Research Article

The Role of S-Palmitoylation of the Human Glucocorticoid Receptor (hGR) in Mediating the Nongenomic Glucocorticoid Actions

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Abstract

Background: Many rapid nongenomic glucocorticoid actions are mediated by membrane-bound glucocorticoid receptors (GRs). S-palmitoylation is a lipid post-translational modification that mediates the membrane localization of some steroid receptors. A highly homologous amino acid sequence (663YLCM KTLLL671) is present in the ligand-binding domain of hGRα, suggesting that hGRα might also undergo S-palmitoylation.
Aim: To investigate the role of the motif 663YLCMKTLLL671 in membrane localization of the hGRα and in mediating rapid nongenomic glucocorticoid signaling.
Methods and Results: We showed that the mutant receptors hGRαY663A, hGRαC665A and hGRαLL670/671AA, and the addition of the palmitoylation inhibitor 2-bromopalmitate did not prevent membrane localization of hGRα and co-localization with caveolin-1, and did not influence the biphasic activation of mitogen-activated protein kinase (MAPK) signaling pathway in the early time points. Finally, the hGRα was not shown to undergo S-palmitoylation.
Conclusions: The motif 663YLCMKTLLL671 does not play a role in membrane localization of hGRα and does not mediate the nongenomic glucocorticoid actions.

Introduction

Glucocorticoids are steroid hormones that regulate a broad spectrum of physiologic functions essential for life, such as growth, reproduction, behavior, cognition, as well as many immune, metabolic and cardiovascular functions, through their ubiquitously expressed glucocorticoid receptor (hGR) (Charmandari *et al.* 2005, Chrousos *et al.* 2004, Chrousos & Kino 2005, 2007, Nicolaides *et al.* 2010, Rhen & Cidlowski 2005). The hGR belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors. The hGR regulates the transcription rate of many genes either by direct binding to their promoter regions or through interactions with other transcription factors, such as nuclear factor (NF)-κB, signal transducers and activators of transcription (STATs) and activator protein (AP)-1 (Nicolaides *et al.* 2010, Rhen & Cidlowski 2005).

In addition to the genomic actions, glucocorticoids exert rapid, 'non-genomic actions',

Figure 1. Stereotactic conformation of the agonist form of the LBD of hGRα. The yellow arrow indicates the 9-amino acid sequence, 663YLCMKTLL670, which is located on helix 8. H: Helix

which occur within seconds or minutes and are not inhibited by transcriptional and translational inhibitors (Ayroldi *et al.* 2012, Bellavance & Rivest 2012, Lee *et al.* 2012, Losel & Wehling 2003, Song & Buttgereit 2006, Stellato 2004). Examples of non-genomic actions of glucocorticoids include i) the immediate suppression of ACTH secretion from the anterior pituitary gland following a rise in glucocorticoid concentrations (Hinz & Hirschelmann 2000); ii) the increased frequency of excitatory post-synaptic potentials in the hippocampus (Karst *et al.* 2005); the rapid and transient decrease in blood pressure associated with a concomitant increase in coronary and cerebral blood flow in patients with myocardial infarction or stroke (Hafezi-Moghadam *et al.* 2002); and some T cell-related immune functions (Löwenberg *et al.* 2006). These rapid non-genomic actions of glucocorticoids are likely to be mediated by membrane glucocorticoid receptors that transduce the glucocorticoid signal via activation of downstream kinases (Ayroldi *et al.* 2012, Bellavance & Rivest 2012, Lee *et al.* 2012, Losel & Wehling 2003, Song & Buttgereit 2006, Stellato 2004).

Covalent lipid modifications anchor numerous signaling proteins to the cytoplasmic face of the plasma membrane (Linder & Deschenes 2007). S-palmitoylation refers to a reversible thioster linkage of palmitate (a C16 saturated fatty acid) to cysteine (Cys) residues of soluble proteins with hydrophobic moieties. This post-translational modification is catalyzed by membrane-bound palmitoyl-transferases (PATs) and increases protein hydrophobicity, thereby enabling protein binding to membranes. Due to its reversible nature, this reaction provides a powerful on/off switch mechanism through anchoring/de-anchoring of proteins on the plasma membrane (Linder & Deschenes 2007).

Many heptahelical G protein-coupled receptors (GPCR) contain a conserved F(X6)LL sequence, where X is any amino acid and L is leucine or isoleucine. F, LL, and the precise 6-amino acid spacing between F and LL are required for protein export from the endoplasmic reticulum (Duvernay *et al.* 2004). A similar to the F(X6)LL sequence, highly conserved, 9-amino acid motif has been identified in the ligand-binding domains (LBDs) of most steroid receptors, and in particular of the human estrogen receptors (ER) α and β, progesterone receptors A (PR-A) and B (PR-B), and the androgen receptor (AR). In contrast to typical GPCRs, the third amino acid of this motif (YLCMKTLL) in all steroid receptors except the mineralocorticoid receptor (MR) is cysteine. Recent studies have demonstrated that this motif plays an important role in S-palmitoylation, membrane localization and the steroid signaling through activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (Marino *et al.* 2006, Pedram *et al.* 2007). Mutation of the phenylalanine or tyrosine at position -2, cysteine at position 0, and hydrophobic isoleucine/leucine or leucine/leucine combinations at positions +5/6, relative to cysteine, significantly reduced membrane localization, MAPK and PI3K activation, thymidine incorporation into DNA and cell viability stimulated by specific steroid receptor ligands (Pedram *et al.* 2007). The localization sequence mediated palmitoylation of some steroid receptors, which facilitated their caveolin-1 association, subsequent membrane localization, and nongenomic signaling by respective steroids. Therefore, S-palmitoylation within the LBD may be a crucial modification for membrane translocation and function of these steroid receptors (Pedram *et al.* 2007).

