Protein 14-3-3σ Interacts with and Favors Cytoplasmic Subcellular Localization of the Glucocorticoid Receptor, Acting as a Negative Regulator of the Glucocorticoid Signaling Pathway*

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The glucocorticoid receptor (GR) α interacts with the highly conserved 14-3-3 family proteins. The latter bind phosphorylated serine/threonine residues of “partner” molecules and influence many signal transduction events by altering their subcellular localization and/or protecting them from proteolysis. To examine the physiologic role of 14-3-3 on the glucocorticoid-signaling pathway, we studied the nucleocytoplasmic shuttling and transactivation properties of GRα in a cell line replete with or devoid of 14-3-3σ. We found that endogenous 14-3-3σ helped localize green fluorescent protein-fused GRα in the cytoplasm in the absence of ligand and potentiated its nuclear export after ligand withdrawal. 14-3-3σ also suppressed the transcriptional activity of GRα on a glucocorticoid-responsive promoter. Disruption of the classic nuclear export signal of 14-3-3σ inactivated its ability to influence the nucleocytoplasmic trafficking and transactivation activity of GRα, whereas introduction of a mutation inactivating the binding activity of 14-3-3σ to some of its partner proteins did not. 14-3-3σ bound the ligand-binding domain of GRα through its COOH-terminal portion, in a partially ligand-dependent fashion, while it did not interact with “ligand-binding domain” of GRβ at all. These results suggest that 14-3-3σ functions as a negative regulator in the glucocorticoid signaling pathway, possibly by shifting the subcellular localization/circulation of this receptor toward the cytoplasm through its nuclear export signal. Since 14-3-3σ proteins play significant roles in numerous cellular activities, such as cell cycle progression, growth, differentiation, and apoptosis, these actions might indirectly influence the transcriptional activity of GRα. Conversely, through its 14-3-3σ protein interactions, GRα may influence these processes.

The glucocorticoid receptor (GR)1 belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins and mediates

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3 The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid response element; NES, nuclear export signal; DBD, DNA-binding domain; LBD, ligand-binding domain; GFP, green fluorescence protein; EGFP, enhanced GFP; MMTV, mouse mammary tumor virus; AD, activation domain; WT, wild type; KO, knock-out; C, cytoplasmic distribution; N, nuclear localization.

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to be involved in nuclear export of this molecule. A serine residue at position 226 of GRα located in the amino-terminal domain is necessary for phosphorylation by the c-Jun NH$_2$-terminal kinase to facilitate the nuclear export of the GRα, while a 67-amino acid region in the DBD is sufficient to support calcitretin-mediated nuclear export (9, 12). In addition, we previously reported that removal of the LBD from GRs resulted in constitutive localization of this peptide in the nucleus, indicating that the LBD also contributes to nuclear to cytoplasmic translocation of GRs (13).

14-3-3 family proteins constitute a highly conserved family present in high abundance in all eukaryotic cells. They consist of nine isotypes from at least 7 distinct genes in vertebrates and regulate important biologic activities by directly binding to and altering the subcellular localization and/or stability of key molecules in several signaling cascades (14–16). For example, 14-3-3 proteins regulate the apoptosis pathway by binding to BAD and affect the intracellular signaling of several growth factors, including insulin, by interacting with important molecules of their cascades, such as Raf-1, insulin receptor substrate 1 (IRS1), and the forkhead transcription factors (17–21). 14-3-3 proteins also influence other signaling events through physical interaction with members of the protein kinase C family proteins Cbl and polyoma middle-T antigen (15). In addition, 14-3-3 proteins play a critical role in the progression/arrest of the cell cycle by binding to Cdc25C, Wee1, Cyclin B1, and possibly Chk1 (15, 16). Binding of 14-3-3 to Cdc25C sequesters the latter into the cytoplasm and eliminates its phosphatase activity from the nucleus, thus inhibiting cells from progressing through the G$_2$/M check-point (22–24).

