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Functional Characterization of a K140N Human Glucocorticoid Receptor Variant

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Abstract: Glucocorticoids are widely used as potent anti-inflammatory drugs. Glucocorticoids exert their pharmacological effects by binding to glucocorticoid receptor (GR), which promotes expression of its target genes, or suppresses transcription mediated by other transcriptional factors, such as nuclear factor-κB (NF-κB). We had recently found one novel single nucleotide polymorphism, 420G>T (K140N), in the glucocorticoid receptor gene in Japanese subjects. In transiently transfected COS-7 cells, the expression of the K140N variant protein was approximately 14% of the wild type protein, although their mRNA levels were almost equivalent. When the transfected COS-7 cells were treated with a proteasome inhibitor MG-132, the K140N variant protein levels were increased 3-fold whereas those of the wild type were increased by 1.5-fold. Immunocytochemistry revealed that the K140N variant protein was localized similar to the wild type protein. The luciferase reporter assay in COS-7 cells treated with 100 nM dexamethasone showed that the overall luciferase activity of the K140N variant was reduced to approximately 67% of the wild type. Thus, the K140N variation was suggested to influence the response to glucocorticoid treatment.

Key words: Glucocorticoid receptor, single nucleotide polymorphism, amino acid substitution, transcriptional activity, proteasome

INTRODUCTION

Glucocorticoid receptor (GR) is a nuclear receptor activated by glucocorticoids and a regulator of the expression of various genes. Human GRα (hGRα) is encoded by 9 exons, including exon 9α[1]. GRβ is an alternatively spliced form with exon 9β replacing exon 9α, which was identified in glucocorticoid-resistant human multiple myeloma cells and functions as a dominant negative type of hGRα[2]. The 777 amino acid hGRα has many functional domains, which include DNA binding (amino acid residues 421-486), ligand binding (528-720), homodimerization (456-777), Hsp90 binding (568-653), nuclear translocation (479-506 and 526-777) and transactivation domains (77-262, 404-491 and 526-556)[3,4]. In the cytosol, GRs are associated with heat-shock and other proteins[5] and the binding of glucocorticoid leads to their nuclear translocation[6].

Glucocorticoid treatments are effective in many inflammatory diseases and some types of cancers. As for anti-inflammatory effects, GR is thought to exert its pharmacological effects through activation of the glucocorticoid-induced leucine zipper gene and/or suppression of nuclear factor-κB (NF-κB)/activator protein-1 (AP-1)-mediated transcription of genes, such as inflammatory cytokines[7-9]. However, a small number of patients do not respond to clinically relevant doses of glucocorticoids (glucocorticoid resistance)[10]. Several familial mutations in the GR gene have been shown to be associated with corticosteroid resistance or haematological malignancies, but these mutations are relatively rare and thought not to be a common cause of resistance[11]. Recently, genetic polymorphisms in the GR gene have been identified, some of which are relatively frequent[11-13]. For example, a BclI restriction fragment polymorphism and an N363S alteration have been reported to influence the regulation of the hypothalamic-pituitary-adrenal axis and to be associated with changes in metabolism and cardiovascular control[11,12]. Moreover, it has been reported that the I559N GRα variant has a transdominant effect on the wild-type GR by inhibiting its nuclear translocation[14]. Furthermore, the I747M GRα variant causes autosomal dominant glucocorticoid resistance through abnormal interactions with p160 steroid receptor coactivators[15]. We previously reported that the S651F and 2314insA variants showed reduced and almost abrogated transcriptional activities, respectively, correlating with their reduced protein expression levels[16].

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In this study, we assessed expression levels and functional changes of the K140N variant, which we had reported previously[17], in transfected COS-7 cells.