Intriguingly, in the LBD domain of the hGRα protein, a sequence highly homologous to the above-discussed motif is present (663YLCMKTLL670), suggesting that hGRα might also undergo S-palmitoylation (Marino & Ascenzi 2006). This post-translational modification might account for membrane-initiated rapid nongenomic glucocorticoid signaling. The position of this motif in the crystal structure of the LBD of hGRα is shown in Figure 1. The hGRα sequence 663YLCMKTLL670 has been further confirmed to be palmitoylable by analysis with CSS-Palm (available from: http://bioinformatics.lcd-ustc.org/css_palm/) (Marino & Ascenzi 2006).

The aim of our study was to determine the role of the motif 663YLCMKTLLL671 in mediating rapid nongenomic glucocorticoid signaling and to investigate whether the hGRα undergoes S-palmitoylation. We performed *in vitro* studies to determine the specific residues within the 9-amino acid motif of the LBD of hGRα that are crucial for rapid glucocorticoid signaling. Specifically, we investigated whether mutation of the amino acids Y, C and LL at positions -2, 0 and +5/6, respectively, relative to cysteine (C) in the 9-amino acid motif, significantly reduce localization of the receptor to the plasma membrane, palmitoylation, association with caveolin-1, and MAPK and AKT activation.

Materials and Methods

Plasmids

The pRShGRα plasmid expresses hGRα under the control of the Rous sarcoma virus (RSV) promoter.