14-3-3 proteins bind the phosphorylated serine or threonine residues of their partner proteins located within a specific amino acid sequence, RX(S/T)XP, identified as a high-affinity 14-3-3-binding motif (25). They contain nine a-helical structures and form a homo- or heterodimer through their NH$_2$-terminal portions (25–27). Their central third to fifth a-helices create a binding pocket for a phosphorylated serine/threonine residue, and the C-terminal seventh to ninth helices determine the specificity to target peptide motifs (25, 26). 14-3-3 proteins contain one classic NES in their ninth helix, which helps localize 14-3-3-partner protein complexes in the cytoplasm (26, 28).

Recent research indicated that GRα formed complexes with 14-3-3 proteins and Raf-1 (29). Although an early study reported that GRα LBD interacted with 14-3-3p in a ligand-dependent fashion in a yeast two-hybrid assay, a subsequent report indicated that GRα was associated with 14-3-3 proteins, both in the ligand-free and -bound conditions (29, 30). To further investigate the functional contribution of 14-3-3 to the biologic activity of GRα, we examined the subcellular localization and transactivation properties of GRα in a cell line used in its wild type 14-3-3 expression repletion and in its mutant type 14-3-3α-deficient forms (31). We found that endogenous 14-3-3α helps localize ligand-free GRα in the cytoplasm and contributes to nuclear export of GRα after withdrawal of ligand. In addition, endogenous 14-3-3α suppresses ligand-activated GRα-induced transactivation of a glucocorticoid-responsive promoter. These results indicate that 14-3-3α functions as a negative regulator of the glucocorticoid signaling pathway by shifting the subcellular circulation of this receptor toward the cytoplasm.

MATERIALS AND METHODS

Plasmids—pF25-hGRα, which expresses the green fluorescence protein (GFP)-fused human GRα under the control of the cytomegalovirus promoter, was reported previously (13). pRS8GRα, which expresses the human GRα, was a kind gift from Dr. R. M. Evans (Salk Institute, La Jolla, CA). pMMTV-Luc, which expresses luciferase under the control of the glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter, was a generous gift from Dr. G. L. Hager (NCI, Bethesda, MD). pCDNA14-14-3α and pEGFP-C1-14-3α were constructed by subcloning the coding sequence of the human 14-3-3α into pcDNA3/HisMax (Invitrogen) or pEGFP-C1 (Clontech, Palo Alto, CA) in an in-frame fashion, respectively. pCDNA14-14-3α-NES Mut, which expresses 14-3-3α, defective in NES due to mutations replacing leucine at positions 243, 247, and 249 to alanine, was constructed by PCR-assisted mutagenesis using pCDNA14-14-3α as a template. pEGFP-C1-14-3α-NES Mut was constructed using the same procedure employing pEGFP-C1-14-3-3α as a template. pCDNA14-14-3α-S812K, which expresses a 14-3-3α mutant that may have a defective binding site for a phosphorylated serine/threonine residue due to a point mutation that replaces a glutamic acid at position 182 to lysine, was also constructed by PCR-assisted mutagenesis using the same template plasmids (β-Gal, which expresses β-galactosidase under the control of the simian virus 40 promoter, was purchased from Promega (Madison, WI).

pLexA-GRoLBD and -GRβLBD, which express the LexA DBD fusions of the human GRα LBD or GRβ LBD, were constructed by inserting the corresponding cDNA fragments of the human GRα LBD or GRβ LBD into pLexA (Clontech) in an in-frame fashion, respectively. pGAD424-14-3-3p-(1-270) and -(106-270), and pB42AD-14-3-3p-(1-244), -(141-244), -(190-244), -(210-244), -(110-210), and -(1-110), which respectively express the GAL4 or LexA activation domain (AD) fusions of the indicated 14-3-3 fragments, were constructed by inserting cDNA fragments of the indicated regions of 14-3-3α or 14-3-3p into pGAD424 (Clontech) or pB42AD (Clontech), respectively. pSOP-LacZ was purchased from Clontech.

Cell Cultures and Transfections—Human colon cancer-derived HCT116 wild type (WT) and 14-3-3α knock-out (KO) cells were kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD) (31). These cells are defective in functional GRα (data not shown). They were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 1 μg/ml of streptomycin. They were transfected using Lipofectin™ with 1 μg/well of pF25-hGRα and/or 0.3 μg/well of 14-3-3α-expressing plasmids for the study of GRα subcellular localization, as described previously (32). For reporter assays, 0.5 μg/well of pRS8GRα and 0.3 μg/well of 14-3-3α-expressing plasmids together with 1.0 μg/well of pMMTV-Luc and 0.3 μg/well of pSV40-β-Gal were used.

