Glucocorticoid Receptor Mutants Demonstrate Increased Motility Inside the Nucleus of Living Cells: Time of Fluorescence Recovery After Photobleaching (FRAP) Is an Integrated Measure of Receptor Function

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Natural mutations of the human glucocorticoid receptor (GR) isoform α cause the glucocorticoid resistance syndrome. Mutant receptors may have abnormal interactions with the ligand, target DNA sequences, and/or multiple intracellular proteins, as well as aberrant nucleocytoplasmic trafficking. Using fluorescence recovery after photobleaching (FRAP) analysis, all GR pathologic mutant receptors examined, as well as 2 synthetic GR mutants lacking the activation function (AF)-1 or the ligand-binding domain (and hence the AF-2), had defective transcriptional activity and dynamic motility defects inside the nucleus of living cells. In the presence of dexamethasone, these mutants displayed a curtailed 50% recovery time ($t_{1/2}$) after photobleaching and, hence, significantly increased intranuclear motility and decreased "chromatin retention." The $t_{1/2}$ values of the mutants correlated positively with their transcriptional activities and depended on the GR domain affected. GRβ, a natural splice variant of the GR gene, also demonstrated a shorter $t_{1/2}$ than GRα. The motility responsiveness of the natural and artificial mutant receptors examined, and of GRβ, to the proteasomal inhibitor MG-132 also depended on the mutant domain. Thus, mutant glucocorticoid receptors possess dynamic motility defects in the nucleus, possibly caused by their inability to properly interact with all key partner nuclear molecules necessary for full activation of glucocorticoid-responsive genes.

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INTRODUCTION

Glucocorticoid resistance is a rare, familial or sporadic condition characterized by generalized, partial end-organ insensitivity to glucocorticoids caused by mutations in the GR gene (1,2). Affected subjects have compensatory elevations in circulating cortisol and adrenocorticotropic hormone (ACTH) concentrations, which maintain circadian rhythmicity and appropriate responsiveness to stressors, and resistance of the hypothalamic-pituitary-adrenal (HPA) axis to dexamethasone suppression. Generally, there is no overt clinical evidence of hypo- or hypercortisolism, with the exception of fatigue, which has been the presenting symptom in some patients (2). Although adequate compensation is achieved by the elevated cortisol concentrations in the majority of patients, the excess ACTH secretion frequently results in increased production of adrenal steroids with androgenic and/or mineralocorticoid activity (1,2). The former accounts for manifestations of androgen excess, such as ambiguous genitalia in girls; precocious puberty in children; acne; hirsutism; infertility; male-pattern hair loss and menstrual irregularities in women; and adrenal rests in the testes and oligospermia in men (3). Mineralocorticoid activity accounts for symptoms and signs of mineralocorticoid excess, such as hypertension and/or hypokalemic alkalosis (2,3).

The GR gene encodes two 3′ splicing variants, GRα and GRβ, from alternative use of a different terminal exon 9α and β (2,14). Each variant mRNA is translated from at least 8 initiation sites into multiple GRα, and possibly GRβ, isoforms termed A through D3 (15). The originally described GRα A encodes a 777–amino acid protein, whereas the original GRβ A contains 742 amino acids. The first 727 amino acids from the NH_2-terminus are identical in the 2 isoforms (14). GRα possesses an additional 50 amino acids, whereas the GRβ encodes an additional 15 nonhomologous amino acids in their COOH-terminus (14). GRα is the classic receptor that binds with glucocorticoids and transactivates or transrepresses glucocorticoid-responsive promoters (2). On the other hand, GRβ does not bind glucocorticoids and exerts dominant negative effects on GRα, and its physiologic and pathologic roles remain unclear (1). The translational GRα isoforms apparently have varying transcriptional activities, and it is likely that functional differences are present between the putative GRβ translational isoforms as well (15).

GRα in its unliganded but ligand-friendly state is located primarily in the cytoplasm, as part of hetero-oligomeric complexes containing heat shock proteins (HSPs) hsp90, hsp70, and hsp50, and possibly other proteins as well (14). In contrast, GRβ is mainly in the nucleus, also forming a hetero-complex with HSPs (16). After binding to its agonist ligand, the GRα undergoes con-

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Table 1. Profile of GR-related molecules studied

Mutant name	Characteristics	Kd for dexamethasone (compared with wild type GRα)	Binding to GREs	AF-2 binding to p160 coactivators
GRα WT	1	+	+	
GRαR477H	Natural mutant, GRE-binding (–)	1	–	+
GRαV571A	Natural mutant	6	+	+
GRαD641V	Natural mutant	3	+	+
GRαG679S	Natural mutant	2.2	+	+
GRαV729I	Natural mutant	2-3	+	+
GRαI747M	Natural mutant, AF-2 (–)	2.1	+	–
GRαL773P	Natural mutant, AF-2 (–)	2.59	+	–
GRβ	Splicing variant, specific "LBD"	–	+	–
GRα(Δ77-262)	Synthetic mutant, AF-1 (–)	1	+	+
GR(1-514)	Synthetic mutant, LBD (–)	–	–	–

From references 3-5,7-10,12,13,23.

formational changes, dissociates from the HSPs, "homo"-dimerizes, and translocates into the nucleus through the nuclear pore via an active, ATP-requiring process (14). There, the ligand-activated GRα directly interacts with DNA sequences, the glucocorticoid response elements (GREs), in the promoter regions of target genes (14). At that time, the activated GRα attracts nuclear receptor coactivators, through its 2 activation functions AF-1 and -2, which are respectively located in the NH_2-terminal domain and the ligand-binding domain (LBD), as well as other chromatin-remodeling complexes and transcriptional components, including general transcription factors and the RNA polymerase II, to the gene promoter regions, finally modulating the transcriptional rates of glucocorticoid-responsive genes (14). GRα, as well as nuclear receptor coactivators and other components of the transcriptional machinery, are cleared by the proteasomal complex after ubiquitination (17,18). The interaction of GRα with GREs, transcriptional components, and the clearance of the receptor are dynamically regulated within the order of seconds (19-21). The proteasomal pathway facilitates the removal of GRα from the transcriptosome, thus negatively regulating the transcriptional activity of this ligand-dependent transcription factor (18,22).