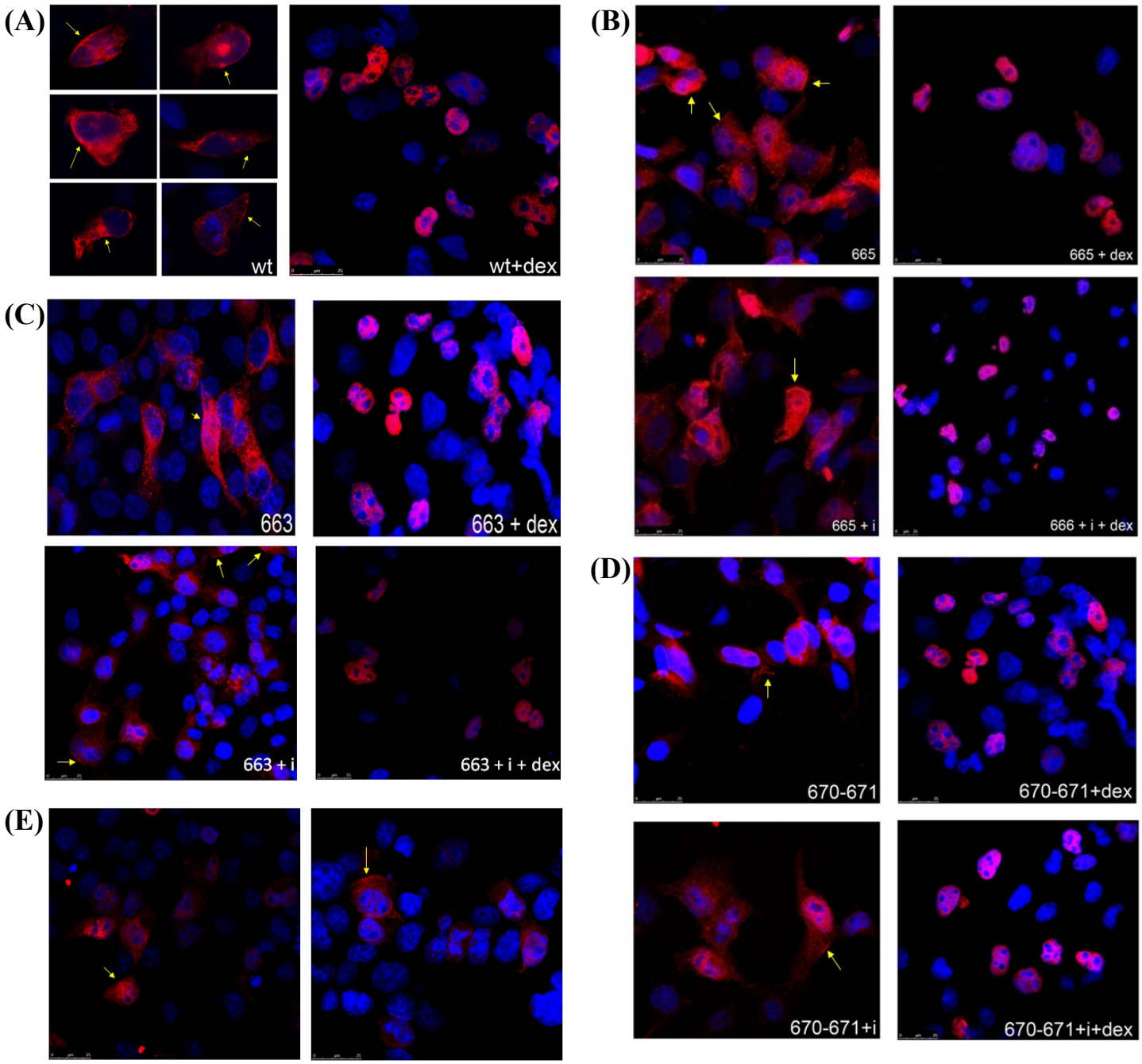

Figure 2. (A-D) Localization of the wild-type hGRα (hGRαWT) or its mutant receptors expressed in COS-7 cells in the absence or presence of dexamethasone. (E) Localization of hGRαWT in the presence of the palmitoylation inhibitor. The localization of hGRα-related proteins was examined with immunofluorescent staining. The yellow arrows point to membrane localization of hGRα. Dex: dexamethasone; i: palmitoylation inhibitor 2-Br.

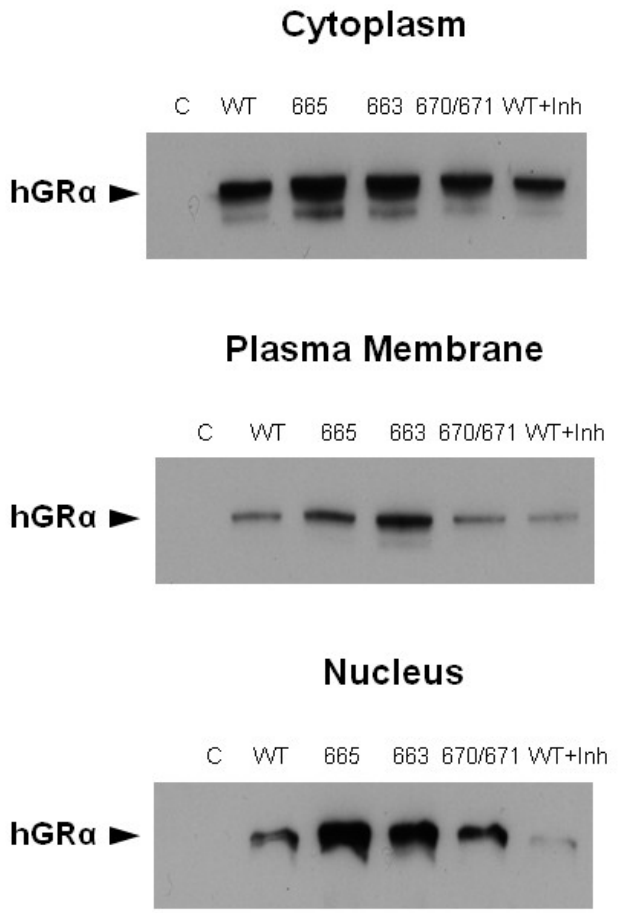

Figure 3. Cytoplasmic, membrane and nuclear localization of the hGRα in the COS-7 cells expressing the wild-type hGRα (hGRαWT) or the mutant receptors. Amounts of hGRα-related proteins were examined in subcellular fractionated samples with Western blotting.

The plasmids pRShGRαY663A, pRShGRαC665A and pRShGRαLL670/671AA were constructed by introducing the indicated mutations into the pRShGRα plasmid using PCR-assisted site-directed mutagenesis (Stratagene, La Jolla, CA, USA). The primers used for site-directed mutagenesis are shown in Table 1. The successful introduction of the indicated mutations into the pRShGRα plasmid was confirmed by sequencing.

Cell cultures

COS-7 embryonic African green monkey kidney cells, which do not express endogenously the glucocorticoid receptor, were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin-streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C and passaged every 3-4 days using trypsin.

Immunofluorescence

COS-7 cells ($3x10^5$) were plated on glass coverslips in 6-well plates. Twenty-four hours later (confluency 90-95%), cells were transiently transfected with pRShGRα, pRShGRαC665A, pRShGRαY663A or pRShGRαLL670/671AA using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, dexamethasone 10^{-6} M was added for 2 hours in specific wells. When indicated, the palmitoyl-acetyl-transferase inhibitor bromohexadecanoic acid (2-Br) (Sigma Aldrich, Gillingham, Dorset, UK) (100 μM, diluted in 100% DMSO) was added 30 min before dexamethasone administration. Subsequently, cells were washed with PBS, fixed with 4% fresh paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.2% Triton-X100 in PBS for 1 min. Prior to antibody incubation, cells were blocked with 2% BSA in PBS at room temperature. Following washing with PBS, cells were incubated with primary anti-hGRα antibody (dilution 1:100 in 0.5% BSA-PBS) (polyclonal anti-rabbit immunoglobulin, Cat No. PA1-516, Thermo Fisher Scientific, Boston, MA, USA) for 2 hours and subsequently with secondary antibody (dilution 1:500 in 0.5% BSA-PBS) (Alexa Fluor® 594 donkey anti-rabbit IgG, Cat No A21207, Invitrogen) for 1 hour at room temperature. Cells were washed with PBS and samples were mounted in 4',6-diamidino-2-phenylindole (DAPI) and stored at 4°C overnight. Samples were analyzed by confocal microscopy (DM IRB, Leica, Wetzlar, Germany). The confluency of the cells was 60-70% on each coverslip.