Detection of Subcellular Localization of GFP-fused GRα and 14-3-3α—Cells were plated on 25-mm dishes and were transfected as described above. 24 h after transfection, the medium was replaced with McCoy’s 5A medium containing 10% charcoal/dextran-treated fetal bovine serum with antibiotics. 48 h after transfection, the cells were analyzed with an inverted fluorescence microscope (Leica DM IRB, Wetzlar, Germany) as described previously (13, 33). 12-Bit black-and-white images were captured using a digital CCD camera (Hamamatsu Photonics K.K., Hamamatsu, Japan). Image analysis and presentation was performed using the Openlab software (Improvision, Boston, MA).

To examine the subcellular distribution of GFP-fused GRα and 14-3-3α, we grew cells expressing plasmids together with 1.0 μg/well of pMMTV-Luc and 0.3 μg/well of pSV40-β-Gal used.

Yeast Two-hybrid Assay—Yeast strain EGY48 (Clontech) was transformed with the lacZ reporter plasmid pSOP-LacZ, pLexA-GRoLBD or -GRβLBD, and the indicated pGAD424-14-3-3αp or pB42AD-14-3-3p, to allow growth in a selective medium in the early stationary phase, permeabilized with CHCl3-SDS treatment, and β-galactosidase activity was measured in the cell suspension using β-galactosidase assay kit (Tropix, Bedford, MA), as described previously (33).
Endogenous 14-3-3σ retains GFP-GRα in the cytoplasm and helps its nuclear export after the dexamethasone withdrawal. A, representative localization of non-ligand-bound GFP-GRα in HCT116 WT (panel a) and 14-3-3σ KO (panel b) cells. WT and KO cells were transfected with pF25-hGRα and intracellular localization of GFP-GRα was detected in an inverted fluorescence microscope. B, KO cells have more non-ligand-bound GFP-GRα in the nucleus than WT cells; supplementation of 14-3-3σ reverses the defect in KO cells. Subcellular localization of non-ligand-bound GFP-GRα was examined in over 100 cells in the above transfected cells. The cells were categorized into five groups depending on the subcellular localization of GFP-GRα as indicated in the x axis and their percentages to the total cell number are shown in the y axis. Bars represent mean ± S.E. N, nuclear localization; C, cytoplasmic localization. C, supplementation of 14-3-3σ in KO cells facilitates the nuclear export of GFP-GRα after the withdrawal of dexamethasone. Subcellular localization of GFP-GRα 8 h after the withdrawal of dexamethasone was examined in over 100 cells and percentages of cells having GFP-GRα mainly in the cytoplasm (categories C and N < C) to complete nuclear localization (N) (Fig. 1B). Using this analysis, HCT116 WT cells had unliganded GFP-GRα mainly in the cytoplasm, while KO cells had more in the nucleus, indicating that endogenous 14-3-3σ retained GFP-GRα in the cytoplasm in the absence of ligand. We also tested nuclear translocation of GFP-GRα in WT cells and KO cells in which 14-3-3σ was supplemented by the transfected expressing plasmid. GFP-GRα entered the nucleus in 10–20 min in response to 10−6 M dexamethasone in both cell types, indicating that the mechanism supporting the nuclear translocation of GRα was intact in these cells (data not shown). We next examined whether 14-3-3σ plays a role in the nuclear export of GRα after withdrawal of ligand. Eight hours after removing 10−6 M dexamethasone from the medium, 32% of HCT116 WT cells had GFP-GRα in the cytoplasm, while almost all KO cells still retained it in the nucleus (Fig. 1C). When the wild type 14-3-3σ was transfected in KO cells, 22% of the cells had unliganded GFP-GRα in the cytoplasm, indicating that transfected 14-3-3σ helped GRα relocate into the cytoplasm after removal of dexamethasone.