Hereditary glucocorticoid resistance syndrome is caused by mutations in GRα isoform produced from the GR gene (1-3). Inactivating mutations within the LBD and the DNA-binding domain (DBD), as well as a 4-bp deletion at the 3'-boundary of exon 6 of the GR gene, have been described in 6 kindreds and 3 sporadic cases (1-13). We recently examined the molecular defects of several GRα mutant receptors that cause familial or sporadic glucocorticoid resistance by examining their ligand-binding activity, cytoplasmic-to-nuclear translocation, and interactions with GREs and p160 type nuclear receptor coactivators and by determining their transcriptional activities (3,5,6,8,9,12,13). In this study, we further assessed the behavior of pathologic and synthetic GR mutants and the natural GRβ isoform inside the nucleus of living cells. We found that the pathologic and synthetic GRα mutants with changes in specific functional domains of the receptor generally demonstrated a defect in their intranuclear motility in vivo and responded differentially to treatment with the proteasomal inhibitor MG-132, one of the known determinants of such motility. These results indicate that pathologic mutant glucocorticoid receptors have a dynamic defect in their "chromatin retention" possibly because of loss of attractive interactions with key nuclear partner molecules necessary for full transcriptional activity.

MATERIALS AND METHODS

Plasmids

pRShGRα, pRShGR(1-514), and pRShGRβ were kindly donated by Dr. R.M. Evans (Salk Institute, La Jolla, CA, USA). pRSVerbA^{-1}, which contains the thyroid receptor cDNA in inverse orientation and was used as a negative control, was also a gift from Dr. R.M. Evans. pF25-hGRα, pF25-hGRβ, and GR(1-514) were described previously (6). pRShGRαR477H, V571A, D641V, G679S, V729I, I747M, L773P, and pF25-hGRαR477H, V571A, D641V, G679S, V729I, I747M, L773P were described previously or produced by inserting the respective point mutations into pRShGRα or pF25-hGRα using PCR-assisted mutagenesis (8,12,13,23). pRShGRα (Δ77-262) and pF25-hGRα(Δ77-262) were constructed by digesting pRShGRα and pF25-hGRα with *Bgl*II and subsequent autoligation. pMMTV-Luc, which contains the full-length mouse mammary tumor virus (MMTV) promoter that drives the luciferase gene, was a gift from Dr. G.L. Hager (National Cancer Institute, Bethesda, MD, USA). pSV40-β-gal was purchased from Promega (Madison, WI, USA).

Cell Cultures and Transfections

African monkey kidney COS7 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units penicillin, and 50 μg/mL streptomycin. They were plated in Delta-T culture dishes (Bioptechs, Butler, PA, USA) and transfected with 1 μg/well pF25-hGR plasmids using Lipofectin, as previously described (24). For the experiments using pMMTV-Luc, the cells were plated in 6-