MATERIALS AND METHODS

Plasmids and transfection: pRShGRα was obtained from the American Type Culture Collection (ATCC, Manassas, VA), which contains the full length coding region of human GRα and the active *Rous sarcoma* virus promoter[18]. An expression plasmid encoding the variant GR (K140N) was prepared with a QuickChange Site-Directed Mutagenesis Kit (Stratagene Co., La Jolla, CA) using the wild type pRShGRα plasmid as a template. The empty vector plasmid used as a control was prepared by removing the hGRα cDNA from the pRShGRα plasmid. phRL-TK (Promega Co., Madison, WI) encoding *Renilla* (sea pansy) luciferase was used for cotransfection and normalization of transfection efficiency.

Cell culture: COS-7 cells were obtained from JCRB (National Institute of Health Sciences, Tokyo, Japan) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100 U mL^{-1} penicillin and streptomycin under an atmosphere of 5% CO_2 at 37°C. For dexamethasone treatment, cells were cultured in DMEM supplemented FBS (10%) that was pretreated with dextran-coated charcoal to remove endogenous glucocorticoids.

TaqMan real-time reverse-transcribed polymerase chain reaction (RT-PCR): COS-7 cells ($1.4x10^5$ cells) were transfected with 300 ng of the wild-type GR expression plasmid pRShGRα or the K140N GR expression plasmid together with 1.2 μg of empty vector using the PolyFect Transfection Reagent (Qiagen GmbH, Hilden, Germany). The empty vector was added to keep the total transfected DNA to 1.5 μg per $1.4x10^5$ cells. Twenty-four hours after transfection, the cells were treated with vehicle (methanol: 0.1% in final) or 100 nM dexamethasone (DEX) and continuously cultured for 24 h. Then the cells were harvested and total RNA was extracted with the RNeasy kit (Qiagen). Five hundred ng of each RNA samples were reverse transcribed using oligo $d(T)_{16}$ primer with TaqMan Reverse Transcription Reagents (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. PCR reactions were performed with GR specific primers and TaqMan probe (Assay ID: Hs00230818_mL, Applied Biosystems Inc.) using TaqMan Universal PCR Master Mix (Applied Biosystems Inc.) with the ABI7700 real-time PCR system. β-actin mRNA expression levels were measured as internal controls. All the GR mRNA expression levels were normalized according to the β-actin mRNA expression levels. The expression levels were quantified from three separate transfection experiments.

Western blot analysis: COS-7 cells ($1.4x10^5$ cells) were transfected with 300 ng of the wild-type GR expression plasmid pRShGRα or the K140N GR expression plasmid together with 1.2 μg of empty vector using the PolyFect Transfection Reagent (Qiagen). Twenty-four hours after transfection, the cells were treated with vehicle (methanol: 0.1% in final) or 100 nM DEX and continuously cultured for 24 h. Then the cells were harvested and the cell pellets were boiled in a protein sample buffer. An equal amount of total protein (50 μg) was separated on an 8% SDS-polyacrylamide gel (Tefuco Co., Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Labo., Hercules, CA, USA). The membrane was blocked with 5% skim milk and incubated with polyclonal rabbit anti-human GR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech., Buckinghamshire, UK) as a secondary antibody. GR protein was visualized with the WestFemto maximum sensitivity substrate (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions. The signals were assessed by densitometric analysis. The average expression levels were obtained from three separate transfection experiments.

Luciferase reporter assay: COS-7 cells ($2.8x10^4$ cells) were cotransfected with 60 ng of the wild-type GR expression plasmid pRShGRα or the K140N GR expression plasmid together with 120 ng of pHH-Luc reporter and 120 ng of phRL-TK. Twenty-four hours after transfection, the cells were treated with vehicle (methanol: 0.1% in final) or 100 nM DEX, then cultured for an additional 24 h. The cells were washed with PBS and the lysates were prepared and the luciferase activities of the prepared lysates were measured using the Dual Luciferase Reporter Assay System (Promega). All transfection efficiencies were normalized according to the *Renilla* luciferase activity. Quantification was done from three independent transfection experiments.