RESULTS

Endogenous 14-3-3σ Helps Non-ligand-bound GRα Remain in the Cytoplasm and Facilitates the Nuclear Export of GRα after Withdrawal of Dexamethasone—To investigate a role of 14-3-3 family proteins on the subcellular localization and transactivation activity of GRα, we employed wild type versus mutant HCT116 cells, in which both alleles of the 14-3-3 gene were destroyed by homologous recombination (31). We first tested the subcellular localization of GFP-GRα in the absence of ligand. GFP-GRα was mainly located in the cytoplasm in the HCT116 WT cells, while substantial amounts of GFP-GRα were found in the nucleus in the 14-3-3σ KO cells (Fig. 1A). To determine this, we examined multiple cells and graded them into five distribution patterns from complete cytoplasmic distribution (C) to complete nuclear localization (N) (Fig. 1B). Statistical analyses was carried out by analysis of variance, followed by Student t test with Bonferroni correction for multiple comparisons.

Using this analysis, HCT116 WT cells had unliganded GFP-GRα mainly in the cytoplasm, while KO cells had more in the nucleus, indicating that endogenous 14-3-3σ retained GFP-GRα in the cytoplasm in the absence of ligand. We also tested nuclear translocation of GFP-GRα in WT cells and KO cells in which 14-3-3σ was supplemented by the transfected expressing plasmid. GFP-GRα entered the nucleus in 10–20 min in response to 10−6 M dexamethasone in both cell types, indicating that the mechanism supporting the nuclear translocation of GRα was intact in these cells (data not shown). We next examined whether 14-3-3σ plays a role in the nuclear export of GRα after withdrawal of ligand. Eight hours after removing 10−6 M dexamethasone from the medium, 32% of HCT116 WT cells had GFP-GRα in the cytoplasm, while almost all KO cells still retained it in the nucleus (Fig. 1C). When the wild type 14-3-3σ was transfected in KO cells, 22% of the cells had GFP-GRα in the cytoplasm, indicating that transfected 14-3-3σ helped GRα relocate into the cytoplasm after removal of dexamethasone.

Endogenous 14-3-3σ Suppresses the Transcriptional Activity of GRα—We then examined the transactivation activity of GRα stimulated with increasing concentrations of dexamethasone in WT and KO cells (Fig. 2). GRα stimulated the MMTV promoter in response to 10−6 M dexamethasone by about 80- and 300-fold in WT cells and KO cells, respectively. The dexamethasone titration curve was shifted upward in the latter cells. Transfection of wild type 14-3-3σ partially reversed this change in KO cells. The EC_{50} (mean ± S.E.; in mM) was 4.01 ± 0.33 and 6.46 ± 1.04 in WT and KO cells, respectively (p > 0.10), whereas the B_{max} (mean ± S.E.; × 10−2 relative luminescence units) was 9.63 ± 8.45 in WT and 8.93 ± 4.25 in KO cells. These results indicate the mechanism supporting the nuclear translocation of GRα was intact in these cells (data not shown).
14-3-3σ Retains GR in the Cytoplasm

The C-terminal Half of 14-3-3σ Interacts with the GRα LBD in a Yeast Two-hybrid Assay—We next examined the interaction of GRα and 14-3-3σ in a yeast two-hybrid assay (Fig. 3A). Administration of dexamethasone stimulated the LexA-DBD-GRαLBD-induced, but not LexA-GRβLBD-induced, β-galactosidase activity by about 3-fold in the EGY48 yeast strain. Co-expression of GAL4-AD fusions of the full-length or the COOH-terminal half of 14-3-3σ enhanced β-galactosidase activity induced by LexA-DBD-GRαLBD in a partially dexamethasone-dependent fashion, whereas it did not affect the activity of LexA-DBD-GRβ LBD. These results indicated that GRα LBD interacted with the COOH-terminal half of 14-3-3σ in a partially ligand-dependent fashion, while GRβ LBD did not. No increase of β-galactosidase activity was observed in the transformed yeast cells, when they were cultured in galactose-deficient medium that did not support the expression of bait proteins (data not shown). This result indicated that expression of GAL4-AD-fused 14-3-3σ did not influence basal promoter activity. We obtained similar results using a plasmid expressing the LexA-DBD fusions of the full-length GRα (data not shown).

Our results showed that 14-3-3σ interacts with GRα in the absence of dexamethasone as well as in its presence. In our system, 14-3-3σ interacted with the GRα LBD in the absence of dexamethasone and the interaction was enhanced in its presence (Fig. 3B). 14-3-3σ fragments, which contained the region from 210 to 240 that corresponds to the ninth α-helix, supported the binding to GRα LBD.