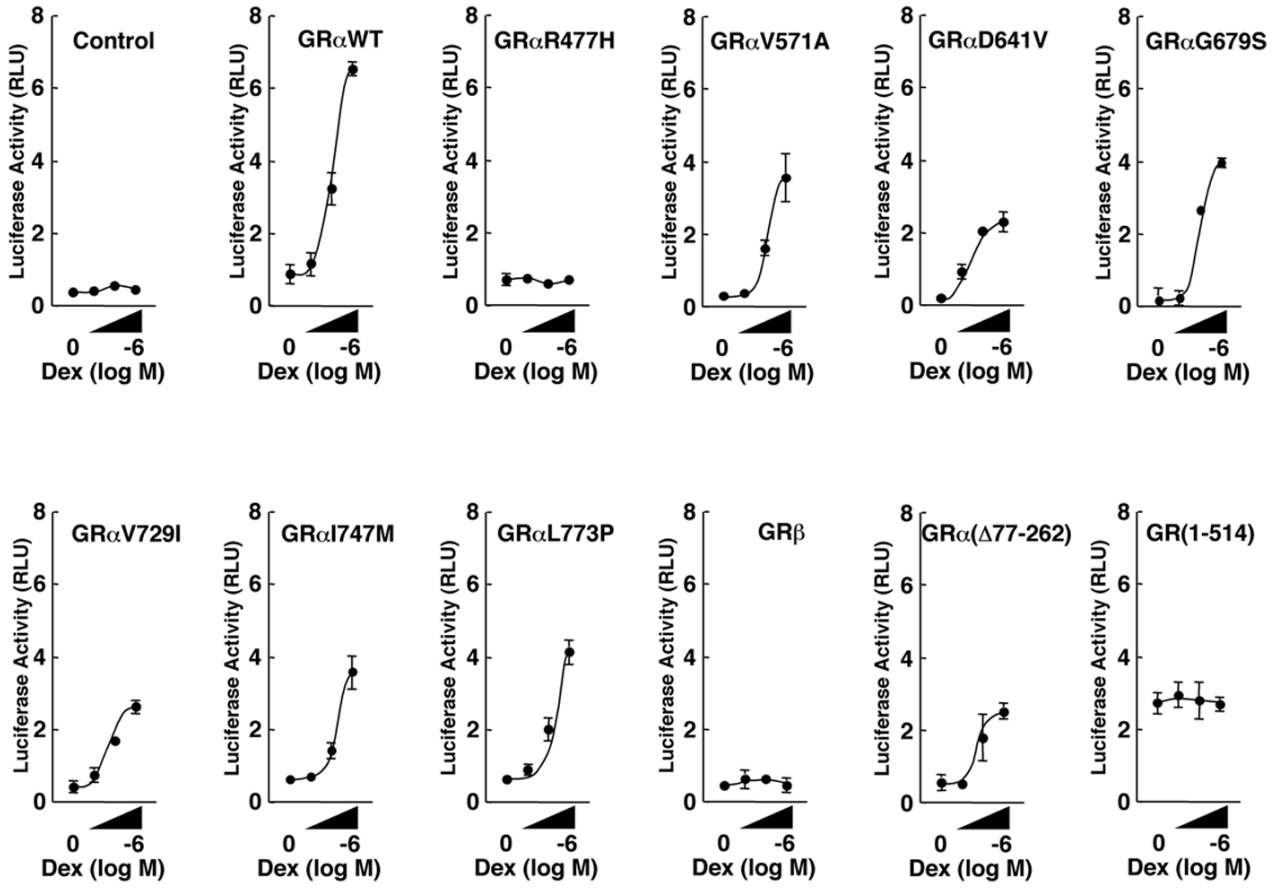

Figure 1. Transcriptional activities of GFP-GRs tested on the MMTV promoter. COS cells were transfected with the indicated GR-expressing plasmid together with pMMTV-Luc and pSV40-β-gal and exposed to increasing concentrations of dexamethasone. Each point indicates the mean ± SE of the luciferase activity corrected for the β-galactosidase activity.

well plates and transfected with 0.2 μg/well pRShGRs together with 1.5 μg/well pMMTV-Luc and 0.3 μg/well pSV40-β-gal. Increasing concentrations of dexamethasone (0, 10^{-10}, 10^{-8}, and 10^{-6} M) were added to the media 24 h after transfection, and cell lysates were harvested after an additional 24 hours. Luciferase and β-galactosidase activities were subsequently determined in the cell lysates as previously described (25).

Confocal Microscopy and Fluorescent Recovery After Photobleaching (FRAP) Analyses

COS7 cells, cultured in Delta-T culture dishes and transfected with the indicated pF25-GRs, were treated for 5 h with 10^{-6} M dexamethasone, 24 h after transfection. Before photobleaching, the media were replaced with Hanks balanced salt solution (HBSS) containing 10% FBS, and the cells were examined under a microscope. Emitted signals were recorded at 37 ± 0.5 °C with the Zeiss LSM510 upright 2-photon meta/Zeiss Axioskop 2 microscope (Carl Zeiss; Thornwood, New York, NY, USA) at the NICHD Microscopy and Imaging Core (National Institute of Child Health and Human Development, Bethesda, MD, USA), with the assistance of Dr. Vincent Schram (24). A water immersion Zeiss Achroplan × 63, 0.9 NA, IR (working distance 2.2 mm) objective lens (Carl Zeiss) was used for image acquisition. Confocal images were built point by point by collecting the intensities from the photo-multiplier tube using Zeiss LSM 5 software version 3.2 (Carl Zeiss). For FRAP, cells were excited with an argon laser at 488 nm, and emission was collected using the Meta detector with custom emission range from 495 to 590 nm. Images were taken every 197 ms at zoom factor 3 and resolution 128 by 128 pixels (pixel size, 0.38 by 0.38 μm; pixel time, 3.84 s). After the first 2 images, a selected rectangular region of fixed size (11.02 by 2.28 μm) in the nucleus was bleached at a set laser power of 15 mW for 50 iterations. Fluorescence in the bleached region was measured as a function of time using the LSM software. To account for bleaching to laser scanning, the intensity of an identical area in a distant nuclear area was also measured with time. In each experiment, 12 to 20 independent cells in 2 to 3 dishes were analyzed.

To correct for differences in expression level between individual cells, fluorescence data for the bleached and control areas in the nucleus were normalized as fractional recovery: $R = (F - F_0)/(F_{infinite} - F_0)$. In addition, results obtained from the bleached area were normalized to those obtained from the control area to account for attenuation of fluorescence due to laser scanning, as

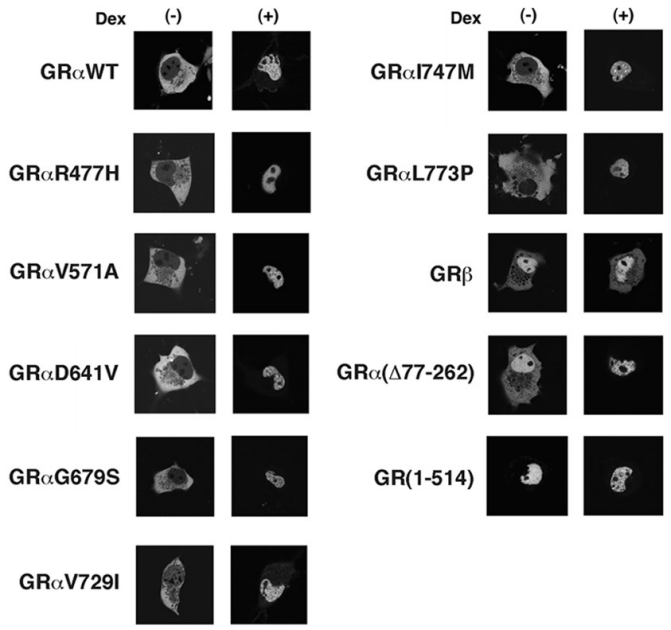

Figure 2. Subcellular localizations of GFP-GRs determined in the absence and presence of dexamethasone. COS7 cells were transfected with the indicated GFP-GR–expressing plasmids, and their confocal images were recorded before and 1 h after addition of 10^{-6} M dexamethasone.