MG-132 treatment of transfected cells: COS-7 cells ($1.4x10^5$ cells) were transfected with 300 ng of the wild-type GR expression plasmid pRShGRα or the K140N GR expression plasmid together with 1.2 μg of empty vector using the PolyFect Transfection Reagent

(Qiagen). Twenty-four hours after transfection, the cells were treated with vehicle (dimethylsulfoxide: 0.1% in final) or 10 μM MG-132 (CALBIOCHEM, SandiegoSan Diego, CA) and continuously cultured for 8 h. Then, the whole cell lysates were prepared and subjected to Western blotting as described above.

Immunocytochemistry: COS-7 cells ($1.4x10^5$ cells) were transfected with 300 ng of the expression plasmid for the wild type or K140N variant GR together with 1.2 μg of empty vector and then cultured for 48 h. The cells were treated with vehicle or 100 nM dexamethasone for 90 min. And then, the cells were fixed, washed and immunostained with anti-GR antibody and Alexa 594-conjugated second antibody as described previously[16]. Immunoreacted GR and the nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) were visualized by fluorescent microscopy.

Statistical analysis: The results of the TaqMan RT-PCR, Western blot analysis and reporter assays were assessed for their statistical significance by One-way analysis of variance, followed by Fisher's Protected Least Significant Difference (PLSD) method or the Student t-test using StatView software (SAS Institute Inc., Cary, NC, USA).

RESULTS

mRNA expression levels of the variant GR K140N in transfected COS-7 cells: To determine expression levels of the variant GR mRNAs, the TaqMan real-time RT-PCR assay was performed using total RNA from COS-7 cells transfected with the wild type or variant expression plasmid (n=3) (Fig. 1). The mRNA expression levels of K140N GR were estimated at 131.3±31.5% in the absence of DEX and 86.8±17.1% in the presence of 100 nM DEX, respectively, when the expression level of the wild type GR (WT) was defined as 100%. There was no significant difference between the levels of WT and K140N regardless of DEX ($p>0.05$). Thus, the mRNA expression level of K140N GR was similar to that of the WT and the addition of DEX did not alter their mRNA expression levels.

Protein expression levels of K140N in whole cell lysates: In order to quantify the expression levels of the variant GR protein, we performed Western blot analysis for the whole cell lysates obtained from transfected COS-7 cells (n=3) (Fig. 2). By densitometric quantification, the protein expression levels of K140N were estimated to be 12.0±2.2% (0 nM DEX, $p<0.0001$) and 14.2±0.7% (100 nM DEX, $p<0.0001$), respectively, when the each WT expression level was defined as 100%. Thus, the K140N protein expression was significantly reduced