For double immunofluorescence staining, cells were co-incubated with 0.5% BSA-PBS containing a combination of primary anti-hGRα antibody (dilution 1:100) (polyclonal anti-rabbit immunoglobulin, Cat No. PA1-516, Thermo Fisher Scientific) and primary anti-caveolin-1 antibody (dilution 1:200) (monoclonal anti-mouse immunoglobulin, Cat No sc-53564, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (placed directly on each coverslip) for 2 hours at room temperature. Following washing with PBS, cells were co-incubated with 0.5% BSA-PBS containing a combination of secondary antibodies (dilution 1:500 each) (Alexa Fluor® 594 donkey anti-rabbit IgG, Cat No A21207, Invitrogen, and Fluorescein goat anti-mouse IgG, Cat No F2761, Invitrogen) (placed directly on each coverslip) for 1 hour at room temperature. Samples were washed with PBS, mounted in DAPI and stored at 4°C overnight. Random fields of samples were selected, the cells with membrane localization of hGRα or co-localization of hGRα with caveolin-1 were counted manually, and the number of these cells was divided by the number of total cells within these fields.

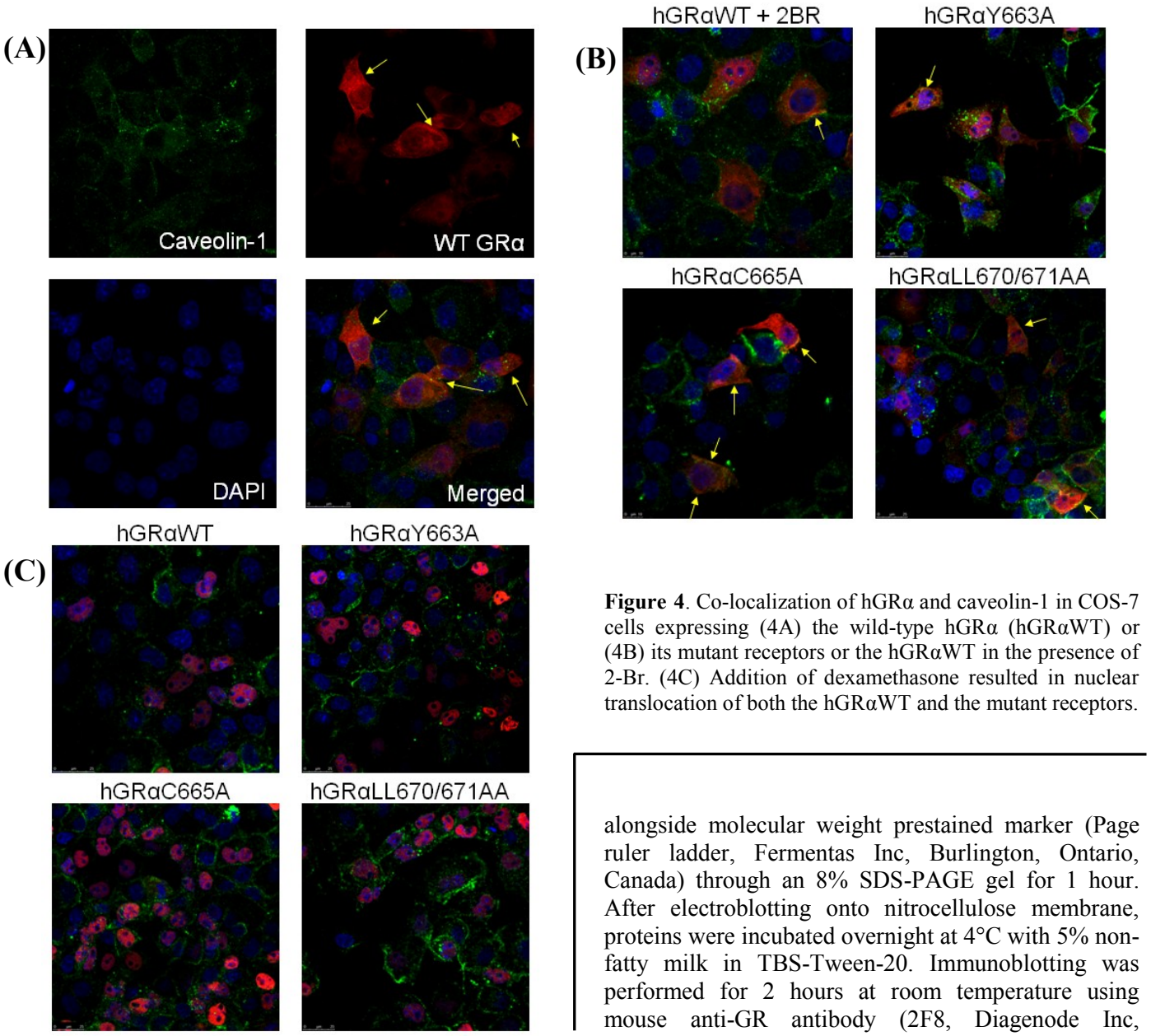

Figure 4. Co-localization of hGRα and caveolin-1 in COS-7 cells expressing (4A) the wild-type hGRα (hGRαWT) or (4B) its mutant receptors or the hGRαWT in the presence of 2-Br. (4C) Addition of dexamethasone resulted in nuclear translocation of both the hGRαWT and the mutant receptors.