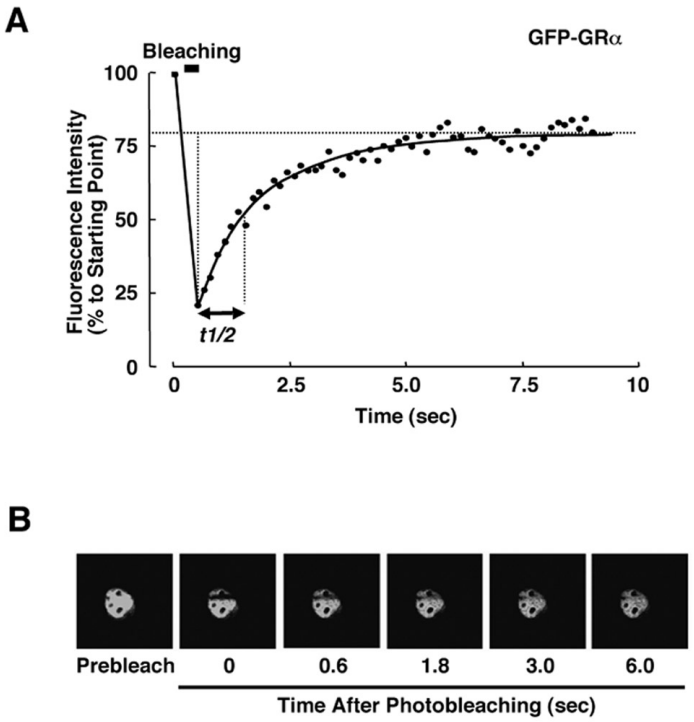

Figure 3. (A) Recovery of GFP-GRα fluorescence intensity in the nucleus after photobleaching and calculation of $t_{1/2}$. COS7 cells were transfected with pRShGRα, treated with 10^{-6} M dexamethasone, and photobleached in the nucleus. Fluorescence intensity was traced time-sequentially, and the recovery $t_{1/2}$ was estimated from the recovery curve produced. (B) Representative photobleaching.

previously described (26). Using the obtained FRAP curve, the $t_{1/2}$ of maximal recovery was determined, which is defined as the time point after bleaching at which the normalized fluorescence has increased to half the amount of the maximal recovery. A representative FRAP curve is shown in Figure 3, with analysis (A) and photobleaching images (B). Recovery $t_{1/2}$ values obtained from 12 to 20 cells for each procedure were statistically analyzed.

Statistical Analyses

Statistical analysis was carried out by unpaired or paired Student *t* test with the two-tailed *P* value. Linear regression was performed by the least-squares method.

RESULTS

Transcriptional Activity and Subcellular Localization of Pathologic Natural and Synthetic GRα Mutants and the Isoform GRβ

We selected 7 pathologic GRα mutants previously associated with the familial or sporadic glucocorticoid resistance syndrome (Table 1). We also tested the natural splicing variant GRβ and 2 synthetic GRα mutants, GRα(Δ77-262) and GR(1-514), devoid of the AF1 transactivation domain and the LBD (and hence the AF-2 domain), respectively. We first examined in parallel and compared the transcriptional activity of these GR-related molecules on the glucocorticoid-responsive MMTV promoter in COS7 cells (Figure 1). As shown previously, GRαV571A, D641V, G679S, V729I, I747M, and L773P all demonstrated reduced transactivation of this promoter. GRαR477H and GRβ showed no transactivation at all. GRα(Δ77-262) demonstrated reduced transactivation, whereas GR(1-514) activated the promoter in a dexamethasone-independent fashion.

We next examined the subcellular localization of these receptors in the absence and presence of dexamethasone (Figure 2). In the absence of the steroid, GRα and R477H, V571A, D641V,

Table 2. $t_{1/2}$ of the natural pathologic and synthetic GRα mutants and of GRβ in FRAP analysis

GRs	$t_{1/2}$, s (mean ± SD)
GRαWT	1.116 ± 0.207
GRαR477H	0.641 ± 0.172[a,b]
GRαV571A	0.729 ± 0.143[a,b]
GRαD641V	0.930 ± 0.094[a,b]
GRαG679S	0.900 ± 0.124[a,b]
GRαV729I	0.825 ± 0.097[c,b]
GRαI747M	0.835 ± 0.125[c,b]
GRαL773P	0.843 ± 0.145[c,d]
GRβ	0.656 ± 0.205[a,d]
GRα(Δ77-262)	0.645 ± 0.101[a,b]
GR(1-514)	0.415 ± 0.121[a]

COS7 cells were transfected with the indicated GFP-GRs exposed to 10^{-6} M dexamethasone and photobleached, and $t_{1/2}$ was measured in 12 to 20 individual cells. Numbers indicate mean ± SE of the recovery $t_{1/2}$ of the fluorescent intensity after photobleaching. [a]$P < 0.01$, [c]$P < 0.05$ compared with GRα WT; [d]$P < 0.05$, [b]$P < 0.01$ compared with GR(1-514).