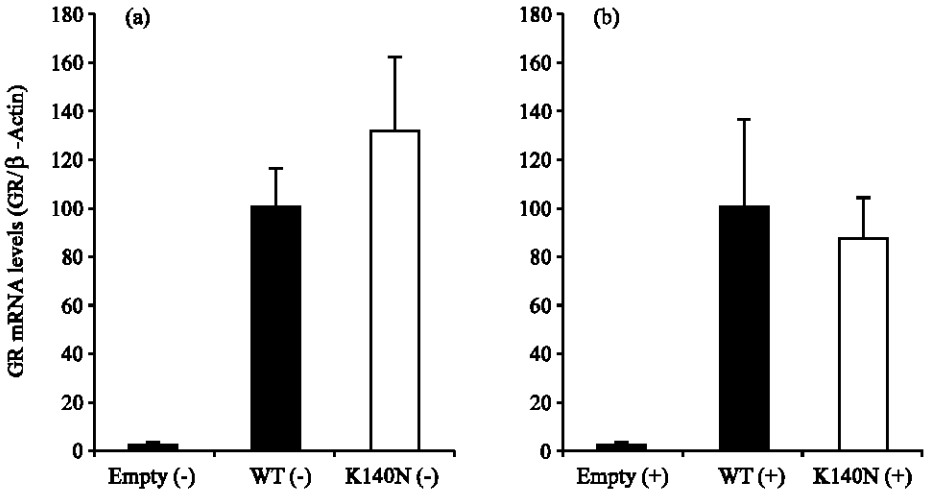

Fig. 1: Quantification of mRNA levels of the wild type (WT) and the K140N variant GRs. COS-7 cells were transfected with the empty vector, WT, or K140N expression plasmid. Twenty-four hours after transfection, the cells were treated with vehicle (-) (Panel a) or 100 nM dexamethasone (DEX) (+) (Panel b) for 24 h. Then, total RNA samples were prepared and the mRNA levels were quantified by TaqMan real-time RT-PCR. GR mRNA expression levels were normalized according to the β-actin mRNA expression levels. Bars represent the mean±SD (n=3)

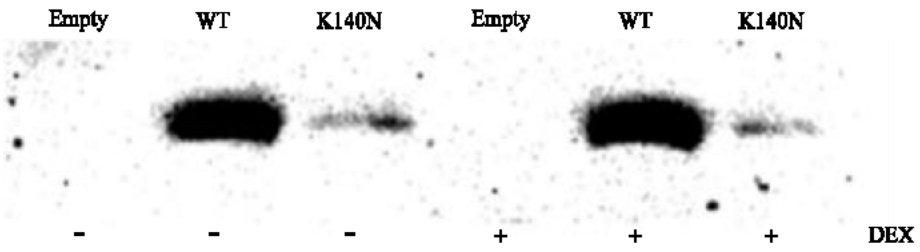

Fig. 2: (a) Western blot analysis of the wild type (WT) and the K140N variant GR proteins. Whole cell lysates were prepared from COS-7 cells, which were transfected with each vector described in the legends of Fig. 1. The lysates were subjected to SDS-PAGE and blotted. One of the representative results from three independent experiments is shown

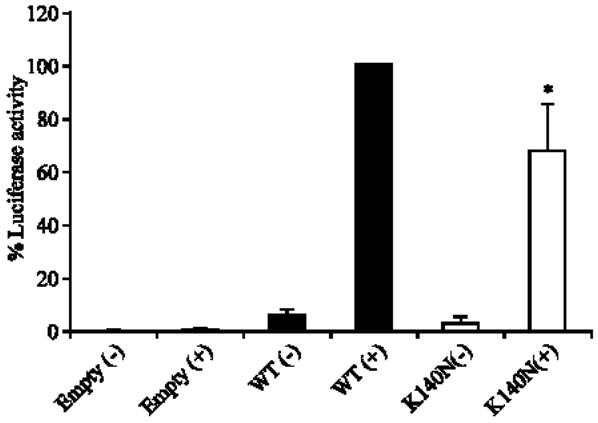

Fig. 3: Transcriptional activity of the wild type (WT) and the K140N variant GR. COS-7 cells were transfected with an expression plasmid, pHH-Luc reporter plasmid and phRL-TK plasmid. Twenty four hours after transfection, the cells were treated with vehicle (-) or 100 nM (+) dexamethasone (DEX) as indicated and continuously cultured for an additional 24 h. Luciferase activity is shown as the percent activity of WT with 100 nM DEX. Significant differences between the luciferase activities of WT and K140N are indicated as an asterisk. Bars represent the mean±SD (n=3). *p<0.05 by Fisher's PLSD Method

both in the presence and absence of DEX. The transfection efficiencies of the K140N variant expression plasmid were almost the same as those of the WT expression plasmid when assessed with the cotransfected phRL-TK (data not shown). From these results, it is likely that the reduction seen in the K140N protein level was caused by protein instability or ineffective translation in COS-7 cells. Furthermore, DEX did not increase both WT and K140N protein levels under present conditions.