 Destruction of 14-3-3σ NES Diminishes the Ability of This Protein to Promote Cytoplasmic Retention/Nuclear Export of GRα and Suppression of GRα Transactivation—14-3-3 proteins may help translocate their partner proteins into the cytoplasm via their classic NES located in their ninth α-helix (16, 26). Thus, we examined the contribution of 14-3-3σ NES on the cytoplasmic retention of GRα, by constructing a plasmid expressing a 14-3-3σ mutant (14-3-3σNES Mut), in which the NES was destroyed by clustered mutations, as a fusion with EGFP (26). The EGFP-fused wild type 14-3-3σ was mainly located in the cytoplasm, while a small fraction of this fusion protein was also observed in the nucleus (Fig. 4A). The EGFP-14-3-3σNES Mut, on the other hand, was distributed more in the nucleus, indicating that the introduced mutations inactivated the NES. Co-expression of 14-3-3σNES Mut did not change the distribution of unliganded GFP-GRα, in contrast to the wild type 14-3-3σ (Fig. 4D). We next examined the effect of this mutant on the nuclear export of GFP-GRα after withdrawal of dexamethasone. Supplementation of the wild type 14-3-3σ brought GFP-GRα into the cytoplasm in 23% of KO cells, while expression of 14-3-3σNES Mut did not change the nuclear export of this receptor (Fig. 4C). In a functional reporter assay, 14-3-3σNES Mut did not suppress GRα-induced transactivation of the MMTV promoter in a dexamethasone titration curve (Fig. 4D). These results suggest that 14-3-3σ retained the ligand-free GFP-GRα in the cytoplasm and helped it through its NES to redistribute in the cytoplasm after the withdrawal of dexamethasone. Since the destruction of NES also abolished the suppressive effect of 14-3-3σ on GRα transactivation, it is possible that 14-3-3σ suppressed GRα-induced transcriptional activity by segregating GRα away from the nucleus.

The Phosphopeptide Binding Activity of 14-3-3σ May Not Be Necessary for Cytoplasmic Retention of GFP-GRα and Suppression of GRα-dependent Transactivation—Since a previous publication indicated that 14-3-3 forms a complex with GRα together with its partner protein Raf-1, we employed a 14-3-3E182K mutant, to examine whether 14-3-3 partner proteins might contribute to the action of 14-3-3σ on the activity of GRα (29). This mutation corresponds to the replacement of a glutamic acid at 180 by a lysine in Drosophila 14-3-3 that inactivates the binding activity of this protein to Raf-1 because of destruction of the phosphopeptide-binding pocket (26). 14-3-3E182K was distributed mainly in the cytoplasm similarly to the wild type 14-3-3σ (Fig. 5A). 14-3-3σE182K preserved property of the wild type 14-3-3σ on the subcellular distribution and transactivation activity of GRα (Fig. 5, B and C), suggesting that association of 14-3-3σ to partner proteins, such as Raf-1, may not be necessary for its effect on these GRα activities.

**DISCUSSION**

GRαs forms heterocomplexes with 14-3-3 and its partner protein Raf-1 in the cytoplasmic fraction of adrenalectomized rat liver shown by immunoaffinity chromatography and co-immunoprecipitation experiments (29). Although these complexes were observed in the absence of ligand, the interaction of constituent molecules was further strengthened by the presence of the hormone. Association of GRα with one of the 14-3-3 proteins, 14-3-3σ, was also found in a yeast two-hybrid screening, using the GRα LBD as a bait (30). In agreement with the above reports, we examined the functional effect of 14-3-3 on the activity of GRα by employing wild type versus mutant HCT116 cells, in which both alleles of the 14-3-3σ gene were destroyed.