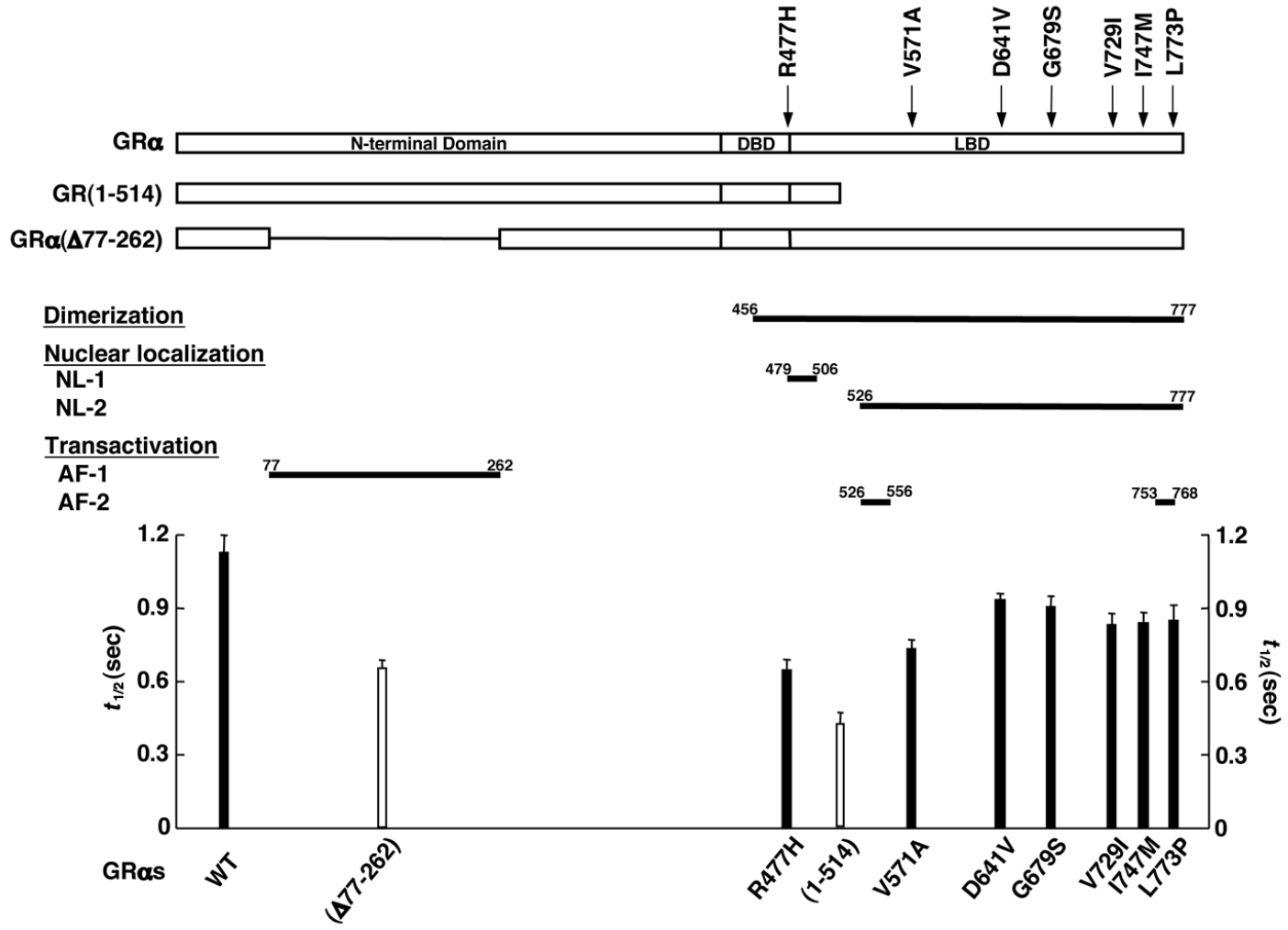

Figure 4. Location of natural pathologic and synthetic GRα mutants in the linearized GRα molecule, domains of known GRα functions, and the recovery $t_{1/2}$ values of these mutants obtained by FRAP analysis. Black bars indicate mean ± SE of $t_{1/2}$ values obtained in the natural pathologic mutants; white bars are those of synthetic mutants.

G679S, V729I, I747M, and L773P mutant GRs were mainly located in the cytoplasm. GRβ and GRα(Δ77-262) were located mainly in the nucleus and to a lesser extent in the cytoplasm. Because GRα(Δ77-262) is devoid of the AF-1 domain, this portion of the GRα apparently contains a function that retains the receptor in the cytoplasm in the absence of ligand. GR(1-514) was exclusively located in the nucleus, suggesting that the GRα LBD is also important for retention in the cytoplasm. One-hour incubation of the cells with dexamethasone caused nuclear translocation of GRα and the mutant GRs R477H, V571A, D641V, G679S, V729I, I747M, L773P, and Δ77-262. GRβ and GR(1-514) showed no response to dexamethasone, maintaining the same subcellular location.

FRAP Analysis of the Pathologic Natural and Synthetic GRα Mutants and the Isoform GRβ

We examined the motility of GR-related molecules in the nucleus of living cells using the FRAP method. A representative FRAP curve of the GFP-GRα and its photobleaching images are shown in Figure 3A and B, respectively. The means ± SE of the recovery $t_{1/2}$ values of these molecules in the presence of dexamethasone are shown in Table 2. All natural pathologic GRα mutants demonstrated a reduction of $t_{1/2}$ values, with GRαR477H, which does not bind to GREs, having the lowest value. All the other pathologic mutant receptors studied had mutations in the LBD. Two of them, GRαI747M and L773P, have a defective AF-2 surface that binds poorly to coactivators, such as p160s and p300/CBP, through the LXXLL motifs of these proteins. GR(1-514), which lacks the entire LBD, demonstrated the shortest recovery time. GRβ also demonstrated a significantly shorter recovery time than the wild-type GRα, whereas it showed a significantly longer recovery time than GR(1-514). GRα(Δ77-262) also demonstrated a shorter recovery time than the wild-type GRα. The means ± SE of the recovery $t_{1/2}$ values of these pathologic and synthetic GRα mutants are shown in Figure 4, with the localization of each mutation in the linearized GRα molecule.