Transcriptional activity of K140N: To investigate the transcriptional activity of the variant GR K140N, COS-7 cells were transfected with the WT or variant GR expression plasmid together with the pHH-Luc reporter. The results of the reporter gene experiments are shown in Fig. 3 (n=3). Transfection of COS-7 cells with the empty vector plasmid alone induced no luciferase activity, even by DEX treatment. When WT was transfected, luciferase activity increased in a dose dependent manner with DEX (data not shown). In contrast, the luciferase activities for K140N treated with 100 nM DEX showed a significant decrease to approximately 68.1±17.1% ($p<0.05$) of WT. Thus, the apparent transcriptional activities of the variant GR did not correlate with their protein expression levels. Normalized with the protein expression levels, the intrinsic transcriptional activity of K140N was calculated to be approximately 4.8-fold of those of the WT.

Effect of MG-132 on the protein levels of WT and K140N: To examine whether the reduced protein levels of the K140N variant is caused by accelerated protein degradation, the transfected cells were treated with a proteasome inhibitor MG-132. The protein levels of the wild type GR were increased to 153.0±7.2% when the cells were treated with MG-132 (Fig. 4). The protein levels of the K140N variant were increased to 299.3±38.9% when the cells were treated with MG-132 (Fig. 4). The recovery of the protein levels of the K140N variant with MG-132 was significant ($p<0.003$ by the Student t-test) as compared with that of WT, suggesting that the reduced protein levels of K140N were partially caused by the increased proteasomal degradation.

Subcellular localization of the variant GR: GR exists in the cytoplasm in a glucocorticoid-free form[19,20]. In order to investigate the subcellular localization of WT and K140N, immunostaining of COS-7 cells transfected with the WT or the K140N variant plasmid was performed (n=3)