Subcellular fractionation

COS-7 cells ($2x10^6$) were plated in 75-cm^2 flasks. Twenty-four hours later, cells were transiently transfected with pRShGRα, pRShGRαC665A, pRShGRαY663A or pRShGRαLL670/671AA using lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were trypsinized and counted. From the total number of cells, $5x10^6$ cells were washed once in PBS and were centrifuged (500 x g) for 3 min. PBS was carefully removed and cell pellets were mixed gently. The subcellular fractionation was performed using the Subcellular Protein Fractionation Kit (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer. Equal amounts of protein (20 µg) were mixed with Laemli buffer (5X), heated at 95°C for 5 min and electrophoresed alongside molecular weight prestained marker (Page ruler ladder, Fermentas Inc, Burlington, Ontario, Canada) through an 8% SDS-PAGE gel for 1 hour. After electroblotting onto nitrocellulose membrane, proteins were incubated overnight at 4°C with 5% non-fatty milk in TBS-Tween-20. Immunoblotting was performed for 2 hours at room temperature using mouse anti-GR antibody (2F8, Diagenode Inc, Denville, USA) (1:500, diluted in 5% non-fatty milk in TBS-Tween-20). After washing with TBS-Tween-20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) (1:5000, diluted in 5% non-fatty milk in TBS-Tween-20) for 1 hour at room temperature. Proteins were visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) and exposed to high performance chemiluminescence film (Kodak, Rochester, New York, USA).

MAPK and AKT kinase assays

COS-7 cells ($3x10^5$) were plated in 6-well plates. Twenty-four hours later, cells were transiently transfected with pRShGRα, pRShGRαC665A,

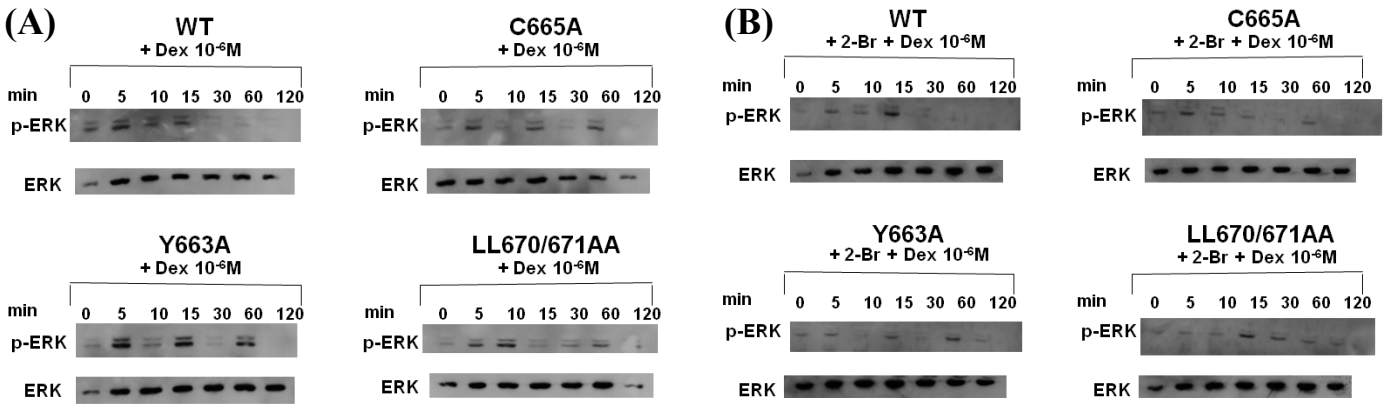

Figure 5. (A) Time-course effects of dexamethasone on the MAPK activity in COS-7 cells expressing the wild-type hGRα (hGRαWT) or its mutant receptors. (B) The effect of 2-Br on the MAPK activity in COS7 cells expressing the wild-type hGRα (hGRαWT) or its mutant receptors. Expression levels of total ERK and p-ERK were examined by Western blotting.