The relation between the transcriptional activities and recovery $t_{1/2}$ values of the wild-type GRα and the natural and synthetic

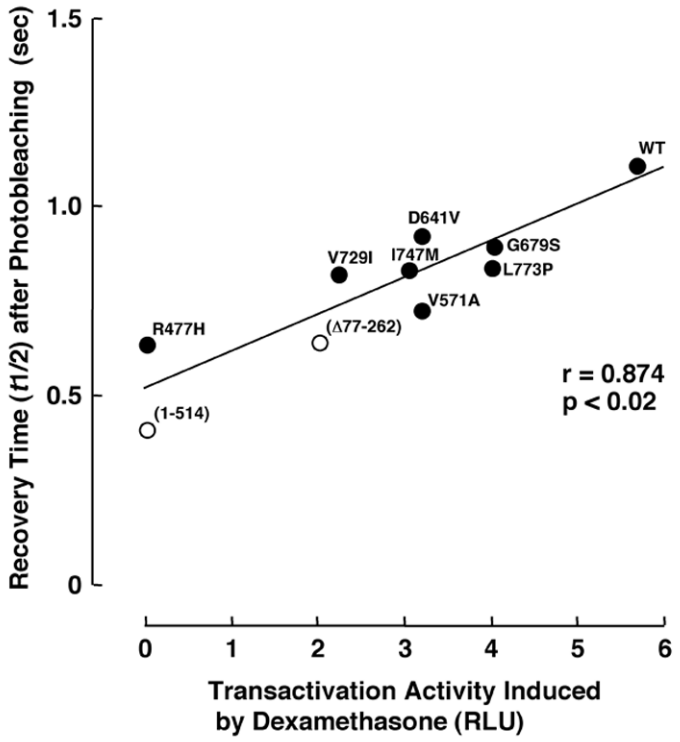

Figure 5. Transcriptional activities of the natural pathologic and synthetic GRα mutants are positively correlated with their recovery $t_{1/2}$ values. The maximal transcriptional activities induced by dexamethasone and recovery $t_{1/2}$ values after photobleaching of GRα and the mutant R477H, V571A, D641V, G679S, V729I, I747M, L773P, (Δ77-262), and (1-514) GRs are plotted at the x- and y-axes, respectively. Closed and open circles indicate the natural and synthetic mutant receptors, respectively. The correlation was tested by the linear regression method.

Table 3. Effect of MG-132 on the $t_{1/2}$ of the natural and synthetic GRα mutants and GRβ in FRAP analysis

	$t_{1/2}$, s (mean ± SD)	
GRs	MG-132 (–)	MG-132 (+)
GRαWT	1.109 ± 0.189	1.510 ± 0.235[a]
GRαR477H	0.641 ± 0.186	0.621 ± 0.152
GRαV571A	0.732 ± 0.157	0.720 ± 0.218
GRαD641V	0.930 ± 0.085	1.302 ± 0.322[a]
GRαG679S	0.896 ± 0.325	1.087 ± 0.322
GRαV729I	0.835 ± 0.154	1.245 ± 0.253[b]
GRαI747M	0.838 ± 0.321	1.181 ± 0.215[b]
GRαL773P	0.852 ± 0.374	1.181 ± 0.247[b]
GRβ	0.649 ± 0.184	0.941 ± 0.121[a]
GRα(Δ77-262)	0.632 ± 0.205	0.830 ± 0.155[b]
GR(1-514)	0.432 ± 0.210	0.370 ± 0.244

COS7 cells were transfected with the indicated GFP-GRs and treated with 1 μM MG-132 for 24 h. Dexamethasone was added to the medium to a final concentration of 10^{-6} M, and photobleaching was performed in 12 to 20 individual cells. Numbers indicate mean ± SE of the recovery $t_{1/2}$ of the fluorescence intensity after photobleaching. [a]$P < 0.01$, [b]$P < 0.05$, calculated by comparing the $t_{1/2}$ values obtained in the absence or presence of MG-132.

mutant receptors hGRαR477H, V571A, D641V, G679S, V729I, I747M, L773P, (Δ77-262), and GR(1-514) was examined by linear regression analysis and showed a significant positive correlation (Figure 5). A separate FRAP study and correlation analysis of findings from the 2nd experiment described below gave almost identical results ($P < 0.02$), suggesting strong consistency in the relation between the transcriptional activity and intranuclear motility effects of neutralizing GR mutations. The FRAP method measurements were highly reproducible, with a very low interassay coefficient of variation (less than 4%).

Influence of the Proteasome Pathway on the Motility of Pathologic Natural and Synthetic GRα Mutants and the Isoform GRβ in FRAP Analyses

The proteasome pathway plays important roles in the transcriptional activity of GRα (17,18,22,27). We examined the effect of the proteasomal inhibitor MG-132 on the motility of natural and synthetic GRα mutants and GRβ inside the nucleus to determine the contribution of the proteasomal pathway to the observed defects of their motility inside the nucleus. We used MG-132 at 1 μM, which can increase motility of the wild-type GRα inside the nucleus without exerting any toxic effects on the cells and does not affect the motility of non–protein-fused GFP alone in the nucleus (data not shown). Treatment of the cells with MG-132 significantly increased the recovery $t_{1/2}$ of the wild-type GRα (Table 3), as reported previously. This treatment also increased the recovery $t_{1/2}$ values of the natural pathologic mutants GRαD641V, V729I, I747M, and L773P, but not those of GRαR477H, V571A, or G679S. MG-132 also increased the recovery $t_{1/2}$ of GRβ and GRα(Δ77-262), but had no effect on that of GR(1-514).

