molecule, which can be easily detected by modifications of proteolysis patterns. In agreement with previous reports (20, 34, 47), we found that dexamethasone-induced conformational changes of the wild-type GR (wt) were characterized by two resistant proteolytic fragments (30 and 27 kDa), whose respective intensities were related to the trypsin
Ligand-induced Conformational Changes of wt and I747T Mutant GR

Table I

| RU486 concentration required to observed half-maximal protection of each resisting fragment for wt GR and I747T |
|-------------------------------------------------|
| wt GR | I747T |
|-------|-------|
| 30-kDa fragment, 10 $\mu$g/ml | $3 \times 10^{-9}$ M | $3 \times 10^{-8}$ M |
| 27-kDa fragment, 50 $\mu$g/ml | $4 \times 10^{-9}$ M | $3 \times 10^{-8}$ M |
| 25-kDa fragment, 100 $\mu$g/ml | $5 \times 10^{-9}$ M | $6 \times 10^{-8}$ M |

Concentration used in the assay. We also found that protection of these fragments was dependent on the dexamethasone concentration used (Fig. 2). At $10^{-7}$ to $10^{-5}$ M, the wt GR was totally converted to a resistant conformation. Although dexamethasone binding was sufficient to protect the mutant I747T from low trypsin concentration digestion (5 $\mu$g/ml; Fig. 2A), a 25–50-fold higher dexamethasone concentration was required to protect the 30- and 27-kDa fragments against high trypsin levels (Fig. 2, B, C, and D). The shift in the dose-response curve for proteolysis protection of I747T was in agreement with the shift in the transcriptional response curve previously reported (42).

It is important to note that at high dexamethasone concentrations, similar protection and transactivation activity levels were reached for I747T and wt GR (42). After cortisol binding, only submaximal protection of these fragments was obtained with the mutant (data not shown), in accordance with the results of a previous study showing submaximal stimulation of a reporter gene (42). It should be also noted that I747T presented an affinity for dexamethasone or cortisol within the same order of magnitude as that obtained for the wt GR (42). Moreover, GR proteins synthetized with rabbit reticulocyte lysate had the same binding affinity as the native protein expressed in cells (51–53). This suggests that the relative affinity of the receptor for the ligand was not directly responsible for the shifted protection curve. Similarly, AF-2 activation domain mouse GR mutants, which are unable to transactivate at concentrations up to $10^{-5}$ M dexamethasone without modification of ligand binding affinity (11), were also unable to generate the same proteolysis pattern as the wt (data not shown). Taken together, the data presented here indicated that the ability of GR to activate transcription was not only dependent on its ability to bind its ligand, i.e., it was also correlated with the ligand binding-induced conformational change of GR characterized by increasing protection of the 30- and 27-kDa species at high trypsin concentrations.

The resistance to trypsin digestion could be a powerful strategy to investigate the involvement of a transcriptionally efficient conformational change by high dexamethasone concentrations. As a similar protection level was obtained for I747T and the wt GR at 0 °C, it is likely that a step in the ligand-induced conformational change occurring at room temperature and probably associated with the stabilization of the conformation was impaired or drastically modified for the mutant. Moreover, our experiments showed that no impaired hsp90 dissociation was involved in the shifted protection curve for the mutant. As reported for the progesterone receptor (20) and the mineralocorticoid receptor (23), the conformational change might have occurred within the heterooligomeric structure, thus inducing hsp release, and is not a consequence of an altered hsp dissociation.