pRShGRαY663A or pRShGRαLL670/671AA using lipofectamine 2000 (Invitrogen), as described above. Twenty-four hours after transfection, the inhibitor 2-Br (100 µM diluted in 100% DMSO) was added in half of the wells. Sixteen hours later, cells were incubated with dexamethasone 10^{-6} M for different periods of time (0, 5, 10, 15, 30, 60 and 120 min). Cells were washed twice with PBS and lysed in 150 µl Complete Lysis-M (Roche, Basel, Switzerland) supplemented with Na_2VO_4 (0.2mM) and NaF (1mM). The homogenates were centrifuged (13,000 rpm at 4°C) for 15 min to obtain whole cell extracts. Western blotting was performed using mouse anti-p-ERK antibody (Cell Signaling, Beverly, MA, USA) (1:1000, diluted in 5% non-fatty milk in TBS-Tween-20) or rabbit anti-p-Akt antibody (Cell Signaling) (1:1000, diluted in 5% non-fatty milk in TBS-Tween-20) for 2 hours at room temperature. After stripping, the membrane was re-blotted using rabbit anti-ERK antibody (Cell Signaling) (1:1000, diluted in 5% non-fatty milk in TBS-Tween-20) or rabbit anti-Akt antibody (Cell Signaling) (1:250, diluted in 5% non-fatty milk in TBS -Tween-20) for 2 hours at room temperature. Following washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, diluted in 5% non-fatty milk in TBS-Tween-20, Dako) for 1 hour at room temperature. Finally, proteins were visualized as described above.

Palmitoylation assays

COS-7 cells ($5x10^6$) were plated in 100-mm cell dishes. Twenty-four hours later, cells were transiently transfected with pRShGRα or $erbA^{-1}$ using lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, supplemented DMEM was replaced by plain DMEM for 1 hour. Cells were then exposed to 9,10-^{3}H(N) palmitic acid (0,1 mCi/mL) and incubated for 2 hours at 37°C. Protein extracts were prepared as previously described, and protein concentrations were estimated with the Bradford protein assays. Equal amounts of protein extracts (500 µg) were used for immunoprecipitation assays. The anti-hGRα or anti-caveolin-1 antibody was bound to magnetic beads for 1 hour at 4°C. Protein extracts were then incubated with anti-hGRα- or anti-caveolin-1-bound magnetic beads overnight at 4°C. The next day the protein immunocomplex was washed six times, mixed with Laemli buffer (5X) and heated for 5 min at 95°C. Equal volumes of each sample were added to 4 ml scintillation fluid, and radioactivity was measured using a β-counter (Beckman LS6000IC counter, Beckman Coulter Inc., Fullerton, CA, USA).

Statistical analysis

All experiments were performed at least three times. Statistical analyses were carried out using the unpaired Student *t* test with a two-tailed P value.

Results

Neither the mutant receptors nor the addition of 2-Br prevented membrane localization of hGRα

To examine the membrane localization of the hGRα, COS-7 cells were transiently transfected with pRShGRα, pRShGRαC665A, pRShGRαY663A or pRShGRαLL670/671AA, and the immunofluorescence microscopy analysis was performed. Membrane localization of the hGRα was observed in 15% of the cells transfected with pRShGRα (Figure 2A) and in 13% of the cells transfected with pRShGRαC665A (Figure 2B), whereas the percentage of membrane localization of the hGRα in cells transfected with pRShGRαY663A or pRShGRαLL670/671AA was 11% in each case (Figures 2C & 2D). Addition of the inhibitor 2-Br did not prevent the membrane

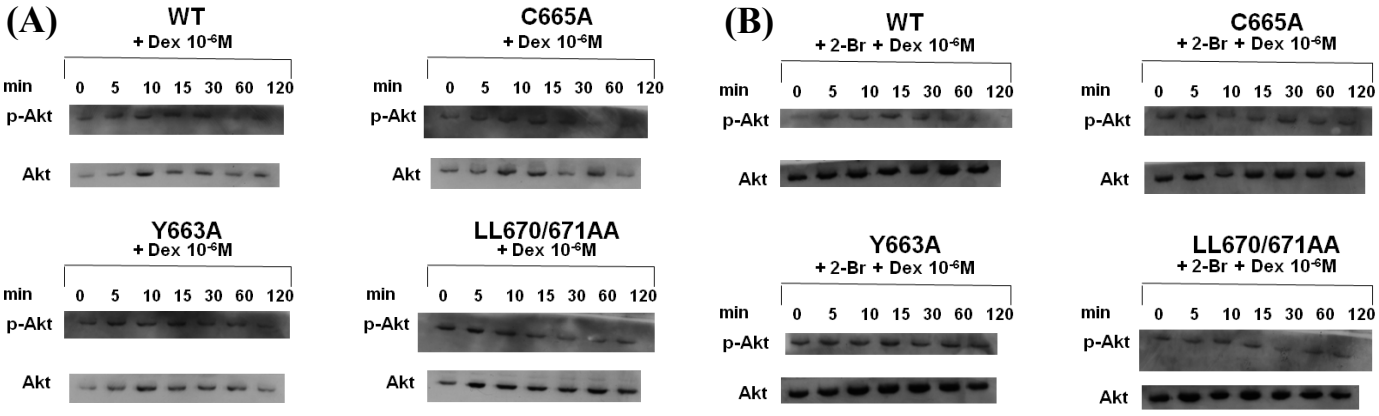

Figure 6. (A) Time-course effect of dexamethasone on the AKT activity in COS-7 cells expressing the wild-type hGRα (hGRαWT) or its mutant receptors. (B) The effect of 2-Br on the AKT activity in COS-7 cells expressing the wild-type hGRα (hGRαWT) or its mutant receptors. Expression levels of the total AKT and p-Akt were examined with Western blotting.

localization in cells transfected either with the wild-type or the mutant receptors (percentage of membrane localization 13%) (Figure 2E). Addition of dexamethasone resulted in nuclear translocation of the hGRα (wild-type or mutant hGRs) (percentage of membrane localization 0%) (Figures 2A-2D). Our immunofluorescence results were further confirmed by subcellular fractionation of COS-7 cells transfected with the pRShGRα or the mutant receptors (Figure 3).