DISCUSSION

The decrease in recovery $t_{1/2}$ inside the nucleus after stimulation with the ligand was a common defect shared by all natural pathologic GRα mutant receptors tested. The transcriptional activities of these mutant receptors correlated positively with their recovery $t_{1/2}$ inside the nucleus, suggesting that increased intranuclear receptor motility—and hence, deficient "chromatin retention"—is an integrated indicator of mutant receptor activity. By chromatin retention we mean restraining of receptor motion by interacting proteins and/or nucleic acids inside the nucleus, which, admittedly, in the absence of experimental evidence, cannot be equated with those pertaining to actively transcribed genes. GRα mutants V571A, D641V, G679S, V729I, I747M, L733P, all of which possess their point mutations at the LBD, demonstrated longer recovery time after photobleaching than GR(1-514), which lacks the entire LBD. Thus, the motility defects of these mutant receptors are partial and allow some interactions with nuclear components that contribute to some retention of the receptor by chromatin. Indeed, there are multiple interactions of the ligand-activated GRα with intranuclear molecules, including components of the nuclear matrix, target DNA sequences, nuclear receptor coactivators, other transcription factors, chromatin remodeling complexes, and/or other components of the transcriptosome, such as RNA-polymerase II and its ancillary factors (Figure 6) (20,21,28,29).

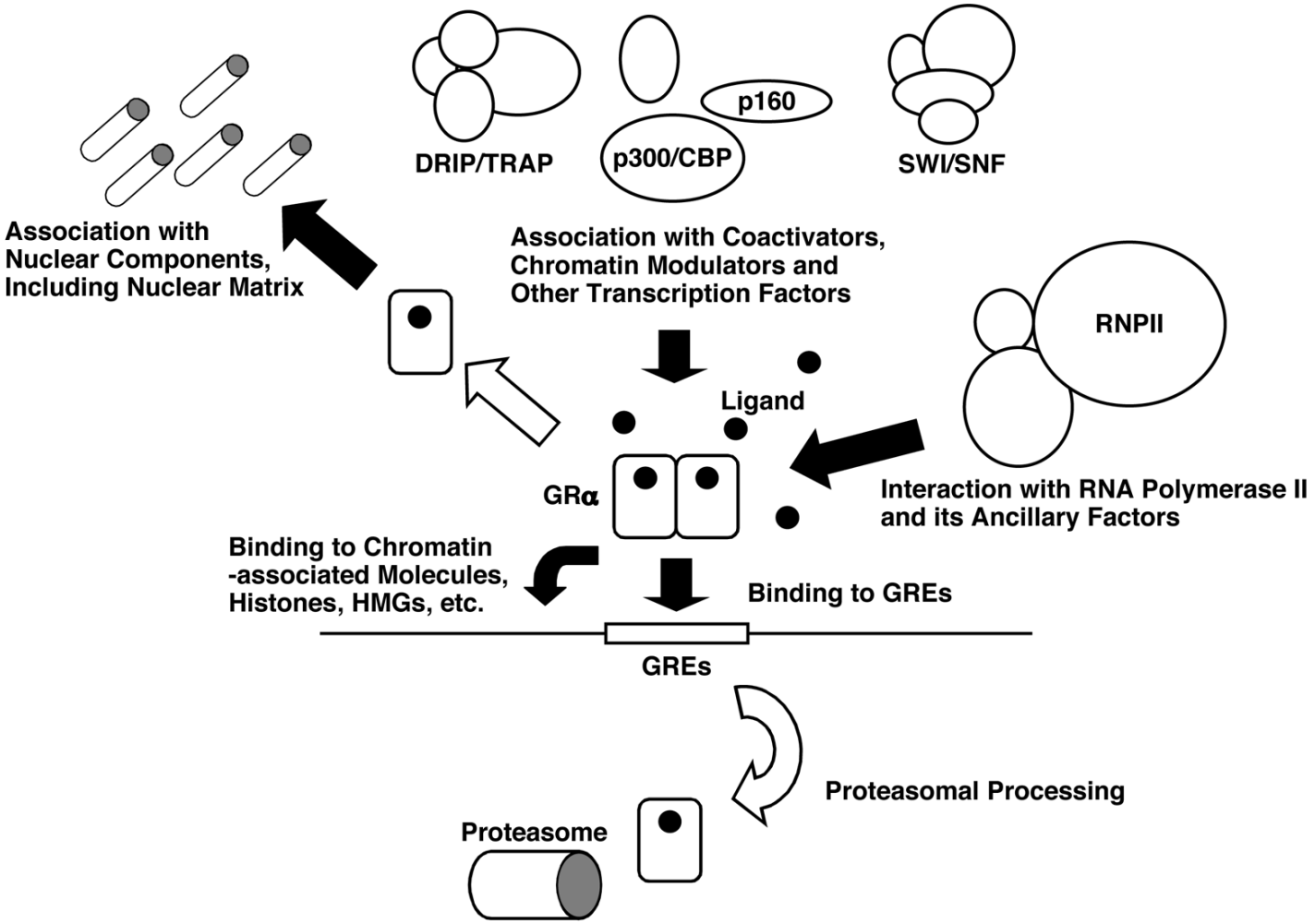

Figure 6. Proposed model of GRα interactions with other molecules in the cell nucleus affecting its chromatin retention time, and the possible impact of receptor mutations on its own intranuclear motility. Different interactions of GRα with intranuclear molecules influence its motility inside the nucleus and, hence, its chromatin retention time. Closed and open arrows indicate potential influences decreasing or increasing GRα motility, respectively. CBP, CREB-binding protein; RNPII, RNA polymerase II.