![Figure 3](http://www.jbc.org/)

**Fig. 3. Interaction of 14-3-3 and GR isoforms α and β in a yeast two-hybrid assay.** A, 14-3-3σ interacts with GRα LBD but not with GRβ LBD via its COOH-terminal portion. Plasmids expressing the indicated bait and prey molecules were co-transfected with p8OP-LacZ in EGY48 strain yeast cells, and their binding activity was tested in the absence or presence of 10−5 M dexamethasone. B, 14-3-3σ interacts with GRα LBD via a portion corresponding to its ninth α-helix. Plasmids expressing the indicated bait and prey molecules were co-transfected with p8OP-LacZ in EGY48 strain yeast cells, and their binding activity was tested in the absence or presence of 10−5 M dexamethasone.
by homologous recombination (31). We found that endogenous 14-3-3σ helped GFP-GRα remain in the cytoplasm in the absence of dexamethasone and supported the nuclear export of GFP-GRα after withdrawal of dexamethasone via its NES. 14-3-3σ, thus, appeared to function as an “attached” partner NES, a finding that might explain the results of previous reports demonstrating that the nuclear export of GRα is sensitive to leptomycin B, even though the GRα molecule does not contain a classic NES (6, 9). We previously reported that deletion of the GRα LBD from GRα localized this GRα fragment in the nucleus (13). Since 14-3-3σ interacts with the LBD, a defect in binding of this domain to 14-3-3σ may explain its particular subcellular distribution.

We also demonstrated that endogenous 14-3-3σ functioned as a negative regulator of GRα-induced transactivation. This activity correlated with the ability of 14-3-3σ to localize unliganded GFP-GRα in the cytoplasm, in the experiment employing the 14-3-3σ mutants NES Mut and E182K. Therefore, it is likely that 14-3-3σ suppresses GRα-induced transactivation by shifting intracellular circulation of GRα toward the cytoplasm, possibly by reducing the chance of ligand-bound GRα to interact with GREs, steroid hormone receptor co-activators, and other related specific or general transcription factors in the nucleus. For instance, GRα dynamically interacts with GREs in living cells, binding to and dissociating from them in the order of seconds (35, 36). In this situation, the drive created by 14-3-3σ, which shifts GRα toward the cytoplasm, may reduce the probability of GRα binding to GREs and, hence, may reduce its transcriptional activity. A similar effect was also observed in a recent report, which showed that c-Jun NH2-terminal kinase suppressed GRα-induced transactivation by phosphorylating serine 216 and facilitating its nuclear export (9).

A previous report demonstrated that overexpressed 14-3-3σ enhanced GRα-induced transactivation of a synthetic GRE-containing heterologous promoter in African monkey kidney-derived COS7 cells, while we showed that endogenous 14-3-3σ

Fig. 4. 14-3-3σNES Mut loses the ability to promote the cytoplasmic retention and nuclear export of GFP-GRα, as well as to suppress GRα-induced transactivation. A, EGFP-14-3-3σNES Mut is more located in the nucleus than the wild type EGFP-14-3-3σ. KO cells were transfected with EGFP-14-3-3σ WT- or NES Mut-expressing plasmids and localization of these EGFP-fused proteins were examined in an inverted fluorescence microscope. B, 14-3-3σNES Mut does not remain non-ligand-bound GRα in the cytoplasm. KO cells were transfected with pP25-hGRα and pCDNA3, pCDNA4-14-3-3σ or pCDNA4-14-3-3σNES Mut and subcellular localization of non-ligand-bound GFP-GRα was examined as indicated in Fig. 1B. C, supplementation of 14-3-3σNES Mut in KO cells does not facilitate the nuclear export of GFP-GRα after withdrawal of dexamethasone. Subcellular localization of GFP-GRα 8 h after the withdrawal of dexamethasone was examined in over 100 cells, and percentages of cells having GFP-GRα mainly in the cytoplasm (categories C and N < C) to total transfected cells were calculated. Bars represent mean ± S.E. *, p < 0.01; n.s., not significant, comparing to KO cells. D, 14-3-3σNES Mut fails to suppress the GRα-induced transactivation of the MMTV promoter. KO cells were co-transfected with plasmids expressing 14-3-3σ WT or 14-3-3σNES Mut together with pShGrα, pMMTV-Luc, and pSV40-β-Gal. The cells were then treated with the indicated concentrations of dexamethasone. Each point represents the mean ± S.E. of luciferase normalized for β-galactosidase activity values. *, p < 0.01, comparing either transfected cell culture to KO non-transfected cells; n.s., not significant, comparing the two transfected lines.