The wild-type hGRα co-localized with caveolin-1 at the plasma membrane

We investigated whether the hGRα co-localizes with caveolin-1 using the double immunostaining method. Caveolin-1 was detected strongly as "dots" on the plasma membrane, as well as weakly in the cytoplasm in the COS-7 untransfected cells, suggesting a role of caveolin-1 in endocytosis/exocytosis processes. Co-localization of the hGRα with caveolin-1 was observed at the plasma membrane of the COS-7 cells transiently transfected either with the hGRα or its mutants at the same percentages as above (Figures 4A & 4B). Addition of the inhibitor 2-Br did not prevent the co-localization of hGRα with caveolin-1 (Figure 4B), whereas addition of dexamethasone resulted in nuclear translocation of hGRα without any co-migration with caveolin-1 (Figure 4C).

The mutant receptors preserved their ability to induce rapid nongenomic glucocorticoid actions through MAPK activation

To examine the role of the 663YLCMKTLLL671 motif in the induction of rapid nongenomic glucocorticoid actions, kinase signaling assays were performed in COS-7 cells transiently transfected either with the pRShGRα or the mutant receptors. Addition of dexamethasone for different periods of time resulted in a biphasic activation of the MAPK activity through induction of ERK phosphorylation at 5 and 15 minutes, either in the absence or presence of 2-Br (Figures 5A, 5B). All mutant receptors tested did not prevent this dexamethasone-induced biphasic MAPK activation; however the double-mutant receptor hGRαLL670/671AA activated this kinase pathway at 5 and 10 minutes (Figures 5A, 5B). Interestingly, the activity of the AKT pathway was not induced by the dexamethasone-activated hGRα or the mutant receptors both in the absence or presence of the palmitoylation inhibitor (Figures 6A, 6B).

The hGRα did not undergo S-palmitoylation

To examine whether the hGRα was a palmitoylated protein, COS-7 cells transiently transfected with the pRShGRα or erbA^{-1} were incubated with 9,10-^{3}H(N) palmitic acid. The immunoprecipitated caveolin-1 was used as positive control, whereas the empty vector was used as a negative control. Caveolin-1 was shown to incorporate 9,10-^{3}H(N) palmitic acid, whereas the wild -type hGRα did not incorporate the tritiated substrate, indicating that the hGRα did not undergo S-palmitoylation (Figure 7).

Discussion

In the present study, we investigated the role of the highly conserved motif 663YLCMKTLLL671 of the LBD of hGRα in mediating the rapid, nongenomic glucocorticoid actions. We demonstrated that this 9-amino acid motif did not play a role in membrane localization of the hGRα and did not influence the co-localization of the receptor with caveolin-1 or the ligand-induced activation of MAPK pathway. We also showed that the hGRα did not undergo S-palmitoylation.

The 663YLCMKTLLL671 motif did not mediate the membrane localization of hGRα, granted

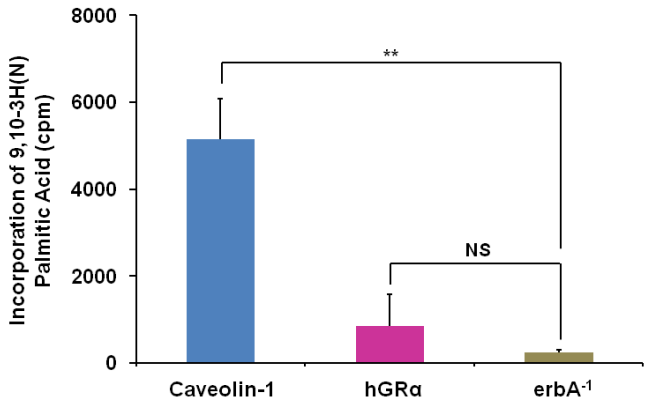

Figure 7. Palmitoylation of wild-type hGRα and caveolin-1. Caveolin-1 incorporated 9,10-^{3}H(N) palmitic acid, whereas the wild-type hGRα (hGRαWT) did not incorporate the tritiated substrate. Bars represent mean ± SEM of at least three independent experiments. **: P<0.01, n.s.: not significant, compared to control.

that the mutant receptors pRShGRαC665A, pRShGRαY663A and pRShGRαLL670/671AA were localized at the plasma membrane in the same percentage compared to that of the wild-type hGRα. Moreover, this motif was not responsible for co-localization of hGRα with caveolin-1. Indeed, in the absence of ligand, all the mutant receptors were still co-localized with caveolin-1 at the membrane, while addition of dexamethasone triggered their nuclear translocation. Furthermore, the activation of MAPK signaling pathway was not influenced by the mutant receptors, while neither the wild-type receptor nor the mutant receptors activated the PI3K/AKT pathway in COS-7 cells. These findings clearly showed that the 9-amino acid motif lying between amino acids 663 and 671 of the hGRα LBD did not influence the membrane localization of the receptor or the MAPK-mediated nongenomic glucocorticoid signaling.