Even chaperones, such as hsp90 and hsp23, also influence the mobility of the GRα inside the nucleus (18). Most of these interactions are ligand dependent; thus, the affinity of GRα to the agonist ligand strongly influences this process, with higher affinities associated with slower movement inside the nucleus (26). Many of the natural GRα mutant receptors tested have lower affinity to dexamethasone; hence, the defective ability of some of these receptors to interact with the ligand might contribute to their increased motility inside the nucleus.

GRαR477H and the mutant GRαs I747M and L773P are, respectively, defective in binding to GREs and to the LXXLL motifs of nuclear receptor coactivators, whereas they all preserve their ability to bind ligand (8,12). The defective interaction of these receptors with GREs or coactivators appears to influence their motility inside the nucleus. GRα(Δ77-262), which is devoid of the AF-1 domain, also demonstrated significantly shorter recovery $t_{1/2}$ than the wild-type receptor, indicating that defective interaction of GRα with nuclear molecules through this domain also contributes to the reduced motility of the receptor. This domain interacts with numerous transcription-related molecules, including nuclear receptor coactivators such as p160 type proteins and p300/CBP and chromatin-remodeling complexes such as the SWI/SNF and the vitamin D receptor–interacting protein/thyroid hormone receptor-associated protein (DRIP/TRAP) complexes (8,14,30-32). Thus, interaction of the receptor to some or all of these transcriptional intermediate molecules through the AF-1 domain may also contribute to the nuclear motility of GRα.

The proteasome pathway plays important roles in transcription regulation promoted by numerous *trans*-acting molecules. Nuclear receptors, including GRα and the estrogen, progesterone, thyroid hormone, retinoic acid, and peroxisome proliferator activated receptors, as well as other transcription factors, such as p53, cJun, cMyc, and E2F-1, are ubiquitinated and subsequently degraded by the proteasome (33,34). The transcriptional intermediate molecules—such as nuclear receptor coactivators, chromatin remodeling factors, and some chromatin components, such as histone H1 and HMG proteins—are also ubiquitinated and lysed by the proteasome (33-35). Moreover, the proteasome inter-

acts with the COOH-terminal tail of RNA polymerase II and is directly associated with the promoter regions of several genes, influencing their transcriptional activities (36). Thus, ubiquitination and subsequent processing of these molecules by the proteasome appear to regulate the transcriptional activity of GRα, possibly by facilitating rapid turnover of promoter-attracted and -associated molecules, including GRα, finally down-regulating the transcriptional activity of this receptor. In agreement with these observations, inhibition of proteasomal activity by MG-132 reduced the motility of the wild-type GRα and its natural mutants GRαD641V, V729I, I747M, and L773P. This compound, however, did not affect the motility of GRαR477H, V571A, and G679S.

Because GR(1-514) did not respond to MG-132, it is obvious that the LBD of GRα is necessary for the proteasome to process the receptor and to influence its intranuclear motility. These results also indicate that mutations variously influence the interactions of the mutant receptors with the proteasomal pathway. Because all of the mutant receptors examined demonstrated decreased chromatin retention in the nucleus, different portions of GRα appear to mediate ligand-induced reduction of receptor motility compared with increased turnover by the proteasomal pathway via entirely distinct mechanisms. MG-132 failed to reduce the motility of GRαR477H, suggesting that, in addition to the LBD, the DBD is necessary for the proteasome to mobilize GRα inside the nucleus. That this mutant receptor does not bind GREs (11,23) suggests that binding of the receptor to these DNA sequences might be required for the proteasome to mobilize GRα. Indeed, the proteasome pathway seems to exert much of its effect on GRα after the latter's binding to GREs and after the modulation of the chromatin structure has been induced, possibly removing ligand-activated GRα from the transcriptosome on target DNA (18,22).

p160 Nuclear receptor coactivators and p300/CBP all interact with GRα at its 2 transactivation domains, AF-1 and AF-2 (8,14). That GRα mutants I747M and L773P or GRα(Δ77-262), which respectively have defective AF-2 or AF-1 domains, were sensitive to MG-132 suggests that interaction of the receptor with these coactivators may not strongly contribute to proteasome-mediated mobilization of the receptor (8,37,38). Interestingly, these coactivators are processed in the proteasome-rich ND10 bodies, where we had demonstrated earlier that the wild-type GRα, but not the GRαI747M mutant, colocalizes with nuclear receptor coactivators (8,37,38). That GRβ, which has a unique, non–ligand binding "ligand-binding domain" (14), was sensitive to MG-132, indicates that the "LBD" of this enigmatic splicing isoform participates in its proteasome-mediated mobilization. Very few activities mediated by the GRβ "LBD" are known; for example, it does not bind agonist ligands, does not support cytoplasmic-to-nuclear translocation, and does not stimulate GRE-mediated transcription (14,39,40), while the same domain is unable to interact with nuclear receptor coactivators (41). Yet, in cDNA microarray studies, stably transfected GRβ altered the expression of a unique complement of genes with little overlap with that generated by the ligand-activated GRα (42). Our FRAP results suggest a role of the GRβ "LBD" module in mediating the proteasomal clearance of this receptor isoform. These results might also imply that, if GRβ were transcriptionally active, its rapid clearance from its "target" promoters by the proteasome would probably assist in the termination of its activity.

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