Fig. 5. 14-3-3σE182K retains its cytoplasmic retention of GFP-GRα activity and suppresses GRα-induced transactivation. A, 14-3-3σE182K is located mainly in the cytoplasm. KO cells were transfected with EGFP-14-3-3σWT- or E182K-expressing plasmids, and localization of EGFP-fused proteins was examined in an inverted fluorescence microscope. B, 14-3-3σE182K retains non-ligand-bound GFP-GRα in the cytoplasm. KO cells were transfected with pP25-hGRα and pCDNA3, pCDNA4-14-3-3σ or pCDNA4-14-3-3σE182K, and the subcellular localization of non-ligand-bound GFP-GRα was examined as indicated in Fig. 1B. C, 14-3-3σE182K suppresses GRα-induced transactivation of the MMTV promoter. KO cells were co-transfected with plasmids expressing 14-3-3σ WT or 14-3-3σE182K together with pShGrα, pMMTV-Luc, and pSV40-β-Gal. The cells were then treated with the indicated concentrations of dexamethasone. Each point represents the mean ± S.E. of luciferase normalized for β-galactosidase activity values. *, p < 0.01, comparing either transfected cell culture to KO non-transfected cells; n.s., not significant, comparing the two transfected lines.
suppressed GRa transactivation in HCT116 cells (30). This discrepancy may result from differences in the experimental systems employed, including the type of cell lines, the different 14-3-3 isoforms and overexpression versus normal expression versus knock-out of 14-3-3. Indeed, overexpression of a protein may sometimes cause artificial effects (37). We examined 14-3-3 “loss of function” by employing the KO cells and showed that the physiologic activity of 14-3-3a is that of a negative regulator of the glucocorticoid signaling pathway.

We demonstrated that the GRa LBD interacts with 14-3-3a as well as a partially ligand-dependent fashion. A previous report indicated that this domain of GRa interacted with 14-3-3 in an absolutely ligand-dependent fashion in the same LexA yeast two-hybrid system (30). Differences in the GRa fragments employed or yeast strains used in the assay might have led to the different results. Since a recent report also demonstrated partial ligand-dependent interaction between GRa and 14-3-3 in a semiquantitative coimmunoprecipitation assay, it is likely that GRa and 14-3-3 proteins associate with each other in the absence of ligand. In contrast to the GRa LBD, the GRa “LBD” did not interact with 14-3-3a at all. Since GRa is constitutively located in the nucleus, the inability of GRa to interact with 14-3-3a might, to some extent, contribute to its constitutive nuclear localization (13, 38–40).

Our results employing 14-3-3a-E182K suggest that the association of known partner proteins with 14-3-3a may not be necessary for this molecule to influence the subcellular localization of GRa and the suppression of its transactivation. However, an E180K mutation in Drosophila 14-3-3a, which corresponds to the E182K mutation in human 14-3-3a, abolishes the interaction of this protein with Raf-1 and BAD, but preserves that with IRS-1, indicating that the E182K mutation in 14-3-3a might not completely exclude the interaction of this 14-3-3a subtype with all partner proteins (26, 41). In agreement with the above-indicated evidence, about half of the 14-3-3-partner proteins use a different phosphopeptide-binding motif to interact with 14-3-3, suggesting that a single surface of 14-3-3a in the phosphopeptide-binding pocket, which the E182K mutation destroys, may not support its association generally with all partner proteins. Further experiments are required to address this issue.

In summary, endogenous 14-3-3a functions as a negative regulator of GRa-induced transactivation, most likely by shifting the subcellular distribution and circulation of GRa toward the cytoplasm. These results indicate that change in the intracellular concentration as well as the subcellular distribution of 14-3-3a may contribute to the altered sensitivity of tissues to glucocorticoids seen in several physiologic and pathologic conditions (1). Since 14-3-3 proteins are involved in a broad array of cellular activities, such as cell cycle progression, growth, differentiation, and apoptosis, these activities might indirectly influence the transcriptional activity of GRa, by changing the availability of 14-3-3a and/or altering partner proteins associated with 14-3-3a. On the other hand, the opposite may be true. Ligand-bound GRa may influence these cellular processes by segregating and/or influencing 14-3-3a and partner molecules.