To examine whether the hGRα undergoes S-palmitoylation by incorporating palmitic acid to the cysteine residue of the motif 663YLCMKTLLL671, we used the palmitoylation inhibitor 2-Br in some experiments and we performed palmitoylation assays using 9,10-^{3}H(N) palmitic acid. The membrane localization of the receptor and the nongenomic glucocorticoid signaling were not affected by the presence of the inhibitor, suggesting that this lipid post-translational modification did not play an important role for these actions. In addition, palmitoylation assays showed that compared with caveolin-1, which is a known palmitoylated protein, the hGRα did not incorporate palmitic acid.

We have chosen to investigate whether the 663YLCMKTLLL671 was a palmitoylated motif, because this amino acid sequence was highly homologous to the conserved peptide located in the LBD of ERα and ERβ, PR-A and PR-B, and the AR. Pedram *et al.* (2007) demonstrated that these steroid receptors underwent S-palmitoylation through thioester linkage between palmitic acid and the cysteine residue located within the 9-amino acid motif. They showed that S-palmitoylation facilitated membrane localization, co-localization with caveolin-1, thymidine incorporation into DNA, cell viability, and MAPK and PI3K activation. In subsequent studies, they demonstrated that heat shock protein 27 promoted S-palmitoylation of the above steroid receptors and was required for their membrane localization (Razandi *et al.* 2010). They also showed that DHHC-7 and -21 were the specific PATs that catalyzed S-palmitoylation of ER, PR and AR (Pedram *et al.* 2012) and revealed the important role of estrogen membrane signaling in organ development and metabolism (Pedram *et al.* 2013, 2014). Based on these findings, we hypothesized that the hGRα might be a palmitoylated protein mediating the rapid nongenomic glucocorticoid signaling. However, our results showed that the motif 663YLCMKTLLL671 was not palmitoylated.

Our findings concur with two recent studies (Deng *et al.* 2015, Samarasinghe *et al.* 2011). In the latter study, Samarasinghe and colleagues (2011) investigated the role of nongenomic glucocorticoid actions in regulating the gap junction-mediated intercellular communication and neural progenitor cell proliferation. They demonstrated that a short time exposure of neural progenitor cells to dexamethasone reduced the gap junction-mediated intercellular communication through a signaling pathway initiated by membrane-bound glucocorticoid receptors, which co-localized with caveolin-1 and cellular Src (c-Src) kinase, and triggered a MAPK-mediated phosphorylation of connexin 43, an important gap junction protein (Samarasinghe *et al.* 2011). To examine whether this membrane localization of the receptor was palmitoylation-dependent, they expressed the hGRαC665A in CHO cells and found no difference in the number of cells with membrane localized hGRα, suggesting that the receptor might not undergo S-palmitoylation (Samarasinghe *et al.* 2011). The second study by Deng *et al.* showed that the endogenously expressed GRα in 4B cells did not incorporate tritiated palmitic acid, indicating that the GRα did not undergo S-palmitoylation (Deng *et al.* 2015). Moreover, they showed that substitution of the highly conserved cysteine 683 to alanine preserved membrane localization of the receptor, suggesting that this amino acid did not play a role in the localization of the GRα at the plasma membrane (Deng *et al.* 2015). However,

they demonstrated that leucines located within this 9-amino acid motif were essential for interaction with HSP90 and for ligand-binding, thereby influencing the transcriptional activity of the GR (Deng *et al.* 2015). Although this study showed for the first time that GR was not a palmitoylated protein, the authors did not further investigate the role of this 9-amino acid motif in nongenomic glucocorticoid actions. Our study shows that neither point mutations in this motif nor the addition of 2-Br influenced the biphasic activation of MAPK pathway, which is involved in mediating the nongenomic glucocorticoid effects.

Delineating the molecular mechanisms of nongenomic glucocorticoid actions is extremely important, because membrane-initiated glucocorticoid signaling pathways are implicated in several pathophysiologic mechanisms. Given that several cardioprotective and immunosuppressive actions of glucocorticoids are non-genomic (Hafezi-Moghadam *et al.* 2002, Löwenberg *et al.* 2006), it is likely that the development of specific agonists and antagonists with well-characterized effects, which are able to modify discrete glucocorticoid-induced cellular functions, will help significantly towards the therapeutic management of myocardial infarction, stroke and several immunological conditions.

In summary, we demonstrated that the 663YLCMKTLLL671 did not play a role in membrane localization of the hGRα and did not influence the co-localization of the receptor with caveolin-1 or the ligand-induced activation of the MAPK pathway. We also showed that the hGRα did not undergo S-palmitoylation. The membrane localization of the receptor, as well as the molecular mechanisms underlying the nongenomic glucocorticoid actions still remain an enigma. Further studies are necessary to investigate these research questions and to explore their clinical implications.

Conflicts of interest

None.

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