Taken together these data suggested that acquisition of a transcriptionally efficient conformational change is a multistep process with a dynamic dimension (Fig. 7). An intermediary complex would first be generated by ligand binding (Fig. 7A, step 1). The second step, occurring rapidly at room temperature, would convert the intermediary complex into a more stable conformational state, corresponding to the transcriptionally efficient conformation (step 2). We hypothesized that the activated receptor could be stable enough to exist in a transient unliganded activated form (step 3). Taking into account the observation that the I747T substitution has little effect on affinity, we propose that this substitution does not affect intermediary complex formation (Fig. 7B; step 1) but alters the stability of the activated receptor conformation. High ligand concentrations are required to permit a rapid ligand reassociation to the unstable activated receptor and so permit conservation of a sufficient concentration of efficient conformation to transactivate. Interestingly the Tyr-537 mutation within the ER (corresponding to Phe-749 for human GR) confers constitutive activity (54), probably by affecting the specific stabilization of active arogen receptor conformation (55). Conversely, a destabilizing effect of mutation I747T in GR might induce an unstable active conformation. Moreover, the mutation occurring at the same position in the androgen receptor (Val-889) in the case of nearly complete androgen insensitivity (56, 57) could also imply destabilization of the active conformation, because this mutant showed a slight increase in its dissociation rate, with a drastic modification of EC50 in transactivation assays. As mentioned by the authors, an increase in denaturation could also be involved because dissociation of the ligand could lead to rapid receptor denaturation because of instability of the activated conformation. High ligand concentrations, by increasing the ligand association rate, could protect against receptor denaturation and are required to obtain full activity.

Contrary to that observed with dexamethasone, the RU486 binding-induced conformation characterized by three main resistant fragments (30-, 27-, and 25-kDa species) was not affected by the mutation. Western blot experiments using a specific antibody recognizing a C-terminal epitope indicated that the RU486-induced conformational change affected the C-terminal tail of the GR differently (data not shown). Similar results were recently reported with rat GR (29). The H12 helix, which contains this region (for reviews and references, see Ref. 58), lies outside the LBD body after RU486 binding, rendering it accessible to proteases. A recent crystallographic analysis of the ligand binding domain of ER in the presence of the antagonists raloxifene revealed that H12 helix can adopt a distinct antagonist position (18). Because (i) the position of the C-terminal tail of the LBD was the major difference between dexamethasone- and RU486-induced conformational changes and (ii) the mutation altered only the structural modifications induced by agonist binding, we postulated that the second step of the agonist conformational change process especially involves the positioning of the H12 helix. Mutation of isoleucine 747, which is located in the loop between helix H11 and H12, might alter the displacement of H12. It has been postulated that folding back of the H12 helix after agonist binding generates a surface for interactions with various co-activators correlated with a functional AF-2 activity (for reviews and references, see Refs. 43 and 44). These co-activators could act, as proposed by Henttu et al. (59), as bridging proteins between the receptor and the basal transcriptional machinery, and they are likely involved in stabilization of the conformation. In contrast, most of these coactivators are unable to interact with receptors after RU486 binding (60–62), in accordance with the inactivity of AF-2 (63).

Finally, mutations in the loop between helix H11 and H12 could specifically alter the conformation process induced by agonist binding, probably by altering the position of H12 helix, and this positioning is affected differently depending on the amino acids. The analysis of the transactivation function correlated with the analysis of the receptor sensitivity to proteolysis could be a powerful strategy to investigate the involvement
of each amino acid of the H11-H12 loop in the ligand-induced conformational change.

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FIG. 7. An equilibrium model for hormone-dependent conformational changes. In the absence of hormone, receptors exist as an aporeceptor complex with hsp90 and other proteins in a conformation permissive for hormone binding but weakly resistant to trypsin digestion (Fig. 7A). After hormone binding, an intermediary complex (step 1) is immediately transformed via an activated receptor complex (Fig. 7B) to an activated receptor complex, with I747T, the rate of transformation of the intermediary complex, normally obtained after ligand binding (step 1) to activated holo-receptor, might be decreased (step 2). Moreover, after ligand dissociation (step 3), the activated aporeceptor might be less stable and so might persist for less time than the wt.

A wt GR

| Unprotected conformation | Protected conformation |
|--------------------------|-----------------------|
| 1. | 2. |
| 3. | 4. |
| 5. | 6. |

B I747T

| Unprotected conformation | Protected conformation |
|--------------------------|-----------------------|
| 1. | 2. |
| 3. | 4. |
| 5. | 6. |

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