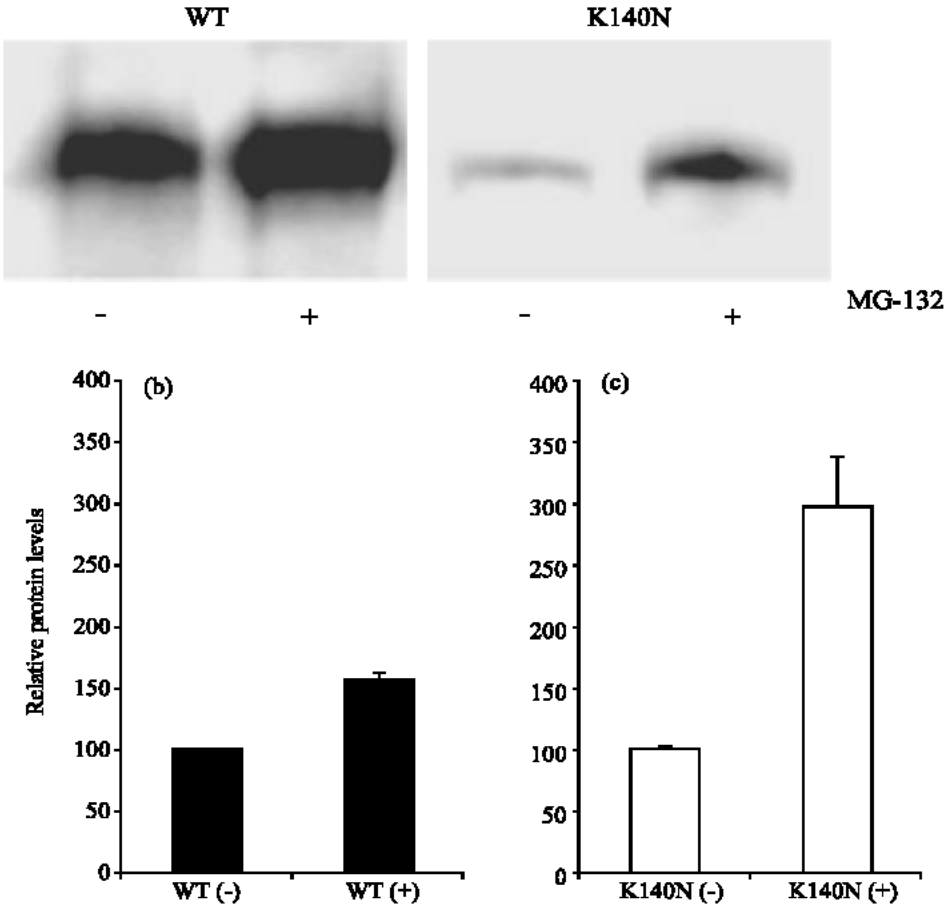

Fig. 4: Effects of MG-132 on the protein levels of the wild type (WT) and the K140N variant GRs. (a) COS-7 cells were transfected with the WT or the K140N expression plasmid. Twenty-four hours after transfection, the cells were treated with vehicle (-) or 10 μM MG-132 (+) for 8 h. Then, the whole cell lysates were subjected to Western blot analysis. One of the representative results from three independent experiments is shown. (b) and (c) The bands in Fig. 4a were quantitated by densitometry. Protein expression level of the WT (b) and the variant (c) sample treated with vehicle was defined as 100. Bars represent the mean±SD (n=3)

(Fig. 5). When the WT-transfected cells were not treated with DEX, the expressed receptors were localized in the cytoplasm. In contrast, when cells were treated with 100 nM DEX, most of the wild type GR proteins were localized in the nucleus. The localization patterns of the K140N variant proteins were almost similar to those of the WT (Fig. 5).

DISCUSSION

Glucocorticoid receptor (GR) is a DNA-binding transcriptional regulatory protein and also acts as a modulator of transcription by interaction with other transcription factors. To date, it has been shown that NF-κB and GR have opposing actions in the modulation of the immune/inflammatory responses[21]. NF-κB induces the expression of proinflammatory genes, while GR suppresses immune function, in part, by reducing the expression of these genes. The molecular mechanism of GR function is still controversial, but its direct binding to the NF-κB p65 subunit is thought to be involved in the subsequent suppression of NF-κB-mediated transactivation[7,8,22]. In addition, GR also transactivates several genes including the recently identified glucocorticoid-induced leucine zipper gene, which interferes with the function of NF-κB and AP-1. Thus, glucocorticoid-induced transactivation is also involved in its anti-inflammatory effects[9,23].

In order to detect novel single nucleotide polymorphisms (SNPs) of the GR gene, the exonic regions of the GR gene for 265 Japanese subjects and found a

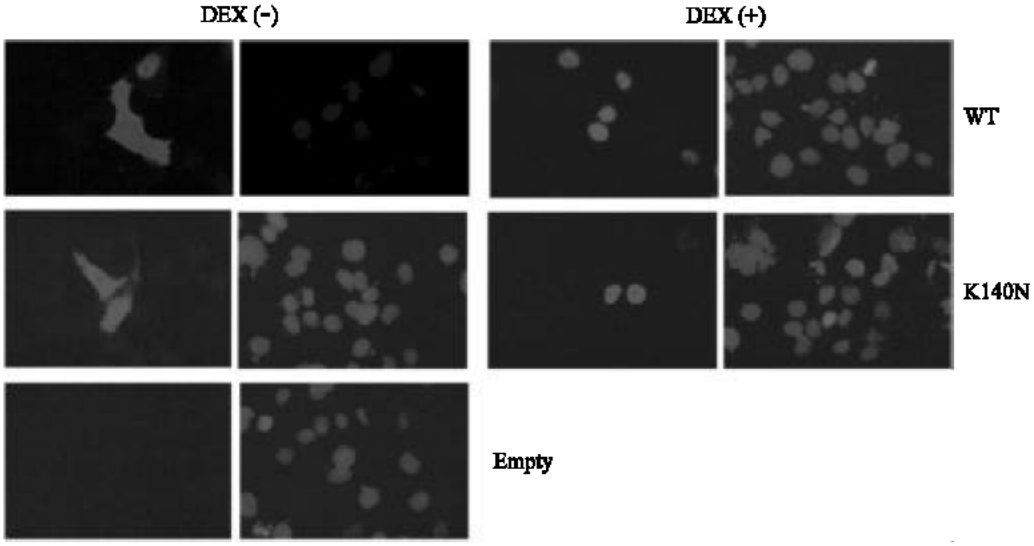

Fig. 5: Cellular localization of the wild type (WT) and K140N variant GR proteins in COS-7 cells. COS-7 cells were transfected with the WT or the K140N variant expression plasmid in the absence (-; the left two columns) or presence (+; the right two columns) of 100 nM dexamethasone (DEX). The transfected cells were immunostained with anti-GR antibody and Alexa 594-conjugated second antibody. Photographs in the left columns show the immunoreacted WT and K140N variant protein (red). Photographs in the right columns show DAPI-stained nuclei (blue). The empty plasmid-transfected cells (negative control) were also immunostained (empty). Data from one of the two independent transfections are shown

SNP 420 G>T (K140N) at a 0.004 frequency[17]. In this study, we assessed the functional effects of this nucleotide variation.

The protein level and transcriptional activity of the K140N variant were reduced to 14 and 67% of the wild type, respectively, without any changes in mRNA levels (Fig. 1-3). Thus, it was expected that the reduced protein level of K140N might have been caused by protein degradation. This was confirmed by treatment of the transfected cells with a proteasome inhibitor MG-132 (Fig. 4). The recovery for the K140N variant protein by MG-132 was higher than that for the WT, suggesting that the variant protein is more rapidly degraded by proteasomes than the WT protein in transfected COS-7 cells.

The protein levels of K140N did not correlate with the apparent transcriptional activity. Thus, by normalization with the expressed protein levels, the intrinsic transcriptional activity of K140N was estimated to be 4.8-times higher than that of the WT. Lys-140 is located in the activation function 1 (AF-1) domain. This domain (amino acid residues 77-262) is involved in a major transactivation function, interaction with c-jun and the synergy limiting activity[24-26]. Previous analysis revealed that the deletion of residues 131-147 did not alter the transactivation properties[27]. In the same report, however, it was shown that the deletion of residues 98-113 or 150-170 resulted in a 2-fold activations. Therefore, it was suggested that the two deletion mutant proteins did not bind negative regulatory factors and showed increased transcriptional activities. Furthermore, Hittelman *et al.*[25] have reported that tumor susceptibility gene 101 (TSG101) protein interacts specifically with the AF-1 domain and represses the GR-mediated transactivation. The K140N variant protein has a substitution in the AF-1 domain and its intrinsic activity was increased. Therefore, we evaluated the effect of overexpression of TSG101 on the transcriptional activity of the WT and K140N variant in COS-7 cells. As previously reported[25], the transcriptional activity of WT was repressed by co-transfecting of the TSG101 expression plasmid in a dose dependent manner (data not shown). The transcriptional activity of K140N variant was also repressed in a similar manner to WT (data not shown). These data indicate that the K140N protein can respond to the repressive action by TSG101 similar to the WT.

To evaluate subcellular localization of the K140N protein, we performed immunostaining of the transfected COS-7 cells (Fig. 5). The localization of K140N protein was almost similar to the WT irrespective of the presence of DEX, suggesting that the K140N

substitution did not affect the subcellular localization. Thus, at present, the mechanism of the increased intrinsic transcriptional activity of K140N is unclear.

In conclusion, we evaluated functional properties of a naturally occurring variant of glucocorticoid receptor in transfected COS-7 cells. The levels of K140N variant protein were decreased to 14% of the wild type GR, without any changes in its mRNA expression. However, the intrinsic transcriptional activity of the K140N variant was increased to approximately 4.8-fold of that of the wild type. The overall transcriptional activity of K140N in the transfected cells was reduced to approximately 67% of the wild type. These data suggest that the K140N variation might affect glucocorticoid response.

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